

Development of Ag-based Quantum Dots for Optical Imaging and Multimodal Therapy of Cancer

by

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Therapy of Cancer**

Koç University

Graduate School of Sciences and Engineering

This is to certify that I have examined this copy of a doctoral dissertation by

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*To the soul of
all 176 innocent passengers and crews of the
Flight PS752
that lost their lives in airplane crash....*



ABSTRACT

Development of Ag-based Quantum Dots for Optical Imaging and Multimodal Therapy of Cancer

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Luminescent quantum dots (QDs) with size-tunable, strong, and long-lived emission properties have attracted significant attention over the past decades. Their theranostic potential has been verified in various studies demonstrating strong optical labelling coupled with drug/gene delivery in diagnosis and treatment of diseases. However, there is still no clinical use of QDs due to their cytotoxicity. Ag-based QDs are one of the most promising QDs with exceptional cytocompatibility compared to heavy metal containing QDs such as PbS, PbSe, and CdHgTe with emission in the medical imaging window, which is near-infrared region.

In this thesis work, both synthesis of promising Ag-chalcogenide QDs and their application in cancer diagnosis and therapy with high specificity and efficacy were intended, which are urgent needs of fight against cancer. Therapeutic approaches adopted here, included phototherapies and its combination with chemotherapy and tumour specificity were achieved with receptor targeting.

The first approach was further developed into targeted PDT/chemotherapy of colorectal cancer using anionic Ag₂S QDs with NIR emission. Ag₂S QDs tagged with Cetuximab for EGFR targeting and loaded with ALA and 5FU provided significant toxicity in EGFR(+) cells. Different pH-sensitive ALA release levels were achieved by electrostatic loading of the ALA to the QDs or conjugation via amide or hydrazone bonds. QDs with electrostatically loaded ALA provided fastest release and quick PpIX formation, hence determined as the most efficient. Selective delivery of Cet conjugated QDs via receptor-mediated endocytosis which can be tracked with strong NIR signal of the QDs, improved the treatment efficacy in EGFR(+) cells. After a short incubation time (4 h) followed with single and short irradiation of SW480 cells treated with these theranostic particles at 100

$\mu\text{g/mL}$ $[\text{Ag}]/0.688 \text{ mM ALA}$ the viability dropped down to 38% (420 nm, 5 min) and 5% (630 nm, 1 min). Toxicity was correlated well with high levels of reactive-oxygen species (ROS) and apoptotic/necrotic cell death.

The major synthetic goal achieved here is aqueous, one step, simple, and economical synthesis of non-toxic, highly stable, strongly luminescent, and small Ag-chalcogenide QDs. The QD portfolio developed here include Ag_2S QDs with glutathione (GSH) coating which has 70% quantum yield, NIR emission, and excellent stability, and AgInS_2 QDs luminescent in the visible region with glutathione coating which have 21% QY, and enhanced stability. Despite the great success of Ag_2S QDs, since most available microscopes, lamps, and LEDs are functioning in the visible range, small and stable AgInS_2 QDs with emission in the visible region was also developed here. Then, delivery of therapeutic cargo to tumour cells with such optically trackable QDs as well as the therapeutic potential of the QDs were evaluated. Ag-chalcogenide QDs with strong absorbance in the visible region and near-infrared coupled with strong luminescence, were evaluated as photothermal therapy (PTT) agents within this thesis. While Ag_2S -GSH was found as a strong PTT agent providing image-guided PTT demonstrating a great theranostic potential by itself without using any other therapeutic agent, AgInS_2 -GSH was poor.

PTT potential of Ag-chalcogenides was further exploited for image-guided targeted combination therapy (PTT/chemotherapy) by decorating the particles with folic acid and methotrexate (MTX) in the treatment of folate receptor overexpressing cancer cells. QD internalization was tripled by FR targeting and the PTT/MTX combination therapy reduced the viability of FR(+) HeLa cells about 80% after only 4 h incubation followed with a single irradiation at 808 nm for 10 min (700 mW, 1.82 W/cm^2) at $1.25 \mu\text{g MTX/mL}$: $25 \mu\text{g Ag/mL}$ concentration in the case of Ag_2S -GSH. AgInS_2 QDs were effective in imaging and drug delivery, but lacked PTT contribution.

The last contribution of this thesis to literature is the development of stable and strongly luminescent anionic and cationic AgInS_2 QDs with anionic 2-mercaptopropionic acid (2MPA) and polyethyleneimine/2MPA (PEI) coating, respectively, in a one-step aqueous method without using a dopant or an inorganic shell. These QDs with the same core but a different coating was also used to investigate the best surface chemistry to deliver ALA to tumour cells. AgInS_2 were electrostatic loaded with ALA to improve PDT of HT29 and SW480 cells. It was found out that despite both particles reduce the ALA dose for

effective cell killing, anionic particles with intrinsically better cytocompatibility can provide better PDT efficiency.

This research study led to the successful demonstration of highly luminescent, stable, and cytocompatible Ag-based QDs for either NIR or visible regions, which can pave the way towards efficient optical imaging. Moreover, the demonstrated targeted anionic and cationic QDs loaded with chemotherapeutic drugs and photosensitizer in this thesis can be utilized for combinational therapy of cancer.



ÖZETÇE

Optik Görüntüleme ve Multi-Modlu Kanser Tedavisi için Ag Tabanlı Kuantum Noktalarının Geliştirilmesi

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Parçacık boyutlarına göre ayarlanabilen, güçlü ve uzun ömürlü emisyon özelliğine sahip ışıyan kuantum noktaları (QD'ler), geçtiğimiz on yılda önemli ölçüde dikkat çekmiştir. QD'lerin teranostik potansiyelleri, hastalıkların tanı ve tedavisinde ilaç/gen taşınmasında kullanılmaları ve güçlü optik etiketlemeyi mümkün kılmaları açısından çeşitli çalışmalarda doğrulanmıştır. Ancak, sitotoksiteleri nedeniyle QD'lerin klinik kullanımı hala mümkün değildir. Ag tabanlı QD'ler, yakın kızılötesi bölge olan tıbbi görüntüleme penceresinde emisyonlu PbS, PbSe ve CdHgTe gibi ağır metal içeren QD'lere kıyasla olağanüstü biyouyumluluğa sahip en umut verici QD'lerden biridir.

Bu tez çalışmasında, kanserle mücadelenin acil ihtiyaçları olan teşhis ve tedavide kullanılmak üzere gelecek vaat eden Ag-kalkojenit QD'lerin sentezi ve yüksek özgüllük ve etkinlik ile bu alanlarda uygulanması amaçlandı. Burada benimsenen terapötik yaklaşımlar, fototerapileri ve bunların kemoterapi ile kombinasyonlarını içeriyor olup, ek olarak tümör spesifikliği, reseptör hedefleme ile elde edildi.

İlk yaklaşımda, NIR dalga boyunda ışıyan anyonik Ag₂S QD'ler kullanılarak kolorektal kanseri hedefleyen PDT/kemoterapi için QD'ler geliştirildi. EGFR hedeflemesi için Cetuximab ile etiketlenen ve ALA ve 5FU ile yüklenen Ag₂S QD'ler, EGFR (+) hücrelerde önemli ölçüde toksisite sağlamıştır. ALA'nın QD'lere elektrostatik yüklenmesi veya amid veya hidrazon bağlarıyla kovalent konjugasyonu yapılarak, farklı pH değerlerine duyarlı ALA salınım seviyeleri elde edildi. Elektrostatik olarak yüklü ALA'ya sahip QD'ler en hızlı salınma ve PpIX oluşumunu sağladı ve bu nedenle en verimli olarak belirlendi. QD'lerin güçlü NIR sinyali ile izlenebilen Cet konjuge parçacıkların, reseptör aracılı endositoz yoluyla seçici olarak verilmesi, EGFR (+) hücrelerinde tedavi etkinliğini arttırdı. Kısa bir inkübasyon süresinden (4 saat) sonra, bu terapötik partiküllerle 100

$\mu\text{g/mL}$ $[\text{Ag}]/0.688 \text{ mM}$ ALA ile tedavi edilen SW480 hücrelerinin 420 nm 'de, 5 dakika ve 630 nm , 1 dakika olmak üzere tek ve kısa uyarımının ardından, canlılık sırasıyla %38'e ve %5'e düştü. Toksisite, yüksek seviyelerde reaktif oksijen türleri (ROS) ve apoptotik/nekrotik hücre ölümü ile korelasyon gösterdi.

Burada ulaşılan ana sentetik hedef, toksik olmayan, oldukça kararlı, güçlü ışıyan ve küçük Ag-kalkojenit QD'lerin sulu çözeltide, tek adımlı, basit ve ekonomik sentezidir. Geliştirilen QD portföyü, %70 kuantum verimi, NIR emisyonu ve mükemmel stabiliteye sahip glutatyon (GSH) kaplı Ag_2S QD'leri ve %21 kuantum verimine sahip GSH kaplı görünür bölgede ışıyan ve gelişmiş stabiliteye sahip AgInS_2 QD'leri içerir. Ag_2S QD'lerin büyük başarısına rağmen, mevcut mikroskopların, lambaların ve LED'lerin çoğu görünür aralıkta çalıştığından, görünür bölgede emisyonlu küçük ve kararlı AgInS_2 QD'ler de geliştirildi. Daha sonra, terapötik ajanların bu tür optik olarak izlenebilir QD'lere sahip tümör hücrelerine iletilmesi ve bu QD'lerin terapötik potansiyeli değerlendirildi. Görünür bölgede ve NIR'da güçlü absorbans ve güçlü ışınma özelliği olan Ag-kalkojenit QD'ler bu tez kapsamında fototermal terapi (PTT) ajanları olarak değerlendirildi. Ag_2S -GSH, başka herhangi bir terapötik ajan kullanmadan kendi başına büyük bir terapötik potansiyel sergileyen görüntü kılavuzlu PTT sağlayan güçlü bir PTT ajanı olarak bulunurken, AgInS_2 -GSH bu açılardan zayıftı.

Ag-kalkojenitlerin PTT potansiyeli, kanser hücrelerinin aşırı eksprese ettiği folat reseptörünün hedeflenmesi amaçlanarak, partiküllerin folik asit ve metotreksat (MTX) ile dekore edilmesi yoluyla görüntü kılavuzluğunda hedefli kombinasyon terapisi (PTT/kemoterapi) ile değerlendirildi. Folat reseptörlerinin hedeflemesi ile QD'lerin hücre içine alımı üç katına çıkarıldı ve PTT/MTX kombinasyon terapisi, 808 nm 'de 10 dakika süreyle tek bir lazer uyarımı ile (700 mW , 1.82 W/cm^2) $1.25 \mu\text{g}$ MTX/mL: $25 \mu\text{g}$ Ag/mL Ag_2S -GSH kullanılarak FR (+) HeLa hücrelerinin canlılığını sadece 4 saatlik inkübasyondan sonra yaklaşık %80 azalttı. AgInS_2 QD'ler görüntüleme ve ilaç taşınmasında etkiliydi, ancak PTT katkısından yoksundu.

Bu tezin literatüre son katkısı, anyonik 2-merkaptopropiyonik asit (2-MPA) ve katyonik polietilenimin (PEI) ile kaplı kararlı ve güçlü ışıyan anyonik ve katyonik AgInS_2 QD'lerin sulu bir çözeltide, tek aşamalı, katkı maddesi veya inorganik dış kaplama kullanılmadan sentezlenmesidir. Aynı çekirdeğe sahip ancak farklı bir kaplamayla sentezlenen bu QD'ler, ALA'yı tümör hücrelerine iletmek için en iyi yüzey kimyasını araştırmak için de kullanıldı. AgInS_2 QD'lere, HT29 ve SW480 hücrelerinin üzerinde PDT'yi geliştirmek

iin ALA elektrostatik olarak yklendi. Etkili hcre ldrme iin her iki partikln de gerekli ALA dozunu dřrmesine raėmen, znde daha iyi biyouyumluluėa sahip anyonik partikllerin daha iyi PDT verimi saėlayabileceėi bulundu.

Bu arařtırma alıřması, verimli optik grntlemeye giden yolu aabilecek NIR veya grnr blgeler iin son derece parlak, stabil ve biyouyumlu Ag tabanlı QD'lerin bařarılı bir řekilde sentezlenebildiėini kanıtladı. Ayrıca, bu tezde kemoteraptik ilalar ve ıřıėa duyarlılařtırıcı molekller yklenmiř, kanser hcrelerini hedefleyen anyonik ve katyonik QD'ler, kanserin kombinasyon terapisi iin kullanılabileceėi gsterildi.



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ABBREVIATIONS

2MPA	2-Mercaptopropionic Acid
5FU	5-Flourauracil
2MPA	2-Mercaptopropionic Acid
ALA	Aminolaevulinic Acid
AgNO ₃	Silver(I)nitride
Ag ₂ S	Silver(I)Sulfide
AS	Silver Sulfide
AIS	Silver Indium Sulfide
bPEI	Branched Polyethyleneimine
BSA	Bovine Serum Albumin
CB	Conduction Band
Cet	Cetuximab
CRC	Colorectal Cancer
Cys	Cysteamine
DAPI	4',6-diamidino-2-phenylindole
DIC	Differential Interference Contrast Average
Dh	Average Hydrodynamic Size
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic Ncid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DOS	Density of States
DOX	Doxorubicin
EBR	Electron Bohr Radius
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
EPR	Enhanced Permeability and Retention
E _g	Band gap Energy
E _u	Urbach Energy
EtOH	Ethanol

FA	Folic Acid
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate
FR	Folate Receptor
FTIR	Fourier Transform Infrared Spectra
FWHM	Full Width at Half Maximum
GSH	Glutathione
h	Planck's constant
HCT116	Human Colon Carcinoma
HeLa	Human Cervical Carcinoma
HSAB	Hard Soft Acid Base
IC ₅₀	Half-maximal Inhibitory Concentration
ICP-MS	Inductively Coupled Plasma-Mass Spectrometer
IR	InfraRed
ITC	Isothermal Titration Calorimetry
kDa	Kilo Dalton
LDS 798	4-[4-[4-(dimethylamino)phenyl]-1,3-butadienyl]-1-ethyl quinolinium perchlorate
LDH	Lactate Dehydrogenase
m _e	Effective mass of electron
m _h	Effective mass of hole
MTX	Methotrexate
MTT	Thiazolyl blue tetrazolium bromide
Mw	Molecular Weight
NaOH	Sodium Hydroxide
Na ₂ S	Sodium sulfide
NC	Nano-Crystal
NIH3T3	Mouse embryo fibroblast cell line
NIR	Near InfraRed
NIRQD	Near Infrared Emitting Quantum Dots
PBS	Phosphate Buffer Saline
PDA	Polydopamine
PDT	Photodynamic Therapy

PEG	Polyethylene Glycol
PEI	Polyethyleneimine
PL	Photo-Luminescence
PLQY	Photo-Luminescence Quantum Yield
PpIX	Protoporphyrin IX
PS	Photosensitizer
PTT	Photothermal Therapy
QD	Quantum Dots
QY	Quantum Yield
ROS	Reactive Oxygen Species
-SH	Thiol
SOD	Superoxide Dismutase
TAA	Thioacetamide
TEM	Transmission Electron Microscopy
UV	Ultra-Violet
VB	Valence Band
Vis	Visible
PEI	Root Mean Squared Error
VB	Valence Band
XPS	X-Ray Photoelectron Spectroscopy
XRD	X-Ray Diffraction

Chapter 1

INTRODUCTION

1.1 Quantum dots

In the last two decades, great attention has been directed on the new class of nanostructured semiconductors, quantum dots (QDs), for several size dependent unique features such as size dependent optical properties in the nanometer range [1], [2]. QD is a nanocrystalline semiconductor typically in the range of 2-10 nm in diameter with the limited number of electrons that result in discrete quantized energy levels.

The synthesis of PbS QDs was initially discovered more than 2000 years ago using PbO, Ca(OH)₂, and water [3]. In the early 20th century, semiconductor particles incorporated in silicate glasses were used in optics as filters. In 1981, Ekinov and Onushchenko were reported a blue shift in the optical spectrum of nanometer-sized CuCl in silicate glass [4]. A year later, in 1982, Efros and Efros declared the hypothesis of quantum size effects indicating the change in the colour of glass by either changing the size or stoichiometry of CdS_xSe_{1-x} [5]. Several experimental and theoretical research on these nanostructured particles has been elevated to explore many basic properties of QDs. The most widely exploited property is their size dependent fluorescence.

1.2 Quantum Confinement and Band gap

Materials can be divided into three well-known categories based on their conductivity: conductors (metals), semiconductors, and insulators according to the energy difference between their valance and conduction bands [3]. The highest electronic energy level entirely occupied by electrons at room temperature is called valence band (VB). Conduction band (CB) is the lowest electronic energy level above the VB that is not filled with electrons. When an electron in the VB gains energy, either by absorption of a photon or by voltage or by heat, electrons jump from VB to CB, resulting in a positively charged hole in the VB. The separation of these VB-CB is called band gap and the energy difference between them is called band gap energy which is expressed in electron volt

(eV). The type of materials is described based on this band gap energy.

As in Bohr model of an atom, electron orbits the hole at some average distance and holds them together by coulombic attraction. If one carrier approaches the other, they may form an electron-hole pair, i.e. an exciton, whose energy is a few meV less than the band gap. The distance between the electron and hole pair is termed as exciton Bohr radius (EBR), which is critical for confinement in semiconductors [3]. A strong confinement is obtained when the size of the QDs is smaller than EBR. Generally, nano-sized QDs have discrete energy levels that confine the motion of electrons and holes in all three directions. Moreover, the electronic structure of the confined QDs is intermediate between that of a single molecule and the bulk material. Consequently, the band gap energy of the QDs become size dependent. The relationship between the size and the band gap energy of the QDs was stated by Brus equation [6]:

$$\Delta E = \frac{\hbar^2 \pi^2}{8R^2} \left[\frac{1}{m_e} + \frac{1}{m_h} \right] - 1.8 \frac{e^2}{\epsilon_{Ag_2S} 4\pi \epsilon_0 R} \quad \text{Equation 1-1}$$

where $E_g(\text{Bulk})$ is the band gap energy of the semiconductor, $\hbar$ is the Planck's constant, m_e and m_h are the mass of the excited electron and hole, e is the charge of an electron, ϵ is the dielectric constant of the material and R is the radius of the QDs. This equation offers a convenient method to estimate the theoretical particle size from the band gap energy instead of particle size measured by TEM, SEM, XRD or other characterization tools.

Generally, band gap energy increases with decreasing size of QDs, which brings out the most valuable property of QDs: size tunable optical properties which made them invaluable and an enabler in many applications [7], [8]. The method of synthesis, QD composition, and precursors can be modified for size tunable optical properties of QDs emitting over a broad wavelength range from visible to IR.

1.3 Luminescence Properties

The main merit of QDs compared to the organic fluorophores is non-blinking PL and outstanding photostability of QDs over time [3], [9]. The non-blinking optical signal is essential for tracking while photostability enables long operational time. Absorption of a photon by QDs with sufficient energy, equal or bigger than its band gap energy, promotes an electron from VB to the CB, creating a hole in the valence band (Figure 1-1). The

electron and hole may form an exciton, as discussed above. Since electrons are not stable in excited energy states, they tend to drop back to VB. This is called “relaxation”. The excess energy between VB and CB may be radiative (emits photon) or nonradiative (vibrational relaxation, photochemical interaction). The “Stokes shift” is a phenomenon that the wavelength of the emitted photon is longer than the wavelength of absorbed light considering the energy lost due to vibrational relaxations and solvent interaction.

Some of the radiative emissions are briefly discussed below.

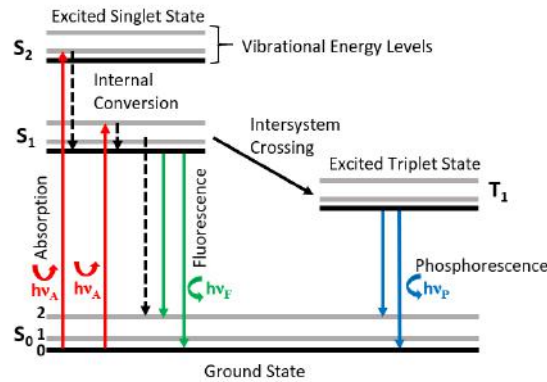


Figure 1–1: Jablonski diagram for absorption, fluorescence, and phosphorescence.

1.3.1 Radiative Relaxation

Radiative relaxations of QDs are due to band-edge or near band-edge transitions or defect quantum states. These emissions are summarized as follows:

Band-Edge Emission: Band edge and near band edge emissions are the most common radiative relaxation processes in semiconductors which indicates the recombination of an excited electron in CB with a hole in VB leading into near band edge emission with energies lower than the band gap [3].

Defect Emission: Localized impurity states in the band gap may lead to the radiative emissions as well. Defect states, which lie inside the band gap, can act as a donor (has excess electrons) or an acceptor (has a deficit of electrons) [3]. Electrons or holes are attracted to these donors and acceptors due to the Coulombic attraction. The defect states can be either shallow, where their energies are near the CB or VB-edge, or deep levels. Typically, the shallow defects exhibit radiative recombination while the deep levels are long-lived and hence, mostly experience nonradiative recombination.

1.4 Synthesis of QDs

Inorganic core of semiconductor QDs is responsible for the size tunable emission and the organic coating on the surface of QDs which may be called as the ligand as well, protect the core from the medium, control the crystal growth, passivate the growing crystal surface, functionalize the surface of QDs, and prevent its oxidation and agglomeration during/after synthesis [7], [10]. Large surface to volume ratio of the QDs despite the use of passivation methods may cause defect states on the surface of the QDs. The surface states act as trap states which generally increase the rate of nonradiative recombination that may degrade the optical and electrical properties of QDs. However, the trap states can also lead to radiative recombination and enhance the optical properties of the QDs.

The synthesis techniques are divided into two categories: top-down synthesis process and bottom-up approach [3]. Top-down approaches include molecular beam lithography, reactive ion etching and/or wet chemical etching to form the QDs with the diameter of ~ 30 nm from a bulk semiconductor. Controlled shapes and sizes of the QDs with the desired geometries are achievable. Incorporation of impurities and structural imperfections are the major drawbacks of these approaches.

In bottom-up process, different self-assembly methods have been used to prepare QDs following a chemical reduction, and they may be subdivided into wet-chemical and vapor-phase methods [3]. Wet chemical methods mainly follow the precipitation process, which involves both nucleation and limited growth of nanoparticles. Nucleation can be categorized as homogeneous, heterogeneous, or secondary nucleation. Size, shape, and composition of the QDs may be controlled by varying factors such as temperature, concentration and ratios of precursors, solvent, and the stabilizer. Some of the common synthesis processes like microemulsion, sol-gel, competitive reaction chemistry, hot-solution decomposition, microwaves, and electrochemistry are generally categorized as wet-chemistry. The most frequently used wet-chemistry approaches are the high-temperature synthesis in organic solvents and the aqueous synthesis of QDs at lower temperatures. Layers are grown in an atom-by-atom process to produce QDs in vapor-phase methods. Therefore, self-assembly of QDs arises on a substrate without any patterning. Self-assembly of nanostructures in material grown by molecular beam epitaxy (MBE), sputtering, liquid metal ion sources, or aggregation of gaseous monomers are generally categorized under these methods.

QDs prepared in organic solvents are usually hydrophobic since they were coated with

ligands consisting of a surface binding moiety and a long hydrocarbon tail to suspend them in the solvent and therefore unsuitable for biological applications. Photophysical and photochemical properties of QDs such as emission wavelength, quantum yield (QY), photostability, water dispersibility, oxidation stability, particle size, and surface functional groups play a pivotal role in QDs biological performance. Therefore, post-synthetic procedures must be employed to obtain an aqueous colloidal solution of these QDs. Ligand exchange is the most used strategy where the hydrophobic surface ligands are entirely replaced with hydrophilic ones. Another strategy is the encapsulation of QDs by amphiphilic molecules to make them water soluble. These methods, especially the ligand exchange, cause more surface defects and usually reduces the emission intensity, and some aggregation which both affects the luminescence and biodistribution of QDs [11].

QDs synthesized directly in water using appropriate capping agents such as thioglycolic acid (TGA), 3- mercaptopropionic acid (3MPA), 2-mercaptopropionic acid, l-cysteine, glutathione, meso-2,3-dimercaptosuccinic acid (DMSA), 1-thioglycerol (1-TGL), peptides, water-soluble polymers (PEG, polyethyleneimine (PEI), poly(acrylic acid), etc.) enable them to passivate the crystal surface and provide a colloidal solution in water. Since there is no loss of the luminescence or aggregation due to post-synthetic ligand exchange, the aqueous synthesis of QDs is more practical for QDs developed for biological applications. Besides, aqueous synthesis is performed at lower temperatures in a safe solvent which is water and usually from less toxic precursors than the high-temperature synthesis in organic solvents.

1.4.1 Nucleation and growth mechanisms of nanoparticles

Nucleation and growth mechanisms take place during the crystallization process to form nanoparticles. Atoms of reactant phase transform into the product phase in the form of cluster in nucleation step due to the supersaturation. Supersaturation ($\Delta\mu$) is the difference in chemical potential between the solution and bulk ($\Delta\mu = \mu_s - \mu_c$). If $\Delta\mu > 0$, the system is supersaturated, and nucleation/growth processes are favourable. When $\Delta\mu < 0$, the dissolution process is favourable. In the crystal growth step, atoms are incorporated onto the nuclei's surface and increase its size. Surface reaction and monomer's diffusion to the surface controls the growth mechanism. There are two widely used theories to explain the difference in the sizes of particles during growth, LaMer and Ostwald Ripening theories.

LaMer explains the nucleation and growth in three steps: (I) Rapid increase in the concentration of monomers in the solution, (II) a burst nucleation as a result of concentration increases that stops the nucleation after this point due to very low concentration of monomers, and (III) diffusion-controlled growth of nuclei. According to the LaMer diagram shown in Figure 1-2a, the nucleation and the growth steps should be separated in time to obtain crystals with monodisperse size distribution. Therefore, minimizing the nucleation time with a slow growth process provides many nuclei at the same size and separates nucleation and growth temporally.

Another accepted theory for growth, Ostwald Ripening, was defined in 1900 and is based on the differences in the solubility of particles. Generally, small particles with high surface to volume ratio have higher surface energy, which is not thermodynamically favourable, and tend to dissolve. Thus, larger particles grow at the expense of smaller particles to reduce the surface energy (Figure 1-2b). Diffusion is a predominant growth mechanism and is controlled by surface energy. The difference in the chemical potential at the solid/liquid interface for smaller particles induces a concentration gradient from smaller to larger particles. This phenomenon allows the growth of larger particles more efficiently than the smaller ones.

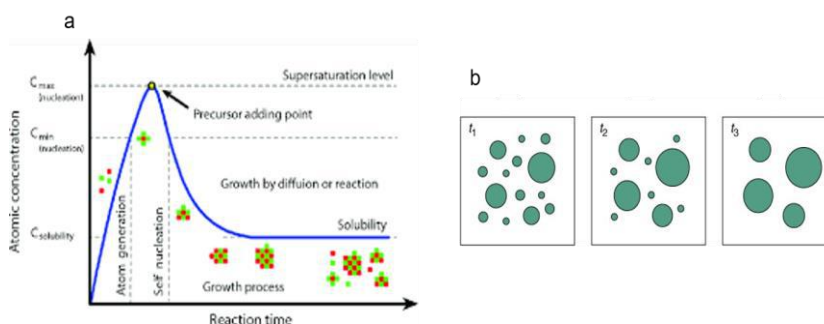


Figure 1–2: (a) Schematic representation of three stages during the nucleation and growth processes suggested by LaMer. Zone I: Increase in the concentration of the monomers in the solution, zone II: nucleation, zone III: growth. (b) Schematic illustration of Ostwald Ripening, showing an increase in the particle mean size without changing the volume fraction of the coercive phase in time ($t_1 < t_2 < t_3$).

1.5 Biomedical Applications of QDs

Unique properties of QDs were exploited in various practical applications such as photovoltaic systems (i.e. solar cells), light-emitting diodes, optical amplifiers, memory elements, photodetectors, lasers, biotechnology, and medicine. Semiconductor QDs can be utilized for fluorescent labelling of cells, specific organelles, or cellular molecules.

Most of the optical bioimaging are based on traditional organic fluorescence dyes which have several drawbacks associated with their instability and photobleaching, emission in the visible region where tissue has autofluorescence, sensitivity to the environmental conditions like pH, narrow excitation window which makes simultaneous excitation of multiple dyes difficult, and broad emission spectra with a long tail in red region which creates spectral cross talk between different detection channels and makes it difficult to quantitate the amount of different probes. On the other hand, large absorption coefficient, size-tunable light emission, high QYs, photostability and resistance to photobleaching, and broad excitation window with narrow emission peaks of QDs make them an excellent candidate for bioimaging and superior to organic fluorophores.

QDs with the emission from red to blue are commercially available, however, they have limited use. Ultraviolet (UV) and visible light can be absorbed by several organic molecules such as DNA, collagen, elastin, proteins, Nicotinamide adenine dinucleotide (NADH), and Flavin adenine dinucleotide (FAD) and be emitted by NADH, FAD, proteins, and DNA (Figure 1-3a, b) [2]. In addition to the absorption, tissue autofluorescence of collagen, NADPH, fatty acids, and porphyrin in the visible spectrum reduces the contrast and require high amounts for good signal-to-background intensity. Besides, QDs with emission in the visible spectrum require excitation at short wavelengths, even in UV, which cannot penetrate deep into the tissue. Lastly, excitation in the UV is less desirable due to potential UV-related damage to the healthy tissue. Hence, QDs with strong emission in the near-infrared region (NIR) are the most promising compositions for practical deep-tissue optical imaging. The region between 650-950 nm (NIR I) is accepted as the ‘diagnostic windows’, and 1000-1350 nm (NIR II) is highly promising as well, not only due to deeper penetration depth but also suppressed autofluorescence (Figure 1-4a).

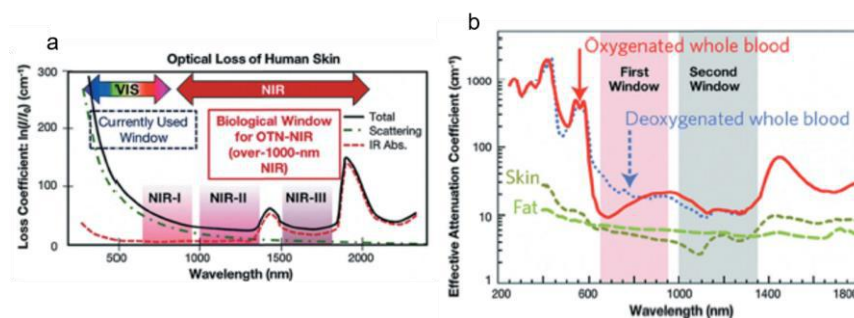


Figure 1–3: (a) Human skin absorption spectra in the of NIR-I, NIR-II, and NIR-III regions. (b) Absorption and scattering spectra from oxygenated blood, deoxygenated blood, skin, and fatty

tissue [2].

First examples to NIR emitting QDs were based on toxic Pb, Hg, and Cd-containing compositions. For example, CdTe/CdS core/shell QDs have been synthesized by Tao et al. to detect and monitor the changes of Cu^{2+} concentration with a low detection limit through *in vitro* and *in vivo* fluorescent imaging (Figure 1-5a) [12]. He et al. prepared protein conjugated $\text{Zn}_x\text{Hg}_{1-x}\text{Se}$ QDs for imaging of different cancer cells (Figure 1-5b) [13].

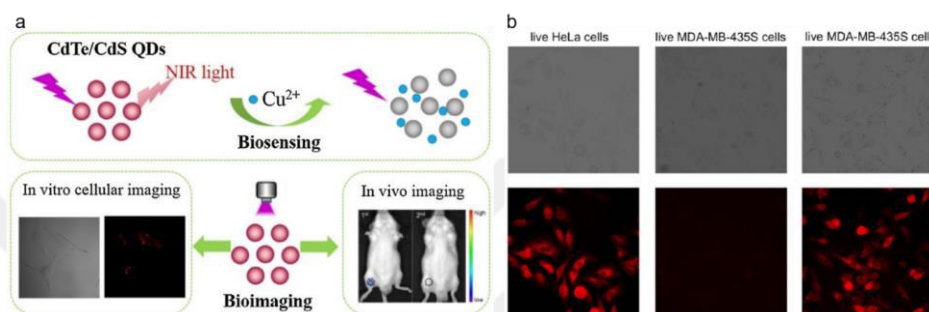


Figure 1–4: The near-infrared QDs based detection of Cu^{2+} and monitoring the change of Cu^{2+} concentration *in vitro* and *in vivo* through fluorescent imaging [12]. (b) Bright field and fluorescence images of cells incubated with $\text{Zn}_x\text{Hg}_{1-x}\text{Se}$ QDs [13].

However, leaching heavy metals cause significant toxicity, and therefore it is unlikely to see one of these QDs in the clinic. This created a new era in QD science, and a great effort was devoted to the development of heavy-metal free QDs over the past decades. Group III-V QDs (InAs, InP) are heavy-metal free QDs with the emission mostly in the range of 700-1400 nm. However, expensive and hazardous precursors, $(\text{TMS})_3\text{P}$ and $(\text{TMS})_3\text{As}$, limit the use of these QDs and make them quite expensive. Carbon dots are a new class of QDs with emission mostly in the visible spectrum but lack standardized synthetic methods for large-scale production and toxicity is still a big concern that needs further investigations. In addition, many of these QDs were synthesized in an organic solvent that requires additional step to suspend them in water which usually caused a reduction in optical properties of QDs and sometimes aggregation.

Ag_2S QDs with strong emission in the NIR are quite promising, especially due to ease of synthesis in water and high biocompatibility. NIH/3T3 mouse fibroblast cells were labelled with 2-mercaptopropionic acid (2MPA) coated Ag_2S QDs show a strong NIR optical signal and a great contrast with almost no background signal originating from the cells (Figure 1-3a) [14]. Figure 1-3b shows fluorescent staining of the cell cytoplasm with polyethyleneimine/L-cysteine (PEI/Cys) coated Ag_2S QDs again with a strong signal with

no background which is critical for diagnosis [15]. Additionally, high surface to volume ratio of QDs enables the conjugation of multiple therapeutic agents like chemotherapeutic drugs, targeting ligands, etc. to enhance the treatment efficacy and specificity. Figure 1-3c-i, ii shows enhanced accumulation and entrapment of Ag_2Se and Ag_2Se -PEG QDs tagged with an antibody (Cetuximab) in the orthotopic tongue tumour xenograft with epithelial growth factor receptor overexpression and in normal nude mice without tumour (Figure 1-3c-iii). For group i, ii, the fluorescent signal in tongue begun to appear after 7 h due to the presence of targeting agent (Cet) which provided tumour specific diagnosis [16]. On the other hand, the fluorescence signal at tongue in group iii (without tumour) decreased over time and disappeared after 13 h. Similarly, A549 and H1299 cells were labelled using Cetuximab conjugated Ag_2S -2MPA/PEI conjugates in Figure 1-3d [17]. The images imply excellent applicability and great potential of QDs in optical bioimaging and diagnosis.

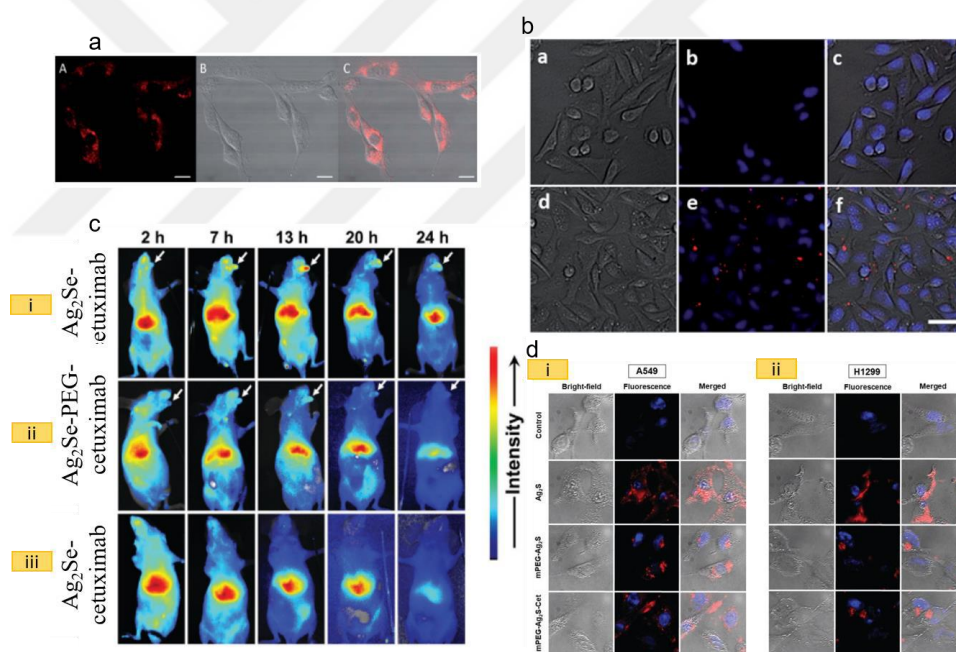


Figure 1-5: (a) Cellular uptake and localization of Ag_2S QDs by NIH/3T3 mouse fibroblast cells (200 mg/mL QDs, 24 h incubation) [15]. (b) Cellular localization of PEI/Cys coated Ag_2S NIRQDs by HeLa cells [14]. (c) Time course of *in vivo* NIR fluorescence images of (i), (ii) orthotopic tongue cancer-bearing and (iii) normal nude mice in the supine position [16]. (d) Fluorescence microscopy images of A549 (i) and H1299 (ii) cells incubated with Ag_2S QDs at 5 mg/mL Ag concentration for 24 h [17].

1.5.1 Toxicity of QDs

Talking about QD toxicity can be somewhat confusing since QDs vary in size and composition [2]. Generally, the toxicity of the QDs is attributed to several factors,

including individual QDs' physicochemical properties and environmental conditions. Mainly, the size, surface coating (hence the surface charge), the intracellular localization and concentration as well as the solubility of the QD leaching out ions are crucial factors affecting the toxicity. Lovric et al. studied the cytotoxicity of CdTe QDs with different sizes (2.3 and 5.7 nm) on PC12 and N9 cells at the same concentrations [18]. A higher toxicity was observed with the smaller QD in both cell lines which was attributed to its penetration into the nucleus while the larger ones were in the cytosol. The mechanisms of cell death were considered to be due to the presence of free Cd, free radical formation, or interaction of QDs with intracellular components leading to loss of their functions [18], [19]. And of course, once the QD has such intrinsic toxicity, it increases further with increasing dose [18], [19]. Similarly, Chen and co-workers exhibited that the toxicity of CdTe/CdS (core-shell) and CdTe/CdS/ZnS (core-shell-shell) QDs arise from the release of Cd^{2+} ions and intracellular distribution of the nanoparticles in cytoplasmic and perinuclear area [7], [20].

The mechanism of cell death highly depends on the distribution of the nanoparticles in different part of the cells and the activation of various pathways [21]. In a study of Yan et al., they have shown the localization of mercaptosuccinic acid (2-sulfanylbutanedioic acid)-capped QDs in mitochondria and activation of toxicity due to the release of cytochrome C through activation of mitochondrial death pathway (apoptosis intrinsic pathway) and induction of apoptosis [21], [22].

Coating QD crystals with different organic coatings does not only allow their suspension in aqueous media but may reduce their toxicity, as well. Indeed, the capping agent of QDs has a great impact on application of the QDs, their biodistribution, and toxicity [18], [19]. For example, the cytotoxicity of cationic Trolox (a water-soluble vitamin E) and N-acetylcysteine (NAC), and anionic bovine serum albumin (BSA) coated CdTe QDs PC12 and N9 cells was assessed under the same conditions [23]. Sub-cellular localization of BSA coated QDs in cytosol, particle's negative charge, and its ability for metal-binding sites reduced the release of Cd^{2+} and subsequently prevent cytotoxicity. Although both Trolox and NAC have positive charges, NAC coated QDs showed less toxicity. The reason could be attributed to the fact that NAC is a free radical scavenger that prevents the toxicity of CdTe QDs. Moreover, mercapto group of NAC could allow the absorption of it to the surface of QDs and improve the stability. Thus, Both NAC and BSA but not Trolox coated QDs significantly reduced the QD toxicity suggesting the effect of charge,

surface coating, and their biodistribution on CdTe QD cytotoxicity.

Another, very important aspect of QD affecting its toxicity is the stability, both *in vivo* and during storage. QD coatings might be labile, degrading and thus exposing potentially toxic capping materials or leaching out of the toxic ions such as Cd^{2+} [21]. Derfus et al. reported that if water solubilized tri-n-octylphosphine oxide (TOPO)-capped CdSe QDs with mercaptoacetic acid (MAA) coating were exposed to air for 30 min before MAA coating, the cell viability was decreased significantly from 98 to 21% at 62.5 $\mu\text{g/mL}$ QD concentration due to the oxidation of the surface of CdSe QDs [21], [24].

Overall, many factors are affecting the toxicity of the QDs, and hence each QD should be evaluated carefully and thoroughly. Besides, QD stability, half-lives, and long-term effects in the the environmental is not well-known yet but is a concern [18]. However, adverse effects of II-IV and IV-VI binary QDs such as Cd, Pb and Hg-chalcogenides changed the course of research towards non-toxic binary and ternary I-VI and I-III-VI QDs as alternatives that will be discussed further in details [2], [18].

1.6 Binary Ag-based QDs

Silver-chalcogenide QDs have narrow band gap and long wavelength emission. The band gap of Ag_2S , Ag_2Se , Ag_2Te are 0.9 eV, 0.15 eV, 0.67 eV and their luminescence in the NIR may be tuned by size and composition. In addition to great advantage of NIR luminescence, the ultralow solubility of Ag-chalcogenides (e.g., $K_{\text{sp}}(\text{Ag}_2\text{S})=6.3\times 10^{-50}$; $K_{\text{sp}}(\text{Ag}_2\text{Se})=2.0\times 10^{-64}$) prevents Ag^+ release and therefore renders them highly biocompatible compared to Cd-based QDs suffering greatly from Cd-based toxicity ($K_{\text{sp}}(\text{CdS})=8.0\times 10^{-27}$; $K_{\text{sp}}(\text{CdSe})=6.31\times 10^{-36}$). These two major features of Ag-chalcogenides make them excellent QDs for optical bioimaging and QD based medical applications.

Among Ag-chalcogenide QDs, monoclinic α - Ag_2S QDs have been developed more rapidly and have been utilized in various biological applications. Brelle et al. reported the first Ag_2S semiconductor colloidal nanoparticles coated with cysteine and glutathione (GSH) with the average diameter of 9 nm but with no detectable luminescence at room temperature [25]. In 1997, hydrophobic Ag_2S nanoparticles were synthesized by Pileni et al. with different length alkyl chain thiols having the uniform sizes of less than 10 nm [26]. Ag_2S QDs were reported for the first time in 2004 by Zhao and co-workers in a pure dodecanethiol solvent. However, no information on fluorescence properties of these

particles were mentioned [27], [28]. Finally, Wang et al. reported the first photoluminescent Ag₂S QDs emitting at 1058 nm in 2010 using single source of precursors [27], [28]. The synthesis of Ag₂S QDs have been developed afterwards, and different methods have been utilized over the past decades.

Although, nanoparticles synthesized in organic solvents showed good optical properties, crystallinity, and size distribution, their synthesis procedure require high temperature mostly and further transfer to aqueous media before biological applications. Therefore, aqueous synthesis of QDs have been extensively studied specially for bio-applications, in the last 10 years. Yang et al. reported the synthesis of water-soluble BSA coated Ag₂S QDs with tunable emission between 1294-1050 nm by tuning the size between 6.8 to 1.6 nm [29]. Then, endothelial growth factor (VEGF) antibody was conjugated to Ag₂S-BSA QDs for targeted cancer imaging.

Ag₂S QDs are also efficiently employed as therapeutic agents besides their diagnostic/imaging properties. Hu et al. developed Ag₂S-PEG loaded with anti-cancer drug doxorubicin (DOX) [30]. DOX was rapidly released from the Ag₂S cargoes into cancer cells due to the acidic microenvironment of tumour, giving rise to significant tumour inhibition. Tumour growth was reduced after injection of the Ag₂S-PEG-DOX QDs in mice bearing MDA-MB-231 breast tumour xenograft.

Synthesis of different Ag₂S QDs for various applications were developed in our group over the past years. Hocaoglu et al. synthesized highly luminescent 2-mercaptopropionic acid (2MPA) coated Ag₂S QDs in water with the emission in the range of 780-950 nm and with the quantum yields (QY) ~7-39% by change in reaction conditions such as pH, Ag/S ratio, and temperature [14]. The prepared QDs exhibited remarkable cytocompatibility even at high concentrations. In another study, utilization of an FDA approved heavy metal chelating drug, meso-2,3-dimercaptosuccinic acid (DMSA), as a coating of Ag₂S QDs exhibited high biocompatibility and strong fluorescence in HeLa cells indicating the potential of the QDs as theranostic nanoparticles [31].

To reduce the cytotoxicity and increase the selectivity of particles to the site of tumour, PEGylation and/or conjugation of targeting agents can be utilized. From this perspective, Asik et al. synthesized folic acid (FA) conjugated Ag₂S-PEG loaded with doxorubicin (DOX) to use them for optical imaging and targeted chemotherapy of cancer [32]. Change in the precursors' ratio, PEG molecular weight, and pH resulted in the QDs with the

emission from 775 to 930 and the QY between 2-65%. Attaching FA and loading with DOX improved the cell death to 50% in folate receptor (FR) positive HeLa cells at 15 nM (Figure 1-6b). Similarly, Duman et al. developed folic acid (FA)-conjugated cationic Ag₂S-2MPA/PEI QDs loaded with DOX. The increase in cell uptake by ~3-folds and reduction the IC₅₀ to 2.5 µg Ag/mL in FR positive HeLa cells compared to FR negative A549 cells proved the therapeutic effect of the resulting QDs (Figure 1-6 c-e) [33].

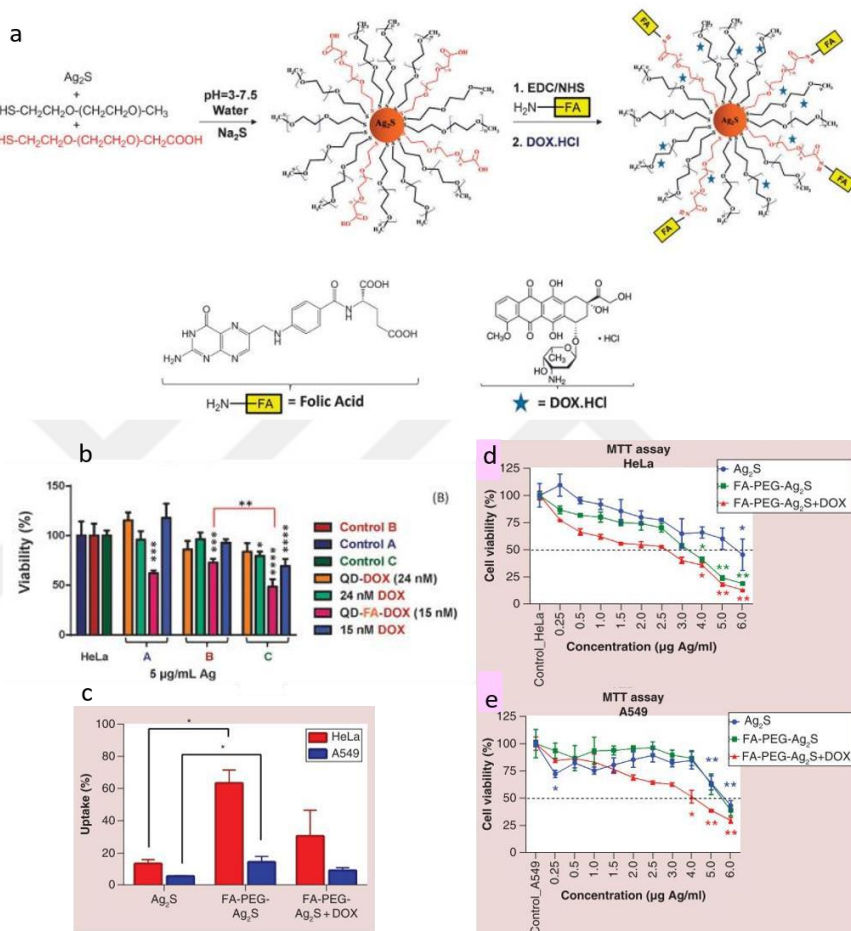


Figure 1-6: (a) Schematic representation of aqueous synthesis of Ag₂S-PEG-FA-DOX QDs [32]. (b) *In vitro* viability of HeLa cells with QDs at 5 mg Ag/mL and free DOX (15 and 24 nM) after 24 h. Incubation of nanoparticles and DOX was performed (A) in complete DMEM, (B) in folic acid free medium with 2 mM folic acid and (C) in folic acid free medium [32]. (c) Cellular uptake of Ag₂S-2MPA/PEI QDs with/without FA and DOX at 2 µg Ag/mL after 1 h [33]. Cell viability of (d) HeLa and (e) A549 cells treated with Ag₂S-2MPA/PEI QDs after 48 h [33].

Buz et al. reported the synthesis of water-soluble N-acetyl-L-cysteine-coated Ag₂S (NAC-Ag₂S) with strong emission in NIR (748-840 nm) as theranostic nanoparticles. The resulting QDs showed slight cytotoxicity to breast and cervical cancer cells but not to lung derived cells despite the higher uptake [34]. Since NAC is a free radical scavenger, reactive oxygen species (ROS) decreased in cells treated with the QDs, but the cell death still increased due to the DNA damage and apoptosis (Figure 1-7). They demonstrated

the strong *in vitro* signal in NIR with selective therapeutic effect.

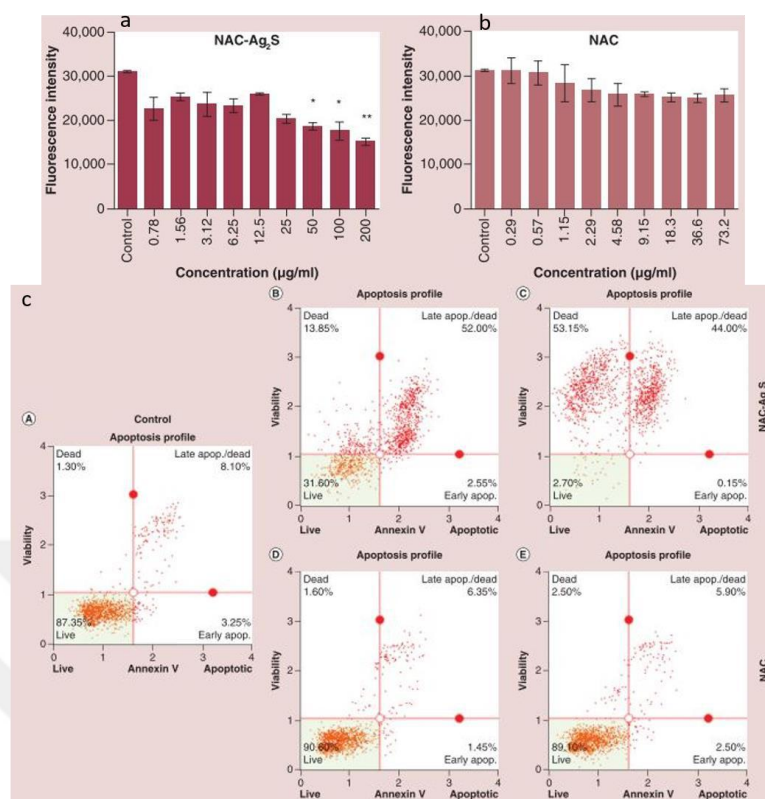


Figure 1-7: Reactive oxygen species downregulation by N-acetyl-L-cysteine-coated Ag₂S quantum dots and equivalent free N-acetyl-L-cysteine in MCF-7 cells measured by 2',7'-dichlorofluorescein diacetate assay 48 h after. (c) Determination of apoptosis in MCF-7 cells treated with NAC-Ag₂S quantum dots or free NAC for 48 h. Assessment [34].

1.7 Ternary Ag-based QDs

Cd-chalcogenide containing semiconductors were the first colloidal nanoparticles synthesized with controllable size about 30 years ago [35]. Understanding of the quantum-confinement phenomena and subsequently the size-dependent optical and electronic properties of the nanoparticles were the major issues at the beginning. Since then, different complex of binary semiconductors such as group II-VI, IV-VI, III-V, I-VI, and II-V and then ternary and quaternary semiconductors consisting of three or four elements have been developed to tune properties [36], [37], [38]. Multicomponent nanocrystals with both stoichiometric and non-stoichiometric compositions result in composition-dependent structural, optical, and electronic properties [39]. Although most of the literature is based on stoichiometric I-III-VI nanomaterials such as CuInS₂, CuInSe₂, AgInS₂, etc., non-stoichiometric QDs, such as In-rich ones, may sometimes show stronger emission than stoichiometric compositions, but the reason is not yet well-known [35].

The structure of I-III-VI group semiconductors (CuInS₂, AgInS₂, etc.) derives from the

zinc blend structure of the II-VI binary semiconductors such as ZnS or CdTe [38], [40]. In both II-VI and I-III-VI groups, the anionic sites are the same, however the cationic sites are occupied by group II metals in binary materials while in ternary ones the cationic sites are occupied by both group I metals (e.g., Ag or Cu) and group III metals (e.g., In, Al, Tl, or Ga) [35], [37]. As an example, when Ag:In ratio in Ag-In-S complex is lower than 1 it is called “In-rich”, and when the ratio is higher than 1 it is “Ag-rich”, and when the ratio is 1, it is referred as “stoichiometric” composition. Figure 1-8 shows the various atomic defects in the structure of non-stoichiometric Cu-In-S composition. Cu vacancies are formed in the case of “In-rich” while the formation of antisite and interstitial Cu defects are realized in “Cu-rich” composition.

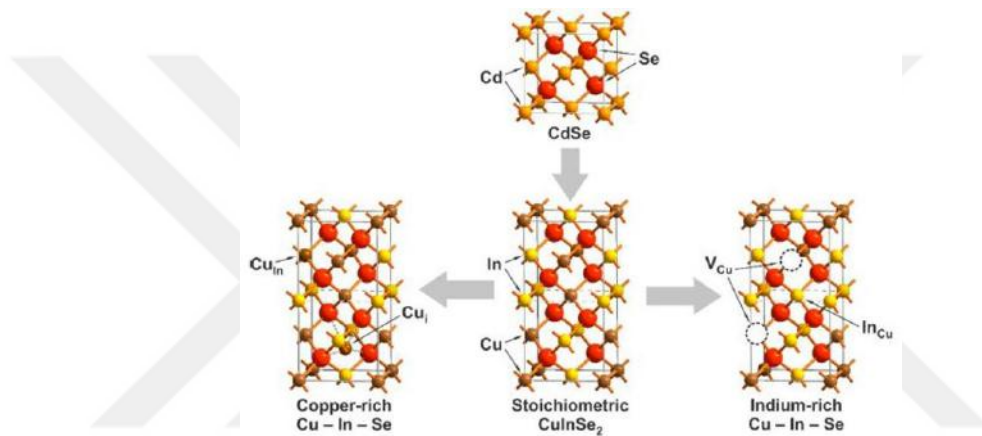


Figure 1–8: Structural relation between CdSe and Cu-In-Se semiconductors (V_{Cu} denotes Copper vacancy; Cu_{In} and In_{Cu} , copper and indium antisite defects; Cu_i , copper interstitial defect).

In the case of ternary I-III-VI nanocrystals, besides the differences in the nature of the atomic composition and size, the stoichiometric differences also change the bandgap and provide defect-related emission and hence opportunity to tune the optical properties (Figure 1-9a) [35]. For instance, In-rich compositions of Ag-In-S show a wider band gap, larger Stokes shift, and long-lived charge carriers and blue shifted emission than stoichiometric ones (e.g., $AgIn_5S_8$ vs $AgInS_2$). Figure 1-9b also shows composition dependent photoluminescence quantum yield (QY) of Ag-In-S QDs, suggesting Ag:In ratio of 0.2 as the best for the highest QY (about 20%) [35]. In the case of higher ratios, high concentration of Ag vacancies creates defect states (e.g., antisite and interstitial Ag atoms) within the band gap, responsible from non-radiative relaxations which reduced the QY. Therefore, while designing complex compositions with specific optical properties, stoichiometry must be taken into account.

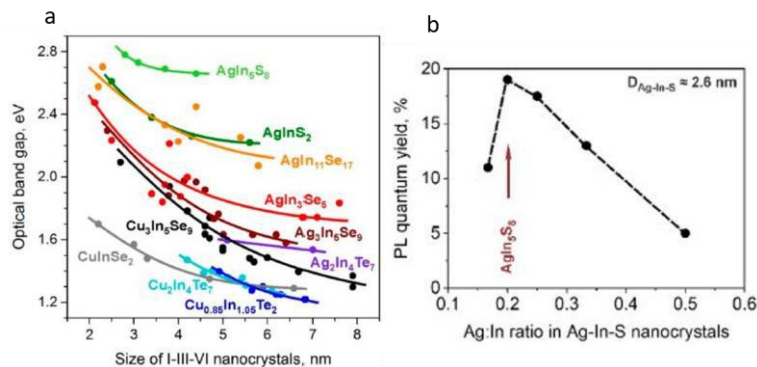


Figure 1–9: (a) Size dependences of optical band gaps for selected compositions of I–III–VI nanocrystals. (b) Composition dependences of photoluminescence efficiency for various I–III–VI nanocrystals [35].

1.7.1 Hard and soft acid bases (HSAB) concept

In the synthesis of complex compositions such as ternary QDs, the reactivity of each species should be considered carefully. Similarly, the same is valid in the synthesis of all stable QDs, HSAB theory was introduced by R. Pearson in 1963 describing the reactions in inorganic and coordination chemistry and the stability of compounds [35]. Reactants are classified in two major groups: acids (acceptor electrons) and bases (donor electrons) in the Lewis sense [40]. Then, both acids and bases as well as ligands were classified as hard (high charge density, small ionic radius, low polarizability) and soft (low charge density, large ionic radius, high polarizability). The HSAB theory states that the faster reactions and more stable compounds are formed from the interaction of hard-hard or soft-soft acid-base than hard-soft mixtures (Figure 1-10) [35], [38]. Selection of the hard-soft mixtures results in faster decomposition of the compounds and/or at lower temperature than hard-hard or soft-soft mixtures. This concept is adoptable to colloidal synthesis of nanocrystals wherein metal cations are the acids, and anions (e.g., chalcogens) and coordination ligands are bases. The softness of the chalcogens increases proportionally with ionic radius ($\text{S}^{2-} < \text{Se}^{2-} < \text{Te}^{2-}$) (Figure 1-10) [35], [40]. In that case, for example, quite stable Ag_2S QDs coated with thiolated ligands can be achieved using soft acid (Ag^+), soft base (S^{2-}) and soft ligand (HS-R). This becomes quite important in the synthesis of ternary compositions such as Ag-In-Se where Ag^+ and Se^{2-} were soft acid and base, but In^{3+} was a hard acid, making the incorporation of In difficult.

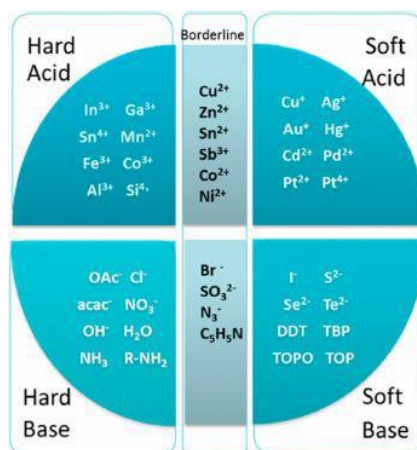


Figure 1–10: Examples of hard and soft acids and bases, used in colloidal synthesis of nanocrystals [35].

1.8 Photodynamic Therapy

Cancer, one of the major causes of disease-related deaths, is a great threat to human lives and leads to a large number of total deaths each year (almost 17% in 2018) [41], [42]. However, traditional therapeutic techniques to fight cancer, such as surgery, chemotherapy, radiotherapy, and more recently immunotherapy are usually associated with broad collateral damage and side effects that could decrease the efficiency of the treatment [43]. For instance, radiotherapy and chemotherapy are usually needed after surgery to completely discard the tumour cells [44]. Chemotherapeutic drugs usually cause severe damage to the healthy cells since it lacks tumour specificity, sometimes at a level that requires termination of the therapy [45]. Radiotherapy, on the other hands, is more local but needs should be well focused to the tumour to minimize the damage to the normal cells on the way and may result in damage to healthy tissue and radiodermatitis during or after the treatment. Consequently, the development of advanced and selective therapeutic strategies for cancer to improve the therapeutic outcome with minor or no damage to healthy tissues has been a hot topic in the last decades [44].

Photodynamic therapy (PDT) is a compelling therapeutic method for the treatment of cancers due to its minimal invasiveness, high selectivity, low side effect, no cumulative toxicity and no significant development of resistance [43]. PDT involves the accumulation of a photosensitizer (PS) in the tumour tissue and its activation with light irradiation at a specific wavelength in the presence of molecular oxygen resulting in phototoxicity. [46], [45]. PS itself is minimally toxic, the excitation wavelength is usually UV or visible but highly focused, therefore PDT offers a great locality with minimal off-

site toxicity.

PDT involves the photo-activation of a PS which has two electrons with the opposite spins (singlet state) [47]. Photoirradiation activates the PS by exciting the electron into the high-energy orbital (excited singlet state). This is an unstable and short-lived specie that loses its energy via radiative or non-radiative decay. The excited singlet oxygen may undergo an intersystem crossing whereby the spin of the electron inverts to form a long-lived excited triplet state [46], [45]. The excited triplet state can undergo two types of reactions: In reaction type I, it reacts with the cell membrane, molecule, or a substrate to create a radical ion (Figure 1-11). Alternatively, in type II, the excited triplet state can form excited state-singlet oxygen by directly transferring its energy to molecular oxygen [48], [47]. PS may undergo either type I or type II reactions or both depending on the type of PS, the concentration of substrates, and oxygen [46].

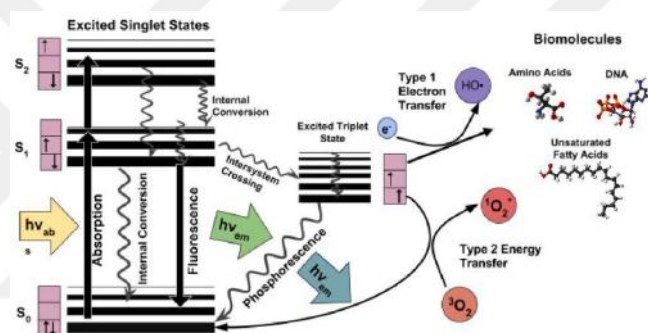


Figure 1–11: Modified Jablonski diagram showing the principle of PDT [49].

Electron transfer from the triplet state to molecular oxygen in type I path produces superoxide anion. This superoxide itself is not fatal in the biological system, but it can react to produce hydrogen peroxide and oxygen that can be catalysed by the enzyme superoxide dismutase (SOD) [46]. Superoxide has a pivotal role in the production of the highly reactive hydroxyl radical ($\text{HO}^\bullet$). In this process, superoxide donates one electron to the metal ions (like Fe^{3+}) that act as the catalyst to convert hydrogen peroxide (H_2O_2) into the hydroxyl radical ($\text{HO}^\bullet$). This reduced metal (Fe^{2+}) then catalyses the breaking of the unsaturated double bonds, including proteins, unsaturated fatty acids, and cholesterol [47], [50]. The peroxy radical is highly reactive and can react with another organic substrate and causes much more damage in a chain reaction. Highly reactive singlet oxygen diffuses over short distances i.e., $\sim 0.02\text{--}1.00\ \mu\text{m}$ with the half-life in the order of $<40\text{--}4000\ \text{ns}$ [46], [45].

These reactive oxygen species (ROS) formed by photoactivation of the PS rapidly react

with the biological molecules and substances, damaging the mitochondria, lysosomes, endoplasmic reticulum, and ultimately triggering necrosis, apoptosis, local ischemia (due to occlusion of neoplastic vessels) as well as subsequent immunological reactions [51]. Therefore, PDT creates high local phototoxicity at the local tumour site and minimizes the risk of damage to the normal tissues.

The “photodynamic effect” was first described in 1904 when Von Tappeiner and Jodblauer started to explain the oxygen-consuming chemical reactions/fluorescence in protozoa after the administration of aniline dyes [52], [53]. The following year, Von Tappeiner and Jesionek utilized 5% eosin as a topically applied PS to treat skin cancer, condylomata lata, and lupus vulgaris [52], [54]. In 1948, hematoporphyrin was found to be concentrated selectively in neoplastic, embryonic, and traumatized tissues by Figge and co-workers and then became a standard PS for PDT [52]. Dougherty et al. used photoactivation by red light for the treatment of skin cancer and other malignancies in 1978 [54]. Since then, PDT was utilized for a variety of non-oncologic conditions, including atherosclerosis, infectious diseases, and rheumatoid arthritis and cancers such as lung, colon, skin, pleura, genitourinary tract, brain, and eye [54], [55], [56].

Effective PDT treatment requires the sufficient penetration of the light into the diseased tissue and accumulation of sufficient amounts of PS at the tumour site [57]. When light penetrates through tissue, the absorption, refraction, transmission, and reflection may occur [58]. Regarding the fact that optical properties of tissues are hardly modifiable, selection of light with an appropriate wavelength for biological application should be considered, but this would also be limited with the absorption characteristic of the PS [44]. Absorption of the light by the PS should be enough to generate the triplet state as well as transfer this energy to a molecular oxygen to form singlet oxygen state. These are important factors that must be taken into consideration for effective PDT [46].

Lasers, light-emitting diodes (LEDs), and lamps are the main types of light sources utilized for PDT applications and a tabulated summary of advantages and disadvantages of each light source is provided in Table 1-1 [57]. The choice of light source depends on the location of tumour, type of PS, and required light dose. Generally, optical fiber-based devices due to the precise delivery of light to the target tissues are more favourable [57]. In general, lamps and LEDs are more widely used light sources due to low cost. At short wavelength end, the absorption of light by tissue chromophores together with the water

absorption at wavelengths greater than 1300 nm with lower penetration of light into the tissue has led to the concept of “optical window” [46]. Among all lights with different wavelengths, 600-1300 nm is called “optical window” with an optimal range between 600-900 nm [46], [57].

Table 1-1: Advantages and disadvantages of different light source [45], [57].

Light source	Advantages	Disadvantages
LED	Cost effective, small instrument, directly used for endoscopic/interstitial application	Low power, large beam divergence, broad spectral width, thermal effects during/after using
Laser	High power, monochromatic light	Expensive, large instrument
Lamp	Cost effective, simple design, broad field irradiation	Large beam divergence, broad spectrum, needs optical filtering, may lead coupling loss with light guides

Type of PSs has a great impact on the degree and type of damage incurred upon PDT. Effective PS should have low levels of dark toxicity, high purity, long shelf-life, water solubility or solubility in a harmless aqueous solvent mixture, preferential uptake by the desired tissue or tumour, proper administration method (such as topical or oral), rapid clearance from the body but suitable circulation time to achieve high dose at the site that will be irradiated, and absorption of light preferentially in the range of red and beyond for tissue penetration [45], [48]. Excitation at short wavelengths prevent light penetration, and may cause skin photosensitivity while excitation at long wavelengths may not provide sufficient energy for triplet state to transfer energy to the ground state oxygen to excite it [57], [46].

PSs can be categorized into three groups. First generation PS molecules are naturally occurring porphyrins, including hematoporphyrin derivatives (HPD) that were developed in the 1970s and early 1980s [57]. They have a strong absorption at about 400 nm but limited absorption in the red spectral region where the light penetration in tissue is better [45], [48]. In addition, their synthesis procedure is quite complex, and it is very difficult to purify them. Their molar extinction coefficient is low, therefore higher doses are needed for effective PDT. To overcome the deficiencies of the first generation PSs, the second generation PSs which can be activated at wavelengths >600 nm have been developed since late 1980s [45], [48]. Their major drawback is the lack of specificity, therefore the third generation PSs were developed by the modification of second generation PSs for improved accumulation of the PSs at the tumour site [48], [59]. Cheng et al. used PEGylated gold nanoparticles (GNPs) loaded with phthalocyanine 4 (Pc4) as

a PDT agent to improve efficient drug release *in vitro* [60]. Faster diffusion of NPs and drug release due to the presence of Pc4 caused the accumulation of nanoparticles into the cytoplasm of the tumour cells. Chang group reported the enhancement in internalization of the GNPs and PDT effect by conjugating epidermal growth factor peptide-targeted (third generation PSs). A summary of most widely used/studied PSs is provided in Table 1-2.

Development of small, stable, and trackable nanoparticles created a potential for improved PDT by delivering the PS with nanoparticles to the tumour site more effectively due to EPR effect and via custom-made nanoparticles via receptor targeting. They are capable of delivering targeted PS to the site of tumour and lastly, nanomaterials higher surface area allows loading sufficient amount of drug and PSs, which enhance cell uptake and PDT efficiency [61], [62], [63].

Table 1-2: Properties of typical PSs.

PS	PS generation	λ_{abs} (nm)	λ_{exc} (nm)	Irradiation (mW/cm ²)	Dose	site	ref
Photofrin	1 st	652, 514	630	50, 70	5 mg/mL body weight	Shoulder and flank	[64]
HPD	1 st	630	630	25-100	5-20 µg/mL	Lung	[65]
Talaporfin sodium	2 nd	664	664	100 J/cm ²	40 mg/m ²	Bile duct	[66]
5-aminolevulinic acid	2 nd	265, 520	635	75	500, 1000 µM	Skin	[67]
m-THPC	2 nd	650	650	10	1 mg/mL body weight	Bile duct	[68]
Hypericin	2 nd	610	610	2.33	0.1-1.1 µM	Breast	[69]
Chlorin e6-cetuximab loaded micelle	3 rd	405, 650	650-670	19.5	0.42-0.173 µM	Skin, colon	[70]
bovine serum albumin and Chlorin e6 functionalized CuFeS₂	3 rd	671	671	1000	1 mg/mL	Breast	[71]

QDs with the optical imaging ability are attractive nanocarriers for PSs, which also serves for image-guided PDT (Table 1-3) [72], [73]. The organic coating of QDs makes it possible to functionalize the surface with PSs and tumour targeting molecules as well as

drugs for multimodal therapy [73].

There are few cases, where QDs acted as a PS. Cheng et al, reported that polydopamine (PDA) conjugated PEGylated Ag₂S QDs do generate ¹O₂ under 808 nm laser irradiation [74]. These Ag₂S QDs showed strong optical signal in HeLa cells for the imaging, with negligible dark toxicity.

Ag₂S QDs can be utilized as either the photosensitizer (PS) or the cargo for conjugation of PSs in PDT. In the study of Cheng and co-workers, they have utilized the capability of Ag₂S QDs in killing tumour cells in the photodynamic way using NIR light irradiation [74]. They have shown that the relative excitation of probe is less than traditional PS in this case and with the help of polydopamine (PDA), the induced ROS increased by 1.69 times. Concurrently, PDA itself was oxidized to quinone and was reacted with oxygen molecules resulted in the formation of active superoxide ions, promoting the PDT effect. As a consequence, phototoxicity of Hela, MCF7, and 4T1 cells treated with QD-PDA at 808 nm laser was dropped to 50%.

Taken together, QDs can be employed as multifunctional vehicles combining diagnostic and therapeutic applications in a single entity (Table 1-3).

Table 1-3: QDs in PDT applications.

Materials	Size (nm)	Dose	Irradiation (mW/cm ²)	Time (min)	Wavelength (nm)	Cell type	Ref
CDs	2.5	50, 100 µg/mL	100, 2000	10	635, 800	HeLa	[75]
RNA modified CDs	3.6	0-7.5 µg/mL	10	5	550	MCF7, HeLa, 4T1, HepG2	[76]
CdSe/ZnS QD-Aluminium disulfonated phthalocyanine	-	50 nM	-	-	532	MCF7	[77]
GQDs, GOQDs	1.5-5.5	0.23, 0.46 µM	1.03	1	365	MCF7, B16F10	[78]
N-GQDs-APTES-DOX	4	0-10 µg/mL	6.8	20	622	MDA-MB-231	[79]
GQDs-PpIX-aptamer	8	100 µg/mL	160	10	635	A549, MCF7	[63]
CuInS ₂ /ZnS) QDs-ALA	-	1-2 mM	100	2	800, 1300	MCF7	[80]
ZnSe/ZnS-Chlorin e6	4.3	0.25-0.5 µM	40	10	405	EAC	[81]
Ag ₂ S-PDA	20	0-400	1000	10	808	HeLa,	[74]

	μg/mL					MCF7, 4T1	
UCNPs- graphitic carbon nitride QDs	27	3-250 μg/mL	1500	30	808	CAL27, OEC- M1	[82]

1.9 ALA-mediated PDT

Usually, active PSs are administered to patients. However, their photosensitivity and stability cause some limitations [83], [84]. Use of a prodrug, which is converted to active PS is an alternative approach. The only clinically approved prodrug for PDT is 5-aminolevulinic acid (ALA), a precursor of the natural photosensitizer PpIX [84], [85]. ALA is administrated topically or systematically and penetrates non-selectively into the cells. Then, mitochondrial ALA-synthetase catalysed the production of ALA from glycine and succinyl CoA in the heme biosynthesis pathway (Figure 1-12) [86]. The last step of the heme pathway involves the incorporation of Fe^{2+} into PpIX to form heme, which is the rate-limiting step of the pathway and catalysed by ferrochelatase [87]. Exogenous ALA administration would bypass the feedback inhibition of ALA synthesis in the heme biosynthetic pathway resulting in the higher accumulation of PpIX in the tumour cells and higher efficiency of tumour targeted PDT [86]. Although nearly all cell types express the enzymes involved in the heme pathway, a distinct activity of the enzymes in tumour cells compared to normal cells results in higher PpIX accumulation [87].

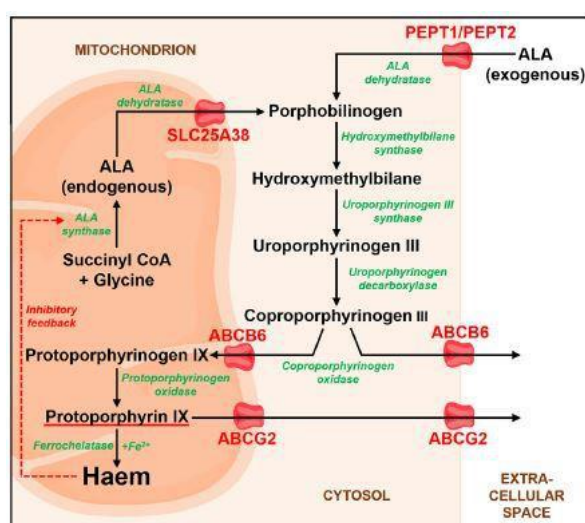


Figure 1–12: The haem biosynthesis pathway. Endogenous ALA is synthesised in the mitochondria and acts as an early substrate for the haem biosynthesis pathway. The penultimate molecule PpIX displays bright red fluorescence when excited by blue/violet light. Exogenous

ALA preferentially accumulates in neoplastic cells leading to an accumulation of PpIX that can be exploited for biophotonic diagnostics [88].

ALA-mediated PDT, the elucidation of the mechanism of ALA-PDT, and the improvement of its therapeutic activities have been investigated over the past decades [86], [89]. ALA clears from the body quickly, hence is quite advantages compared to other PSs. But, the polarity and zwitterionic nature of ALA in physiological pH, which accounts for its low lipid solubility, limit its bioavailability at the same time [86]. In response, modification of ALA to improve its cellular permeability, stability in physiological pH, and selectivity are important challenges to extend the clinical use of ALA-PDT. Two most successful ALA derivatives are methyl ester (methyl aminolevulinate, MAL) and hexyl ester (hexyl aminolevulinate, HAL) [89]. Attaching a carbon chain to ALA molecules results in increased lipophilicity and subsequently, higher membrane permeability. Nowadays, ALA mediated PDT is an FDA approved method for the treatment of several malignant conditions such as actinic keratosis, Bowen's disease, bladder cancer, etc [86], [90]. Osiecka et al. showed that 60-75% of the patients using Levulan[®]-PDT (ALA-PDT) totally cured basal-cell carcinoma [91]. In another study, Jeffes utilized 20% ALA with blue light illumination in patients with actinic keratosis [92]. Complete clearance of actinic keratosis was seen in 88% of the cases using ALA-PDT (10 J/cm²). Addition of dimethyl sulfoxide (DMSO) and ethylenediaminetetraacetic acid (EDTA) to ALA may improve its delivery to mice skin [93]. Conjugation of ALA to the nanoparticles is another solution suggested to improve the efficiency of ALA-PDT recently [94], [95], [96].

The organic coating of QDs makes it possible to functionalize the surface with PSs, drugs, or targeting molecules for multimodal therapy. In some cases, QDs can be used as a donor in fluorescence resonance energy transfer (FRET) to transfer the energy to an acceptor molecule (PS in this case), which allows excitation of the PS at a longer wavelength than its absorption peak [77], [97]. 5-Aminolaevulinic acid (ALA) conjugated CuInS₂/ZnS (CIS/ZnS) QDs as the multi-photon excitation PDT photosensitizer was prepared by Feng and coworkers at longer laser wavelength to improve the therapeutic effect due to the higher penetration depth and less damage to the organism (Figure 1-13a) [80]. It was concluded that the CIS/ZnS-ALA QDs killed MCF7 cells under low power NIR femtosecond laser (800 nm and 1300 nm) whereas, there was no significant PDT effect for free ALA due to the lack of absorption of ALA in this region (Figure1-13b). Once the

QDs are excited, absorbed energy by QDs were transferred to ALA, enables them to produce ROS and leads to cell death.

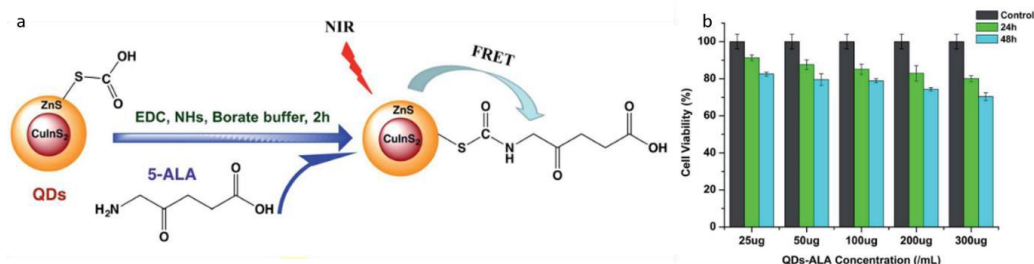


Figure 1–13: (a) Schematic of the covalent linkage of the CIS/ZnS QDs to ALA. (b) Cell viability of MCF7 cells treated with CIS/ZnS–ALA for 24 h and 48 h [80].

1.10 Photothermal Therapy

For many years, thermal therapy, which means increasing the tissue temperature above the normal level for a defined period of time, has been a technique used in different areas. Based on the magnitude of temperature increase and duration, thermal therapy can be categorized into three different groups: (i) diathermia, (ii) hyperthermia, and (iii) thermal ablation. The major issue to keep the temperature increase limited to the diseased area [98]. Therefore, photothermal therapy (PTT), wherein the temperature increase is only limited to the irradiated area, has received a great deal of attention. PTT is a light-based technique used mostly in acne treatment in the clinics. However, due to poor blood supply and hence, low heat tolerance of tumour compared to normal tissue, the technique is very promising to kill the cancer cells selectively. PTT employs light absorbing dyes to achieve photothermal damage. So, irradiation of tumours infiltrated with light absorbing agents, heats up to tumour [99]. Temperature increase known as hyperthermia results in irreversible cell damage as a result of loosening of cell membranes and denaturation of proteins. Irradiation at NIR wavelengths results in more penetration of the light both through blood and soft tissues and increases the success of the phototherapy. The crucial point is to achieve sufficiently high temperatures with stable, small, and biocompatible agents at low laser powers and short irradiation time, which is comfortable for the patient.

The interaction of light and tissues should be considered, as well. Light may alter biological activities, enzyme activities, or may result in catalysis [100] and may cause thermal damage to the healthy tissue [58], [101]. Hence, irradiation wavelength, laser power, and irradiation time are critical factors that should be chosen carefully [44]. NIR region is considered as the biological window for diagnosis and therapy of the deep-seated lesions, since it can pass through blood and soft tissues and increases the success of the

therapy [102], [103]. Nanoparticles, with sufficient absorbance in the NIR, are promising photosensitizers for near-IR photothermal effect which can be triggered at around 808 nm or 980 nm [44], [102].

Laser power is another critical factor. According to the American National Standard for the Safe Use of Lasers, utilization of NIR laser with power density $\sim 0.33 \text{ W cm}^{-2}$ for the 808 nm and $\sim 0.726 \text{ W cm}^{-2}$ for the 980 nm are safe [104], [101]. Exposure time and focal spot size also affect the interaction and based on the application; all the above-mentioned parameters need to be taken into account to assess the biosafety of the laser procedure.

Different nanoparticles such as gold, copper, and iron oxide nanoparticles, dyes, graphene, and carbon nanotubes, and QDs were evaluated for PTT, among these, Ag_2S QDs are very attractive due to NIR imaging opportunity, strong absorbance in NIR, and high biocompatibility [44], [105]. Table 1-4 summarizes some of the key studies on Ag_2S -based PTT. In a study conducted by Gao et al., they have shown high photothermal conversion capabilities of Ag_2S QDs for specific cancer imaging and PTT [106]. They conjugated the designed aptamer (Apt43) as template to the QDs that consists of an aptamer S2.2 sequence for specifically recognizing the cancer cells and an 18-cytosine (18-C) extending spacer for growing Ag_2S QDs. They could ablate the tumours on the nude mice utilizing 100 $\mu\text{g/mL}$ QDs upon irradiation at 808 nm at a power density of 1 W cm^{-2} for 10 min.

Yang et. al synthesized Ag_2S nanodots in hollow serum albumin nanocages with different diameters as multimodal imaging and photothermal therapy agent [107]. The temperature increase induced by Ag_2S with diameters of 4.1, 7.9, and 9.8 nm was 11.8, 19.5, and 32 $^{\circ}\text{C}$, respectively at 1 mM Ag concentration under 785 nm laser irradiation at 1.5 W cm^{-2} for 5 min (Figure 1-14a). Their light-to-heat conversion efficiency, which was between 33.7-35% for all diameters, proved that these nanodots are promising agents for PTT. During laser on/off experiments, the same temperature increase was observed after each cycle indicating photothermal stability of these nanodots, as well. The *in vitro* PTT potential of these particles were demonstrated on 4T1 murine breast tumour cells and a significant reduction in cell viability at 0.17 mM Ag after 3 min irradiation was reported (Figure 1-14b). In the *in vivo* experiments, 7.7 and 12.5 $^{\circ}\text{C}$ temperature increase in the tumour after 5 min irradiation was observed at 10 and 30 $\mu\text{mol/kg}$ Ag dose, which resulted in significant tumour ablation; however, reoccurrence of the tumour was observed after

8-14 days (Figure 1-14c). Increasing the nanoparticle dose to 50 $\mu\text{mol/kg}$ Ag caused 19 $^{\circ}\text{C}$ increase in the tumour temperature and total tumour ablation with no hepatic or renal toxicity and no tumour regrowth.

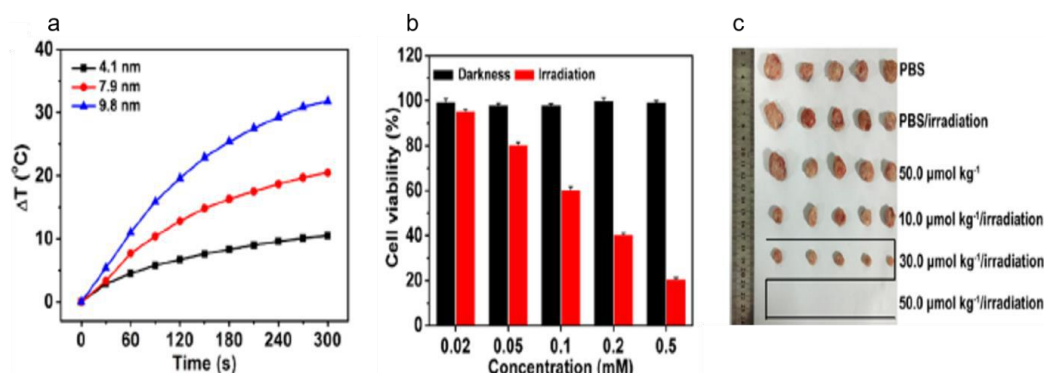


Figure 1-14: (a) The temperature elevation of nanodots with different sizes, (b) cell viability, and (c) tumour volume after PTT treatment based on different concentrations [107].

In another study, Zhao et. al prepared Ag_2S QD-polypeptide hybrid nanogel (Ag_2S QD@PC₁₀ARGD) for NIR imaging, photoacoustic imaging, and PTT [108]. The photothermal potential of Ag_2S QD@PC₁₀ARGD under 810 nm laser irradiation at 2.5 W/cm^2 with different concentrations (0.06-1 mg/mL Ag ions) for 8 min was shown in Figure 1-15a. For example, photothermal conversion efficiency of the particles at 1 mg/mL was 28.7%. In *in vivo*, the temperature increase was 60.7 $^{\circ}\text{C}$ after 2 min laser exposure causing tumour ablation as a result of necrosis (Figure 1-15b) without any abnormality in organs.

These initial studies point out the clinical potential of Ag_2S NIR emitting QDs as cancer theranostics combining PTT and diagnosis in a single structure due to efficient light to heat conversion as well as strong NIR emission (Table 1-4).

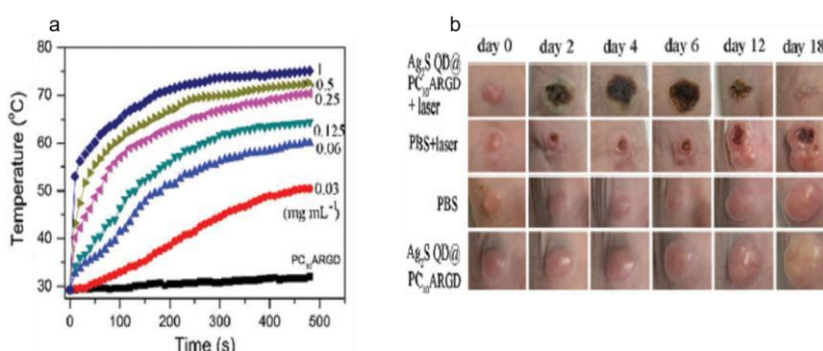


Figure 1-15: (a) The temperature elevation of Ag_2S QD@PC₁₀ARGD at different concentrations and (b) photographs of tumour-bearing mice [108].

Table 1-4: Ag-based nanoparticles in PTT applications.

Particles	Size	Dose	Irradiation	Time	Wavelength	T	Ref
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	(nm)		(W/cm ²)	(s)	(nm)	(°C)	
Apt-Ag₂S	2-2.5	100 µg/mL	1	720	808	29	[106]
Ag-Ag₂S-CdS	15.6	0.5 mg/mL	0.5 W	300	980	4.7	[109]
Ag₂S nanodots	4.1	1 mM [Ag]	1.5	300	785	11.8	[107]
	7.9	1 mM [Ag]	1.5	300	785	19.5	
	9.8	1 mM [Ag]	1.5	300	785	32	
Ag₂S QD@PC₁₀ARGD	4.5	1 mg/mL Ag	2.5	480	810	~ 45	[108]
Ag₂S@DP-FA	50 200	2.5 mg/mL	1.8	300	808	~ 45	[105]

1.11 Proposal and Aim of the Thesis

Cancer is one of the first three reasons for death worldwide, and its early detection is extremely critical for good prognosis and effective therapy of carcinomas. Most cancers are detected late, which makes its cure difficult. It would be ideal for detecting even a single tumour cell by imaging modalities, but not yet possible. More sensitive and tumour specific diagnostic tools are needed. For the treatment, a variety of treatment methods have been adopted: Surgery, chemotherapy and radiotherapy are more conventional and are mostly used in combination based on the aggressivity, locality and the type of cancer. Other relatively new but promising methods include antibody-drugs and immunotherapy. However, still there is an urgent need for improved treatment regimens.

Despite its wide use, chemotherapy suffers from drug resistance, the short lifetime of the chemotherapeutic drugs, lack of tumour selectivity causing low accumulation of drugs in tumour and significant off-site toxicity, therefore ending with an ineffective or incomplete treatment in many cases. Radiotherapy is a more local treatment but should be focused precisely on the tumour tissue to minimize the damage on the surrounding healthy tissue, which still exists. Surgery is invasive but beneficial, but some tumours are anatomically challenging to remove. Surgery is often followed with radiotherapy and chemotherapy for complete cure which is still not curative in case of aggressive cancers. The new immunotherapy is quite promising but also have severe side effects and at the moment shows some inconsistencies. Combining these strategies has been tested over the past decades to improve the prognosis and provide better treatment. Yet still, side effects,

metastasis, and reoccurrence are frequent. Therefore, the development of less or non-invasive alternative treatment modalities with high tumour selectivity and treatment efficacy with fewer side effects is an urgent need in this field. Non-invasive non-ionising light-activated therapies such as PDT or PTT has gained noticeable attention over the past decades due to high locality and fewer side effects. Yet, they need significant improvement for tumour selectivity and reduced side effects, as well.

PDT relies on photoactivation of a PS that generates ROS resulting in usually apoptotic cell death. PTT, on the other hand, initiates the photothermal effect by absorbing particular light irradiation and converting the energy to heat in the lesions, which causes apoptotic/necrotic cell death. Both modalities are local and minimally invasive to the surrounding tissues. Although they may be exploited as a single modality, their combination with chemotherapy can offer a synergistic outcome with minimizing the side effects of each modality. In addition, all three modalities, chemotherapy, PDT and PTT do need higher specificity of the agents, hence should be rendered tumour specific. In this regard, the use of nanoparticles with imaging modality and PTT potential to deliver a chemotherapeutic agent and/or a PS, offers a great advantage and multimodal approach to cancer imaging and therapy. Nanoparticles can provide tumour specificity in a couple of ways. Tagging nanoparticles with antibodies or small molecules with high affinity to the external domain of tumour-specific cell surface receptors and EPR effect accumulating nanoparticles at the tumour site result in high tumour selectivity. Besides, the exploitation of different binding chemistries for drug-nanoparticle conjugates may allow the protection of the drug in physiological pH and triggered release in the tumour, which may reduce side effects and enhance therapeutic outcome.

In selecting appropriate nanoparticles, heavy metal free Ag-based semiconductor QDs with narrow NIR emission, a broad absorption band including the NIR, large molar extinction coefficient, and chemical stability are promising optical imaging agents. Use of Ag-chalcogenides as drug delivery vehicles also provide the opportunity to track the biodistribution of therapeutic agents and possibly the progress of treatment. In case of the phototherapies, delivery of PS with Ag-chalcogenides does provide an opportunity for NIR-image guided localization of the irradiation light, hence image-guided PDT. Additionally, reports indicating PTT potential of Ag₂S QDs, suggests that Ag-chalcogenides may be perfect theranostic nanoparticles for optical imaging coupled with chemotherapy/phototherapy of tumours with high specificity.

Based on these needs in cancer diagnosis and treatments, the goal of this thesis is to develop strongly luminescent, stable, small Ag-chalcogenides in water with a relatively green synthetic approach and with different surface chemistries to provide strong optical contrast for imaging and effective treatment either via PDT, PTT or the combination of phototherapies with chemotherapy.

The first aim of this work is the targeted PDT and PDT/chemotherapy of colorectal cancer using Ag₂S theranostic QDs. For this, NIR emitting Ag₂S QDs with 2-mercaptopropionic acid coating, which was previously developed by Hocaoglu et al. [14] with long-term stability and high QY and no significant cytotoxicity even at high concentrations, were tagged with Cetuximab (Cet) for EGFR targeting is proposed. For targeted PDT, ALA was preferred due to mentioned advantages over other PSs. A study to identify the influence of ALA-QD conjugation chemistry on effective delivery of ALA, generation of PpIX, and the therapeutic outcome was also suggested using either electrostatically loaded ALA or hydrazone and amide linked ALA. For the multimodal treatment of EGFR overexpressing colorectal cancer cells with PDT/chemotherapy combination 5-fluorouracil (5FU) which is a first line chemotherapeutic drug was conjugated to ALA loaded QDs via ester linkages. In vitro evaluation of the idea was tested on colorectal cancer cell lines with different EGFR expression levels and in 2D and more realistic 3D cell cultures. The PDT effect tested both with blue light irradiation (420 nm) for 5 min and red light irradiation (640 nm laser) for 1 min. These wavelengths are suggested since the blue lamp coincides with the Soret band of PpIX but red light is preferred in the clinic.

The second aim is the synthesis of Ag₂S QDs coated with a biologically significant molecule, glutathione (GSH) with strong emission in the medical imaging window (700–900 nm) in a simple, one step and aqueous process, and to evaluate the cytocompatibility, diagnostic potential and PTT potential of these QDs. Aqueous synthesis wherein the reaction variables (temperature, time, pH and sulphur source (Na₂S and thioacetamide)) are changed systematically is suggested to achieve small, stable QDs with strong emission in the NIR. The cytocompatibility and intracellular optical signal generation were evaluated using different cancer cell lines. Light-to-heat conversion by these QDs was first investigated using a 795 nm, continuous-wave fiber coupled diode laser as a function of QD concentration, irradiation time, and input power of QDs in solution; then the PTT potential was demonstrated in vitro.

The third aim of this project is to adopt these GSH-Ag₂S QDs for NIR imaging coupled with targeted PTT/chemotherapy combination. While doing so, development of aqueous AgInS₂ QDs with strongly emission in the visible region, preferentially with a red emission, in a simple aqueous synthesis and investigation of these QDs for optical imaging, PTT and targeted chemotherapy combination, is also aimed. AgInS₂ QDs were less studied in medical applications and their potential in PTT is unknown. This also would provide a broad spectrum of Ag-based QDs for theranostic use, in a comparative study. So, synthesis of GSH coated AgInS₂ QDs without a core/shell structure or Zn doped which are shown as a must in the literature, is suggested, using an aqueous preparation wherein reaction variables are systematically changed. Both QDs were tagged with folic acid (FA) and directly conjugated with methotrexate (MTX) via an amide bond as a drug. Therapeutic effects of the FR-targeting, MTX-loaded QDs were assessed on different cancer cells *in vitro* with and without laser irradiation.

The fourth aim of this thesis work is the synthesis of ternary silver-indium-sulfide (AIS) QDs with different surface coating chemistries as theranostic agents for nanomedicine since they do not contain regulated heavy metals. This part demonstrates the optimum conditions for preparation of highly luminescent, stable, and water soluble AIS QDs with the coating of either polyethyleneimine/2-mercaptopropionic acid (AIS-PEI/2MPA) or 2-mercaptopropionic acid (AIS-2MPA), providing cationic and anionic AIS QDs, respectively. In such a synthetic method, several variables were studied to achieve the desired properties with small hydrodynamic size and colloidal stability. These include In/Ag/S, Ag/coating ratio, synthesis duration, temperature, and pH as the basic synthetic parameters. These QDs were further loaded with zwitterionic ALA as a prodrug for PDT, exploiting the zwitterionic nature of the ALA, to identify the best nanoparticle chemistry for delivery of ALA for further studies. The influence of particle charges on the pro-drug loading, cell uptake, and subsequently PDT effects were determined using 420 nm blue light in 2D and 3D cell cultures.

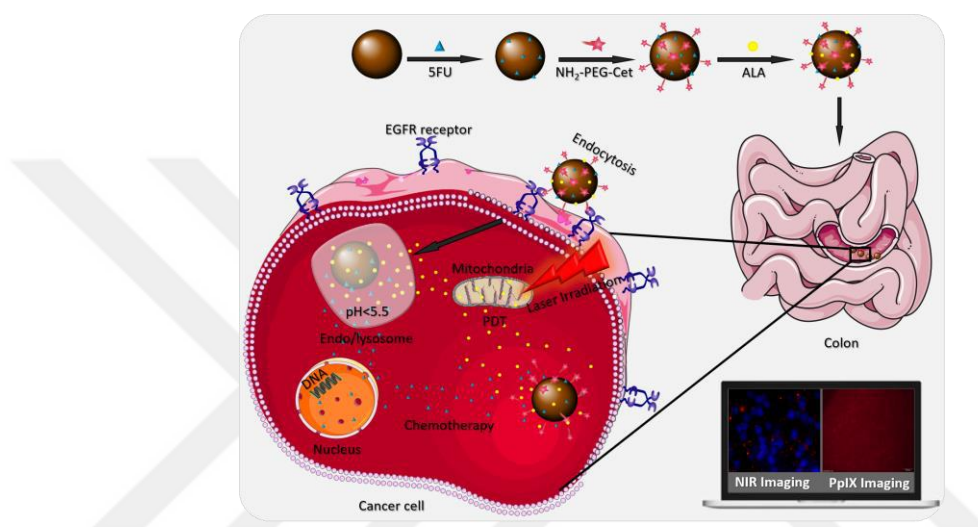
Chapter 2

Targeted ALA-PDT and Chemotherapy Combination for Cancer Treatment

2.1 Abstract

Colorectal Cancer (CRC) show poor prognosis with drug-resistance, severe off-site drug-toxicity, and a low survival rate. 5-Aminolevulinic acid (ALA) based photodynamic therapy (PDT) is very popular in recent years to treat several cancers but not yet well investigated for CRC ALA is converted to photosensitiser, protoporphyrin (PpIX), most effectively by the cancerous cells and cause phototoxicity when irradiated with a non-ionising light, providing some specificity and locality to the treatment. But ALA-PDT requires enhanced accumulation of ALA at the tumour site as well. Herein, theranostic nanoparticles offering tumour specificity, enhanced PDT or PDT-chemotherapy combination and optical imaging in the near-infrared (NIR) are described for EGFR(+) CRC cells. These are composed of Ag₂S quantum dots (AS-2MPA) conjugated with Cetuximab (Cet) and loaded with ALA or ALA/5-fluorouracil (5FU). SW480, HCT116, and HT29 cells with decreasing EGFR expression level were studied. Cet-conjugated QDs endowed excellent targeting of the high EGFR expressing cells and showed a strong intracellular signal for optical detection. The efficiency of the cells in PpIX production decreased in the order of SW480-HT29-HCT116; hence therapeutic efficacy of the theranostic QDs were evaluated in 2D cell cultures and 3D spheroids of SW480 and HT29. PDT was performed at 420 nm (blue lamp-5 min) and 640 nm (diode laser-1 min) using QDs with either electrostatically loaded, hydrazone or amide linked ALA to achieve a different level of pH-sensitive release. AS-2MPA-ALA-electrostatic always performed better than free ALA due to improved uptake and the other ALA conjugated QDs with fastest ALA release in acidic pH. SW480 cells were more sensitive to PDT and reduced IC₅₀ below 0.347 mM [ALA] even after 4 h incubation. In 2D and 3D cell cultures, AS-2MPA-ALA-electrostatic-Cet+420 nm irradiation reduced the viability below 30% in SW480 and 40% in HT29 which was further reduced below 18% and 30%, respectively,

with codelivery of ALA/5FU. Long incubation time and 640 irradiations both cells (2D) caused dramatic toxicity reaching near-complete elimination of viable cells at 0.347 mM [ALA]/15 $\mu\text{g/mL}$ [5FU]. Toxicity was correlated with high levels of reactive-oxygen species (ROS) and apoptotic/necrotic cell death. Hence, both AS-2MPA-ALA-Cet based PDT and AS-2MPA-ALA-Cet-5FU based Chemo/PDT combination therapy coupled with strong NIR tracking of the QDs demonstrate an exceptional therapeutic effect on CRC cells and an excellent potential for synergistic multistage tumour targeting therapy (Scheme 2-1).



Scheme 2-1: Targeted Near Infrared AS QDs for Optical Imaging and Targeted Chemo-Photodynamic Therapy of Colorectal Cancer.

2.2 Introduction

Colorectal cancer (CRC) is the third-most diagnosed cancer and the fourth leading cause of cancer deaths worldwide [110], [111]. Surgical resection is potentially curative at the early-stage, at advanced-stages when the tumour is metastatic or inoperable, patients have a 5% survival rate in 5 years. Most common treatment methods of inoperable cases are radiotherapy and conventional chemotherapy utilising drugs such as 5-fluorouracil (5FU), oxaliplatin, and capecitabine. 5FU is one of the widely used drugs but suffers from low lipophilicity, non-selective biodistribution, low bioavailability, and various side effects [112], [44], [62]. Therefore, the development of highly sensitive detection techniques for early diagnosis and targeted treatments of CRC are urgent demands in the field.

Light-based therapies are exciting alternative treatment methods that provide high locality. Photodynamic therapy (PDT), which involves photoexcitation of a photosensitiser (PS) at a specific wavelength to generate toxic reactive oxygen species

(ROS), is already used in the clinic for the treatment of some cancerous and non-cancerous indications [113], [114], [115]. Low dark toxicity of such PSs, presence of a “turn on” mechanism with localised light and a short travel distance of ROS reduce the off-site toxicity while increasing the locality of the treatment. Ability to repeat PDT without cumulative toxicity and its effectiveness in even chemo- and radio-resistant cells are quite attractive features of PDT, rendering it as a promising single and adjuvant therapy. PDT is mostly used for melanoma, head and neck, pancreatic, and bladder cancers [62], [116], [117]. There are only a few examples of PDT of CRC [118], [119], [120], [121]. In a clinical study performed with 23 patients with advanced CRC, Photofrin II-based PDT effectively reduced complications and hospital stay and increased the survival time [121]. Redoporphirin-based PDT of CT29-tumour xenografts caused complete regression of the tumour in 83% of the treated mice [122]. However, PDT has several limitations as well. These include long-term photosensitivity, short excitation wavelengths, poor PS solubility/stability, quick clearance, and low tumour selectivity [117], [123]. Therefore, selective and high accumulation of the PSs at the tumour site, just like the chemotherapeutic agents, and also solubilisation/stabilisation of the PSs in the circulation are urgent needs of PDT for improved therapeutic outcomes [122], [124].

FDA approved 5-aminolevulinic acid (ALA)-induced protoporphyrin (PpIX) is now popular for PDT [125]. Compared to other PSs, ALA offers lower toxicity, faster clearance and hence less photosensitivity for patients. ALA is a natural precursor of the fluorescent protoporphyrin IX (PpIX) in the heme biosynthetic pathway, where the transient formation of PpIX occurs in mitochondria before its conversion to photoinactive heme by ferrochelatase [126], [127]. Exogenous administration of ALA cause accumulation of high levels of PpIX which can be exploited for PDT and fluorescence imaging due to red luminescence of PpIX [128], [129]. ALA is a low molecular weight, highly water soluble zwitterionic molecule with rapid body clearance, which reduces the patients' photosensitivity but also causes relatively low bioavailability [89]. Different approaches have been taken to enhance the bioavailability of ALA such as esterification with long-chain fatty acids to enhance lipophilicity, conjugation to dendrimers, and its loading to liposomes or nanoparticles (NPs) [125], [62], [130]. Xu *et al.* showed that ALA loaded electrostatically to cationic Au nanoparticles provides higher phototoxicity to K562 (chronic myelogenous leukemia) cells than free ALA [131]. Although very limited, there are some promising examples to ALA-PDT of CRC but mostly performed with free

ALA [132], [133], [134], [135]. Chung *et al.* incorporated ALA to methoxy(polyethylene glycol)/chitosan (PEG-Chito), and showed a 2-folds increase in PpIX formation compared to free ALA (0.1 mM) that subsequently caused 50% more phototoxicity in CT26 cells after irradiation at 635 nm with 1.2 J/cm² light dose [119]. ALA-PDT is mostly performed at 400-420 nm which corresponds to the intense Soret porphyrin absorption band or 635 nm which corresponds to the long-wavelength band that is commonly used in clinic [131], [133], [136]. Despite lower absorption of PpIX at 635 nm, long-wavelength has better safety and deeper penetration.

Use of nanocarriers usually enhances the therapeutic outcome by altering the cell penetration pathway of small drugs, but the desired high specificity of the tumour therapy may be achieved with nanocarriers tagged with monoclonal antibodies that selectively bind to tumour-specific biomarkers. There are several examples wherein antibody targeting of PS loaded nanocarriers improve the phototoxicity significantly [137], [138]. The EGFR (ErbB1, HER1) is a transmembrane glycoprotein with a regulatory carboxyl-terminal part that belongs to HER family receptors. HER family receptors consist of four related receptors: the EGFR (ErbB1/EGFR/HER1), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4) [139], [140]. The N-terminal extracellular domain of EGFR binds to epidermal growth factor (EGF), transforming growth factor- α (TGF), and amphiregulin leading to activation of the intracellular tyrosine kinase region of EGFR, autophosphorylation, and initiation a cascade of intracellular events [140], [141]. Cetuximab (IMC-C225, Erbitux[®]) is a chimeric human murine derivative IgG1 monoclonal antibody (mAb) which binds to the accessible extracellular domain of the EGFR [142]. It consists of two identical light and two heavy chains held together by covalent and non-covalent binding with 152 kDa molecular weight. Cet binds to EGFR with high affinity ($K_d = 0.1-0.2$ nM) which is more than either endogenous ligand, as EGF and TGF- α [140]. Cet has been approved in the EU and US for the use in patients with locally advanced squamous cell carcinoma of the head and neck cancer and in combination therapy of metastatic CRC [139], [143] [144] [145], [146]. About 80% of CRC cells overexpress epidermal growth factor receptor (EGFR) and Cetuximab (Cet), which is an immunoglobulin G1 mouse-human chimeric monoclonal IgG1 antibody, binds to the external domain of epidermal growth factor receptor (EGFR) with 10 times higher affinity than the epithelial growth factor [147]. Therefore, Cet is used clinically as a targeted antibody drug for metastatic CRC in combination with chemotherapy and also

as tags to deliver nanocarriers to EGFR (+) tumour cells [137], [148], [144], [145], [146].

For the delivery of ALA at therapeutic doses to the CRC, we investigated the use of Cet conjugated Ag₂S quantum dots (QDs). Because, Ag₂S QDs have been shown as non-toxic *in vitro* and *in vivo* due to low solubility [72], [14], [30], and have a strong luminescence in near-infrared (NIR), which is well suited for medical imaging due to deeper tissue penetration and a higher signal-to-background ratio as a result of lower tissue autofluorescence in the NIR [149]. This is more advantages than red fluorescence (peaking at 635 nm) of PpIX at 405 nm excitation which has been exploited for intra-operative fluorescence imaging of tumours [150], [151], but suffers from photobleaching [152], [153]. Ag₂S QDs have better resistance to photobleaching, allowing longer operational time and can be excited at longer wavelengths to separate the wavelengths for imaging and PDT. Here, anionic Ag₂S QDs coated with 2-mercaptopropionic acid (2MPA) (AS-2MPA), which is one of the most stable and luminescent (39% QY) examples with excellent cyto- and hemocompatibility, is exploited [154], [155].

Herein, we propose the use of Cet conjugated AS-2MPA QDs loaded with ALA and a first-line anticancer drug, 5FU, to achieve tumour-specific, image-guided PDT/chemotherapy combination in EGFR(+) CRC cell lines. This approach will provide: (a) higher tumour accumulation of ALA and 5FU due to active EGFR targeting of the QDs, and (b) protection of the ALA and 5FU in the circulation but a selective and rapid release of 5FU and ALA from QDs into the acidic tumour microenvironment; (c) increase the PDT efficiency and locality since PDT is a focal therapy and is effective at the zone which retains sufficient PS and receives sufficient light dose; (d) ability to track the location of QDs due to strong, stable NIR optical signal in the medical-imaging window along with image-guided therapy option, and finally (e) a synergistic therapeutic effect in the treatment of CRC with the combination of chemotherapy and ALA-based PDT in targeted cells and should reduce the effective dose of the therapeutic agents.

In the design of such theranostic nanoparticles, Cet was conjugated to Ag₂S-2MPA QDs (AS-2MPA) via a PEG spacer, 5FU was conjugated via ester linkages, and ALA was loaded to Ag₂S in three different ways to determine the most efficient approach to produce high levels of PpIX: *electrostatically, via pH-sensitive hydrazone linkages or amide bonds*. These modes of conjugation were proposed to allow the release of the 5FU and ALA as a response to intrinsic stimuli such as pH, esterase and amidase enzymes and protect them in the physiological pH. In the *in vitro* studies, low, intermediate, and high-

EGFR overexpressing CRC cell lines, HT29, HCT116, and SW480 cells [141], were used to confirm the EGFR targeting of Cet conjugated QDs, and their imaging potential. We also investigated the time and cell line dependence of ALA to PpIX conversion to elucidate the PDT potential of these cell types. The therapeutic effect of EGFR targeted ALA-PDT monotherapy at both 420 nm and 630 nm irradiation and ALA-PDT/5FU combination therapy of CRC with the designed theranostic QDs were evaluated both in 2D cell cultures and more complex 3D spheroids.

2.3 Materials and Methods

2.3.1 Materials

All chemicals were of analytical grade or highest purity. Silver nitrate (AgNO_3), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), Sulfo-N-hydroxysuccinimide (sulfo-NHS) and 5-fluorouracil were purchased from Sigma–Aldrich. Sodium sulfide (Na_2S) was obtained from Alfa Aesar. 2-Mercaptopropionic acid (2-MPA), acetic acid (CH_3COOH), sodium hydroxide (NaOH), and Erbitux were provided from Merck. 5-aminolevulinic acid hydrochloride (ALA) was bought from research products international (USA). Heterobifunctional crosslinker NH_2 -poly(ethylene glycol)-maleimide (NH_2 -PEG-MI) with an average molecular weight of 2 kDa was obtained from Sunbio (Orinda, USA) and Polysulfone filtration membrane (3, 10 and 100 KDa) from Sartorius (Germany). 20 kDa Slide-A-lyzer dialysis cassette, live/dead cell viability, and Alamar Blue reagents were bought from Thermo Fisher Scientific (USA). The Annexin V-FITC early apoptosis detection kit was purchased from Cell Signaling Technology (UK).

Roswell Park Memorial Institute (RPMI) 1640 medium (with L-glutamine and 25 mM HEPES) 1640 and McCoy's 5A (Modified) medium were obtained from Multicell, Wisent Inc. (Canada). Fetal bovine serum was provided from Capricorn Scientific GmbH (Germany). Trypsin–EDTA and penicillin streptomycin solutions were purchased from Wisent Inc. (Canada). Thiazolyl blue tetrazolium bromide (MTT) and phosphate buffered saline (PBS) tablets were purchased from Biomatik Corp. 80% Rat Tail Collagen Type I was bought from First Link UK Ltd. Paraformaldehyde solution 4 % in PBS was obtained from Santa Cruz Biotechnology, Inc. (USA). 96-well plates were purchased from Nest Biotechnology Co. Ltd. (China). Human breast adenocarcinoma cells (HT29 and SW480) cells were given as a gift from the Gozuacik Lab (Sabanci University, Istanbul, Turkey)

for this study. HCT116 cells were purchased from ATCC (Ireland).

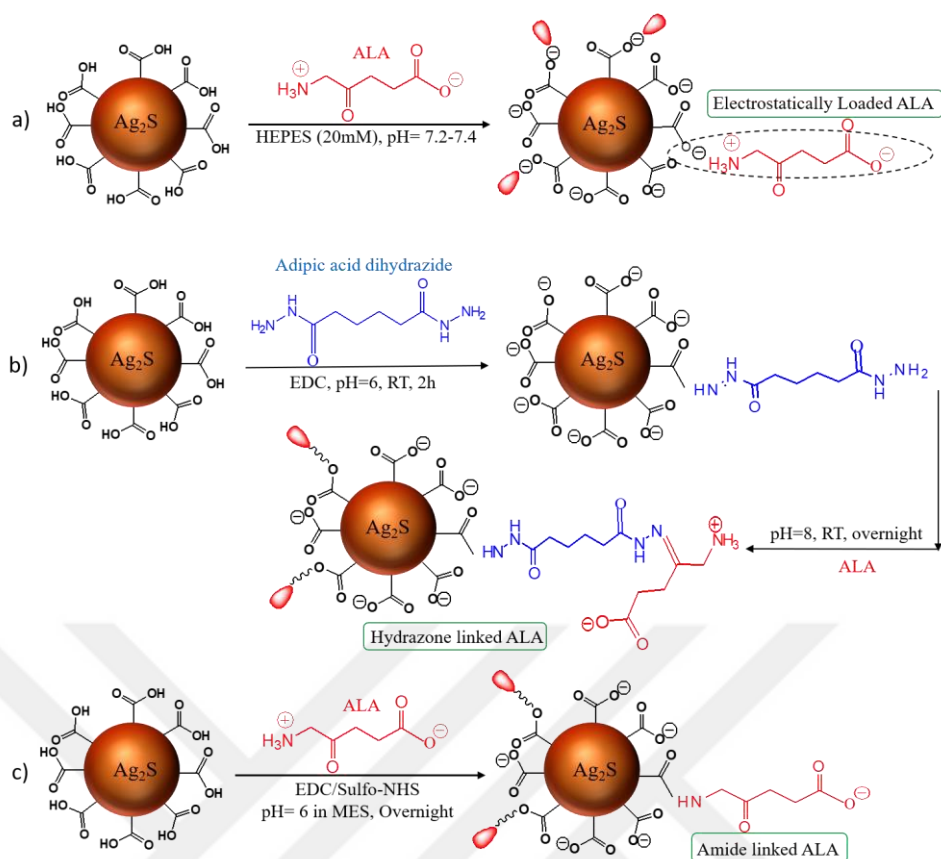
2.3.2 Synthesis of 2MPA coated AS QDs

AS-2MPA QDs were prepared as described in the literature [14]. As an example, 111.15 μL of 2MPA was dissolved in 75 mL of deoxygenated water in a round-bottomed flask under argon, then the pH of the solution was adjusted to 7.5 using NaOH and CH_3COOH (1 M). 42.5 mg of AgNO_3 was added to this solution, and the pH was adjusted to 7.5 again. Here, 4.9 mg of Na_2S for Ag/S molar ratio of 4/1 was dissolved in 25 mL of deoxygenated water, separately and added gently to the initial solution under vigorous mechanical stirring (500 rpm) for 90 min at room temperature (RT). QD solutions were washed with deionized water using Sartorius centrifugal filters (3 kDa cut-off) to remove excess unreacted molecules, and stored in the dark at 4 $^\circ\text{C}$.

2.3.3 Electrostatic loading of ALA onto AS-2MPA surface

ALA was loaded electrostatically to AS-2MPA in an amount that is equivalent to 30 mol% of carboxylic acid groups of the AS-2MPA determined by TGA analysis (Figure S1a). Isothermal titration calorimetry (Affinity ITC, USA) experiment was performed to confirm complete binding. For this, 1 mL aliquots of QD (5 mg/mL in HEPES) were titrated with ALA (total of 240 μL from 3 mg/mL in HEPES) at the rate of 15 $\mu\text{L/s}$. The temperature of the solution in the titration cell was maintained at 25 $^\circ\text{C}$ throughout the experiments. The results are reported as the change in enthalpy per titration (μcal) *versus* the concentration of added ALA after each injection. Continues binding exotherm throughout the titration (Scheme 2-2a), confirmed complete binding of ALA to QDs electrostatically. Therefore, after the electrostatic loading of ALA, no washing was performed

Based on the ITC data, 7.5 mg ALA dissolved in 20 mM HEPES to adjust the pH to 7.2-7.4 and 0.6 mL AS-2MPA (8.5 mg/mL) in 0.4 mL HEPES solution were both filtered from 0.2 μm sterile filter. These solutions were mixed at RT for 30 min to prepare the ALA loaded AS-2MPA (Scheme 2-2a). Loading was done freshly before cell studies and kept in the dark at 4 $^\circ\text{C}$ until use.



Scheme 2-2: Synthesis of different methods of ALA conjugated AS-2MPA QDs.

2.3.4 Coupling of ALA to AS-2MPA via hydrazone linkage

Adipic acid dihydrazide (ADH) (9.5 mg) was added to 25 mg AS-2MPA (contains 0.146 mmol $-\text{COOH}$) in DI water. The pH of the reaction mixture was adjusted to 6 by addition of 1 M CH_3COOH /NaOH. EDC (0.09 mmol) in 1 mL of MES buffer (pH=6, 0.1 M) was added to the reaction mixture. The pH of the reaction was maintained around 6 for carbodiimide reactions by addition of 1 M CH_3COOH , and the reaction was allowed to proceed at RT for 2 h or until no further change in pH was observed. The solution was washed with DI water using Sartorius centrifugal filters (3 kDa cut-off) for 3 times, then the pH of the solution was adjusted to 8-9 by adding 1 M NaOH. 0.044 mmol ALA (equivalent to 30 mol% of the 2MPA content of QDs which was determined by TGA in Figure 2-1) in 1 mL water was added to this solution and left stirring overnight at RT (Scheme 2-2b). Finally, the prepared QD solutions were washed with DI water using Sartorius centrifugal filters (3 kDa cut-off) and stored in the dark at 4 °C. The amount of unbound ALA was calculated from its absorbance at 263 nm using a concentration-dependent absorbance calibration curve for ALA. The bound amount was calculated accordingly (Figure 2-2a).

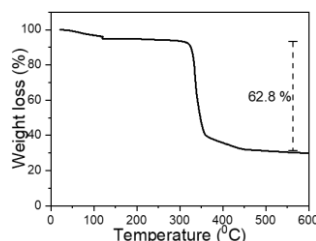


Figure 2–1: TGA of AS-2MPA QDs.

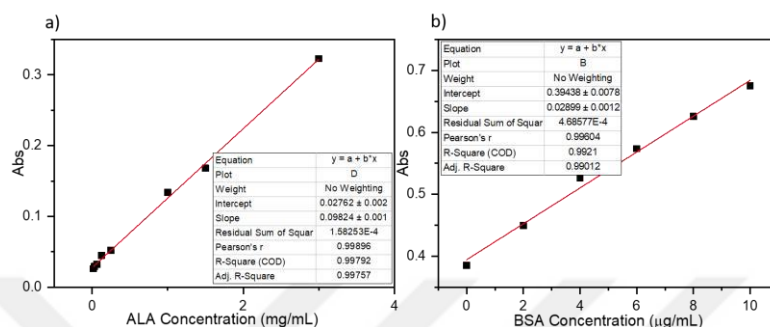


Figure 2–2: Quantification of Cet using Bradford Assay. Standard curve of BSA concentration (μg/mL) versus absorbance at 595 nm. Standard curve of ALA concentration (mg/mL) versus absorbance at 263 nm.

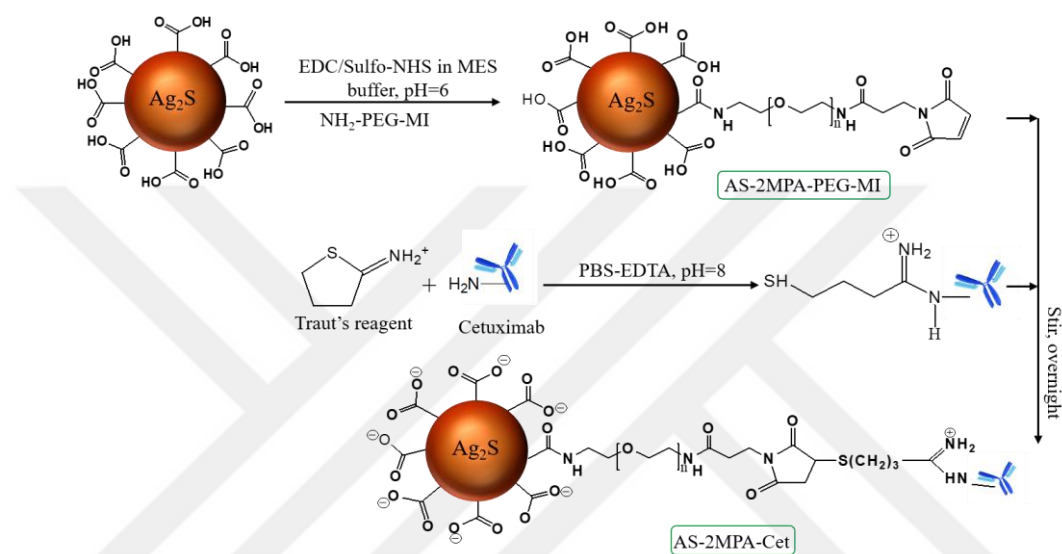
2.3.5 Direct conjugation of ALA to AS-2MPA QDs via amidation

ALA was covalently linked to AS-2MPA QDs as follows: First, 0.088 mmol EDC was added to the AS-2MPA QDs (25 mg QD, 0.146 mmol -COOH) in MES buffer (pH=6, 0.1 M) and stirred for 5 min, then 0.088 mmol sulfo-NHS was added, and the mixture was stirred for 30 min at RT to activate the carboxylic acid groups of QDs. Then, 0.044 mmol ALA was added to the solution of activated QDs, and the mixture was kept stirring overnight for amidation (Scheme 2-2c). Finally, the prepared QD solutions were washed with DI water using Sartorius centrifugal filters (3 kDa cut-off) and stored in the dark at 4 °C. The amount of unconjugated ALA was calculated using the ALA calibration curve, as mentioned above, and the conjugated ALA amount was calculated accordingly (Figure 2-2a).

2.3.6 Conjugation of Cet to AS-2MPA QDs

NH₂-PEG-MI was used as a bridge between the Cet and QDs. AS-2MPA QDs (25 mg in MES buffer-pH 6) were activated with 14.6×10^{-4} mmol of EDC and 14.6×10^{-4} mmol sulfo-NHS as described above. Then, the medium was changed with PBS (1X, pH=7.2-7.4) and 14.6×10^{-6} mmol NH₂-PEG-MI (2kDa) in 1mL PBS buffer was added to the reaction solution. After overnight reaction under mild stirring at RT, QDs were washed with PBS through a 3 kDa cut-off ultracentrifuge tube (Scheme 2-3).

Cet was thiolated to react with maleimide (MI) of the PEG bridge via thiol-ene reaction. For thiolation, Cet (3 mg/mL in PBS) was incubated with 21.75×10^{-4} mmol of 2-iminothiolane solutions (Traut's reagent, 1 mg/mL PBS-EDTA, pH=8) for 2 h at RT, then dialysed in 20 kDa dialysis cassette in phosphate buffer-EDTA (pH=8) for 5-6 h. Freshly prepared thiolated-Cet (Cet-SH) was added to the QD solutions (in PBS, pH=7.2-7.4) and stirred overnight at RT. The mole ratio of Cet-SH: NH₂-PEG-MI was 1:1. Unbound Cet-SH was removed by washing 6 times with PBS through a 100 kDa cut-off Sartorius centrifugal filters.



Scheme 2-3: Synthesis of Cet conjugated AS-2MPA QDs.

Bradford Assay was used to quantify the Cet content of the washed QDs. Bradford Assay is a protein detection method which uses the Coomassie Brilliant Blue G-250 dye as an indicator with red colour in its cationic form ($\lambda_{\text{abs}} = 465$ nm), but when it interacts with a protein it is converted into a non-protonated stable form which has blue colour ($\lambda_{\text{abs}} = 595$ nm). Protein amount can be calculated based on its absorbance at 595 nm. The absorbance values of the reference protein bovine serum albumin (BSA) are given in the Figure 2-3b. A calibration curve was plotted in Figure 2-2b based on these λ_{abs} values.

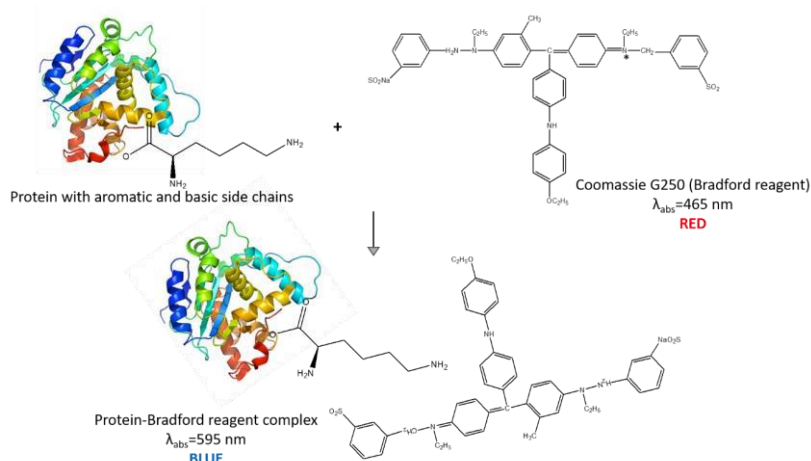


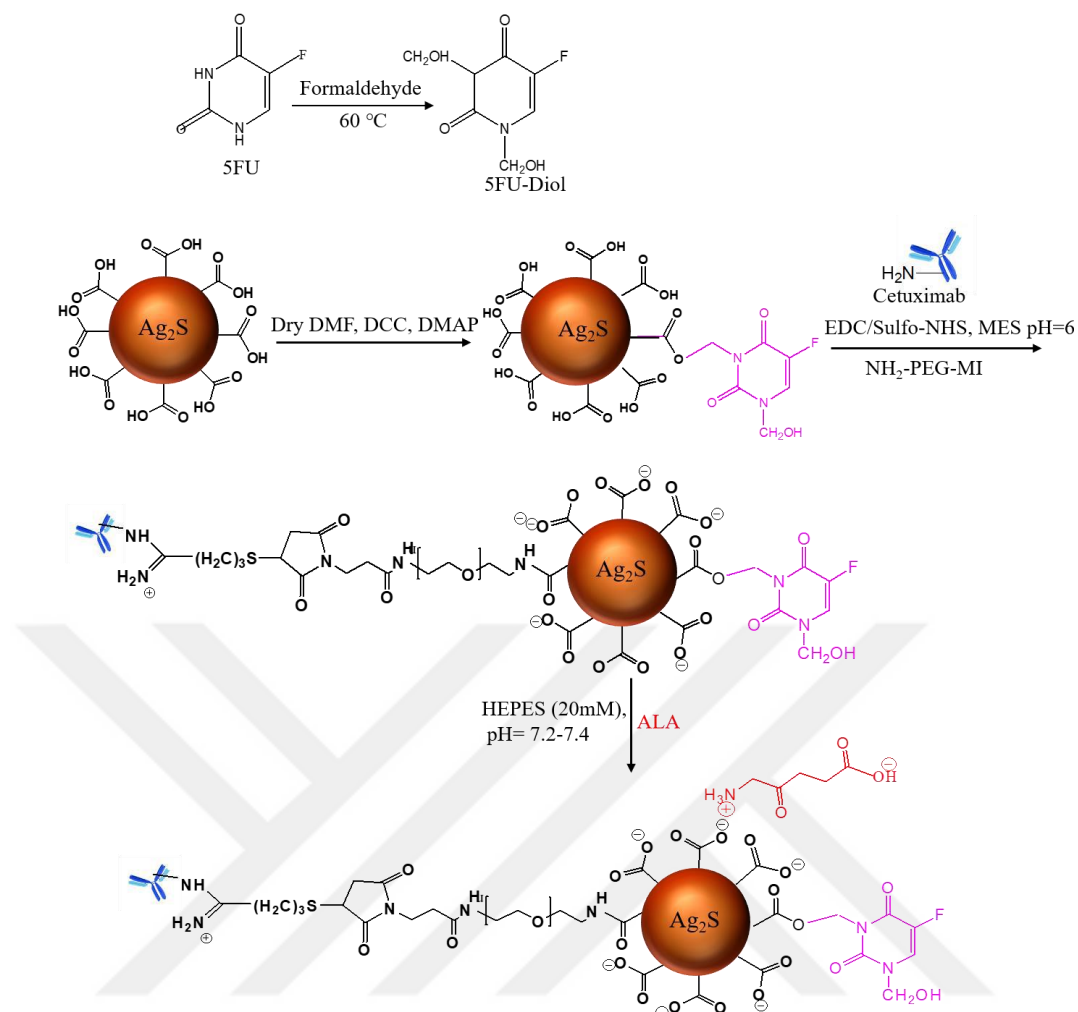
Figure 2–3: Mechanism of action of the Bradford Assay.

The amount of Cet conjugated to the QDs was calculated according to the Bradford assay. Briefly, bovine serum albumin protein at 0, 1, 2, 4, 6, 8, 10, and 20 $\mu\text{g/mL}$ concentrations in PBS was utilised as a standard. Washed product of AS-2MPA-Cet was concentrated and measured as the analyte. 1 ml of each reference and analyte were mixed with 1 mL of Bradford reagent for 10 min at RT. Then, the absorbance at 595 nm was recorded using ultraviolet-visible (UV-vis) spectroscopy (Figure 2-2b).

2.3.7 Conjugation of 5FU to AS-2MPA

First, 5FU was reacted with formaldehyde at 60 $^{\circ}\text{C}$ in an oil bath for 4 h to produce 5FU-diol (Figure S3) [156]. QDs in water were protonated with dropwise addition of acetic acid and precipitated with centrifugation. Isolated QDs were suspended in dry DMF. For esterification reaction, 50 mg QDs in DMF (5 mg/mL), 10.74 mg 5FU-diol (equivalent to 30 mol% of 2MPA content of the QDs) and 2.3 mg DMAP was added and stirred at RT. After the reaction flask was cooled down in an ice bath, 11.66 mg DCC was added to the reaction and stirred for overnight under Ar flow. The solution was mixed with about 200 ml dichloromethane in a separatory funnel and 5FU conjugated QDs were extracted into the about 40 ml aqueous solution. The aqueous phase was washed with DI water from 3 kDa Sartorius centrifugal filters to obtain the final AS-2MPA-5FU QDs. The amount of the unconjugated 5FU present in the filtrate was quantified from the absorbance of 5FU at 279 nm by using a concentration-dependent absorbance calibration curve of 5FU. The conjugated 5FU amount was determined as 15 mol% of 2MPA content of the QDs.

Cet was conjugated to AS-2MPA-5FU, and then ALA was electrostatically loaded following the procedures given above for each of these steps (Scheme 2-4).



Scheme 2-4: Synthesis of 5FU conjugated AS-2MPA-Cet QDs.

2.3.8 Characterization

The absorbance was measured by Shimadzu UV-3600 spectrophotometer in the 300–1000 nm range. The crystal sizes of AS were calculated using the Brus equation [157]:

$$\Delta E = \frac{\hbar^2 \pi^2}{8R^2} \left[\frac{1}{m_e} + \frac{1}{m_h} \right] - 1.8 \frac{e^2}{\epsilon_{Ag_2S} 4\pi \epsilon_0 R} \quad \text{Equation 2-1}$$

Where DE is the band gap energy difference, m_e ($0.286m_0$) and m_h (1.096) are the respective effective electron and hole masses for AS, ϵ_{AS} (5.95) is the dielectric constant of bulk AS and R is the radius of the nanocrystal.

To measure the photoluminescence (PL) intensity, a home-made instrument with a femtowatt sensitive Si detector (Thorlabs PDF10A, $1.4 \times 10^{-15} \text{ W Hz}^{-1/2}$) and a 1/8 Newport Cornerstone 130 monochromator having 600 L/mm grating were performed over the wavelength range of 600-1100 nm. AS-2MPA QDs were excited with a frequency doubled output of a DPSS laser operating at 532 nm excitation wavelength. The luminescence signal was filtered using a 590 nm long-pass filter.

Hydrodynamic size, zeta potential, and polydispersity index (PDI) measurements of the aqueous QDs were determined using a Malvern zetasizer nano ZS. The Ag⁺ content of the QD solutions was employed after treating of samples with sulfuric acid (96%) and nitric acid (65%) (at the ratio of 1:9 v/v) using an Agilent 7700x Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

2.3.9 ALA release

Release of ALA from the QD nanocarrier is a crucial prerequisite for the intracellular generation of PpIX. The rate of release of ALA was investigated under different pH media (pH 5.5 and 7.4) at 37 °C. Typically, AS-2MPA-ALA-Electrostatic, AS-2MPA-ADH, and AS-2MPA-Amide QDs (5 mL) were added to a dialysis bag (MWCO 3500). The dialysis bag was then immersed in 50 mL of PBS (pH 5.5 and 7.4) with constant shaking (150 rpm) at 37 °C. At fixed time intervals, 3 mL of the released solution was taken out, and 3 mL fresh PBS medium was added. The release of ALA was calculated by UV-vis spectrometer using a concentration dependent standard curve for ALA in PBS (pH 5.5 and 7.4) at 263 nm.

2.3.10 5FU release

The release of the 5FU was studied in PBS solution (pH 7.4) at 37°C. 5FU-bound QDs were placed in the dialysis bag (3500 MWCO). In determined time intervals, aliquots were taken and 5FU release followed with UV-vis spectrometer.

2.3.11 Isothermal titration calorimetry (ITC)

An isothermal titration calorimeter (Affinity ITC, USA) was used to measure enthalpy changes resulting from the interaction between ALA and AS-2MPA QDs. 1 mL aliquots of either QDs (5 mg/mL in HEPES) or ALA solution (3 mg/mL in HEPES) were injected sequentially into a titration cell with the rate of 15 µL/s. The temperature of the solution in the titration cell was maintained at 25 °C throughout the experiments. The results are reported as the change in enthalpy per titration (µcal) *versus* the concentration of added ALA after each injection.

2.3.12 Cell culture

Different EGFR overexpressing human colon adenocarcinoma cells (HT29: low, HCT116: intermediate, and SW480: high) and healthy colorectal cells (CCD481) were cultured in complete McCoy's and DMEM media supplemented with 10% fetal bovine

serum and 1% penicillin-streptomycin antibiotic solution according to ATCC recommendations [141]. Trypsin-EDTA was used for cell detachment of the cells.

2.3.13 Manufacture of 3D cancer constructs

The 3D *in vitro* cancer constructs were manufactured following the protocol from RAFT 3D culture systems (Lonza, Slough, UK) [158]. The hydrogels were prepared from a mixture containing 10% 10X MEM (used as colour/pH indicator), 80% Rat Tail Collagen Type I (First Link UK Ltd. Custom Bio-Reagents) before undergoing neutralization using a solution made from 1.65 M NaOH and 840 mM HEPES buffer solution (Thermo Fisher Scientific). The cells were seeded into the collagen at the density of 50000 cells for spheroid constructs at overall collagen mix volumes of 240 μ L per well in a 96-well plate. The constructs were incubated at 37°C for 15 min to set before being subjected to plastic compression using absorbers (Lonza), at RT for a further 15 min. After the removal of the absorbers, fresh medium was added, and cells returned to the incubator.

2.3.14 Determination of cytotoxicity

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay is a widely used method measuring cytotoxicity of the cells usually at a final concentration of 0.2-0.5 mg/mL after 1-4 h incubation [159]. MTT is converted to purple formazan only by mitochondrial enzymes of the viable cells, hence MTT is considered to measure mitochondrial activity. NADH or a similar reducing molecule can transfer electron to MTT and reduce it to formazan (Figure 2-4). Formazan is insoluble inside the cells and deposited near the cell surface in the medium. Therefore, various methods, including the use of acidified isopropanol, DMSO, dimethylformamide, SDS, or combination of them, are utilized to dissolve it before its quantification with its measured absorbance at $\lambda_{\text{abs}}=570$ nm which is proportional to the number of viable cells. Acidification of the solution changes the colour of phenol red to yellow which minimizes its interference with the absorbance of formazan.

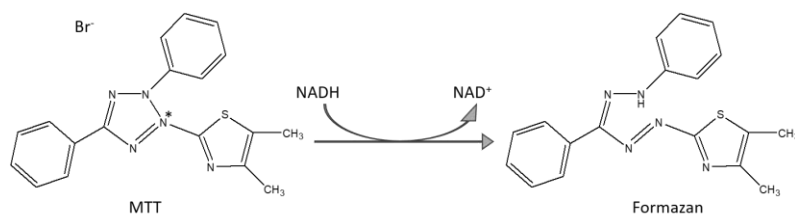


Figure 2–4: Structures of MTT and coloured formazan product.

Cytotoxicity was evaluated by performing the thiazolyl blue tetrazolium bromide (3-(4,5-

dimethyl-thiazol-2yl)-2,5-diphenyltetrazolium bromide, MTT) and Alamar blue assay on HCT116, HT29, and SW480 cell lines before/after PDT. Cells were seeded at a density of 12500 cells/well into 96-well culture plates in culture medium and incubated at 37 °C and 5% CO₂ atmosphere for 24 h. Following incubation, the medium was replenished; cells were treated with QDs (5-300 µg/mL [Ag]) and incubated for 48 h. MTT reaction solution was added to the cells and incubated for 4 more hours. DMSO-ethanol (1: 1 v/v) solution (200 µL) was added into the wells and plates were shake gently for 15 min to dissolve the purple formazan formed as a result of the mitochondrial activity of the viable cells. The absorbance at 600 and 640 nm was measured with a Bio-Tek EL×800 Absorbance Microplate Reader, and the reference absorbance at 640 nm was subtracted from the absorbance at 600 nm. Absorbance values of the QDs were subtracted from the formazan values.

For the Alamar blue assay, the cells were seeded and the QDs were added as described for MTT assay. The working solution of the Alamar blue assay was made by the addition of the dye to fresh culture media with the dye making up 10% of the overall solution. The cells were incubated with the Alamar blue solution for 2 h and the solutions were transferred from each well into a corresponding well in a black well plate (TPP, Sigma Aldrich, UK) for measurements. The fluorescence intensities were measured at wavelengths of (λ_{exc} : 530 nm and λ_{emi} 590 nm) using Fluoroskan ascent FL plate reader (Thermo Labsystems, UK).

2.3.15 Cell uptake

Qualitative Analysis. The *in vitro* cell imaging of QDs was performed on the 2D culture first using a confocal microscope (Leica dmi8/SP8) to show the cell uptake qualitatively. HCT116, SW480, and HT29 cells were grown on a 3 mL glass-bottom petri dishes at a density of 1.5×10^5 cells/well in complete medium, incubated at 37 °C under 5% CO₂ for 24 h. QDs at 100 µg/mL [Ag] was introduced to the cells, and after 4 and 24 h incubation, the cells were washed three times with PBS to remove un-internalised QDs. Then, the cells were fixed with paraformaldehyde solution (4% in PBS) for 20 min. Cells were incubated for an additional 15 min with 2 µg/mL of DAPI, a nuclear staining dye. The cells were rewashed with PBS three times to remove the unbound dye and 1 mL PBS was left in each well to protect the cells against drying.

The fixed cell samples were examined using a confocal microscope (Leica dmi8/SP8)

with two different filters for NIR region (λ_{exc} : 510-550 nm and λ_{em} : 710 nm long pass) and DAPI (λ_{exc} : 325-375 nm and λ_{em} : 435-485 nm). The same experimental procedure was also performed for control cells which were not treated with QDs. Images were processed and merged using ImageJ analysis program.

Quantitative Analysis. The quantitative analysis of QD uptake was performed by the determination of Ag content in the QD treated cells. HCT116, SW480, and HT29 cells were seeded on a 12-well plate at a density of 1.5×10^5 cells/well complete medium and grown for 24 h at 37 °C and 5% CO₂. Next day, the growing cells were incubated with QD samples at 100 µg/mL [Ag] for 4 and 24 h. Then, the cells were washed with PBS to remove un-internalised QDs and were collected after being trypsinised. Cells were digested with acid (HNO₃/H₂SO₄: 9/1 v/v) for about 1 week at RT, and Ag content was determined by ICP-MS (n=3).

Imaging 3D spheroids: The multicellular aggregates of HT29 and SW480 (50000 cells/model) were grown for 7 days. Then, the spheroids were incubated with QDs at 100 µg/mL [Ag] for 4 and 24 h. The spheroids were washed three times with PBS and incubated with PBS for imaging with Olympus fluorescence microscope (10x objective, Olympus BX63) with the following bandpass filters: BF (λ_{exc} : 345 nm and λ_{em} : 455), CY8 (λ_{exc} : 480 nm and λ_{em} : 810 nm) and TRITC (λ_{exc} : 545 nm and λ_{em} : 580-650 nm (band pass filter). The spheroids without QD treatment were used as controls.

2.3.16 Measurement of PpIX formation

HCT116, SW480, and HT29 cells were seeded as described in cytotoxicity study. After 24 h of incubation, the medium was replaced with a serum-free medium containing 5-300 µg/mL QDs to find the optimum time and concentration of ALA. The use of serum-free medium is essential to prevent efflux of PpIX mediated by binding to serum proteins. Untreated cells were used as control. The fluorescence intensity was measured using Synergy H1 microplate reader (Biotek) at excitation and emission wavelengths of 420 and 635 nm every 2 h.

2.3.17 PDT studies

For the *in vitro* PDT studies, HCT116, SW480, and HT29 cells at a density of 12500 cells/well (2D cell culture) and 50000 cells/well (3D models) were seeded in 96-well plates and cultured as described above for 24 h (2D cell culture) or 7 days (3D models).

Cells were treated with QDs between 25-100 $\mu\text{g/mL}$ Ag concentrations or with free ALA (0.173-0.688) for 4 and 24 h. Then, the medium was replenished with serum-free medium, and each well was illuminated with either a blue flatbed lamp with peak emission at 420 nm (7 mW/cm^2 , 2.1 J/cm^2) (Lumisource, PCI Biotech, Oslo, Norway) for 5 min or a 640 nm laser for 1 min from the bottom of the plate. The output beam of 640 nm laser was adjusted with the help of a telescope to match the beam diameter to the diameter of the wells on the 96-well plate. The diode laser delivered a maximum power of 100 mW, corresponding to an irradiance fluence of 0.78 W/cm^2 (the fluence: 15.5 J/cm^2). Light irradiated cells were then incubated for further 24 h before cell viability was determined using standard MTT (2D cell culture) or Alamar blue (3D models) assays. Cells that were not treated with QD, free ALA or light were used as a control. Cells that were not treated with QD or ALA but irradiated were used as a “laser control”.

2.3.18 Measurement of Reactive Oxygen Species (ROS generation)

HT29 and SW480 cells were seeded as described in cytotoxicity study. QDs were added to the cells at Ag concentration of 100 $\mu\text{g/mL}$ for QDs for 24 h. The cells were irradiated using a blue LumiSource-flatbed lamp with peak emission at 420 nm and 7 mW/cm^2 output (PCI Biotech, Oslo, Norway) for 5 min or 640 nm laser for 1 min from the bottom underside of the plate and incubated for further 24 h. The ROS levels in the cells were determined using the Enzo Total ROS Detection Kit following the manufacturer’s instructions using Synergy H1 microplate reader (Biotek) at excitation/emission: 485/538 nm (for 420 nm irradiation) and excitation/emission: 540/570 nm (for 640 nm irradiation).

2.3.19 Live/dead staining

Live/dead cell viability assay was used to determine live and dead cells before and after PDT treatment. HCT116, SW480, and HT29 cells were seeded as described above for 4 h QD incubation. Cells were irradiated with either 420 nm blue light for 5 min or 640 nm laser for 1 min. After 24 h incubation, cells were stained with live/dead assay based on the protocol that is provided by the manufacturers. Finally, images were collected using fluorescence microscope (for 2D: Zeiss Axio observer Z1 and for 3D: EVOS FL colour, Life Technologies, Thermo Fisher Scientific, UK).

2.3.20 Mechanism of death (apoptosis/necrosis studies)

Annexin V-FITC apoptosis detection kit (Abcam, UK) was used to confirm the

mechanism of cell death in 2D and 3D models. The cells were seeded as described in PDT studies in 2D and 3D models with 100 µg/mL QDs. 24 h after being exposed to irradiation with blue lamp, 100µL of 1X Binding buffer was added to the models in each well before adding 1µL of Annexin V-FITC, (Ex/Em 488/525 nm) and 5µL of propidium iodide, (Ex/Em 535/617 nm) to the buffer solution in each of the wells. The models were then imaged using an EVOS fluorescence microscope (EVOS FL color, Life Technologies, Thermo Fisher Scientific, UK).

2.3.21 Statistical analysis

One-way ANOVA with Tukey's multiple comparison test of GraphPad Prism 8 software package was employed for statistical analysis (GraphPad Software, Inc., USA). All data expressed as mean $\pm$ standard deviation (SD), and $p < 0.05$ was considered statistically significant.

2.4 Results and Discussion

2.4.1 Synthesis and characterization of AS-2MPA QDs

NIR emitting AS-2MPA QDs were synthesised from AgNO₃ and Na₂S using 2MPA as a coating at the molar ratios of Ag/S/2MPA:1/4/5 at RT according to the procedure previously published by our group [14]. AS-2MPA QDs have a strong emission at 830 nm (Figure 1a). The crystal size of the Ag₂S-core was calculated as 2.93 nm by the Brus equation. The hydrodynamic size of the AS-2MPA QDs was measured as ~327 nm (number/intensity-based average) by DLS and zeta potential was -35.3 eV (Table 1).

2.4.2 Synthesis and characterization of AS-2MPA-ALA

ALA was loaded to QDs in three different ways: (1) *electrostatically*, which is a single step, simple loading approach. Electrostatic interactions are sensitive to pH, which is low in the tumour microenvironment and the endosomal/lysosomal compartments of the cells, providing an intrinsic stimulus for the release of ALA; (2) via a *pH-sensitive hydrazone linkage*, which should protect the ALA at the circulation but quickly cleave in the low pH areas, as well; (3) via an *amide bond*, which is relatively stable under physiological conditions and hydrolyse slowly but preferentially in the acidic environment of the tumour. In ALA loading, 30% of the surface -COOH groups of the AS-2MPA was targeted to have enough functional groups for Cet and 5FU conjugation to the same QD (Figure 2-5a-f).

2.4.2.1 Electrostatic loading of ALA to AS-2MPA (AS-2MPA-ALA-Electrostatic)

ALA is a zwitterionic molecule with one carboxylic acid and one primary amine groups with the pK_a values of 4.05 and 8.9, respectively. At pH 7.2-7.4, amine group of ALA is protonated, and the electrostatic interaction between NH_3^+ group of ALA and COO^- groups of QDs could allow the electrostatic loading of ALA to the QDs [131] (Scheme 2-2a). Intensity-based hydrodynamic size of the AS-2MPA QDs changed from 12.98 to 19.52 nm in HEPES buffer (pH=7.2-7.4) after loading of 30 mol% ALA (Table 1) and the zeta potential increased from -7.82 (AS-2MPA in HEPES) to -13.60 (AS-2MPA-ALA-Electrostatic) confirming the successful electrostatic loading of ALA. Free carboxylic acid groups of ALA may facilitate an increase in negative charge of the QDs at pH 7.4. Furthermore, the emission spectra of QDs showed a slight red shift and less than ca 10% luminescence reduction after ALA loading. Reduction in the emission intensity is common when QDs are subjected to post-synthetic modifications due to changes in the medium and the surface of QDs.

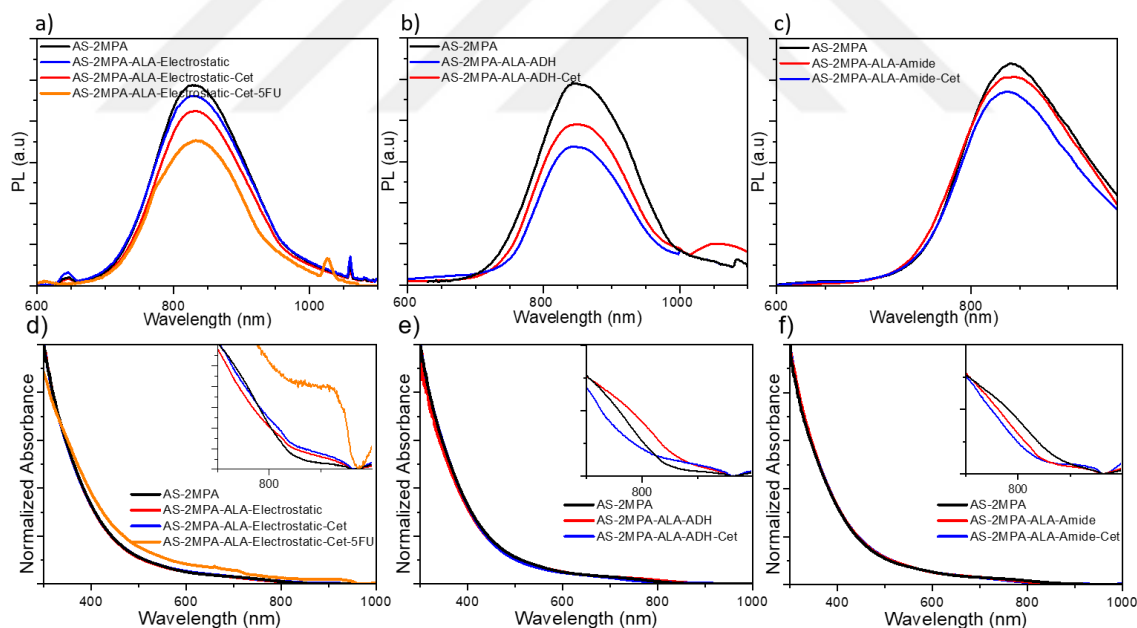


Figure 2-5: Photoluminescence and UV-vis spectra of Cet and ALA conjugated QDs via (a), (d) electrostatic, (b), (e) ADH, and (c), (f) Amide bonds.

To further confirm the interaction between ALA and QDs, Isothermal Titration Calorimetry (ITC) measurements were performed. Titration of QDs at pH 7.4 with 30 mol% ALA in HEPES (pH=7.4) caused observation of exothermic peaks throughout the whole titration, indicating complete binding of ALA to QD (Figure 2-6). Therefore, after

the electrostatic loading of ALA, no washing was performed.

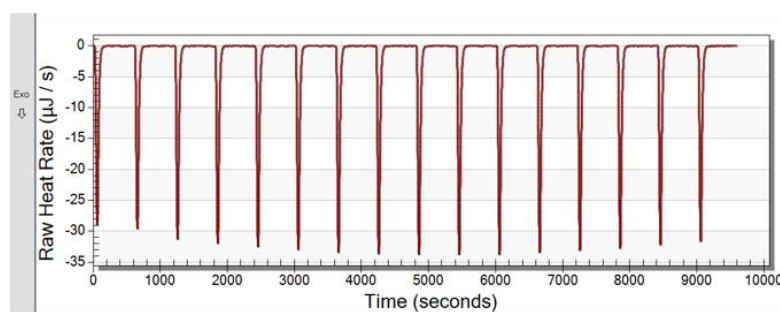


Figure 2-6: Isothermal titration calorimetry (ITC) of AS-2MPA-ALA-Electrostatic QDs in HEPES at pH 7.4.

Table 2-1: PDI, size and zeta potential of all compositions.

Sample	Dh-number ^a (nm)	Dh-intensity ^b (nm)	Zeta potential (mV)	[Cet] μg/mL	[ALA] μg/mL	[Ag] μg/mL	[5FU] μg/mL
ALA in HEPES	0.69	5.78	-7.85	-	-	-	-
AS-2MPA in HEPES	5.35	12.98	-7.82	-	-	100	-
AS-2MPA in water	3.03	27.41	-35.30	-	-	100	-
AS-2MPA in PBS	3.61	42.21	-5.81	-	-	100	-
AS-2MPA-ALA-Electrostatic ^c	3.53	19.52	-13.60	-	25	100	-
AS-2MPA-ALA-ADH ^c	4.81	118.51	-7.70	-	25	100	-
AS-2MPA-ALA-Amide ^c	3.90	112.23	-36.23	-	25	100	-
AS-2MPA-ALA-PEG	6.45	45.11	-7.83	-	25	100	-
AS-2MPA-PEG-Cet ^d	3.31	58.10	-8.87	30	-	100	-
AS-2MPA-ALA-Electrostatic-Cet ^c	5.87	49.83	-12.21	30	25	100	-
AS-2MPA-ALA-ADH-Cet ^d	9.19	139.67	-14.26	30	25	100	-
AS-2MPA-ALA-Amide-Cet ^d	6.37	147.01	-16.67	30	25	100	-
AS-2MPA-ALA-Electrostatic-Cet-5FU ^d	6.27	114.38	-7.78	30	25	100	30.1

^a Hydrodynamic diameter measured by DLS and reported as the number average. ^b Hydrodynamic diameter measured by DLS and reported as the intensity average. ^c Particles are in HEPES buffer. ^d Particles are in PBS (pH=7.4).

2.4.2.2 Covalent conjugation of ALA to AS-2MPA (AS-2MPA-ALA-ADH and AS-2MPA-ALA-Amide)

Conjugation of ALA to QDs via acid-labile hydrazone linkage was done by using an adipic dihydrazide linker which was first conjugated to QD via amidation, and then to ALA (Scheme 2-2b). Conjugation efficiency was determined as about 70%, which means 28 mol% of surface carboxylic acid groups of AS-2MPA were conjugated with ALA. These particles (AS-2MPA-ALA-ADH) have a relatively low zeta potential (-7.70 eV) since -COOH groups were replaced with zwitterionic ALA. Particles were also aggregated slightly indicated by larger hydrodynamic size (118.51 nm), but still, the number-based average was 4.81 nm. There is about 20% reduction in the PL intensity with respect to AS-2MPA, but QDs still have strong luminescence (Figure 2-5c).

ALA was also conjugated to QDs via a more stable amide linkage utilising the well-known activated ester chemistry (Scheme 2-2c). AS-2MPA-ALA-Amide QDs have 26 mol% ALA with respect to its 2MPA content. Their hydrodynamic size was similar to hydrazone linked ones (112.23 nm), suggesting some aggregation, but not dramatic since the number-based average was again small (3.90 nm). The charge of the AS-2MPA-ALA-Amide QDs were similar to AS-2MPA (-35.30 and -36.23 mV). This is reasonable since the amine group of ALA was used for conjugation, and its carboxylic acid groups substitute the lost -COOH from the surface of the QDs. The PL and absorbance spectra remain almost unchanged after the conjugation with only 7% reduction in PL intensity (Figure 2-5c, f). Overall, the emission wavelength did not change significantly, but a typical small loss in the intensity was observed after ALA was loaded or conjugated to QDs.

2.4.2.3 Synthesis and characterization of EGFR targeting QDs (AS-2MPA-ALA-Cet)

Cet was conjugated to 0.01 mol% of the carboxylic acid groups of AS-2MPA via a PEG spacer to increase the accessibility of Cet to EGFRs. A three-step protocol was followed to synthesise QDs bearing both Cet and covalently linked ALA: First, NH₂-PEG-MI (MW 2000 Da) was conjugated to QDs via amidation, then ALA was conjugated to QDs, and in the last step thiolated Cet was conjugated to QDs via thiol-ene chemistry (Scheme S2-3). In the case of electrostatically loaded ALA, first Cet conjugated QD was prepared (Scheme 2-3), and then ALA was loaded. The efficiency of antibody conjugation to QDs

was determined as 94-97% by Bradford assay. As shown in Table 1, Cet conjugation increases the hydrodynamic size of the ALA loaded QDs, slightly. The smallest one was again the ALA loaded one (50 nm) and the ADH and amide linked ones were larger (140-150 nm), with a negative surface charge (ca -12 to -17 mV). But, the number based average sizes are again 6-9 nm. Overall, these particles are small enough to study targeted delivery of cargo exploiting by receptor-mediated internalisation of Cet conjugated QDs. Impact of Cet conjugation on luminescence was again a small drop with respect to AS-2MPA QDs in an amount of 12, 20, and 13% in the case of AS-2MPA-ALA-Electrostatic-Cet, AS-2MPA-ALA-ADH-Cet, and AS-2MPA-ALA-Amide-Cet QDs, respectively (Figure 2-5).

2.4.2.4 Cetuximab conjugated QDs for simultaneous delivery of ALA and 5FU (AS-2MPA-ALA-Cet)

In the case of the QDs designed for targeted chemotherapy and PDT combination, only electrostatic loading of ALA was studied. 5FU was conjugated to AS-2MPA QDs via ester bonds with 50% efficiency producing particles with 15 mol% 5FU with respect to its carboxylic acid groups of the QDs. Then, Cet was conjugated (0.01 mol%) followed by ALA's electrostatic loading (30 mol%). The emission peak position of these AS-2MPA-ALA-Electrostatic-Cet-5FU QDs did not shift, but the intensity was reduced about 30% with respect to AS-2MPA (Figure 2-5a), which was accompanied with an increase in the hydrodynamic size from 49.8 to 114.4 nm with an expected drop in the zeta potential since -COOH groups are consumed (Table 2-1). Overall, the proposed EGFR targeting AS-QDs delivering 5FU and ALA were successfully synthesised in small sizes with strong emission at about 830 nm.

2.4.3 ALA release from QDs

Conjugation of hydrophilic ALA to the QDs is designed to restrict its release at physiological pH, but promote it in the acidic microenvironment of the tumour and within the endosome/lysosomes. Therefore, the release rate of ALA in PBS at pH 5.5 and 7.4 from all three ALA loaded QDs were determined at 37 °C in 48 h period. Protonation of carboxylic acids at acidic pH would weaken the electrostatic interaction between ALA and anionic QDs, resulting in its release over time. Electrostatically loaded ALA showed about 18% initial burst release from QDs at pH 5.5 followed by sustained release reaching to about 50% at 32 h (Figure 2-7a). At pH 7.4, the initial release was 12%, reaching a

plateau at 24% in about 4 h and finally to 30% in 32 h. This data represents adequate protection in the circulation with an enhanced release in acidic conditions.

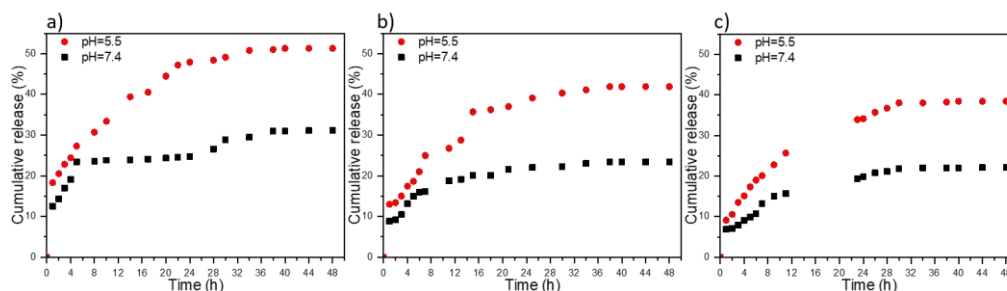


Figure 2–7: Release behaviour of (a) AS-2MPA-ALA-Electrostatic, (b) AS-2MPA-ALA-ADH, and (c) AS-2MPA-ALA-Amide at 37 °C at pH 5.5 and 7.4.

In AS-2MPA-ALA-ADH QDs, the initial burst release was lower at both pH values (Figure 2-7b). Total ALA release at neutral pH was only 19% within 12 h, but was ca 30% at pH 5.5 due to acid hydrolysis of the hydrazone bond. A plateau was reached after 30 h and ca 42% and 24% release at pH 5 and 7. ALA release from AS-2MPA-ALA-Amide QDs was the slowest as expected since the amide bond is the strongest bond used here at both pH values. In 24 h, 22% and 34% ALA was released at pH 7.4 to 5.5, respectively, but in 4 h the release was about 13 and 8% at acidic and neutral pH (Figure 2-7c). This pH-dependent ALA release is very desirable for cancer therapy since it minimises the amount of drug release during blood circulation while it introduces a sufficient amount of drug inside cancer cells.

Overall, as shown in Figure 2-7, the ALA release at pH 5.5 after 4 h was 25, 18, and 15 and after 24 h was 48, 39, and 34% from AS-2MPA-ALA-Electrostatic, AS-2MPA-ALA-ADH and AS-2MPA-ALA-Amide QDs, respectively. The release is faster from the electrostatically loaded QDs and slower with the covalently conjugated one, which slows down with the increasing stability of the bonds. This result demonstrates that all three conjugation methods could be tested for ALA-based PDT.

2.4.4 5FU release from QDs

When compared with the literature [160], [161], [162], [163], the release of the drug is extremely fast as much as the free drug (Figure 2-8). However, this fast release was needed for the combination therapy with the ALA [112], [161].

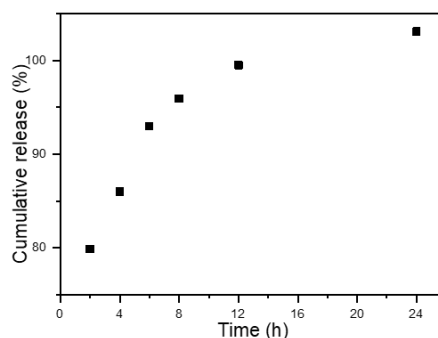


Figure 2-8: (f) Release behaviour of 5FU bound AS-2MPA-5FU in PBS at pH 7.4 at 37 °C.

2.4.5 Evaluation of EGFR targeting

EGFR targeting ability of Cet conjugated QDs were examined in three different cell lines with different EGFR expression levels. HT29, HCT116, and SW480 cell lines are low, intermediate, and high EGFR expressing cell lines, respectively. The internalisation of QDs by these three cell lines was determined qualitatively by fluorescence microscopy and quantitatively by ICP-MS after 4 and 24 h incubation of cells with QDs at 100 µg/mL [Ag] dose (Figure 2-9). Short incubation times were used to differentiate receptor-mediated endocytosis from passive uptake of QDs by the cells. According to ICP, which quantified the internalised QDs based on intracellular Ag concentration, none of these three cell lines differentiated the AS-2MPA from AS-2MPA-ALA family after 4 h incubation, indicating that ALA loading by either method did not affect the cellular uptake of the QDs (Figure 2-9a). On the other hand, SW480 cells accumulated 2-3 folds more AS-2MPA-ALA-Cet than HT29 and HCT116 cells but did not differentiate the Cet conjugated QDs in terms of ALA-loading chemistry. This is in agreement with the fluorescence microscopy images of QD treated cells after 4 h incubation. SW480 cells show a strong intracellular optical signal of QDs, while there is only a couple of luminescent cells in HCT116 and HT29 cell lines (Figure 2-9b-d).

After 24 h incubation, passive uptake of all QDs by all three CRC cell lines regardless of their EGFR-expression level was seen, as expected (Figure 2-10a-c). Collectively, these data indicate that Cet conjugation enables targeted delivery of QDs selectively to high EGFR-overexpressing SW480 cells. Therefore, there is a strong possibility to deliver high amounts of the pro-drug ALA specifically to high EGFR-expressing cancer cell lines in a short time and detect a collection of the QDs at the tumour site via optical imaging in the NIR, followed up by image-guided irradiation of the highlighted tissue

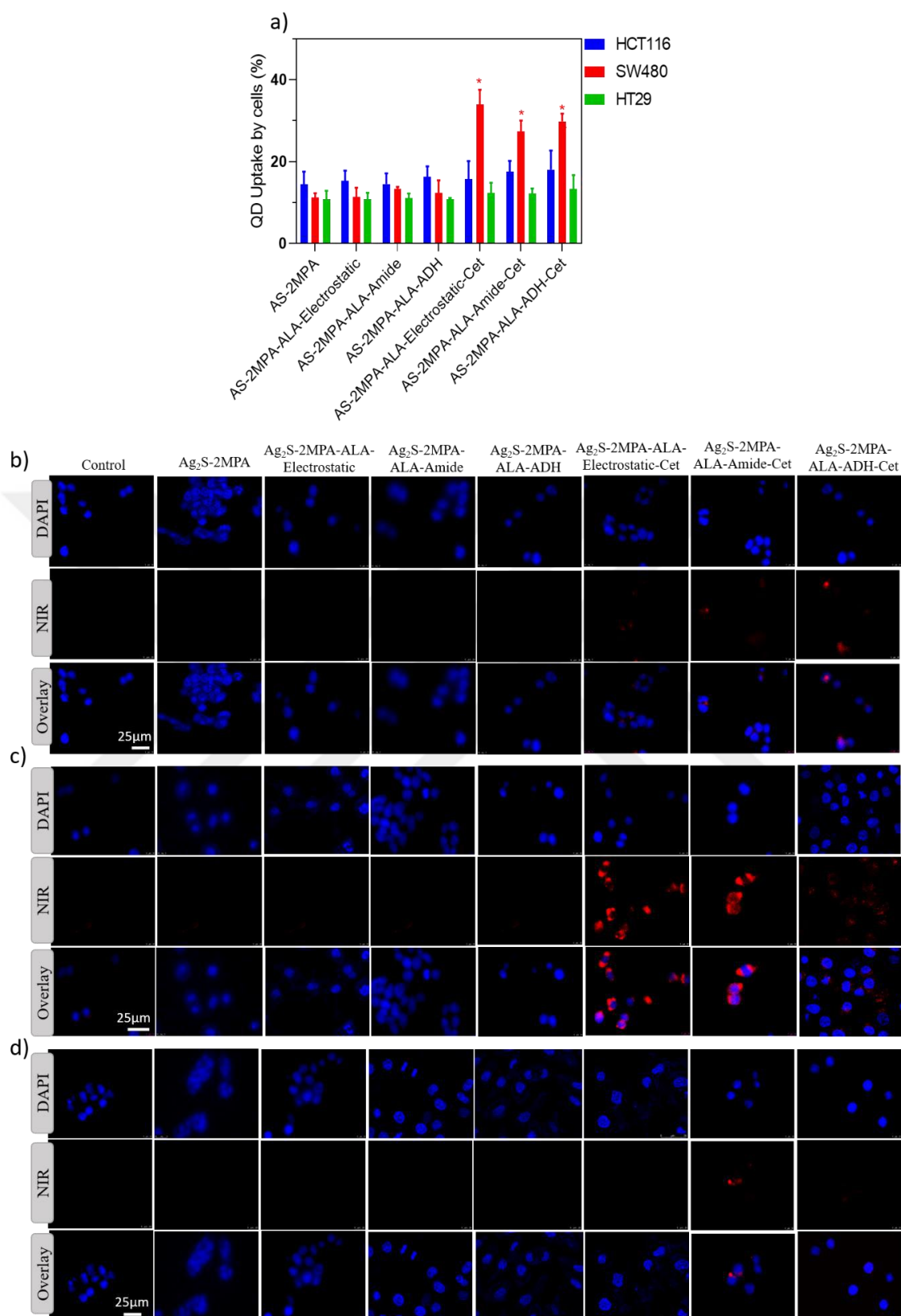


Figure 2-9: (a) Intracellular quantification of QDs in HCT116, SW480, and HT29 cells treated with QDs at 100 $\mu\text{g/mL}$ Ag concentration for 4 h. Quantification was based on intracellular Ag concentration determined by ICP-MS. The data are expressed as mean $\pm$ S.D. ($n = 3$), ($p < 0.05$). Fluorescence microscopy images of (b) HCT116, (c) SW480, and (d) HT29 cells incubated with different conjugates of AS-2MPA QDs for 4 h and control cells. Red: Luminescence from QDs

(excitation/emission:510-550/710 nm long-pass filter), Blue: Nuclear stain (excitation/emission: 325-375/435-485 nm). Scale bar for all figures is 25 μ m.

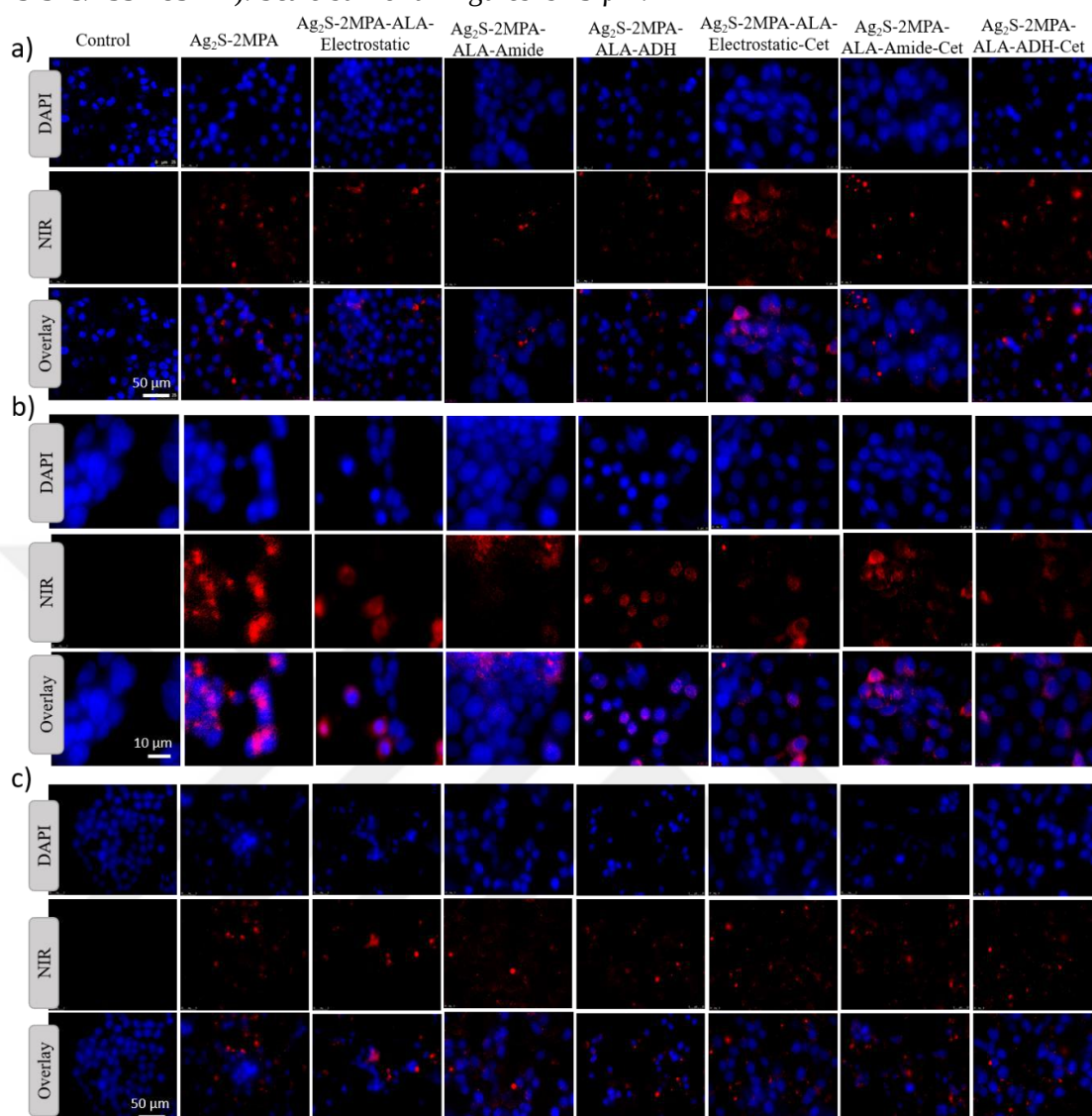


Figure 2-10: Fluorescence microscopy images of (a) HCT116, (b) SW480, and (c) HT29 cells incubated with different conjugates of AS-2MPA QDs for 24 h and control cells. Red: Luminescence from QDs (excitation/emission:510-550/710 nm long pass filter), Blue: Nuclear stain (excitation/emission: 325-375/435-485 /nm). Scale bar is 50, 10, and 50 μ m, respectively.

2.4.6 Evaluation of dark toxicity of AS-2MPA conjugates

Dark toxicity of all QDs produced here on all three CRC cell lines were evaluated by MTT and Alamar blue viability assays after 48 h incubation of QDs with the cells. Viability of a healthy colorectal cell line (CCD481) treated with these QDs was also determined to compare the selective cytotoxic activity of QDs to CRC cell lines. The dose-dependent toxicity was studied in the range of 25-500 μ g/mL based on the Ag content of different QDs, which was determined by ICP-MS, to keep the number of particles constant for each QD (Figure 2-11, Figure 2-12, Figure 2-13).

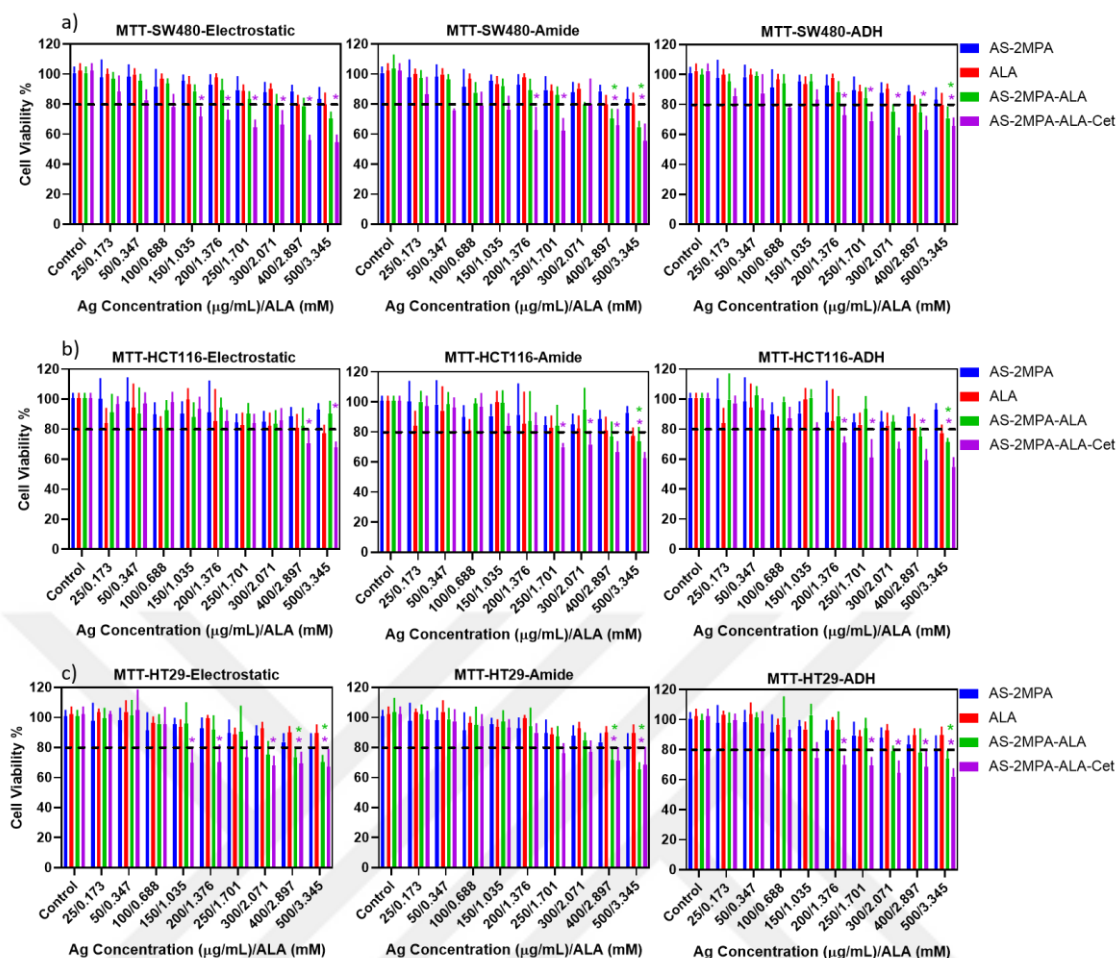


Figure 2-11: Viability of (a) HCT116, (b) SW480, and (c) HT29 cells treated with AS-2MPA QD conjugates at 25-500 $\mu\text{g/mL}$ [Ag] after 48 h incubation in 2D cell culture determined by MTT assay ($n=4$).

Considering the viability above 80% as non-toxic based on ISO 10993-5, ALA, AS-2MPA, and all three different ALA-conjugated QDs (AS-2MPA-ALA) exhibited no significant cytotoxicity up to 300 $\mu\text{g/mL}$ [Ag] based on MTT assay (Figure 3, S4). Furthermore, the *in vitro* cytotoxicity of all groups of QDs was dose-dependent on each cell line. Cet conjugation to QDs enhanced the cytotoxicity and showed dose-dependent and a more potent anticancer effect on SW480 cell lines than HCT116 and HT29. Viability of Cet conjugated QDs dropped down to ca 60-70% at 200 $\mu\text{g/mL}$ in all cell lines after 48 h (Figure 2-11). The increased cytotoxicity seen in Cet conjugated QDs agrees with the higher uptake of the particles by high- EGFR expressing SW480 cells (Figure 2-9).

5FU conjugation to EGFR targeting, ALA loaded QDs (AS-2MPA-ALA-Electrostatic-Cet-5FU) increased the cytotoxicity further in SW480 and HT29 cells (Figure 2-11, Figure 2-12, Figure 2-13). AS-2MPA-ALA-Electrostatic-Cet-5FU decreased the viability

of SW480 cells at 100 $\mu\text{g/mL}$ [Ag] to ca 65%, which corresponds to ca 30 $\mu\text{g/mL}$ [5FU] (Figure 2-12a). This indicates that Cet conjugated QDs can deliver 5FU to strongly EGFR targeting cells and an effective release of 5FU from the particles take place, causing some toxicity, which will be improved with the combination of PDT.

More than 90% of the healthy CRC cells (CCD481) survived at high dosages of the QDs (200 μg Ag/mL and ca 60 μg 5FU/mL) after 48 h incubation (Figure 2-12c), suggesting a great potential of these QDs as a chemo/phototherapy agent against cancer cells but not normal cells.

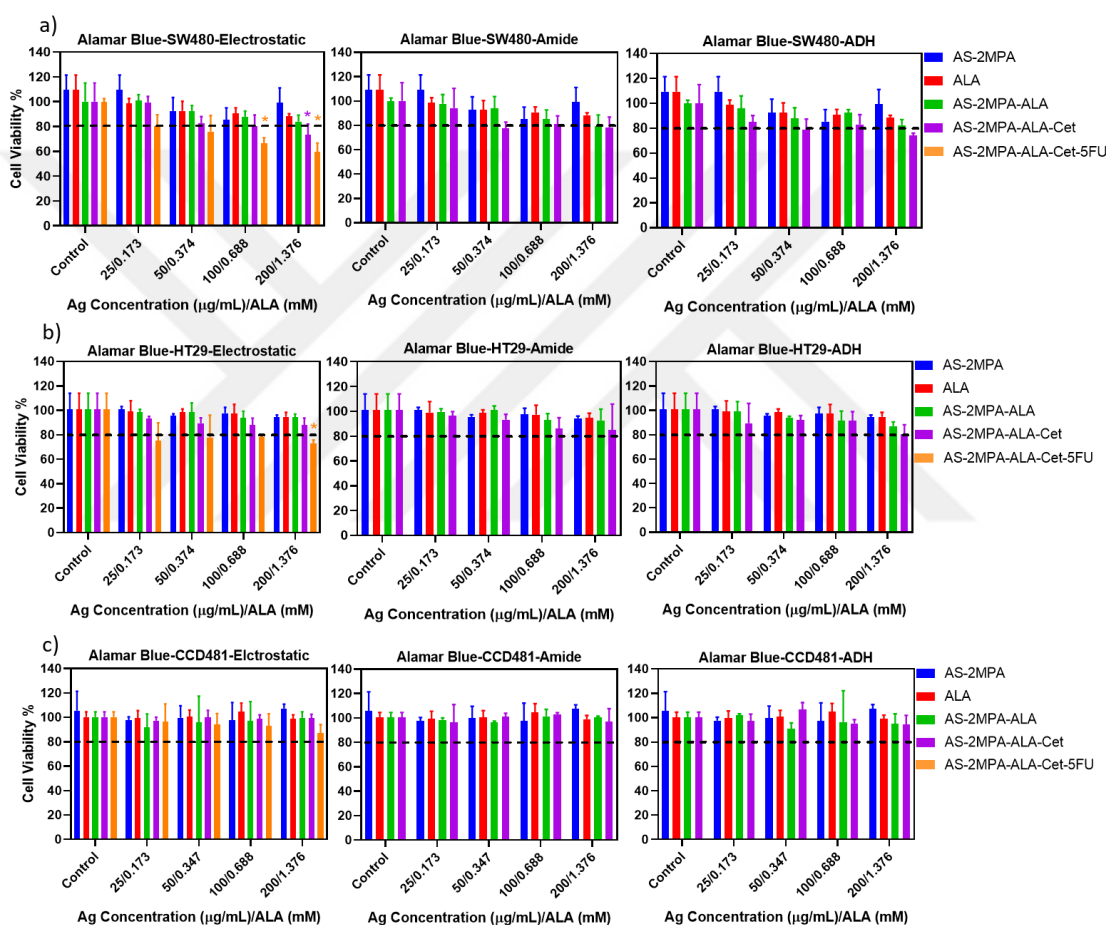


Figure 2-12: Viability of (a) SW480 and (b) HT29 cells treated with AS-2MPA QD conjugates at 25-200 $\mu\text{g/mL}$ [Ag] after 48 h measured by Alamar blue assay ($n=3$). (c) Viability of CCD481 cells (healthy colorectal cells) treated with AS-2MPA QD conjugates at 25-200 $\mu\text{g/mL}$ [Ag] after 48 h measured by Alamar blue assay ($n=3$).

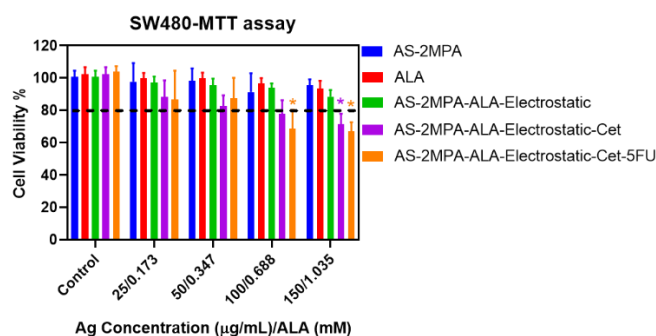


Figure 2–13: Viability of SW480 cells treated with AS-2MPA QD conjugates at 25-150 µg/mL [Ag] after 48 h incubation measured by MTT assay (n=4).

2.4.7 Intracellular PpIX generation

Effective ALA-based PDT requires efficient conversion of the prodrug into the active photosensitiser PpIX via heme pathway [164]. Cells vary in their ALA-PpIX conversion efficiency due to differences in their metabolic activity, iron metabolism, the number of mitochondria, and enzyme levels [165]. The efficiency of SW480, HCT116, and HT29 cells to convert ALA to PpIX was first determined using free ALA as a function of ALA concentration (25-800 µM) and incubation time (2-48 h). In all three cell lines, the mean fluorescence intensity of intracellular PpIX increased with ALA concentration up to a point (200-600 µM [ALA]), above which PpIX signal decreases, which may be due to enzyme saturation [165], [166]. This trend and concentration range agree well with the literature [167], [166], [67], [92]. Intracellular PpIX concentration also increased with the incubation time as expected [167], [67].

More importantly, a clear cell-type dependent difference in intracellular PpIX signal intensity was observed (Figure 2-14a). SW480 cells are the most efficient with the strongest PpIX signal, which is twice as strong as signal observed in HT29 cells. PpIX generation by HCT116 was extremely poor. Hence, SW480 is not only an excellent cell line for selective delivery of ALA but also to effectively convert it to PpIX for targeted enhanced PDT.

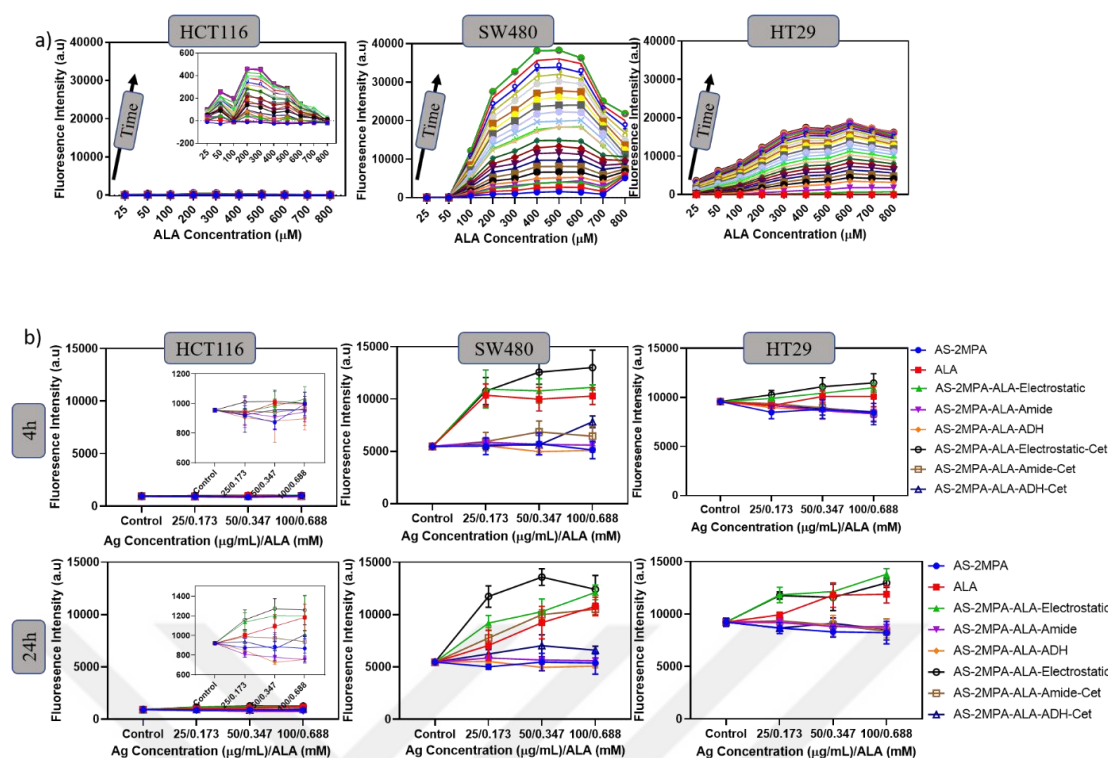


Figure 2-14: (a) Intracellular formation of PpIX from free ALA in HCT116, SW480, and HT29 cells during 48 h incubation. Measurements were done in 2 h intervals (n=4). (b) Intracellular formation of PpIX in HCT116, SW480, and HT29 cells after 4 and 24 h incubation with QDs or free ALA (PpIX λ_{exc} : 420 nm and λ_{em} : 635 nm).

Same study was performed with ALA loaded QD compositions between 0.173-0.688 mM [ALA] after short and long incubation times (4, 24 h) to elucidate the impact of EGFR targeting and the ALA loading chemistry on intracellular PpIX level of the three CRC cells (Figure 2-14b). Highest PpIX level was observed in SW480 cells at both incubation times, and the most effective AS-2MPA-ALA QD producing more PpIX than ALA was the AS-2MPA-ALA-electrostatic. This is supported by the highly efficient ALA-PpIX conversion in SW480 cells demonstrated above (Figure 2-14a) and the faster release of the prodrug from this conjugate with respect to covalently conjugated ones (Figure 2-7). PpIX level of the SW480 cells increased further and significantly above the levels of free ALA after Cet conjugation, clearly demonstrating the benefit of EGFR targeting. Impact of Cet conjugation on QDs had a slight effect on intracellular PpIX levels of the treated HT29 cells due to low-EGFR expression. But QDs with electrostatically loaded ALA provided slightly better intracellular PpIX accumulation than free ALA in HT29 cells, as well. No significant levels of PpIX were achieved in HCT116 cells, as expected. Therefore, HCT116 was eliminated from the rest of the study.

Fluorescence microscopy confirmed an increasing level of PpIX in SW480 cell line

treated with AS-2MPA-ALA-electrostatic and AS-2MPA-ALA-electrostatic-Cet compared to free ALA (Figure 2-15). NIR optical signal detected in CY8 channel confirmed QD internalisation parallel to PpIX generation, and improved internalisation with Cet conjugated QDs. Besides, 5FU conjugation to these QDs did not cause a detectable change in the uptake or the PpIX production as can be visualised from the intensity of the signal in TRITC and CY8 channels.

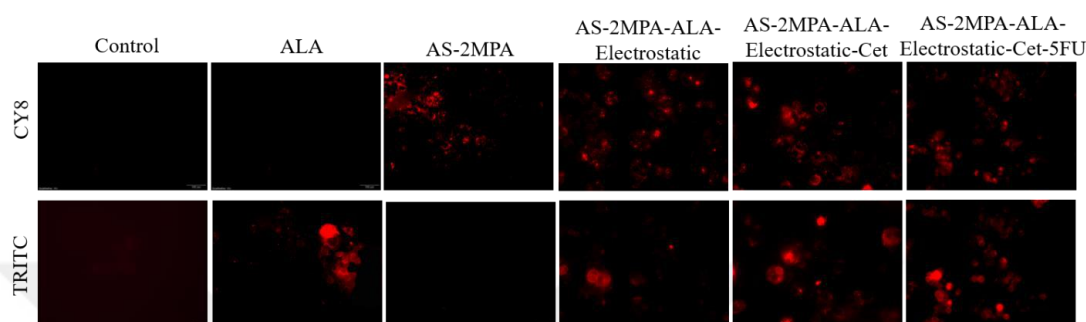


Figure 2-15: Fluorescence imaging of control cells, ALA, AS-2MPA, AS-2MPA-ALA-Electrostatic, and AS-2MPA-ALA-Electrostatic-5FU in 2D cell culture of SW480 cells. Samples at 100 µg/mL [Ag] were incubated with cells for 24 h. CY8 (λ_{exc} : 480 nm and λ_{em} : 810 nm) and TRITC (λ_{exc} : 545 nm and λ_{em} : 580-650 nm (band pass filter)).

In the 3D spheroids, QDs were incubated with the SW480 (for 4 and 24 h) and HT29 (for 24 h) cells to investigate further the cellular uptake and PpIX production in a more complex model. Bright fluorescence signals of the QDs (CY8 filter) and PpIX (TRITC filter) were observed in both SW480 and HT29 spheroids which indicated the successful intracellular uptake of QDs, ALA delivery and subsequent PpIX generation (Figure 2-16). Images also show that ALA was mainly taken up by the cancer cells than becoming bound in the collagen matrix, and much higher fluorescence was observed in the cancerous mass than in the surrounding. Control collagen spheroids without QD or its ALA conjugates showed no autofluorescence. High uptake of Cet-conjugated QDs was observed in SW480 spheroids even after 4 h as expected from its proven targeting ability in 2D cell cultures. Besides, the strongest PpIX signal was detected from 3D-cultures treated with AS-2MPA-ALA-electrostatic-Cet, as in the case of 2D cell cultures. Intracellular PpIX level of HT29 3D-cultures were lower despite of 24 h incubation due to relatively poor/slow ALA-PpIX conversion efficiency of this cell line (Figure 2-9, Figure 2-14, Figure 2-16). Moreover, the free ALA is localized selectively by cancer cells in 3D cells with cancer/normal cells in Figure 2-16 a, b.

Cumulative data here point out that loading ALA to AS-2MPA QDs tagged with Cet

improves the PpIX levels of the cells in an EGFR expression level-dependent manner and depending on the efficiency of the cells to convert the prodrug to active photosensitiser, and electrostatic loading of ALA is the most effective in delivery and release, facilitating the endogenous generation of high concentrations of PpIX.

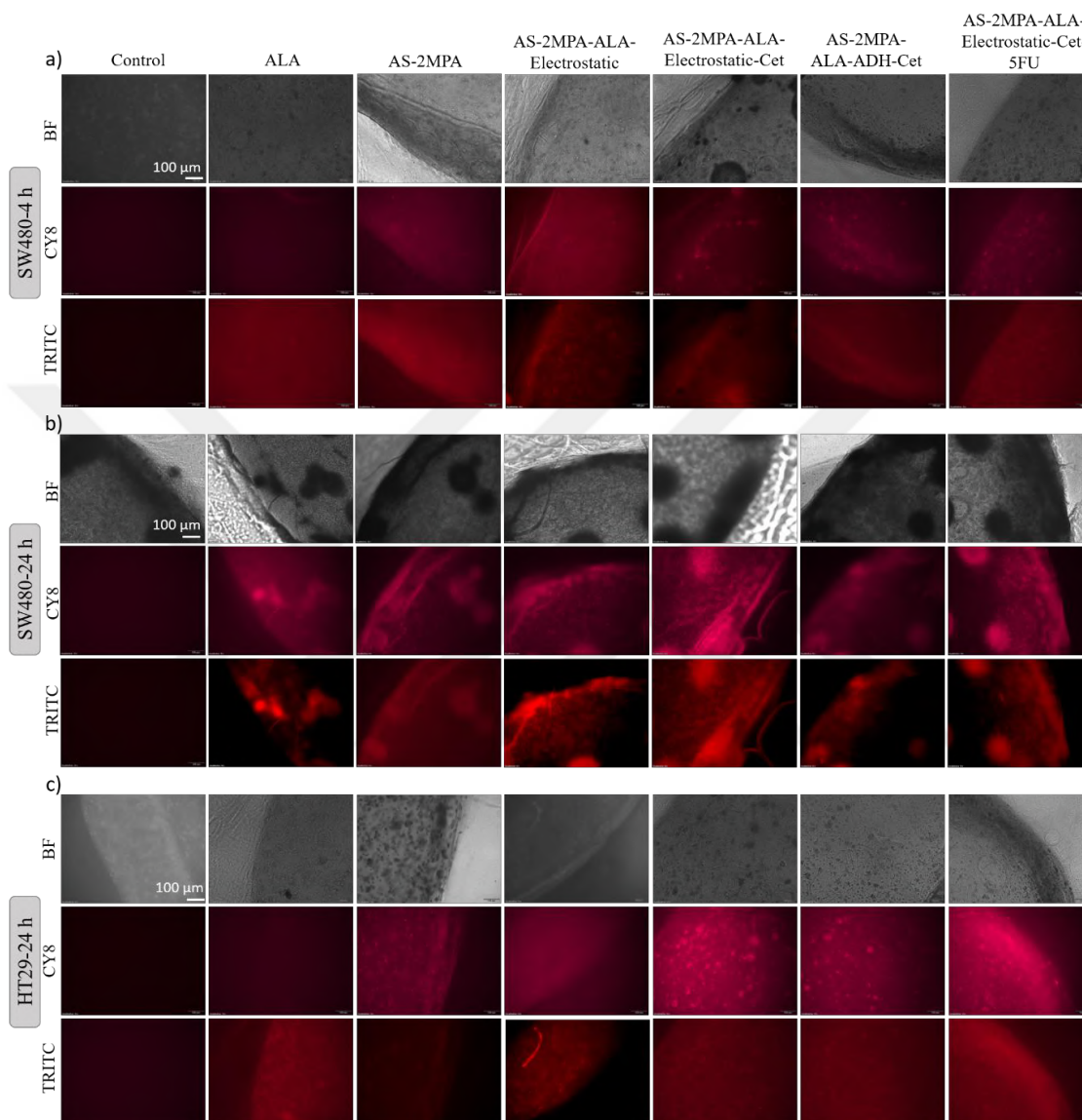


Figure 2-16: Intracellular quantification of QDs in SW480 after (a) 4h and (b) 24h, and (c) HT29 cells after 24h incubation of the NPs at 100 $\mu\text{g/mL}$ Ag concentration. CY8 (λ_{exc} : 480 nm and λ_{em} : 810 nm) and TRITC (λ_{exc} : 545 nm and λ_{em} : 620 nm). The scale bar is 100 μm .

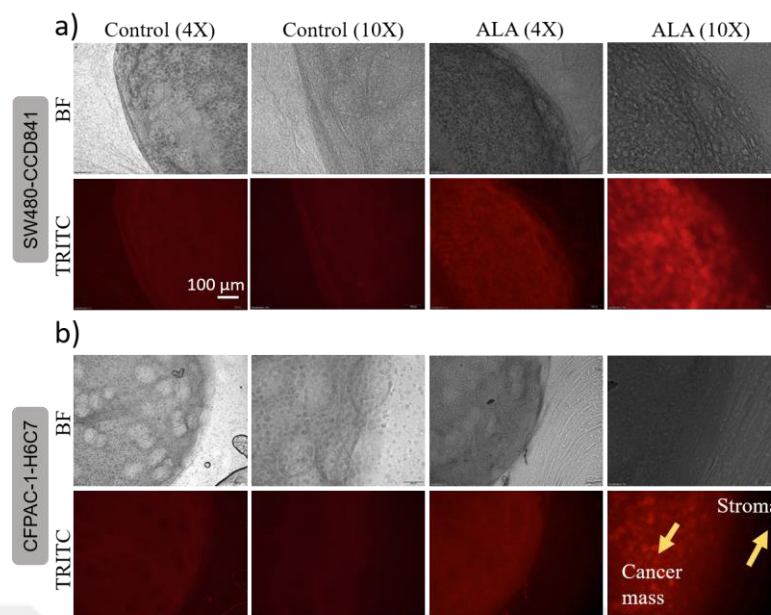
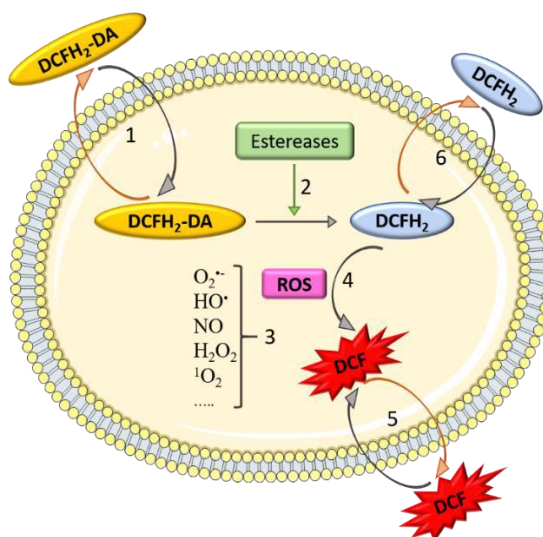


Figure 2–17: Intracellular quantification of QDs in (a) SW480 and CCD841 (normal colon cells) and (b) CFPAC1-H6C7 (normal human pancreatic duct epithelial) cells after 24h incubation with free ALA at 100 µg/mL Ag concentration. TRITC (λ_{exc} : 545 nm and λ_{em} : 620 nm). The scale bar is 100 µm.

2.4.8 ROS generation

A variety of active molecules derived as a result of molecular oxygen metabolism are called reactive oxygen species (ROS). ROS refers to a series of highly active and small molecules such as superoxide anion ($\text{O}_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\text{HO}^{\cdot}$), hydroperoxides, singlet oxygen ($^1\text{O}_2$), alkoxy radical ($\text{RO}^{\cdot}$), peroxy radical ($\text{ROO}^{\cdot}$), etc [46], [168]. Although ROS are produced in normal oxygen metabolism like the superoxide during electron escaping from mitochondrial respiratory chain, it was shown that they are involved in a variety of other cellular processes from cell proliferation to apoptosis [45], [168].

Although there is no “ideal” fluorescent probe detection of ROS based on the oxidation of the non-fluorescent reagent 2',7'-dichlorodihydrofluorescein (DCFH_2) to the highly fluorescent 2',7'-dichlorofluorescein (DCF) is one of the most widely used methods [168], [169]. The mechanism of action for DCFH_2 -DA (diacetate) is described schematically in Scheme 2-5. (1) The non-fluorescent DCFH_2 -DA pass through the lipophilic cell membrane. (2) The chemical activation and catalysis processes hydrolyse DCFH_2 -DA to DCFH_2 by esterase that is still non-fluorescent but is now membrane-impermeable. (3) DCFH_2 reacts with cellular ROS to form the highly fluorescent DCF [168].



Scheme 2-5: The formation mechanism of DCF in cells [168].

In a PDT process, the photoexcited photosensitizer molecule can transfer its energy to surrounding oxygen molecules to generate cytotoxic singlet oxygen ($^1\text{O}_2$), and/or undergo electron or proton transfer reactions with biomolecular substrates that lead to other cytotoxic reactive oxygen species (ROS) [170]. Therefore, the ability of the photosensitizer to generate ROS is one of the crucial factors that determine the efficacy of PDT. Intracellular PpIX should produce ROS in toxic level for an effective ALA-based PDT. SW480 and HT29 cells were treated with ALA, AS-2MPA-ALA, AS-2MPA-ALA-Cet, and AS-2MPA-ALA-Cet-5FU QDs at 100 $\mu\text{g/mL}$ [Ag] dose (0.688 mM [ALA]) and were exposed to light irradiation. Typically, blue light is used for the FDA approved ALA (Levulan) prodrug, whereas most other indications, especially multimodal therapies, use red light [92], [136], [91]. Therefore, PDT studies were performed with two different light sources: 1) 420 nm blue lamp for 5 min and, 2) 640 nm laser for 1 min. Then, ROS levels of the treated cells were measured from the green fluorescence intensity of ROS-oxidized 2',7'-dichlorofluorescein (DCFH) (Figure 2-18). AS-2MPA did not produce any ROS by itself, confirming ALA as the sole photosensitiser if irradiated at these wavelengths. In general, fast ALA releasing AS-2MPA-ALA-Electrostatic QDs produced more ROS than the covalently conjugated ones. The release of ALA from electrostatically loaded QDs (48%, 24 h) at acidic pH was more than hydrazone (38%, 24 h) and amide (34%, 24 h) conjugates resulting in the higher generation of PpIX and hence ROS (Figure 2-7, Figure 2-18). But, Cet conjugation increased the ROS levels of the cells treated with all QDs. Despite the low EGFR level of HT29, 24 h incubation allows significant QD internalisation (Figure 2-10). More efficient ALA-PpIX conversion of SW480 provides

higher ROS level than HT29 cells, which is best seen with electrostatically loaded ALA. Taken together, the nanocarriers can serve as promising photosensitising agents for PDT at both 420 nm and 640 nm.

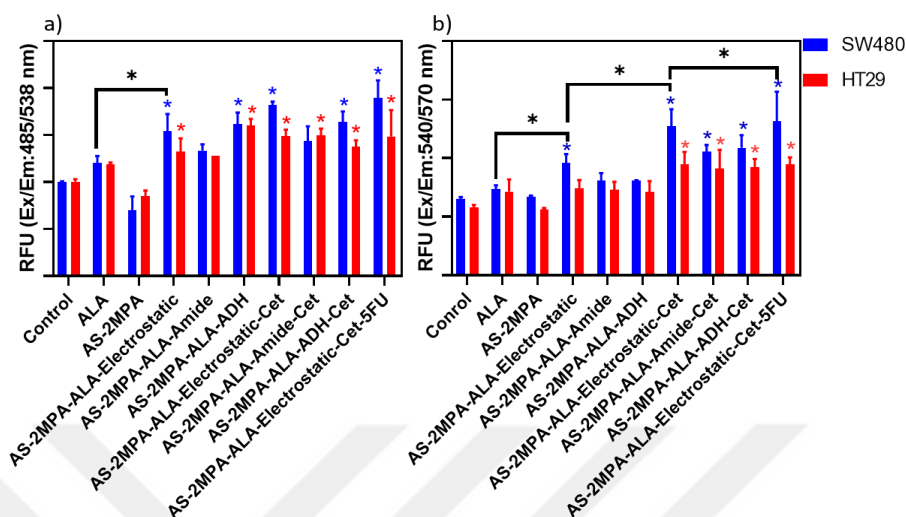


Figure 2-18: ROS production of SW480 and HT29 cells upon incubation with ALA, AS-2MPA, AS-2MPA-ALA, and AS-2MPA-ALA-Cet QDs at 100 $\mu\text{g/mL}$ [Ag] after 24 h incubation upon illumination of (a) blue lamp for 5 min and (b) 630 nm laser for 1 min. The data are expressed as mean $\pm$ S.D. ($n = 3$), ($p < 0.05$).

2.4.9 PDT studies

Phototoxicity of the QD compositions were evaluated in 2D and 3D cell cultures. Cells were treated with QDs between 25-100 μg [Ag]/ml corresponding to 0.173-0.688 mM [ALA] for 4 h (SW480) and 24 h (SW480 and HT29). At these concentrations, no significant dark toxicity was observed. Short incubation time was not tested for HT29 due to lower uptake and poorer ALA-PpIX conversion compared to SW480 cells. Treated cells were irradiated with a 420 nm blue lamp for 5 min and 640 nm laser for 1 min, separately. No phototoxicity was observed on either cell line when only subjected to irradiation (laser control) confirming that the laser protocol itself is safe. No toxicity was observed on cells treated with AS-2MPA+light, indicating QDs do not cause phototoxicity in the absence of ALA (Figure 2-19, Figure 2-20). This is in agreement with the no ROS generation in the absence of ALA (Figure 2-18). Hence, any observed cytotoxicity will be related to ALA-PDT. Irradiation of cells treated with ALA or ALA loaded QDs showed incubation time, dose, cell type, ALA loading/conjugation chemistry, and irradiation wavelength-dependent phototoxicity.

Table 2-2: the half maximal effective inhibitory concentration (IC₅₀) of AS-2MPA, AS-2MPA-ALA, AS-2MPA-ALA-Cet, AS-2MPA-ALA-Cet, and AS-2MPA-ALA-Cet-5FU QDs after PDT

at 420 and 630 nm in ALA concentration (mM).

Samples	SW480 cells, 4h		SW480 cells, 24h		HT29 cells, 24 h	
	420nm	630nm	420nm	630nm	420nm	630nm
ALA	-	-	-	-	-	-
AS-2MPA	-	-	-	-	-	-
AS-2MPA-ALA-Electrostatic	-	0.137	0.135	0.126	0.491	0.122
AS-2MPA-ALA-ADH	0.398	-	0.682	0.284	-	-
AS-2MPA-ALA-Amide	-	-	0.368	0.545	-	-
AS-2MPA-ALA-Electrostatic-Cet	-	0.129	0.106	0.104	0.524	0.098
AS-2MPA-ALA-ADH-Cet	0.359	0.257	0.120	0.187	-	0.368
AS-2MPA-ALA-Amide-Cet	-	0.347	0.233	0.178	-	0.395
AS-2MPA-ALA-Electrostatic-Cet-5FU	0.329	0.106	0.101	0.092	0.299	0.084

Treatment at 420 nm (Figure 2-19): Both free ALA and AS-2MPA-ALA compositions showed a higher phototoxic effect on SW480 cells than HT29 as expected based on better efficiency of the former to produce PpIX (Figure 2-19). In general, QD with electrostatically loaded ALA was the most effective, followed by the hydrazone and amide linked ones, respectively, parallel to the ALA release rate from these conjugates.

In short incubation time, the phototoxicity of free ALA was insignificant with viabilities at and above 80% within the whole concentration range. Similarly, QDs with covalently conjugated ALA did not cause significant phototoxicity in SW480 cells after 4 h incubation, suggesting the insufficient level of released ALA and intracellular PpIX accumulation at short incubation time. However, QDs with electrostatically loaded ALA reduced the viability of this cell line to 53% at 0.347 mM ALA concentration after irradiation. This was reduced further to 43% when the particle was conjugated with Cet (AS-2MPA-ALA-Cet-electrostatic) increasing intracellular ALA concentration and to 38% when 5FU (15 µg/mL) was co-loaded, exhibiting a stronger therapeutic effect via combination therapy. Considering that free 5FU reduced the viability to 71 and 52 % at 10 and 25 µg/mL after 48% incubation (Figure 2-21), data present a clear synergistic effect.

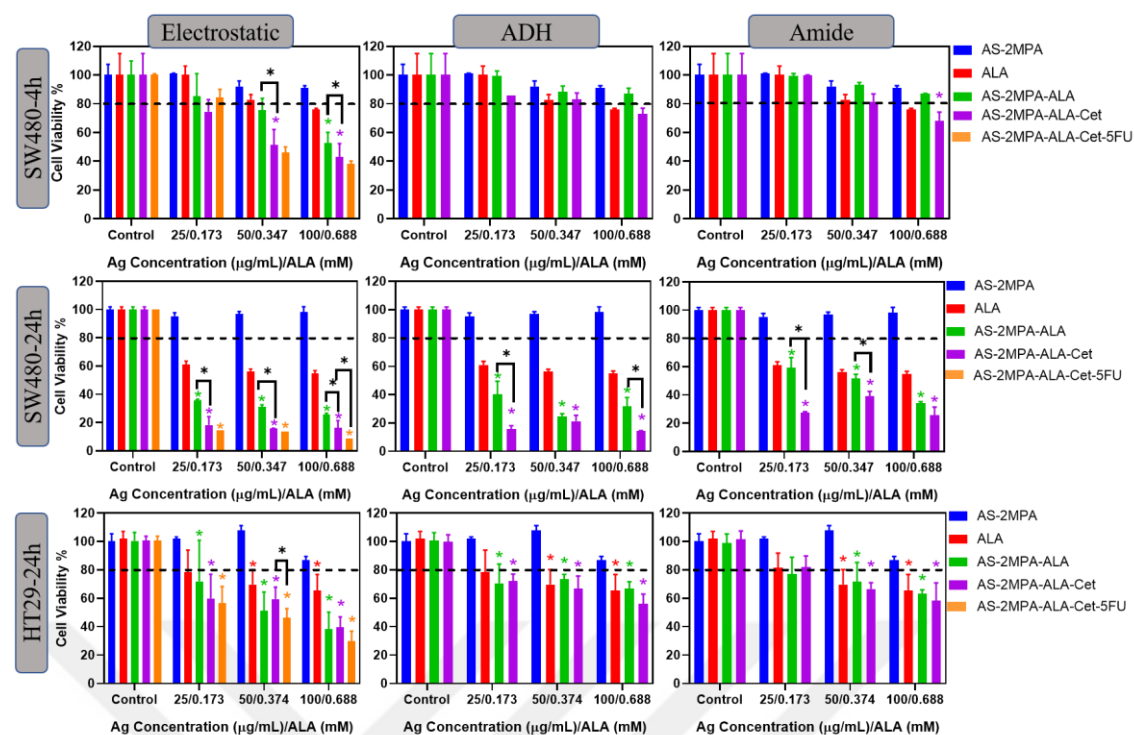


Figure 2-19: Viability of AS-2MPA, AS-2MPA-ALA, AS-2MPA-ALA-Cet, and AS-2MPA-ALA-Cet-5FU QDs in SW480 (4 and 24h) and HT29 (24h) cell lines treated with QDs illuminated at 420 nm blue light for 5 min in 2D cell culture. MTT assay were used measurement of cell viability. The data are expressed as mean $\pm$ S.D. ($n = 4$), ($p < 0.05$).

Treatment at 640 nm (Figure 2-20): An interesting observation is the higher phototoxicity observed after 640 nm irradiation of cells treated with ALA delivering QDs, while still no phototoxicity was observed in the absence of ALA. This was most significant in the case of SW480-4 h and HT29-24 h incubation, where the phototoxicity was lower compared to SW480-24 h incubation (Figure 2-20) which was already very efficient with more than 80% reduction in viability with the targeted QDs. Although still mostly above 80%, free ALA seems to reduce the viability at 640 nm slightly better than 420 nm. According to Wawrzyniec *et al.* SW480 cells treated with 0.5 mM ALA at 10 and 30 J/cm² (wavelength 600–720 nm) have a viability around 85% which is comparable to the free ALA toxicity seen here, after 640 nm irradiation [171]. Although the blue light (420 nm) which hits the Soret band of the PpIX seems more suitable for ALA-PDT due to stronger absorbance, significantly higher fluence obtained with 640 nm-diod laser (15.5 J/cm²) than the one at 420 nm-lamp (2.1 J/cm²) coupled with less interaction of the long-wavelength light with biological species could provide superior PDT. Several studies have demonstrated ALA-based PDT using different light sources such as lamps, LEDs, or lasers [57], [172] and suggested that the coherent emission of the laser along with deeper tissue penetration of red light improves the PDT efficiency

with 640 nm lasers [172], [173], [174].

Short incubation followed by 640 nm irradiation of the cells treated with AS-2MPA-ALA-Electrostatic and AS-2MPA-ALA-Electrostatic-Cet QDs reduced the viability of SW480 cells below 40% even at the lowest concentration, indicating strong phototoxicity generated at 640 nm reaching to 11% with Cet conjunction at 0.688 mM [ALA]. On the other hand, free ALA+640 nm light reduced the viability to about 80%. Loading of ALA (0.1 mM) to PEGylated chitosan doubled the toxicity in CT26 CRC cell line after irradiation with 635 nm LED [175]. Compared to this, more dramatic enhancement over ALA-phototoxicity was observed with QD loaded with ALA and even more with Cet targeting confirming the success of the design presented here. Combination therapy increased the toxicity further, almost to the level of complete loss of viability even after 4 h incubation at 15.05-30.1 $\mu\text{g/mL}$ 5FU/0.374-0.688 mM [ALA] concentration. Enhanced phototoxicity over free ALA was also observed at the same conditions (4h+640 nm) with AS-2MPA-ALA-ADH, and AS-2MPA-ALA-Amide as well, while they were not very useful at 420 nm irradiation. They have also displayed improved phototoxicity when conjugated with Cet reaching to IC₅₀ and below at and above 0.347 mM ALA concentration.

In the case of HT29 cells, a dramatic improvement in the therapeutic outcome was observed at 640 nm irradiation. Even AS-2MPA-ALA-ADH and AS-2MPA-ALA-Amide caused more than 60% loss of viability when tagged with Cet after irradiation. All QD compositions with electrostatically loaded ALA, caused more than 60% reduction in viability, reaching to almost complete loss of viability with Cet conjugation and combination therapy at all concentrations. At the lowest concentration, combination therapy seems to improve the toxicity over the PDT-monotherapy more significantly, since at higher doses PDT already caused dramatic toxicity alone. This level of toxicity is quite encouraging for CRC. The most relevant study on ALA-PDT of HT29 cell line, reported about 50% cell killing at 1 mM ALA concentration with blue light irradiation and about 20% with red light at 3 J/cm² using LEDs [133] which provided 88% tumour inhibition after three treatments of 250 mg ALA/kg-blue light irradiation (32 J/cm²). Therefore, targeted ALA-based PDT and its combination with 5FU improved the known ALA-PDT of CRC cells dramatically.

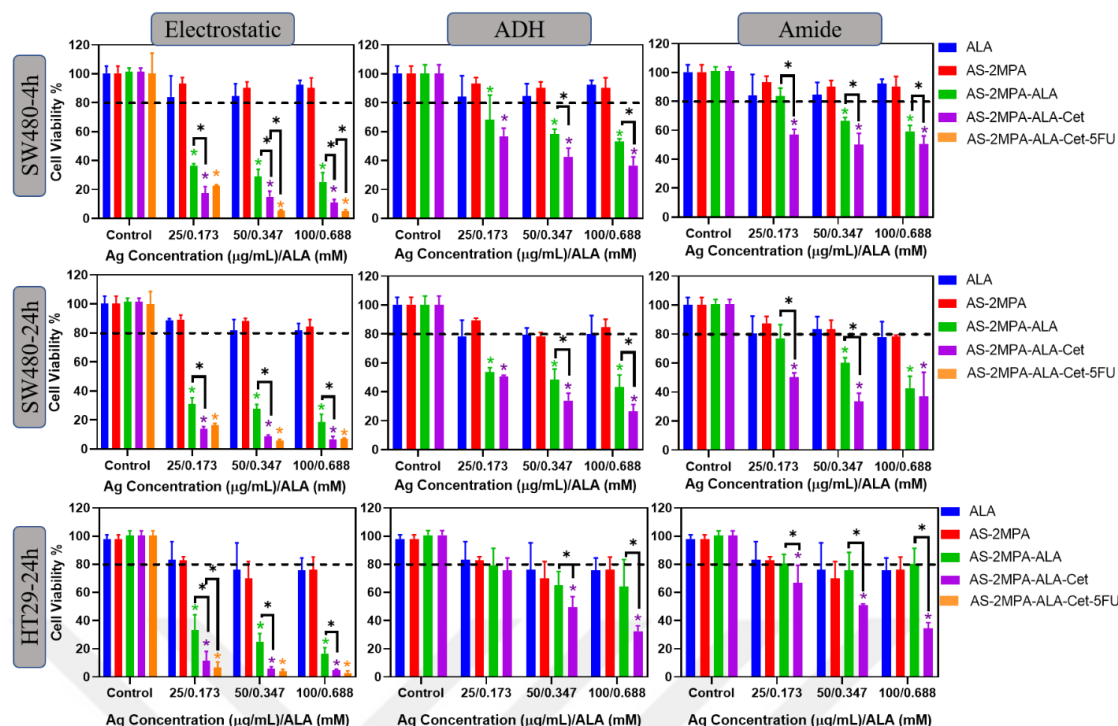


Figure 2–20: Viability of AS-2MPA, AS-2MPA-ALA, AS-2MPA-ALA-Cet, and AS-2MPA-ALA-Cet-5FU QDs in SW480 (4 and 24h) HT29 (24h) cell lines treated with QDs illuminated at 640 nm laser for 1 min in 2D cell culture. MTT assay were used measurement of cell viability. The data are expressed as mean $\pm$ S.D. (n = 4), (p < 0.05).

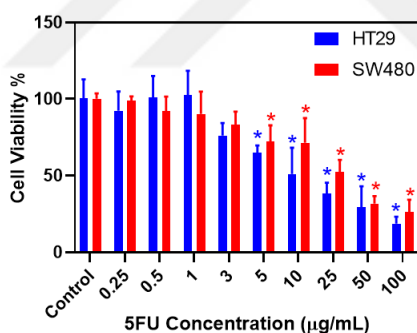


Figure 2–21: Viability of SW480 and HT29 cells treated with free 5FU at after 48 h incubation measured by MTT assay (n=3).

2.4.10 Effect of combination of targeted PDT and chemotherapy

Prompted by the efficacy of the QD nanocarriers in the 2D cell cultures, we also investigated their PDT efficacy on 3D spheroids grown in compressed collagen scaffolds. Improvement of cell organisation, enhancement of expression of differentiated functions, multicellular resistance, and limited drug penetration are some advantages of using 3D models over 2D cell culture [176]. Therefore, 3D models provide a more representative model for determining the therapeutic response *in vitro*. Since MTT is not suitable to check viability in 3D cell cultures, Alamar blue was used to determine the viability of

cells before and after PDT in 3D spheroids. The 3D spheroid models were incubated with all QD groups at 100 $\mu\text{g/mL}$ [Ag] dose for 24 h, then irradiated with 420 nm blue light for 5 min. Dramatic reduction in viable cell population was observed in both SW480 and HT29 3D spheroids upon light irradiation (Figure 2-22). Viability of SW480 cells in spheroids treated with free ALA, AS-2MPA-ALA-Electrostatic, AS-2MPA-ALA-ADH, and AS-2MPA-ALA-Amide were 93-96% in dark but was reduced to 59, 47, 67 and 57% after PDT treatment. Electrostatically loaded ALA was again most efficient and better than free ALA that is in agreement with data obtained by 2D cell culture. Yet, when QDs were directed to cells with Cet conjugation, all QDs were significantly more effective than free ALA, and live-cell fraction was only 28, 29, and 35 %, for the electrostatically loaded, ADH, and amide linked conjugates, respectively.

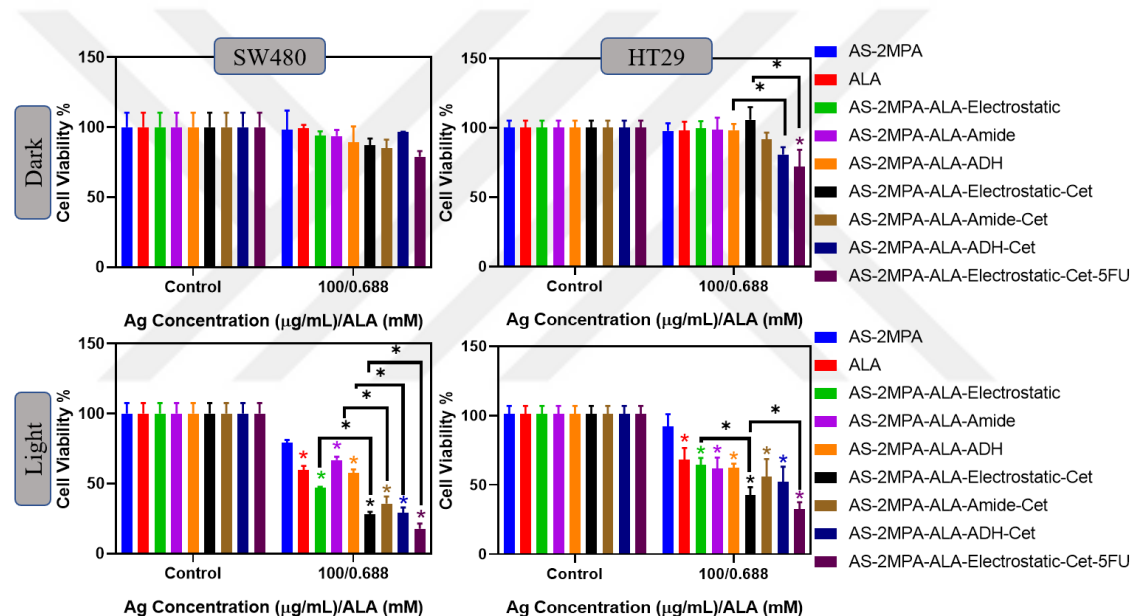


Figure 2-22: (a) Viability of AS-2MPA, AS-2MPA-ALA, AS-2MPA-ALA-Cet, and AS-2MPA-ALA-Cet-5FU QDs in SW480 and HT29 3D spheroid models treated with [Ag]: 100 $\mu\text{g/mL}$ QDs for 24 h and illuminated with 420 nm blue light for 5 min. Alamar blue assay were used measurement of cell viability. The data are expressed as mean $\pm$ S.D. (n = 3), (p < 0.05).

In HT29 3D spheroids, AS-2MPA-ALA did not show a clear advantage over free ALA, but Cet conjugated QDs triggered a greater cell killing and more effective PDT with 42, 52, and 56% viable cell count when treated with electrostatically loaded, ADH, and amide conjugated QDs, respectively. In both 3D spheroids, combination therapy (targeted chemo/PDT) almost doubled the toxicity over targeted PDT monotherapy, and the viability was reduced to 18 and 32% in SW480 and HT29 spheroids, respectively. All trends are in agreement with 2D data. But in general, toxicity was lower in 3D spheroids, which is possibly due to higher chemo/phototherapy resistance of spheroids than 2D

culture [177].

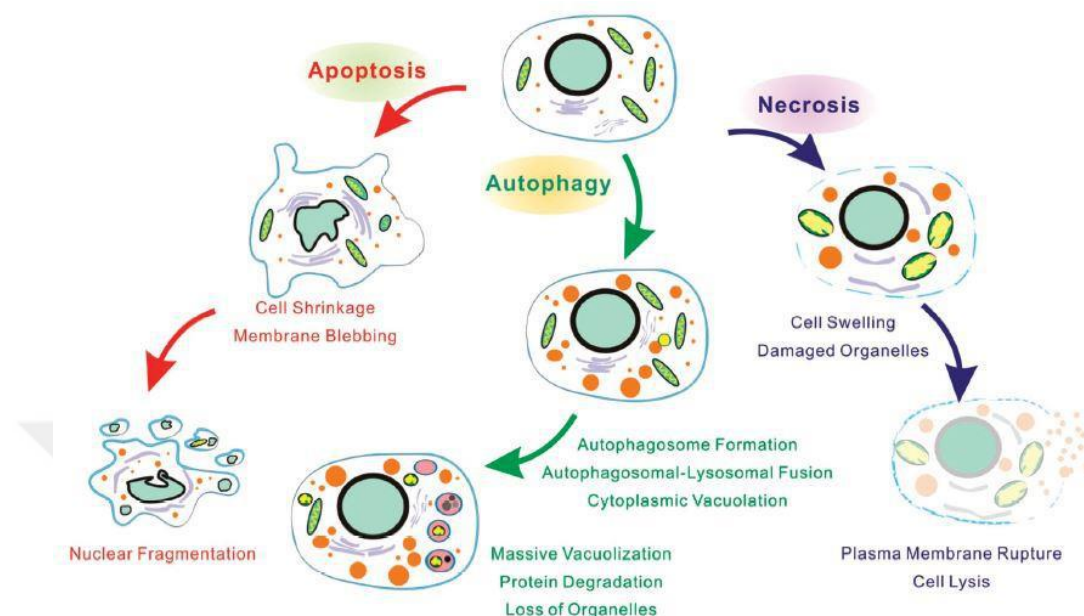
2.4.11 Mechanisms of Death

2.4.11.1 Apoptosis/necrosis

In 1972, Kerr and co-workers utilized the word “apoptosis” for the first time to describe the change in the morphology of dead cells [178], [179]. This was characterized with cellular fragmentation, nuclear and cytoplasmic condensation in which these fragments and apoptotic bodies were degraded by phagosomes without any inflammation (Scheme 2-6) [179]. The term apoptosis comes from a Greek word meaning “falling off” as leaves from a tree. The word ascribes a controlled physiological process of removing organism’s components without damage to the other organisms. Apoptosis can be triggered by: 1) intrinsic stimuli via mitochondrial signalling pathway and 2) extrinsic stimuli by cell surface receptors such as Tumour Necrosis Factor- α (TNF- α), First Apoptosis Signal (FAS), and TNF-Related Apoptosis Inducing Ligand (TRAIL) [59], [179]. In either case, a family of cysteine aspartyl proteases known as caspases or cysteine-dependent aspartate specific proteases leads to mitochondrial membrane permeabilization, cellular fragmentation, and chromatin condensation resulting in the destruction of cells [51], [178]. The caspases are divided into two groups: those that are involved in apoptosis (caspase 2, 3, 6, 7, 8, 9, and 10) and those related to caspase 1 (caspase 1, 4, 5, 13, 14, 11, 12) whose primary role is in cytokine processing during inflammatory reactions [51], [178]. Characteristic morphology of apoptosis includes rounding up of the cells, condensation of chromatin, nucleus fragmentation, and shedding of apoptotic bodies. While necrosis is characterized by swelling of organelles, such as mitochondria and endothelial reticulum, rupture of plasma membrane, and lysis of the cells mostly with inflammatory reactions [59], [178]. Mechanistically, necrosis is generally associated with the inflammation and not activation of caspases. Briefly, Samali and co-workers have simplified the caspase-dependent apoptosis with the following morphological features [180]:

- Cytoplasmic and nuclear condensation
- Chromatin cleavage
- Formation of apoptotic bodies
- Maintenance of an intact plasma membrane

- Exposure of surface molecules targeting cell corpses for phagocytosis (phosphatidylserine)
- Proteolytic activity of certain caspases (caspase 2, 3, 6, 7, 8, 9, and 10)



Scheme 2-6: Multifunctional features of apoptosis, autophagy, and necrosis [179].

There are a variety of techniques and reagents to study the cell death. But, most of these methods are not adequate enough to prove the mechanism of cell death when used alone [178]. Therefore, a couple of experiments are performed mostly based on measurement of particular characteristics associated with cell death such as DNA fragmentation, apoptotic body formation, and caspase-dependent measurement to report the extent of apoptosis and necrosis [51]. A variety of fluorescent dyes and histological stains can be utilized to demonstrate the cell death mechanism. However, identifying the exact mechanism of death is sometimes complicated simply by visualizing the morphological changes. In particular, it is hard to differentiate the morphology of condensed chromatin in oncotic cells from apoptotic cells. Thus, several methods underlying biochemical processes have been developed [178]. Detection of DNA fragmentation is one of the widely used techniques in cell death studies using gel electrophoresis or commercial kits. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) methods show the activity of the terminal deoxynucleotidyl transferase enzyme to label the 3' ends of DNA strand breaks, which can be identified in individual nuclei by microscopy [178], [181]. Another method focuses on the determination of the structural integrity of the plasma membrane as a hallmark of necrosis. This technique basically,

depends on the release of cytosolic enzymes, including lactate dehydrogenase and uptake of membrane-impermeant dyes such as trypan blue, propidium iodide, and 7-aminoactinomycin D upon structural damage of the plasma membrane [159], [178]. As mentioned earlier, the externalization of phosphatidylserine changes the asymmetry of its distribution in apoptotic cells hence is also used in the identification of the cell death mechanisms. Phosphatidylserine exposure on the outer leaflet of the plasma membrane can be recognized by phagocytes as a signal of dying cells [178]. Loss of phospholipid asymmetry is characterized using Annexin V experimentally by flow cytometry or fluorescent microscopy. The translocation of phosphatidylserine on cells' surface is an early apoptosis event while the plasma membrane still intact [51], [178].

Based on observed toxicity of the targeted PDT via ALA delivering QDs and the targeted combination therapy in SW480 2D cell cultures and 3D spheroids, contribution of apoptotic/necrotic cell death in these therapeutic approaches were studied using Annexin V-FITC/PI co-staining assay. Cells were treated with QDs at 100 $\mu\text{g/mL}$ [Ag] for 24 h and then were irradiated at 420 nm (5 min) (Figure 2-23). Apoptotic cells were indicated with green fluorescence, and cells that lost cell membrane integrity showed red fluorescence.

Accordingly, cells treated with either free ALA or QD conjugates mostly have red luminescence suggesting late apoptotic/necrotic cell death and QD conjugates were more effective than free ALA in 2D cell cultures. In 3D spheroids mostly apoptotic cells were observed with an increasing amount of late/apoptotic/necrotic cells with Cet conjugated QDs. Hence, both in 2D and 3D, a clear advantage of targeted delivery of ALA to CRC cells and combination therapy in driving cells to apoptotic cell death was observed.

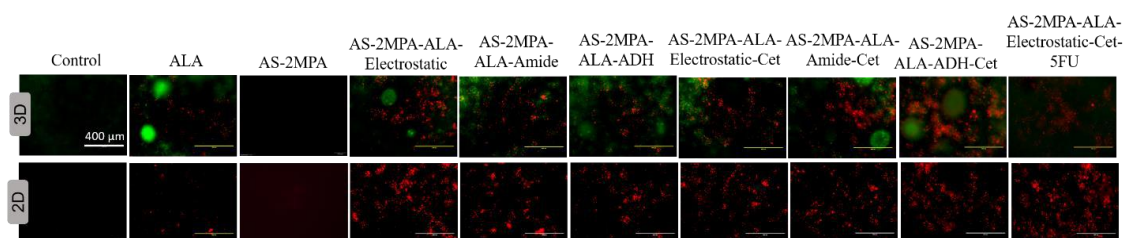


Figure 2–23: Apoptotic and necrotic induced cell death in 2D and 3D compressed constructs of SW480 cells. Comparison of cell death pathways induced after PDT (420 nm with blue lamp for 5 min) with ALA, AS-2MPA, AS-2MPA-ALA, and AS-2MPA-ALA-Cet at 100 $\mu\text{g/mL}$ [Ag] after 24 h incubation. Annexin V was used to indicate apoptosis (green) and propidium iodide was used to indicate necrosis (red).

2.4.11.2 Live/dead assay

The high toxicity of PDT and PDT/chemo combination observed in 2D and 3D models were further confirmed with live/dead assays using Calcein-AM/propidium iodide (PI) co-staining. This study was performed after 4 h incubation of the cells with the test materials to highlight the clear benefit of targeting and observe cell-based and QD-conjugate chemistry-based differences. In the absence of laser, none of the reagents caused a significant cell killing, and in the absence of ALA, no photo-damage was observed due to irradiation at 420 nm or 640 nm in either 2D or 3D models since all these cells showed a vivid green fluorescence indicating live cells (Figure 2-24, Figure 2-25). This confirms that any dead cell population in all other cases will be due to PDT and combination therapy. As seen in Figure 2-24, a significant population of the red luminescent dead cell population was observed in SW480 cells in 2D cell culture, and when ALA was delivered with Cet conjugated QDs, while cells treated with free ALA show mostly green fluorescence of live cells. Besides, the greatest cell killing was observed with EGFR targeting QDs delivering both ALA and 5FU, in agreement with viability assays and highlighting the superiority of both targeting and combination therapy over monotherapy. In HT29 cells, only AS-2MPA-ALA-Cet and AS-2MPA-ALA-ADH-Cet provided a noticeable dead cell population, while the combination therapy was the most successful approach again to achieve an appreciable cell killing.

Imaging of the 3D spheroids was more challenging since focusing is more difficult (Figure 2-25). But overall, significant concentrations of dead cells were observed in spheroids treated with the Cet conjugated particles, especially with the electrostatically loaded or hydrazone linked ALA, and more in SW480 spheroids, as in the case of 2D cell cultures. Also, the combination therapy showed a clear benefit in both cell lines.

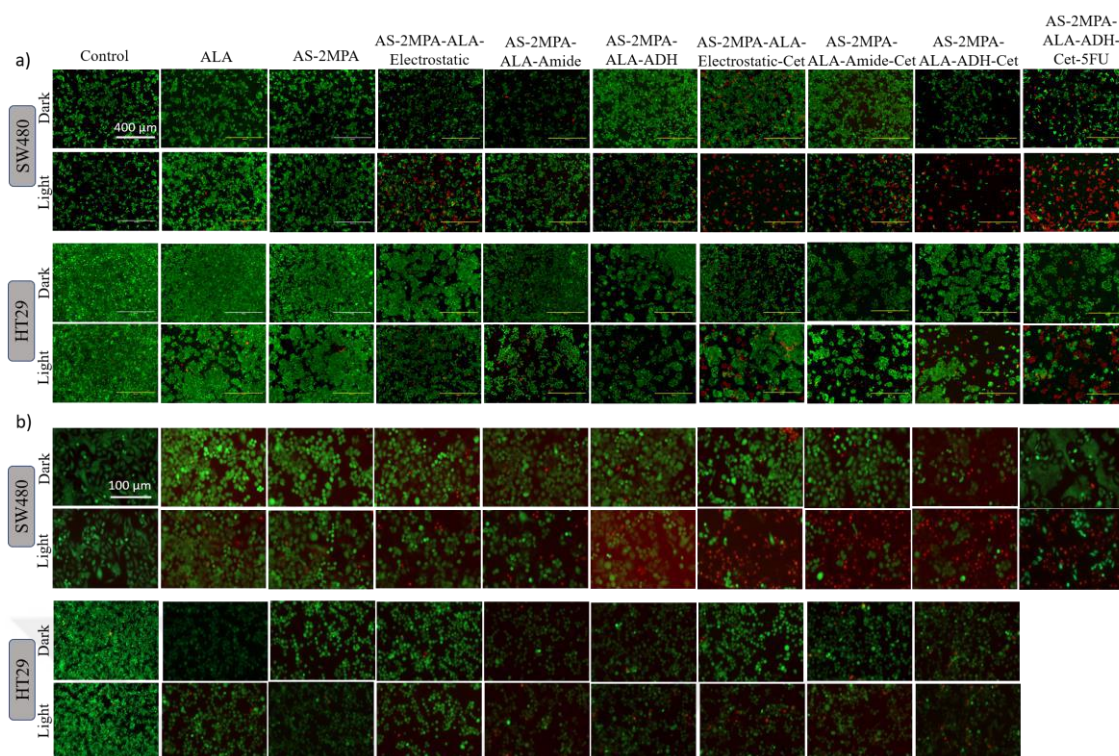


Figure 2–24: Live/dead images of SW480 and HT29 in 2D cell cultures. The cultures were treated with free ALA, AS-2MPA, AS-2MPA-ALA, AS-2MPA-ALA-Cet, and AS-2MPA-ALA-Cet-5FU QDs at 100 $\mu\text{g/mL}$ [Ag] after 4 h incubation with/without PDT illuminated at (a) 420 nm with blue lamp for 5 min and (b) 640 nm laser for 1 min.

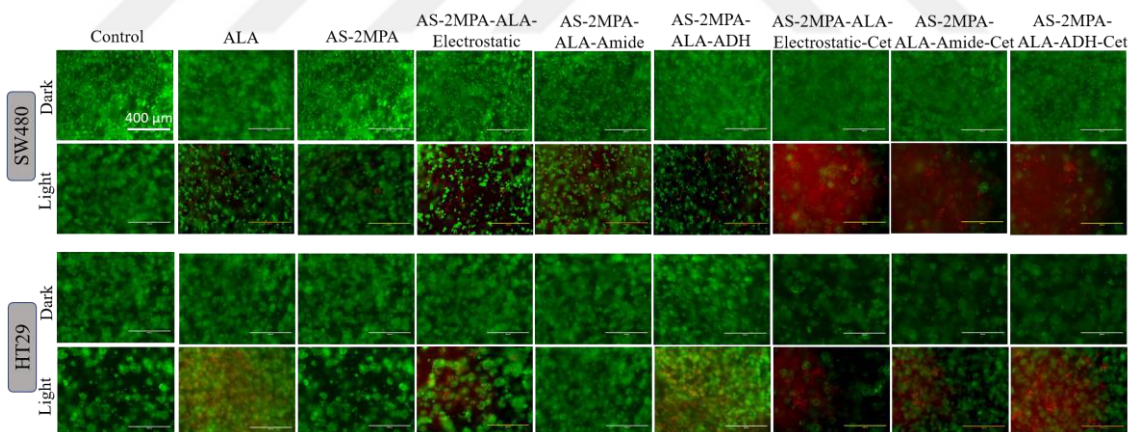


Figure 2–25: Live/dead images of 3D SW480 and HT29 spheroids. The cultures were treated with free ALA, AS-2MPA, AS-2MPA-ALA, AS-2MPA-ALA-Cet, and AS-2MPA-ALA-Cet-5FU QDs at 100 $\mu\text{g/mL}$ [Ag] after 4 h incubation with/without PDT illuminated at 420 nm with blue lamp for 5 min.

2.5 Conclusion

EGFR targeting theranostic nanoparticles based on Cet conjugated AS-2MPA QDs with NIR emission was developed to deliver ALA to EGFR(+) CRC cell lines for effective PDT and ALA/5FU combination for the combined chemotherapy/PDT of CRC. All QDs emit strongly around 830 nm and have small sizes, ideal for *in vivo* deep-tissue imaging

and molecular targeting.

The *in vitro* studies performed in 2D and 3D cell cultures demonstrate expression level dependent EGFR targeting of the AS-2MPA-ALA-Cet QDs in decreasing order of SW480, HCT116 and HT29 and strong intracellular NIR signal with good contrast with the background. Intracellular PpIX level of the cells increased with incubation time and the ALA concentration, which was most efficient in 200-600 μ M range. Meanwhile, these cell lines also differ in ALA-PpIX conversion efficiency. SW480 is the most effective, HCT116 is the least effective cell line. Cells with faster and more effective PpIX production are better candidates for PDT wherein short time intervals between the administration of the PS and light exposure is advantages. Therefore, PDT and combination therapy were studied in SW480 and HT29 cell lines.

Higher levels of PpIX and then ROS formation were achieved in SW480 cells using AS-2MPA-ALA-electrostatic-Cet due to higher cellular uptake, faster ALA release than covalently conjugated ALA, especially in acidic pH, and most efficient ALA-PpIX conversion. This was translated into high phototoxicity in SW480 cell lines even after 4 h incubation at ALA concentrations as low as 0.347 mM after 5 min irradiation at 420 nm and 0.173 mM at 1 min 640 nm irradiation, with less than 50 and 20% viable cell count, respectively. Cells treated in the same way but with free ALA maintained 80% viability at 0.173-0.688 mM [ALA], pointing out the dramatic enhancement in ALA-PDT of SW480 cells. Longer incubation time (24 h) allows more particle uptake and more PpIX generation due to higher ALA dose and more time to convert it to PpIX, hence improved the phototoxicity significantly in SW480 cells: Near-complete loss of viability at 0.688 mM [ALA] and less than 20% viable cell count at lower concentrations were achieved after 420 nm irradiation. Excitation at 640 nm improved the phototoxicity dramatically, especially at the low dose, killing more than 80% of the cells. Lower absorbance of PpIX at 640 nm, which is the preferred wavelength in the clinic, seem to be overcome with the use of a coherent light source, which did not cause any phototoxicity in the absence of ALA. Accumulation of high levels of ALA, fast PpIX formation and high phototoxicity archived with short irradiation at 640 nm at ALA concentrations lower than typical 1 mM are exciting for the PDT of EGFR(+) CRC tumours. Although HT29 was more challenging to treat due to poor ALA-PpIX conversion efficiency, irradiation at 420 nm after 24 h incubation with AS-2MPA-ALA-Cet-electrostatic at 0.688 mM [ALA] resulted in 60% loss in viable cell count and caused almost complete loss of viability after 640 nm

irradiation.

Targeted combination therapy provided with AS-2MPA-ALA-Cet-5FU QDs enhanced toxicity further, especially when PDT-monotherapy did not cause severe phototoxicity, namely in HT29 and SW480 (short incubation), and eliminated almost all cells at and above 0.374 mM ALA/15 ug/mL 5FU doses. Successful therapy of CRC cells was also confirmed at 0.688 mM [ALA] after 24 h incubation of all QDs in 3D spheroids of the cells, which is a more complex structure. In particular, AS-2MPA-ALA-Cet-5FU reduced the cell viability of 3D SW480 spheroids almost 2-folds compared to PDT-monotherapy after 420 nm irradiation and reduced the viability to 18% in SW480 and 32 % in HT29. The therapeutic effect was confirmed as apoptotic/necrotic cell death in both 2D and 3D spheroids.

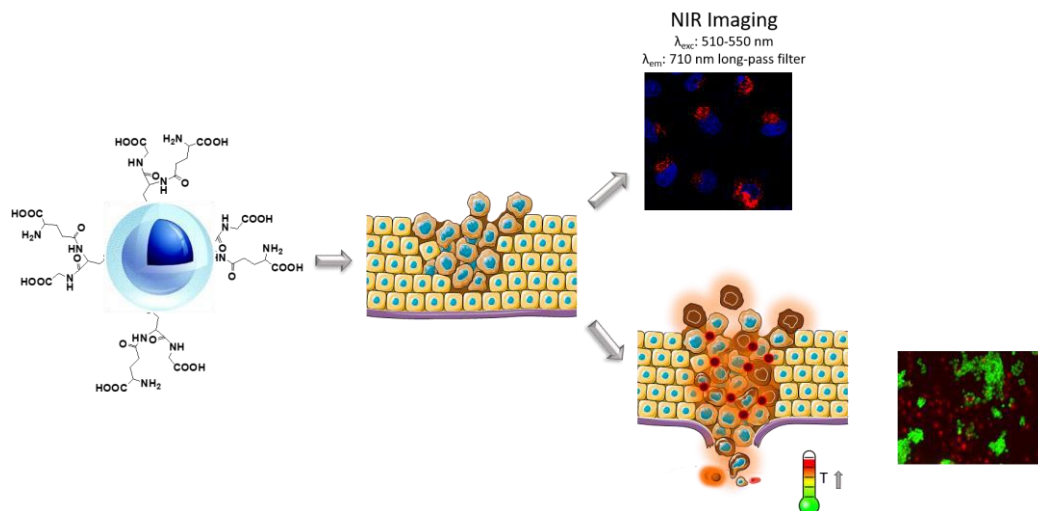
In case of the amide and hydrazone linked ALA, although the response is somewhat slower than electrostatically loaded ALA, significantly better toxicity than free ALA was achieved with EGFR targeting QDs especially after long incubation time and irradiation at 640 nm with the diode laser.

Overall, evidence provided both in 2D and 3D cell cultures confirm that the strategy proposed here and demonstrated with AS-2MPA-ALA-Cet-5FU nanoparticles providing a highly selective, local and effective therapy coupled with NIR and image guidance in addition to red luminescence of PpIX is highly promising for the treatment of EGFR(+) CRC. In further *in vivo* studies, these particles are expected to enhanced tumour accumulation via multilevel targeting: firstly, active tumour targeting via overexpression of EGFR, and secondly passive tumour targeting owing to the EPR effect that favours higher retention of nanoparticles in tumours versus normal adjacent tissue. Rapidly dividing cells such as tumour cells provide more efficient conversion of ALA to PpIX, which also confers some further PDT selectivity *in vivo*, and indeed makes it an attractive prodrug. Finally, fast release in the acidic microenvironment adds on to selectivity of the treatment. So, one could say that there are 4 mechanisms operating in selective PDT of tumours with these QDs. Although these theranostic nanoparticles were tested on EGFR(+) CRC cells, they can also be adopted for different tumour types and chemotherapy combination

Chapter 3:
**Ag₂S-Glutathione Quantum Dots for NIR Image
Guided Photothermal Therapy**

3.1 Abstract

Aqueous synthesis of glutathione (GSH) coated AS quantum dots (QD) with strong emission in the medical imaging window (700–900 nm) was performed from AgNO₃. Variations in temperature, time, pH and sulphur source (Na₂S and thioacetamide) were studied to tune the emission wavelength and intensity of the QDs. The optimum synthesis conditions to produce the smallest QDs (ca. 7 nm) with the highest quantum yield (70%) and excellent stability were determined as 50 °C and pH 10 where Na₂S was used in 2 h reactions. The cytotoxicity was evaluated with MTT assay on the HT29 and MCF7 cancer cell lines and indicated no significant toxicity of the QDs up to 200 mg/mL. The Photothermal Therapy (PTT) potential was first investigated using a 795 nm, continuous-wave fiber coupled diode laser using aqueous solutions of QDs and then on HT29 and MCF7 cells. QD (150 mg/mL) treated MCF7 cells almost quantitatively died after 10 min irradiation at 795 nm (700 mW, 1.82 W/cm²) and the viability of HT29 cells dropped to 40%. Most cell death was late apoptotic/necrotic. This study indicates that such a laser irradiation procedure and the QDs are safe, yet their combination provides dramatic cell death upon short laser treatment. PTT coupled with strong intracellular optical signal promotes AS-GSH QDs as potential theranostic nanoparticles (Scheme 3-1).



Scheme 3-1: Synthesize of ultrasmall, colloidally stable, and biocompatible AS-GSH QDs for NIR image guided-long wavelength PTT.

3.2 Introduction

In recent years, there have been many efforts to synthesize quantum dots emitting in the near-infrared region (NIRQDs) due to their great potential in optical imaging/diagnosis [14], [182]. Emission in the range of 700–900 nm is medically relevant since the scattering, absorbance and autofluorescence of living tissue is minimal in this window and the penetration depth is significantly better [182]. In terms of quantum dot composition, due to the Cd and Pb related reactive oxygen species (ROS) generation and toxicity, Cd and Pb free QDs are highly desired [183]. CuS, CuInS, AS and AgInS₂ are emerging, promising QDs [183], [184], [28]. Studies on AS have also shown that they are highly biocompatible [15], [33], [31].

The biocompatibility, hydrodynamic size, colloidal stability and optical properties of AS QDs depend on the coating that is used to passivate the crystal surface and suspend the nanoparticles in a carrier fluid [31]. Coating molecules also plays a crucial role in biodistribution. In this regard, biomolecules are promising candidates for the development of highly biocompatible nanoparticles. However, this should be studied carefully, as well. For example, very recently, we have discovered that N-acetylcysteine (NAC) coated AS could pose selective dose dependent toxicity to some cancer cell lines [34]. This is quite interesting, considering that NAC is an important antioxidant and often used to reverse the toxicity of other QDs in *in vitro* studies or used as a biocompatible coating to render Cd-based QDs nontoxic [185], [186].

Glutathione (GSH) is a tripeptide (γ-Glu-Cys-Gly) and another antioxidant with a

significant role in protection of cells from oxidative damage and maintenance of homeostasis [187]. GSH has a free thiol and hence is an alternative structure that may be utilized as a coating for quantum dots since thiol has a strong affinity to Ag [149], [188]. Tan et al. reported the intracellular synthesis of AS QDs in cultured hepatocellular carcinoma cells (HepG2 cells) treated with silver nitrate and sodium sulfide [189]. Endogenous GSH in the cells coordinated with the nanoparticles during the crystal formation and provided AS QDs with an average size of 5.45 nm and emission at 945 nm with a quantum yield (QY) of 1.56%. Although this method is interesting, it is far from providing a controllable and scalable process for the synthesis of AS-GSH. Brelle et al. synthesized AS QDs through a two-step procedure, wherein AS-GSH clusters were formed and treated with Na₂S solution [25]. The average particle size of these QDs was 9 nm with aggregation and no data on toxicity or biomedical use was provided. Wang et al. demonstrated size tunable synthesis of AS-GSH with emission between 624 and 724 nm using S in hydrazine hydrate as a sulphur source and reported that no luminescence was observed if Na₂S is used. This emission range is hardly matching with the biological imaging window and the QDs had a QY between 0.1 and 1.2% [190]. Tan et al. utilized high temperature decomposition of GSH to produce AS-GSH in ethylene glycol without using any other sulphur source [191]. This approach produced 8 nm crystals with emission at 1100 nm, which is beyond the medical imaging window. Hence, although there are a few examples of AS-GSH in the literature, these examples are either not applicable for large scale synthesis or provide QDs with aggregation, poor luminescence and emission wavelengths outside of the accepted imaging window (700–900 nm). Besides, the toxicity of AS-GSH has not been studied deeply, yet.

Very recently, it was also reported that the broad absorbance of AS QDs may be utilized for photothermal therapy (PTT). PTT is a relatively new approach providing a local therapy for improved therapeutic outcome with reduced or no off-target effects [44]. In PTT, a local temperature increase is induced by photoexcitation of a sensitizer. Hence, the stability of the sensitizer, excitation wavelength, penetration depth in the tissue and necessary laser intensity to create a local temperature increase are important issues [58]. Although there are examples of effective PTT induced by nanomaterials such as graphene, carbon nanotubes, and metallic nanoparticles (gold, iron, and copper), clinical applications of photothermal therapy are still limited [192], [193], [194], [195], [196], [197], [198], [199]. Recently, AS QDs emerged as a promising PTT agent since they

absorb the visible and part of the NIR I region, allowing excitation at safe long wavelengths. Furthermore, their luminesce in the NIR region provides a straightforward detection method to locate the QDs and therefore the tumour. Hence, AS is quite promising as a theranostic PTT agent which will allow also image-guided PTT. Examples of AS based PTT are very few. Gao et al. demonstrated successful thermal ablation of the MCF7 cell line exposed to AS-Apt43 (quantum yield 1–1.3%, emission between 978 and 1179 nm) and 808 nm laser irradiation at 1 W/cm² for 12 min [200]. Successful thermal ablation of a tumour xenograft in nude mice was also demonstrated at a QD concentration of 100 mg/mL. Cu–AS/PVP was developed for photoacoustic imaging (PAI) guided PTT, which enhanced the efficiency (58.2%) and selectivity of the *in vivo* therapy [201]. The PTT potential of AS-GSH has not been studied, yet.

Herein, a simple, one step, aqueous synthesis of GSH coated AS QDs is described first. The influence of reaction variables such as different sulphur sources, reaction temperature, time and pH on the optical properties of the nanoparticles was investigated in order to explore the possibility of tuning the optical properties and to develop small, stable, AS-GSH QDs with strong emission in the medical imaging window. All of these variables are known as major factors that would affect the particle properties and hence should be studied thoroughly to understand the design space and to find the recipe for the best possible QD of this composition. Next, the dose dependent toxicity of the QDs was determined using the human breast adenocarcinoma (MCF7) and human colon adenocarcinoma (HT29) cancer cell lines in *in vitro* experiments. Lastly, utilizing the absorbance of the QDs in the NIR, the PTT potential of the AS-GSH QDs was investigated in aqueous solutions and effective PTT was demonstrated on the MCF7 and HT29 cancer cell lines *in vitro* for the first time in the literature.

3.3 Materials and Methods

3.3.1 Materials

All chemicals were of analytical grade or the highest purity. Silver nitrate (AgNO₃) and thioacetamide (TAA) were purchased from Sigma-Aldrich. Sodium sulfide (Na₂S) was obtained from Alfa Aesar. 2-Glutathione (GSH), acetic acid (CH₃COOH) and sodium hydroxide (NaOH) were provided by Merck (Germany) and the polysulfone filtration membrane (3, 10 and 100 KDa) by Sartorius (Goettingen, Germany). Roswell Park Memorial Institute (RPMI) 1640, 1X medium (with L-glutamine and sodium

bicarbonate), McCoy's 5A (modified) medium and HEPES 1 M solution were obtained from Multicell, Wisent Inc. (Canada). Fetal bovine serum was provided by Capricorn Scientific GmbH (Germany, www.capricorn-scientific.com). Trypsin–EΔTA and penicillin streptomycin solutions were purchased from Wisent Inc. (Canada). Thiazolyl blue tetrazolium bromide (MTT) and phosphate buffered saline (PBS) tablets were obtained from Biomatik Corporation (Canada). MCF7 and HT29 were given as a gift from the Gozuacik Lab (Sabanci University, Istanbul, Turkey). The live/dead cell viability assay was purchased from Thermo Fisher Scientific. LysoTracker yellow HCK-123 (Invitrogen) was purchased from Thermo Fisher Scientific (USA). The Annexin V-FITC early apoptosis detection kit was purchased from Cell Signalling Technology (UK).

3.3.2 Synthesis of GSH coated AS QDs

42.5 mg (0.25 mmol) of AgNO₃ was dissolved in 75 mL of deoxygenated water in a round-bottomed flask under argon. GSH (0.5 mmol) was added to this solution and the pH was adjusted to either 4.5, 7 or 10 using NaOH and CH₃COOH (1 M). Then, 0.125 mmol of the sulphur source (Na₂S or TAA) according to the desired Ag/S ratio of 2/1 was dissolved in 25 mL of deoxygenated water and added to the silver salt–GSH solution under vigorous mechanical stirring. The sulphur source was either added at room temperature, 30 or 50 °C. The progress of crystal growth was monitored using the photoluminescence of samples removed from the reaction mixture at different times. At the end of the reaction, the QD solutions were washed with deionized water using Sartorius centrifugal filters (3 kDa cut off) and stored in the dark at 4 °C.

3.3.3 Characterization of the QDs

Fourier-transform infrared spectroscopy (FTIR) with an attenuated total reflection (ATR) attachment (Thermo Scientific Nicolet iS10) was used to determine the functional groups in the wavenumber range of 400–4000 cm⁻¹. The hydrodynamic size and zeta potential of the aqueous QDs were determined using a Malvern Zetasizer Nano ZS. The Ag content of the QD solutions was determined after treating the samples with a 9:1 volume ratio of nitric acid: sulphuric acid using an Agilent 7700x Inductively Coupled Plasma Mass Spectrometer (ICP-MS). The organic content of the QDs was measured by thermogravimetric analysis (TGA, TGA 500). The samples were heated from room temperature to 1000 °C at a heating rate of 10 °C min⁻¹ under a nitrogen atmosphere. The absorbance was measured with a spectrophotometer (Shimadzu UV-3600) in the 300–

1000 nm range using aqueous QD solutions. Particle sizes were determined by using the Brus equation [157]:

$$QY = \left(\frac{\text{Grad QD}}{\text{Grad Reference}} \right) \left(\frac{\eta_{\text{water}}}{\eta_{\text{MeOH}}} \right)^2 \times 100 \quad \text{Equation 3-1}$$

where DE is the band gap energy, R is the radius of the nanocrystal, m_e (0.286 m_0) and m_h (1.096) are the effective electron and hole masses for AS, respectively, and ϵ_{AS} (5.95) is the relative dielectric constant [157]. For the photoluminescence (PL) measurements, a homemade instrument with a femtowatt sensitive Si detector (Thorlabs PDF10A, 1.4×10^{-15} W/Hz^{1/2}) and a 1/8 Newport Cornerstone 130 monochromator having a 600 L per mm grating was used. Excitation at 532 nm was performed with the frequency doubled output of a DPSS laser. A 590 nm long pass filter and 0.5 nm slit width were used in the measurements.

The QY of the AS-GSH QDs was calculated with respect to the QY of LDS 798 NIR dye (QY in MeOH = 14%) based on the procedure detailed in the literature using the integrated area under the PL curve of the QDs and dye, prepared at five different concentrations with absorbance below 0.1 at the excitation wavelength [157]. To determine the crystal structure of the QDs, a Bruker D2 Phaser Benchtop X-Ray Diffraction (XRD) system with Cu Ka radiation ($\lambda = 1.5406$ Å) was used between 2 θ angles of 10° and 80°. For the X-ray photoelectron spectroscopy (XPS) of the QDs, a Thermo Scientific XPS was used with Al K α monochromatic radiation (1486.3 eV) with a 400 mm X-ray spot size and 50.0 eV pass energy, corresponding to a resolution of roughly 0.5 eV. A 3×10^{-9} mbar base pressure and 1×10^{-7} mbar experimental pressure were achieved. The C 1s peak set to 285.0 eV was used as the reference signal.

3.3.4 Cell culture

Human breast adenocarcinoma cells (MCF7) and human colon adenocarcinoma cells (HT29) were cultured in RPMI and McCoy's complete medium, respectively, supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin antibiotic solution according to ATCC recommendations. Cells were incubated at 37 °C under 5% CO₂ and trypsin–EDTA was used for cell detachment.

3.3.5 Determination of cytotoxicity

The most stable and most strongly luminescing AS QDs (QD2) were used in the *in vitro*

studies. The viability of MCF7 (breast cancer) and HT29 (colorectal cancer) cells was determined using the standard thiazolyl blue tetrazolium bromide (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT) assay. 12,500 cells per well were seeded into 96-well culture plates in culture medium and incubated at 37 °C under a 5% CO₂ atmosphere for 24 h. Then, the cells were treated with fresh media containing QDs (10 to 300 mg/mL Ag) and incubated for 24 h. After washing the cells with 2×PBS (1 M), 50 mL MTT solution and 150 mL complete media were added to the cells and incubated for 4 hours. The cells were treated with DMSO–ethanol (1 : 1 v/v) solution (200 mL) and the plates were shaken gently for 15 min to dissolve the purple formazan formed as a result of the mitochondrial activity of the viable cells. The absorbance at 600 and 630 nm was measured with a BioTek EL×800 microplate reader and the reference absorbance at 630 nm was subtracted from the absorbance at 600 nm. The absorbance values of the QDs ($abs_{600nm} = 0.0578$ and $abs_{630nm} = 0.0412$) were subtracted from the formazan values. The experiments were repeated five times. The relative cell viability was calculated using the following equation:

$$\text{cell viability (\%)} = [\text{absorbance of treated cells} / \text{absorbance of control}] \times 100$$

Equation 3-2

3.3.6 Fluorescence imaging

MCF7 and HT29 cells were grown on a 3 mL glass-bottom Petri dish at a density of 15×10^6 cells per well in complete RPMI and McCoy's culture medium. After 24 h, nanoparticles were added to the cells at a 50 mg/mL Ag dose and incubated for 24 h. Then, the medium was replenished, and the cells were washed with 3×PBS (1 M). Paraformaldehyde was added to each well and the samples were stored in the dark for 20 min to fix the cells. Following this procedure, the paraformaldehyde was discarded, and each well was washed with 3×PBS (1 M). MCF7 and HT29 cells were incubated for an additional 15 min with 2 mg/mL of DAPI, a nuclear staining dye. These cells were rewashed with 3×PBS (1 M) to remove unbound dye and 3 mL PBS (1 M) was left in each well to protect the cells against drying. The fixed cell samples were examined under oil at 63X magnification (Leica DMi8/SP8). Two different filters that were specific for the NIR region (λ_{exc} : 510-550 nm and λ_{em} : 710 nm long pass) and DAPI (λ_{exc} : 325-375 nm and λ_{em} : 435-485 nm) were used to image the cell nuclei and QDs. The same experimental procedure was also performed for control cells which were not treated with

nanoparticles.

In order to stain the lysosome, LysoTracker yellow HCK-123 (Invitrogen) was used. The cells were seeded in a 3 mL glass bottom Petri dish as described above. They were incubated with QD2 at a 50 mg/mL Ag concentration for 24 h, and then washed with 3×PBS (1 M) and stained with LysoTracker yellow according to the manufacturer's instruction. The cells were examined without being fixed using a confocal microscope (Leica DMI8/SP8) with different filters for FITC (λ_{exc} : 460-500 nm and λ_{em} : 512-542) and NIR emission (λ_{exc} : 510-550 nm and λ_{em} : 710 nm long pass).

3.3.7 Live/dead assay

Live/dead cell viability assay was used to determine live and dead cells before and after PTT treatment. MCF7 and HT29 cells were seeded as described in the viability test. Then, nanoparticles were added to the cells at 150 mg/mL Ag and incubated further for 24 h. The cells were irradiated for 10 min at 1.82 W/cm². After 24 h of incubation, the cells were stained with live/dead assay staining solution based on the protocol that is provided by the manufacturer. Finally, images were collected using a fluorescence microscope (Zeiss Axio Observer Z1).

3.3.8 Photothermal heating procedure for quantum dots in solution and *in vitro* PTT studies

Photothermal heating of aqueous QD solutions (0.75 mL) with QD concentration in the 1–10 mg/mL range was investigated by using a continuous wave (CW) fiber-coupled diode laser at 795 nm (FAP-79-25C-800-B, Coherent, Inc.). In the first set of experiments, the temperature rise (ΔT) was measured at different QD concentrations in polystyrene cuvettes by irradiating them for 20 min with 800 mW power. In the second set of experiments, the QD concentration was kept at 7 mg/mL and the temperature rise was measured as a function of laser power ranging from 300 to 800 mW. For the *in vitro* PTT studies, MCF7 (12,500 cells per well) and HT29 (10,000 cells per well) cells were seeded in 96-well plates and cultured as described above. QDs were added to each well at different Ag concentrations and incubated for 24 h. Then, the medium was replenished with fresh medium and each well was irradiated with 700 mW laser power at 795 nm from the bottom of the plate for 10 min. The corresponding laser intensity was 1.82 W/cm². The temperature of the environment was kept constant at 37 °C during laser irradiation. The laser irradiated cells were then incubated for 24 h before cell proliferation

was determined using the standard MTT protocol.

3.3.9 Cell apoptosis assay

HT29 and MCF7 cells were seeded in 96-well plates at a density of 12,500 cells per well and cultured for one day and then were incubated with/without QD2 at a 100 mg/mL Ag concentration for 24 h. After the medium was changed for a fresh one, some of these cells were irradiated at 795 nm for 10 min at a power density of 1.82 W/cm² and further incubated for 24 h. Then, the cells were collected and stained with an Annexin V-FITC early apoptosis detection kit (Cell Signalling Technology, UK) according to the manufacturer's instruction and analysed by flow cytometry (Attune NXT focusing cytometer, Thermo Scientific, UK). Data were processed with FlowJo (USA) and untreated cells were used as a control.

3.3.10 Statistical analysis

The statistical significance of the observed differences was determined using Kruskal–Wallis one-way ANOVA with Dunn's multiple comparison test of the GraphPad Prism 8 software package (GraphPad Software, Inc., USA). All data were represented as mean ± standard deviation (SD), and $p < 0.05$ was considered statistically significant.

3.4 Results and Discussions

3.4.1 Synthesis and characterization of AS-GSH NIRQDs

AS QDs were prepared directly in water with the addition of Na₂S or TAA to a GSH–silver salt mixture at different pH values and temperatures in order to evaluate the emission tunability and to find the best recipe to produce small, stable and strongly luminescent QDs. The reaction parameters and characteristic features of all samples which were prepared in this study are summarized in Table 3-1.

Table 3-1: Properties of GSH coated AS QDs

Code	Sulphur Source	T ^a (°C)	pH	λ_{abs}^b (nm)	Size ^c (nm)	$\lambda_{\text{em(max)}}^d$ (nm)	FWHM ^e (nm)	Dh-number ^f (nm)	Dh-intensity ^g (nm)	Zeta pot. (mv)
QD1	Na ₂ S	RT	10	906	2.94	848	65.9	4.3 ± 0.2	169.8	-42.9 ± 5.5
QD2	Na ₂ S	50	10	923	3.01	897	140.6	3.8 ± 0.1	6.7	-21.5 ± 3.1
QD3	Na ₂ S	50	4.5	910	2.96	786, 898	257.9	7.2 ± 6.2	202.8	-33.3 ± 2.1
QD4	Na ₂ S	50	7	911	2.96	899	148.65	5.1 ± 0.4	287.3	-14.9 ± 7.3

QD5	TAA	50	10	937	3.06	828	76.6	5.1 ± 3.9	137.3	-33.6 ± 9.2
QD2*	Na ₂ S	50	10	910	2.96	799	187.4	3.2 ± 0.3	36.1	-24.3 ± 2.4

^a Temperature. ^b Absorbance onset. ^c Crystal diameter calculated by the Brus equation. ^d Emission maxima.

^e Full-width at half-maximum of the emission peak. ^f Hydrodynamic diameter measured by DLS and reported as the number average. ^g Hydrodynamic diameter measured by DLS and reported as the intensity average. Hydrodynamic size and zeta potentials were measured after washing and adjusting the pH to 7.5.

* Unwashed sample.

The particle growth and change in the emission peak with time under different synthesis conditions were monitored to determine the optimum reaction time which results in the strongest emission. The colour of the reaction mixture turned from yellow to dark brown with time (Figure 3-1a). Aliquots were taken from these reactions at different time points and the particle growth and luminescence intensity were monitored with a spectrofluorometer. A red shift with some broadening in the photoluminescence (PL) spectra was observed with time, reaching a maximum intensity, and then dropping down at extended reaction times up to 2.5 h (Figure 3-1b). Particle growth with time causes a red shift in the emission peak, but as the particles grow larger defects may occur which cause nonradiative recombination of photogenerated excitons.

The influence of the reaction pH on the particle properties was studied at a growth temperature of 50 °C and at an Ag/S ratio of 2/1. The QDs synthesized at pH 7 (QD4) and pH 10 (QD2) have emission maxima at about 897–899 nm and similar emission profiles but the latter had about three times stronger emission intensity (Figure 3-1c). According to the pK_a of the carboxylic acid groups of GSH (pK₁ = 2.12 and pK₂ = 3.53), at pH 7 (QD4) there would be a negative charge density on the surface and at pH 10 the amine and carboxylic acid groups of GSH would be all deprotonated. Indeed, the reactions at pH 4.5 and 7 start out blurry, indicating that the initial complex is not completely soluble at these pH values, but the reactions at pH 10 are completely clear, indicating full solubility, which would enhance the reaction kinetics. The hydrodynamic size, measured by DLS based on the intensity, indicates aggregation of QDs at pH 4.5 and 7 (QD3 and QD4), which decreases the stability of the QDs with time (Table 3-1). Deprotonation of the thiol group of GSH enhances passivation of the AS surface with Ag–S bond formation. Hence, the QDs produced at pH 10 are smaller and have the strongest PL intensity. On the other hand, the QDs synthesized at pH 4.5 (QD3) had a very broad emission peak with about two peak maxima at 786 and 898 nm and lost colloidal stability after 1 month.

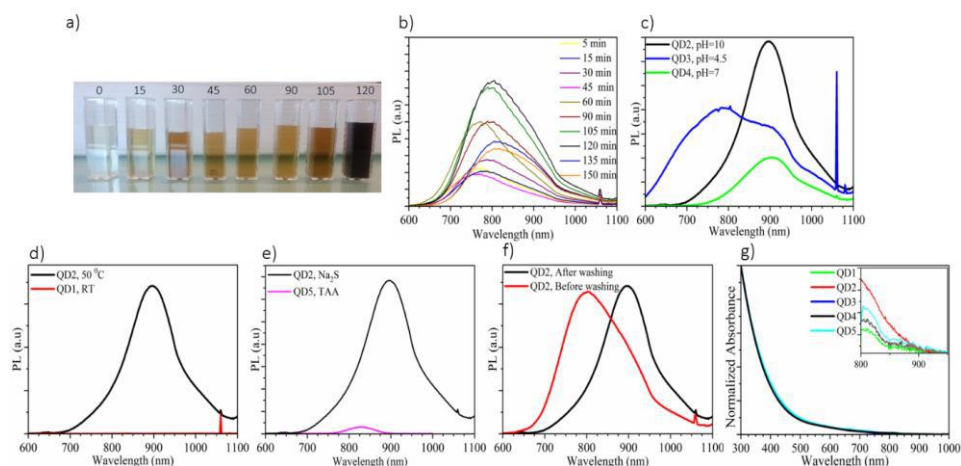


Figure 3–1: a) Colour of the reaction mixture after Na₂S addition to Ag⁺-GSH solution between 0–2 h reaction time. Absorbance calibrated photoluminescence spectra of b) aliquots taken from QD2 reaction, c) QD2, QD3, QD4 synthesized at different pH for 2 h (PL spectra were taken after particles were washed and the solution pH was adjusted to 7.5 to prevent differences in intensity based on pH), d) QD1 and QD2 synthesized at different temperatures for 2 h, e) QD2 and QD5 synthesized with different sulphur sources for 2 h, f) QD2 before and after washing steps, g) Absorbance spectra of the QD1, QD2, QD3, QD4, and QD5 prepared at different reaction parameters.

Another factor affecting the particle growth is the reaction temperature (Figure 3-1d). A blue shift in the absorbance onset on decreasing the temperature from 50 °C (QD2) to RT (QD1) provided a reduction in the crystal size from 3.01 nm to 2.94 nm and a small blue shift in the peak maximum (from 897 nm to 848 nm) but with a dramatic loss in emission intensity, providing no effective luminescence (Table 3-1 and Figure 3-1d). Increasing the reaction temperature usually causes the formation of larger crystals, since the solubility of the initial particles is higher at higher temperatures, but also particles may crystallize better, which enhances the emission intensity, as well [202].

Another way to slow down the reaction and tune the particle size and emission wavelength may be the use of a slow sulphur releasing source, TAA. Under similar conditions replacement of Na₂S with TAA shifted the emission maximum from 897 (QD2) to 828 nm (QD5) with a dramatic loss in the intensity (Figure 3-1e). The slower sulphur release of TAA also slowed down the particle growth, producing the most luminescent particles at a relatively longer time compared to Na₂S, i.e. in 120 min for QD2 versus 180 min for QD5 (Figure 3-1b and Figure 3-2).

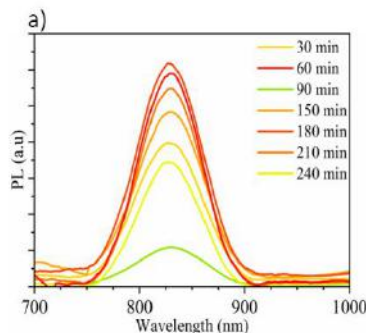


Figure 3–2: Absorbance calibrated photoluminescence spectra of a) QD5 synthesized with TAA at 50 °C at different time.

After synthesizing the QDs, washing is another factor which affects the particle properties, especially their stability, hydrodynamic size, and optical properties. A major change in the maximum emission peak, FWHM and hydrodynamic size of the nanoparticles was observed after washing (Figure 3-1f and Table 3-1). It seems like that the low wavelength region of the PL emission peak is eliminated after washing and hence the average seems to be shifted to longer wavelengths. The number based hydrodynamic size increased slightly, which agrees well with the PL spectra, but the intensity based hydrodynamic size is reduced from 36.1 to 6.7 nm after washing, indicating elimination of small aggregates.

All QDs have a negative zeta potential due to the presence of two carboxylic acid and one amine functionality (Table 3-1). Overall, QD1 and QD2 prepared at pH 10 with Na₂S have the smallest hydrodynamic size; the QDs prepared at 50 °C and pH 10 (QD2 and QD5) have the largest crystal sizes. QD2 emerged as the most luminescent QD with the smallest hydrodynamic size (3.8 nm number average, 6.7 nm intensity average). Therefore, further studies and characterization were performed with QD2.

TEM analysis of QD2 indicates spherical crystals with an average size of 3.16 nm with a narrow size distribution between 2 and 4 nm (Figure 3-3a and b). XRD patterns revealed that the crystal structure of these QDs at room temperature is monoclinic α -AS (JCPDS: 14-72) (Figure 3-3c). XPS analysis also confirms the chemical composition of AS-GSH. The Ag peaks at a BE of 367.73 (3d_{5/2}) and 373.72 (3d_{3/2}) eV correspond to the +1 oxidation state (Figure 3-3d). The sulphur peaks at a BE of 163.39 (2p_{3/2}) and 161.51 (2p_{1/2}) eV correspond to S coming from GSH and from Ag–S–Ag, respectively (Figure 3-2e). The N 1s region can be fitted using two peaks at 399.45 and 401.85 eV corresponding to NH and NH₃ of GSH (Figure 3-3f). After background subtraction and

correction for atomic sensitivity factors, the area under each curve was used to obtain quantitative information, which indicated a Ag/S ratio of 2.1/1, which is in agreement with the theoretical ratio of 2/1 and the ratio obtained by ICP (ICP: Ag/S: 1.75/1). FTIR demonstrates the presence of characteristic 1570 and 1510 cm⁻¹ bands of GSH on the nanoparticles and the absence of S–H stretching at 2530 cm⁻¹, confirming Ag–S binding (Figure 3-3g). TGA analysis performed on QD2 indicates 50.6% organic content (Figure 3-3h).

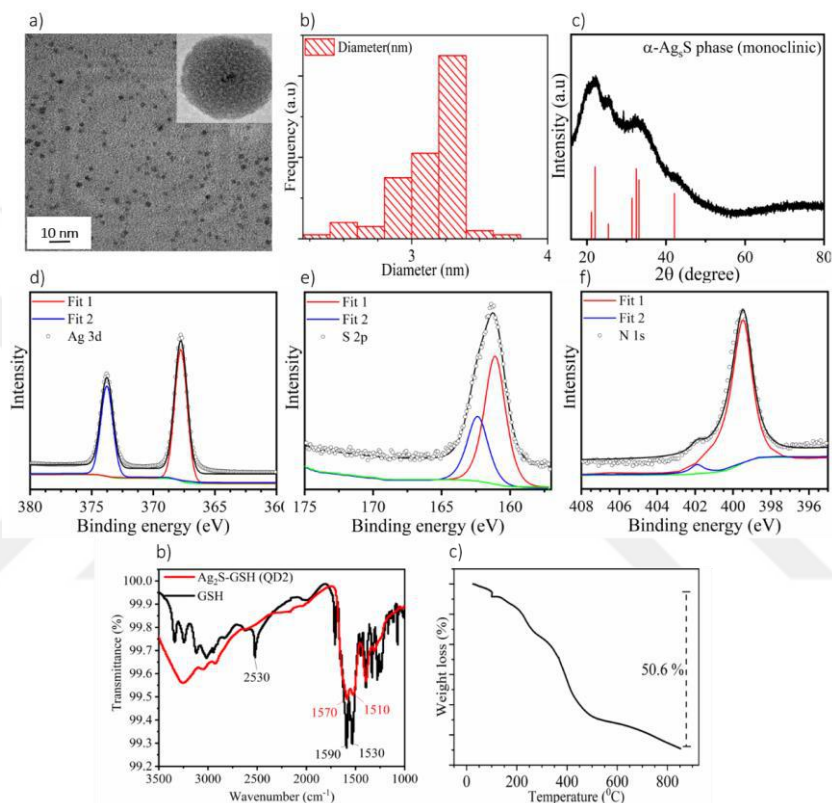


Figure 3–3: a) TEM image and b) the corresponding histogram of QD2, c) XRD pattern of the QD2 at room temperature, d) Ag 3d, e) S 2p and f) N 1s XPS spectra of QDs. g) FTIR spectrum of QD2 and glutathione and h) Thermogravimetric analysis (TGA) of QD2.

3.4.2 Long term stability of AS-GSH QDs

The shelf-life of QDs is very important. Aqueous QD2 suspensions after being washed and adjusted to pH 7.4 were kept in the refrigerator and analysed at different time points to determine the changes that may occur in their size, stability or emission intensity (Table 3-2 and Figure 3-4a-c). They stayed well suspended without any change in their colour in the 12 months that was monitored (Figure 3-4c). Within this time, the inorganic core size of QD2, which was calculated with the Brus equation, and the position of the emission maximum stayed the same. The hydrodynamic size based on intensity increased from 6.7 to 48.9 nm, indicating slight aggregation, and the QY decreased from 70% to 33% in one

year. However, this is one of the highest QYs reported for AS QDs even after 1 year [34], [32]. Also, compared to analogous QDs with only a 1.56% QY produced by Tan et al. which were successfully used for *in vivo* NIR optical imaging in animal models, these AS-GSH QDs offer very practical and strong emission in the medical imaging window. Such a drop in QY over time is quite normal and usually attributed to oxidation of surface ligands and desorption, which can support slight aggregation, as well.

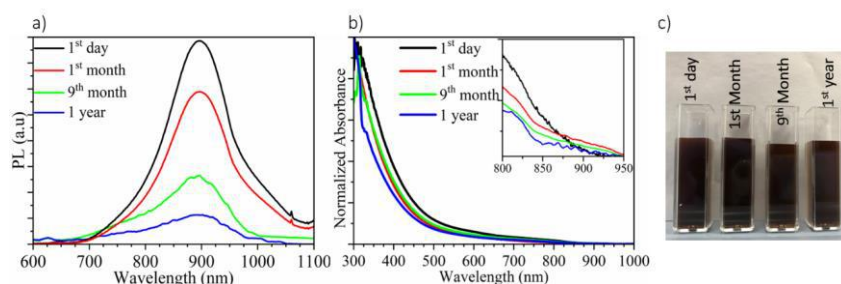


Figure 3-4: a) Absorbance calibrated photoluminescence and b) absorbance spectra of QD2 from the 1st day to one year after synthesis, c) Colloidal stability of the solution of AS-GSH after 1 year.

Table 3-2: Time dependent changes in the particle properties*

Time	$\lambda_{\text{abs(max)}}^a$ (nm)	Size ^b (nm)	$\lambda_{\text{em(max)}}^c$ (nm)	Dh-number ^c (nm)	Dh-intensity ^d (nm)
1 st day	923	3.01	891	6.7	3.8 ± 0.1
1 st month	923	3.01	891	12.8	4.1 ± 0.2
9 th month	946	3.09	891	30.2	4.0 ± 0.1
1 year	946	3.09	897	48.9	4.6 ± 0.4

* QD2 was used for this experiment. ^a Absorbance onset. ^b Crystal diameter calculated using the Brus equation. ^c Hydrodynamic diameter measured by DLS and reported as the number average. ^d Hydrodynamic diameter measured by DLS and reported as the intensity average.

3.4.3 Evaluation of the *in vitro* cytotoxicity of AS-GSH QDs

The most strongly luminescing AS QDs (QD2) were used in the *in vitro* studies. The proliferation rate of the MCF7 and HT29 cell lines after 24 h incubation with QDs in a broad concentration range (10–300 mg/mL) [Ag], corresponding to 5 mg/mL QDs at the highest [Ag], was determined with MTT assay (Figure 3-5). Suggesting a correlation between viability and proliferation and considering viability above 80% as non-toxic based on ISO 10993-5, QD2 showed excellent biocompatibility within this dose range.

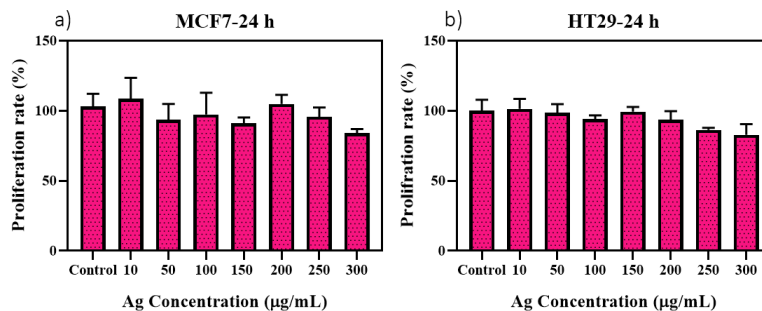


Figure 3–5: Proliferation of a) MCF7 and b) HT29 cells treated with QD2 at Ag concentration of 10–300 µg/mL after 24 h incubation measured by MTT assay. Untreated cells were used as the control. * indicates statistical significance between the control and QD treated cells ($p < 0.05$).

3.4.4 *In vitro* optical imaging

To examine the NIR imaging potential of the AS-GSH QDs (QD2), MCF7 and HT29 cells were incubated at a 50 mg/mL Ag dose for 24 h. The fluorescence microscopy images of the cells treated with QD2 in Figure 3-6a, b revealed high intracellular uptake and lysosomal co-localization of nanoparticles in both cell lines. This is in agreement with the literature [36], [203], [204].

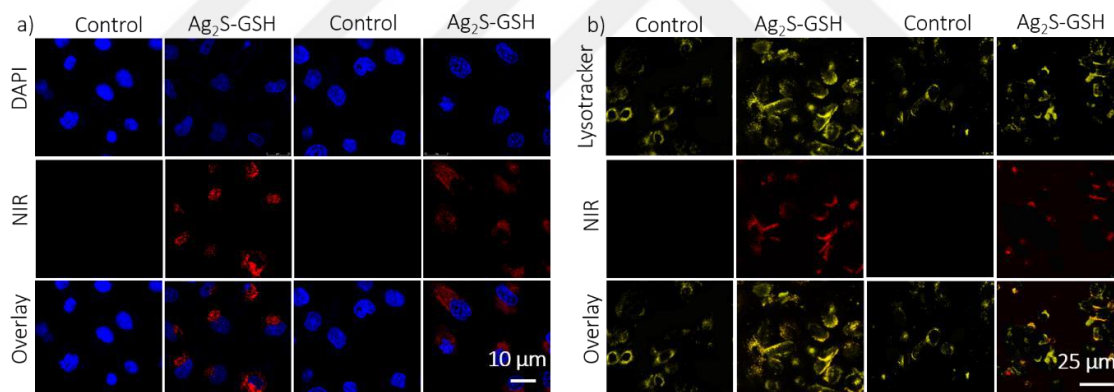


Figure 3–6: a) Fluorescence microscopy images of fixed HT29 and MCF7 cells treated with/without QD2 after 24 h incubation with the cells at a 50 mg/mL Ag concentration. Blue: DAPI nuclear stain; red: QD2. b) Fluorescence microscopy images of HT29 and MCF7 cells treated with QD2 at a 50 mg/mL Ag concentration for 24 h and stained with LysoTracker yellow. Yellow: LysoTracker, red: QD2.

3.4.5 *In vitro* PTT results

The photothermal effect of the AS-GSH QDs was first tested in aqueous solutions as a function of quantum dot concentration (Figure 3-7a), irradiation time and input power (Figure 3-7b). The temperature rise (ΔT) in the solutions increased with the QD concentration (1–10 mg/mL). In particular, ΔT was 8, 20, 21.6 and 21.8 °C for QD concentrations of 1, 4, 7 and 10 mg/mL after 20 min irradiation of the solutions with 800

mW power at 795 nm (Figure 3-7a). In these measurements, the spot radius of the laser was 3.5 mm, giving an irradiation intensity of 2 W/cm². Note that above a 7 mg/mL QD concentration, no further increase in ΔT was observed. Therefore, higher QD concentrations were not tested. Over the course of 20 min irradiation, the maximum temperature rise was obtained at about 10 min. The temperature increases of the control (DI water) were negligible, indicating that the observed temperature rise was predominantly due to the QDs. The impact of the laser input power on ΔT was also studied at a 7 mg/mL QD concentration. Here, a maximum ΔT of 7.2, 12.7 and 21.7 °C was recorded with 300, 500 and 800 mW laser powers, respectively. The maximum temperature rise is nearly proportional to the irradiation power as expected. Three cycles of laser on/off experiments were performed, and the same amount of temperature increase was observed after each cycle (Figure 3-7c), indicating the stability and potential of reusability of the QDs after irradiation. The photothermal conversion efficiency of the AS-GSH QDs at 7 mg/mL was determined as 80% by using the equation derived by Roper et al. using following formula [205]:

$$\eta = \frac{ha(T_{max}-T_{amb})-Q_{dis}}{P(1-10^{-A_{795nm}})} \quad \text{Equation 3-3}$$

where h is the heat transfer coefficient, a is the irradiated area, T_{max} is the equilibrium temperature, T_{amb} is the ambient temperature, and Q_{dis} is the heat dissipated due to light absorption in the polystyrene cell. P and A_{795nm} appearing in the denominator represent the laser power and absorbance of the Ag₂S-GSH QDs, respectively, at 795 nm.

ha can be calculated by using the following formulas:

$$ha = \frac{\sum m_i C_{p,i}}{\tau_s} \quad \text{Equation 3-4}$$

$$t = -\tau_s \ln \theta \quad \text{Equation 3-5}$$

$$\theta = \frac{T-T_{amb}}{T_{max}-T_{amb}} \quad \text{Equation 3-6}$$

Above, m , C_p , t , τ_s are the mass of sample, the thermal capacity, cooling time after irradiation, and the sample system time constant, respectively. The quantity Q_{dis} was determined by using the following formula which accounts for the absorption of the laser power within water and the polystyrene cuvette:

$$Q_{dis} = ha(T_{max,water} - T_{amb,water}) \quad \text{Equation 3-7}$$

The values of $T_{\max}$, T_{amb} , τ_s , m_{cuvette} , m_{sample} , C_{cuvette} , C_{water} were 45 °C, 25 °C, 220.8 s, 1.9110 g, 0.7513 g, 1.4 J/g °C, and 4.186 1.4 J/g °C, respectively. Therefore, h_a was calculated as 26.4 mW/°C. Q_{dis} was determined to be 0 because there was no significant temperature change in the water at these pump power levels. By substituting these parameters into Equation 3-3, the PTT efficiency was determined to be 80%. This efficiency is noticeably higher than most of the values reported for AS in the literature. Dong et al. reported a PTT efficiency of 58.2% at 808 nm for Cu-AS/PVP.30 AS nanodots with different diameters (4.1–9.8 nm) provided photothermal conversion efficiencies around 33.7–35% (785 nm) [107]. Comparable photothermal efficiencies were observed with Apt43- AS QDs (23.8%, 808 nm) [108] and AS QDs coated with dendritic mesoporous silica (28.35%, 808 nm) [36].

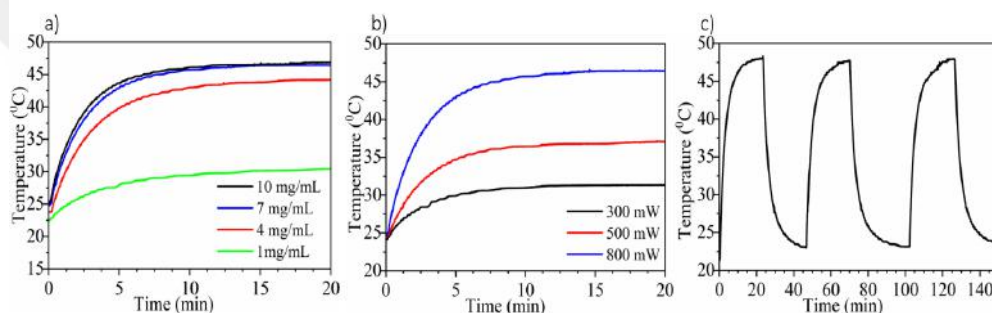


Figure 3–7: Time dependent change in the temperature of AS-GSH solutions a) at different QD concentrations irradiated at 800 mW, b) irradiated at different powers (7 mg/mL QD) and c) after laser on (20 min)/off (20 min) cycles (7 mg/mL, 800 mW). Excitation wavelength=795 nm.

In vitro PTT experiments were performed on the HT29 and MCF7 cell lines. Cells treated with QDs at non-toxic doses (25–150 mg/mL Ag dose range) were irradiated for 10 min with 700 mW laser power (intensity = 1.82 W/cm²) at 795 nm and the cell proliferation was determined 24 h after laser treatment using MTT assay. Control cells (not treated with QDs) were also irradiated under the same conditions. The proliferation rate of MCF7 cells dropped to 75, 68, 52, and 6% at 25, 50, 100, and 150 mg/mL [Ag], respectively, while there was no reduction in the control cells (Figure 3-8a). HT29 cells were more resistant to PTT and their viability decreased to 57 and 40% only at 100 and 150 mg/mL [Ag] after laser irradiation (Figure 3-8b). This is indeed quite a successful result considering that both in our hands and in the literature HT29 is more resistant to PTT. Ghorbani et al. reported a 22±1% and 37±2% reduction in the viability of HT29 and MCF7 cells treated with 231.25 mg/L gallic acid coated SPIONs and exposed to 12 min irradiation at 730 nm (124.8 J/cm²) [206]. Chen et al. achieved 35% cell death only after treating HT29 cells with gold nanorods conjugated with carbonic anhydrase IX (CAIX),

which targeted the nanoparticles to hypoxic areas, and subjected to 2 min laser treatment with a 760 nm 12 W/cm² laser [207].

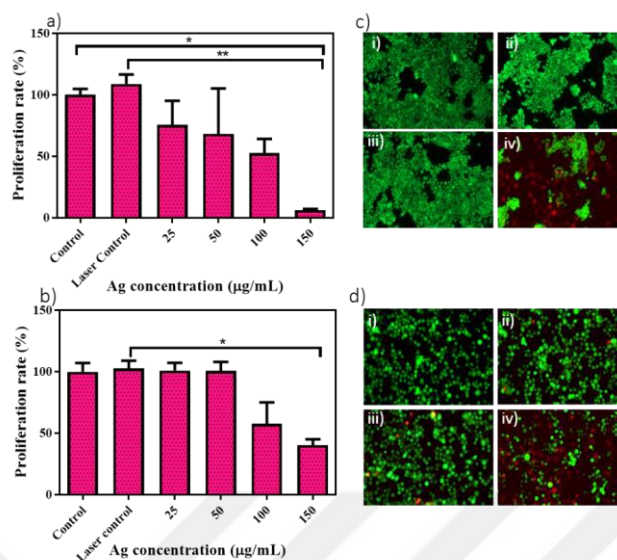


Figure 3–8: Proliferation of a) MCF7 and b) HT29 cell lines treated with AS-GSH and laser at different Ag concentrations determined with MTT assay. Control indicates untreated cells. Laser control indicated cells treated only with laser irradiation for 10 min (700 mW, 1.82 W/cm²). Microscopy images of c) MCF7 and d) HT29 cells treated with (i) nothing, (ii) laser control, (iii) nanoparticle (150 µg/mL [Ag]), and (iv) nanoparticle (150 µg/mL [Ag]) and laser irradiation (700 mW, 1.82 W/cm²) after live/dead staining.

To confirm the MTT results, cells treated with QDs at a 150 mg/mL [Ag] dose for 24 h, with and without laser irradiation, were stained with a live/dead assay kit where red and green fluorescence indicate dead and live cells from ethidium homodimer-1 and calcein-AM staining, respectively. Fluorescence microscopy images supported that the QDs alone or only laser treatment at these doses do not cause any significant cell death (Figure 3-8c(ii, iii) and d(ii, iii)). However, dramatic cell death was achieved when QD treated cells were also exposed to laser irradiation in the case of both the MCF7 and HT29 cell lines (Figure 3-8c(iv) and d(iv)).

Later, the cell death mechanism was studied further using an Annexin-V/FITC-PI kit and flow cytometry. AS-GSH QDs at 100 mg/mL [Ag] caused about 10% cell death, mostly necrotic and late apoptotic, in both cell types (Figure 3-9), which is in agreement with the MTT results (Figure 3-5) and the literature.²⁹ Upon laser irradiation of QD2 treated cells, the percentage of live cells dropped down to 54% and 50% for HT29 and MCF7, respectively. These values agree with the MTT data, as well (Figure 3-8a, b). Late apoptotic and necrotic cell death are found to be dominant, which agrees well with the literature [36], [108], [208]. The major difference between the two cell lines is the

relatively larger amount of necrotic HT29 cells (26%) versus more apoptotic (early and late apoptotic) MCF7 cells (33%) (Figure 3-9). The cell type has a great impact on the mechanism of cell death, which may be investigated further. These results clearly demonstrate that AS-GSH QDs significantly kill tumour cells upon NIR laser irradiation and are promising candidates for image guided PTT.

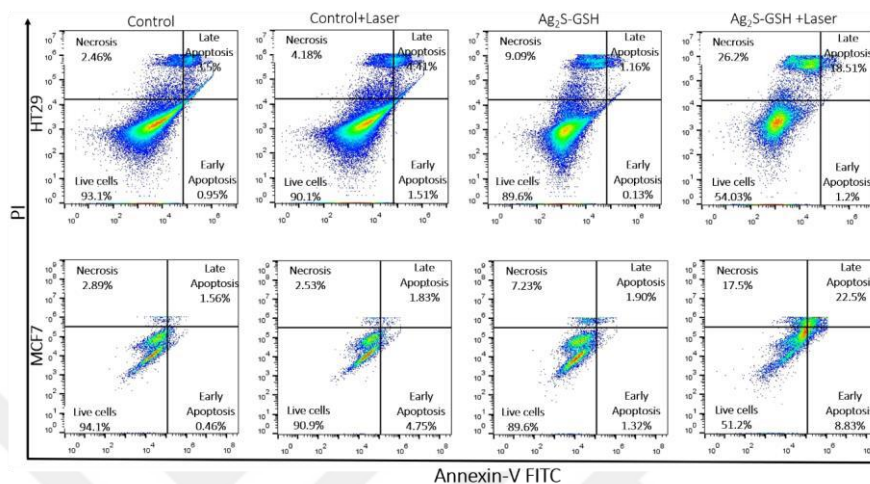


Figure 3–9: Flow cytometry analysis of apoptotic HT29 and MCF7 cell lines treated with AS-GSH and laser at 100 µg/mL [Ag]. Control indicates untreated cells. Control + Laser indicated cells treated only with laser irradiation for 10 min (700 mW, 1.82 W/cm²).

3.5 Conclusion

Aqueous colloidal AS-GSH QDs with excellent colloidal and good optical stability over a year were obtained with emission maxima between 850 and 950 nm by changing the reaction temperature, pH, time for growth and the S-source. Reactions run at 50 °C and pH 10 for 2 h using Na₂S produced un-aggregated QDs with the smallest hydrodynamic size of ca. 7 nm with emission maxima at ca. 900 nm and a 70% QY. These are the smallest AS QDs coated with GSH with the strongest luminescence in the literature, to the best of our knowledge, and were produced in a very simple way. AS-GSH QDs were well internalized by HT29 and MCF7 cancer cells and provided strong optical signal in the NIR by eliminating the cell autofluorescence and showed no cytotoxicity up to a 200 mg/mL dose in either cell line.

Laser treatment of AS-GSH QD solutions at 795 nm demonstrated a dose and laser power (300 to 800 mW) dependent increase in the solution temperature, which can be achieved over and over again with the same magnitude in repeated irradiation of the same solution. In the *in vitro* studies, a dose dependent, dramatic drop in viability was seen in both the HT29 and MCF7 cell lines using a safe laser irradiation procedure (10 min, 700 mW, 1.8

W/cm²). Almost complete elimination of viable cells in the MCF7 cell line and a 60% reduction in viability in the HT29 cell line, which is usually more resistant to PTT [107], were achieved at a 100 mg/mL dose with a single laser treatment. Live/dead assay confirmed that QD + laser treatment causes the death of these tumour cells and Annexin-V/FITC-PI staining indicated late apoptotic and necrotic cell death. The ability to achieve such significant cell death with otherwise safe nanoparticles after local and single irradiation is very promising for NIR image-guided local therapies for cancer. Considering the popularity of targeted therapies, which require small QDs such as these ones and the rise of the photoacoustic imaging potential of AS QDs, these AS-GSH QDs possess great theranostic potential.

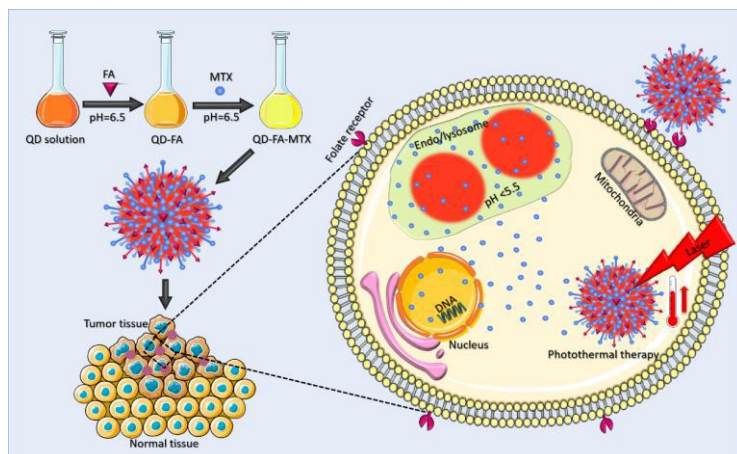


Chapter 4:

**Folic Acid Targeted Ag₂S-Glutathione Quantum Dots
for the Combination of NIR Image Guided
Chemo/Photothermal Therapy**

4.1 Abstract

Multifunctional quantum dots (QDs) with photothermal therapy (PTT) potential loaded with an anticancer drug and labelled with a targeting agent can be highly effective nano-agents for tumour specific, image-guided PTT/chemo combination therapy of cancer. Ag-chalcogenides are promising QDs with good biocompatibility. Ag₂S QDs are popular theranostic agents for imaging in near-infrared with PTT potential. However, theranostic applications of AgInS₂ QDs emitting in the visible region and its PTT potential need to be explored. Here, we first present a simple synthesis of small, glutathione (GSH) coated AgInS₂ QDs with peak emission at 634 nm, 21% quantum yield, and excellent long-term stability without an inorganic shell. Ag₂S-GSH QDs emitting in the near-infrared region (peak emission=822 nm) were also produced. Both QDs were tagged with folic acid (FA) and conjugated with methotrexate (MTX). About 3-fold higher internalization of FA-tagged QDs by folate-receptor (FR) overexpressing HeLa cells than HT29 and A549 cells was observed. Delivery of MTX by QD-FA-MTX reduced the IC₅₀ of the drug from 10 µg/mL to 2.5-5 µg/mL. MTX release was triggered at acidic pH, which was further enhanced with local temperature increase created by laser irradiation. Irradiation of AgInS₂-GSH QDs at 640 nm (300 mW) for 10 min, caused about 10°C temperature increase but did not cause any thermal ablation of cells. On the other hand, Ag₂S-GSH-FA based PTT effectively and selectively killed HeLa cells with 10 min 808 nm laser irradiation via mostly necrosis with an IC₅₀ of 5 µg Ag/mL. Under the same conditions, IC₅₀ of MTX was reduced to 0.21 µg/mL if Ag₂S-GSH-FA (Scheme 4-1).



Scheme 4-1: Multifunctional QD with FA targeted PTT potential loaded with an MTX [209].

Introduction

Cancer is one of the leading causes of death worldwide, and fights against cancer requires better early detection, selective and more efficient therapeutic methods [210], [211]. Signs of success in advanced techniques enabling simultaneous diagnosis and therapy have carried the anti-cancer research into a new dimension [32], [211], [212]. Advances in the discovery of cancer biomarkers also play a pivotal role in early diagnosis, development of antibody drugs, and targeted drug delivery systems.

Folate receptor (FR) is a cell surface receptor which is one of the cancer biomarkers with many cysteine residues [212]. FRs are overexpressed in malignant epithelial cells and make it possible to differentiate them from normal cells [210]. Folic acid (FA) is a popular ligand for selectively binding to FR with a high binding affinity ($K_d \sim 10^{10}$ M) [33], [213]. FA and its conjugates are selectively internalized by cancer cells overexpressing FR via receptor mediated endocytosis [214]. Zhang et al. designed highly biocompatible folate-targeted carbon dots with the average particle sizes of 4.1 nm for cancer diagnosis [210]. Methotrexate (MTX) is an analogous structure to folic acid and internalized by FR and has a high affinity to dihydrofolate reductase (DHFR) and folates to prohibit proteins, DNA, and RNA inside the cancer cells [215]. MTX is one of the most widely used anti-inflammatory, anti-proliferative, immunosuppressant drug, and one of the earliest anticancer drugs [216], [217]. Since FR is exceptionally upregulated in malignant cells compared to healthy cells, it is possible to differentiate the cancer cells by detecting FR expression using a nanocarrier for the therapeutic agents, such as MTX, to overcome severe side effects and improve the efficiency of chemotherapy [110], [210]. Various

elegant nanocarriers such as nanoparticles [210], polymeric micelles [218], and inorganic nanomaterials [110] have been developed. Among them, fluorescent nanomaterials are especially promising since they allow optical tracking coupled with enhanced tumour uptake via enhanced permeability and retention effect (EPR) or tumour specific delivery of the drugs when tagged with receptor specific ligands [211], [219].

Luminescent semiconductor quantum dots (QDs) are popular fluorescent nanostructures that are exploited for diagnostic or theranostic purposes due to their narrow emission, broad absorption window, large molar extinction coefficient, which make them an encouraging optical imaging agent over organic fluorophores [14], [15], [220]. Heavy metal free Ag₂S near-infrared QDs (AS NIRQDs) offering strong emission in the NIR window and high cytocompatibility with ultralow solubility ($K_{sp}=6.3 \times 10^{-50}$) became one of the most promising QDs over the last decade [149], [34]. Glutathione (GSH) which is an antioxidant that protects the cells from oxidative damages and binds effectively to chalcogenides through its thiol, has been an interesting coating material for QDs [149], [221]. Recently, Hashemkhani et al. demonstrated that GSH coated AS QDs offer strong intracellular NIR emission and also provide a strong photothermal effect when irradiated at 808 nm [72], [222].

PTT is a local laser therapy technique used mostly in acne treatment in the clinics, but it is being investigated for cancer therapy as well [44]. Light to heat conversion via irradiation of photosensitizers increases the local temperature to a therapeutic level, which may cause cell membrane damage and denaturation of proteins [99]. The main advantage of PTT over the chemotherapeutic drugs is the fact that the photosensitizer does not become active or toxic unless locally irradiated. Several important issues in designing materials for PTT are biocompatibility of the photosensitizer, effective light-to-heat conversion with short irradiation at low laser powers to avoid damage of healthy tissue in the path of the light, and use of visible or NIR wavelength for deeper penetration of light [43], [44]. One attractive approach in phototherapeutic methods is image-guided application of light which allows focusing the light only to the target-region with high photosensitizer accumulation. Couple of efforts offer CT-PTT using Au or WS₂ nanoparticles [223], [224] or MRI-PTT using Fe₃O₄, MnSe, MnO₂ [225], [226], [227], [228]. However, if optical imaging is coupled with PTT, then the whole operation would be light-based and may be even performed with a single device. This would provide

attractive simplicity, safety and reduce the cost of application. Therefore, PTT potential of AS-GSH QDs at long irradiation wavelengths (i.e., 808 nm) allowing deeper light penetration and minimization of scattering events, coupled with NIR imaging are beneficial for practical applications and clinic translation [229]. Indeed, a combination of chemotherapy and PTT may be achieved by drug loaded nanoparticles. Lue et al. combined chemotherapy and PTT in NIR region for the treatment of cancer using folic acid tagged-black phosphorus QDs loaded with doxorubicin [219]. The thermal ablation of cancer cells was demonstrated at a concentration of 50 µg/mL QD concentration (808 nm, 1 W/cm², 10 min). Li et al. reduced the viability of cancer cells significantly using 808 nm laser treatment for PTT (1 W/cm², 5 min) with NIR laser for magnetic polydopamine coated with hyaluronic acid-methotrexate at the nanoparticles concentration of 200 µg/mL [230].

Recently, ternary group I–III–VI QDs such as CuInS₂, CuInSe₂, and AgInS₂ with visible emission have been investigated as well [231]. Part of the reason for this is the lack of clinical devices for NIR optical imaging and the fact that many *in vivo* imaging instruments and traditional analysis devices such as fluorescent microscopes and FACs systems work in the visible region. Heavy metal-free AgInS₂ (AIS) with small bandgap energy between 1.87 and 2.03 eV is a potential candidate for biological studies [221], [232]. Currently, most of highly luminescent AIS QDs were synthesized in non-polar organic solvents and/or at high reaction temperatures and usually with a ZnS shell to achieve strong emission [221], [233]. There are some efforts to synthesize AIS QDs directly in water using water soluble coating materials such as mercaptopropionic acid and polyethyleneimine [233], [234]. GSH coated AIS QDs that were synthesized in water was also reported but with 3% quantum yield, which increased to 15% only after ZnS shell deposition [221]. In another study, GSH coated quaternary Zn-AIS QDs were produced in water with 10-30% quantum yield (with respect to R6G) [235]. Unlike AS NIRQDs, there are no reports on the PTT potential of AIS QDs.

Here, one of the aims of this study is to develop GSH coated AS and AIS QDs that are luminescent in the NIR and visible region, respectively, to deliver MTX specifically to folate receptor overexpressing cancer cell lines to reduce the effective therapeutic dose of the toxic drug, as well as to reduce site toxicity, and to investigate the potential FR targeted chemo/ PTT combination. The use of two different QDs (AS and AIS) allows

different optical ranges for imaging and phototherapy. For this purpose, synthesis of small, stable, and highly luminescent GSH coated AIS QDs in aqueous media was achieved first. AS-GSH QDs were synthesized based on the reported procedure [72]. FA was conjugated to QDs via a PEG spacer, and MTX was covalently linked to GSH via an amide bond. The FR targeting, optical imaging potential and dark-cytotoxicity of these QDs were investigated on FR (+) HeLa, and FR (-) HT29 and A549 cell lines. The PTT potential of AIS was determined for the first time using 640 nm laser with a power of 300 mW. pH and laser triggered release of MTX was determined. Therapeutic effects of the FR-targeting, MTX-loaded QDs were assessed on HeLa and HT29 cells *in vitro* with and without laser irradiation. Impact of laser irradiation on cell membrane integrity, generation of reactive oxygen species, and on cell death mechanisms was investigated on HeLa cells treated with AS QD family. Such nanoparticles with rational design have great potential as a new therapeutic platform that deserves further investigation.

4.1.1 Folic acid and its mechanism of action

Folic acid (FA) is a water-soluble vitamin B that was first isolated from spinach leaves, and its name was driven from the Latin word for leaf (folium). Since the synthesis of folates in human bodies is not possible, they rely on dietary intake to derive sufficient amount of vitamins [236]. FA was therapeutically used by Lucy Wills in 1931 for the first time to treat tropical macrocytic anemia [236]. FA has an essential role in DNA synthesis, therefore take a role in the production and maintenance of new cells [237]. Folate is responsible for the transfer of carbon groups which are required in DNA synthesis and proliferation, as well. Therefore, the lack of FA may cause inadequate nucleic acid synthesis and impairs cell division [238]. Several mechanisms are underlying the beneficial effects of FA [237], [238]. 1) FA reacts with dihydrofolate reductase (DHFR) to form dihydrofolate (DHF) utilizing nicotinamide adenine dinucleotide phosphate (NADPH) enzyme (Figure 4-1). Then, DHF is reduced to highly active tetrahydrofolate (THF) using DHFR and NADPH. THF serves as a carbon source. 5, 10-methylene THF, 5-methenyl THF, and 10-formyl THF metabolized to pyrimidine and purine, essential bases in DNA synthesis. 2) FA has a homocysteine lowering effect. Homocysteine is a sulphur-containing amino acid that is metabolized to methionine by methionine synthase in the presence of vitamin B12 and FA as a methyl donor [239]. The methyl group produced in this pathway is utilized in DNA methylation to synthesize pyrimidine and

purine.

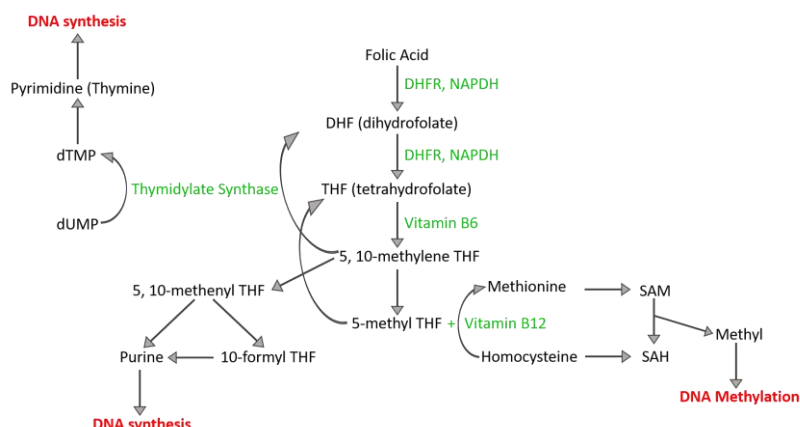


Figure 4–1: Folic acid and cellular mechanism.

4.1.2 Methotrexate and its mechanism of action

MTX is an antifolate drug with the structure similar to FA and folinic acid (5-formyl-tetrahydrofolic acid) that inhibits a couple of folate-dependent metabolic pathways (Figure 4-2) [240]. In general, MTX is administered weekly to patients with 7.5-25 mg/week dosage either orally or intramuscularly. The half-life of MTX in the serum is 6-8 h after drug administration [240]. Once MTX is absorbed, it is metabolized to more active polyglutamate derivatives that can inhibit other folate-dependent pathways such as thymidylate synthetase, and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase. MTX inhibits DHFR, which reduces FA to DHF and THF and consequently inhibits purine and pyrimidine synthesis [238], [240]. Inhibition of AICAR transformylase increases the AICAR levels with additional inhibition of adenosine deaminase (ADA). MTX increases the adenosine concentration that can bind to many transmembrane-spanning adenosine surface receptors (such as A₁, A_{2α}, A_{2β}, and A₃) [238]. Binding of adenosine to A₂ and A₃ receptors inhibits the proliferation of lymphocytes and consequently suppresses the inflammation. Possibly, a combination of these mechanisms are responsible for the anti-inflammatory effects of MTX [238]. As mentioned above, methionine is an essential sulphur-containing amino acid that is a substrate for cysteine. In the conversion of homocysteine to methionine, FA and vitamin B12 are utilized to produce methyl groups for DNA methylation. MTX therapy impairs the methionine pathway and therefore inhibits the DNA synthesis, as well [240].

Patients taking MTX are more likely to stop therapies due to the adverse effects of MTX

rather than lack of efficiency. Hence, it has been documented over the past 30 years that either FA or folinic acid (Leucovorin[®]) supplementation helps to prevent MTX associated toxicities [237], [238]. Besides, concomitant use of MTX with either FA or folinic acid has no impact on MTX therapy's efficacy. The American College of Rheumatology (ACR) guidelines in 2002 state that the use of FA or folinic acid prevents MTX therapy complications [238]. But, high dosage of folinic acid results in the reduction of MTX uptake that could impair the MTX's anti-inflammatory effects. Therefore, patients are advised to take folinic acid at least 12 h after MTX therapy [238], [240].

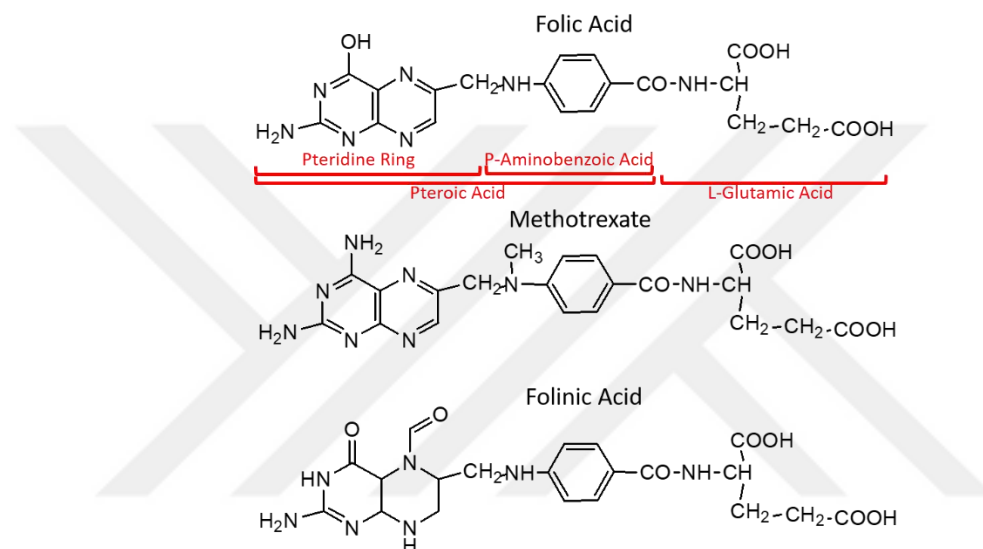


Figure 4–2: Structures of folic acid, methotrexate, and folinic acid.

4.2 Materials and Methods

4.2.1 Materials

All reagents were of analytical grade or of the highest purity. Silver nitrate (AgNO₃) and In(NO₃)₃.xH₂O, folic acid and 2-(N-morpholino)ethanesulfonic acid (MES) were purchased from Sigma-Aldrich. Sodium sulfide (Na₂S) and 1-ethyl-3-(3-dimethylamino propyl) carbodiimide (EDC) were purchased from Alfa Aesar (UK). Methotrexate, glutathione (GSH), sodium hydroxide (NaOH), ethanol, acetic acid (CH₃COOH), and N-hydroxysuccinimide (NHS) were purchased from Merck (USA). Amine-polyethylene glycol-valeric acid (NH₂-PEG-VA) (3400 MW) was purchased from Laysan Bio Inc. (AL, USA). The polysulfone filtration membranes (3 and 30 KDa) were purchased from Sartorius (Goettingen, Germany). The live/dead cell viability assay was purchased from Thermo Fisher Scientific. CytoTox-one homogeneous membrane integrity assay (LDH

assay) was purchased from Promega (USA). The Annexin V-FITC early apoptosis detection kit was purchased from Cell Signaling Technology (UK).

Roswell Park Memorial Institute (RPMI) 1640 and McCoy's 5A (Modified) medium were obtained from Multicell, Wisent Inc. (Canada). Fetal bovine serum and l-glutamine were purchased from Capricorn Scientific GmbH (Germany). Trypsin-EDTA and penicillin-streptomycin solutions were provided from Wisent Inc. (Canada). Thiazolyl blue tetrazolium bromide (MTT) and phosphate buffered saline (PBS) tablets were purchased from Biomatik Corp. (Canada). Paraformaldehyde solution 4 % in PBS was obtained from Santa Cruz Biotechnology, Inc. (USA). 12-well and 96-well plates were purchased from Nest Biotechnology Co. Ltd. (China). HeLa human cervical cancer cells, A549 adenocarcinoma human alveolar basal epithelial cells, and HT29 human caucasian colon adenocarcinoma cells were provided by the Gozuacik Lab (Sabanci University, Istanbul, Turkey).

4.2.2 Synthesis of AS-GSH QDs

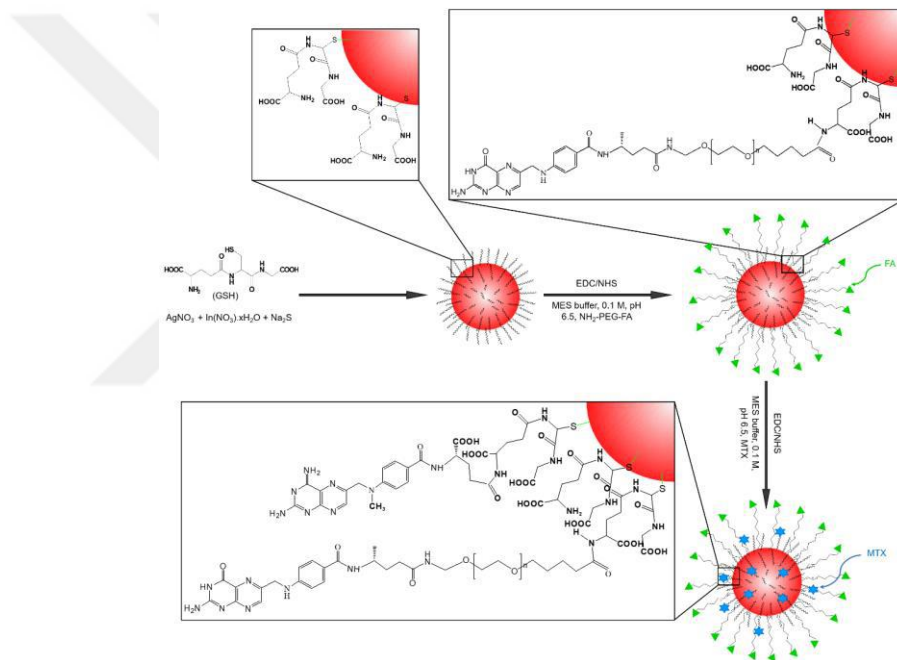
AS-GSH QD was synthesized based on our previous work. Briefly, AgNO₃ and GSH in the mole ratio of Ag/GSH: 1/2 were dissolved in 75 mL deoxygenated water in a round bottom flask under argon. The pH of the solution was adjusted to 10 using NaOH and CH₃COOH (1M) at 50 °C. Na₂S was first dissolved in 25 mL deoxygenated water, then was added to the reaction mixture under vigorous mechanical stirring at 500 rpm. Reaction kept stirring for 2h. At the end, prepared quantum dot solutions were washed and stored as described above.

4.2.3 Preparation of FA-PEG-COOH

Folic acid was conjugated to NH₂-PEG-VA (MW 3400 Da) by EDC/NHS reaction in MES buffer (0.1 M, pH 6.5). First, folic acid (0.75 mmol) was dissolved in 10 mL of MES buffer (0.1 M, pH 6.5) and activated with EDC (3 mmol) and NHS (3 mmol) for 30 minutes. Then, NH₂-PEG-VA (0.075 mmol) was added to the reaction solution and agitated overnight at room temperature under dark condition. After conjugation of FA to NH₂-PEG-VA, the solution was washed with deionized water via Sartorius Ultra centrifugal filters (30 kDa cut off) and stored at -20 °C after being lyophilized.

4.2.4 Preparation of FA tagged QDs

After activation of FA-PEG-COOH (0.02 mmol) with EDC (0.2 mmol) and NHS (0.2 mmol) in 10 mL of MES buffer (0.1 M, pH 6.5) for 30 minutes, QD (50 mg) was added and was stirred at RT for 24 h. Finally, the resulting particles were washed with deionized water using Sartorius Ultra centrifugal filters (30 kDa cut off). The amount of FA conjugated to the nanoparticles was calculated according to the absorbance of FA at 320 nm using a concentration dependent absorbance calibration curve for folic acid (Figure 4-3a).



Scheme 4-2: Synthesis of MTX conjugated and FA tagged QDs.

4.2.5 Preparation of FA tagged and MTX conjugated QDs

AIS-GSH-FA and AS-GSH-FA QDs (50 mg) were taken into MES buffer (0.1 M, pH 6.5). 10.5 mg EDC and 6.3 mg NHS were added to the reaction mixture and stirred for 30 min at room temperature. The resulting solution was then mixed with 2.5 mg of MTX and gently stirred overnight in the dark at room temperature. Conjugated QDs were washed from centrifugal filters (30 kDa cut off) with deionized water for at least 5 times to remove the unconjugated MTX from AIS-GSH-FA-MTX and AS-GSH-FA-MTX (Scheme 4-2). The amount of unloaded MTX was calculated from the MTX absorbance at 301 nm utilizing a calibration curve created with free MTX (Figure 4-3b) and the

loading efficiency (LE) was calculated using the following equation:

$$LE\% = \frac{\text{Weight of loaded drug}}{\text{Weight of drug loaded QDs}} \times 100 \quad \text{Equation 4-1}$$

For drug release studies, AIS-GSH-MX and AS-GSH-MTX were also synthesized following the same procedure starting with 50 mg QDs and conjugating 2.5 mg MTX.

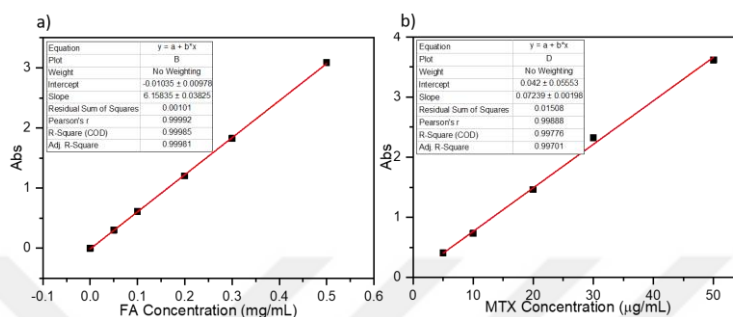


Figure 4-3: Concentration dependent absorbance calibration curve for (a) FA at $\lambda_{\text{abs}}=320$ nm and (b) MTX at $\lambda_{\text{abs}}=301$ nm.

4.2.6 Characterization

To measure the photoluminescence (PL) intensity, an Agilent Cary Eclipse fluorescence spectrophotometer was used at 480 nm and 532 nm excitation with the emission in the range of 500-900 nm and 600-1100 nm for AIS-GSH and AS-GSH QDs, respectively. The absorbance was measured with a Shimadzu UV-3600 spectrophotometer in the 300–1000 nm range. The quantum yield (QY) was calculated based on the procedures in the literature using Rhodamine 6G as reference according to the following equation:

$$QY = \left(\frac{\text{Grad QD}}{\text{Grad Rhodamine 6G}} \right) \left(\frac{\eta_{\text{water}}}{\eta_{\text{EtOH}}} \right)^2 \times 100 \quad \text{Equation 4-2}$$

Grad is the ratio of fluorescence intensity to the absorbance of the corresponding sample and Rhodamine 6G dye, which was used as a reference, and η is the refractive index of the solvents.

Hydrodynamic size and zeta potential measurements of the aqueous QDs were determined by dynamic light scattering (DLS) using Malvern zetasizer nano ZS. The Ag⁺ content of the QD solutions was determined after treating samples with nitric acid and sulfuric acid (9:1 v/v) using an Agilent 7700x Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Thermo Scientific Nicolet iS10 instrument (FTIR) was utilized in the wavenumber region from 650 to 4000 cm⁻¹ with 4 cm⁻¹ resolution for functional

group analysis. For X-Ray photoelectron spectroscopy (XPS) measurements, a Thermo Scientific K-Alpha XPS with Al K-alpha monochromatic radiation (1486.3 eV) was used. Dried samples were placed on an adhesive carbon tape and exposed to 50.0-eV X-rays with a 400- μ m spot size and a resolution of roughly 0.5 eV. All spectra were corrected with respect to C1s peak at 284.5 eV. To determine the crystal structure of AIS QDs, a Bruker D2 Phaser Benchtop X-Ray Diffraction (XRD) system with Cu K- α radiation ($\lambda=1.5406$ Å) was used between 2θ angles of 10° and 80° . The organic content of the QDs was measured by thermogravimetric analysis (TGA, TGA 500). The samples were heated from room temperature to 1000 °C at a heating rate of 10 °C/min under a nitrogen atmosphere.

4.2.7 Drug release behaviour in the simulated physiological solution

To investigate the *in vitro* drug release from these QDs, 2mL of MTX-conjugated QDs were put into dialysis bags (MWCO 3.5 kDa) and placed in 50 mL PBS at pH 7.4 and pH 5.5 and stirred at 100 rpm at 37 °C for 95 h. At different time intervals, 2 mL aliquots were replaced with fresh buffer and the aliquots were used to determine the released MTX from the absorbance at 301 nm by using the calibration curve. In order to determine the effect of temperature increase upon PTT on the release of MTX from the QDs, the same experiment was repeated at pH 5.5 with 10 min laser irradiation at 808 nm (700 mW laser power) for AS-GSH-MTX and 640 nm (300 mW laser power) for AIS-GSH-MTX. Results are reported as the average and the standard deviation of two runs.

4.2.8 Cell culture

HeLa human cervical cancer cells, A549 adenocarcinoma human lung epithelial cells and HT29 human colon adenocarcinoma were grown at 37°C under 5% CO₂ in RPMI and McCoy's medium with 10 % fetal bovine serum, 2 % l-glutamine (200 mM) and 1% penicillin-streptomycin.

4.2.9 Determination of cytotoxicity

In vitro cytotoxicity of QDs was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) viability assay. HeLa, A549, and HT29 cells were seeded in 200 μ L of culture medium at a density of 12500 cells/well in 96-well plates, incubated for 24 h at 37 °C in a 5% CO₂ atmosphere. QD doses were added at different concentrations based on the concentration of the cations obtained by ICP-MS, for 4, 24

and 48 h. After incubation, 50 μ L of MTT solution (5 mg/mL in PBS, pH 7.4) was added to each well with 150 μ L of culture medium and incubated for 4 more hours. Purple formazan crystals that appear in the mitochondria of living cells were dissolved with DMSO/EtOH (1:1 v/v) solution. The absorbance intensity was read by a microplate reader (BioTek ELx800 Absorbance Microplate Reader) at 600 nm with a reference wavelength of 630 nm. Treated cells without MTT reagent were used to correct the absorbance intensities. The relative cell viability of treated cells was calculated with respect to the untreated control cells using the following equation:

$$\text{cell viability (\%)} = [\text{Absorbance treated cells} / \text{Absorbance control}] \times 100 \quad \text{Equation 4-3}$$

4.2.10 Intracellular uptake of QDs

HeLa, A549, and HT29 cells were seeded on 3 mL glass-bottom petri dishes at a density of 1.75×10^5 cells/well in complete medium and grown for 24 h at 37 °C and 5% CO₂. Next day, the growing cells were incubated with nanoparticles at 25 μ g/mL cation concentration for 4 h. Then, the medium was replenished, and the cells were fixed with paraformaldehyde solution (4% in PBS) for 20 min. After washing with PBS (three times), cells were incubated for an additional 15 min with 2 μ g/mL of DAPI nucleus dye. The cells were rewashed three times with PBS to remove the unbound dye and left in 2 mL PBS to prevent drying. Cells without any QD treatment were used as controls. The fixed cell samples were examined using confocal microscope (Leica dmi8/SP8) with different filters for NIR region (λ_{exc} : 510-550 nm and λ_{em} : 710 nm long pass), Alexa 488 (λ_{exc} : 488 nm and λ_{em} : 580-780) and DAPI (λ_{exc} : 325-375 nm and λ_{em} : 435-485 nm). The same experimental procedure was also performed for control cells which were not treated with nanoparticles. Images were processed and merged using ImageJ analysis program.

The quantitative analysis of QD uptake was performed by the determination of cation content in cells after incubation of cells with the QDs. HeLa, A549, and HT29 cells were prepared as in microscopy image analysis and incubated with QD samples for 4 h. Then, the cells were washed with PBS to remove un-internalized nanoparticles by the cells and were collected after trypsinization. Acid digestion procedure was performed (suprapure nitric acid 65% and suprapure sulphuric acid 96%), and cation content was determined by ICP-MS (n=3).

4.2.11 Laser irradiation protocol and PTT studies

PTT studies were conducted in an experimental setup which consisted of two different diode lasers operating at the wavelengths of 640 nm and 808 nm. The output beam of each laser was adjusted with the help of a telescope to match the beam diameter to the diameter of the wells on the 96-well plate. The diode laser operating at 640 nm delivered a maximum power of 300 mW, corresponding to an irradiance fluence of 0.78 W/cm². The second laser at 808 nm was operated up to a maximum output power of 700 mW (corresponding fluence=1.8 W/cm²). For the photothermal heating experiments, the aqueous solutions (0.75 mL) containing AIS-GSH (with concentrations of 3, 6, and 9 mg/mL) were put inside a polystyrene cuvette and irradiated at 640 nm for 10 minutes. The temperature rise was measured with a k-type thermocouple at room temperature. For the *in vitro* PTT studies, HeLa and HT29 cells (12500 cells/well) were seeded in 96-well plates and cultured as described above. QDs were added to each well at different cation concentrations in the range of 5-100 µg/mL and incubated for 4 and 24 h. Cell irradiation experiments were conducted inside a plexiglass box which was kept at 37 °C with a temperature controller. During these experiments, the 96-well plates were placed inside this box and irradiated with either the 640-nm or 808-nm diode laser. Laser irradiated cells were then incubated further for 24 h before cell viability was determined using the standard MTT protocol.

4.2.12 Mechanism of death studies

4.2.12.1 LDH assay, live/dead assays, and determination of intracellular ROS levels

Cells were prepared as described above and treated with QDs at 10 µg/mL [Ag] dose for 4 h. Then, the medium was replaced with a fresh one and cells were irradiated with 808 nm laser for 10 min, as described above. For the LDH assay (Promega) and determination of intracellular ROS levels, the irradiated cells were incubated for an additional 24 h. LDH levels were determined based on the protocol provided by the manufacturer and using the emission at 590 nm with 560 nm excitation. The ROS levels in the cells were determined using the Enzo Total ROS Detection Kit following the manufacturer's instructions from the 570 nm emission at 540 nm excitation. Fluorescence was read using Synergy H1 microplate reader (Biotek). For the live/dead assay, irradiated cells were co-

stained with the dyes of the kit as described by the manufacturer (Thermo Fischer Scientific). Finally, images were collected using a fluorescent microscope (Zeiss Axio observer Z1).

4.2.12.2 Cell apoptosis assay

HeLa cells were treated with QDs at 10 µg/mL [Ag] concentration for 4 h with/without laser exposure at 808 nm with 700 mW for 10 min and were further incubated for 24 h. Then, the cells were collected and stained with PI and Annexin V-FITC of the early apoptosis detection assay kit (Cell signaling technology, UK) according to the manufacturer's instruction and finally analysed by flow cytometry (Attune NXT focusing cytometer Thermo Scientific, UK). The data were processed with FlowJo (USA).

4.2.13 Statistical analysis

Statistical evaluation of data was carried out using multiple Dunn's comparison test of GraphPad Prism 8 software package (GraphPad Software, Inc., USA). P-value < 0.05 was accepted for statistical significance. All quantitative data were expressed as mean ± standard deviation (S.D.).

4.3 Results and Discussions

4.3.1 Synthesis and characterization of AIS-GSH NIRQDs

In this study, we first explored the synthesis of AIS-GSH QDs in water, based on our previous procedure optimized for AS-GSH QDs [240], [222]. The AIS-GSH QDs were prepared at cation/S ratio of 2/1, Ag/In ratio of 1/4 and GSH/cation ratio of 2/1 at 100 °C in water from Ag and In salts using Na₂S at two different pH values (10 and 8.5). Figure 4-4a-d shows the time dependent changes in the PL and UV-visible (UV-vis) spectra of the QDs during the synthesis. No change in the peak position was observed but there was an increase in the emission intensity at pH 10 with time, reaching its maximum at about 120 min (Figure 4-4e). At pH 8.5, a slight red shift in peak position coupled with an increase in the emission intensity was observed after 10 min, but both properties stayed the same in the next 2h, which is in agreement with the growth behaviour reported by Luo et al. [221].

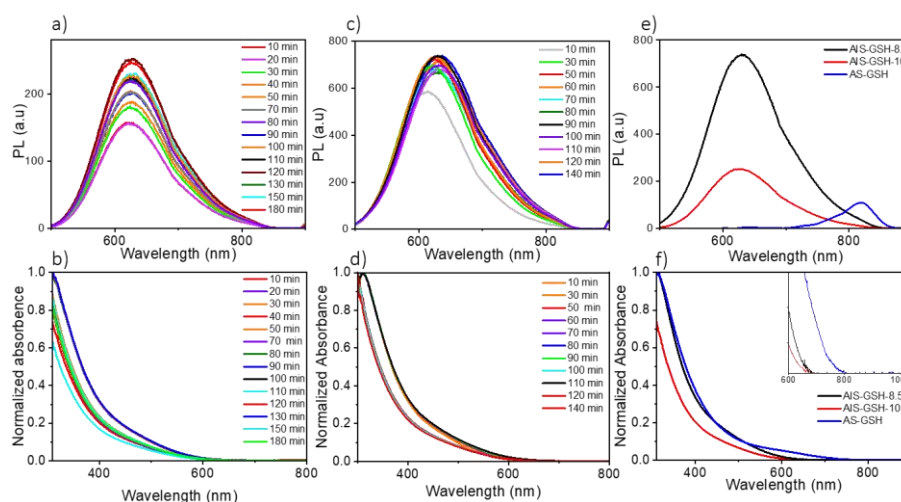


Figure 4-4: The PL and UV-vis spectra of the (a), (b) AIS-GSH-10 and (c), (d) AIS-GSH-8.5 QDs prepared at different conditions pH=10 and pH=8.5, respectively. (e) The PL and (f) UV-vis spectra of the AS-GSH and AIS-GSH QDs prepared at different conditions.

When the most luminescent AIS-GSH QDs synthesized at two different pH values were compared, a clear pH dependence was seen (Figure 4-4e). At about the same peak position, AIS-GSH QDs prepared at pH 8.5 had about 3.5 times stronger PL intensity with a QY of 21.1% with respect to rhodamine 6G. QY of the AIS-GSH QDs prepared at pH 10 was 8.4% while Luo et al. reported the QY of 3% for their most luminescing AIS-GSH QDs [221]. Luo et al. utilized an S-rich reaction with cation/S ratio of 0.24 and GSH/cation ratio of 4. Excess S may cause defects and excess GSH beyond a certain point may cause surface distortions or influence surface binding, which may all cause non-radiative relaxation of excited electrons [34]. The reason for higher QY in pH 8.5 compared to 10 could be attributed to the protonation state of the functional groups of GSH. The apparent pK_a values for the thiol and the amine groups of GSH are 8.66 and 9.6. At pH 10, the thiol is deprotonated, thereby more reactive to bind or coordinate with metal ions [241], [242] but amino groups are also deprotonated. This may compete with the chelation of the cations, bind to crystal surface and cause surface defects that would cause a reduction in QY [241]. At pH 8.5, some of the thiol groups deprotonate, but the amines are protonated, which would reduce aggregation, its surface binding and chelation. Hydrodynamic sizes of the AIS QDs synthesized at pH 10 has a large fraction with an average ca. 101 nm based on the scattered light intensity despite the high surface charge, which indicates some aggregation during crystal growth (Table 4-1). Taken together, growth time and reaction pH influenced the relative intensity of the emission but not its position. Ag/In/S/GSH ratio of 1/4/2.5/10 and reaction at pH 8.5 provided a

AIS-GSH with 21% QY, very small hydrodynamic size (6.01 nm number based, 28.82 nm intensity based) and strong negative surface charge (-38.3 mV). This recipe improved the quality of the known AIS-GSH dramatically. AIS-GSH-8.5 was used in the remaining experiments and was named as AIS-GSH in the rest of the study.

Table 4-1: Effect of the different reaction variables on the optical properties of AIS-GSH QDs.

	Ag/S	Ag/In	λ_{emi} (nm)	λ_{abs} (nm)	QY (%)	E_g^a (eV)	FWHM ^b (nm)	Dh- number ^c (nm)	Dh- intensity ^d (nm)	Zeta potential (mV)
AS-GSH	1/0.5	-	822	804	-	1.54	62.63	3.8±0.7	29.7±2.1	- 21.5±3.4
AIS-GSH-10	1/2.5	1/4	624	660	8.4	3.61	129.47	8.2±3.2	101.53±9.1	-37.57 ± 5.7
AIS-GSH-8.5	1/2.5	1/4	634	681	21.1	3.31	137.01	6.01±0.3	28.82±2.3	- 38.3±8.4

^a Bandgap energy by Tauc plot. ^b Full-width at half-maximum of the emission peak. ^c Hydrodynamic diameter measured by DLS and reported as the number average. ^d Hydrodynamic diameter measured by DLS and reported as the intensity average.

Compared to AS-GSH QDs, there is a large blue shift in both emission peak position and absorption onset of AIS-GSH nanoparticles, as expected (Figure 4-4e, d). AS-GSH QDs are luminescent in the NIR while AIS-GSH QDs are luminescent in the visible region. Bandgap energies of the three QDs calculated from the absorption spectra using Tauc equation (Figure 4-5a, b Table 4-1) shows an increase from 1.54 (AS-GSH QDs) to 3.31 eV (AIS-GSH-8.5 QDs) that leads to a blue shift in the absorption and emission spectra. In addition, the best AIS-GSH produced here has about seven times stronger luminescence than AS-GSH QDs. Doh et al. demonstrated that the addition of a different cation like In in Ag₂S causes an increase in the bandgap and has a great impact on the size and optical properties of QDs (QY_{AS}=0.2% and QY_{AIS}=12%) [243]. This was explained by the recombination of electron-hole pairs captured at the defect sites of small size ternary QDs, which provides a relatively high QY [244].

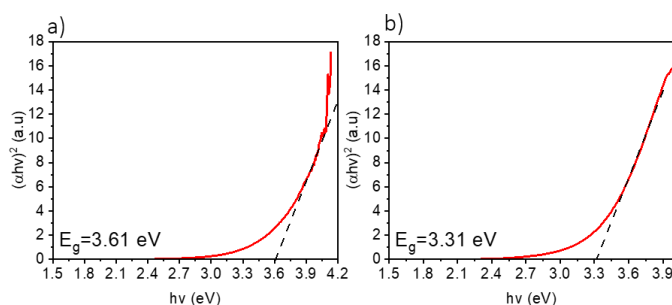


Figure 4-5: Bandgap determination by Tauc plot for (e) AIS-GSH-10, (f) AIS-GSH-8.5 QDs.

FTIR analysis confirmed GSH binding to AIS surface (Figure 4-6a). The broad absorption band at 3280 cm⁻¹ and 1578 cm⁻¹ indicate the presence of OH and carboxylate groups. The absence of S-H stretching band which is around 2500-2700 cm⁻¹ confirms the binding of GSH to AIS core from its thiol [222]. XPS provided more information about the structure (Figure 4-6b-e). Peaks at the binding energy (BE) of 367.10 eV (3d5/2), 373.09 eV (3d3/2), 444.05 eV (3d5/2), and 451.51 eV (3d3/2) correspond to the reported BE peaks of Ag and In in AIS, respectively [15]. The peaks at 161.10 (2p3/2) eV and 162.38 (2p1/2) eV are typical for the chalcogenide S. N 1s at 399.21 and 401.11eV stands for NH and NH₃ of GSH. After background subtraction and correction for atomic sensitivity factors, the area under each curve provided information about the amounts of these elements. Ag/In/S ratios were calculated as 1/3.2/2.2, respectively. Ratios calculated from ICP-MS are in agreement with XPS results with Ag/In/S: 1/3.5/2 (experimentally, these ratios were Ag/In/S: 1/4/2.5). Broad diffraction peaks detected via XRD analysis are attributed to tetragonal AIS QDs (Figure 4-6f) [245]. TGA analysis performed on AIS-GSH QD indicates 39.8% organic content (Figure 4-6g).

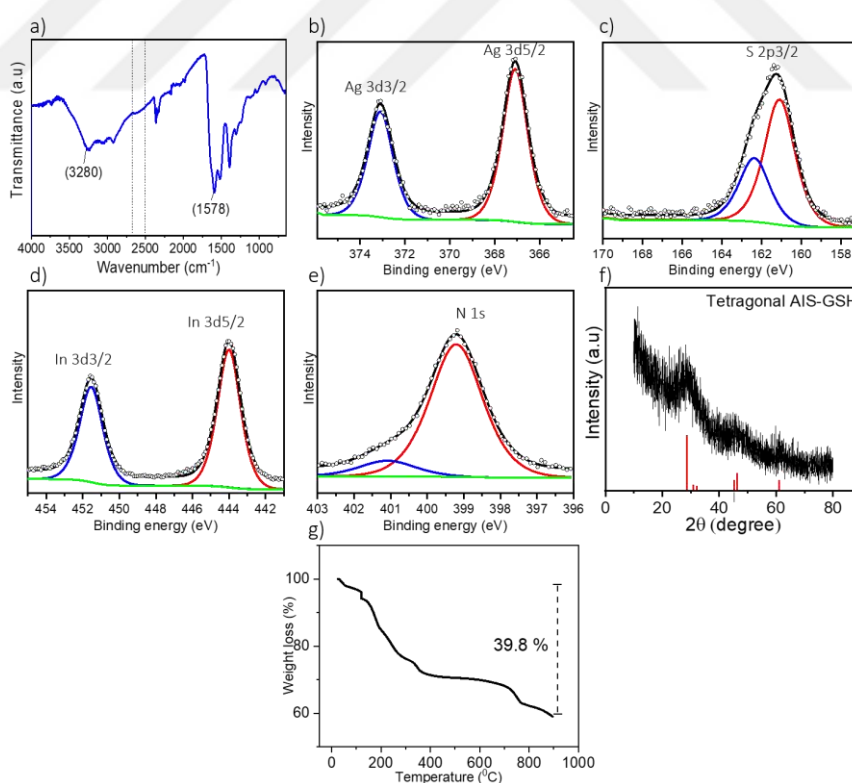


Figure 4-6: (a) FTIR, (b) Ag 3d, (c) In 3d, (d) S 2p, and (e) N 1s XPS spectra of AIS-GSH QDs. (f) XRD spectra and (g) TGA of AIS-GSH QDs.

4.3.2 Long term stability of AIS-GSH QDs

To investigate the optical and colloidal stability of the QDs stored at 4 °C after washing, changes in the absorbance/emission and hydrodynamic size were monitored over a period of 6 months. The results in Figure 4-7a, b and Table 4-2 revealed that the emission maxima and absorption peak did not change significantly after 6 months. The hydrodynamic size based on intensity increased from 28.82 to 40.21 nm, indicating a slight aggregation which may also account for the drop in the emission intensity. However, this may be due to some oxidation of the surface ligands. After all, particles were colloiddally stable with no precipitation or change in colour (Figure 4-7c). Hu et al. reported aqueous AIS QDs with 21.6% QY with about 10 weeks stability [246]. In addition, Liu et al. reported 3 months of colloidal stability for the aqueous AIS/ZnS core/shell QDs [247]. Compared to AIS QDs produced in the literature, these AIS-GSH QDs offer much longer shell life with a strong emission after 6 months and no precipitation. Therefore, such stability combined with simple composition, preparation, and high QY makes these AIS-GSH QDs great candidates for biomedical applications with longer shelf life.

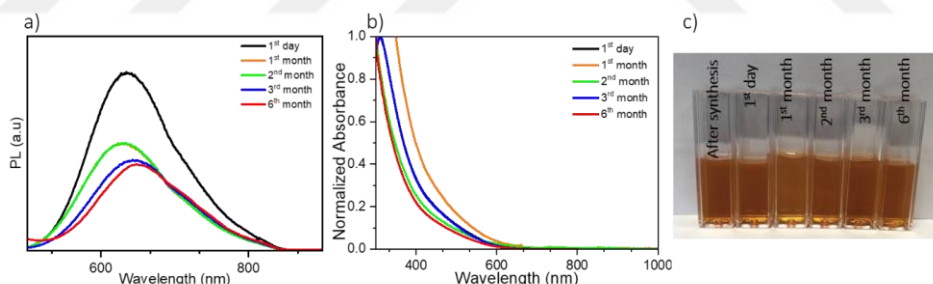


Figure 4–7: (a) PL spectra and (b) normalized absorbance spectra of AIS-GSH QDs from the 1st day to 6th month after synthesis. (c) Picture of AIS-GSH QDs at different time points following its synthesis showing its colloidal stability.

Table 4-2: Time dependent changes in particle properties.

	$\lambda_{\text{abs}}^{\text{a}}$ (nm)	E_{g}^{b} (eV)	λ_{emi} (nm)	Dh-number ^c (nm)	Dh-intensity ^d (nm)	Zeta potential (mv)
AIS-GSH-1st day	681	3.31	634	6.01±0.3	28.82±2.3	-38.3±8.4
AIS-GSH-1st month	730	3.24	630	6.16±1.1	23.32±2.9	-36.5±2.9
AIS-GSH-2nd month	714	3.28	630	6.71±0.3	40.92±8.9	-44.1±6.8
AIS-GSH-3rd month	714	3.28	644	7.01±0.3	42.53±5.7	-46.3±3.2
AIS-GSH-6th month	717	3.28	648	6.97±0.8	40.21±4.5	-39.9±8.8

^a Absorbance onset. ^b bandgap energy by Tauc plot. ^c Hydrodynamic diameter measured by DLS and reported as the number average. ^d Hydrodynamic diameter measured by DLS and reported as the intensity average.

4.3.3 Preparation and characterization of FA and MTX conjugated QDs

In this study, both the AS-GSH QDs which were prepared and optimized based on our previous published work and the newly synthesized AIS-GSH were conjugated with FA and MTX to compare their efficiency in FR targeted combination therapy and also to provide an option for both visible range and NIR optical imaging modality (Scheme 4-2). FA was readily combined with other molecules through gamma-carboxyl group, resulting in no reduction in its affinity to FR [210]. In order to increase the accessibility of FA to the folate receptors, NH₂-PEG-VA (MW 3400 Da) was used as a spacer between the crystal surface and FA. Initially, carboxylic acid of FA was activated by EDC/NHS in MES buffer (0.1 M, pH 6.5) and then was reacted with NH₂-PEG-VA. Then, the carboxylic acid of the produced FA-PEG-VA was coupled with the amine group of GSH via EDC/NHS activation. Finally, MTX was conjugated to QDs via direct amidation. This kept MTX away from FA during its binding to FR.

FA conjugation to PEG and QDs were confirmed spectroscopically from the absorbance of FA at 370 nm (Figure 4-8a, c). Based on a calibration curve prepared from the concentration dependent absorption of free FA, conjugation efficiency of FA to AIS-GSH and AS-GSH were determined as 97.3% and 93.6%, respectively. This resulted in ~60% drop in the emission intensity of both QDs with a slight red shift (Figure 4-8b, d). Reduction in the emission intensity is typical when QDs are subjected to post-synthetic modifications due to changes in the medium and the surface of QDs. An increase in the intensity based hydrodynamic size from ca 30 nm to 75 nm indicates some aggregation, and this may have an influence on the drop in emission intensity, as well (Table 4-3).

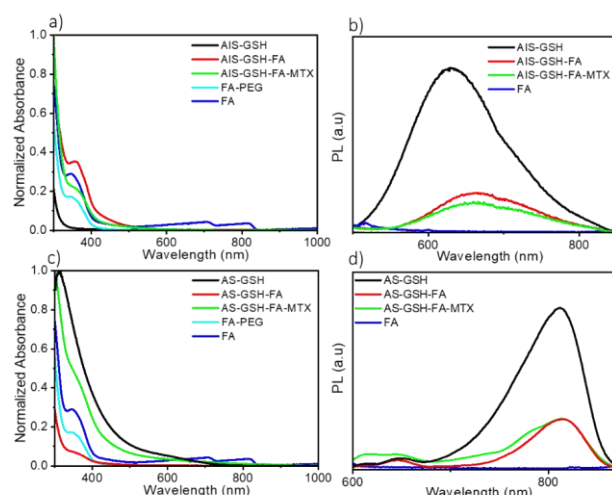


Figure 4-8: (a), (c) The UV-vis, (b), (d) PL spectra of AIS and AS QDs, respectively.

MTX conjugation to AIS-GSH-FA and AS-GSH-FA were 60.23 and 54.4%, respectively. MTX has absorbance peaks at 301 and 360 nm, which is not distinguishable when conjugated to QDs due to the broad absorption of QDs (Figure 4-8a, c). Therefore, MTX conjugation efficiency was determined from the absorbance of unbound MTX. MTX conjugation increased the intensity based hydrodynamic size to 110-140 nm, which represents some agglomeration, with an expected drop in the zeta potential. However, this was not accompanied by a further change in the emission property of either QDs.

Table 4-3: Hydrodynamic properties of MTX and FA conjugation to AS-GSH and AIS-GSH QDs.

Samples	PDI ^a	Dh-number ^b (nm)	Dh-intensity ^c (nm)	Zeta potential (mv)
AS-GSH	0.43	3.8±0.7	29.7±2.1	-21.5±3.4
AS-GSH-FA	0.28	6.34±0.6	75.36±10.6	-27.03±1.9
AS-GSH-FA-MTX	0.18	7.4±1.2	113.5±20.9	-15.03±0.4
AIS-GSH	0.15	6.01±0.3	28.82±2.3	-38.3±8.4
AIS-GSH-FA	0.31	6.51±1.9	76.34±3.6	-38±1.3
AIS-GSH-FA-MTX	0.29	6.64±1.1	141.71±7.2	-24.6±6.9

^a Polydispersity index, ^b Hydrodynamic diameter measured by DLS and reported as the number average. ^c Hydrodynamic diameter measured by DLS and reported as the intensity average.

4.3.4 Investigation of the PTT potential of AIS-GSH QDs

In order to evaluate the PTT potential of AIS-GSH QDs, the temperature increase of aqueous solutions between 3-9 mg/mL QD concentrations were determined during 10 min irradiation at 640 nm with a power of 300 mW, as described in the PTT protocol. As shown in Figure 4-9, the temperature increase (ΔT) was about 10 °C, which does indicate mild hyperthermia. Photothermal efficiency is calculated using the following formula:

$$\eta = \frac{ha(T_{max}-T_{amb})-Q_{dis}}{P(1-10^{-A_{640nm}})} \quad \text{Equation 4-4}$$

where h is the heat transfer coefficient, a is the irradiated area, T_{max} is the maximum equilibrium temperature, T_{amb} is the ambient temperature, and Q_{dis} is the heat dissipated due to light absorption in the polystyrene cell containing water without nano particles. P and A_{640nm} appearing in the denominator represent the incident laser power and absorbance of the AIS-GSH QDs, respectively, at 640 nm.

ha can be calculated by using the following formulas:

$$ha = \frac{\sum m_i c_{p,i}}{\tau_s} \quad \text{Equation 4-5}$$

$$t = -\tau_s \ln \theta \quad \text{Equation 4-6}$$

$$\theta = \frac{T-T_{amb}}{T_{max}-T_{amb}} \quad \text{Equation 4-7}$$

Above, m , C_p , t , τ_s are the mass of sample, the corresponding specific heat capacity, the time during the cooling process, and the cooling time constant, respectively. The quantity Q_{dis} was neglected at 640 nm. For AIS-GSH QDs with a concentration of 6 mg/mL, the measured values of T_{max} , T_{amb} , τ_s , m_{water} , C_{water} , $m_{cuvette}$, $C_{cuvette}$, and A_{640nm} were 31.4 °C, 24 °C, 180 sec, 0.75 g, 4.186 J/g °C, 0.7 g, 1.4 J/g °C, and 0.47, respectively. By substituting these parameters into Equation S1, the PTT efficiency was determined to be 85.5%. The photothermal effect and temperature increase of AS-GSH QDs have been previously investigated by Hashemkhani et al. [72]. At 808 nm irradiation, AS-GSH QDs provided a ΔT around 20 °C with a PTT efficiency of 80 %.

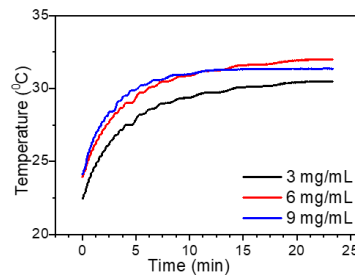


Figure 4-9: Concentration dependent change in the temperature of AIS-GSH solutions at 640 nm laser source with 300 mW laser power for 10 min.

4.3.5 Drug release behaviour in the simulated physiological solution

Release of MTX from these QDs were monitored via dialysis at 37 °C for 95 h at pH 5.5

and 7.4 mimicking endosomal and physiological pH values. The drug release behaviour was closely related to the pH and possibly to laser irradiation since temperature increase can facilitate hydrolysis [248]. Therefore, the release profiles of MTX in PBS at different pH values with/without laser, exposure were examined. MTX release from either QDs at pH 7.4 was less than 10% after 30 h, indicating the high stability of MTX-QD bond at physiological pH (Figure 4-10a, b). At pH 5.5, which is similar to endosomal pH, a significant release of MTX starts and reaches 17% in 48 h and 19% in 90 h, indicating slow acidic breakdown of the amide bond. This is in agreement with the studies of Kohler et al. which studied the release of amide bonded MTX, as well [249], [250]. Even faster release may be expected in the *in vitro* and *in vivo* experiments where pH drops down further to 4.5-4.0 in the late endosomes [251]. Once internalized in the acidic compartments of the cells such as lysosomes, acidic pH and proteases break down the amide bond releasing MTX faster.

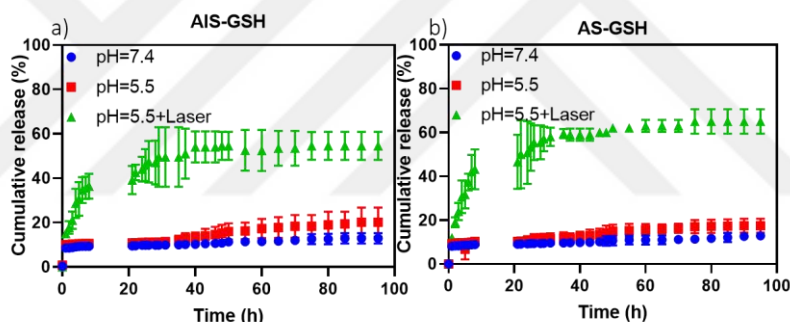


Figure 4-10: Drug release profiles of (a) AS-GSH-MTX in PBS solution at pH 7.4 and 5.5 with/without laser illumination at 808 nm for 10 min (700 mW, 1.82 W/cm²), at 37 °C for 95 h. (b) AIS-GSH-MTX in PBS solution at pH 7.4 and 5.5 with/without laser illumination at 640 nm for 10 min (300 mW, 0.78W/cm²), at 37 °C for 95 h.

In addition to the pH sensitivity, the influence of laser irradiation, which causes local temperature increase, on the release rate of MTX from QDs was also studied at pH 5.5. As shown in Figure 4-10a, b, a dramatic enhancement of drug release was observed after a single 10 min irradiation either at 808 nm for AS-GSH or 640 nm for AIS-GSH. After a steady MTX release in the first 24 h reaching to 45-55%, a more sustained release was observed. About 58 and 49% MTX was released from AS-GSH-MTX and AIS-GSH-MTX QDs, respectively, 30 h after laser irradiation, whereas only 10-12% MTX was released without the laser treatment at pH 5.5. As shown in Figure 4-9, after laser irradiation of AIS-GSH QDs at 640 nm for 10 min, the temperature increased to ca 32 °C at 6 mg/mL QD concentration, while the temperature in AS-GSH QDs increased to ca 45

°C at the same condition [72]. According to MTX release profile of both QDs, local temperature increases of this amount, effectively speeded up the release rate, slightly better with AS-GSH QDs as shown in Figure 4-10a, b (MTX release in AS-GSH-MTX: 65% and in AIS-GSH-MTX: 54.5% after 40h). Cheison et al. [252] reported higher hydrolysis of peptides and amide bonds at 40-45 °C than 30-35 °C in short incubation times, while Robinson and co-workers [253] reported increasing hydrolysis rate of amide bonds under acidic conditions with increasing incubation times. These reports support our observations. Overall, these findings indicate that exogenous laser irradiation and the endogenous lower pH in endo-lysosome facilitates the breakdown of the amide bond and increases the release rate of the MTX from QDs. Liu et al. [254] demonstrated that the drug release of doxorubicin loaded hydrogel at pH 6.5 with and without laser has negligible impact on the drug release (drug release with/without laser was almost 100% after 15 h) while Wu et al. [211] showed the drug release accelerated from 60 to 92% after NIR laser irradiation at pH 5 after 24 h. Faster drug release from the irradiated samples provides an attractive turn-on mechanism which can be exploited to induce strong toxicity in the irradiated tumour but much less damage to the non-irradiated healthy tissue.

4.3.6 Determination of cytotoxicity

To evaluate the biocompatibility of these QDs, the viability of cells treated with these nanoparticles were determined by MTT assay in a dose dependent manner (Figure 4-11, 12). HeLa, A549, and HT29 were used as model cancer cell lines, which differ in their FR expression levels. Cation content of QDs, determined by ICP-MS, were used as the basis of dose to keep the particle content constant between different compositions of a given QD. Viability was determined after 4, 24, and 48 h incubation of cells with QDs in order to differentiate receptor mediated uptake from the passive uptake of QDs by the cells. Free MTX was also tested for comparison. The viability of the cells was determined based on the formation of purple formazan by metabolically active cells in the MTT assay. No reduction in cell viability was observed in the cell lines treated with MTX, AIS-GSH or AS-GSH for 4 h (Figure 4-11, d, g and Figure 4-12a, d, g). In the case of HeLa cell line, a dose dependent toxicity was observed after 24 and 48 h incubation with AIS-GSH, which has started at around 25 µg/mL cation concentration, but the viability stayed above 80% until 200 µg/mL and reached the half-maximal effective inhibitory concentration

(IC₅₀) at 300 µg/mL after 48 h (Figure 4-11b-c). FA tagging of AIS-GSH increased the toxicity and showed a dose and time dependent reduction in viability of HeLa cells with FR overexpression. Viability was ca 80% at 100 µg/mL at all incubation times but dropped to 75% and ca 40% at 300 µg/mL in 4 h and 24-48 h incubations, respectively. Results of 24 and 48 h incubations were similar with IC₅₀ at ca 200 µg/mL. MTX loading to FR targeting QDs improved the toxicity dramatically over unloaded QDs and free MTX at long incubation times. No significant difference between AIS-GSH-FA and AIS-GSH-FA-MTX was observed after 4 h incubation, except at the highest dose, most probably due to insufficient drug release. On the other hand, at longer incubation times, targeted delivery of MTX to FR positive HeLa cells via AIS-GSH-FA QDs, reduced the IC₅₀ of MTX to 5 and about 2.5 µg MTX/ml in 24 and 48 h respectively, reaching to about 85-90% reduction in viability at 15 µg/mL MTX while 50% of the HeLa cells were still viable when treated with free MTX. This is in agreement with about 50% reduced IC₅₀ of DOX when delivered to HeLa cells by FA conjugated cationic AS, where the drug was electrostatically loaded to QD [33]. It is quite attractive to see effective intracellular unloading of MTX at a reasonable time to improve the therapeutic outcome as good as electrostatically loaded drugs. Indeed in some cases, while targeting is achieved, the efficacy of nanoparticle-delivered drug may be lower due to ineffective release [214].

A549 and HT29 cell lines, which lack folate receptors, were more resistant to MTX and AIS-GSH QDs in general (Figure 4-11d-i). MTX is an analogue of folic acid that inhibits dihydrofolate reductase; hence it is less toxic to FR negative cell lines [216]. After 4 h incubation, none of the tested materials showed toxicity to either A549 or HT29 cell lines. Viability at 24 and 48 h incubations were again similar. The response of cells to AIS-GSH and AIS-GSH-FA QDs were similar at long incubation times and dropped the viability to ca 80% at the highest concentration. MTX loading to AIS-GSH-FA reduced viability but only as much as free MTX with not much dose dependence between 2.5-15 µg/mL MTX providing 25-40% reduction in viability.

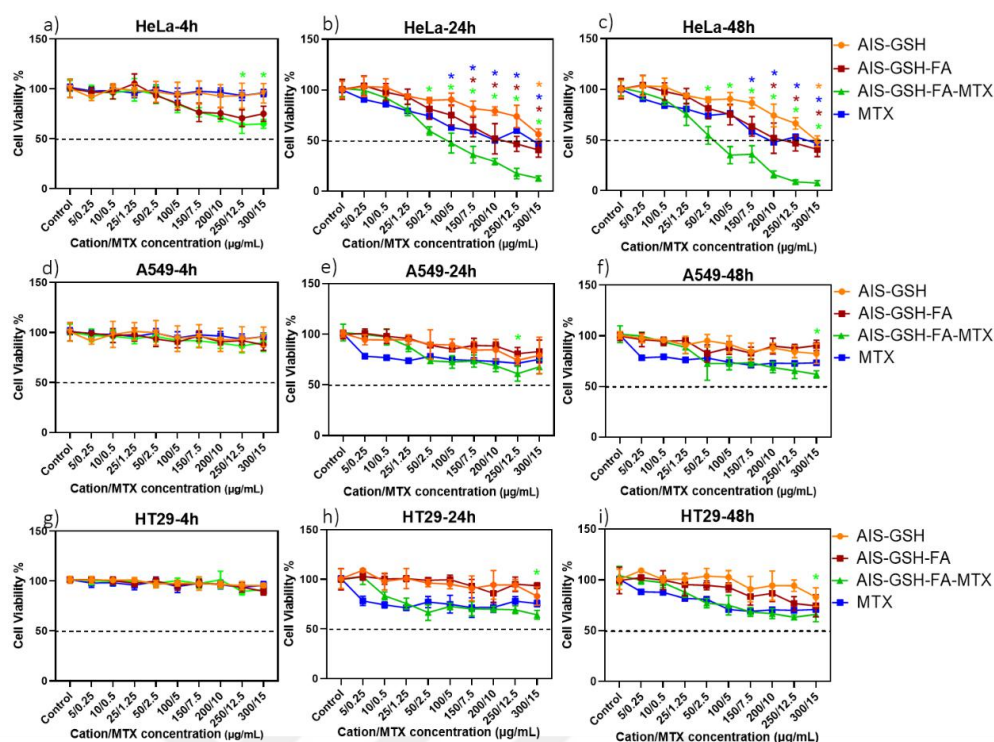


Figure 4-11: Viability of (a), (b), (c) HeLa, (d), (e), (f) A549 and (g), (h), (i) HT29 cells treated with AIS QDs or MTX after 4, 24 and 48 h incubation measured by MTT assay. Untreated cells were used as control. The data are expressed as mean $\pm$ S.D. ($n = 4$), ($p < 0.05$).

Influence of AS-GSH based compositions on cell viabilities are similar to those observed with AIS-GSH family (Figure 4-12). Again AS-GSH QDs were more toxic to HeLa cells than A549 and HT29. Only AS-GSH-FA and AS-GSH-FA-MTX QDs showed significant toxicity starting at around 50 μg cation/mL dose after 4 h incubation with maximum 38% reduction in viability at 300 $\mu\text{g}/\text{mL}$ (Figure 4-12a). But they were not that different from each other just like in the case of AIS-GSH family. AS-GSH started to show toxicity at longer incubation times and 100 $\mu\text{g}/\text{mL}$ with still 65% viability at the highest dose (Figure 4-12b, c). FA conjugation reduced viability significantly due to enhanced FR mediated uptake with an IC_{50} around 250 $\mu\text{g}/\text{mL}$. This is slightly higher than the IC_{50} of AIS-GSH-FA, indicating slightly more toxic nature of AIS. When MTX was loaded to FR targeting AS-GSH-FA, IC_{50} was dropped down to 5 μg MTX/mL at long incubation times (Figure 4-12b, c). While short incubation times prove FR mediated uptake, long incubation times provide enough time for drug release.

AS-GSH based QDs were less toxic to A549 and HT29 cell lines, as in the case of AIS-GSH family (Figure 4-12d-i). No significant toxicity was observed after 4 h incubation of the reagents with these cell lines. A549 did not differentiate AS-GSH and AS-GSH-

FA and viability was above 75% after long incubation times with the studied doses (Figure 4-12d-f). In the case of HT29, FA conjugation increased the toxicity slightly, and viability stayed above 70%. MTX loading to AS-GSH-FA did not improve toxicity beyond free MTX in either cell lines. Indeed, MTX showed relatively non-dose dependent viability at about 27% in A549 cells and slight dose dependence in HT29 with maximum 30% reduction at the highest concentration.

These data clearly demonstrate that FR targeting of MTX with FA conjugated QDs effectively and selectively reduce the therapeutic dose of the drug in FR overexpressing HeLa cells. QD based toxicity seen in A549 and HT29 cells at long incubation times and high concentrations are related to the passive uptake of particles by the cells, which is non-selective. While evaluating the toxicity of QDs which lack MTX, it should be noted that quite a high concentration of nanoparticles was used here. For example, thioglycolic acid coated and gelatin stabilized AgInSe/ZnSe was tested on HeLa and HT29 cells only up to 100 µg nanoparticle/mL to keep viability above 80% [255]. Similarly, FA conjugated black phosphorous was tested on HeLa and A549 cell lines but only up to 100 µg nanoparticle/ml to again keep the viability above 80% [219]. Pluronic F127 encapsulated AIS QDs were tested up to 500 µg/mL on PANC-1 cells and showed viability above 70% after 48 h incubation [256].

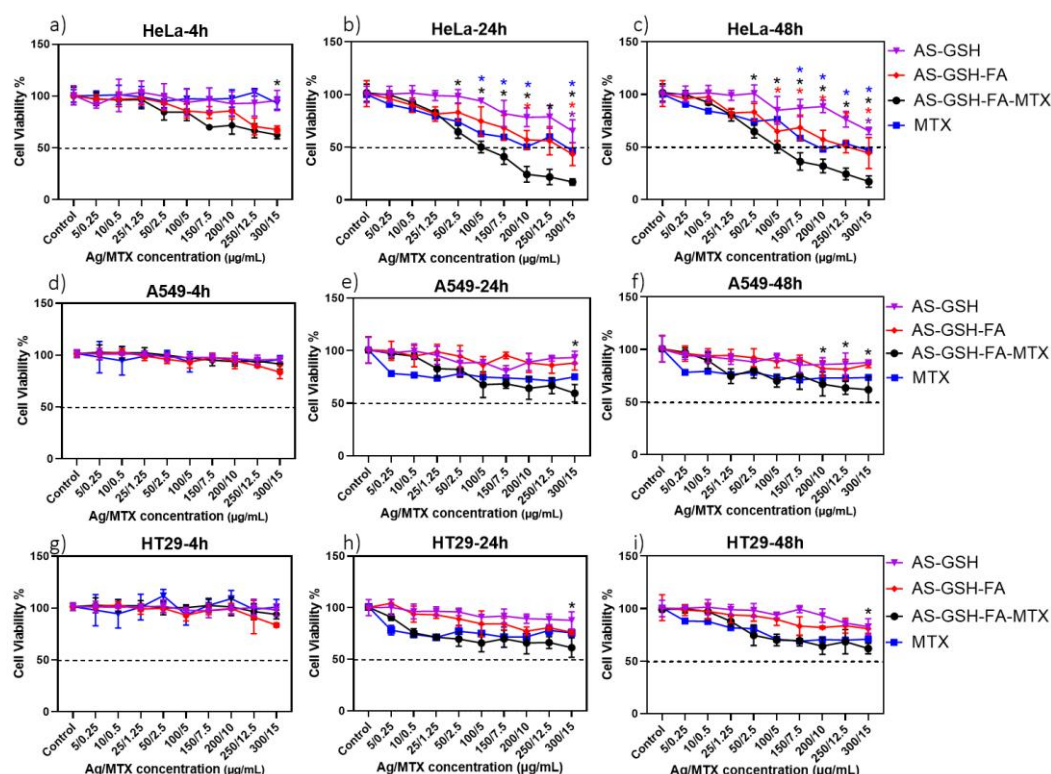


Figure 4-12: Viability of (a), (b), (c) HeLa, (d), (e), (f) A549 and (g), (h), (i) HT29 cells treated with AS QDs or MTX after 4, 24 and 48 h incubation measured by MTT assay. Untreated cells were used as control. The data are expressed as mean $\pm$ S.D. ($n = 4$), ($p < 0.05$).

4.3.7 Cellular uptake

The internalization of these QDs by these three cell lines was investigated quantitatively and qualitatively by ICP-MS and fluorescence microscopy techniques. To differentiate the FR mediated endocytosis versus passive uptake, cells were incubated with the QDs only for 4 h at a nontoxic dose (25 $\mu\text{g/mL}$). Ag and In amount of the cells, which were determined by ICP-MS, indicated no significant difference between the cells treated with AIS-GSH or AS-GSH, but HeLa internalized about 20% while A549 and HT29 internalized about 10% of the applied QDs. A major difference was observed when FA was conjugated to QDs. About 3-folds higher uptake of FA conjugated QDs by HeLa cells compared to A549 and HT29 cells was observed (Figure 4-13a, b) confirming FR mediated uptake of FA tagged QDs by FR-overexpressing HeLa cells. MTX loading did not influence this selective uptake, proving the effectiveness of the nanoparticle design where MTX was covalently conjugated to the surface bound GSH while FA was kept away from the surface with a flexible PEG linker to facilitate its FR binding. These results

successfully support higher toxicity of QDs to HeLa cells, especially after FA conjugation. One concern in the design could have been the involvement of MTX in FA-FR binding. But uptake data shows no significant reduction in the uptake of QD-FA-MTX compared to only QD-FA after 4 h (Figure 4-13a, b). This coupled with no toxicity (Figure 4-11, Figure 4-12) and only about 7% MTX release in 4 h from QDs (Figure 4-10a, b) all support each other and suggested that free MTX is not available in high amount and it does not hinder FA interaction with FR receptors within 4 h.

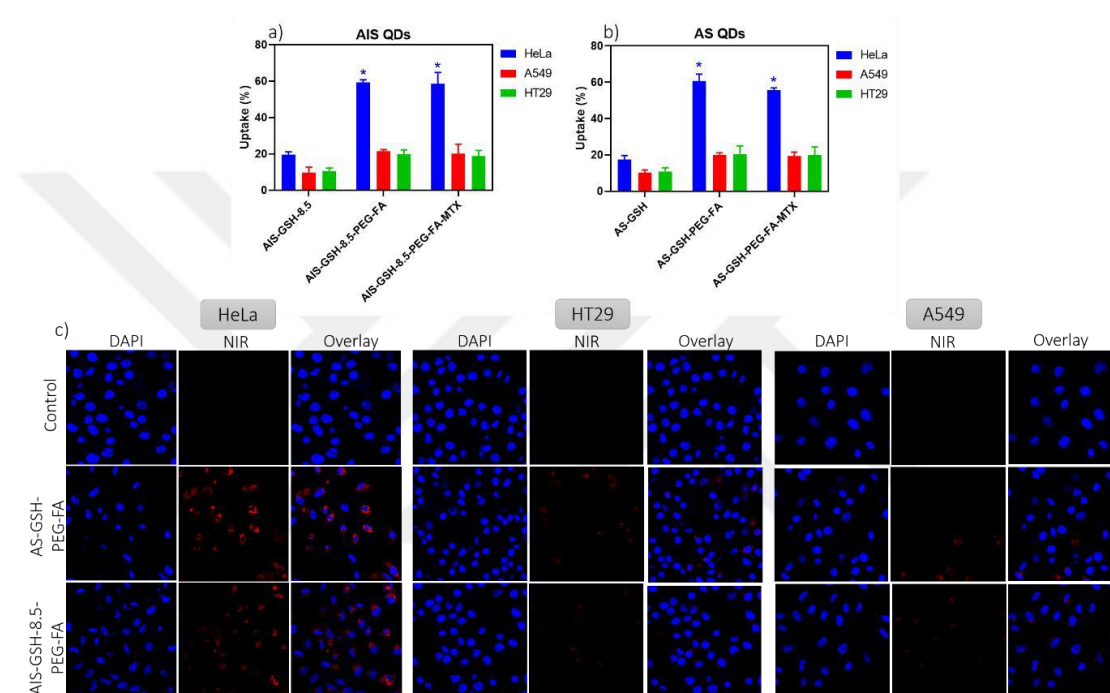


Figure 4–13: Intracellular quantification of (a) AIS QDs and (b) AS QDs in HeLa, A549, and HT29 cells based on the total Ag and In ion concentrations measured from the cell lysates by ICP-MS. Samples at 25 μ g/mL cation concentration were incubated with cells for 4 h. The data are expressed as mean $\pm$ S.D. (n = 3), (p < 0.05). (c) Fluorescence microscopy images of HeLa, HT29, and A549 cells after 4 h incubation with QDs at cation concentration of 25 μ g/mL. Control indicates the cells that were not treated with QDs. Red: Luminescence from QDs (AS-GSH QDs: excitation/emission:510-550/710 nm long-pass filter and AIS-GSH QDs: excitation/emission:488/580-780 nm), Blue: Nuclear stain (excitation/emission: 325-375/435-485 nm).

Fluorescence microscopy images of AS-GSH-FA and AIS-GSH-FA show that FA conjugated QDs are internalized in a dramatically higher amount by the FR-positive HeLa cells as indicated by the red intracellular optical signal generated by the QDs. These images also confirm that these QDs provide a strong intracellular signal which may be exploited for optical detection and/or diagnosis.

4.3.8 *In vitro* combined PTT and chemotherapy

As mentioned before, Hashemkhani et al. have previously studied the photothermal effect of AS-GSH QDs as a function of quantum dot concentration, irradiation time, and laser input power at 808 nm [72]. Considering the low temperature increase (ΔT about 10 °C) and lack of effective PTT potential of AIS-GSH QDs (Figure 4-9, Figure 4-14), combination therapy was only studied with AS-GSH QDs. Since uptake and toxicity results of A549 and HT29 cell lines are quite similar, only FR positive HeLa and FR negative HT29 cell lines were used in the combination therapy. AS-GSH QDs were used in the range of 5-100 $\mu\text{g/mL}$ and incubated for 4 and 24 h. Cells treated with QDs were irradiated for 10 min with 700 mW of laser power at 808 nm. The viability of cells was determined after 24 h incubation following the laser treatment. Cells that were not treated with QDs but irradiated were used as laser control and showed no reduction in viability, indicating that laser treatment by itself is safe. Overall, there is a dramatic reduction in viability of HeLa cells after laser irradiation, pointing out a PTT effect and a dramatic reduction in IC₅₀ when MTX loaded FR targeting QDs were used, proving an enhanced therapeutic effect of PTT/MTX combination (Figure 4-15a-d).

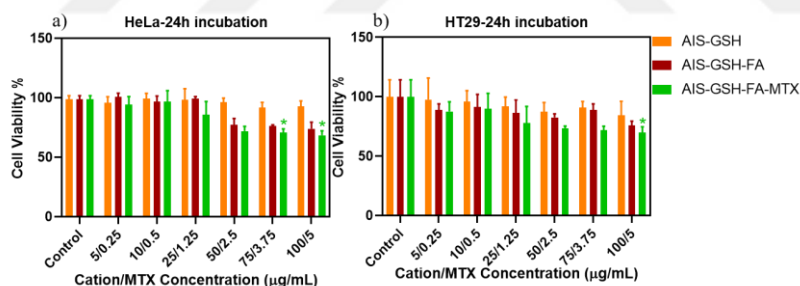


Figure 4–14: Viability of (a) HeLa and (b) HT29 cell lines treated with AIS-GSH, AIS-GSH-FA, and AIS-GSH-FA-MTX upon laser irradiation after 24 h incubation of QDs at different [cation] determined with MTT assay, respectively. Control indicates untreated cells. Laser control indicated cells treated only with laser irradiation at 640 nm for 10 min (300 mW).

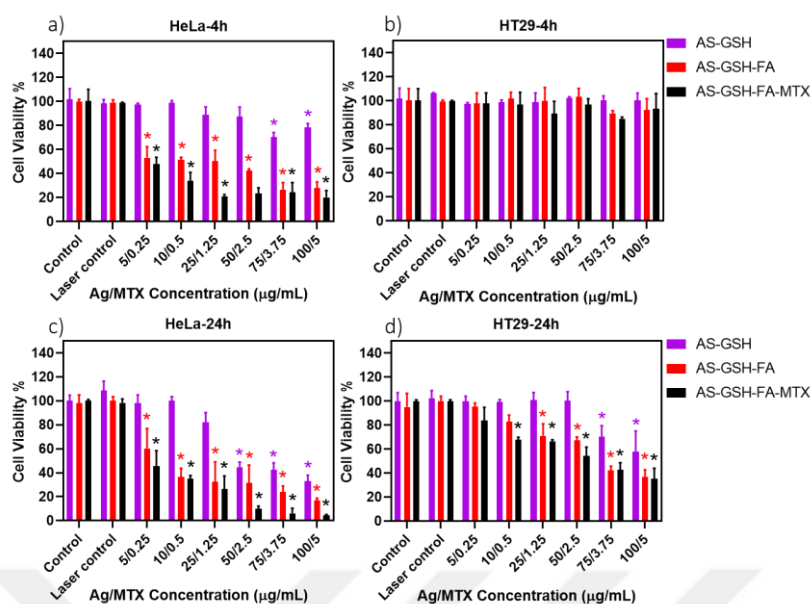


Figure 4–15: Viability of (a), (c) HeLa and (b), (d) HT29 cell lines treated with AS-GSH, AS-GSH-FA, and AS-GSH-FA-MTX and laser after 4 and 24 h incubation of QDs at different [Ag] determined with MTT assay, respectively. Control indicates untreated cells. Laser control indicated cells treated only with laser irradiation at 808 nm for 10 min (700 mW, 1.82 W/cm²).

Laser treatment after 4 h incubation did not cause any phototoxicity in HT29 cells, but about 25% drop in viability at 75-100 μg cation/mL in HeLa cells, which may be explained with higher uptake of HeLa even in the absence of FA (Figure 4-15a, b). Viability of HeLa cells treated with AS-GSH-FA in the absence of laser treatment was 98% at 5 μg Ag/mL but dropped down to 53% after irradiation due to the PTT effect of these QDs. Indeed, only 25% of the HeLa cells were viable at 100 μg Ag/mL concentration after PTT when QDs were tagged with FA, suggesting a therapeutic utility even in the absence of a toxic drug. Combination of PTT and MTX was observed clearly with AS-GSH-FA-MTX with viability around 25% between 5-100 μg Ag/mL after 4 h incubation, compared to 95 and 75% viability at 5 and 100 μg Ag/mL in the absence of laser treatment (Figure 4-12a, Figure 4-15a). Longer incubation times increased the uptake and drug release; therefore, phototoxicity also increased with increasing incubation time. In HeLa cells, 24 h incubation followed by laser irradiation caused significant phototoxicity even with untargeted AS-GSH with viability below 50% at an above 50 μg Ag/mL. At this dose, about 70 and 90% drop in viability was observed with AS-GSH-FA+laser and AS-GSH-FA-MTX+laser treatments. AS-GSH-FA-MTX+laser reduced the IC₅₀ for MTX below 0.25 μg/mL (0.21 μg MTX/mL) and provided an almost complete loss of viability at and above 2.5 μg MTX/mL.

Short incubation time did not cause any phototoxicity to QD treated HT29 cells in agreement with uptake studies. Some phototoxicity in HT29 cells, which were irradiated after 24 h incubation was also observed since more particles would be internalized by the cells via passive uptake at extended exposure times (Figure 4-15b, d). A significant drop in viability was observed at 75 $\mu\text{g Ag/mL}$ and close to 60% viability at 100 $\mu\text{g Ag/mL}$ when HT29 cells were treated with AS-GSH+laser. FA conjugation and MTX binding increased the toxicity further when coupled with PTT but with no significant contribution from MTX, similar to dark toxicity results.

These results suggest effective FR targeting of AS-GSH-FA, which provides significant phototoxicity at safe concentrations, and the therapeutic effect was enhanced dramatically with PTT/MTX combination at relatively low MTX concentrations. Combination therapy provided IC₅₀ below 0.25 $\mu\text{g MTX/mL}$ while it was 5 $\mu\text{g/mL}$ in the absence of PTT after 24 h incubation (Figure 4-12, Figure 4-15a-d). This IC₅₀ is noticeably lower than most of the values reported for FA conjugated nanoparticle in the literature. Luo et al. achieved 50% cell death after treating HeLa cells with 25 $\mu\text{g/mL}$ black phosphorous conjugated with FA and subjected to 10 min laser treatment with 808 nm, corresponding to a fluence of 1 W/cm² laser [219]. Moreover, Wu et al. could not induce significant toxicity in MCF7 cells using FA conjugated NIR-triggered amphiphilic block copolymer nanoparticles at 2 $\mu\text{g/mL}$ with 808 nm laser irradiation (1 W/cm²) after 6 min [211]. AS-GSH-FA-MTX QD showed highly promising results after 4 and 24 h for the application in cancer treatment due to the remarkable tumour targeting ability, low dark toxicity, excellent PTT effect, and especially drug release triggered in acidic pH and enhanced after laser exposure. Pandey et al. [257] achieved the cell viability of 80% in RAW264.7 cells using nanoGold-core multifunctional dendrimer loaded with IR780 and MTX at a concentration of 1 mg/mL MTX even after NIR laser irradiation. According to Zhang et al. [258] the viability of HeLa cells treated with functionalized graphene oxide loaded with MTX at 15 $\mu\text{g MTX/mL}$ dropped from ca 50% to 20% using NIR laser at 808 nm for 5 min. Also, Fallah et al. [259] reported a reduction in the viability of MCF7 cells after incubation with MTX conjugated gold nanorods at nanoparticles concentration of 50 $\mu\text{g/mL}$ from 60 to 20% using 1062 nm laser irradiation for 4 min (125 mJ/pulse). Results provided in Figure 4-15a-d show that combination of AS-GSH-FA-MTX with PTT provides a dramatic anti-proliferative activity on FR(+) cells even at low

concentrations and after short incubation times, but does also cause significant toxicity if FR(-) cells are exposed to QDs for a long time and irradiated. However, the latter does not show much impact of the MTX.

PTT is a harmless therapy due to its higher selectivity, non-ionizing nature of the NIR light, and lower side effects to the normal cells. Therefore, the effect of nanoparticles with/without laser irradiation on the viability of the normal myoblast cells was also tested. The cell viability data on C2C12 healthy myoblast cells demonstrates the viability higher than 80% over the period of 48 h (Figure 4-16a, b). Collectively, these results demonstrated AS-GSH nanoparticles as a potential theranostic agent with potentially no significant toxicity to healthy cells.

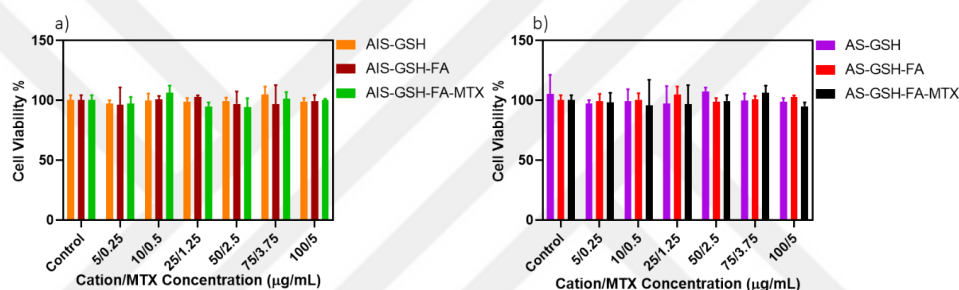


Figure 4–16: Viability of C2C12 cells (normal myoblast cells) treated with (a) AIS-GSH, AIS-GSH-FA, AIS-GSH-FA-MTX and (b) AS-GSH, AS-GSH-FA, AS-GSH-FA-MTX QDs after 48 h incubation measured by MTT assay compared to untreated cells. The data are expressed as mean $\pm$ S.D. (n = 3). (p<0.05).

4.3.9 Investigation of cell death mechanism

4.3.9.1 LDH assay, live/dead assays, and determination of intracellular ROS levels

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme found in many different cells [260]. LDH is rapidly released into the cell culture medium when the cellular membrane is damaged, a key feature of necrosis [260], [261]. Therefore, LDH assay is used to assess the cytotoxicity based on loss of membrane integrity [193], [262]. No difference between the LDH levels of control and QD (10 μg Ag/mL) treated HeLa cells were observed after 4 h incubation. Laser irradiation increased the LDH level of AS-GSH-FA and AS-GSH-FA-MTX treated HeLa cells 2 and 3 folds, respectively. No significant difference between QD compositions and no influence of laser irradiation on LDH release from HT29 cells were observed (Figure 4-17a). These are in agreement with reduced

proliferation in HeLa upon FA and MTX conjugation coupled with laser irradiation as determined by MTT assay, and no dark toxicity or phototoxicity observed in HT29 cell line (Figure 4-15a-d).

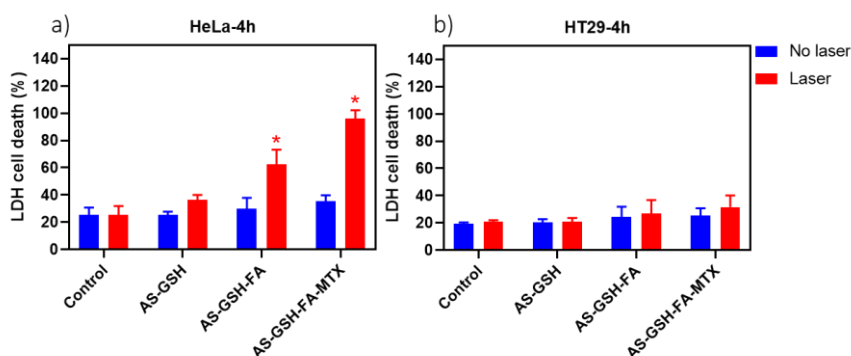


Figure 4–17: LDH release of (e) HeLa and (f) HT29 exposed to 10 µg/mL [Ag] of AS-GSH, AS-GSH-FA, and AS-GSH-FA-MTX QDs after 4 h incubation with/without laser treatment at 808 nm for 10 min (700 mW, 1.82 W/cm²). The data are expressed as mean ± S.D. (n = 3), (p<0.05).

To evaluate the possible ROS formation due to laser irradiation which would contribute to the loss of viability and membrane disruption, levels of the intracellular reactive oxygen species (ROS) were measured before and after laser treatment of 10 µg Ag/mL QD treated cells for 24 h at excitation/emission wavelengths of 540/570 nm. Figure 4-18 reveals that none of the AS-GSH based QDs or laser treatment alone or in combination produced significant levels of ROS.

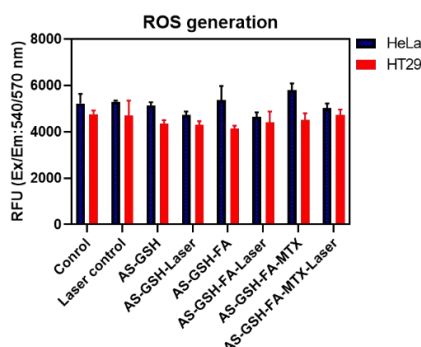


Figure 4–18: ROS level generated by AS-GSH QDs on HeLa and HT29 cells at 10 µg/mL [Ag] after 24 h incubation of the nanoparticles before and after laser treatment (808nm, 700 mW, 10 min) at excitation/emission: 540/570 nm (n=3).

Reduction in the viability of treated cells was further supported with live/dead cell assays where live and dead cells could be identified by co-staining with calcein AM and ethidium homodimer-1, respectively. Cells were treated with AS-GSH based QDs at 10 µg/mL [Ag] for 4 h before staining. Control cells and AS-GSH treated HeLa and HT29 cells

before and after laser treatment were all stained green indicating all live cells (Figure 4-18a). Only a few red (dead) cells were observed in AS-GSH-FA and AS-GSH-FA-MTX treated HeLa cells before laser treatment. After being subjected to the NIR laser irradiation, a large number of dead HeLa cells with a significant increase in the dead cell portion with subsequent FA and MTX conjugation as a result of enhanced uptake by targeting, enhanced drug release, and PTT combination. In agreement with the uptake, MTT, and LDH assay results, HT29 cells were almost entirely live even after the laser irradiation of AS-GSH based QD treatments. The results mentioned above suggest that the AS-GSH-FA-MTX does kill the FR-overexpressing cancer cell line effectively and selectively at a low dose, in short incubation time, and with a safe laser procedure via targeted chemo/phototherapy combination. Besides, AS-GSH-FA QDs can provide an efficient image-guided PTT at NIR wavelength as a single treatment, as well.

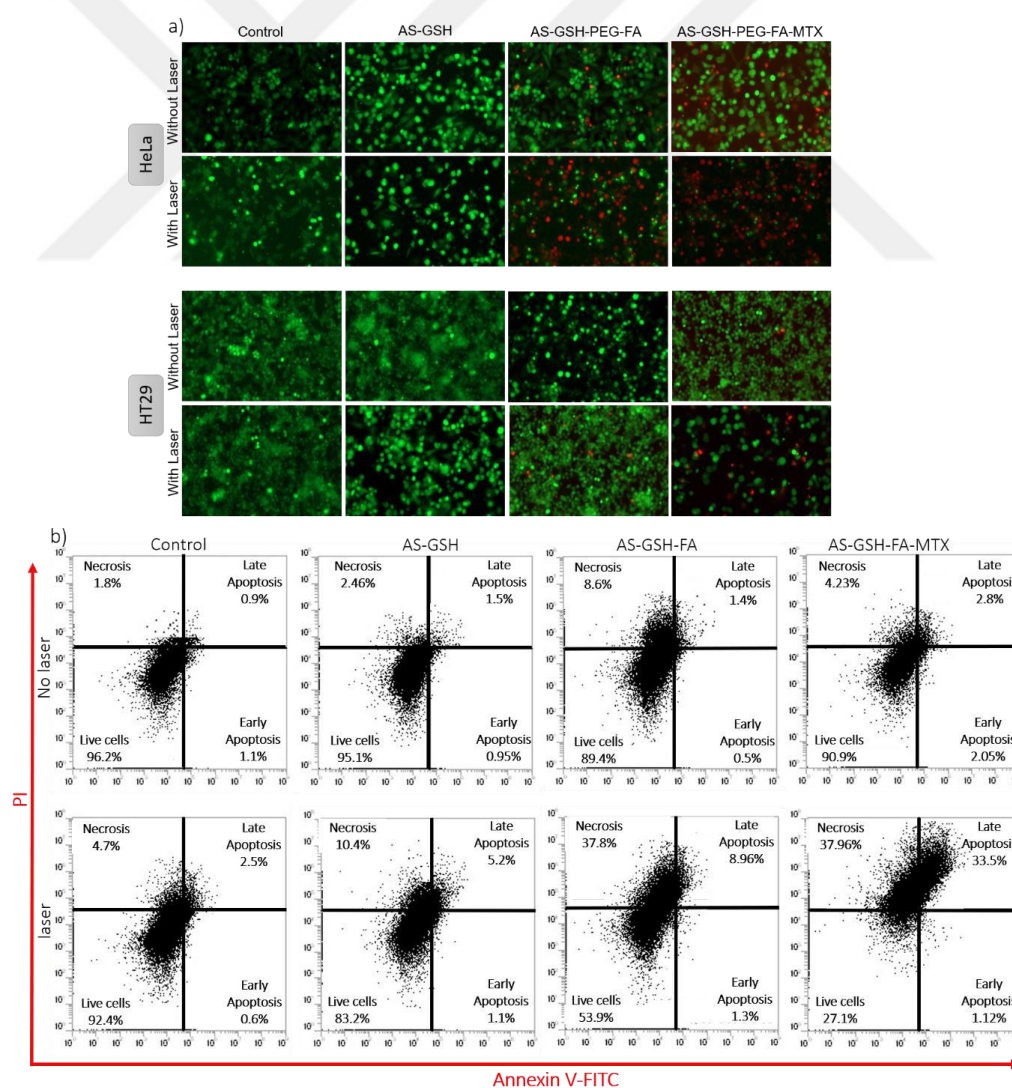


Figure 4–19: (a) Microscopy images of HeLa and HT29 cells treated with 10 µg/mL [Ag] for 4 h with/without laser irradiation (700 mW, 1.82 W/cm², 10 min) after live/dead staining. (b) Flow cytometry profiles of HeLa cell lines treated with AS-GSH, AS-GSH-FA and AS-GSH-FA-MTX QDs at 10 µg/mL [Ag] dose and/or 10 min laser irradiation at 808 nm (700 mW). “Control” indicates untreated cells. “Control + Laser” indicates cells treated only with laser irradiation.

4.3.9.2 Cell apoptosis assay

The cell death was further corroborated by co-staining of the treated cells with Annexin V-FITC and propidium iodide (PI). Annexin V is a phosphatidylserine binding protein indicating mostly apoptotic cell death, which makes phosphatidylserine accessible. PI is a nuclear stain indicating necrosis since it requires the destruction of the cell membrane to reach nuclei. Since the target cells were HeLa and strong PTT and PTT/MTX therapy were observed on them, this assay was only performed on the targeted HeLa cells treated with AS-GSH, AS-GSH-FA, and AS-GSH-FA-MTX at 10 µg/mL for 4 h. Stained cells were analysed by flow cytometry. Laser irradiation in the absence of QDs or when coupled with un-targeted AS-GSH QDs did not show significant cell death. Targeting these QDs with FA to HeLa cells, provided about 10% mostly necrotic (8.6%) and late apoptotic (1.4%) cell death (Figure 4-19b). Conjugation of MTX to the targeted QDs just changed the distribution of phenotypes with about 2% early apoptotic cells summing up to 10% dead cells. Laser irradiation of AS-GSH-FA treated cells increased the cell death dramatically mostly by a dramatic increase in necrotic (37.8%) cells with the contribution of late apoptotic death (9%). The scattered plot shows about the same amount of necrotic population coupled with a dramatically enhanced late apoptotic population (33.5%) when cells were treated with AS-GSH-FA-MTX+Laser. These results clearly indicate necrotic cell death as a response to PTT which is supported by no ROS generation but increased LDH release upon laser irradiation, which is a hallmark of necrosis, coupled with the apoptotic cell death due to MTX when AS-GSH-FA-MTX+Laser treatment was applied. PTT/MTX combination coupled with targeting demonstrated high levels of cell death which is very exciting and promising for further studies.

4.4 Conclusion

Two FR-targeting Ag based QDs were designed for a combination of optical imaging, tumour-specific delivery of MTX and PTT. AS-GSH QDs with emission at 822 nm were prepared following our previous procedure [72]. Aqueous AIS-GSH QDs emitting at 634 nm with QY of 21% were synthesized without using Zn dopant or ZnS shell at pH 8.5 with the optimized mole ratio of Ag/In/S/GSH: 1/4/2.5/10. Moreover, AIS-GSH QDs

exhibited excellent colloidal stability and strong emission for over 6 months. These features demonstrate a definite improvement over existing AIS-GSH QDs. Both AS-GSH and AIS-GSH QDs showed appreciable cytocompatibility to HeLa, HT29, A549, and C2C12 cell lines and provided a strong intracellular optical signal, hence have a potential for medical imaging. Both QDs were tagged with FA with a PEG spacer and conjugated with MTX via an amide bond directly. FA tagged QDs showed an apparent specificity for FR (+) HeLa cells over HT29 and A549 cell lines in the *in vitro* studies. Therefore, MTX delivered with FA tagged QDs reduced viability of HeLa cells in a dose and time dependent manner, reducing the MTX IC₅₀ to 5-2.5 µg/mL in 24-48h.

Amide bond protected MTX in the physiological pH, but hydrolysed slowly in the acidic medium, which was enhanced after laser irradiation due to increased temperature of the medium. This is excellent for the release of the toxic MTX specifically in the acidic tumour microenvironment and during PTT. In its aqueous solutions, AIS-GSH QDs showed ca 10 °C ΔT when irradiated at 640 nm (300 mW) for 10 min, compared to ca 20 °C increase achieved with 10 min irradiation of AS-GSH QDs at 808 nm (700 mW). In the *in vitro* PTT studies, AIS-GSH family did not show significant phototoxicity. AS-GSH-FA acted as an effective PTT agent at 808 nm (700 mW, 1.82 W/cm², 10 min) irradiation driving HeLa cells to necrotic death, but AS-GSH-FA-MTX/laser treatment reduced IC₅₀ of MTX to 0.21 µg/mL from 10 µg/mL (for free MTX) and provided almost a complete elimination of cells above 2.5 µg MTX/mL. Study of the cell death mechanisms, ROS and LDH levels of treated cells supported PTT coupled with chemotherapy. Near-complete death of HeLa cells was achieved with collaborative action of enhanced localization in FR(+) cells, enhanced hydrolysis of pH-sensitive amide bonds for the release of MTX at high temperature upon PTT and phototoxicity of AS-GSH QDs. In the *in vivo* studies, small size of the QDs should allow effective tumour targeting via FR-FA interaction, escaping from RES system, slow release of MTX at physiological pH should keep the toxic cargo from off-site toxicities and fast release of MTX in acidic microenvironment of the tumour which increases dramatically with laser irradiation should allow high levels of MTX when and where needed. This should reduce the effective dose significantly.

Overall, AIS-GSH-FA-MTX is a promising theranostic agent. Its mild hyperthermia activity may be exploited further as a turn-on mechanism for temperature-sensitive drug

release. AS-GSH-FA-MTX offers excellent properties, such as NIR luminescence and PTT at NIR wavelengths, high tumour specificity and therapeutic efficacy in PTT/Chemotherapy combination.

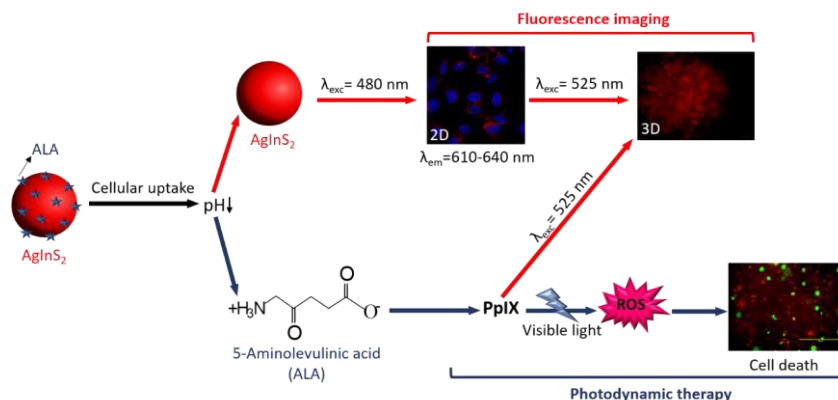


Chapter 5:

Anionic and Cationic AgInS₂ Quantum Dots

5.1 Abstract

Silver-indium-sulfide quantum dots (AIS QDs) are promising theranostic agents for nanomedicine since they do not contain regulated heavy metals. The present study demonstrates the optimum conditions for preparation of highly luminescent, stable and water soluble AIS QDs with a coating of either polyethyleneimine/2-mercaptopropionic acid (AIS-PEI/2MPA) or 2-mercaptopropionic acid (AIS-2MPA), providing cationic and anionic AIS QDs, respectively. Small AIS QDs with long-term stability and high quantum yields (19-23%) were achieved at the molar ratio of Ag/In/S: 1/10/10 in water without any dopant or a semiconductor shell. These nanoparticles were further loaded with zwitterionic 5-aminolevulinic acid (ALA) as a prodrug for photodynamic therapy (PDT). Both QDs provided enhanced ALA release at acidic pH, but protected the prodrug at physiological pH, which is advantageous for tumour delivery. Ligand composition found to be more critical in cellular internalization and dark toxicity, rather than drug loading and release, limiting acceptable dose of the cationic AIS QDs compared to the anionic ones. PDT efficacy of ALA loaded AIS QDs was tested under 5 min irradiation at 420 nm (7 mW/cm²) in 2D monolayers and 3D constructs of HT29 and SW480 human colon adenocarcinoma cancer cell lines. At low doses, ALA delivery by the AIS QDs, which does not cause any phototoxicity, elicited significant cell death as a result of enhanced ROS generation and apoptotic/necrotic cell death. A 50% ALA loading on AIS-2MPA QDs+PDT combination was highly effective in killing HT29 and SW480 cells, demonstrating IC₅₀ values below 0.1 mM [ALA]. Combined with simple synthetic methods, the strong intracellular photoluminescence of AIS QDs both in 2D and 3D constructs, good biocompatibility of especially the anionic AIS QDs, and the enhanced PDT efficiency, show that AIS-2MPA QDs are highly promising theranostic QDs (Scheme 5-1).



Scheme 5-1: Use of ALA loaded cationic and anionic AIS QDs for visible light PDT coupled with QD based optical imaging in the medical imaging window.

5.2 Introduction

Semiconductor quantum dots (QDs) have attracted great interest as a novel class of promising optical probes in the past two decades [14], [149]. They exhibit many advantages compared to the bulk materials, such as size-dependent photoluminescence (PL), high PL quantum yields (QY), sharp and symmetrical fluorescence peaks, broad excitation spectra, large Stokes shifts and long-lasting luminescence which is valuable for medical imaging [72], [73]. Recently, heavy metal free ternary I–III–VI₂ QDs such as CuInS₂ [263], CuInSe₂ [264], and AgInS₂ [265] have been intensively investigated. AgInS₂ (AIS) QDs are quite promising due to their relatively wide bandgap ranging from 1.87 to 2.03 eV that provide tuneable luminescence in the visible region, especially at and above 600 nm suitable for medical imaging [264], [266]. Besides, more stable nature and synthesis at lower temperatures are important advantages of AIS over CuInS₂, making it quite attractive for medical purposes. Synthetic procedures for high-quality ternary I–III–VI₂ QDs usually involve non-polar organic solvents, which might limit the direct applications of these QDs in biological applications [267], [268]. Cai *et al.* [266] developed an organometallic high-temperature method for the synthesis of AIS QDs with a long lifetime and 74% QY in organic solvents for solar cell application. Recent efforts towards AIS synthesis in aqueous medium are quite valuable considering numerous advantages over high heat synthesis in organic solvents, which produces hydrophobic QDs. However, aqueous protocols usually produce AIS QDs with much lower QY, hence usually, a ZnS shell or Zn-Ag-In-S (ZAIS) quaternary systems are preferred [265], [267], [235]. Deng *et al.* [265] produced glutathione (GSH) coated ZAIS QDs in water with tunable PL in the range of 550–605 nm and QY between 10–30% (with respect to R6G).

Luo *et al.* [221] synthesized GSH coated AIS nanocrystals in water at 95 °C with a maximum QY of 3%. They investigated the influence of pH, GSH/In, In/Ag, and In/S ratios on the optical properties of AIS. It was shown that pH 8.5 and a high GSH/In ratio could increase the PL intensity (peak position was not changed), while the ratios of In/Ag and In/S could affect the emission wavelength and intensity. ZnS shell deposition improved QY to 15%. Raevskaya *et al.* [221] reported the preparation of water soluble AIS nanoparticles with branched polyethyleneimine (PEI) with maximum QY of 20% (with respect to anthracene) when Ag/In/S ratio of 1/5/5 was used and only after 2h post-synthesis thermal treatment at 100 °C. Hence, there is still a clear need to develop simple, economical, aqueous synthetic protocols for the preparation of functional, stable, and highly luminescence AIS with emission at and above 600 nm for medical applications.

Photodynamic therapy (PDT) is a light-based medical treatment that relies on the retention of photosensitizers (PSs) in tumour tissues, which can be activated at a specific wavelength to provide a therapeutic effect at the irradiated area. PSs that are activated by a specific wavelength in the presence of molecular oxygen generate singlet oxygen and other reactive oxygen species (ROS), which are cytotoxic to the cells nearby due to the rapid and dramatic increase in oxidative stress. Traditional PSs are usually unstable, poorly soluble in water, and easily affected by the internal environment and molecules in the body [44]. 5-Aminolevulinic acid (ALA), is a prodrug and the precursor of protoporphyrin IX (PpIX), which is a natural PS, and a precursor of heme biosynthesis pathway [269]. ALA is used both for PDT and fluorescence-based diagnosis of different cancers owing to efficient metabolism to the fluorescent PpIX photosensitizer in malignant tumour cells. However, ALA has relatively low bioavailability and cellular uptake owing to its zwitterionic nature. Hence, for better therapeutic outcomes, its derivatization and loading to nanoparticles have been proposed. Preparation of ALA derivatives with improved bioavailability and lipophilicity via esterification, its peptide esters, dendritic and liposomal formulations have been investigated [270], [271], [272]. Chung *et al.* were able to increase cellular uptake of ALA by conjugating it to methoxy polyethylene glycol/chitosan [119]. Indeed, the utilization of nanoparticles in ALA based PDT not only improves the stability of PS but could also be tuned to deliver it to the target cells in high concentration to improve the therapeutic effect and to evade efflux transporters that mediate drug resistance [119]. Wang *et al.* [273] increased the circulation time and stability of ALA by loading it into HER2 targeted aldehyde-functionalized

hyaluronic acid and hydroxyethyl chitosan nanoparticles. They showed that less than 40% of ALA was released at physiological pH after 45 h whereas the half-life of free ALA under the same conditions is less than an hour [273], [274].

ALA may be conjugated to nanoparticles or it may be electrostatically loaded to charged nanoparticles [272]. One advantage of electrostatic loading is the fact that it can be done in fewer steps. Procedures that involve multiple solvent exchanges, surface functionalization, washing, and etc., usually affect the luminescence intensity and hydrodynamic size of QDs negatively. Another advantage of electrostatic loading is usually pH dependent, quick release with higher efficiency, especially in acidic media, which are important for clinical applications. The few examples of electrostatic loading in the literature utilized positively charged gold nanoparticles [131], [275]. We suggest that ALA can be loaded electrostatically to both cationic and anionic nanoparticles due to its zwitterionic nature. Current studies related to the relationship between cellular uptake and physicochemical properties of nanoparticles in the absence of targeting agents imply that the access of QDs into cells is principally governed by size and charge of the nanoparticles [276], [277]. Negatively charged proteoglycans are supposed to attract positively charged QDs toward the cell membrane [278]. Hence, negatively charged QDs would be repelled, resulting in a lower uptake by the cells. However, the influence of surface charge of the nanoparticles on cellular uptake of non-targeted QDs are still disputed. Some papers report that negatively charged nanoparticles are endocytosed more than positively charged QDs, although there is also evidence that positively charged QDs exhibit increased endocytic uptake [279], [280], whilst other studies show no correlation of the QD surface charge with endocytosis [281]. For the present study, on the other hand, the conjugation efficiency and drug release kinetics of the zwitterionic ALA from cationic and anionic QDs may be quite different.

Herein, we developed both cationic and anionic AIS QDs with strong luminescence in one step aqueous synthesis without Zn incorporation or ZnS shell, simply by utilizing either PEI/2MPA or 2MPA as stabilizers, and loaded them with ALA as potential theranostic agents for tumour therapy. In this design QDs will protect the ALA and cause its accumulation in the tumours due to EPR effect and endocytic cell internalization evading the efflux transporters; provide a strong and long-lived QD-based luminescence highlighting the region of accumulation; release ALA in the tumours selectively, hence cause a significant increase in the tumour PpIX levels, which will cause enhanced cell

death when irradiated [282].

We have previously demonstrated the advantage of using PEI/2MPA mixture over PEI coating on the luminescence properties of Ag₂S [182]. Likewise, the advantage of 2MPA over 3MPA in luminescence intensity and colloidal stability has been demonstrated on CdS QDs, and this coating was successfully utilized in Ag₂S QDs, as well [154]. AIS QDs prepared from silver nitrate, indium (III) nitrate hydrate and sodium sulfide, in different Ag/In/S/coating ratios, provide stable cationic and anionic AIS QDs emitting from green to orange-red. *In vitro* fluorescence, cytotoxicity and cell internalization of both cationic and anionic AIS QDs were examined in 2D and 3D-spheroid models of HT29, HeLa, SW480 and HCT116 cell lines, to evaluate their imaging potential. Then, potential for ALA-based PDT was investigated. ALA loading and pH dependent release efficiency from both anionic and cationic QDs, as well as PDT potential of AIS/ALA QDs compositions upon 5 min 420 nm (UV lamp) irradiation, were examined (Scheme 5-2a). The promising results have opened possibilities for the bio-labeling and image-guided PDT of cancer utilizing these AIS/ALA QDs.

5.3 Materials and Methods

5.3.1 Materials

Silver nitrate (AgNO₃), In(NO₃)₃.xH₂O and branched polyethyleneimine (PEI) (Mw 25 kDa) were purchased from Sigma-Aldrich (Germany). Sodium sulfide (Na₂S) was purchased from Alfa-Aesar. Sodium hydroxide (NaOH), 2-mercaptopropionic acid (2MPA), ethanol and acetic acid (CH₃COOH) were purchased from Merck (USA). 5-Aminolevulinic acid hydrochloride (ALA) was bought from Research Products International (RPI-USA). The LDS 798 near-IR laser dye was purchased from Exciton, Inc., (USA). Ultraceltrifuge tubes with polysulfone filtration membranes (3 KDa) were purchased from Sartorius (Germany). All chemicals were analytical grade and used without any further purification.

Roswell Park Memorial Institute (RPMI) 1640 and McCoy's 5A (Modified) medium were obtained from Multicell, Wisent Inc. (Canada). 10x Minimum essential medium (MEM) (used as colour/ pH indicator) and HEPES buffer solution were purchased from Gibco by Life Technologies, Thermo Fisher Scientific (UK). Fetal bovine serum and l-glutamine were purchased from Capricorn Scientific GmbH (Germany). 80% Rat Tail Collagen Type I was bought from First Link UK Ltd. Trypsin-EDTA and penicillin-

streptomycin solutions were bought from Wisent Inc. (Canada). Thiazolyl blue tetrazolium bromide (MTT), Alamar blue and phosphate buffered saline (PBS) tablets were purchased from Biomatik Corp. (Canada). Paraformaldehyde solution 4% in PBS was obtained from Santa Cruz Biotechnology, Inc. (USA). 96-well plates were purchased from Nest Biotechnology Co. Ltd. (China). HeLa (human epithelial carcinoma cell line), HT29 and SW480 cells were provided by Gozuacik Lab (Sabanci University, Istanbul, Turkey) for this study. HCT116 cells were purchased from ATCC (Ireland).

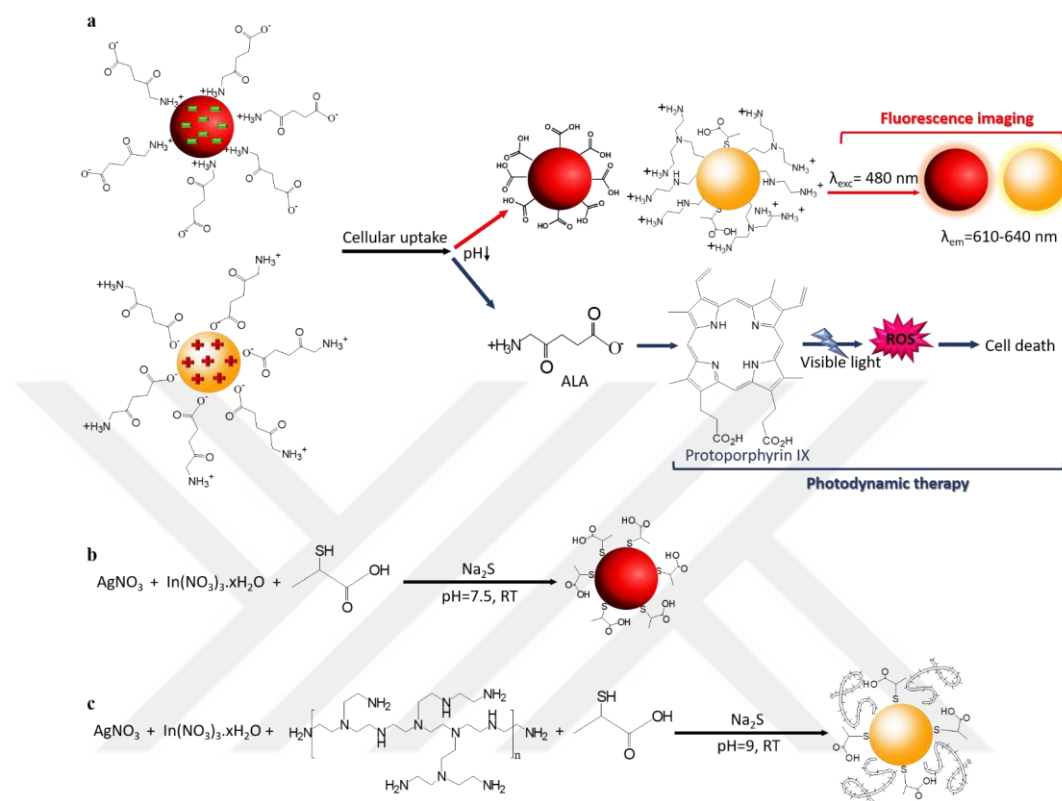
5.3.2 Synthesis of AIS-2MPA QDs

2MPA coated AIS QDs were prepared at different Ag/In and Ag/S ratios as summarized in Table 5-1, keeping the 2MPA/cations mole ratio fixed at 5. All reactions were performed under Argon and at room temperature. Briefly, 2MPA in the desired amounts based on the stoichiometries listed in Table 5-1 (2MPA/S: 1/0.25-1/3.3 molar ratio), was dissolved in 75 mL deoxygenated water in a round bottom flask. pH of this solution was adjusted to 7.5 using NaOH and CH₃COOH solutions (1 M), then AgNO₃ and In(NO₃)₃.xH₂O were added. As an example, 0.022 mmol AgNO₃, 0.22 mmol In(NO₃)₃.xH₂O and 1.2 mmol 2MPA were utilized for the molar ratio of Ag/In: 1/10. The pH of the final solution was adjusted to 7.5 and then charged with 25 mL aqueous solution of Na₂S (0.22 mmol Na₂S for the molar ratio of Ag/In/S: 1/10/10) under vigorous mechanical stirring at 500 rpm. Samples were removed from the reaction mixture at different time points to track the particle growth. Prepared quantum dot solutions were washed with deionized water using centrifugal filters (3 kDa cut off) and stored in the dark at 4 °C (Scheme 5-2b).

5.3.3 Synthesis of AIS-PEI/2MPA QDs

PEI/2MPA coated AIS QDs were prepared at different Ag/In and Ag/S ratios as summarized in Table 5-2 at coating/cations mole ratio of 5. All reactions were performed under Argon and at room temperature. 4/1 mol ratio of PEI/2MPA was used in all reactions based on the amine/thiol content of the species. Cation sources (AgNO₃, In(NO₃)₃.xH₂O) and Na₂S were dissolved in 75 ml and 25 mL of deoxygenated water, respectively, in separate round bottom flasks. In a typical reaction for molar ratios of Ag/In/S: 1/10/10, 0.2 mmol PEI and 0.05 mmol 2MPA were added to the 0.022 mmol AgNO₃ and 0.22 mmol In(NO₃)₃.xH₂O solution. The pH of the final solution was adjusted to 9 using NaOH and CH₃COOH solutions (1 M). Na₂S solution (0.22 mmol Na₂S for the

molar ratio of Ag/In/S: 1/10/10) was added to this reaction mixture under vigorous mechanical stirring at 500 rpm. Samples were removed during the reaction at different time points to follow the particle growth. In the end, quantum dot solutions were washed and kept as described above (Scheme 5-2c).



Scheme 5-2: (a) Use of ALA loaded cationic and anionic AIS QDs for visible light PDT coupled with QD based optical imaging in the medical imaging window. Synthesis of (b) AIS-2MPA and (c) AIS-PEI/2MPA QDs.

5.3.4 Characterization

X-ray photoelectron spectroscopy (XPS) using Thermo Scientific K-Alpha XPS with Al K-alpha monochromatic radiation (1486.3 eV) was used for compositional analysis of the crystals. Dried samples were placed on an adhesive carbon tape and exposed to 400 μm X-ray spot size with 50.0 eV pass energy, corresponding to a resolution of roughly 0.5 eV. All spectra were corrected with respect to C1s peak at 284.5 eV. To determine the crystal structure of AIS QDs, Bruker D2 Phaser Benchtop X-Ray Diffraction (XRD) system with Cu K-α radiation (λ=1.5406 Å) was used between 2θ angles of 10° and 80°. Hydrodynamic sizes, zeta potentials and polydispersity index (PDI) of the colloidal AIS QDs were assessed with Malvern zetasizer nano ZS. The Ag and In content of the colloidal AIS QDs were determined quantitatively by Agilent 7700x Inductively Coupled Plasma Mass Spectrometry (ICP-MS) after samples were treated with nitric acid: sulfuric

acid (9:1 v/v). This was performed after sterile filtration of the colloidal solutions in order to have exact concentrations used in the *in vitro* studies. Functional group analysis of QDs was performed on a Thermo Scientific Nicolet iS10 instrument (FTIR) in the wavenumber region from 650 to 4000 cm⁻¹ with resolution 4 cm⁻¹. The organic content of the QDs was measured by thermogravimetric analysis (TGA, TGA 500). The samples were heated from room temperature to 1000 °C at a heating rate of 10 °C/min under a nitrogen atmosphere.

Photoluminescence (PL) spectra of AIS QDs in the range of 500-900 nm were recorded on an Agilent Cary Eclipse Fluorescence Spectrophotometer at 480 nm excitation. The PL lifetimes were measured using Edinburgh FLS100 Spectrometer. Absorbance spectra were recorded using Shimadzu UV-3600 spectrophotometer in the 300–1000 nm range. The quantum yield (QY) was calculated based on the procedures in the literature using Rhodamine 6G as reference according to the following equation [283]:

$$QY_{\text{sample}} = QY_{\text{reference}} \left(\frac{Grad_{\text{sample}}}{Grad_{\text{reference}}} \right) \left(\frac{n_{\text{sample}}}{n_{\text{reference}}} \right)^2 \quad \text{Equation 5-1}$$

Grad is the ratio of fluorescence intensity to the absorbance of the corresponding sample, and *n* is the refractive index of the solvent.

5.3.5 Cell culture

All culture media were supplemented with 10% fetal bovine serum, 2% l-glutamine (200 mM) and 1% penicillin-streptomycin. HT29, SW480 and HCT116 were cultured in McCoy's and HeLa cells were cultured in RPMI 1640 complete medium in a 5% CO₂-humidified incubator at 37 °C.

5.3.6 Formation of 3D spheroid constructs

In vitro 3D spheroid constructs were prepared following the protocol from RAFT 3D culture systems (Lonza, Slough, UK). The hydrogels were prepared from a mixture containing 10% 10x MEM (used as colour/pH indicator), 80% Rat Tail Collagen Type I (First Link UK Ltd. Custom Bio-Reagents) and neutralized with a solution made from 1.65 M NaOH and 840 mM HEPES buffer (Thermo Fisher Scientific). The cells were seeded into the collagen at densities of 50000 cells for spheroid constructs at overall (cells and collagen mix) volumes of 240 µL per well in a 96-well plate. The constructs were incubated at 37 °C for 15 min to set before being subjected to plastic compression using

absorbers (Lonza) for a further 15 min at room temperature. After the removal of the absorbers, fresh medium was added, and wells were placed into the incubator.

5.3.7 Cytotoxicity assay

MTT metabolic activity assay was used to determine the dark cytotoxicity of the AIS-2MPA and AIS-PEI/2MPA QDs. All QD colloidal solutions were sterilized by passing through sterile 0.2 μm filters. HT29, HeLa, SW480 and HCT116 cells were seeded into a 96-well plate at a density of 12500 cells/well and cultured at 37 °C in 5% CO₂ atmosphere. The medium was replaced with a fresh medium after an overnight exposure with QDs in cations concentrations of 0.025-100 $\mu\text{g/mL}$ as determined by the ICP analysis. After 48 h incubation, the medium was replenished, and cells were washed with 3 $\times$ PBS. After the addition of 50 μL of MTT reagent (5 mg/mL) and 150 μL medium to each well, purple formazan crystals formed in 4 h. Cells were washed with 3 $\times$ PBS, and then the crystals in each well were dissolved with ethanol:DMSO (1:1 v/v) solution by shaking gently for 15 min. Relative cell viability was determined based on the absorbance intensity measured at 600 nm with a reference reading at 630 nm using a microplate reader (BioTek ELx800 Absorbance Microplate Reader). Cells with no treatment were used as controls. Treated cells without MTT reagent were used for the correction of absorbance intensities. All reported data is the average of 4 replica.

5.3.8 Cell uptake and imaging

The first experiments were performed on 2D cell culture and then on 3D spheroids consisting of multicellular aggregates that grew over 7 days before treatment. HT29, HeLa, SW480 and HCT116 cells were seeded on 3 mL glass-bottom petri dishes at a density of 1.75×10^5 cells/well in complete medium and grown for 24 h at 37 °C and 5% CO₂. On the next day, the growing cells were incubated with nanoparticles at 50 and 2.5 $\mu\text{g/mL}$ cations concentration of AIS-2MPA-3 and AIS-PEI/2MPA-1 QDs for 24 h. Then, the medium was replenished, and cells were fixed with paraformaldehyde solution (4% in PBS) for 20 min. After being washed with PBS, cells were incubated for an additional 15 min with 2 $\mu\text{g/mL}$ of DAPI nucleus dye. The cells were rewashed three times with PBS to remove the unbound dye and left in 2 mL PBS to prevent drying. Cells without any QD treatment were used as controls. The fixed cell samples were examined using a confocal microscope (Leica dmi8/SP8) with different filters for Alexa 488 (λ_{exc} : 488 nm and λ_{em} : 580-780) and DAPI (λ_{exc} : 325-375 nm and λ_{em} : 435-485 nm). The same

experimental procedure was also performed for control cells that were not treated with nanoparticles. Images were processed and merged using ImageJ analysis program. The quantitative analysis of QD uptake was performed by the determination of cations (Ag and In) content in QD treated cells. HT29, HeLa, SW480 and HCT116 cells were prepared similarly as for microscopy image analysis and incubated with QD samples for 4 h. Then, the cells were washed with PBS to remove un-internalized nanoparticles and, after trypsinization, the supernatant was collected. An acid digestion procedure (suprapur nitric acid 65% and suprapur sulphuric acid 96%) was performed, and the cations concentration of each sample was determined by ICP-MS (n=3).

To determine the intracellular localization of QDs within 3D constructs, separate constructs of HT29 and SW480 cell lines were prepared (50000 cells/model). The constructs were incubated with AIS-2MPA-3 (50 and 2.5 $\mu\text{g/mL}$ [cations]) and AIS-PEI/2MPA-1 (2.5 $\mu\text{g/mL}$ [cations]) and their ALA-conjugates for 24 h. The constructs were washed with PBS three times and incubated with PBS for fluorescence imaging using Olympus fluorescence microscope (10x objective, Olympus BX63) with the following filters: BF (λ_{exc} : 345 nm and λ_{em} : 455) and TRITC (λ_{exc} : 545 nm and λ_{em} : 620 nm). Samples without QD treatment served as a control.

5.3.9 Electrostatic loading of ALA to AIS QDs

ALA (3 mg/mL) was dissolved in 20 mM HEPES buffer, its pH was adjusted to 7.2–7.4 and finally filtered from a 0.2- μm filter. The AIS-2MPA-3 and AIS-PEI/2MPA-1 QDs were also, separately, filtered from a 0.2- μm filter. Subsequently, 0.3 and 0.5 mmol ALA solutions were mixed with 1 mmol AIS-2MPA-3 and AIS-PEI/2MPA-1 suspensions. The mixtures were stirred at 150 rpm at room temperature. ALA amounts were calculated as 30 mol% and 50 mol% of the functional groups available on QDs, determined by TGA. After loading, nanoparticles were stored at 4°C.

5.3.10 Isothermal titration calorimetry (ITC)

An isothermal titration calorimeter (Affinity ITC, USA) was used to measure enthalpy changes resulting from the interaction between ALA and either AIS-2MPA or AIS-PEI/2MPA QDs. 1 mL aliquots of either QD or ALA solution (1mg/mL, pH 7.4) were injected sequentially into a 1.5 mL titration cell every 10 s. The temperature of the solution in the titration cell was maintained at 25 °C throughout the experiments. The results are reported as the change in enthalpy per titration (μcal) versus the added ALA

concentration after each injection.

5.3.11 *In vitro* ALA release

The release of the loaded ALA from the AIS QDs was investigated at pH 5.5 and 7.4 at 37 °C. Typically, 5 mL AIS-2MPA-3-50%ALA or AIS-PEI/2MPA-1-50%ALA was added to a dialysis bag (MWCO 3500) and immersed in 50 mL of PBS (pH 5.5 or 7.4) with constant shaking (150 rpm) at 37 °C. At fixed time intervals, 3 mL of the released solution was removed, and 3 mL fresh PBS medium was added. The amount of ALA in the removed samples was determined by absorbance at 263 nm and using a concentration dependent absorbance standard curve prepared for ALA in PBS (pH 5 or 7.4).

5.3.12 Measurement of PpIX formation

HT29, HeLa, SW480 and HCT116 cells were seeded at a density of 12500 cells/well in 96-well plates and incubated for 24 h in a complete medium. The medium of some wells containing cells was then replaced with a serum-free medium containing 0.002-0.688 mM ALA to be used as control while other wells were subjected to AIS-2MPA-3 and AIS-PEI/2MPA-1 containing the corresponding amount of ALA. Untreated cells were used as the drug-free control. The fluorescence intensity was measured using a Synergy H1 microplate reader (Biotek) at excitation and an emission wavelength of 420 nm and 635 nm, respectively, after 48 h incubation with ALA or nanoparticles.

5.3.13 PDT studies

For the *in vitro* PDT studies, HT29 and SW480 cells were seeded at a density of 12500 cells/well (2D cell culture) and 50000 cells/well (3D models) in 96-well plates and cultured as described above for 24 h (2D cell cultures) and 7 days (3D models). QDs containing different concentrations of ALA in the range of 0.018-0.688 mM were added to each well and incubated for 24 h. Then, the medium was substituted with a serum-free medium and each well was illuminated with a blue LumiSource-flatbed lamp with peak emission at 420 nm and 7 mW/cm² output (PCI Biotech, Oslo, Norway) from the bottom of the plate for 5 min. Light irradiated cells were then incubated for another 24 h before cell viability was determined using the standard MTT protocol (2D cell cultures) and Alamar blue assay (3D models). Viability of cells that were treated the same way except laser treatment were also determined. Cells that were not treated with no ALA or QD treatment were used as controls.

5.3.14 Intracellular ROS generation

To assess ROS generation as a result of irradiation, HT29 and SW480 cells were seeded in 96-well plate at a density of 12500 cells/well (2D cell cultures) and incubated in complete media for 24 h. Then, QDs were added to the cells at a cation concentration of 50 and 2.5 $\mu\text{g/mL}$ of AIS-2MPA-3 and AIS-PEI/2MPA-1 QDs for 24 h, respectively. The cells were then also irradiated using a blue LumiSource-flatbed lamp with peak emission at 420 nm and 7 mW/cm^2 output (PCI Biotech, Oslo, Norway) for 5 min and incubated for another 24 h. ROS levels of the treated cells were determined using the Enzo Total ROS Detection Kit following the manufacturer's instructions using a Synergy H1 microplate reader (Biotek) at excitation/emission wavelengths of 485/538 nm.

5.3.15 Live/dead assay

A Live/dead cell viability assay (Thermo Fisher Scientific, UK) was utilized to determine the live and dead cell population after ALA, QD and ALA loaded QD treatment before and after irradiation. HT29 and SW480 cells were seeded following the procedure explained for ROS assay. After irradiation at 420 nm for 5 min, cells were stained with live/dead assay based on the protocol that is provided by the manufacturer. Finally, images were collected using an inverted fluorescence microscope (EVOS FL color, Life Technologies, Thermo Fisher Scientific, UK).

5.3.16 Termination of apoptosis/necrosis

Annexin V-FITC apoptosis detection kit (Abcam, UK) was used to confirm the mechanism of cell death in 2D and 3D models. The cells were seeded as described above. 24h after being exposed to irradiation at 420 nm, 100 μL of 1X Binding buffer was added to the models in each well before adding 1 μL of Annexin V-FITC (Ex/Em 488/525 nm) and 5 μL of propidium iodide (Ex/Em 535/617 nm) to the buffer solution in each well. Stained cells were then imaged using an EVOS fluorescence microscope (EVOS FL color, Life Technologies, Thermo Fisher Scientific, UK).

5.3.17 Statistical analysis

One-way ANOVA with Tukey's multiple comparison test was used for statistical analysis from GraphPad Prism 8 software package from GraphPad Software, Inc., USA. All data expressed as mean $\pm$ standard deviation (SD), and $p < 0.05$ was considered statistically significant.

5.4 Results and Discussions

5.4.1 Synthesis and characterization of AIS-2MPA QDs

One of the major hurdles in nanotechnology is the stability of nanoparticles [221]. 2MPA was shown to be a good coating material since it dramatically increases the stability of the nanoparticles and enhances the luminescence intensity [14], [284]. Hence, anionic AgInS₂ (AIS) QDs were synthesized under aqueous conditions in the presence of 2MPA at room temperature at different Ag/In/S/2MPA ratios and its influence on the optical properties and stability of QDs were determined (Table 5-1, Figure 5-1, Figure 5-2). All emission peaks were quite broad with FWHM between 79-219 nm (Table 5-1). Such a broad emission profile (Figure 5-1, Figure 5-2) coupled with featureless absorbance spectrum of such ternary QDs are attributed not only to size distribution but also to compositional inhomogeneity of the crystals, structural defects and related mid-gap energy states, resulting in not only band-edge but also donor-acceptor recombination and trap state emissions [235], [244]. In general, these AIS-2MPA QDs show a major peak maximum around 630-660 nm and a shoulder at a longer wavelength 705-715 nm. This is in line with reported emission profiles for AIS QDs and above-mentioned explanation about radiative events [235], [265], [285]. Bandgap energy determination of AIS is relatively difficult due to the uncertainty in electron-hole mass values and due to long absorbance tail [245]. Bandgaps of these QDs were calculated from the absorption spectra by replotting the data in the coordinates of the Tauc equation were found as 2.10-2.34 eV (Figure 5-3, Table 5-1). These values agree well with the E_g reported for AIS [286], [287]. On the other hand, when these values are evaluated a certain error originating from the long absorbance tails masking the exact position of the band edge should be taken into account [286], [288]. Therefore, the Urbach energy is also considered as a more appropriate way to calculate the band gap after replotting the absorption spectra. Such long absorbance tails are attributed to the presence of localized defect states within the crystals providing sub-band states and hence long absorbance tails and broad emissions [288]. The absorption tail is called Urbach tail and is associated with the Urbach energy (E_u) that can be calculated using:

$$\alpha = \alpha_0 + \exp\left(\frac{E}{E_u}\right) \quad \text{Equation 5-2}$$

where α is the absorption coefficient, E is the photon energy ($h\nu$), and E_u is the Urbach

energy. By plotting the $\ln(\alpha)$ against E , Urbach energy can be calculated from the reciprocal of slope of the linear fit (Figure 5-4a-e) [287]. The Urbach energies of these AIS QDs are between 137-172 meV and show an increasing trend with decreasing emission maxima, which suggests more disorder in crystals as the size decreases. These E_g and Urbach energies are in agreement with the values reported in the literature for different anionic AIS QDs [233], [288].

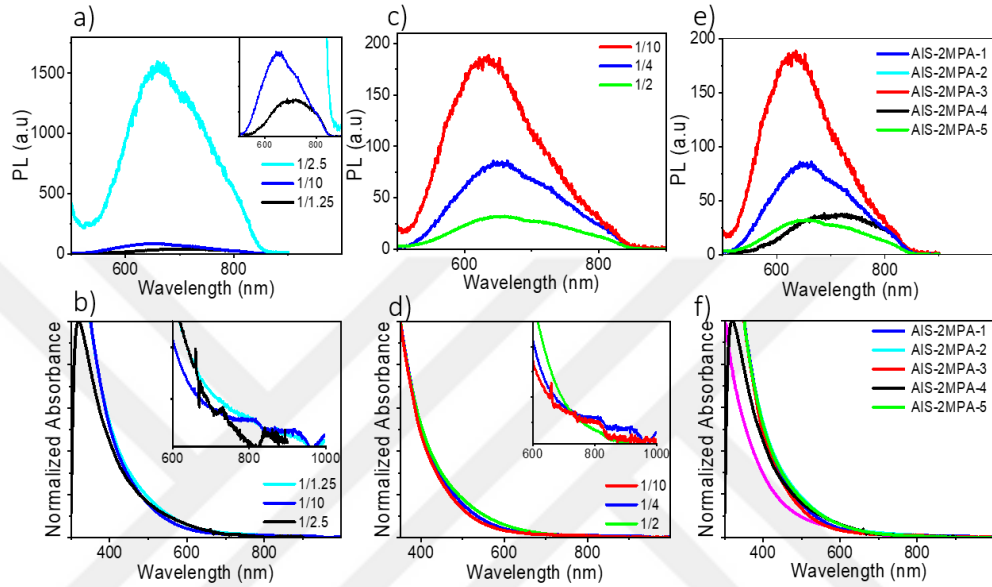


Figure 5-1: (a) The PL (b) and UV-vis spectra of AIS-2MPA QDs prepared with constant molar ratio of Ag/In: 1/4, cations/coating: 1/5 and different molar ratios of Ag/S. (c), (d) The PL and UV-vis spectra of the AIS-2MPA QDs prepared with constant molar ratio of Ag/S: 1/10, cations/coating: 1/5 and different molar ratios of Ag/In. (e), (f) The PL and UV-vis spectra of all AIS-2MPA QDs prepared at different conditions.

Table 5-1: Effects of the different reaction variables on the properties of AIS-2MPA QDs.

Sample	AIS-2MPA-1	AIS-2MPA-2	AIS-2MPA-3	AIS-2MPA-4	AIS-2MPA-5
Ag/S ratio	1/10	1/2.5	1/10	1/1.25	1/10
Ag/In ratio	1/4	1/4	1/10	1/4	1/2
Cation/S ratio	0.5	2	1.1	4	0.3
$\lambda_{\text{emi}}^{\text{a}}$ (nm)	634	660	630	701	649
$\lambda_{\text{abs}}^{\text{b}}$ (nm)	727	940	941.5	795.5	890
Band gap (eV)	2.10	2.13	2.15	2.34	2.15
E_{u}^{c} (meV)	172	168	170	137	161
FWHM (nm) ^d	160.84	190.01	79.61	202.64	218.97
Dh-number (nm) ^e	46.03	5.80	5.23	103.67	22.59
Dh-intensity (nm) ^f	80.65	3.34	74.13	117.90	67.97

Zeta potential (mv)	-38.13	-50.23	-42.01	-43.80	-41.57
QY (%)	10.57	-	19.38	5.57	4.98

^a Emission maxima. ^b Absorbance onset. ^c Urbach Energy, ^d Full-width at half-maximum of the emission peak. ^e Hydrodynamic diameter measured by DLS and reported as the number average. ^f Hydrodynamic diameter measured by DLS and reported as the intensity average.

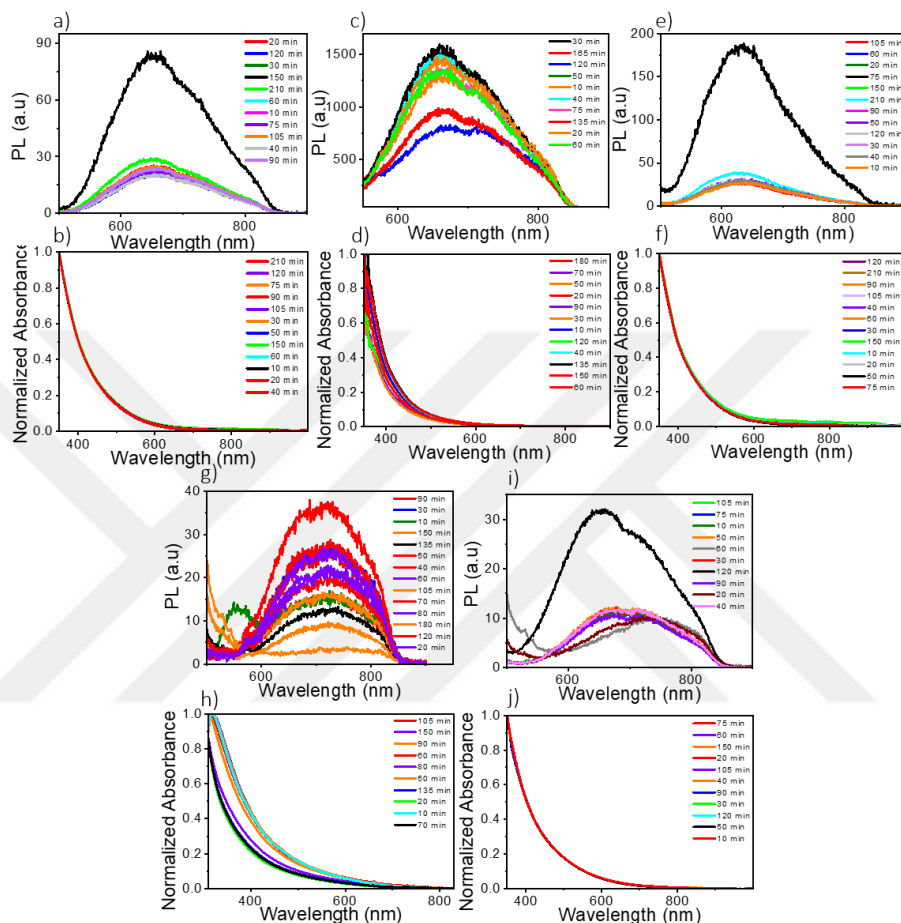


Figure 5-2: Time dependent changes in the photoluminescence spectra and UV-vis spectra of (a), (b) AIS-2MPA-1, (c), (d) AIS-2MPA-2, (e), (f) AIS-2MPA-3, (g), (h) AIS-2MPA-4 and (i), (j) AIS-2MPA-5 during the synthesis.

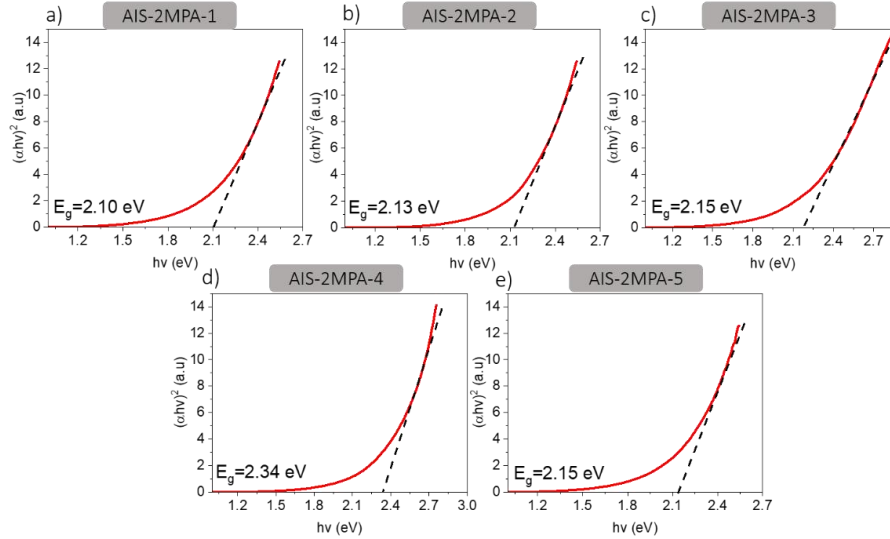


Figure 5–3: Tauc plots for (a) AIS-2MPA-1, (b) AIS-2MPA-2, (c) AIS-2MPA-3, (d) AIS-2MPA-4 and (e) AIS-2MPA-5 QDs.

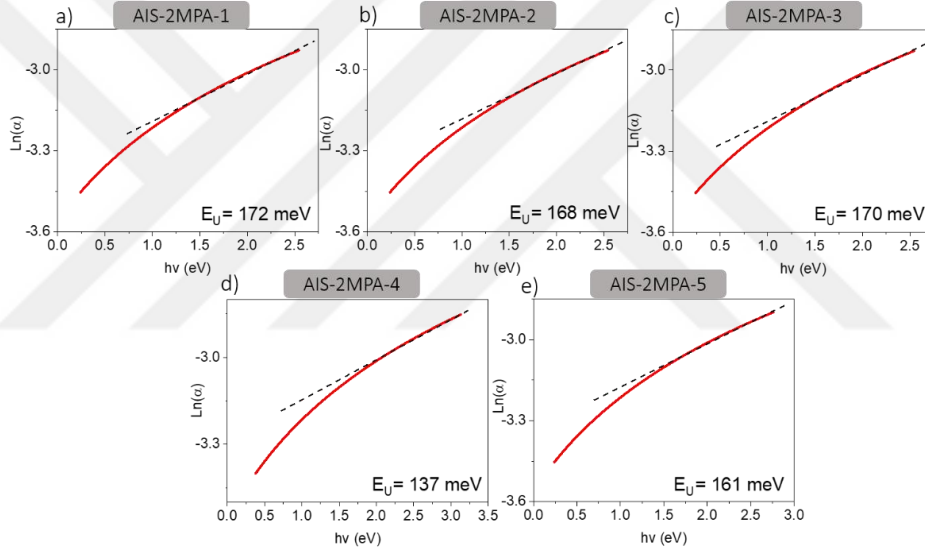


Figure 5–4: Urbach energies of (a) AIS-2MPA-1, (b) AIS-2MPA-2, (c) AIS-2MPA-3, (d) AIS-2MPA-4 and (e) AIS-2MPA-5 QDs.

5.4.2 Influence of experimental variables on the optical properties of AIS-2MPA QDs

5.4.2.1 Influence of Ag/S molar ratio

To evaluate the effect of the Ag/S ratio (1/1.25, 1/2.5, 1/10) on the optical properties of the produced QDs, varying amounts of the sulfur precursor were used while the other variables were fixed (Ag/In: 1/4, cations/coating: 1/5) (Table 5-1). Increasing the amount of sulfur precursor blue shifted the major peak maxima from 701 nm to 634 nm with an accompanying blue shift in the absorbance spectra (Figure 5-1a, b).

These three recipes have indeed cation/S ratio of 0.5, 2 and 4 for AIS-2MPA-1, AIS-

2MPA-2 and AIS-2MPA-4, respectively. So, in a way, as Ag/S ratio decreased, the cation/S ratio also decreased, and a resultant blue shift in emission maxima was observed. This was also accompanied by a stronger emission and a higher QY. These observations are in line with GSH coated ZAIS reported by Deng et al. [235] and PEI coated AIS reported by Raevskaya et al. [233]. E_g decreased and E_u increased with the decreasing Ag/S ratio. Relatively large E_u 's and broad long-wavelength emission tail may support significant emissions from trap states. Here, Ag/S ratio of 1/2.5 yielded the highest PL intensity, however, due to the lack of colloidal stability during the washing step, they could not be studied further. Since the QDs produced at Ag/S molar ratio of 1/10 were the most luminescent of the stable QDs, this ratio was kept fixed to study the influence of In/S ratio on QD properties.

5.4.2.2 Influence of Ag/In molar ratio

To further investigate the opportunity to tune the optical properties of AIS-2MPA QDs, the ratio of Ag/S was fixed to 1/10 while the Ag/In ratio was varied as 1/2, 1/4 and 1/10 (molar ratio of cations/coating: 1/5) (Figure 5-1c, d). Although emission peaks were broad again, increasing In content (decreasing Ag/In ratio) caused a slight blue-shift in the emission maxima from 649 to 630 nm. This trend is in agreement with earlier reports [221], [289]. The impact of the Ag/In ratio was more on the emission intensity rather than the peak position or size and the strongest emission was achieved at Ag/In ratio of 1/10. This ratio also corresponds to cations/S ratio of 1.1 (AIS-2MPA-3) while others had a ratio of 0.5 (AIS-2MPA-1) and 0.3 (AIS-2MPA-5). Cation rich formulation in this set may provide a better surface binding of the 2MPA and hence elimination of surface defects. Indeed, when the emission profile of these three QDs are examined, relatively strong intensity of the long-wavelength emission peaks are noticed, while AIS-2MPA-3 has a strong emission peak at 630 nm with quite a weak contribution of the long-wavelength emission (Figure 5-1c). This may be due to reduced defects and related emissions that appear as the long-wavelength peak in these ternary QDs [221].

In order to follow the growth of the particles, aliquots were taken from the reaction mixture at discrete time points and their optical properties were examined, as it is believed that crystal growth kinetics play an important role in tuning particle size and emission [31]. Prolonged reaction times could influence crystallinity, change the crystal composition, and promote particle growth via Ostwald ripening process [221], [290].

However, at extended reaction times, oxidation of the ligands, agglomeration of the nanoparticles, the formation of defects may result in a decrease of luminescence intensity [291]. Figure 5-2 shows the time-dependent changes in the emission and absorbance profile of the AIS-2MPA QDs. Nearly no change in the absorption onset was observed with the increase of growth time. Reaction duration mostly influenced the emission intensity, rather than the peak maxima.

The photoluminescence QY of the AIS-2MPA-1 and AIS-2MPA-3 QDs are about 10 and 19% (Table 5-1). Hence, the best recipe for the synthesis of colloiddally stable and highly luminescence anionic AIS-2MPA has the molar ratio of Ag/In/S: 1/10/10. Therefore, a new batch of AIS-2MPA-3 was synthesized and used in all *in vitro* studies with emission maxima at 634 nm, 5.38 nm (number-based hydrodynamic size) diameter and -37.5 mV zeta potential.

5.4.2.3 Structural characterization of AIS-2MPA QDs

Structural characterization of AIS-2MPA was performed using AIS-2MPA-3. Broad diffraction peaks detected via XRD analysis are attributed to tetragonal AIS QDs (Figure 5-5a) [221]. FTIR spectrum of this QD, provided information about the coating molecule (Figure 5-5b). The disappearance of S-H stretching band, which would be between 2500 2540 cm⁻¹, indicates the binding of 2MPA to AIS core by its thiol [14], [267]. The stretching band of the C=O observed at 1567 cm⁻¹ is consistent with the carboxylic acid interacting with the crystal surface. The broad absorption band at 3300 cm⁻¹ can be attributed to the OH group, and the band at 1395 cm⁻¹ correspond C–O stretching vibrations of 2MPA bound to AIS crystals, respectively [267], [291]. XPS analysis provided further information about the composition (Figure 5-5 c-e). Ag 3d core level peaks at the binding energy (BE) of 367.10 eV (3d5/2) and 373.14 eV (3d3/2) fit well to the Ag⁺. In 3d peaks at BE of 444.06 eV (3d5/2) and 451.58 eV (3d3/2) fits to In³⁺ of AIS. Two different peaks of S at BE of 161.05 (2p3/2) eV and 162.30 (2p1/2) eV match with the S–Ag and sulfur of 2MPA [235], [283]. According to the XPS data, Ag/In and Ag/S ratio were 1/7.5 and 1/11.5 which were consistent with the Ag/In ratio of 1/7 and Ag/S ratio of 1/12 determined by ICP. TGA analysis performed on AIS-2MPA-3 QD indicates 62.8% organic content (Figure 5-5f). TEM images of these QDs indicate small particles with average diameter of 5.85±0.74 nm (Figure 5-5g, h).

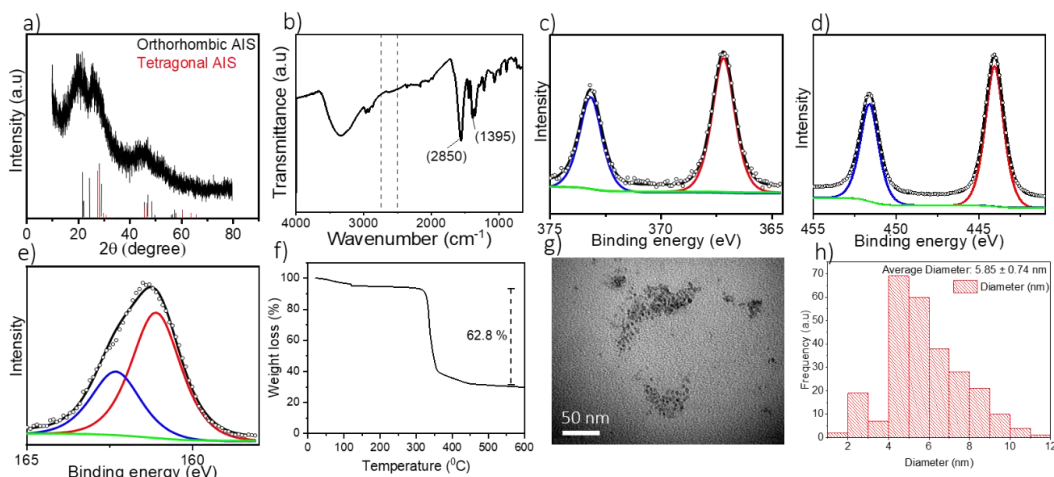


Figure 5-5: (a) XRD, (b) FTIR, (c) Ag 3d, (d) In 3d, (e) S 2p XPS spectra of AIS-2MPA-3 QDs. (f) Thermogravimetric analysis (TGA) of AIS-2MPA-3 QDs. (g) TEM image and (h) histogram of AIS-2MPA-3 QDs.

5.4.3 Synthesis and characterization of AIS-PEI/2MPA QDs

In the development of PEI coated Ag₂S, we have previously shown that PEI coating does not produce luminescent particles, but PEI/2MPA does [154], [182]. The most stable and luminescent Ag₂S-PEI/2MPA QDs (AS-PEI/2MPA) were synthesized at pH 9 at an Ag/coating molar ratio of 1/5 and PEI/2MPA ratio of 4/1 [182]. Based on this knowledge, cationic AIS QDs were prepared with PEI/2MPA coating in water with slow addition of sulfide to the salt mixtures [31], [266], [289]. The introduction of Na₂S into the solution containing Ag/In-PEI/2MPA produced a cloudy solution with yellow to brown coloration depending on the molar ratio of the components. Emission peak profiles of AIS-PEI/2MPA QDs resemble those observed for AIS-2MPA: broad emission peaks with a long wavelength shoulder or tail due to various radiative coupling events involving trap states and donor-acceptor pair recombination (Figure 5-6a, b). With different recipes wherein stoichiometry of Ag/In/S was changed, AIS-PEI/2MPA QDs emitting in the red-NIR region with an emission peak maxima between 774 nm and 617 nm were obtained (Table 5-2, Figure 5-6c-h, Figure 5-7). Bandgap energies of these cationic AIS QDs were calculated between 2.02-2.74 eV from the Tauc plots (Figure 5-8) and Urbach energies between 138-168 meV (Figure 5-9). Indeed, these energies are in agreement with the only reported PEI coated AIS [233].

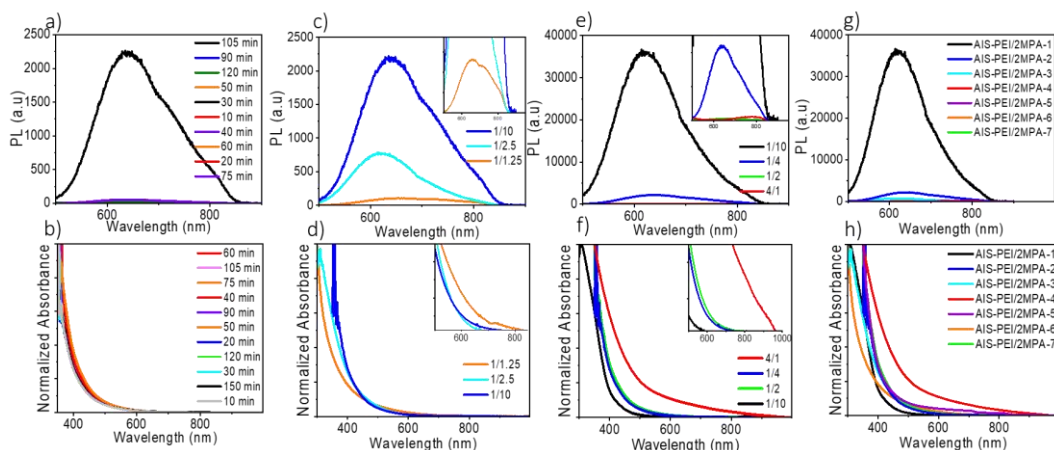


Figure 5-6: (a), (b) UV-vis spectra of the AIS-PEI/2MPA-2 samples withdrawn from the reaction mixture at different time points. (c), (d) The PL and UV-vis spectra of AIS-PEI/2MPA QDs prepared with the constant molar ratio of Ag/In: 1/4, cations/coating: 1/5 and different molar ratios of Ag/S. (e), (f) The PL spectra of the AIS-PEI/2MPA QDs prepared with the constant molar ratio of Ag/S: 1/10, cations/coating: 1/5 and different molar ratios of Ag/In. (g), (h) The PL spectra of all AIS-PEI/2MPA QDs prepared at different conditions.

Table 5-2: Effect of the different reaction variables on the properties of AIS-PEI/2MPA QDs

Sample	AIS- PEI/2M PA-1	AIS- PEI/2M PA-2	AIS- PEI/2M PA-3	AIS- PEI/2M PA-4	AIS- PEI/2M PA-5	AIS- PEI/2M PA-6	AIS- PEI/2M PA-7
Ag/S ratio	1/10	1/10	1/2.5	1/10	1/5.5	1/1.25	1/10
Ag/In ratio	1/10	1/4	1/4	1/0.25	1/10	1/4	1/2
Cations/S ratio	1.1	0.5	2	0.5	2	4	0.3
$\lambda_{\text{emi}}^{\text{a}}$ (nm)	617	640	618	774	618	655	638
$\lambda_{\text{abs}}^{\text{b}}$ (nm)	602	742	662	960	907	821	721
Band gap (eV)	2.74	2.4	2.57	2.02	2.15	2.12	2.24
E_{u}^{c} (meV)	138	149	144	167	168	151	150
FWHM (nm) ^d	146.64	176.5	146.87	105.13	138.6	239.82	188.85
Dh-number ^e (nm)	7.66	12.73	10.45	11.85	-	8.20	57.93
Dh-intensity ^f (nm)	27.91	26.28	37.96	60.40	-	17.56	99.06
Zeta potential (mV)	10.67	8.02	9.84	16.43	-	20.97	-4.95
QY (%)	20.28	15.61	7.87	-	-	-	-

^a Absorbance onset. ^b Emission maxima. ^c Urbach Energy. ^d Full-width at half-maximum of the emission peak. ^e Hydrodynamic diameter measured by DLS and reported as the number average. ^f Hydrodynamic diameter measured by DLS and reported as the intensity average.

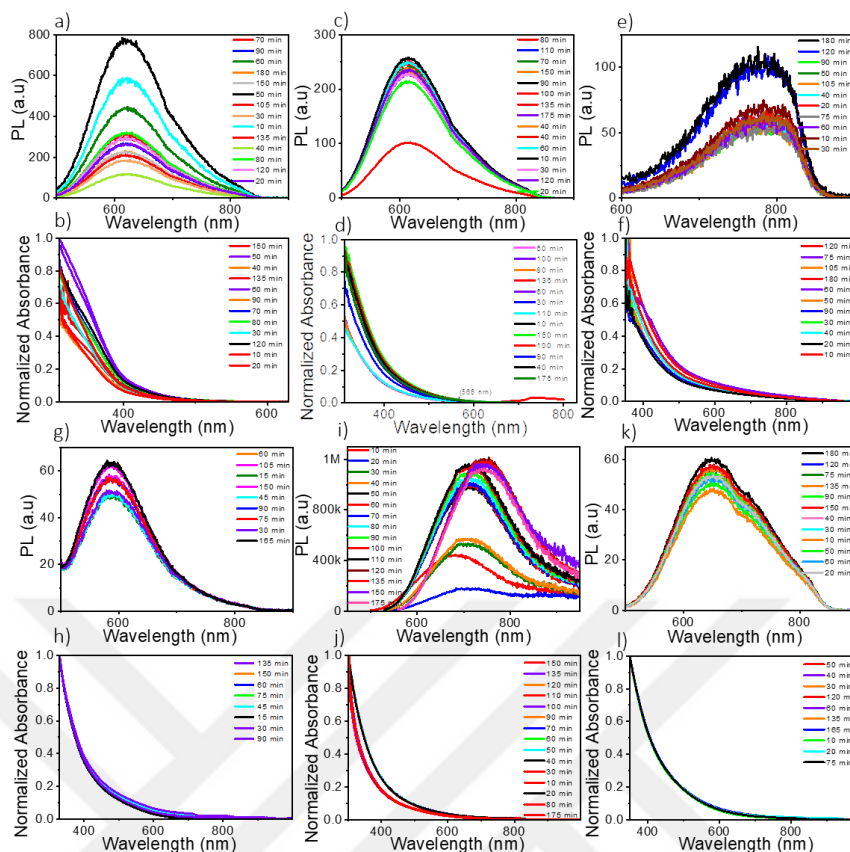


Figure 5-7: Time dependent changes in the PL and UV-vis spectra of (a-b) AIS-PEI/2MPA-1, (c-d) AIS-PEI/2MPA-3, (e-f) AIS-PEI/2MPA-4, (g-h) AIS-PEI/2MPA-5, (i-j) AIS-PEI/2MPA-6 and (k-l) AIS-PEI/2MPA-7 QDs during the reaction.

5.4.4 Influence of experimental variables on the optical properties of AIS-PEI/2MPA QDs

5.4.4.1 Influence of Ag/S molar ratio

The colloidal AIS-PEI/2MPA QDs synthesized with different Ag/S ratios at constant Ag/In ratio of 1/4 and cations/coating ratio of 1/5, exhibited emission peaks with a maximum between 618-655 nm (AIS-PEI/2MPA-1, 3 and 6) with no significant tuning of the peak position. The dramatic difference in the PL intensity indicates stronger emission with decreasing Ag/S ratio reaching its maximum at Ag/S ratio of 1/10 (Figure 5-6c). Thus, this ratio is chosen as the optimum Ag/S ratio for the following experiments. Although there is no clear correlation between the PL peak maxima, bandgap energy, Urbach energy, and the Ag/S ratio (Figure 5-6c, d, Figure 5-8, Figure 5-9), in general, bandgap widening with decreasing Ag/S ratio was observed within this series. These three recipes are all cation rich, but excess Ag may be another source of defects increasing the E_u [292], [293].

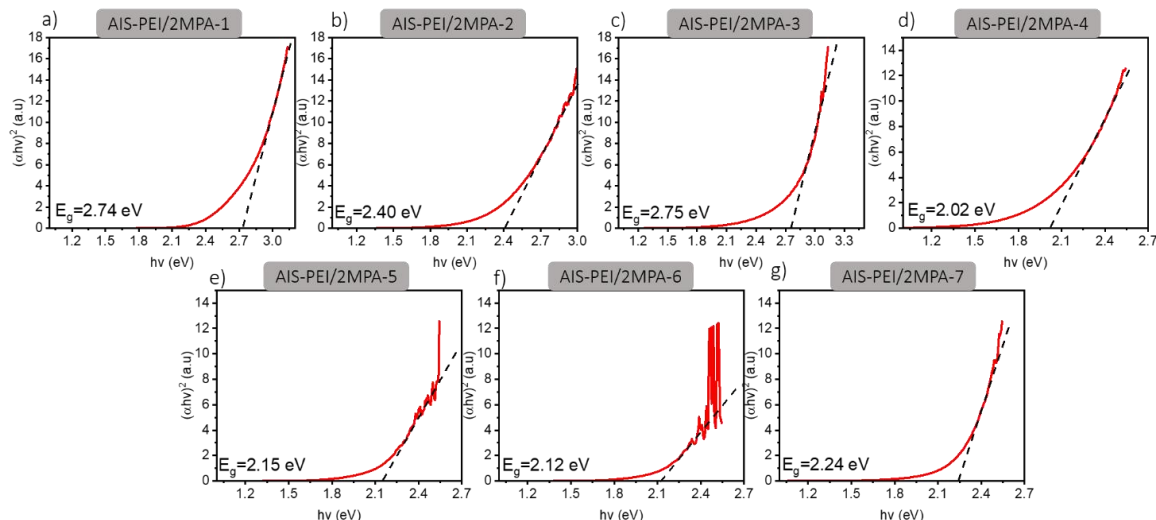


Figure 5-8: Tauc's plots for (a) AIS-PEI/2MPA-1, (b) AIS-PEI/2MPA-2, (c) AIS-PEI/2MPA-3, (d) AIS-PEI/2MPA-4, (e) AIS-PEI/2MPA-5, (f) AIS-PEI/2MPA-6 and (g) AIS-PEI/2MPA-7 QDs.

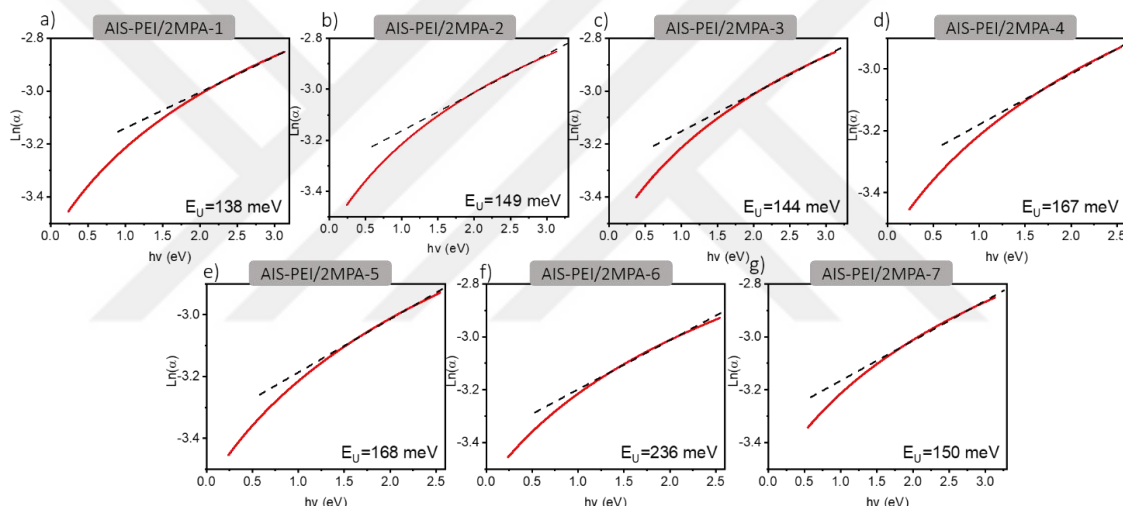


Figure 5-9: Urbach energies of (a) AIS-PEI/2MPA-1, (b) AIS-PEI/2MPA-2, (c) AIS-PEI/2MPA-3, (d) AIS-PEI/2MPA-4, (e) AIS-PEI/2MPA-5, (f) AIS-PEI/2MPA-6 and (g) AIS-PEI/2MPA-7 QDs.

5.4.4.2 Structural characterization of AIS-PEI/2MPA QDs

The XRD pattern of AIS-PEI/2MPA-1 consists of the characteristic peaks of the tetragonal and orthorhombic AIS structure (Figure 5-10a) [244]. FTIR spectrum of the AIS-PEI/2MPA-1 (Figure 5-10b) indicated the presence of stretching vibrations at 1585 and 1405 cm⁻¹, which are generally assigned to binding mode of carboxylates to a surface or to a cation. Bands at 3387 cm⁻¹ and 2850-2960 cm⁻¹ correspond to stretching modes of N-H and C-H bonds of PEI, respectively [268]. The absence of the thiol peak in the range of 2500-2550 cm⁻¹ confirms the covalent conjugation of the inorganic core to 2MPA via its thiol [291]. XPS analysis provided further information about AIS-PEI/2MPA-1 QDs.

XPS spectra obtained in Figure 5-10c-e confirmed the presence of the following elements: Ag 3d_{5/2} and 3d_{3/2} at BE of 372.92 and 336.94 eV, In 3d_{5/2} and 3d_{3/2} at BE of 451.38 and 443.79 eV and S 2p_{3/2} and 2p_{1/2} at BE of 161.82 and 160.60 eV. These are consistent with the reported values for AIS QDs and the ones observed for AIS-2MPA (Figure 5-10c-e) [293]. N 1s region could be fitted to two peaks at 398.20 and 401.12 eV, which correspond to tertiary, secondary and primary amines of PEI (Figure 5-10f). The high binding energy N 1s peak indicates primary amine binding to crystal surface [182]. The atomic ratio of elements calculated from the area under each curve indicated Ag/In/S ratio of 1/9/10, which is consistent with the theoretical ratio of 1/10/10. This ratio is also in agreement with the atomic ratio measured by ICP as Ag/In/S=1/8.8/10.8. TGA analysis performed on AIS-PEI/2MPA-1 QD indicates 58.6% organic content (Figure 5-10g). TEM images of these QDs also indicate small particles with average diameter of 6.12±1.65 nm (Figure 5-10h, i).

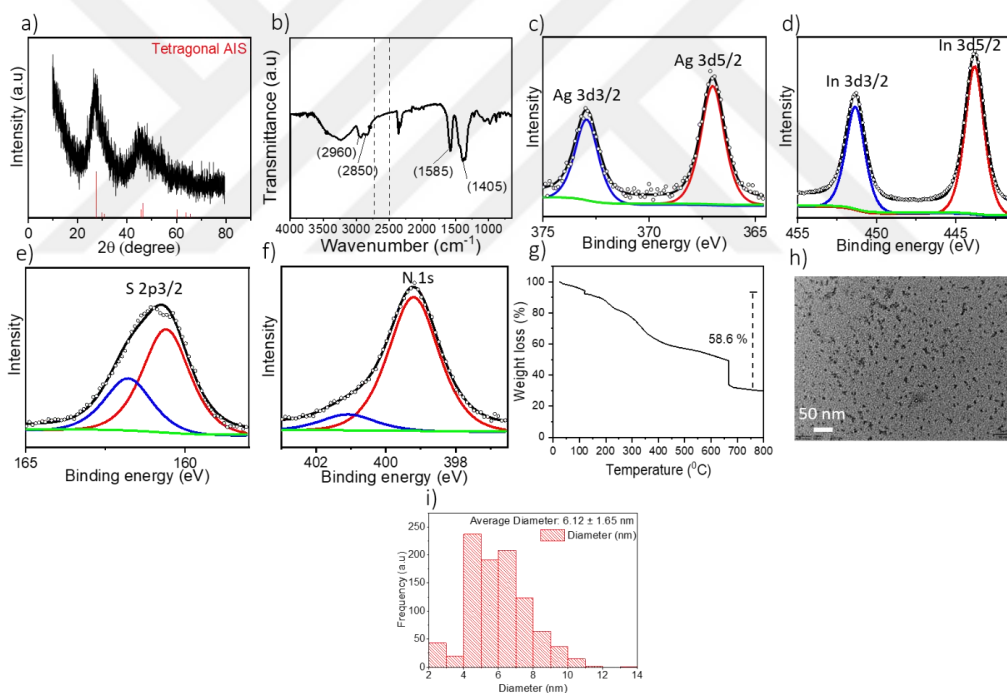


Figure 5–10: (a) XRD, (b) FTIR and (c) Ag 3d, (d) In 3d, (e) S 2p, (f) N 1s XPS spectra of AIS-PEI/2MPA-1 QDs. (g) Thermogravimetric analysis (TGA) of AIS-PEI/2MPA-1 QDs. (h) TEM image and (i) histogram of AIS-PEI/2MPA-1 QDs.

5.4.5 Colloidal stability of QDs

The shelf-life of nanoparticles is critical for any practical application. Generally, the PL intensity of QDs decreases over time due to the desorption of the ligands and/or oxidation, which may also cause an increase in the hydrodynamic size and precipitation [182]. Emission and absorbance characteristics (PL and UV-vis spectra), hydrodynamic size and

zeta potential of the most luminescent QDs over a period of 3 months were tracked to determine the stability of colloidal anionic and cationic AIS produced here (Figure 5-11 and Table 5-3). Colloidal QDs were stored at 4 °C after being washed. The emission intensity of both QDs increased with time similar to Ag₂S-2MPA which was reported before (Figure 5-11a, e) [15]. To better understand the time-dependence of PL intensity, the kinetics of the photogenerated charge carriers were studied using time-resolved PL spectra of these QDs.

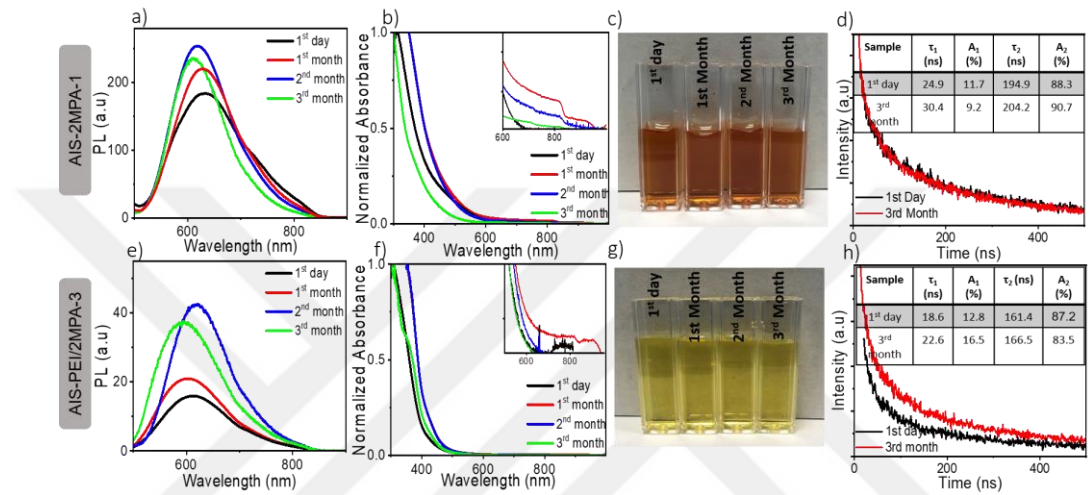


Figure 5-11: (a), (e) PL spectra and (b), (f) normalized UV-vis spectra and (c), (g) PL lifetime of AIS-2MPA-3 and AIS-PEI/2MPA-1 QDs at 1st day and 3rd month of their synthesis, respectively. Inset shows the fit parameters of the decay curves. (d), (h) colloidal stability of AIS-2MPA-3 and AIS-PEI/2MPA-1 QDs at different time points following its synthesis.

All the decay profiles were fitted according to the function of $[I(t): A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)]$, where τ_1 and τ_2 are the decay times, and A_1 and A_2 are the fractional contributions of the two components [264]. Generally, the fast component, τ_1 represents the bandgap recombination and slow component, τ_2 is related to the defect-related radiative recombination of carriers [235]. They were also suggested as high energy, intrinsic defect related, donor-acceptor coupling versus low energy, surface effect related emissions for AIS QDs [286]. For these AIS QDs both τ_1 (18-25 ns) and τ_2 (161-165 ns) are relatively short than most AIS reported in the literature [244], [233]. The average lifetimes calculated from $\langle \tau \rangle = (A_1 \tau_1^2 + A_2 \tau_2^2) / (A_1 \tau_1 + A_2 \tau_2)$ are also relatively shorter than AIS QDs, which also have lower QYs than the ones produced here (inset table-Figure 5-11c, g) [264]. Contribution of τ_1 , which was around 24.9 ns and 18.6 ns, was 9 % and 13 % for AIS-2MPA-3 and AIS-PEI/2MPA-1 QDs, respectively. After 3 months τ_1 became longer, 30.4 and 22.6 ns, with an increased contribution, which is about 11.7 % and 16.5

% for the anionic and cationic AIS QDs, respectively. The ratio of A_2/A_1 decreased from 9.9 to 7.6 in AIS-2MPA-3 and from 6.8 to 5 in AIS-PEI/2MPA-1 QDs after 3 months, indicating more contribution of the band edge and/or intrinsic defects than surface defects with time [289]. This was accompanied by a slight blue shift in the emission maxima from 632 nm to 612 nm for the anionic and from 617 nm to 593 nm for the cationic AIS QDs (Table 5-3). The enhanced contribution of fast component usually accompanied with a blue shift in the emission maxima of AIS or ZAIS QDs, indicates a lesser contribution of or just say fewer surface defects [233], [244]. The hydrodynamic size of the QDs showed a slight increase after 3 months (Table 5-3). However, high surface charge of both AIS-2MPA-3 (-44.56 eV) and AIS-PEI/2MPA-1 QDs (11.75 eV) prevented any significant aggregation/precipitation and they have maintained small hydrodynamic sizes that may be desired for any biological application (Table 5-3).

Table 5-3: Time dependent changes in particle properties ^a

	λ_{emi} (nm) ^b	λ_{abs} (nm) ^c	Dh- number (nm) ^d	Dh- intensity (nm) ^e	Zeta potential (mv)
AIS-2MPA-3-1st day	634	727	5.38	38.17	-37.53
AIS-2MPA-3-1st month	627	951	3.98	29.98	-38.10
AIS-2MPA-3-2nd month	617	906	4.31	62.60	-39.40
AIS-2MPA-3-3rd month	612	855	4.92	38.70	-44.56
AIS-PEI/2MPA-1-1st day	617	630	7.63	27.91	10.67
AIS-PEI/2MPA-1-1st month	603	969	8.95	38.89	9.92
AIS-PEI/2MPA-1-2nd month	620	666	8.34	55.40	9.45
AIS-PEI/2MPA-1-3rd month	593	630	9.22	38.11	11.75

^a These QDs were synthesized according to recipes of AIS-2MPA-2 and AIS-PEI/2MPA-1; therefore, particle properties are slightly different than those reported in Table 1 and 2. ^b Emission maxima. ^c Absorbance onset. ^d Hydrodynamic diameter measured by DLS and reported as the number average. ^e Hydrodynamic diameter measured by DLS and reported as the intensity average.

In summary, we can say that the particles are colloiddally stable and no aggregation, precipitation or quenching was observed after 3 months (Figure 5-11d, h), but instead, an enhancement in the luminescence intensity was detected (Figure 5-11a, e). Hence, they are very suitable for exploitation in the *in vitro* studies.

5.4.6 Electrostatic loading of ALA to AIS-2MPA-3 and AIS-PEI/2MPA-1

ALA has one carboxylic acid and one primary amine groups with pK_a values of 4.05 and 8.9, respectively. ALA should be zwitterionic at pH 7.2-7.4, and the electrostatic interaction between the -COO⁻ group of AIS-2MPA-3 QDs and -NH₃⁺ group of ALA could allow electrostatic loading of ALA to the anionic QDs. Two compositions were

prepared with 30 mol% and 50 mol% ALA loading (with respect to 2MPA content of the QD) to AIS-2MPA-3. This increased the intensity-based hydrodynamic size of the AIS-2MPA-3 QDs from 35 nm to 62 and 50 nm, respectively, in HEPES buffer (pH 7.2) (Table 5-4). This indicates some aggregation; however, the number-based size average does not indicate a dramatic aggregation (4.40 nm versus 5.29-5.97 nm). Zeta potential of the QDs changed slightly from -39 eV to -41 and -44 eV for 30% and 50% ALA loading, respectively (Table 5-4). Although, carboxylate groups of the QDs are consumed by the NH₃⁺ groups of ALA, -COO⁻ groups of ALA reside on the periphery after binding, therefore, the zeta potential did not change much. In the case of AIS-PEI/2MPA-1 QDs, at pH 7.2 in HEPES buffer, the -NH₃⁺ groups of QDs are electrostatically bound to the -COO⁻ groups of ALA. This increased the charge of AIS-PEI/2MPA-1-30%ALA and AIS-PEI/2MPA-1-50%ALA QDs from ca 10 to 24 and 30 mV, respectively (Table 5-4). This prevented aggregation and the hydrodynamic size stayed around 27-33 nm with reduced size distribution (Table 5-4). Again, the number-based average hydrodynamic size increased slightly from 7.6 nm to 10.0 nm and 9.2 nm with ALA loading (Table 5-4). Overall, after ALA loading both QDs maintained small hydrodynamic sizes and their original charge character as desired for this study.

Table 5-4: Composition and properties of ALA loaded AIS-2MPA-3 and AIS-PEI/2MPA-1 QDs used in the *in vitro* studies.

Sample	PDI ^a	Dh-number ^b (nm)	Dh-intensity ^c (nm)	Zeta potential (mv)	[cation] (μg/mL)	[ALA] (mM)
AIS-2MPA-3	0.39	4.40	34.77	-38.80	10	-
AIS-2MPA-3-30%ALA	0.37	5.29	62.10	-40.70	10	0.068
AIS-2MPA-3-50%ALA	0.29	5.97	50.60	-43.95	6	0.068
AIS-PEI/2MPA-1	0.23	7.66	27.91	10.67	10	-
AIS-PEI/2MPA-1-30%ALA	0.21	10.07	27.41	24.03	10	0.068
AIS-PEI/2MPA-1-50%ALA	0.18	9.20	33.01	29.91	6	0.068

^a Polydispersity index, ^b Hydrodynamic diameter measured by DLS and reported as the number average, ^c Hydrodynamic diameter measured by DLS and reported as the intensity average. All QDs are in HEPES at pH=7.2.

Isothermal Titration Calorimetry (ITC) has been used to further study the interaction between ALA and the QDs. Titration of QDs at pH 7.2 for AIS-2MAP-3 and AIS-PEI/2MAP-1 with 50 mol% ALA dissolved in HEPES buffer created an exotherm, which is an indication of binding (Figure 5-12a, b). The exotherm regularly decreased with the

addition of ALA and reached almost to a constant value at the last injection. This suggests ALA fully binds to both cationic and anionic AIS QDs at the studied doses. Yet, binding exotherm is much stronger in the case of cationic AIS QDs, indicating stronger binding (Figure 5-12a, b).

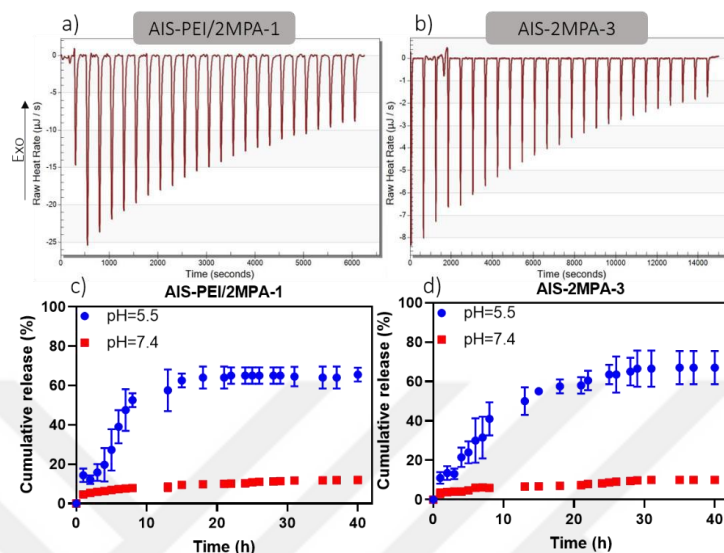


Figure 5-12: Isothermal titration calorimetry of (a) AIS-PEI/2MPA-1 and (b) AIS-2MPA-3 with 50 mol% ALA in HEPES at pH 7.2. *In vitro* release profiles of (c) AIS-PEI/2MPA-1-50%ALA and (d) AIS-2MPA-3-50%ALA in PBS solution at pH 7.4 and 5.5, 37 °C for 40 h (n=3).

5.4.7 *In vitro* release of ALA

Best drug delivery vehicles supposed to bind strongly to the drug at the physiological pH and then release it effectively at the diseased site [294]. Since ALA is loaded to QDs via electrostatic interaction, its release should be affected by pH changes. AIS-2MPA-3-50%ALA and AIS-PEI/2MPA-1-50%ALA were incubated in PBS at acidic and neutral pH (5.5 and 7.4) for 40 h at 37 °C. Protonation of carboxylic acids in acidic pH would weaken the electrostatic interaction between the anionic QD and ALA, causing its release. In the case of cationic QDs with PEI content, protonation of amines and the 2MPA component will also weaken the interactions as ALA may have started to protonate as well. In the *in vitro* studies, the effective release of anionic cargo from PEI-containing delivery vehicles are also associated with the proton-sponge effect. Here, both QDs kept the release of ALA $\leq 12\%$ at pH 7.4 in 24-40 h (Figure 5-12c, d). In acidic media, a small burst release around 10% was followed by a sustained release profile. In general, release from the cationic QDs was slightly faster, reaching to $\sim 30\%$ in 5 h and to a maximum value of $\sim 67\%$ in 18 h. ALA release from the anionic QD was about $\sim 24\%$ in 5 h and $\sim 60\%$ in 18 h in acidic pH (Figure 5-12c, d). The enhanced ALA release in the acidic

media should facilitate the conjugates' therapeutic efficacy under the acidic tumour microenvironment *in vivo*. Xu *et al.* have demonstrated that ALA conjugated to gold nanorods via hydrazone linkage, which hydrolyzes under acidic tumour environment, release 71% of ALA at pH 5 in 24 h, and only 6% of ALA at neutral pH [295]. It is quite promising to see similar pH selectivity in release with simply electrostatically loaded ALA to our QDs. ALA release was much less in amount with poor pH dependence from self-assembled polysaccharide-based nano-complexes reported by Wang *et al.*, which trapped ALA non-covalently. They reported 15 and 20% release of ALA in 5 h and 30% and 50% after 24 h at pH 7.4 and pH 5.5, respectively [273]. Di Martino *et al.* observed a fast release of ALA from cationic polysaccharide [296].

5.4.8 Evaluation of the *in vitro* dark cytotoxicity of QDs

Cytotoxicity of AIS-2MPA-3 and AIS-PEI/2MPA-1 QDs and their ALA-conjugates on a variety of tumour cells were determined using 2D cell culture of HT29, HeLa, SW480 and HCT116 cell lines by MTT assay (Figure 5-13). QD doses were based on the total cation concentration of each QD determined by ICP-MS and studied in the range of 0.25-100 µg/mL. AIS-2MPA-3 QDs did not show any significant cytotoxicity in this range after 48h incubation (Figure 5-13a-d). The AIS-PEI/2MPA-1 QDs significantly impaired the cell viability of all tested cell lines at concentrations above 2 µg/mL cation (Figure 5-13e-h). PEI has a dose and molecular weight dependent toxicity due to its cationic nature which is suppressed significantly by adsorption on QD surface [182]. Hence, cationic nanoparticles are expected to be more toxic. Free ALA did not show any substantial cytotoxicity in either of the tested cell lines up to 0.688 mM, which is the highest concentration that was delivered at 100 µg/mL cation concentration. Although cell viability slightly decreased when exposed to ALA loaded cationic QDs compared to free QDs, ALA association with QDs did not lead to a dramatic difference between different formulations. The influence of these QDs on the viability of healthy colorectal cells was also tested. There was no loss in the viability of CCD841 cells that were exposed to free ALA, AIS-2MPA-3 QDs (up to 200 µg/mL [cations]) or ALA loaded AIS-2MPA-3 QDs (Figure 5-14a). In the case of AIS-PEI/2MPA-1 QDs and its ALA loaded formulations, a significant drop in the cell viability was observed at 10 µg/mL cation concentration, which is mostly due to cationic nature of the QDs since ALA did not cause any reduction in cell viability (Figure 5-14b).

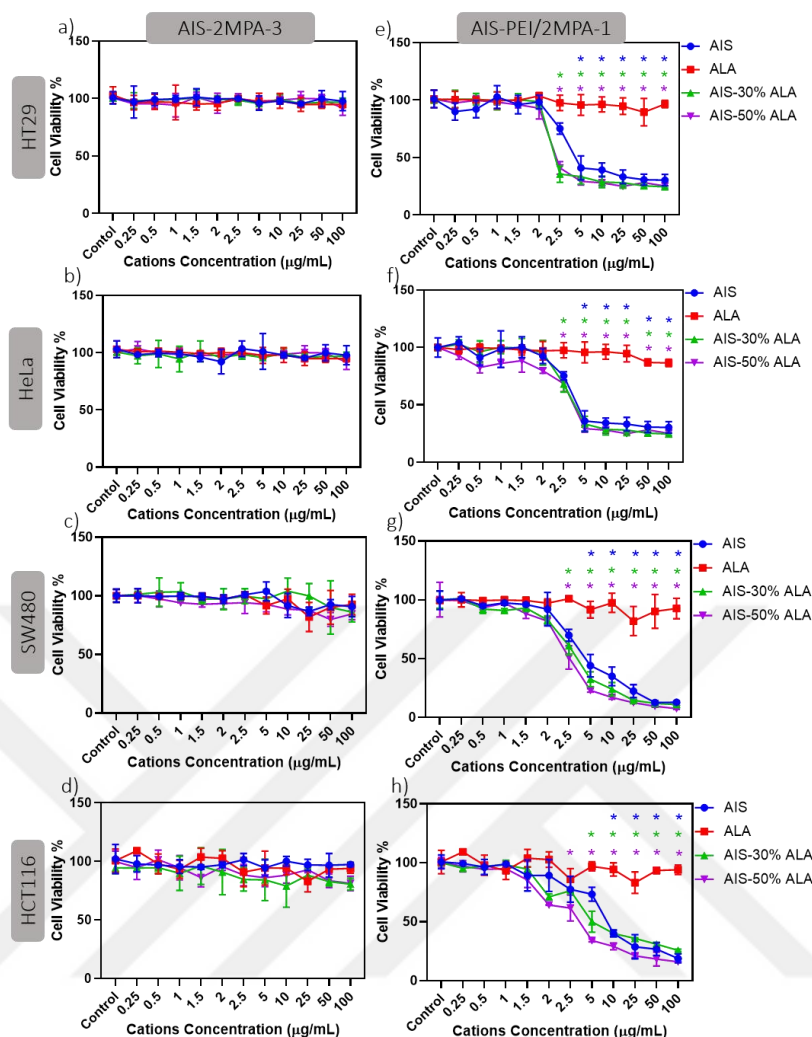


Figure 5–13: Viability of HT29, HeLa, SW480 and HCT116 cells treated with (a-c) AIS-2MPA-3 (d-f) AIS-PEI/2MPA-3 QDs after 48 h incubation, determined by MTT assay. Untreated cells were used as controls. The data are expressed as mean $\pm$ S.D. ($n = 4$). ($p < 0.05$).

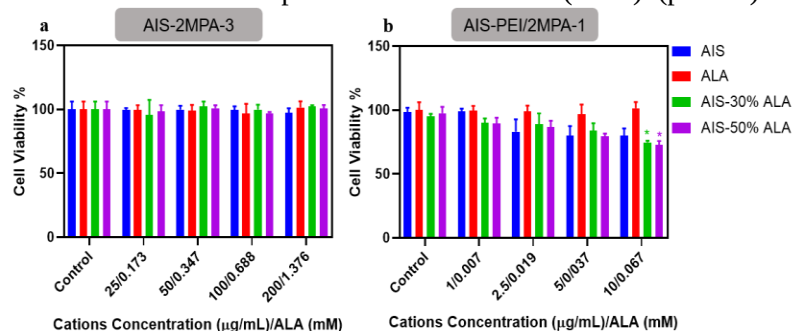


Figure 5–14: Viability of CCD841 cells (healthy colorectal cells) treated with (a) AIS-2MPA-3, AIS-2MPA-3-30%ALA, AIS-2MPA-3-50%ALA QDs and (b) AIS-PEI/2MPA-1, AIS-PEI/2MPA-1-30%ALA, AIS-PEI/2MPA-1-50%ALA QDs after 48 h incubation, measured by MTT assay. Untreated cells were used as control. The data are expressed as mean $\pm$ S.D. ($n = 4$). ($p < 0.05$).

5.4.9 *In vitro* cell uptake and optical imaging

HT29, HeLa, SW480 and HCT116 cells treated with 2 $\mu\text{g/mL}$ [cation] of AIS-2MPA-3

and AIS-PEI/2MPA-1 QDs for 24 h exhibited intense intracellular optical signal indicating effective internalization of both QDs to each cell line (Figure 5-15a-d). The intensity and clarity of the intracellular signal support the potential of these QDs for optical-medical imaging. There seems to be more luminescence in the cells treated with the cationic QDs. Hence, to quantify the uptake of QDs by these cell lines, the Ag and In concentration in the QD treated cells was determined by ICP-MS. After 24 h incubation with QDs (2 $\mu\text{g/mL}$) about 20-25% of the AIS-PEI/2MPA-1 and 10-15% of AIS-2MPA-3 QDs were internalized by different cell lines according to ICP-MS analysis of lysed cells with no significant difference between different cell lines (Figure 5-15e). In addition to the more toxic nature of PEI than 2MPA, this higher uptake of the cationic QDs may also contribute to higher toxicity of the AIS-PEI/2MPA-1 QDs.

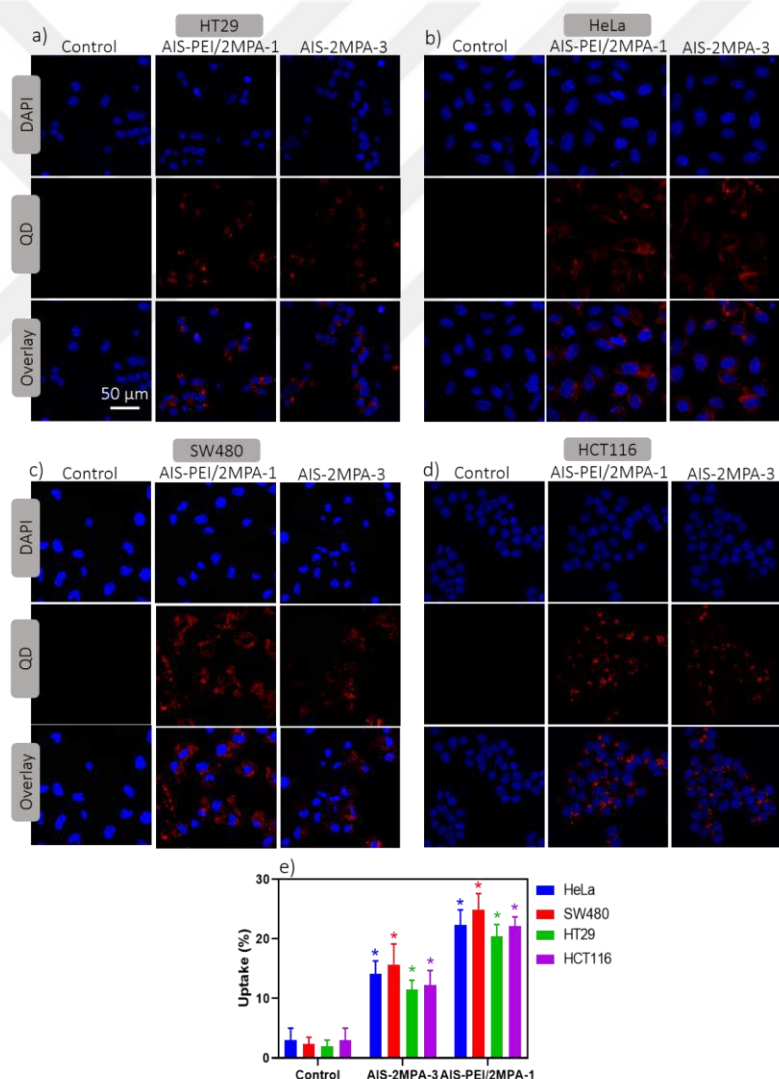


Figure 5–15: Cellular localization of control cells, AIS-PEI/2MPA-1 and AIS-2MPA-3 QDs by (a) HT29, (b) HeLa, (c) SW480 and (d) HCT116 cells. Samples at 2 $\mu\text{g/mL}$ cations concentration were incubated with cells for 24 h in 2D culture. Red: Luminescence from QDs

(excitation/emission:488/580-780 nm), Blue: Nuclear stain. (e) Intracellular quantification of AIS-PEI/2MPA-1 and AIS-2MPA-3 QDs in HeLa, SW480, HT29 and HCT116 cell lines in 2D culture. Measurements were done by ICP-MS instrument. Samples at 2 $\mu\text{g/mL}$ cations concentration were incubated with cells for 24 h. The data are expressed as mean $\pm$ S.D. ($n = 3$), ($p < 0.05$).

Fluorescence microscopy was also conducted on 3D spheroids of HT29 and SW480 cells with AIS-PEI/2MPA-1 (2 $\mu\text{g/mL}$) and AIS-2MPA-3 QDs (2 and 50 $\mu\text{g/mL}$) and their ALA loaded formulations (Figure 5-16a, b). Both QDs showed strong luminescence and in the case of anionic ones, the optical signal seems to be stronger as the concentration increased. ALA loaded versions seems to provide more signal, especially in SW480, which is probably due to PpIX generation which luminescence at around the same wavelengths with these AIS QDs. Overall, bright red fluorescence observed in the 3D matrix originates from the embedded cells rather than the collagen matrix. These strong, AIS based intracellular luminescence observed within 3D matrix further support the candidacy of these QDs for optical imaging and/or utilization of such nanoparticles for image-guided therapy.

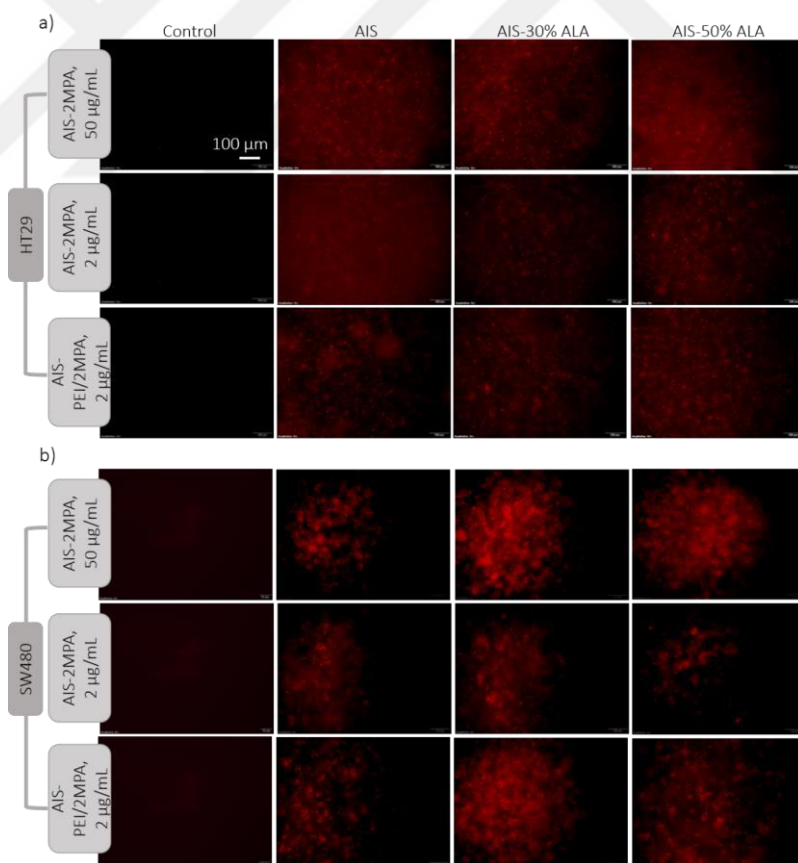


Figure 5–16: Cellular localization of 3D-spheroid constructs of control cells, AIS-PEI/2MPA-1 and AIS-2MPA-3 QDs by (a) HT29 and (b) SW480 cells. Excitation: 545 nm, emission 620 nm.

5.4.10 *In vitro* PDT results

ALA can act as a prodrug of a strong photosensitizer, PpIX, which upon irradiation produces highly toxic ROS, causing apoptosis/necrosis. After the internalization of ALA conjugates by the cells, ALA was converted to PpIX via the heme-synthesis pathway [297]. Therefore, delivery of ALA into cells in a therapeutic dose, its release and its intracellular conversion to PpIX in cancer cells are crucial steps for effective PDT. Fluorescence of PpIX at 635 nm can be used to determine the intracellular PpIX amount. However, since QDs have absorbance at the excitation wavelength of 420 nm and luminescence at the same region with PpIX, it interfered with the measurements (Figure 5-17). Yet, an indication of higher PpIX generation with QD loaded ALA was observed in addition to a slight dose and cell type dependence mostly at the high concentrations. Some differences between cell lines are possible since the metabolism of ALA could be different and conversion of ALA to PpIX involves ALA release and conversion to PpIX by mitochondrial enzymes.

5.4.11 *In vitro* PDT studies

Intracellular PpIX levels of treated HT29, HeLa, SW480 and HCT116 cells were determined from the PpIX fluorescence intensity at 635 nm with 420 nm excitation recorded on a microplate reader. However, since AIS QDs have absorbance and emission at these wavelengths, it interferes with PpIX measurements. In order to see the significance of AIS QD based delivery on the PpIX levels under these circumstances, a broad range of ALA concentration was needed; hence, the non-toxic AIS-2MPA-3 was used for a dose dependent study in the range of 0.018-0.668 mM ALA. Cells without any treatment were used as control. Especially in SW480 cell lines, a stronger PpIX signal with 50% ALA loading to AIS QDs was clearly seen. Since the AIS content for the equivalent amount of ALA is lower when 50% ALA loading is considered compared to 30% ALA loading, QD interference with the measurement is lower (Table 5-4). So, especially in SW480 there is a clear advantage of delivering ALA to cells with high ALA content AIS QDs (Figure 5-17).

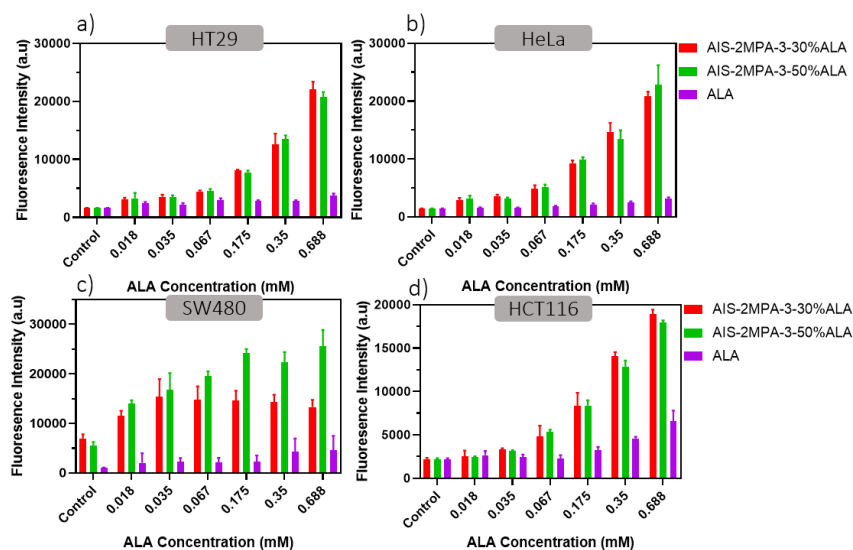


Figure 5–17: Intracellular PpIX levels of (a) HT29, (b) HeLa, (c) SW480 and (d) HCT116 cells after 48 h incubation with free ALA and ALA loaded AIS-2MPA-3 QDs in serum-free media. The data are expressed as mean $\pm$ S.D. (n = 4).

In vitro PDT studies were performed both on 2D and 3D cell cultures using blue light (420 nm, 7 mW/cm²) for 5 min. Since MTT is not suitable to check viability in 3D cell cultures, Alamar blue was used to determine the viability of cells before and after PDT (Figure 5-18). The viabilities of 2D-cell cultures after PDT were also tested with MTT assay (Figure 5-13). All doses were given in terms of ALA in Figure 5-18 since it is critical to compare the phototoxicity of free ALA versus QD delivered ALA. Corresponding QD concentration are given in Table 5-4. All PDT studies were performed on two cell colorectal cancer cell lines: SW480 and HT29.

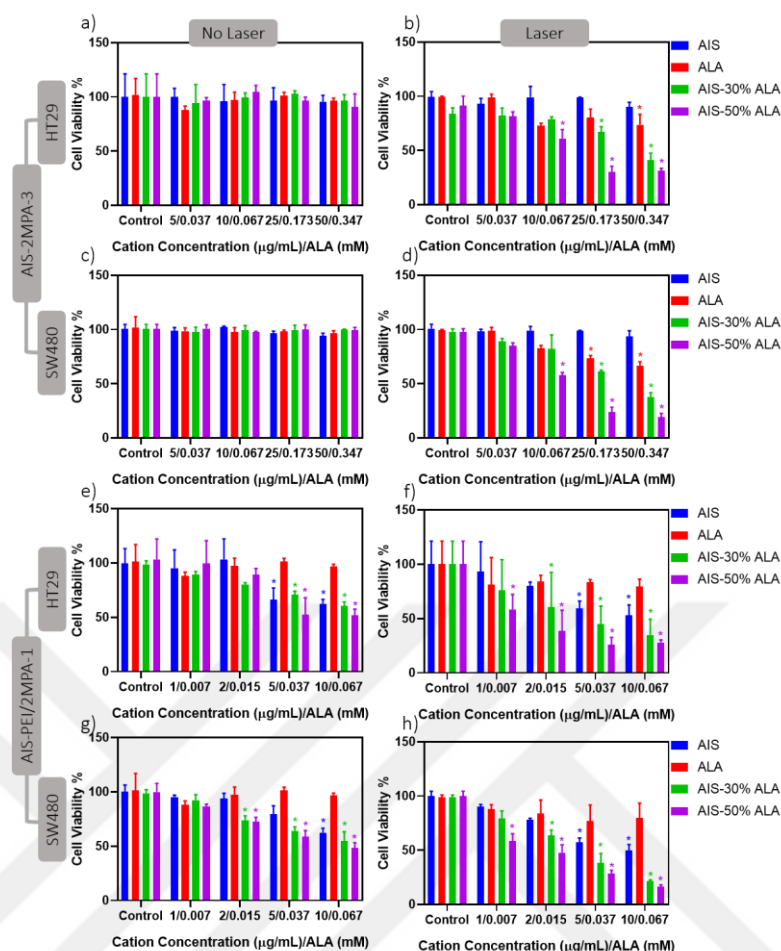


Figure 5–18: Viability of (a), (b) HT29 and (c), (d) SW480 cells treated with AIS-2MPA-3, AIS-2MPA-3-30%ALA, AIS-2MPA-3-50%ALA QDs and (e), (f) HT29 and (g), (h) SW480 cells treated with AIS-PEI/2MPA-1, AIS-PEI/2MPA-1-30%ALA, AIS-PEI/2MPA-1-50%ALA QDs after 24 h incubation with and without PDT measured by Alamar blue compared to untreated cells.

Anionic AIS-2MPA-3 and its ALA loaded versions showed no dark toxicity in the studied range (Figure 5-18a, c). ALA created effective toxicity upon irradiation only at the highest dose, 0.347 mM in HT29 cell line and at and above 0.173 mM in SW480 cell line. However, ALA loaded AIS-2MPA-3 QDs induced greater phototoxicity in a dose and ALA loading dependent manner. Viability of HT29 cells dropped to 41% (30% ALA loading) and 31% (50% ALA loading) at 0.347 mM ALA, respectively, while free ALA provided 74% viability at this dose. The effect was more dramatic on SW480 cells with 38% (30% ALA loading) and 19% (50% ALA loading) viability, while free ALA provided 67% viability at 0.357 mM ALA. Since no phototoxicity was detected in cells treated with AIS-2MPA-3, enhanced phototoxicity should indicate better ALA-PDT when delivered with these nanoparticles (Figure 5-18b, d). AIS-2MPA-3-30%ALA provided IC₅₀ at 0.291 mM and 0.249 mM in HT29 and SW480 cells. This was improved

by using AIS-2MPA-3-50%ALA QDs: IC₅₀ was 0.103 mM and 0.093 mM for HT29 and SW480 cells, respectively.

Cationic AIS-PEI/2MPA QDs and their ALA loaded versions were tested between 0.007-0.015 mM ALA dose (0.6-2.24 µg/mL [cation]) due to QD based dark toxicity at higher concentrations (Figure 5-13). At these concentrations, ALA or the cationic AIS QDs showed no dark toxicity in either cell line (Figure 5-18e, g). Significant dark toxicity was only recorded at 0.015 mM ALA in SW480 cell line. Irradiation of AIS-PEI/2MPA-1 treated cells did not cause a significant change in the viability with respect to the dark toxicity of these cationic QDs. At these concentrations, ALA did not cause notable phototoxicity either. However, phototoxicity of ALA delivered by AIS-PEI/2MPA-50%ALA QDs effectively reduced viability even at 0.007 mM ALA dose to 57 and 58% in HT29 and SW480 cell lines, respectively (Figure 5-18f, h). At 0.015 mM ALA concentration, the viability of HT29 cells were 60 and 38% when treated with AIS-PEI/2MPA-1-30%ALA and AIS-PEI/2MPA-1-50%ALA QDs, respectively. SW480 cell line showed a similar trend with 63 and 47% viability after treatment with AIS-PEI/2MPA-1-30%ALA and AIS-PEI/2MPA-1-50%ALA QDs. IC₅₀ of AIS-PEI/2MPA-1-50%ALA QDs for HT29 and SW480 cells were 0.010 mM and 0.012 mM [ALA], respectively, while AIS-PEI/2MPA-1-30%ALA QDs couldn't hit the IC₅₀ in the studied range after PDT. All these data indicate much better delivery of ALA into the cells with the nanoparticles and its effective conversion to PpIX, resulting in a dramatic improvement in PDT of tumour cells at low ALA concentrations after single and short irradiation at 420 nm.

All of the further *in vitro* experiments were performed at 0.347 mM ALA and corresponding concentrations of AIS-2MPA-3 QDs as well as at 0.015 mM ALA and corresponding amounts of AIS-PEI/2MPA-1 QDs, since at these doses, 2D experiments indicated no dark toxicity but significant phototoxicity on both cell lines (Figure 5-19a-d).

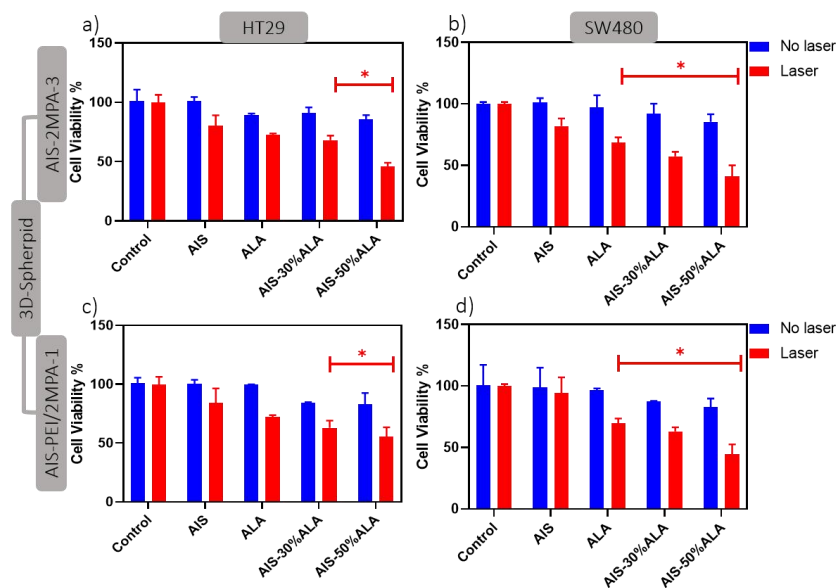


Figure 5–19: Viability of 0.347 mM ALA of AIS-2MPA-3, AIS-2MPA-3-30%ALA and AIS-2MPA-3-50%ALA QDs on (i) HT29 and (j) SW480 3D-spheroid models and 0.015 mM ALA of AIS-PEI/2MPA-1, AIS-PEI/2MPA-1-30%ALA and AIS-PEI/2MPA-1-50%ALA on (k) HT29 and (l) SW480 3D-spheroid models treated with QDs and illuminated at 420 nm blue light for 5 min. PDT control indicated cells treated only with 420 nm irradiation for 5 min. The data are expressed as mean $\pm$ S.D. ($n = 3$), ($p < 0.05$).

PDT was also tested on 3D spheroid constructs of the HT29 and SW480 cells treated with ALA, AIS QDs and their ALA loaded formulations. Free ALA (0.015 and 0.347 mM) reduced the viability to ~70-75% in both HT29 and SW480 cells after irradiation. Both QDs improved the ALA based PDT: The viability of the cells treated with AIS-2MPA-3-50%ALA QDs (0.347 mM ALA) reduced the viability of HT29 cells to 46% and SW480 cells to 40% (Figure 5-19a, b). In case of the cationic AIS-PEI/2MPA-3-50%ALA QDs, irradiation of the treated spheroids reduced the viability of HT29 and SW480 cells to 55% and 44%, respectively, at 0.015 mM ALA concentration (Figure 5-19c, d).

Since irradiation of free ALA and free QDs did not create significant phototoxicity and ALA loaded ones showed dose-dependent toxicity, ROS generation due to irradiation of intracellular PpIX should be the primary factor responsible from the reduction of cell viability. To determine the amount of ROS generated after blue light illumination, a cell-permeable nonfluorescent dye 2',7'-dichlorofluorescein diacetate (DCFDA) is utilized as the ROS indicator. The dye is oxidized to a green fluorescent dichlorofluorescein (DCF) by the ROS. After incubation of HT29 and SW480 cells with ALA, QDs and ALA loaded the QDs, the production of ROS is followed by the green fluorescence intensity at 538 nm (Figure 5-20a, b). QD irradiation did not generate any ROS, which supports no

phototoxicity observation in the absence of ALA. ALA loaded QDs showed significantly more ROS generation compared to free ALA in both cell lines, especially with 50% ALA loading to QDs. The amount of the ROS generated by ALA loaded cationic versus ALA loaded anionic QDs are not significantly different, although anionic ones carried more ALA at the studied doses of the two QDs (0.0.015 versus 0.347 mM ALA). The release of ALA from cationic QDs after 24 h was only about 10% more than the ALA released from the anionic QDs (Figure 5-12c, d). At especially low ALA concentrations, the amount of PpIX produced from 0.015 and 0.347 mM ALA is similar (Figure 5-17). These two data support a similar amount of ROS generated by ALA delivered by either delivery vehicle upon irradiation. When phototoxicity of ALA loaded QDs at similar ALA concentrations, but cationic versus anionic QD delivery is considered, more toxicity via cationic delivery vehicle is observed. For example, at 0.067 mM ALA delivered by AIS-PEI/2MPA-1-50%ALA versus AIS-2MPA-3-50%ALA, the viabilities of both cells were around 15-30% versus 20-30%, respectively. Considering no dark toxicity of the anionic QD but significant dose-dependent cytotoxicity of the cationic QD, the anionic AIS-2MPA QDs are better delivery vehicles when the above discussion is also taken into account.

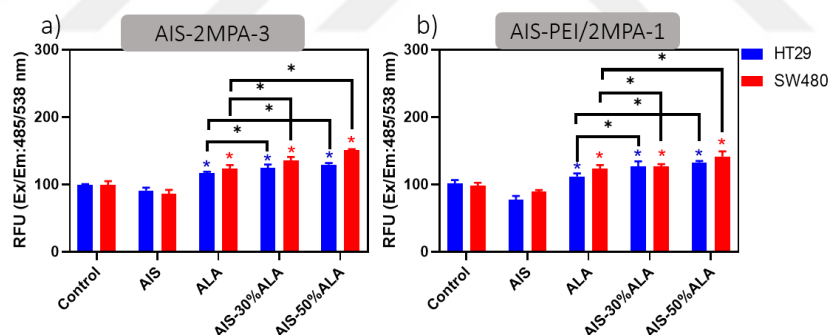


Figure 5-20: ROS production upon 24 h incubation with (e) AIS-2MPA-3, AIS-2MPA-3-30%ALA and AIS-2MPA-3-50%ALA QDs at 50 μ g/mL and (f) AIS-PEI/2MPA-1, AIS-PEI/2MPA-1-30%ALA and AIS-PEI/2MPA-1-50%ALA at 2 μ g/mL upon blue-light irradiation for 5 min. The data are expressed as mean $\pm$ S.D. (n = 3), (p < 0.05).

To further confirm the therapeutic efficiency of the nanoparticles, and PDT dependent cell death, a calcein-AM/propidium iodide (PI) staining was used. No dead cells were seen in the absence of laser treatment at the studied doses of QDs and ALA. Treatment with cationic or anionic AIS QDs followed by irradiation did not cause any cell death either. Few dead cells were observed when treated with ALA loaded cationic AIS QDs only in SW480 cell line, but not with ALA loaded anionic AIS QDs. However, ALA loaded cationic and anionic AIS QDs exhibit significantly higher cell death after

irradiation both in 2D cell cultures and 3D spheroid constructs of both cell lines. Figure 5-21a and b clearly shows more cell death when AIS-2MPA-3-50%ALA QDs was used, which is consistent with the cell viability results and measured ROS levels.

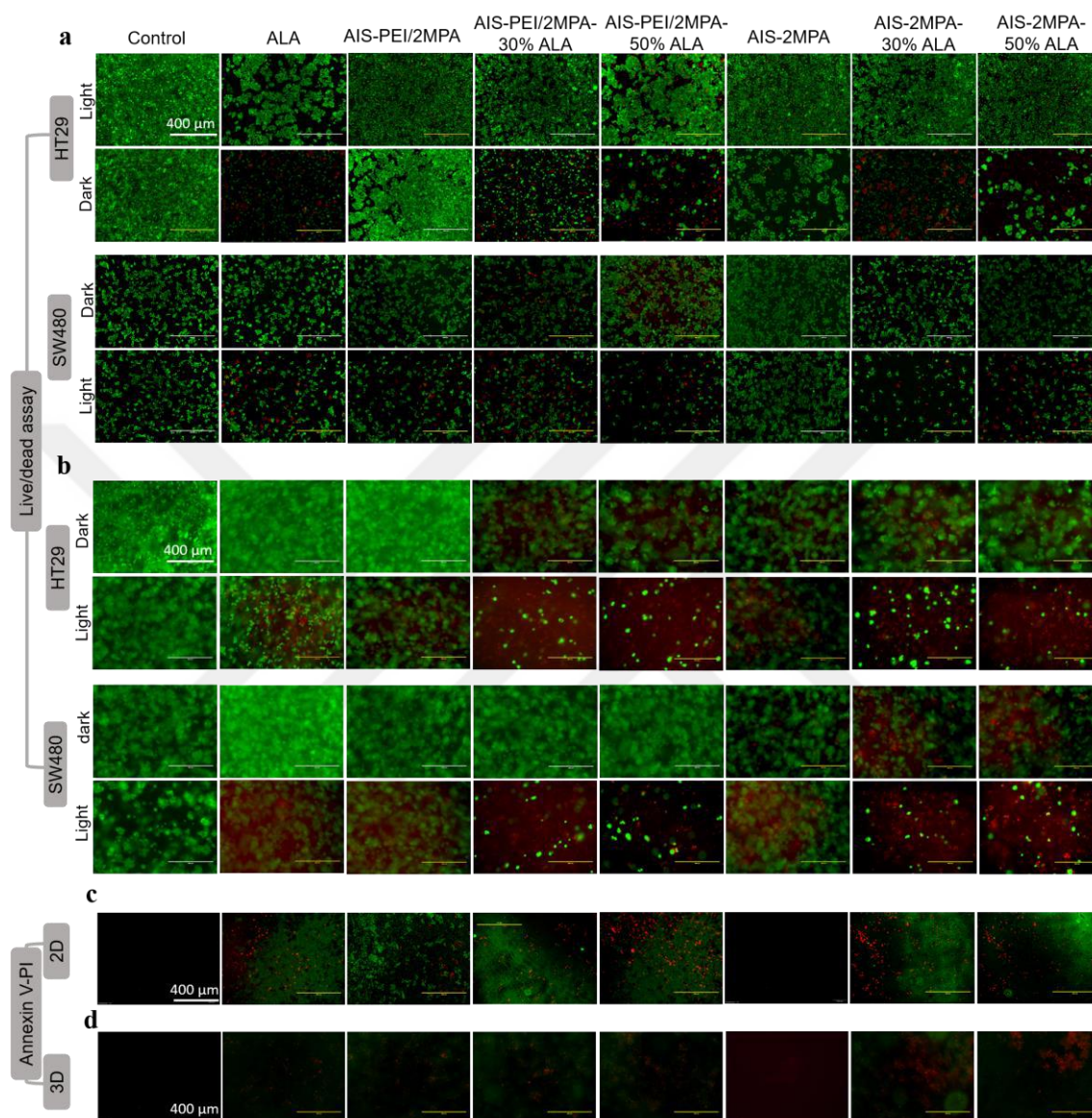


Figure 5–21: Live/dead images of HT29 and SW480 (a) 2D and (b) 3D-spheroid cultures. The cultures were treated with free ALA (0.347 mM), AIS-2MPA-3, AIS-2MPA-3-30%ALA and AIS-2MPA-3-50%ALA QDs at 50 μg/mL and free ALA (0.015 mM), AIS-PEI/2MPA-1, AIS-PEI/2MPA-1-30%ALA and AIS-PEI/2MPA-1-50%ALA at 2 μg/mL after 24 h incubation with/without PDT illuminated at 420 nm with a blue lamp for 5 min. Apoptotic and necrotic induced cell death in (c) 2D and (d) 3D cultures of SW480 cells. Comparison of cell death pathways induced after PDT (420 nm with a blue lamp for 5 min) with free ALA (0.347 mM), AIS-2MPA-3, AIS-2MPA-3-30%ALA and AIS-2MPA-3-50%ALA QDs at 50 μg/mL and free ALA (0.015 mM), AIS-PEI/2MPA-1, AIS-PEI/2MPA-1-30%ALA and AIS-PEI/2MPA-1-50%ALA at 2 μg/mL. Annexin-V was used to indicate apoptosis (green) and propidium iodide was used to indicate necrosis (red). ‘Control’ indicates untreated cells. Scale bar for all images is 400 μm.

To determine the apoptotic/necrotic cell death caused by the PDT treatment, SW480 cells

in 2D and 3D models were stained with Annexin-V for the detection of apoptosis (shown in green) and PI for detection of necrosis (shown in red), 24 h after illumination. ALA showed apoptotic cell death in 2D and a combination of apoptotic and necrotic cell death in 3D. Dramatic enhancement in the apoptotic cell population was observed with ALA loaded AIS QDs both in 2D and 3D cell cultures (Figure 5-21c, d). QDs with 50% ALA loading caused a higher population of necrotic/apoptotic cells than 30% ALA loaded ones. Necrotic cells were more visible in 3D spheroid and more with cationic nanoparticles (Figure 5-21d).

5.5 Conclusion

AIS-2MPA and AIS-PEI/2MPA QDs were successfully prepared in aqueous solutions via the reaction between indium nitrate, silver nitrate and sodium sulfide at room temperature in the presence of 2MPA or PEI/2MPA as the capping/stabilizing agents for the first time in the literature. The best precursor stoichiometry for the synthesis of both 2MPA coated anionic AIS and PEI/2MPA coated cationic AIS QDs with small sizes (less than 8 nm) and strong emission with QYs 19.38% (AIS-2MPA-3) and 20.28% (AIS-PEI/2MPA-1) was determined as Ag/In/S: 1/10/10. These are one of the highest reported QYs for AIS with no Zn dopant or ZnS Shell, to the best of our knowledge. Moreover, these QDs showed excellent colloidal stability with enhanced emission intensity over time. *In vitro* studies showed that both AIS QDs are internalized by a variety of cancer cells (HeLa, HCT116, SW490, and HT29) and exhibit strong intracellular luminescence, proving their efficiency in medical imaging. Cationic AIS QDs were internalized slightly more than the anionic ones by all cell types and were more toxic, as well. While AIS-2MPA QDs caused no significant cytotoxicity up to 100 µg/mL [cation], AIS-PEI/2MPA QDs reduced viability of cells above 2 µg/mL [cation].

AIS QDs were loaded with ALA electrostatically at two different concentrations to combine their imaging potential with a therapeutic modality and to improve the efficiency of ALA-based PDT. Both cationic and anionic AIS QDs successfully loaded with 30% and 50% ALA with respect to coating amounts of the QDs. Both QDs released ALA preferentially in acidic pH (up to 80% after 30 h) with less than 10% release at physiological pH. Hence, the acidic tumour microenvironment could enhance the ALA release at the tumours selectively, thereby enhancing tumour localization, causing enhanced conversion of ALA to PpIX with also the aid of different levels of haem

biosynthetic enzymes in tumour cells (eg. ferrochelatase) which favour higher tumour PpIX levels. All PDT experiments were performed at up to 50 µg/mL cations/0.688 mM ALA concentrations in the case of AIS-2MPA QDs, and at 2 µg/mL cation/0.015 mM ALA concentration in case of AIS-PEI/2MPA QDs, using 2D cell culture and 3D spheroid constructs of HT29 and SW480 cell lines. Lower doses were studied with the cationic AIS QDs since their dark toxicity is higher than the anionic ones. This finding suggests the possibility of using AIS-2MPA-3-50%ALA QDs as a promising construct for PDT using 420 nm blue lamp for 5 min at high concentrations. In both 2D and 3D spheroid models of the SW480 cell line, ALA conjugated AIS-2MPA and AIS-PEI/2MPA QDs lead to necrosis after 24 h. Considering the surface charge and coating of the nanoparticles, low concentration of AIS-PEI/2MPA-1-50%ALA QDs and high concentration of AIS-2MPA-3-50%ALA QDs were found to exhibit significant cell toxicity, cell uptake, ALA loading, and ROS generation with/without PDT, which also makes them a promising candidate for further image-guided local therapies for cancers.

In the clinic, tumour selectivity of such multimodal theranostic nanoparticles will be aided by the EPR effect which accumulates nanoparticles and hence ALA at the tumour, and the enhanced conversion of ALA to PpIX in tumour cells will be achieved with the faster release of ALA at the acidic tumour microenvironment coupled with higher conversion of ALA to PpIX in tumour cells, providing higher toxicity to tumour cells when irradiated. Strong luminescence of the AIS QDs would also provide image-guided irradiation of the area occupied by the nanoparticles and hence ALA, which would aid the operator to decide when to irradiate and highlight the sensitized region for high-level of locality in the treatment, which is undoubtedly very desirable for the improved therapeutic outcome with reduced side effects.

Chapter 6:

Conclusion and Future Perspectives

With the rapidly increasing demands of therapeutic materials, interest in nanomaterials with multimodal therapeutic ability is also enhanced. QDs with strong luminescence in either visible or NIR regions suitable for the medical devices and higher penetration depth are considered as the promising materials especially in cancer therapy. However, there are still some concerns about the biocompatibility and efficiency of the QDs *in vitro* and *in vivo*, which limits their widespread bio-applications. In this thesis, we introduced different types of Ag-based QDs with high cytocompatibility and efficiency, which can be utilized for cancer treatment. Using the targeting agents to improve the nanoparticles' uptake in short incubation time as well as utilizing chemotherapeutic drugs could enhanced the efficiency of the treatment. Moreover, local phototherapy, PTT and/or PDT, showed great promise for reducing the side effects of the chemotherapeutic drugs and killed the cancer cells at very low concentrations compared to either free drugs or bare QDs.

To summarize, in this thesis work:

- Different aqueous cationic and anionic Ag-based QDs were developed in very simple and one step synthetic methods. Anionic AS-2MPA QDs were synthesized according to Hocaoglu et al. [14] with molar ratios of Ag/S/2MPA:1/4/5 at RT for 2 h. To evaluate the PDT efficacy, different methods were utilized to graft ALA to the QDs; electrostatic, hydrazone, and amide bond. To improve the biocompatibility and accessibility of the QDs, PEG spacer was utilized to connect Cet to the QDs and then to EGFRs. Finally, 5FU was bounded to the final particles using esterification reaction. The prepared anionic AS-2MPA-ALA-Cet-5FU nanoparticles (-7.8 eV zeta potential) with the size of ca 6.5 nm showed great cytocompatibility up to 150 µg Ag/mL with the pH-triggered ALA release in acidic media. Moreover, the release of different ALA-conjugates exhibited faster hydrolysis of ALA from the electrostatic one compared to hydrazone and amide bonds.

- AS-2MPA-ALA-Cet-5FU utilized the passive uptake using EPR effect and active tumour targeting via EGFR overexpression in 2D and 3D cell cultures. Another factor to enhance PDT efficacy is the higher hydrolysis of ALA under acidic tumour microenvironment and subsequently, conversion of inactive ALA into the active PpIX. Combination targeted chemo/PDT increased the generation of ROS and apoptotic/necrotic cells upon utilizing either 420 or 640 nm irradiation, indicating remarkable cell death utilizing AS-2MPA-ALA-Cet-5FU QDs+PDT.

- Aqueous AS-GSH QDs with excellent colloidal and optical stability over a year, emission maxima at ca. 900 nm, and 70% QY were synthesized at 50 °C and pH 10 for 2 h. *In vitro* cell viability results exhibited remarkable cytocompatibility of AS-GSH QDs using MCF7, HT29, A549, HeLa, and C2C12 cells without any significant cytotoxicity up to 200 µg Ag/mL. AS-GSH QDs displayed an excellent cell internalization with the endosomal localization and strong luminescence. The dramatic reduction in the viability of MCF7 (ca. 5%) and HT29 (ca. 35%) was seen after laser irradiation (700 mW, 1.8 W/cm² for 10 min) at 150 µg Ag/mL with late apoptotic and necrotic cell death.

- Colloidally stable AIS-GSH QDs emitting at 634 nm were synthesized without using Zn dopant or ZnS shell at pH 8.5 with the molar ratio of Ag/In/S/GSH: 1/4/2.5/10 and 21% QY. To improve the specific uptake and target the cancer cells, both AIS-GSH and AS-GSH QDs were conjugated to FA via a PEG spacer and then loaded with an anticancer drug, MTX. FA targeted AS-GSH QDs with the loading of MTX upon PTT at 808 nm enhanced the specific uptake by FR (+) HeLa cells, the passive uptake by EPR effect, improved the MTX release under acidic tumour microenvironment and PTT. Another point to be emphasized is that AS-GSH-FA-MTX reduced IC₅₀ of MTX from 10 µg/mL to 0.21 µg/mL in HeLa cells after irradiation at 808 nm (700 mW, 1.82 W/cm², 10 min) driving HeLa cells to necrosis. This reduced IC₅₀ is very crucial in reduction of side effects of chemotherapy while improving its efficacy in the targeted sites.

- Lastly, anionic AIS-2MPA and cationic AIS-PEI/2MPA QDs were synthesized without any Zn/ZnS precursors at different conditions in aqueous media for the first time in the literature. Both AIS-2MPA and AIS-PEI/2MPA QDs showed strong luminescence emitting at 610-660 nm with the sizes less than 8 nm and QYs 19.38% (AIS-2MPA-3) and 20.28% (AIS-PEI/2MPA-1) at the molar ratios of Ag/In/S: 1/10/10. *In vitro* cell internalization of the QDs by a variety of cancer cells (HeLa, HCT116, SW490, and

HT29) exhibited slightly higher accumulation of the cationic AIS-PEI/2MPA QDs over anionic AIS-2MPA QDs and were more toxic, as well. However, cell viability results of AIS-2MPA QDs showed good cytocompatibility up to 100 µg/mL [cation] while AIS-PEI/2MPA QDs hit the IC₅₀ at 2-2.5 µg/mL [cation].

- To improve the therapeutic efficacy of the nanoparticles, AIS QDs were loaded electrostatically with ALA at two different concentrations (30 and 50% ALA with respect to coating amounts of the QDs) for multimodal image guided PDT. Both QDs showed enhanced ALA release in acidic pH preferentially and resulted in the higher accumulation of ALA in acidic tumour microenvironment and consequently higher conversion of ALA to PpIX using haem biosynthetic enzymes. Both 2D and 3D cell culture experiments showed significant reduction in the viability of the cells after PDT at 420 nm for 5 min leading to necrosis after 24 h. Low concentration of AIS-PEI/2MPA-1-50%ALA QDs and high concentration of AIS-2MPA-3-50%ALA QDs were found to perform remarkable cytotoxicity, cell uptake, ROS generation, and necrosis after irradiation at 420 nm, which showed their potential for further image guided PDT.

Overall, our works hold several benefits in targeted therapies. All different aqueous AS-2MPA, AS-GSH, AIS-GSH, AIS-2MPA, and AIS-PEI/2PA QDs were synthesized in green and simple methods with strong luminescence. These biocompatible QDs having the emission in either visible or NIR window paved the ways for the use in clinical devices which are mostly in the visible region and NIR window with the deeper penetration. They provided excellent theranostic platform with the ability to use as a PTT agent and to deliver targeting agents, anticancer drugs, and PDT agents to the site of tumours for the local therapies of cancers.

For further applications, *in vivo* studies of the prepared QDs can be conducted. Most of the cancers have heterogeneous receptor overexpression and need advanced molecular targeting strategies. Dual conjugation of targeting agents such as Herceptin and Cet together may improve the therapeutic efficacy and therefore overcome the failure in targeting detection.

6.1 Spin-off the thesis

Interaction of QDs with enzymes are not well explored but quite important to understand and assess their fate *in vivo*. AS-GSH QDs were utilized in different studies that will be described further in brief.

6.1.1 AS-GSH QDs, a new substrate for glutathione reductase enzyme

Under oxidative stress, GSH oxidizes (to GSSG) and then reduced back by glutathione reductase (GR) enzyme to maintain the balance of GSH/GSSG ratio (Figure 6-1a). Activity of AS-GSH QDs as a substrate-analogue for GR enzyme was determined in a collaboration with Prof. Nuray Ulusu and Ms. Duygu Aydemir, to provide a new perspective in evaluating QDs in medical applications, and to explore its enzyme activity and possibility to study its efficiency in level detection. Figure 6-1b, c showed about 15% reduction in the PL intensity after addition of enzyme and NADPH to AS-GSH QDs. This effect seems to be time dependent and dropped to about 50% after 5 min.

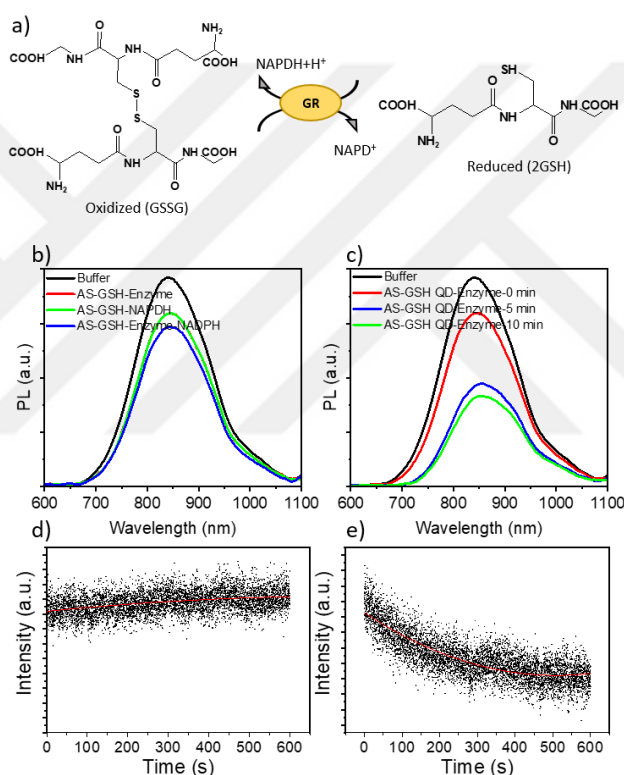


Figure 6–1: a) b) Photoluminescence spectra of the AS-GSH QDs in sodium phosphate buffer before and immediately after adding 8.5 μM GR enzyme, 0.2mM NADPH and the combination. c) Time dependent change in the photoluminescence of AS-GSH QDs after GR enzyme addition. Kinetic photoluminescence spectra for d) AS-GSH QD and e) AS-GSH QD with GR enzyme in buffer under continues illumination at 532 nm for ten minutes.

Kinetic photoluminescence measurements were also conducted to investigate the time dependency of the PL quenching. AS-GSH QDs suspended in deionized water was irradiated for 10 min continuously, and the change in the PL intensity at the peak wavelength recorded to determine the photostability of the QDs as a control (Figure 6-1e). No loss in the intensity was detected. A slight photo-brightening was observed during

irradiation, which might be related to passivation of dangling bonds via photooxidation. This indicates that AS-GSH QDs have an excellent photostability. After GR/NADPH addition to AS-GSH, a time dependent drop in the emission intensity reaching 24.8% with 20 nm red-shift in peak maxima was observed (Figure 6-1c, d, e). The major drop occurs within the first 300s (Figure 6-1f), which is consistent with the results shown in Figure 6-1c.

Influence of GR enzyme, NADPH, GSSG, GSH, and AS-GSH concentration were studied in separate reactions (Figure 6-2). Since GSSG is the original substrate of GR enzyme, at the optimum substrate (1 mM) and NADPH (0.2 mM) concentration, we have accepted the GR activity of GSSG as 100% (Figure 6-2j). Based on this, GSH showed 4% activity while AS-GSH showed 15% enzymatic activity as a substrate, which is significant enough to consider AS-GSH as a substrate for GR enzyme (Figure 6-2j).

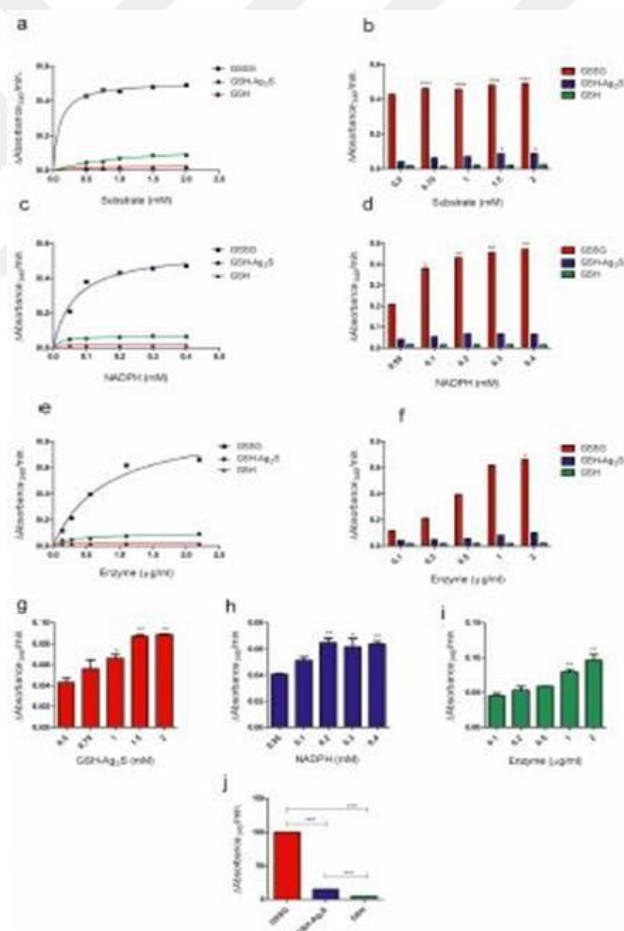


Figure 6–2: a, b) Influence of substrate type and concentration on the GR activity at 0.5 $\mu\text{g/ml}$ GR and 0.2 mM NADPH concentration. c, d) Influence of NADPH concentration on the GR activity at 0.5 $\mu\text{g/ml}$ GR and 1 mM substrate concentration. e, f) Influence of GR concentration on the enzyme activity at saturating NADPH concentration of 0.2 mM and 1 mM substrate concentration. Influence of the (g) AS-GSH (0.2 mM NADPH and 0.5 $\mu\text{g/ml}$ GR), (h) NADPH

(1mM AS-GSH and 0.5 μ g/ml GR) and (i) enzyme concentration (0.2 mM NADPH and 1mM AS-GSH) on GR activity. j) Comparison of GR enzyme activity of GSH and AS-GSH with respect to the activity of 1mM GSSG with 0.2 mM NADPH (* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.0001$).

Overall, as expected, GSH did not act as a substrate for GR at any condition tested and GSSG was confirmed as a good substrate while AS-GSH was discovered as a new GR enzyme substrate, which is new in the literature, and would be interesting for many further studies in the related area.

6.1.2 *In vitro* interaction of glutathione S-transferase-pi enzyme with AS-GSH QDs

In this study, we demonstrated that AS-GSH QDs act as a substrate analogue of glutathione S-transferase (GST) enzymes for the first time in the literature. The GSTs belong to a major group of detoxification enzymes involved in the detoxification metabolism responsible for the protection of cells against reactive oxygen species (ROS) or electrophiles. GST isozymes are impaired in various diseases such as neurological diseases and cancer. We evaluated the interaction of GST-pi enzyme with AS-GSH QDs, which have never been studied in the literature before.

As shown in Figure 6-3a, b, the emission intensity of AS-GSH QDs increase with the concentration. When 1-Chloro-2,4-dinitrobenzene (CDNB) was added into the AS-GSH QDs, a noticeable dose-dependent decrease in the emission intensity was observed (Figure 6-3c). However, GST addition to CDNB-QD mixture, reduced the drop in the emission intensity (Figure 6-3d). This indicates a possible interaction of CDNB with the GSH coating of QDs, which is partially prevented at high doses of GST. Interaction between CDNB-GST-QD is quite quick as can be seen in Figure 6-3e-h. Right after mixing all three components, more than 50% drop in emission intensity of QDs were observed. This drop was 80% after 5 min after mixing and did not change significantly afterwards.

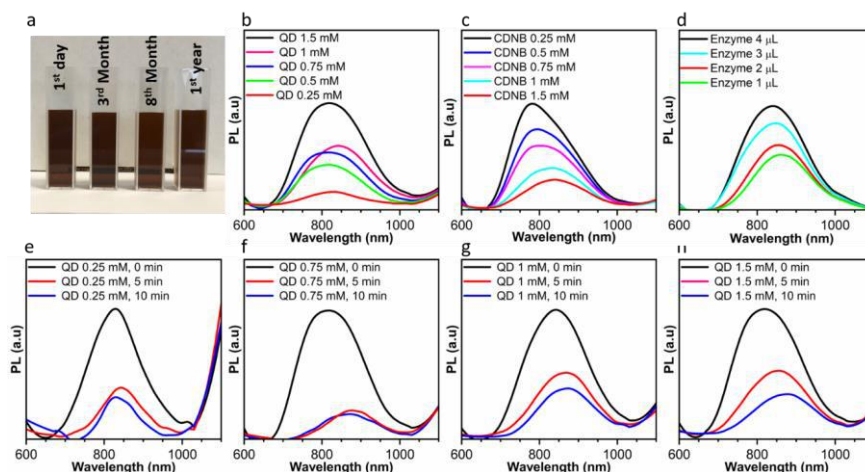


Figure 6–3: (a) Pictures of AS-GSH QD over a period of 1 year. Photoluminescence spectra of (b) QDs at different concentration, (c) 1 mM QD with different concentrations of CDNB, (d) 1 mM QD with 1 mM CDNB and different concentrations of GST enzyme. Photoluminescence spectra of 1 mM CDNB- 0.4 mg/ml GST enzyme with (e) 0.25 mM QD, (f) 0.75 mM QD, (g) 1 mM QD, and (h) 1.5 mM QD after 0, 5, and 10 min of mixing.

Ductal adenocarcinoma cell line CFPAC-1 and liver lysates were used as different GST isoenzyme sources to test the activity of AS-GSH QDs as a substrate analogue of all these GST enzymes. Luminescence of different concentrations of AS-GSH QDs was determined in rat liver and CFPAC-1 lysates in the presence of 1mM CDNB, and increasing emission intensity was observed with increasing QD concentration (Figure 6-4a, b). The influence of the time of measurement after mixing on the emission of QDs was studied at 0, 5, and 10 min after enzyme addition. For instance, Figure 6-4b-e displayed about 30 and 50% reduction in the emission intensity after 5 and 10 min in different QD concentration, respectively.

Figure 6-4 exhibited the reaction of AS-GSH QDs with rat liver and CFPAC-1 cell lysates at different concentrations. GST enzymes are multifunctional enzymes, which have the capacity to conjugate GSH with a variety of compounds.

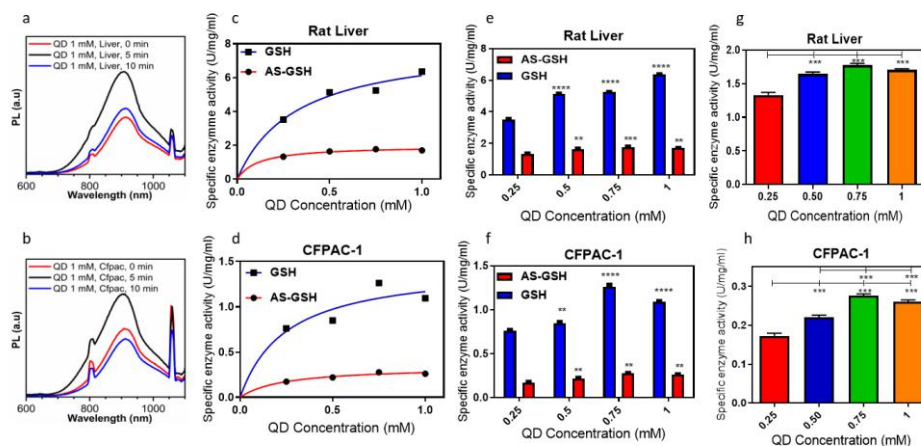


Figure 6-4: Photoluminescence spectra of 1 mM CDNB and (a) liver lysate and (b) CFPAC-1 cell lysate (1.3 mg/ml protein concentration) mixed with 1 mM QD right after mixing (0 min), 5 and 10 min after mixing. Impact of the AS-GSH QD concentration (1 mM CDNB, 10 μ l of liver lysate (24 mg/ml protein concentration) and CFPAC-1 cell lysate (1.3 mg/ml protein concentration) was investigated separately. We used both Michaelis Menten graph (c,d) and bar graphs (e, f, g, h) to show comparison of GST activity between natural substrate and substrate analogue, AS-GSH QD (**** $p \leq 0.0001$).

Changes in the luminescence intensity and increased absorbance at 340 nm, specific for the CDNB-SG conjugate, indicate the enzyme activity in the presence of these QDs, as well. It is only natural that the activity of QDs is not as dramatic as GSH since QDs lack free -SH as the GSH is bound to nanoparticle surface from the -SH group. Also, diffusion of the enzyme to the surface of AS-GSH QDs is probably difficult, limiting the extent of the reaction. Besides, if the enzyme activity would remove the surface bound GSH in large quantities, it would be natural to expect colloidal instability and precipitation, which was not the case.

This enzyme's primary role is the detoxification of xenobiotics and toxic endogenous compounds; therefore, GST enzymes have a broad number of substrates and show some substrate promiscuity, which may explain the action of AS-GSH QDs as GST substrate analogue [298].

6.1.3 Evaluation of biocompatibility of AS-GSH QDs

Investigation of the toxicity of the nanomaterials are necessary to use them in the medical field and biomedical applications. Thus, in this study we investigated biocompatibility of the AS-GSH QDs *in vitro* using 293T and CFPAC-1 cell lines. As shown in Figure 6-5a, b, no significant reduction in the viability of CFPAC-1 and 293T cells were observed until 200 μ g/ ml [Ag] dose which indicates good biocompatibility. To test cellular

internalization, qualitative analysis of the intracellular uptake of AS-GSH QDs were performed utilizing fluorescence microscopy (Figure 6-5c). Efficient cellular internalization and endosomal localization of QDs with reasonable luminescence intensity were observed according to the confocal images proving their potential as an optical probe. Afterwards, both cell lines were treated with AS-GSH QDs to monitor their morphology under the light microscope after 24 and 48 h (Figure 6-6 a, b). AS-GSH QDs exhibited no change in cell morphology indicating the nanoparticles' biocompatibility.

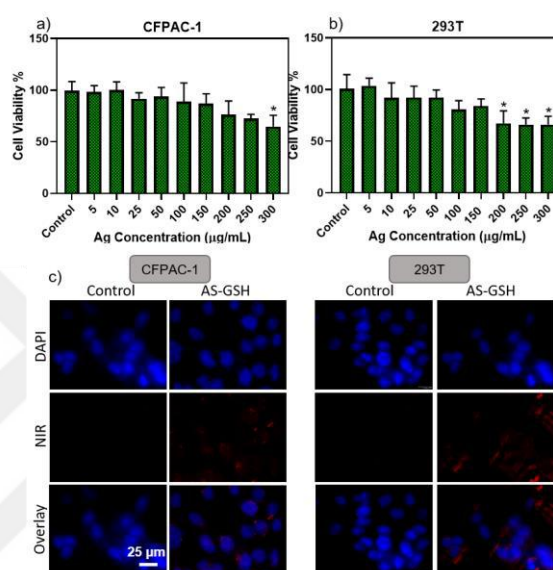


Figure 6–5: Viability of (a) CFPAC-1 and (b) 293T cells treated with AS-GSH QDs at Ag concentration of 5-300 µg/mL after 24 h incubation measured by MTT assay ($p < 0.05$). (c) Fluorescence microscopy images of control and cellular localization of CFPAC-1 and 293T treated with 100 µg Ag/ml after 24 h.

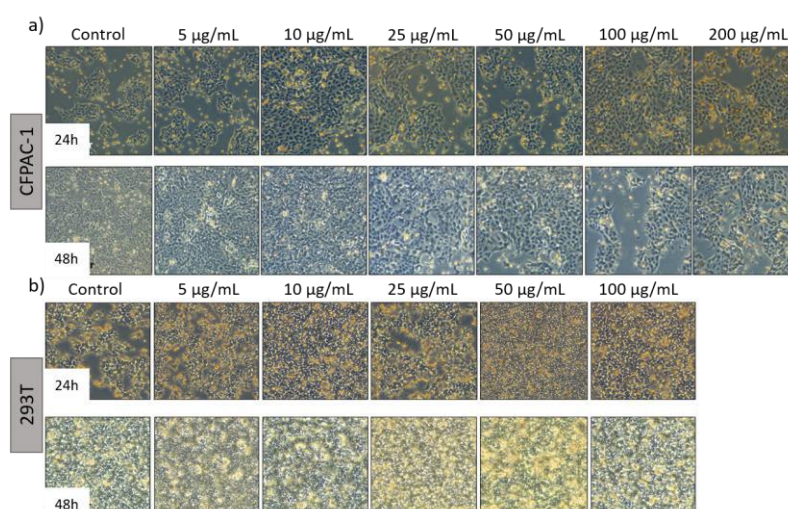


Figure 6–6: Light microscopy images of (a) CFPAC-1 and (b) 293T cells treated with of AS-GSH QD QDs after 24 and 48 h.

Toxic QDs are known by their contribution to the elevated levels of oxidative stress in

the biological environments resulting in the toxicity and even cell death [299], [21], [300]. Furthermore, oxidative stress is considered as the major modulator when biological system exposed to the nanomaterials [301]. Oxidative stress is described by the imbalance between the antioxidant defense and reactive oxygen species (ROS). Lower levels of oxidative stress can induce detoxification via antioxidant enzymes, however higher levels of it cause cytotoxicity, inflammation, tissue damage, and apoptosis [302]. Thus, in this study glutathione-dependent and independent antioxidant enzyme activities (G6PD, 6-PGD, GR, and GST) were investigated. G6PD and 6PGD enzymes are responsible in the production of NADPH, which is a reducing molecule for the anabolic processes active in the elimination of the oxidative radicals. Furthermore, NADPH is vital for the cytochrome p450 systems responsible for the detoxification of xenobiotics including drugs, nanoparticles, and chemotherapeutics. Also, $\text{NADPH} + \text{H}^+$ is required for the reduction of the GSSG into the GSH, which is processed by GR and GST enzymes [302], [21]. G6PD, 6-PGD, GR, and GST enzyme activities significantly increased upon AS-GSH QDs treatment. However, these changes did not result from the oxidative stress, since AS-GSH QDs can be used as substrates of the GST and GR enzymes, that results in the increased concentration of NADP which is a substrate for G6PD (Figure 6-7a, b) [149], [73]. Increase in the G6PD and 6PGD enzymes result in the production of NADPH which is necessary for the GR enzyme activity. Also, GR enzyme is responsible for the reduction of the GSSG into the GSH which is one of the substrates of the GST enzyme. Thus, changes in the G6PD and/or 6PGD enzymes are correlated with the fluctuations in the GR and GST activities. Moreover, since AS-GSH QDs can be used as GR and GST substrate analogue, increase in both enzyme activities can be related with the treatment [149], [73], [303]. Overall, there was no dramatic loss in the antioxidant enzyme activity of 293T and CFPAC-1 cells using AS-GSH QDs indicating their biocompatibility.

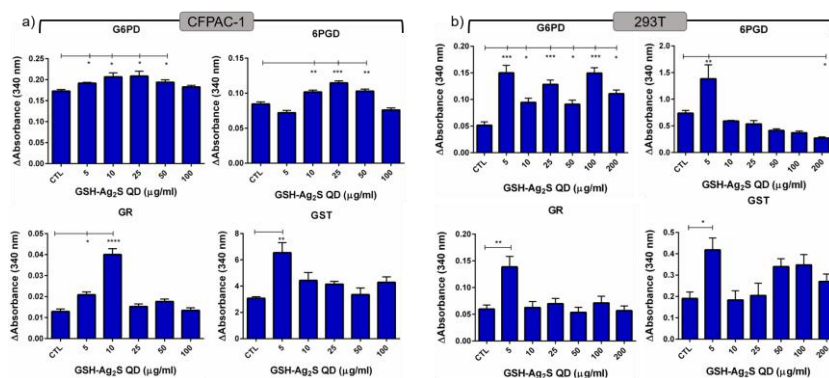


Figure 6–7: Antioxidant enzyme activities in the control and AS-GSH treated (a) CFPAC-1 and

(b) 293T cell lines. All groups are compared to control group and each other (n=6).

Another important point for nano-toxicological research is the investigation of the releasing toxic elements from nanoparticles into the biological microenvironment. Therefore, the level of trace element and mineral were determined in CFPAC-1 and 293T cells treated with AS-GSH QDs using ICP-MS. Ion signaling including K, Na, Ca, and Mg is vital for the cellular metabolism via crosstalk between protein, ion- and small molecule signaling [304]. Mg is essential for both G6PD and 6PGD activities and its concentration was fluctuated upon QD treatment in both cell lines (Figure 6-8a, b). This data is consistent with the G6PD and 6PGD activities, where increased levels of both enzymes resulted in the decreased concentrations of the Mg in both cell lines (Figure 6-8). Unlike highly toxic Ag^+ ions, AS QDs are quite non-toxic due to their ultraslow solubility product constant ($k_{sp} = 6.3 \times 10^{-50}$). Ag concentration was not detected in either cell lines after QD treatment which indicates no Ag release into the cells from the QDs supporting non-toxic nature of these QDs. Intracellular and extracellular K are responsible for the regulation of the physiological properties of the cells [305]. Furthermore, K regulates membrane potential of organelles and ion homeostasis that further impacts intracellular Ca concentrations [306], [305], [307]. Figure 6-9 shows that K and Ca concentrations fluctuated upon AS-GSH QDs that can be resulted from balancing cellular membrane potential and Ca signaling.

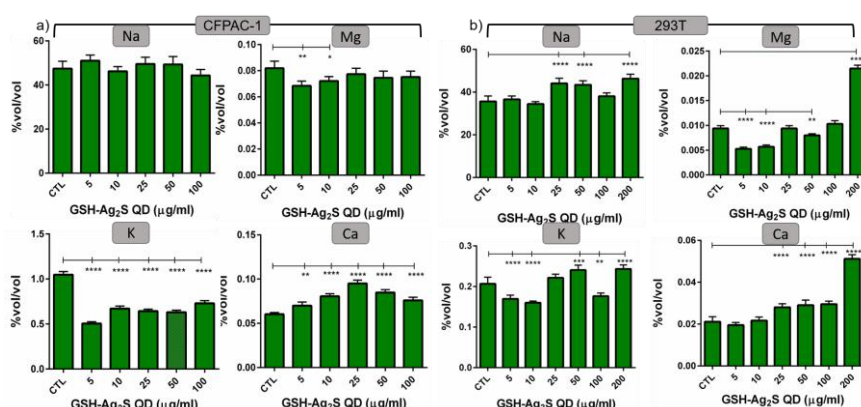


Figure 6–8: Mineral analysis of control and AS-GSH treated (a) CFPAC-1 and (b) 293T cell lines. All groups are compared to control group and each other.

Taken together AS-GSH QDs exhibited no dramatic loss in the cell viability and antioxidant enzyme activity which support its biocompatibility. Thus, AS-GSH QDs can be used for bio-detection of enzymes and/or optical imaging and/or for targeted therapy in the future.

6.2 Publications and Conferences

Publications

- **M. Hashemkhani**, A. Muti, A. Sennaroglu, H. Y. Acar, “Multomodal image-guided folic acid targeted quantum dots for photothermal therapy and selective metotheraxate delivery” *J. of Photochemistry and Photobiology b: Biology*, November 2020
- **M. Hashemkhani**, K. Bilici, A. Muti, A. Sennaroglu, H. Y. Acar, “Ag₂S-Glutathione quantum dots for NIR image guided photothermal therapy,” *New Journal of Chemistry*, March 2020.
- D. Aydemir, **M. Hashemkhani**, H. Y. Acar, N. N. Uluşu, “Evaluation of the biocompatibility of the GSH-coated Ag₂S quantum dots *in vitro*: A perfect example for the non-toxic optical probes”, *Molecular Biology Reports*, May 2020.
- P. L. Labib, E. Yaghini, **M. Hashemkhani**, B. R. Davidson, A. J. MacRobert, M. Loizidou, H. Y. Acar, S. P. Pereira, “Targeting of epidermal growth factor (EGFR)-positive pancreatic cancer cell lines with cetuximab-conjugated near-infrared silver sulfide quantum dots”, *17th International Photodynamic Association World Congress*, August 2019.
- D. Aydemir, **M. Hashemkhani**, H. Y. Acar, N. N. Uluşu, “*In vitro* interaction of glutathione S-transferase-pi enzyme with glutathione-coated silver sulfide quantum dots: A novel method for biodetection of glutathione S-transferase enzyme,” *Chem. Biol. Drug Des.*, no. June, pp. 2094–2102, 2019.
- D. Aydemir, **M. Hashemkhani**, E. G. Durmusoglu, H. Y. Acar, N. N. Uluşu, “A new substrate for glutathione reductase: Glutathione coated Ag₂S quantum dots,” *Talanta*, vol. 194, 2019.
- **M. Hashemkhani**, M. Loizidou, A. J. MacRobert, H. Y. Acar, “Development of anionic and cationic AgInS₂ quantum dots for fluorescence imaging and ALA delivery for effective photodynamic therapy”, *Journal of Materials Chemistry B* (Under revision)
- **M. Hashemkhani**, G. Demirci, A. Muti, A. Sennaroglu, Layla Mohammad-Hadi, Elnaz Yaghini, M. Loizidou, A. J. MacRobert, H. Y. Acar, “Development of

targeted near infrared Ag₂S quantum dots for optical imaging and targeted photodynamic therapy”, *Nanoscale (Submitted)*

Conferences

- Multimodal image-guided folic acid targeted Ag₂S quantum dots for photothermal therapy and selective methotrexate delivery, International Conference on Nanomedicine and Nanobiotechnology (ICONAN 2019), Munich, Germany, October 2019; *Oral presentation*
- Development of targeted near-infrared Ag₂S-2MPA quantum dots for optical imaging and photodynamic therapy, 17th International Conference of Photobiology (Light and Life), Barcelona, Spain, August 2019; *Oral presentation*
- Development of Glutathione-Ag₂S near-infrared emitting quantum dots for optical imaging and photothermal therapy, International Conference on Nanomedicine and Nanobiotechnology 2018 (ICONAN 2018), Rome, Italy, September 2018; *Oral presentation*
- Development of Glutathione-Ag₂S near-infrared emitting quantum dots as an optical imaging agent, 13th Nanoscience and nanotechnology Conference (NanoTR13), Antalya, Turkey, September 2017; *Poster presentation*

APPENDIX A

Calculation of particle size for Ag₂S QDs using Brus equation

To calculate the size of the QDs, their band gap is calculated based on the absorption edge from the absorbance spectrum (Figure A-1).

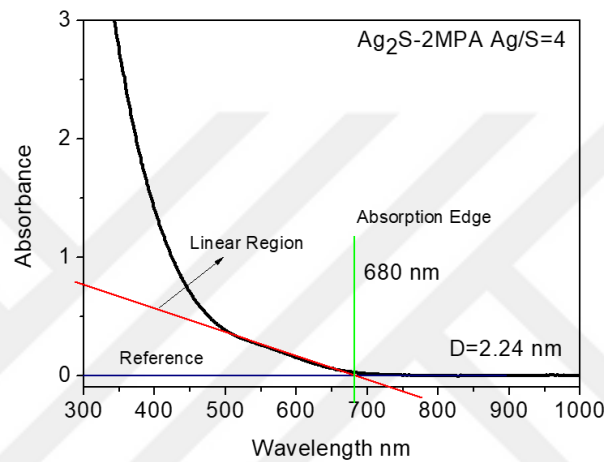


Figure A-1: Absorbance spectrum of Ag₂S QDs for determination of crystal band gap.

A reference line is drawn where the absorption is zero and a tangent line is drawn to the excitonic peak. The intersection of these two lines represents the absorption edge which corresponds to the band gap of QD. Then, the size of the QD can be calculated using Brus equation (equation 2-1):

$$\Delta E = \frac{\hbar^2 \pi^2}{8R^2} \left[\frac{1}{m_e} + \frac{1}{m_h} \right] - 1.8 \frac{e^2}{\epsilon_{Ag_2S} 4\pi \epsilon_0 R}$$

m_e (0.286 m_0) and m_h (1.096) are the respective effective electron and hole masses for Ag₂S. ϵ_{Ag_2S} (5.95) is the dielectric constant. Band gap energy of bulk ($E_g(\text{Bulk})$) Ag₂S is 0.9 eV. Calculated particle sizes are listed on Table A-1 for different absorption wavelengths and corresponding band gap energies.

Table A-1: Calculated particle size of Ag₂S QDs using Brus equation.

Wavelength (nm)	Diameter (nm)	Bandgap (eV)
340	1.4032	3.6517
341	1.4056	3.641
342	1.4081	3.6303

Appendix A. Calculation of particle size for Ag₂S QDs using Brus equation

343	1.4105	3.6197
344	1.413	3.6092
345	1.4154	3.5988
346	1.4179	3.5884
347	1.4204	3.578
348	1.4228	3.5677
349	1.4253	3.5575
350	1.4277	3.5474
351	1.4302	3.5372
352	1.4326	3.5272
353	1.4351	3.5172
354	1.4375	3.5073
355	1.44	3.4974
356	1.4424	3.4876
357	1.4448	3.4778
358	1.4473	3.4681
359	1.4497	3.4584
360	1.4522	3.4488
361	1.4546	3.4393
362	1.4571	3.4298
363	1.4595	3.4203
364	1.4619	3.4109
365	1.4644	3.4016
366	1.4668	3.3923
367	1.4692	3.383
368	1.4717	3.3738
369	1.4741	3.3647
370	1.4766	3.3556
371	1.479	3.3466
372	1.4814	3.3376
373	1.4839	3.3286
374	1.4863	3.3197
375	1.4887	3.3109
376	1.4911	3.3021
377	1.4936	3.2933
378	1.496	3.2846
379	1.4984	3.2759
380	1.5009	3.2673
381	1.5033	3.2587
382	1.5057	3.2502
383	1.5081	3.2417
384	1.5106	3.2333
385	1.513	3.2249
386	1.5154	3.2165
387	1.5178	3.2082

Appendix A. Calculation of particle size for Ag₂S QDs using Brus equation

388	1.5203	3.1999
389	1.5227	3.1917
390	1.5251	3.1835
391	1.5275	3.1754
392	1.53	3.1673
393	1.5324	3.1592
394	1.5348	3.1512
395	1.5372	3.1432
396	1.5396	3.1353
397	1.5421	3.1274
398	1.5445	3.1195
399	1.5469	3.1117
400	1.5493	3.1039
401	1.5517	3.0962
402	1.5542	3.0885
403	1.5566	3.0808
404	1.559	3.0732
405	1.5614	3.0656
406	1.5638	3.0581
407	1.5663	3.0505
408	1.5687	3.0431
409	1.5711	3.0356
410	1.5735	3.0282
411	1.5759	3.0209
412	1.5783	3.0135
413	1.5807	3.0062
414	1.5832	2.999
415	1.5856	2.9917
416	1.588	2.9846
417	1.5904	2.9774
418	1.5928	2.9703
419	1.5952	2.9632
420	1.5977	2.9561
421	1.6001	2.9491
422	1.6025	2.9421
423	1.6049	2.9352
424	1.6073	2.9282
425	1.6097	2.9213
426	1.6121	2.9145
427	1.6145	2.9077
428	1.617	2.9009
429	1.6194	2.8941
430	1.6218	2.8874
431	1.6242	2.8807
432	1.6266	2.874

Appendix A. Calculation of particle size for Ag₂S QDs using Brus equation

433	1.629	2.8674
434	1.6314	2.8608
435	1.6339	2.8542
436	1.6363	2.8476
437	1.6387	2.8411
438	1.6411	2.8346
439	1.6435	2.8282
440	1.6459	2.8218
441	1.6483	2.8154
442	1.6508	2.809
443	1.6532	2.8026
444	1.6556	2.7963
445	1.658	2.7901
446	1.6604	2.7838
447	1.6628	2.7776
448	1.6652	2.7714
449	1.6677	2.7652
450	1.6701	2.7591
451	1.6725	2.7529
452	1.6749	2.7468
453	1.6773	2.7408
454	1.6797	2.7347
455	1.6821	2.7287
456	1.6846	2.7227
457	1.687	2.7168
458	1.6894	2.7109
459	1.6918	2.705
460	1.6942	2.6991
461	1.6967	2.6932
462	1.6991	2.6874
463	1.7015	2.6816
464	1.7039	2.6758
465	1.7063	2.67
466	1.7087	2.6643
467	1.7112	2.6586
468	1.7136	2.6529
469	1.716	2.6473
470	1.7184	2.6416
471	1.7208	2.636
472	1.7233	2.6305
473	1.7257	2.6249
474	1.7281	2.6194
475	1.7305	2.6138
476	1.733	2.6083
477	1.7354	2.6029

Appendix A. Calculation of particle size for Ag₂S QDs using Brus equation

478	1.7378	2.5974
479	1.7402	2.592
480	1.7426	2.5866
481	1.7451	2.5812
482	1.7475	2.5759
483	1.7499	2.5705
484	1.7523	2.5652
485	1.7548	2.5599
486	1.7572	2.5547
487	1.7596	2.5494
488	1.7621	2.5442
489	1.7645	2.539
490	1.7669	2.5338
491	1.7693	2.5287
492	1.7718	2.5235
493	1.7742	2.5184
494	1.7766	2.5133
495	1.7791	2.5082
496	1.7815	2.5032
497	1.7839	2.4981
498	1.7864	2.4931
499	1.7888	2.4881
500	1.7912	2.4831
501	1.7937	2.4782
502	1.7961	2.4733
503	1.7985	2.4683
504	1.801	2.4634
505	1.8034	2.4586
506	1.8059	2.4537
507	1.8083	2.4489
508	1.8107	2.444
509	1.8132	2.4392
510	1.8156	2.4345
511	1.8181	2.4297
512	1.8205	2.4249
513	1.823	2.4202
514	1.8254	2.4155
515	1.8278	2.4108
516	1.8303	2.4061
517	1.8327	2.4015
518	1.8352	2.3969
519	1.8376	2.3922
520	1.8401	2.3876
521	1.8425	2.3831
522	1.845	2.3785

Appendix A. Calculation of particle size for Ag₂S QDs using Brus equation

523	1.8474	2.3739
524	1.8499	2.3694
525	1.8523	2.3649
526	1.8548	2.3604
527	1.8573	2.3559
528	1.8597	2.3515
529	1.8622	2.347
530	1.8646	2.3426
531	1.8671	2.3382
532	1.8695	2.3338
533	1.872	2.3294
534	1.8745	2.325
535	1.8769	2.3207
536	1.8794	2.3164
537	1.8819	2.3121
538	1.8843	2.3078
539	1.8868	2.3035
540	1.8893	2.2992
541	1.8917	2.295
542	1.8942	2.2907
543	1.8967	2.2865
544	1.8991	2.2823
545	1.9016	2.2781
546	1.9041	2.2739
547	1.9066	2.2698
548	1.909	2.2656
549	1.9115	2.2615
550	1.914	2.2574
551	1.9165	2.2533
552	1.9189	2.2492
553	1.9214	2.2452
554	1.9239	2.2411
555	1.9264	2.2371
556	1.9289	2.233
557	1.9314	2.229
558	1.9338	2.225
559	1.9363	2.2211
560	1.9388	2.2171
561	1.9413	2.2131
562	1.9438	2.2092
563	1.9463	2.2053
564	1.9488	2.2014
565	1.9513	2.1975
566	1.9538	2.1936
567	1.9563	2.1897

Appendix A. Calculation of particle size for Ag₂S QDs using Brus equation

568	1.9588	2.1859
569	1.9613	2.182
570	1.9638	2.1782
571	1.9663	2.1744
572	1.9688	2.1706
573	1.9713	2.1668
574	1.9738	2.163
575	1.9763	2.1593
576	1.9788	2.1555
577	1.9813	2.1518
578	1.9838	2.1481
579	1.9863	2.1443
580	1.9889	2.1406
581	1.9914	2.137
582	1.9939	2.1333
583	1.9964	2.1296
584	1.9989	2.126
585	2.0014	2.1223
586	2.004	2.1187
587	2.0065	2.1151
588	2.009	2.1115
589	2.0115	2.1079
590	2.0141	2.1044
591	2.0166	2.1008
592	2.0191	2.0973
593	2.0217	2.0937
594	2.0242	2.0902
595	2.0267	2.0867
596	2.0293	2.0832
597	2.0318	2.0797
598	2.0343	2.0762
599	2.0369	2.0727
600	2.0394	2.0693
601	2.042	2.0658
602	2.0445	2.0624
603	2.0471	2.059
604	2.0496	2.0556
605	2.0522	2.0522
606	2.0547	2.0488
607	2.0573	2.0454
608	2.0598	2.0421
609	2.0624	2.0387
610	2.0649	2.0354
611	2.0675	2.032
612	2.0701	2.0287

Appendix A. Calculation of particle size for Ag₂S QDs using Brus equation

613	2.0726	2.0254
614	2.0752	2.0221
615	2.0778	2.0188
616	2.0803	2.0155
617	2.0829	2.0123
618	2.0855	2.009
619	2.088	2.0058
620	2.0906	2.0025
621	2.0932	1.9993
622	2.0958	1.9961
623	2.0983	1.9929
624	2.1009	1.9897
625	2.1035	1.9865
626	2.1061	1.9833
627	2.1087	1.9802
628	2.1113	1.977
629	2.1139	1.9739
630	2.1165	1.9708
631	2.119	1.9676
632	2.1216	1.9645
633	2.1242	1.9614
634	2.1268	1.9583
635	2.1294	1.9552
636	2.132	1.9522
637	2.1346	1.9491
638	2.1373	1.946
639	2.1399	1.943
640	2.1425	1.94
641	2.1451	1.9369
642	2.1477	1.9339
643	2.1503	1.9309
644	2.1529	1.9279
645	2.1556	1.9249
646	2.1582	1.9219
647	2.1608	1.919
648	2.1634	1.916
649	2.1661	1.9131
650	2.1687	1.9101
651	2.1713	1.9072
652	2.174	1.9043
653	2.1766	1.9013
654	2.1792	1.8984
655	2.1819	1.8955
656	2.1845	1.8926
657	2.1872	1.8898

Appendix A. Calculation of particle size for Ag₂S QDs using Brus equation

658	2.1898	1.8869
659	2.1925	1.884
660	2.1951	1.8812
661	2.1978	1.8783
662	2.2004	1.8755
663	2.2031	1.8727
664	2.2057	1.8698
665	2.2084	1.867
666	2.2111	1.8642
667	2.2137	1.8614
668	2.2164	1.8586
669	2.2191	1.8559
670	2.2217	1.8531
671	2.2244	1.8503
672	2.2271	1.8476
673	2.2298	1.8448
674	2.2324	1.8421
675	2.2351	1.8394
676	2.2378	1.8366
677	2.2405	1.8339
678	2.2432	1.8312
679	2.2459	1.8285
680	2.2486	1.8258
681	2.2513	1.8232
682	2.254	1.8205
683	2.2567	1.8178
684	2.2594	1.8152
685	2.2621	1.8125
686	2.2648	1.8099
687	2.2675	1.8072
688	2.2702	1.8046
689	2.2729	1.802
690	2.2757	1.7994
691	2.2784	1.7968
692	2.2811	1.7942
693	2.2838	1.7916
694	2.2866	1.789
695	2.2893	1.7864
696	2.292	1.7839
697	2.2948	1.7813
698	2.2975	1.7788
699	2.3002	1.7762
700	2.303	1.7737
701	2.3057	1.7711
702	2.3085	1.7686

Appendix A. Calculation of particle size for Ag₂S QDs using Brus equation

703	2.3112	1.7661
704	2.314	1.7636
705	2.3167	1.7611
706	2.3195	1.7586
707	2.3223	1.7561
708	2.325	1.7536
709	2.3278	1.7512
710	2.3306	1.7487
711	2.3333	1.7462
712	2.3361	1.7438
713	2.3389	1.7413
714	2.3417	1.7389
715	2.3445	1.7365
716	2.3473	1.734
717	2.35	1.7316
718	2.3528	1.7292
719	2.3556	1.7268
720	2.3584	1.7244
721	2.3612	1.722
722	2.364	1.7196
723	2.3668	1.7173
724	2.3696	1.7149
725	2.3725	1.7125
726	2.3753	1.7102
727	2.3781	1.7078
728	2.3809	1.7055
729	2.3837	1.7031
730	2.3866	1.7008
731	2.3894	1.6985
732	2.3922	1.6961
733	2.395	1.6938
734	2.3979	1.6915
735	2.4007	1.6892
736	2.4036	1.6869
737	2.4064	1.6846
738	2.4093	1.6823
739	2.4121	1.6801
740	2.415	1.6778
741	2.4178	1.6755
742	2.4207	1.6733
743	2.4236	1.671
744	2.4264	1.6688
745	2.4293	1.6665
746	2.4322	1.6643
747	2.4351	1.6621

Appendix A. Calculation of particle size for Ag₂S QDs using Brus equation

748	2.4379	1.6599
749	2.4408	1.6576
750	2.4437	1.6554
751	2.4466	1.6532
752	2.4495	1.651
753	2.4524	1.6488
754	2.4553	1.6466
755	2.4582	1.6445
756	2.4611	1.6423
757	2.464	1.6401
758	2.4669	1.638
759	2.4698	1.6358
760	2.4727	1.6336
761	2.4757	1.6315
762	2.4786	1.6294
763	2.4815	1.6272
764	2.4845	1.6251
765	2.4874	1.623
766	2.4903	1.6209
767	2.4933	1.6187
768	2.4962	1.6166
769	2.4992	1.6145
770	2.5021	1.6124
771	2.5051	1.6103
772	2.508	1.6083
773	2.511	1.6062
774	2.514	1.6041
775	2.5169	1.602
776	2.5199	1.6
777	2.5229	1.5979
778	2.5259	1.5959
779	2.5289	1.5938
780	2.5319	1.5918
781	2.5349	1.5897
782	2.5378	1.5877
783	2.5408	1.5857
784	2.5439	1.5836
785	2.5469	1.5816
786	2.5499	1.5796
787	2.5529	1.5776
788	2.5559	1.5756
789	2.5589	1.5736
790	2.5619	1.5716
791	2.565	1.5696
792	2.568	1.5676

Appendix A. Calculation of particle size for Ag₂S QDs using Brus equation

793	2.5711	1.5657
794	2.5741	1.5637
795	2.5771	1.5617
796	2.5802	1.5598
797	2.5832	1.5578
798	2.5863	1.5559
799	2.5894	1.5539
800	2.5924	1.552
801	2.5955	1.55
802	2.5986	1.5481
803	2.6016	1.5462
804	2.6047	1.5442
805	2.6078	1.5423
806	2.6109	1.5404
807	2.614	1.5385
808	2.6171	1.5366
809	2.6202	1.5347
810	2.6233	1.5328
811	2.6264	1.5309
812	2.6295	1.529
813	2.6326	1.5272
814	2.6357	1.5253
815	2.6389	1.5234
816	2.642	1.5215
817	2.6451	1.5197
818	2.6483	1.5178
819	2.6514	1.516
820	2.6546	1.5141
821	2.6577	1.5123
822	2.6609	1.5104
823	2.664	1.5086
824	2.6672	1.5068
825	2.6704	1.5049
826	2.6735	1.5031
827	2.6767	1.5013
828	2.6799	1.4995
829	2.6831	1.4977
830	2.6863	1.4959
831	2.6895	1.4941
832	2.6927	1.4923
833	2.6959	1.4905
834	2.6991	1.4887
835	2.7023	1.4869
836	2.7055	1.4851
837	2.7087	1.4834

Appendix A. Calculation of particle size for Ag₂S QDs using Brus equation

838	2.712	1.4816
839	2.7152	1.4798
840	2.7184	1.4781
841	2.7217	1.4763
842	2.7249	1.4746
843	2.7282	1.4728
844	2.7314	1.4711
845	2.7347	1.4693
846	2.738	1.4676
847	2.7412	1.4658
848	2.7445	1.4641
849	2.7478	1.4624
850	2.7511	1.4607
851	2.7544	1.459
852	2.7577	1.4572
853	2.761	1.4555
854	2.7643	1.4538
855	2.7676	1.4521
856	2.7709	1.4504
857	2.7742	1.4487
858	2.7775	1.4471
859	2.7809	1.4454
860	2.7842	1.4437
861	2.7875	1.442
862	2.7909	1.4403
863	2.7942	1.4387
864	2.7976	1.437
865	2.801	1.4353
866	2.8043	1.4337
867	2.8077	1.432
868	2.8111	1.4304
869	2.8145	1.4287
870	2.8178	1.4271
871	2.8212	1.4255
872	2.8246	1.4238
873	2.828	1.4222
874	2.8314	1.4206
875	2.8349	1.4189
876	2.8383	1.4173
877	2.8417	1.4157
878	2.8451	1.4141
879	2.8486	1.4125
880	2.852	1.4109
881	2.8555	1.4093
882	2.8589	1.4077

Appendix A. Calculation of particle size for Ag₂S QDs using Brus equation

883	2.8624	1.4061
884	2.8658	1.4045
885	2.8693	1.4029
886	2.8728	1.4013
887	2.8763	1.3997
888	2.8798	1.3982
889	2.8833	1.3966
890	2.8868	1.395
891	2.8903	1.3935
892	2.8938	1.3919
893	2.8973	1.3903
894	2.9008	1.3888
895	2.9043	1.3872
896	2.9079	1.3857
897	2.9114	1.3841
898	2.915	1.3826
899	2.9185	1.3811
900	2.9221	1.3795
901	2.9256	1.378
902	2.9292	1.3765
903	2.9328	1.3749
904	2.9364	1.3734
905	2.94	1.3719
906	2.9436	1.3704
907	2.9472	1.3689
908	2.9508	1.3674
909	2.9544	1.3659
910	2.958	1.3644
911	2.9616	1.3629
912	2.9653	1.3614
913	2.9689	1.3599
914	2.9726	1.3584
915	2.9762	1.3569
916	2.9799	1.3554
917	2.9836	1.354
918	2.9872	1.3525
919	2.9909	1.351
920	2.9946	1.3495
921	2.9983	1.3481
922	3.002	1.3466
923	3.0057	1.3451
924	3.0094	1.3437
925	3.0131	1.3422
926	3.0169	1.3408
927	3.0206	1.3393

Appendix A. Calculation of particle size for Ag₂S QDs using Brus equation

928	3.0243	1.3379
929	3.0281	1.3365
930	3.0319	1.335
931	3.0356	1.3336
932	3.0394	1.3322
933	3.0432	1.3307
934	3.047	1.3293
935	3.0507	1.3279
936	3.0545	1.3265
937	3.0584	1.3251
938	3.0622	1.3236
939	3.066	1.3222
940	3.0698	1.3208
941	3.0737	1.3194
942	3.0775	1.318
943	3.0814	1.3166
944	3.0852	1.3152
945	3.0891	1.3138
946	3.093	1.3124
947	3.0968	1.3111
948	3.1007	1.3097
949	3.1046	1.3083
950	3.1085	1.3069
951	3.1124	1.3055
952	3.1164	1.3042
953	3.1203	1.3028
954	3.1242	1.3014
955	3.1282	1.3001
956	3.1321	1.2987
957	3.1361	1.2974
958	3.14	1.296
959	3.144	1.2947
960	3.148	1.2933
961	3.152	1.292
962	3.156	1.2906
963	3.16	1.2893
964	3.164	1.2879
965	3.1681	1.2866
966	3.1721	1.2853
967	3.1761	1.2839
968	3.1802	1.2826
969	3.1842	1.2813
970	3.1883	1.28
971	3.1924	1.2787
972	3.1965	1.2773

Appendix A. Calculation of particle size for Ag₂S QDs using Brus equation

973	3.2006	1.276
974	3.2047	1.2747
975	3.2088	1.2734
976	3.2129	1.2721
977	3.217	1.2708
978	3.2212	1.2695
979	3.2253	1.2682
980	3.2295	1.2669
981	3.2336	1.2656
982	3.2378	1.2643
983	3.242	1.263
984	3.2462	1.2618
985	3.2504	1.2605
986	3.2546	1.2592
987	3.2588	1.2579
988	3.263	1.2567
989	3.2673	1.2554
990	3.2715	1.2541

APPENDIX B

Calculation of band gap and Urbach energy in ternary AIS QDs

The electron and hole effective masses are rarely reported for AIS compounds and even if they are reported the values for tetragonal structure ($m_e=0.15m_0$, $m_h=1.36m_0$) differs from orthorhombic one ($m_e=0.15m_0$, $m_h=1.9m_0$) [308]. Thus, calculation of the band gap of AIS QDs using Brus equation does not provide a good correspondence to the experimental data. To determine the optical band gap in semiconductor AIS QDs, Tauc plot is mostly utilized. In general, Tauc plot shows the energy of the light ($h\nu$) versus $(\alpha h\nu)^{1/r}$. Where α is the absorption coefficient and r denote the nature of the transition:

$r = 1/2$ for direct allowed transitions

$r = 3/2$ for direct forbidden transitions

$r = 2$ for indirect allowed transitions

$r = 3$ for indirect forbidden transitions

The intercept of the linear region of the plot corresponds to the optical band gap.

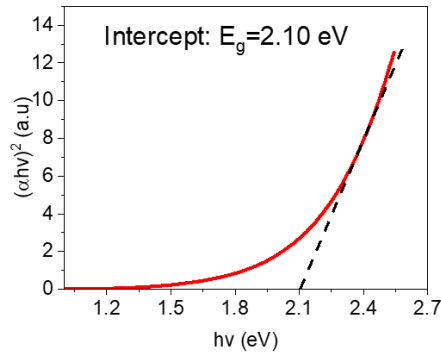


Figure B-1: The Tauc plot the AIS QDs.

Although, direct transition of AIS QDs is quite tricky due to the presence of disorders near the absorption band edge, r in the case of AIS QDs assumed to be $1/2$ [308], [309].

To differentiate the fundamental absorption caused from the tail absorption “Urbach energy” was defined. Band tail states are localized between VB and CB as a consequence of disorders, e.g., thermal, impurities, structural, and/or compositional disorders [310].

The Urbach energy was assumed as the width of the tail of localized defect states within the band gap that was defined as an exponential distribution. When AIS QDs are irradiated with light of a specific wavelength, defect states trap the excited electrons, preventing their direct transition from CB and consequently, creating the band tail in the absorption spectra. This absorption tail is called Urbach tail and is associated with the Urbach energy. The Urbach energy can be calculated according to Equation B-1 [310]:

$$\alpha = \alpha_0 + \exp\left(\frac{E}{E_u}\right) \quad \text{Equation B-1}$$

Where the Urbach energy can be calculated by plotting $\ln(\alpha)$ (α is proportional to absorbance of F(R)) versus E . The reciprocal of the slope in the linear fit region gives the Urbach energy.

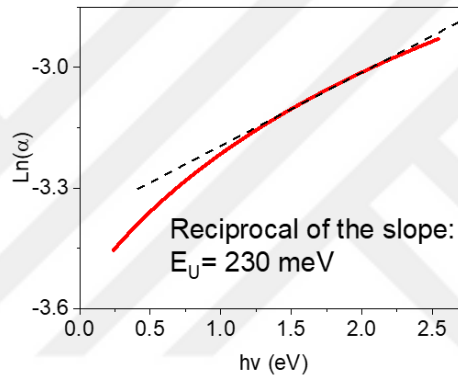


Figure B-2: The Urbach energy of AIS QDs.

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