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ACIBADEM MEHMET ALİ AYDINLAR UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES

**DESIGN OF A VCAM-1 TARGETED DRUG DELIVERY
SYSTEM TO DIMINISH OXIDATIVE STRESS IN
ATHEROSCLEROSIS AND ITS IN VITRO EVALUATIONS**

RABİA GÜNER
M.Sc. THESIS

DEPARTMENT OF BIOMEDICAL ENGINEERING

SUPERVISOR
Asst. Prof. Özgül Gök

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TEZ ONAY SAYFASI



DECLARATION

I declare that this thesis work is my own work, I had no unethical behavior at any stage from the planning to the writing of the thesis, I obtained all the information in this thesis in accordance with academic and ethical rules, and I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

30.05.2023

Rabia Güner

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LIST OF ABBREVIATIONS

11β-HSD1	11 Beta-Hydroxysteroid Dehydrogenase Type 1
ACE	Angiotensin-Converting Enzyme inhibitors
ACN	Acetonitrile
AE-MA	2-Aminoethyl Methacrylate Hydrochloride
Afbs	Affibodies
ALA	α -Lipoic Acid
AMF	Alternative Magnetic Field
AMMs	Atherosclerotic Molecular Markers
AT1	Angiotensin receptor
CPT	Camptothecin
DCM	Dichloromethane
DDSs	Drug Delivery Systems
DIPA	Diisopropylamine
DMAP	4-(Dimethylamino) Pyridine
DMSO	Dimethyl Sulfoxide
DOX	Doxorubicin
dPGS	Dendritic Polyglycerol Sulfate
DSPE	1, 2-Distearoyl-Sn-Glycero-3-Phosphoethanolamine
EC	Endothelial Cells
EC-SOD	Extracellular Superoxide Dismutase
EMO	Emodin
EPR	Enhanced Permeability and Retention
ER	Estrogen Receptor
FA	Folic Acid
FRs	Folate Receptors
GC	Glucocorticoid
GSH	Glutathione
HER2	Human Epidermal Growth Factor Receptor 2
HOCl	Hypochlorous Acid
HPMA	N- (2-Hydroxypropyl) Methacrylamide

HSA	Human Serum Albumin
HUVEC	Human Umbilical Vein Endothelial Cells
LA3-MA	Tri-Lipoic Acid Methacrylate
LCST	Low Critical Solution Temperature
LDL	Low-Density Lipoprotein
MA	Methyl Ester
MAA	Methacrylic Acid
MAC	Methacryloyl Chloride
MDA	Malondialdehyde
MeOH	Methanol
MET	Metformin
MMP-2	Matrix Metalloproteinases-2
MNPs	Magnetic-responsive Nanoparticles
mPEGA	Methoxy Polyethylene Glycol Acrylate
MSNs	Mesoporous Silica Nanoparticles
NHS	N-Hydroxy Succinimide
NOX	Nitric Oxide
NPs	Nanoparticles
Nrf-2	Nuclear Factor E2-Related Factor-2
OEAM	Ortho Ester Diacrylamide
Ox-LDL	Oxidized Low-Density Lipoprotein
PCL	Poly- ϵ -Caprolactone
PDAC	Pancreatic Ductal Adenocarcinoma
PDCs	Polymer-Drug Conjugates
PEG	Polyethylene Glycol
PEG-DiMA	Poly (Ethylene Glycol) Dimethacrylate
Penta-MA	Pentaerythritol-Methacrylate
PGA	Poly (Glycolic Acid)
PHBV	3-Hydroxybutyric Acid-co-Hydroxy Valeric Acid
PLA	Poly (Lactic Acid)
PLGA	Poly (Lactide-co-Glycolide)
PMMA	Poly (Methyl Methacrylate)

PNPs	Polymeric Nanoparticles
PSMA	Polystyrene Maleic Anhydride
PT	Pentaerythritol Tetra (3-Mercaptopropionate)
PTX	Paclitaxel
RAFT	Reversible Addition Fragmentation Chain Transfer
RC	Regenerated Cellulose
RGD	Arginyl Glycyl Aspartic Acid
ROS	Reactive Oxygen Species
RT	Room Temperature
TBA	Thiobarbituric Acid
TDDSs	Targeted Drug Delivery Systems
TLC	Thin-Layer Chromatography
TMB	3,3',5,5'-Tetramethylbenzidine
VCAM-1	Vascular Adhesion Molecule-1

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SUMMARY

Design of A VCAM-1 Targeted Drug Delivery System to Diminish Oxidative Stress in Atherosclerosis and Its In Vitro Evaluations

Atherosclerosis has been the most significant disease which has over 50% of total mortality in developed countries. It is an age-dependent disease that can start at earlier ages and progresses year by year. The disease can be asymptomatic for many years and generally, for several cases, it shows its first symptom at the advanced stage which can cause critical arterial occlusion (blockage) with some serious effects. Due to the high incidence of those serious outcomes of atherosclerosis and the lack of knowledge of atherosclerotic plaque mechanism, there is a corresponding increase in demand for drug therapies. Particularly drug therapies that are not targeted to damaged sites lead to toxicity and side effects for healthy tissues and organs. Thus, it becomes very obvious that there is a need for targeted therapies that deliver drug molecules only to sites where disease conditions are seen. Within the scope of this thesis, novel antioxidant (α -lipoic acid)-containing drug molecules (LA3-MA) were successfully synthesized and incorporated into PEG-based nanogels. To target the atherosclerotic region, anti-VCAM1 antibodies were conjugated on the surface of the nanogel. It was expected that anti-VCAM1 antibody-bounded nanogels reduce oxidative stress, inflammation, and oxidation of the lipids in atherosclerosis.

Keywords: Targeted Drug Delivery Systems, Atherosclerosis, α -Lipoic Acid, Nanogel, Surface Functionalization, Anti-VCAM1 Antibody, Cy5 Fluorescent Dye

ÖZET

Ateroskleroz, gelişmiş ülkelerde toplam mortalitenin %50'den fazlasına sahip en önemli hastalıklardan biridir. Daha erken yaşlarda başlayabilen ve yıldan yıla ilerleyen yaşa bağlı bir hastalıktır. Hastalık yıllarca asemptomatik olabilir ve genellikle birçok vakada ilk semptomunu ileri evrede gösterir ve bazı ciddi etkilerle birlikte kritik arteriyel oklüzyona (tıkanmaya) neden olabilir. Aterosklerozun bu ciddi sonuçlarının yüksek insidansı ve aterosklerotik plak mekanizması hakkındaki bilgi eksikliği nedeniyle, ilaç tedavilerine olan talepte bir artış vardır. Özellikle hasarlı bölgelere hedeflenmeyen ilaç tedavileri, sağlıklı doku ve organlar için toksisiteye ve yan etkilere yol açmaktadır. Bu nedenle, ilaç moleküllerini yalnızca hastalık durumlarının görüldüğü bölgelere ileten hedefe yönelik tedavilere ihtiyaç olduğu çok açıktır. Bu tez kapsamında, yeni antioksidan (α -lipoik asit) içeren ilaç molekülleri (LA3-MA) başarıyla sentezlendi ve PEG bazlı nanojellere etegre edildi. Aterosklerotik bölgeyi hedeflemek için, nanojelin yüzeyi anti-VCAM1 antikoruyla konjuge edildi. Anti-VCAM1 antikoruyla bağlı nanojellerin, aterosklerozda oksidatif stresi, enflamasyonu ve lipidlerin oksidasyonunu azaltması beklendi.

Anahtar Kelimeler: Hedefli İlaç Taşıyıcı Sistemler, Ateroskleroz, α -Lipoik Asit, Nanojel, Yüzey İşlevselleştirme, Anti-VCAM1 Antikoru, Cy5 Floresan Boya

1. BACKGROUND AND AIM OF THE STUDY

The aim of this thesis is to develop a targeted drug delivery system for the treatment of atherosclerosis. This system was expected to diminish oxidative stress, inflammation, and oxidation of the lipids due to atherosclerosis.

Within the scope of this thesis, for this targeted drug delivery system, (Poly (ethylene glycol) which is a biocompatible and hydrophilic polymer was used mainly to prepare nanoparticles in the form of nanogels. A novel drug-monomer lipoic acid (which is a kind of anti-oxidant) containing drug (LA3-MA) was synthesized and characterized and was covalently bonded by ester bonds to nanogels to be released in a controlled and prolonged fashion. While anti-VCAM1 antibody conjugation was used for targeting to atherosclerotic region, Cy5 fluorescent dye functionalization was used for the visibility of the nanogels for in vitro assays. Furthermore, In vitro 2D inflammation model was developed with the HUVEC cells to mimic an atherosclerotic environment, and the cytotoxicity, IL-6, and MDA assays were performed for testing the effectiveness of prepared anti-VCAM1 and Cy5- bounded nanogels.

By this proposed drug delivery system, drug molecules (lipoic acid molecules) are expected to be accumulated only at the atherosclerotic region on the vessel wall eliminating unwanted side effects and toxicity to healthy tissues and organs, and released in a slow and sustained manner.

2. INTRODUCTION

2.1. Drug Delivery Systems (DDSs)

Drug delivery systems are defined as a drug administration method to the human body for therapeutic purposes. Compared to traditional method which is time-consuming and expensive, recent drug delivery systems have been getting an interest due to their several beneficial properties. For example, drugs can be released in a slow and controlled manner and can be targeted to disease areas (1–3).

2.1.1. EPR Effect

Despite the discovery of thousands of new targeted drug delivery systems, there is still a lack of efficient and selective targeting on the site of solid tumors or inflammation. In order to execute the anticancer or anti-inflammatory activities on the site of tumor or inflammation, there is a certain length of time and effective concentration level for the drugs. The mechanism which is called the enhanced permeability and retention (EPR) effect leads to increased vascular permeability by an accumulation of macromolecular compounds, high molecular weight pro and nontargeted drugs, on the site of cancer or inflammation. Thus, efficient targeting can be achieved by the EPR effect (4).

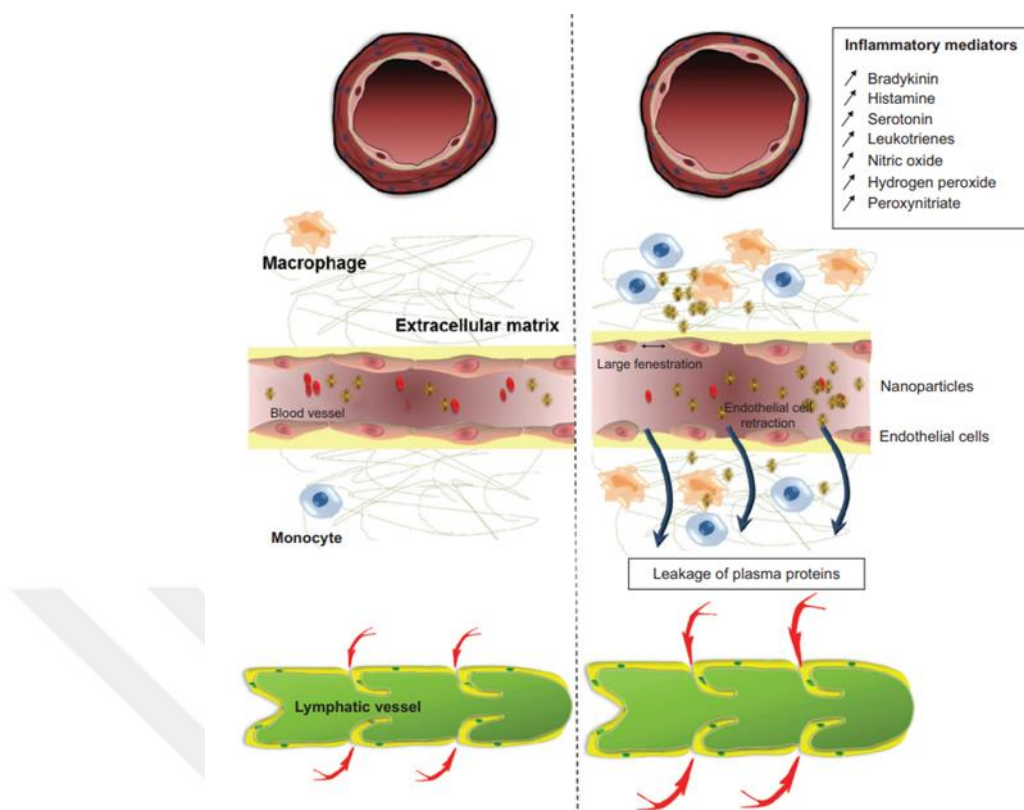


Figure 1. The EPR effect on inflammatory tissue. The release of inflammatory mediators from inflammatory tissue results in an increase in the EPR effect, moreover, dilating blood vessels and increasing blood flow (4).

In solid tumors or cancer, some properties such as spatial configuration, size and surface charge of drugs or drug carriers are significant for EPR effect. In one of the studies, it was found that HPMA copolymers, macromolecular drugs, which are above 40 kDa were accumulated in solid tumors successfully (5). Furthermore, in another study, the EPR effect was found to be the most effective in solid tumors when nanoparticles between 100 and 200 nm in size are used so that they can avoid filtration in liver and spleen (6,7).

In addition to cancer, the EPR effect which induces vascular permeability is also developed in inflammatory conditions by the contraction of the immune cells lined in the capillaries (Figure 1.). The inflammation is induced by the expression of inflammatory mediators which are nitric oxide, hydrogen peroxide, peroxynitrite,

serotonin, bradykinin, leukotrienes and histamine. These inflammatory mediators cause a rise in intra-endothelial gaps or fenestrations. Especially in inflammatory tissue, by these fenestrations, the nanoparticles can be extravasated. In inflammatory conditions, vascular permeability is also induced by the pathogen itself, via secreting some factors. In addition to that, the expression of some inflammatory biomarkers (e.g., reactive oxygen species, ROS) allows some nanomedicines to actively release their encapsulated drugs which are sensitive to these biomarkers (8,9).

2.1.2. Targeted Drug Delivery Systems (TDDSs)

Despite of having several advantages in drug delivery systems (DDSs), there is still a need for key adjustments in some drug delivery systems such as self-regulated insulin delivery, protein delivery, delivery of hardly-soluble drugs and targeted drug delivery systems (10).

Targeted drug delivery to specific site of the tissue or organ can be achieved by nanotechnology-based drug delivery systems. The DDSs that use nanoparticles as a drug delivery agents have several opportunities such as giving drugs to release in a controlled and sustained manner and allowing drugs to have response to specific stimuli (e.g., pH) (11).

TDDSs are aimed to deliver drugs to specific site of the tissue or organ rather than whole organ or body and regulate and manage immunogenicity, non-specific toxicity, pharmacodynamics, pharmacokinetics and the identification of therapeutic agents (12). Furthermore, the final goal in TDDSs is enhancing the efficacy of the treatment while decreasing the side effects. Overall, TDDSs is far different compared to traditional and conventional DDSs. For instance, while in traditional and conventional

DDSs, drugs are absorbed through biological membranes, in TDDSs drugs are released to specific site in a dosage form (13).

The targeting of drugs to specific site of tissue or organ is based on the concept of “magic bullet” which is a century ago, Paul Ehrlich proposed this idea of utilizing "magic bullets" to selectively target a pathogen without damaging the host organism. Ehrlich developed his idea for the "magic bullet" in two stages: first of all, he screened for toxic drugs, then he modified those toxic drugs to make them more targeted and less harmful. By deploying a lethal action is only on the site of a pathogen or disease rather than on the site of host organism's healthy tissue or organ, not only increases affinity, but also results in less toxicity. This magic concept of his further led to discovery of today's “nanomedicine”. However, this success of his concept still a challenge when it is implemented in clinics due to its difficulty in having a stable form while the drug is released on the specific site without any non-specific interaction and immunogenicity, efficacy in targeting specifically on the site of diseased tissue or organ and finding the right and efficient medication to treat the diseases (14,15).

In terms of the need for TDDSs, there are four main constraints in conventional or traditional DDSs which are in pharmacokinetics; the drugs may have a low half-life and high volume of distribution, in pharmacodynamics; they may have low specificity and low therapeutic index, in pharmaceutical; they may have low stability and solubility, while in pharmacotherapeutics; the drugs may have distributed in high doses, have adverse effects. Moreover, patients' poor compliance with drugs can affect medications overall (13).

The key principle of TDDSs is delivering an adequate or higher amount of drug to the targeted site while decreasing or diminishing the drug amount on non-targeted sites of the tissues or organs. Behind this principle, it is aimed to lower the side effects

due to excess amounts of drugs, non-targeted doses and multi-targeting of the drugs while adjusting the optimal therapeutic effect (16).

There are several types of targeted drug delivery systems depending on the targeting: Active and passive, first-, second-, third-, and fourth-order, local and systemic, inverse, dual, double, and combination, location-based and disease-based, physical, chemical, and biological targeting (16). While passive targeting aims for systemic circulation and is a result of physicochemical reactions of drug molecules or drug carriers, active targeting occurs via specific binding elements on drug carriers (17).

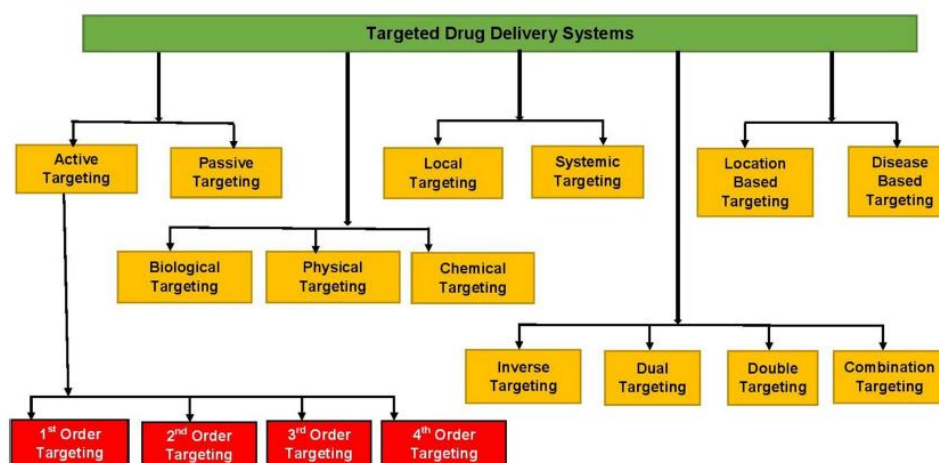


Figure 2. Different kinds of targeted drug delivery systems (13).

2.1.3. Active Targeting

In drug delivery systems, targeting nanocarriers/nanoparticles (NPs) to specific sites of tissues or organs is very important for their success as a drug delivery system. In addition to the physicochemical properties of nanocarriers/nanoparticles, the surface modification of NPs (e.g., modification with biological ligands) is a major targeting element. The surface modifications of NPs can be achieved by binding

biological ligands to specific/targeted receptors that are located on the surface of the specific cells. Moreover, these biological ligands can enhance the uptake of surface-modified NPs which is called “active targeting” of NPs (18).

Numerous types of biological ligands have been studied for active targeting such as polysaccharides, nucleic acids, peptides, proteins and small molecules. In order to enhance binding and cellular uptake, compared to singular ligands, the increase in the density of binding ligands can be more advantageous. In general, biological ligands can be bonded to nanoparticles by either physical adsorption or chemical conjugation or can be directly linked to components in the nanoparticles such as polymers (19,20).

Proteins and polysaccharides compared to other biological active-targeting ligands; antibodies are the most studied ones when it comes to specific-receptor targeting (21). Along with their advantageous properties such as having higher specificity due to their larger size and therapeutic purposes, antibodies exhibit a disadvantage in which their larger size limits the density of antibodies on the surface of nanoparticles (22,23).

One of the engineered proteins which are smaller than antibodies and have high affinity is called Affibodies (Afb) (24). In one of the studies, they functionalized the surface of camptothecin (CPT) loaded mesoporous silica nanoparticles (MSNs) with human epidermal growth factor receptor 2 (HER2) binding affibodies by the corona shield concept. With this concept, the study resulted in colloidal-stabilized NPs that enhanced blood circulation time without any need for conventional polyethylene glycol (PEG) coating. In the cellular-uptake assay, HER2-receptor-overexpressing SK-BR-3 breast cells showed higher uptake compared to control MCF-10A cells. In *in vivo* experiments, in SK-BR-3 tumor-bearing mice, the results showed that compared to the control of PEG-coated NPs, a higher level of Afb-functionalized NPs accumulated in tumor tissue. All in all, in *ex vivo* experiments, it was found that Afb-

functionalized NPs could escape from reticuloendothelial organs and inhibited tumor growth by almost 90% (25).

Compared to other biological ligands, peptides have more benefits such as better stability, having easily conjugated on NPs at a higher density due to their small size and their production cost is lower than other targeting ligands (26). One of the well-known tumor-targeting peptides that bind to integrins, the arginyl glycyl aspartic acid (RGD), is an overexpressed peptide in vascular endothelial cells of tumor tissues (27). The RGD peptide is derived from several cell attachment proteins such as laminin, fibronectin and vitronectin (28). In one of the recent studies, RGD peptide was used as a targeting ligand for size-shrinkable NPs. For deeper penetration into tumor tissue, they developed large-sized gelatin NPs which have doxorubicin (DOX) and metformin (MET) loaded and RGD functionalized small-sized NPs on their surface. While large-sized gelatin NPs can be degraded by matrix metalloproteinases-2 (MMP-2), small-sized NPs which are functionalized with RGD can potentially penetrate deeply into the tumor. As a result, both large-size gelatin NPs and RGD functionalized small-sized NPs enhanced the accumulation in both 4T1 and CT26 tumor tissues (29).

Another potential good candidate for active targeting ligands is aptamers which have some advantages; they are biodegradable, small in size, highly sensitive and have immunogenicity (30). In a study, Adipo8 (Ap) which is a DNA aptamer that has a high affinity to mature white adipocytes was used as an active targeting ligand for obesity treatment. They functionalized the surface of PEG-PLGA NPs with Adipo8 aptamer by EDC/NHS method and used emodin (EMO) as a drug to inhibit 11 beta-hydroxysteroid dehydrogenase type 1 (11β -HSD1) which is a key controlling enzyme for glucocorticoid (GC) activation in adipocytes (31). The excess amount of glucocorticoid (GC) in adipocytes is one of the main factors for obesity (32). It was found that not only Ap-EMO-NPs reduce the expression of 11β -HSD1 but also selectively bind to adipocytes (33).

Folic acid (FA) is well-known and one of the most common biological and active targeting ligands. Most solid tumor cells and macrophages have overexpression of folate receptors, making them eligible for active targeting by a small molecule like “folic acid”. For instance, FA functionalized nanospheres that are made by PEG functionalized poly (3-hydroxybutyric acid-co-hydroxy valeric acid) (PHBV) and inside of nanospheres; there is paclitaxel (PTX) loaded copper oxide (CuO) NPs. According to in vitro experiments with breast cancer cells (e.g., MCF-7 cells), it was found that FA conjugated PHBV modified PTX loaded CuO NPs showed a higher anticancer effect than free PTX (34).

2.2.Polymeric DDSs

Polymeric drug delivery systems have been studied since the 1980s (35). Since then, synthetic and natural polymers have rapidly emerged in pharmaceutical areas. For the polymeric drug delivery systems, bio-absorbable and biodegradable polymers make several drugs to deliver in the intended areas of the body. For example, hydrogels (e.g., bio-absorbable polymer) that are made of poly (glycolic acid), PGA and poly (lactic acid), PLA and their copolymers have been used as delivery systems for drugs due to their ability to hold components of delivery systems (36,37). Whether it is bio-absorbable or biodegradable polymeric drug delivery systems, the polymeric DDS allows safe passage for drugs to be delivered without any damage to the body. Along with its advantages like enhancing the efficacy and safety of the system by controlling the time, rate and position of the drug in the body, there are several disadvantages such as drug molecules having difficulty entering drug molecules into interested parts of the body (e.g., brain). There are several kinds of polymers for drug delivery purposes. One of the natural polymers chitosan is a cationic polymer derived from chitin by deacetylation reactions and has been used widely in drug delivery systems due to its non-toxicity, biodegradability, biocompatibility, and muco-adhesiveness (38).

2.2.1. Polymer Drug Conjugates

One of the polymeric therapeutics in which the drug molecules are not encapsulated into it but chemically (e.g., covalently) linked to the polymer is called polymer-drug conjugates (PDCs). Depending on the strategy of the conjugation of drug molecules to polymers, it drastically affects the polymer-drug conjugation system's behavior. Therefore, it gives some advantages over pristine drugs such as enhancing drug targeting and bioavailability, enabling drug release without any degradation or in a specific kind of environment (e.g., pH or enzyme), improving hydrophilicity for lipophilic drugs, extending plasma half-life and changing biodistribution (39).

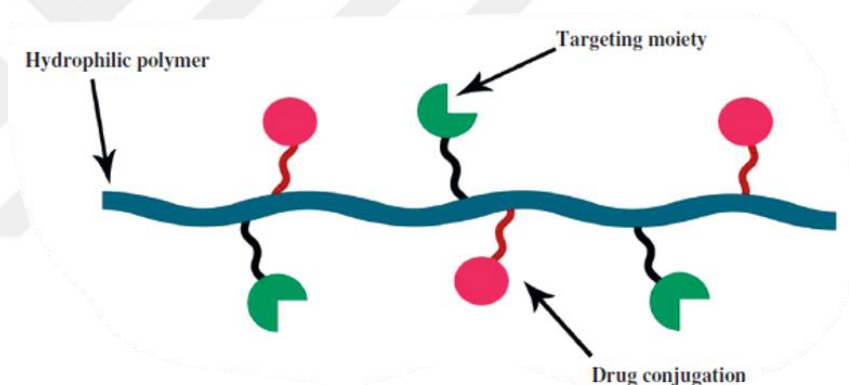


Figure 3. General Scheme of Hydrophilic Polymer-Drug Conjugates (40).

There are several kinds of polymers that are often used in drug-polymer conjugates such as polyglutamic acid (PGA), PEG, and N- (2-hydroxypropyl) methacrylamide (HPMA). One of the most popular strategies for polymer-drug conjugates is PEGylation which demonstrates PEG polymer conjugation to therapeutics (e.g., proteins). There are some benefits in PEG-protein conjugations; the degradation of proteins by enzymes is less likely to happen and the uptake of PEG-protein conjugates by the reticuloendothelial system is decreased (41). In the first generation of polymer-drug conjugates, the most significant ones are camptothecin conjugates (CT-2106), PGA-paclitaxel, and a polystyrene maleic anhydride (PSMA)

- neocarzinostatin conjugate that is approved for hepatocellular carcinoma treatment (42).

The well-known anticancer drug Doxorubicin (DOX) is cardiotoxic and has a low therapeutic index. A lot of drug delivery systems were studied to enhance the targeting of the drug and lower toxicity. In the studies, while HPMA- DOX conjugates were found to increase cardiovascular tolerability (e.g., heart rate and blood pressure), in another study, DOX-HPMA conjugates also caused microscopic damage to heart tissue. Therefore, the prolonged delivery of DOX-HPMA conjugates is a cautionary issue to be taken into (43,44).

Another promising polymer-drug conjugate for anticancer treatment is stimuli-responsive PDCs. In one of the studies, monomethyl auristatin E which is an antimitotic agent conjugated to dendritic polyglycerol sulfate (dPGS) and dendritic polyglycerol (dP) by disulfide linker which is a self-immolative and reductively cleavable linker. The cytotoxicity assays on HeLa (cervix cancer cells) and A549 (lung cancer cells) showed that the cytotoxicity level decreased and moreover, the sulfated conjugates had been found that is more effective than those without sulfate ones (45).

2.2.2. Polymeric Nanoparticles

In recent years, polymeric nanoparticles (PNPs) have been used for DDS due to their unique features such as 'being small-sized nanoparticles. The possibility for controlled release, the integration of therapy and imaging (theranostics), the enhancement of their therapeutic index and bioavailability, and the protection of drugs and other molecules, are all benefits of using polymeric NPs as drug carriers (46,47).

Depending on the morphology of the PNPs, they can be either nanospheres or nanocapsules. In nanocapsules, while the drug is located in the oily or water core, the surrounding polymeric shell protects the drug from the environment and controls the release of the drug. In nanospheres, the drug is located either inside the continuous network of polymers (matrix) or adsorbed onto the surface of the polymer (48,49). Both nanospheres and nanocapsules are considered reservoir systems, are shown in Figure 4.

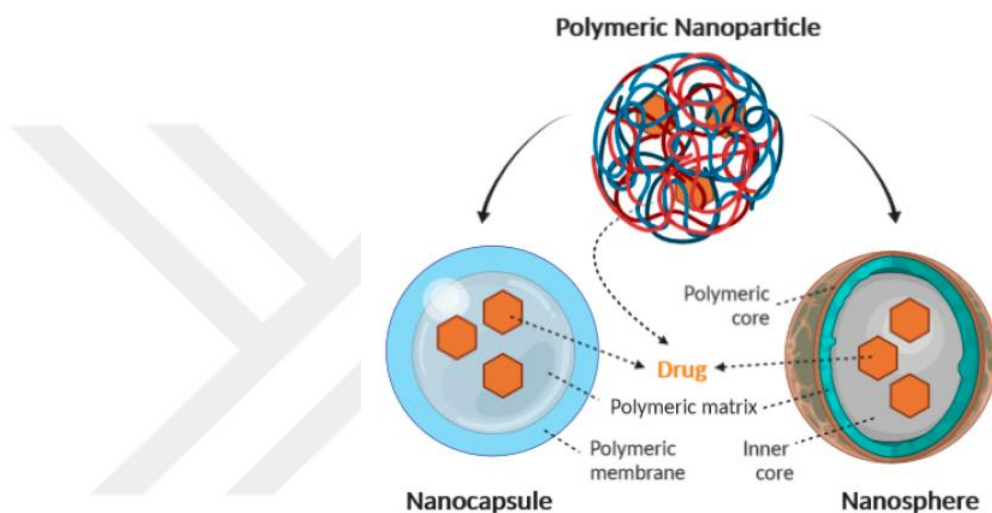


Figure 4. The scheme of nanosphere and nanocapsules (50).

In addition to the morphology of PNPs, there are two types of procedures for PNPs to be loaded with drugs depending on their type and administration ways: polymerization of monomers and dispersion of polymers that is already been formed (51,52). Along with the similarities between both methods such as being the final product of aqueous suspension, ‘the monomer polymerization’ method gives more benefits like allowing better drug loading efficiency without the need for several steps to form PNPs (51,53).

The PNPs can be either made up of non-degradable or degradable polymers. Non-degradable polymers such as polyacrylamide, polyacrylates, polystyrene, and poly (methyl methacrylate) (PMMA) were the first materials utilized in the formation of

PNPs. Although the PNPs made of non-degradable polymer are cleared quickly and efficiently from the body, they show inflammatory reactions and chronic toxicity. In general, although the degradation duration of a non-degradable polymer takes more time than its effective time of application, the PNPS that is made of biodegradable material can be degraded depending on some features such as their structure, size, molecular weight, and the pH or temperature of the external environment. Throughout the years, the studies of PNPs are mainly focused on non-degradable polymers, however, biodegradable polymers have been quite an impact due to their biosafety and biocompatibility(54–56).

Synthetic biodegradable polymers such as poly-polyalkylcyanoacrylates, poly- ϵ -caprolactone (PCL), and poly(lactide-co-glycolide) (PLGA), and natural biodegradable polymers such as alginate, chitosan, albumin, gelatin, and zein have been used to form PNPs (57). The biodegradable PNPs usually show a better profile of drug-release kinetics, less toxicity, and more bioavailability (58,59).

2.2.2.1. Nanogels

Nanogels are nanosized drug- carriers that have received a lot of interest due to their profile of time-controlled or site-specific delivery of bioactive agents (60). As a type of hydrogel, compared to microgels which size in the range of microns (1-350 μm), the nanogels are made of nano-scaled (from 20 to 250 nm) crosslinked and highly swellable-shrinkable polymeric materials (synthetic, natural or combinations of them) which preserve their swelling-shrinking features in different circumstances and allows the encapsulation of various type of molecules such as oligonucleotides and proteins for imaging, drug delivery and diagnostics purposes (61–63). The nanogels are different from other types of NPs due to their several properties such as particle shape and size, and external stimuli (e.g., temperature, pH, ionic strength) sensitivity makes

them efficient for controlled drug release (64). Moreover, nanogels can be customized to be targeted, functionalized and modified for longer circulation periods (65).

Nanogels were either synthesized by the methods of physical self-assembly or chemical crosslinking. While chemical crosslinking which is by covalent binding between polymers and functional groups has better profile of stability than physical crosslinking of nanogels which is obtained by several interactions such as hydrostatic, host-guest and hydrophobic, and Van der Waals forces and hydrogen bonds (66). Even though the nanogels synthesized by chemical crosslinking gives stronger stability, the synthesis process which is done by physical crosslinking has better flexibility due to lack of complex steps (67,68).

For nanogel synthesis, compared to physical crosslinking, chemical crosslinking has more variable and the most established plans. So far, there are four methods for the synthesis of chemically-crosslinked nanogels: click chemistry crosslinking, reversible addition fragmentation chain transfer (RAFT), emulsion polymerization, and photo-induced crosslinking (69,70).

In the last years, the nanogels which is synthesized by click chemistry crosslinking are resulted in excellent selectivity, high yield and reactivity (71). In one of the studies, the pH-responsive nanogel which was prepared by the polymerization of an acid-labile monomer (ortho ester diacrylamide, OEAM) that gave nanogel the feature of pH dependent responsiveness, pentaerythritol tetra(3-mercaptopropionate) (PT) and methoxy polyethylene glycol acrylate (mPEGA). In this polymerization, an efficient method of thiol-ene click chemistry which results in no byproduct was used to obtain the pH-responsive nanogel (72). In emulsion polymerization method, the reaction occurs continuously in oil phase by water emulsification in oil. There are several parameters such as pH, surfactant, crosslinker and monomer ratio can change the size of nanogels (73). In RAFT polymerization, there are bunch of reactions such as chain

transfer, addition and degradation of adduct reactions (reversible) that polymers go through. By RAFT, the polymers' structure can be altered by changing properties, conformation, and the length of the polymer (73).

The photo-induced crosslinking method has become an interest due to its controllable particle size, bacteriostatic effect, and multifunctionality characteristics. In addition, its characteristics that do not need any additives make them a popular method for nanogel preparation. In this method, the nanogel construction is obtained by the formation of intermolecular crosslinking which is initially developed by breaking water molecules into hydrogen atoms and radicals (74). Furthermore, there is another crosslinking method (e.g., optical crosslinking) that uses photo-initiators such as Irgacure 2959. In one of the studies, the Irgacure 2959 was used as a photo-initiator to form docetaxel-loaded lipid-coated nanogel, by this crosslinking the drug molecules were released slower than the ones that were not crosslinked (75).

Stimuli-responsive nanogels have attracted interest because of their features for specific conditions of diseased areas. For example, in a tumor environment, while the glutathione (GSH) concentration and the temperature are high, the pH is low. With these specific conditions, utilizing stimuli-responsive nanogels reduces toxicity to healthy tissues while lowering drug dose and toxicity (75,76). The stimuli-responsive nanogels are preferred highly due to their high drug-loading capacity, stability, and easy preparation. Furthermore, compared to other DDSs, their 3D network structure makes them easily adjustable in any environment to control drug release (77). There are several common stimuli-responsive nanogels such as thermo-responsive, pH-responsive, magnetic-responsive, ultrasound-responsive, and multi-stimuli-responsive nanogel.

Thermo-responsive nanogels have swelling and shrinkage characteristics depending on the temperature of the environment. With these characteristics, the

loaded drug inside nanogel can be released in a controlled manner. One of the commonly used thermo-responsive materials NIPAm is hydrophilic below 32 °C (the lower critical solution temperature, LCST of NIPAm). When the temperature is above its LCST, the NIPAm units become agglomerated and also, stimulate shrinkage (78). With these abilities to shrink and swell, the thermo-responsive nanogel which is made of pNIPAm was loaded with the protein “lysozyme” and showed imprint-release behavior when the temperature changes (79).

In pH-responsive nanogels, the shrinkage and swelling are depended on the change of pH value in the environment. Furthermore, ionization or deionization caused by the pH change alters the form of the nanogel. In research, it was found that the pH of the tumor tissues and the cells (pH: 4.5 to 7.2) is acidic compared to normal human tissues, and the blood (pH: 7.4) (80). In one of the studies, the methyl ester (MA) and methacrylic acid (MAA) monomers were used to form pH-responsive nanogel. These monomers were swollen at the basic pH, while they were shrunken at the acidic pH. This shrinking allowed drug and fluorescent agent (DOX and oligothiophene, TF) entrapment. Besides this, it was seen that the nanogel got shrunken at the acidic pH (5.5), and resulted in increased DOX release (81).

When an alternative magnetic field (AMF) is applied to magnetic-responsive nanoparticles (MNPs), it allows recognition of not only hyperthermia but also magnetic targeting. Magnetic-responsive nanogels are hybrid nanogels which is made of MNPs and thermo-responsive nanogels and also can encapsulate drug molecules (e.g., DOX) because of their 3D structure. Hence, the swelling and shrinking nature of magnetic-responsive nanogels allow the release of drug molecules when AMF is applied (82).

The ultrasound-responsive nanogels are widely used in central nervous system treatment as well as an anticancer treatment. Especially, for anticancer treatment,

acoustic waves are beneficial for penetration in deep areas (83). Having only one responsiveness for the nanogels cannot control drug release effectively compared to multi-responsive ones. Hence, multi-responsive nanogels have gained interest due to variable responsiveness. In one study, the dual-responsive nanogel which is made of crosslinked three types of monomers (PEGMA, MAA, and NIPAm) showed great release and loading efficiency for the drug molecule (84).

2.3. Targeted Nanogels

Nanogels can either be passively targeted by surface, shape, and size adjustments or be actively targeted by changing their surface properties which leads to targeting only diseased areas of the body (85). For the actively-targeted nanogels, the surface modifications require interactions of cellular receptors, and ligands such as peptides, proteins, and polysaccharides (11).

2.3.1. Functionalized Nanogels

In the last decade, folic acid (FA) has become one of the most studied small molecules for the functionalization of nanogels due to its specific interactions with cancerous cells which have overexpressed folate receptors (FRs). Compared to normal healthy tissues, the cancerous tissues (e.g., ovarian) express FRs more and this expression difference is used as a biomarker for the treatment and the diagnosis of cancer. In one of the recent studies, a folic acid-functionalized, fluorescent agent green carbon dots (CD) which was made from grapefruit extract and anti-miR-21 sequence loaded hybrid nanogel showed great surface charge and size for imaging of SKOV3 (human ovarian cancer cell) cancer cells and when compared to FA-free nanogel, it showed greater cellular uptake (86).

In another study, one of the cell adhesion peptides that bind to integrin receptors of the cells and have a superior attraction for cancerous cells cyclic RGD (cRGD) was used as a targeting agent to functionalize pNIPAM and MAA-based nanogel. The cRGD nanogel was loaded with two drug molecules which are Lidocaine and CDDP and was introduced to a breast cancer model. From the results, it was found that the dual drug-loaded cRDG nanogel accumulated in the cancerous region and showed increased targeting (87).

In one of the recent studies, the integrin “ $\alpha v \beta 3$ ” was conjugated to lipid-coated nanogel which had human serum albumin (HSA) in its core as a crosslinked form to carry drug molecules. Several chemotherapeutic agents (e.g., docetaxel) were assessed. Pertaining to Abraxane, taxane-loaded nanogel showed greater antitumor activity, inhibition of metastasis, and tumor growth in pancreas and breast cancerous tissues (88).

2.3.2. Antibody Conjugated Nanogels

When it is compared to the binding sites of the particular receptors, the antibodies are highly utilized as a targeting agent due to their greater affinity and accuracy toward diseased areas (89). Along with their usage as targeting agents, they also work as therapeutic agents in some diseases (e.g., cancer). In one of the recent studies, anti-CD4 conjugated nanogel was used as a cargo element for targeting CD4_{high} T cell lymphoma and CD4⁺ T cells. By antibody conjugation, while the un-intended uptake of nanogels was reduced in CD4 lymphocytes, anti-CD4 conjugated nanogels were taken with greater efficiency by the CD4⁺ T cells (90). In pancreatic ductal adenocarcinoma (PDAC), Sialyl Tn (STn) antigen is expressed at an abnormal level. In another study, the anti-STn antigen-specific antibody conjugated nanogel was used to target PDAC and it was found an enhanced tumor activity and efficient targeting toward the PDAC (91).

2.4.Nanomedicine for Atherosclerosis

Atherosclerosis has been the most significant disease which has over 50% of total mortality in developed countries (92). It is an age-dependent disease that can start at earlier ages and progresses year by year. The disease can be asymptomatic for many years and generally, for several cases, it shows its first symptom at the advanced stage which can cause critical arterial occlusion (blockage) with some serious effects (93,94).

Hypertension, hyperlipidemia, diabetes mellitus, smoking, gender, and genetic disposition are some of the risk factors for Atherosclerosis disease. From the current view of atherosclerosis disease, it is understood that those factors can cause damage to vascular endothelium by lipid penetration into the vascular wall. One of the most significant risk factors is plasma low-density lipoprotein (LDL) level and hyperlipidemia is one of the major contributing factors for atherosclerosis. In addition to LDL, it is found that high content of blood cholesterol also contributes to atherogenesis (95).

Atherosclerosis starts with the accumulation of LDL-cholesterol in the subendothelial area of the arterial wall. Hence, this accumulation which is subendothelial retention is the main reason for atherosclerotic plaque formation or growth. Atherosclerotic plaques can cause thickening of the arterial walls which in mainly large and medium-sized arteries leads narrowing of those arteries. Therefore, a hemodynamic disturbance occurs and moreover, due to some unknown reasons, atherosclerotic plaques can rupture and leads to stroke or myocardial infarction (96,97).

One of the major contributing components to atherosclerosis is oxidative stress which is occurred through chemokine or cytokine expression and pro-inflammatory signaling stimulation (98). In atherosclerotic cardiovascular disease, the risk factors such as hypertension, dyslipidemia, and diabetes are associated with enhanced ROS production in the arterial wall (99,100). The significant atherosclerotic plaque formation molecule which is oxidized low-density lipoproteins (ox-LDL) is accumulated in the arterial wall and leads to the expression of cellular adhesion molecules like P- and S-selectins and vascular adhesion molecule-1 (VCAM-1). These expressions result in leukocyte employment and intrusion in the sub-endothelial region of the vessel wall (101). In the internal side of the vessel wall, monocytes become macrophages, then, by taking modified lipids into themselves, change to foam cells. This foam cell formation is a distinctive character of the initial stage of atherosclerosis (102).

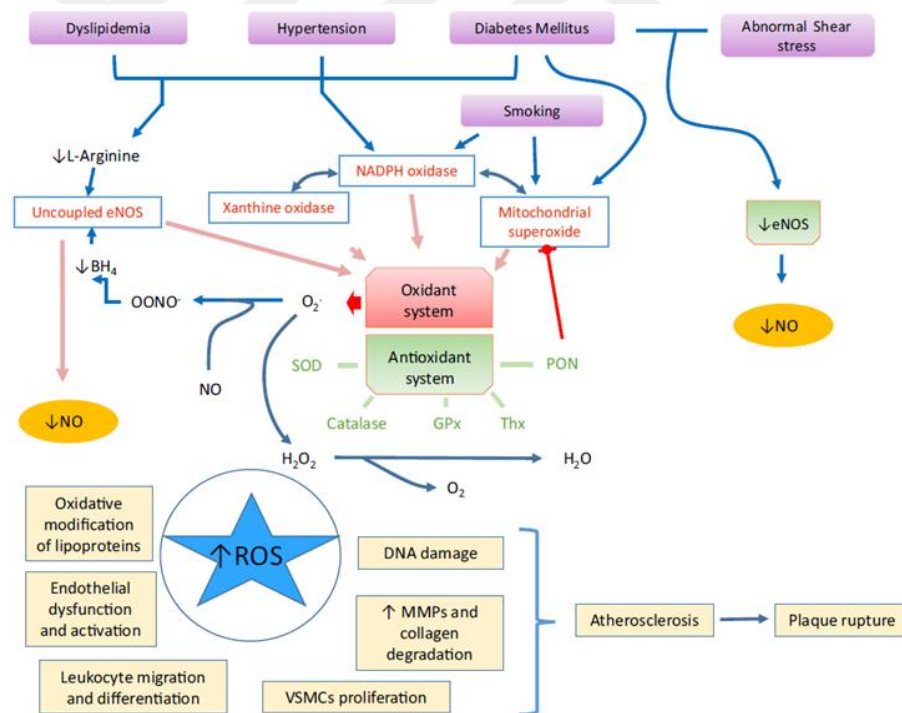


Figure 5. ROS generation mechanism depending on risk factors, oxidant and antioxidant systems and its effect on atherosclerosis (103).

There are several significant enzymes that play a crucial role in ROS generation mechanisms. For instance, NADPH oxidase which is a member of the membrane-

associated enzymes promotes oxidant production in the vascular wall (104). While monocyte or macrophage invasion occurs on the internal side of the vessel wall, immune cells such as T-lymphocytes and mast cells are also been employed. Due to this increased recruitment of immune cells in the atherosclerotic region of the vascular wall, the inflammatory process is induced, ROS is generated and some cytokine is released. For example, IL-1 β promotes ROS generation by the NADPH oxidase (105).

Due to the high incidence of those serious outcomes of atherosclerosis, there is a corresponding increase in demand for drug therapies. Particularly drug therapies that are not targeted to damaged sites lead to toxicity and side effects for healthy tissues and organs. Thus, it becomes very obvious that there is a need for targeted therapies that deliver drug molecules only to sites where disease conditions are seen.

In order to target Atherosclerosis, Atherosclerotic molecular markers (AMMs) are the primarily used targeting agents. They are also incorporated with some ligands (e.g., therapeutic or imaging) for delivering purposes (106). The targeting can either be in atherosclerotic or intra-plaques which can be reachable via cellular gaps. P- and S-selectin, ICAM-1, VCAM-1, and integrin $\alpha\text{v}\beta 3$ which are up-regulatory cell receptors on the endothelial cells of the internal wall are a few of the appealing targeting agents that are overexpressed in the region of atherosclerosis (107). In addition to these cell receptors, HDL which is a key contributor to the formation of atherosclerotic plaque, and one of the junctional adhesion molecules JAM-A which allow inflammatory cells to reach the area of atherosclerosis are used for targeted therapies of atherosclerosis (108,109).

So far, several kinds of NPs have been designed for the treatment of atherosclerosis. Lipid-based NPs (e.g., liposomes), and polymeric NPs are some of them. In one of the studies, Ferulic acid-based polymeric NPs were designed with an anti-oxidant-polymer core (Ferulic acid) and biodegradable polymeric shell

(polyanhydride ester) that reduced ROS generation and prevented ox-LDL uptake in macrophages (110). In the early stage of atherosclerosis, the significant factors which are monocyte adhesion to endothelial cells (EC) and transmigration are reduced. In another study, Teijin-loaded liposomes which have sensitivity to target “VCAM-1” molecule improved monocyte adhesion to activated EC compared to non-targeted liposomes and Teijin itself (111).

2.4.1. Drugs for Atherosclerosis

Some therapeutics such as angiotensin-converting enzyme (ACE) inhibitors, statins, and angiotensin receptor (AT1) antagonists are commonly employed especially for their antioxidant effects in atherosclerosis treatment (112). For example, ACE inhibitors were observed to reduce nitric oxide (NOX) in the vessel wall by increasing the activity of extracellular superoxide dismutase (EC-SOD) (113). Thus, antioxidants have been getting significant attention due to their effectiveness in atherosclerosis therapy. In recent years, utilizing systemic antioxidants such as vitamin E or C transitioned into using targeted therapies for the treatment of atherosclerosis. Furthermore, the natural components such as polyphenols, glutathione, and N-acetyl cysteine have got an interest due to their non-adverse effect on the body for prolonged atherosclerotic therapies (114).

One of the appealing polyphenolic molecules resveratrol which is in fruits (e.g., berries) has an anti-atherosclerotic character that scavenges oxidants (e.g., peroxynitrite). In preclinical studies, it was found that it has antioxidant effects on several targeting molecules such as estrogen receptor (ER), and nuclear factor E2-related factor-2 (Nrf-2) (115,116).

Another promising anti-atherosclerotic molecule Quercetin has antioxidant and anti-inflammatory effects. In addition to these properties, it impacts lipid metabolism as well. For instance, atherosclerotic rats that were fed by hyper-cholesterol- diet were treated with Quercetin supplements and it was found after the treatment that it lowered the inflammatory molecules (e.g., NOS) (117).

2.4.2. Lipoic Acid Based DDSs

Lipoic acid is a kind of antioxidant that is produced by mitochondria, scavenges peroxy radicals, peroxynitrite, and hypochlorous acid (HOCl), and reduces the progression of atherosclerosis. In addition, lipoic acid also enhances NOS activity and reduces inflammation biomarkers and endothelial impairment (118–121). In one of the studies, the α -lipoic acid (ALA) loaded polymeric nanoparticles which are obtained from 1, 2-Distearoyl-sn-glycero-3- phosphoethanolamine (DSPE) and PEG polymers are used to treat diabetes-mellitus related periodontal loss. While ALA decreased the ROS and inflammation, it induced osteogenesis under lipase stimulated pathogenic environment (122). In another study, the α -tocopheryl and lipoic acid functionalized DOX-loaded liposomal systems were applied to HeLa cells. The DOX-loaded tocopheryl and lipoic acid-functionalized NPs were found to be more effective than the DOX alone (123).

One of the serious neuronal diseases Parkinson's is strongly associated with oxidative stress. The disease occurs via α - synuclein aggregation that leads to neural cell dysfunction (e.g., death). In one of the researches, lipoic acid-gold nanoconjugates were tested on SH-SY5Y cells. It was found that lipoic acid-gold nanoconjugates successfully decreased ROS generation and restored cell injury (124).

3. MATERIALS AND METHODS

3.1. Materials

α -Lipoic Acid (Sigma Aldrich, US), pentaerythritol (Sigma Aldrich, US), anhydrous dimethyl sulfoxide (DMSO, Sigma Aldrich, US), anhydrous dichloromethane (DCM, Sigma Aldrich, US), methacryloyl chloride (MAC, Sigma Aldrich, US), NaHCO₃ (Sigma Aldrich, US), NaCl (Sigma Aldrich, US), Na₂SO₄ (Sigma Aldrich, US), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, Sigma Aldrich, US), N-Hydroxy Succinimide (NHS, Sigma Aldrich, US), 4-(Dimethylamino) pyridine (DMAP, Sigma Aldrich, US), HATU (Sigma Aldrich, USA), Diisopropylamine (DIPA, Thermo Fisher, US), 2-Aminoethyl methacrylate hydrochloride (AE-MA, Sigma Aldrich, US), Concanavalin A. Alexa Fluor 488 (Thermo Fisher, US), anti-VCAM1 (CD106) monoclonal antibody (Sigma Aldrich, US), Cy5 dye (Thermo Fisher, US), Poly(ethylene glycol) dimethacrylate (PEG-DiMA, Mwt: 750 Da, 2 kDa, 6 kDa and 10 kDa, Sigma Aldrich, US), Amicon® Ultra-15 Centrifugal Filter Unit (Mwt cut-off: 30 kDa and 50 kDa, Sigma Aldrich, US) AR1160_DS Boster cell counting kit-8, IL-6 Human ELISA kit (Invitrogen, US), ab113851 DCFDA / H2DCFDA - Cellular ROS Assay Kit (Abcam, US), Lipid Peroxidation (MDA) Assay Kit (Sigma Aldrich, US).

3.2. Drug-Containing Monomer Synthesis and Characterization

The drug-containing monomer, tri-lipoic acid methacrylate (LA3-MA), was synthesized in two main steps. For the first step, the pentaerythritol-methacrylate (Penta-MA) synthesis, 200 mg of pentaerythritol was dissolved in 2 mL of anhydrous dimethyl sulfoxide (DMSO) in an inert environment at room temperature (RT). On the other hand, 77,5 μ L of methacryloyl chloride (MAC) dissolved in 3 mL of anhydrous dichloromethane (DCM) in an inert environment, and the solution was slowly dropped by drop to the first solution for 15 minutes. The reaction was continued for 40 minutes at RT and gradually blurred due to the formation of HCl salt. The HCl salt formed during the reaction was filtered with the help of cotton. The base and DCM volatiles in the obtained clear solution were removed under vacuum at around 30 °C with the help of a rotary evaporator (Buchi).

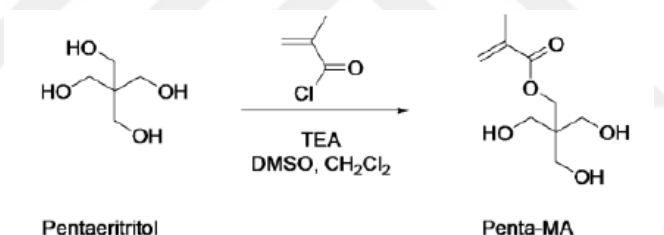


Figure 6. The synthesis step of pentaerythritol-methacrylate (Penta-MA) has a methacrylate group in its structure.

The number of substances in the solution and their comparative polarities were obtained by thin-layer chromatography (TLC) for purification and characterization purposes. Then, the crude was purified by prep-HPLC (Teledyne ISCO). In this purification step, the C18 column was used as a stationary phase and the acetonitrile/deionized water mixture was used as a mobile phase. For further chemical characterization, the LC-MS (50% ACN: 50% H₂O, C18 HPLC column, Thermo

Fisher), LC-MSMS (50% ACN: 50% H₂O, C18 HPLC column, Thermo Fisher), and H-NMR (Bruker) were used.

For the second step of the LA3-MA synthesis, the pre-synthesized 40 mg of Penta-MA was dissolved in 1 mL of anhydrous DMSO in an inert environment. Then, 145,5 mg of lipoic acid (LA), 162,2 mg of EDC, and 86,14 mg of DMAP were dissolved in 9 mL of anhydrous DCM and stirred on a magnetic stirrer for 1 hour at RT. The Penta-MA solution was added dropwise to the mixture of activated LA solution and the reaction continued at RT in the dark for two days. After the reaction, the reaction mixture was diluted with 30 mL of DCM to get rid of DMSO salts and organic base and was extracted with 10% NaHCO₃ solution twice and once with concentrated NaCl solution. The DCM phase was dried with anhydrous Na₂SO₄ salt and filtered. Then, to get rid of the DCM solution, a rotary evaporator was used.

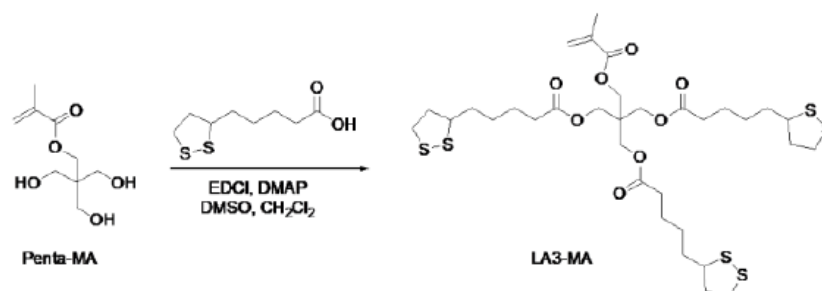


Figure 7. The synthesis step of LA3-MA has a free methacrylate and 3 Lipoic acid groups in its structure.

To detect different substances, free drug molecules, and Penta-MA in this mixture were checked using TLC. Then, the mixture was purified by prep-HPLC. In the purification step, the silica column was used as a stationary phase, and ethyl acetate/hexane was used as a mobile phase. For further characterization, the LC-MS

(50% ACN: 50% H₂O, and C18 HPLC column), LC-MSMS (50% ACN: 50% H₂O, and C18 HPLC column) H-NMR were used.

3.3.Nanogel Preparation and Characterization

3.3.1. Control Nanogel (C-NG) Preparation

Several types of control nanogel (C-NG) were prepared for this project. The different molecular weight of the PEG-DiMA polymer (Mwt: 750 Da, 2 KDa, 6 KDa, and 10 KDa) was subjected to a radical reaction by adding an Irgacure (photo-initiator). The polymer to photo-initiator ratios ([P]: [I]) were 1:50, 1:100, 1:150, and 1:200 by moles, and different amounts of solvents (2 mL, 4 mL, and 8 mL) were tested to determine the most ideal C-NG. The most ideal C-NG was prepared as follows: 27 mg of PEG-DiMA (6 KDa) polymer was dissolved in 8 mL of 10 mM PBS (pH:7.4) for 10 min at 1500 rpm, RT. Then, Irgacure (10 μ L, 20,74 μ g) was added into the solution ([P]: [I] =1:50) and put under 365 nm of UV light for 20 min. After 20 min. of reaction, the prepared nanogel solution was filtered once with 0,22 μ m regenerated cellulose (RC) filters.

3.3.3.1.Stability Test of Control Nanogel (C-NG)

The prepared C-NG was diluted by 1:10 with the total volume in both pH 7.4 10mM PBS buffer solution (modeling blood and healthy tissues in the body) and pH 5.5 acetate buffer solution (modeling inflamed tissues in the body). Half of these prepared solutions were shaken at 200 rpm at room temperature; half of them were incubated at 37 °C, which is body temperature, by shaking at 200 rpm in an orbital

mixer. In this way, the stability of both 1:50 and 1:100 nanogels in two different pH values and two different temperatures was observed. While these nanogel solutions were incubated, samples were taken from them at certain time intervals and nanoparticle sizes were investigated by performing DLS analysis (Wyatt dynopro nanostar).

3.3.4. Drug-Loaded Nanogel Preparation

For the preparation of the drug-loaded nanogels, different % mass (w:w) ratio of LA (5%, 10%, and 20%) was used. The most ideal drug-loaded nanogel was with the 20% LA of the total mass of the nanogel. The most ideal 20% LA-loaded nanogel (NG-LA20) was prepared as follows: 27 mg of PEG-DiMA (6 KDa) polymer was dissolved in 8 mL of 10 mM PBS (pH:7.4) for 10 min at 1500 rpm, RT. Then, 6,75 mg of LA3MA in 80 μ L absolute acetone was added into the solution and stirred for another 10 min. After that, Irgacure (10 μ L, 20,74 μ g) was added into the solution ([P]: [I] =1:50) and put under 365 nm of UV light for 20 min. After 20 min. of reaction, the prepared nanogel solution was filtered once with 0,22 μ m RC filters, then was centrifuged five times with the Amicon filter units (Mw: 30 kDa), was filtered five times with 0,22 μ m RC filters, and was sonicated for five min., respectively.

3.3.4.1.Determination of Drug Release Kinetics from Drug-Loaded Nanogels

Time-dependent drug release profiles of different LA-loaded nanogels (NG-LA5, NG-LA10, and NG-LA20) were studied in different environments (in 10mM PBS solution (pH 7.4) and acetate buffer solution (pH5.5). The NG-LA5, NG-LA10, and NG-LA20 were incubated in both 10 mM PBS solution (pH 7.4) and acetate buffer solution (pH 5.5) and at RT and body temperature (37 °C) separately. Each 1 mL of nanogel solution was placed in dialysis bags (Mwt cut-off: 1 kDa). Then, incubation

was started by placing it in 40 mL of buffer solution. The 'sink' conditions were provided for the release tests by using 40 times more than the volume of the nanogel solution in the bag. The pH value of the PBS solution used in these drug release experiments was 7.4 which mimicked the blood pH conditions, while sodium acetate buffer solution, with a pH value of 5.5, was used to mimic the ambient conditions in the inflammation region.

While these prepared dialysis systems were incubated in an orbital shaker at 37 °C and 200 rpm, 100 µL of the receptor solution was taken at different time intervals and stored at -20 °C. After samples were collected, they were analyzed using the MRM method (with 20 µL injection volume) prepared for LA in an LC-MS/MS device. Then, the free LA concentration in the samples was calculated using the calibration curve.

For another drug release profile of NG-LA20, the prepared nanogel solution was again subjected to drug release experiments in 5 different environments. These environments were; PBS (pH 7.4), PBS (pH 7.4) with esterase enzyme; ACE buffer (pH 5.5); ACE buffer (pH 5.5) with esterase enzyme; Serum (versus PBS). In these experiments, a dialysis membrane with an mwt cut-off value of 1 kDa was used. The 'sink' conditions were provided by placing these receptor solutions in the form of 40 mL each outside of the membrane. Different pH values were provided with PBS and ACE buffer solutions, and since drug molecules in the structure of nanogels were bound to the structure by ester bonds, the experiments were repeated by treating them with an esterase enzyme. In this context, NG-LA20 was incubated in 10 mM PBS (pH: 7.4) and ACE buffer solutions (pH 5.5) separately. In the release system that contained the esterase enzyme, the 1-unit enzyme corresponded to a 1 µmol ester bond. For the release studies with serum, 1 mL of human serum was added into 1 mL of nanogel solution and placed in the dialysis membrane, and dialysis was performed against 10 mM PBS (pH 7.4) as a receptor solution. The prepared dialysis experimental setups were incubated at 37 °C in an orbital mixer at 200 rpm and 100 µL samples were collected from the receptor solution at different time intervals. Collected samples were

kept frozen at -20 °C until analysis and analyzed in LC-MS/MS device. The obtained results were calculated as the percentage of the total drug amount in the amount of nanogel placed in each bag, assuming 100%.

3.3.5. Nanogel Functionalization with Cy5 and Anti-Vcam1 Antibody

3.3.5.1. Amine-Containing Nanogel NG-NH₂ Preparation and Characterization

For the preparation of amine-containing nanogel, 1%, 2% and 4% AE-MA by mole was tested. For the preparation of the most ideal amine-containing nanogel: 27 mg of PEG-DiMA (6 kDa) polymer was dissolved in 6 mL of 10 mM PBS (pH:7.4) for 10 min at 1500 rpm, RT. Then, 2 mL of 2% AE-MA (2,92 mg, 0,008 mmol) was added into the solution and stirred for 10 min. Then, if it was loaded with LA3-MA, 6,75 mg of LA3MA in 80 µL absolute acetone was added into the solution and stirred for another 10 min. After that, Irgacure (20,74 µg, 10 µL) was added into the solution ([P]: [I] =1:50) and put under 365 nm of UV light for 20 min. After 20 min. of reaction, the prepared nanogel solution was filtered once with 0,22 µm RC filters, then centrifuged five times with the Amicon filter units (Mw cut-off: 50 kDa), and filtered five times with 0,22 µm RC filters, respectively.

3.3.5.2. Functionalization of the Nanogels with the Cy5 Fluorescent Dye (NG-LA20-Cy5)

For the preparation of dye-conjugated nanogels, the dye Cy5 which has a free carboxylic acid group in its chemical structure was activated with HATU and DIPA,

and the activated Cy5 molecule participated in the amidation reaction with primary amine groups on the surface of NG-NH₂.

For this conjugation reaction, the mole ratio of amine monomer in NG-NH₂ nanogels was calculated theoretically, and 1: 0,4 (moles) compared to their amines was subjected to conjugation. First of all, Cy5 dye was activated with HATU (0,76 mg, 0,0019 mmol) and DIPA (0,183 mg, 0,0018 mmol), then, NG-NH₂ (1,21 mg, 0,00453 mmol) was added into the activated Cy5 solution at RT in dark for 48 h. After the reaction, to optimize Cy5 functionalized nanogels (NG-Cy5 or NG-LA20-Cy5), the nanogel solution was centrifuged five times with the Amicon filter units (Mw cut-off: 30 kDa) and then, was filtered five times with 0,22 µm RC filters.

In addition, both the Cy5 dye amount and free-LA amount from the same sample was measured using appropriate calibration curves in LC-MS/MS device. To measure these amounts of Cy5 and LA, the nanogels were treated with esterase enzyme (1 unit of enzyme: 1 µmol ester bond ratio) for at least 24 hours. The details of the MRM methods developed for both Cy5 and LA and the calibration curves created are given. The same sample was carried out in 2 different methods with 20 µL injection volume in stationary phase with C18 column and flow rate of 50% ACN: 50%H₂O mobile phase at 0,5 mL/min.

3.3.5.3.Functionalization of the Nanogels with the Anti-VCAM1 Antibody (NG-Cy5-ANT and NG-LA20-Cy5-ANT)

After conjugation of Cy5 dye to the nanogels, remaining free amine functional groups on the surface, 1:0,6 (moles) compared to their amines, anti-VCAM1 antibody was conjugated to the remaining amine groups on the surface of the nanogels (NG-Cy5 or NG-LA20-Cy5). This reaction was performed using EDC/NHS reagents. First

of all, an anti-VCAM1 antibody (2 µg, 10 µL) was taken and added into EDC (21 ng, 0,5 mL) and NHS (14,2 ng, 0,5 mL) solution prepared in 1 mL of 10 mM PBS solution in the ice bath for thirty min. After that, NG-Cy5 or NG-LA20-Cy5 nanogel solution (in 10 mM PBS) was added to the activated antibody solution, and the reaction was continued at RT for 4 h and then, at +4 °C for 18 h in the dark. After the reaction, to optimize anti-VCAM1 antibody functionalized nanogels (NG-Cy5-ANT or NG-LA20-Cy5-ANT), the nanogel solution was centrifuged five times with the Amicon filter units (Mw cut-off: 30 kDa) and then, was sonicated for 10 min. and filtered once with 0,22 µm RC filters, respectively.

3.4. In Vitro Experiments

3.4.1. In Vitro Inflammation Modeling

For *in vitro* inflammation model, 10,000 cells/well (HUVEC cells) were seeded on a flat-bottom 96-well plate and incubated at 37 °C, 5% CO₂ for 24 h. Then, different concentrations of LPS (10, 50, 100 ng/mL) were applied and incubated at 37 °C, 5% CO₂ for 6h. Then, the supernatant of the cells was taken and used for IL-6 ELISA assay.

3.4.2. In Vitro Cytotoxicity Assay (CCK-8)

For the *in vitro* cytotoxicity assay, AR1160_DS Boster cell counting kit-8 was used. For the first day of the experiment, 10,000 cells/well (normal human skin fibroblasts and HUVEC cells) were seeded on a flat-bottom 96-well plate and incubated at 37 °C, 5% CO₂ for 24 h. After that, the medium was discarded and the

wells were washed with 1X PBS (pH:7.4). Then, different kinds of drug groups (Free-LA molecules, NG-LA20, NG-C, NG-LA20-Cy5, and NG-LA20-Cy5-ANT) were applied from the maximum concentration of 21 $\mu\text{g/mL}$ to 0,041 $\mu\text{g/mL}$ by serial dilution and then, the cells were incubated at 37 °C, 5% CO₂. After 24 h of incubation, 10 μL of CCK-8 reagent was added to each well and it was kept for 4 h at 37 °C, 5% CO₂. The results were obtained by a plate reader at 450 nm.

3.4.3. IL-6 Assay

For the IL-6 protein measurement, Human IL-6 ELISA kit was used. For the ELISA kit, the protocol recommended by the manufacturer was followed. Briefly, 50 μL of biotinylated antibody reagent was first added to each well. Then, the IL-6 standards from the kit and the test samples (the medium of 0,5 mM and 2 mM of free-LA and NG-LA20-Cy5-ANT treated LPS-activated HUVEC cells) were added to each well as 50 μL in duplicate. The plate was carefully covered with an adhesive plate cover and tightly sealed from all edges and strips and then incubated for two hours at RT. After two hours, the adhesive plate cover was carefully removed. The plate was then washed three times with the wash solution. After washing, 100 μL of prepared Streptavidin-HRP solution was added to each well. The plate was then carefully covered with an adhesive plate cover, tightly sealed from all edges and strips, and then incubated for thirty minutes at RT. The plate was then washed three times with the wash solution. After washing, 100 μL of TMB (3,3',5,5'-Tetramethylbenzidine) substrate was added to each well. The color reaction was allowed to develop at RT in the dark, and incubated for thirty minutes. Unlike the other stages, the plate was not covered with aluminum foil or a different plate cover. After thirty minutes, the reaction was stopped by adding 100 μL of stop solution to each well. Finally, absorbance values were measured on an ELISA plate reader set to 450 nm and 550 nm. Calculations were made in accordance with the protocol by subtracting the 550 nm values from the 450 nm values to correct the optical defects in the microplate. Using the IL-6 standard and the calibration curve from this protocol, it was calculated how much free IL-6 protein

was in each sample. The values obtained were compared with the control groups which had with and without LPS.

3.4.4. ROS Assay

To determine ROS production, DCFDA / H2DCFDA - Cellular ROS Assay Kit was used. For this assay, the protocol recommended by the manufacturer was followed. First of all, 25,000 cells/well (HUVEC cells) were seeded in a flat-bottom 96-well plate and incubated for 24 h at 37 °C, 5% CO₂. After the incubation, 100 ng/mL LPS was applied per well and cells were incubated for 6 h at 37 °C, 5% CO₂. After the LPS treatment, the medium was discarded and the wells were washed with 1X wash buffer. Then, 100 µL of the DCFDA was added to each well (except for unstained cell wells) and incubated for 45 min. at 37 °C, 5% CO₂ for 24 h in the dark. After 45 min. of incubation, the cells were washed with 1X wash buffer. Then, different kinds of drug groups (Free-LA molecules, and NG-LA20-Cy5-ANT) were applied in two different concentrations (0,5 and 2 mM) for 6 hours. Then, the results were obtained by a microplate reader at Ex/Em: 485/535 nm.

3.4.5. Lipid Peroxidation (Malondialdehyde, MDA) Assay

In order to determine the amount of MDA, the lipid peroxidation assay was performed and the protocol recommended by the manufacturer was followed. After cells were treated with appropriate samples (free-LA and NG-LA20-Cy5-ANT), the medium of the cells was collected and used for this assay. In this kit, lipid peroxidation is determined by the reaction of MDA with thiobarbituric acid (TBA) to form a colorimetric (532 nm)/fluorometric ($\lambda_{\text{ex}} = 532/\lambda_{\text{em}} = 553$ nm) product which is directly proportional to the amount of MDA presence. In this analysis, supernatant solutions were collected from the wells which were also used for IL-6 measurement.

For this purpose, 20 μ L of the collected samples were incubated with sulfuric acid (500 μ L, 42 mM) and Phosphotungstic acid (125 μ L), and then reacted with 2 μ L of BHT (Butylated hydroxytoluene) and 600 μ L of TBA solution, respectively. Afterward, the absorbance values of the samples extracted with n-propanol were measured using a microplate reader.

3.4.6. Visualization of the HUVEC Cells Under Fluorescent Microscopy

To visualize LPS-activated and free-LA and NG-LA20-Cy5-ANT treated HUVEC cells, the cells were fixed. First of all, after the treatment of 24 h and 72 h, the medium of the cells was removed, then, each well was washed with 100 μ L of 1X PBS and was shaken gently. After that, it was incubated at RT for 5 min. Then, the PBS solution was discarded. cold 100 μ L of cold 4% PFA was added slowly to each well and incubated for 10 min at RT. After the incubation, the PFA was removed slowly to not to dislodge cells. Right after, the wells were washed with deionized water and were shaken gently. Then, 100 μ L of cold MeOH was added and incubated at RT for 5 min. After removing MeOH, the cells were fixed and stored at 4 $^{\circ}$ C.

After the fixation, the HUVEC cells were primarily stained with Cocavalin A. Alexafluor 488 to visualize the cellular membrane and then, were secondarily stained with the DAPI 488 to visualize the nucleus of the cell. For this staining, first of all, the cells were washed with 1X PBS three times, then, 100 μ L of Alexafluor 488 (20 μ g/mL) was added to each well and incubated for 45 min. After the incubation, the cells were washed again with 1X PBS three times. After Alexafluor staining, 100 μ L of DAPI (10 μ g/mL) was added into each well and incubated for 5 min. Then, the cells were washed again with 1X PBS three times and were visualized by EVOS M5000 (Thermo Fisher).

4. RESULTS

4.1. Drug-Containing Monomer Synthesis and Characterization

The LA3-MA drug molecule was synthesized in two steps: initial starting molecule Penta-MA and LA3-MA synthesis. To characterize synthesized Penta-MA molecule, prep-HPLC, LC-MS, LC-MSMS, and H-NMR were used.

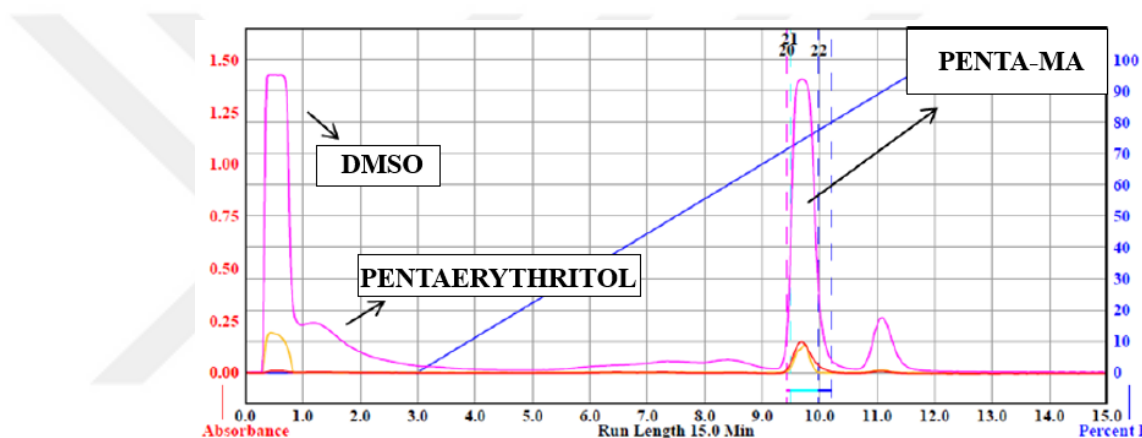
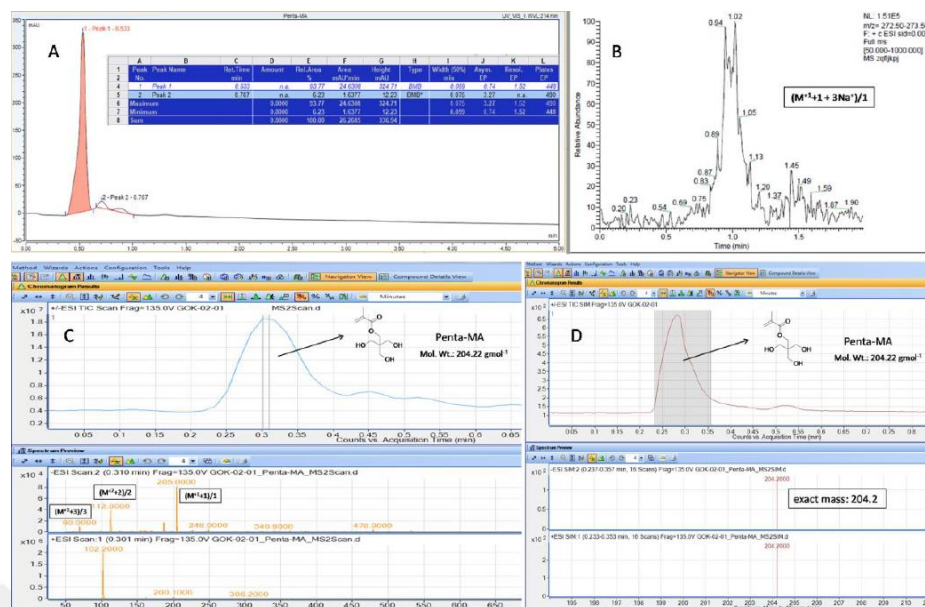


Figure 8. Prep-HPLC chromatogram of Penta-MA

The UV-Vis wavelength of 214 nm and 254 nm were selected for prep-HPLC measurement. The substances were collected separately by following the signals at these nm values during purification. As seen in Figure 8., each peak in the chromatogram was collected separately to detect which substance belongs to the desired crude. After the collection of all peaks, each peak was examined in terms of total molecular weights in LC-MS and LC-MSMS. As a result, it was determined that the substance, which was around 60-70% acetonitrile in the prep-HPLC analysis, was Penta-MA with 84% yield.



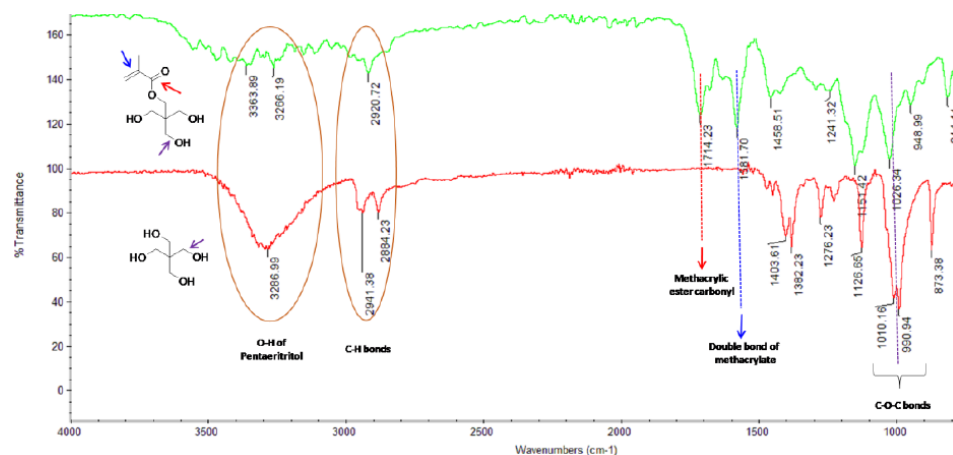


Figure 10. FT-IR Spectra as functional group analysis of Penta-MA.

For the chemical characterization of the Penta-MA molecule, FT-IR spectrophotometer and NMR spectroscopy were used to determine functional groups. In Figure 10., the FT-IR spectrum of the Penta-MA molecule is shown compared to its starting molecule pentaerythritol. It is indicated that O-H ($3400\text{--}3200\text{ cm}^{-1}$), C-H bonds ($2950\text{--}2880\text{ cm}^{-1}$), and C-O-C bonds ($1026\text{--}990\text{ cm}^{-1}$) bonds in the structure of pentaerythritol are also seen in the FT-IR spectrum of Penta-MA molecule. On the other hand, the peaks that belong to only Penta-MA which are the methacrylic ester carbonyl group (1714 cm^{-1}) and the methacrylate double bond (1582 cm^{-1}), proved that Penta-MA synthesized successfully.

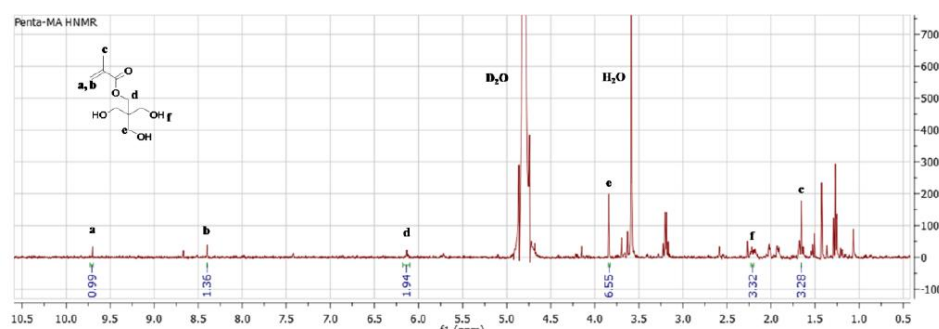


Figure 11. H-NMR analysis of Penta-MA (in D₂O).

In H-NMR analysis of the Penta-MA molecule (Figure 11.), the ratio of the integration values of peaks that belong to the double bond of the ester protons was

shown as 1: 1: 2, indicating that the methacrylate group is included in Penta-MA's chemical structure. Also, the compatibility of these ester proton integration values with the other three -CH₂-OH groups was shown as 2:6 proving that only 1 methacrylate group is included in the Penta-MA structure.

After the LA3-MA synthesis, the reaction mixture was purified using prep-HPLC (Figure 12.). The impurities of the synthesis were observed till 60% ethyl acetate/ 40% hexane polarity and the desired crude, LA3-MA, was seen at around 100% ethyl acetate polarity. The calculated yield which was purified from all impurities was 76%.

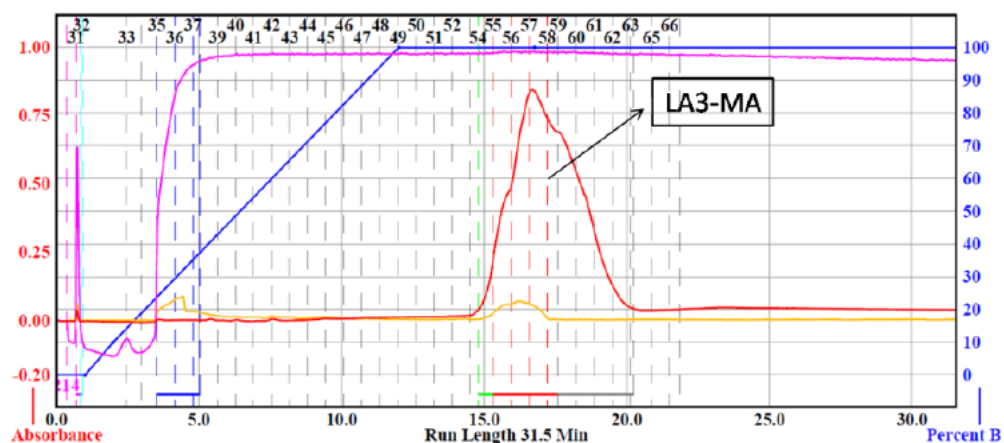


Figure 12. Prep-HPLC chromatogram of LA3-MA.

To confirm the purity and molecular weight of the LA3-MA drug molecule, the LC-MS and LC-MSMS were used. In the LC-MS chromatogram of LA3-MA, it was seen that the obtained purity of the LA3-MA molecule was 99,58% (Figure 13.A). The molecular mass of the LA3-MA (theoretically expected to be 769,2 g·mol⁻¹) was determined as 751 g·mol⁻¹. This result was considered to be the lack of one methacrylic ester group in the LA3-MA molecule. In addition to that, another peak was also been observed, 792,7 g·mol⁻¹, the LA3-MA's 2 Na⁺ ions-bonded version of the LA3-MA drug molecule (Figure 13.B).

To verify the total molecular weight value of LA3-MA, LC-MSMS was used. The total molecular weight of LA3-MA in MS2Scan scanning mode was found to be 792,7 gmol⁻¹ which is the version that holds one sodium ion (23 gmol⁻¹). Also, the mass peak of the +2, ($M^{+2}+2$)/2, 384,8) charged ion state is also seen (Figure 13.C). Additionally, when the MS2SIM target molecular weight detection method was used, the 769,2 gmol⁻¹ molecular weight was observed (Figure 13.D).



Figure 13. LC-MS (A-B) and LC-MSMS (C-D) chromatograms of LA3-MA.

The functional groups in the chemical structure of the LA3-MA, which has three LA and a methacrylate group, were analyzed using an FT-IR spectrophotometer. Figure 14. shows the FT-IR spectra of the LA3-MA molecule in comparison with the starting drug molecule Penta-MA and LA. It is observed that peaks at the frequency of 1741 cm⁻¹, and 1597 cm⁻¹ belong to the methacrylate double bond and the methacrylic ester carbonyl group, respectively. In addition to that, the peaks of C-O (1100 cm⁻¹) which are found only in the Penta-MA structure are also observed in the LA3-MA spectrum. Those prove the methacrylate group in the LA3-MA drug molecule. The symmetrical stretching peaks around 1200-1300 cm⁻¹ indicate that there are aliphatic groups in the LA3-MA drug molecule. The bending peaks of S-S (532 cm⁻¹) and C-S (660 cm⁻¹ and 746 cm⁻¹) which are only seen in the starting free drug molecule LA, are

also observed in the LA3MA drug molecule. Moreover, the absence of the carboxylic acid carbonyl peak at 1682 cm^{-1} which is only seen in free LA structure demonstrated that there is no unreacted and free LA molecule in the LA3-MA drug molecule. All in all, it is proved that both methacrylate and LA molecules are present in the synthesized LA3-MA drug molecule.

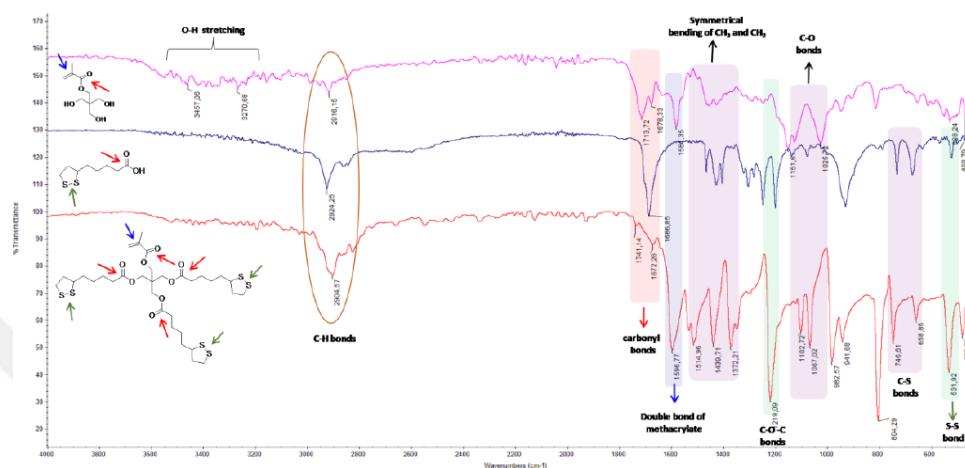


Figure 14. FT-IR Spectra as functional group analysis of LA3-MA.

To show that there is only one methacrylate and three LA molecules in the LA3-MA drug molecule, H-NMR was used. In the H-NMR spectrum of the LA3-MA drug molecule (Figure 15.), it is seen that the double bond peaks at 6,27 and 5,72 ppm, and methacrylic ester protons at 4,17 ppm showed the presence of reactive methacrylate group in the drug molecule. It can be seen that ester protons are in the bond of monomer and the lipoic acid molecules at around 3,7-3,9 ppm. In addition to that, the methacrylic ester peak (two protons) has the h peak (d) in lipoic acid that is seen at 2,07 ppm, (d of d) the integration ratio of three indicates that there are three LA molecules in the LA3-MA. Furthermore, multiplet aliphatic peaks off peaks (1,25 ppm) and g peaks (1,5 ppm) come from the LA molecule and the integration value of each is six, which again indicates that three LA molecules bond to the LA3-MA.

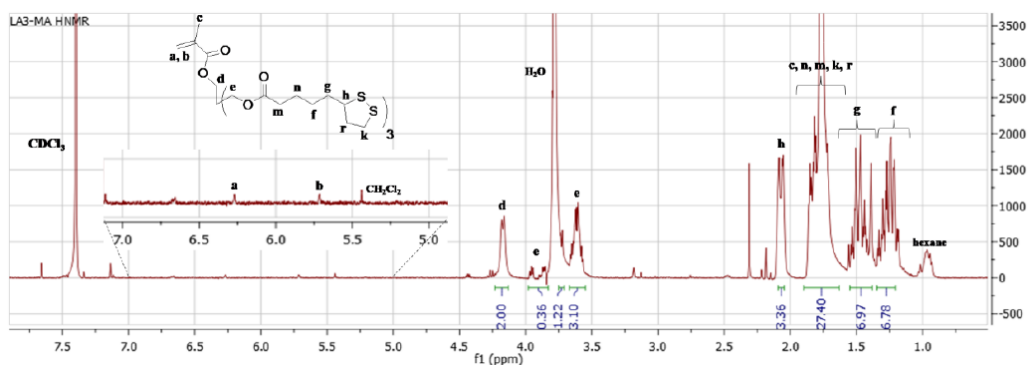


Figure 15. H-NMR analysis of LA3-MA (in CDCl₃).

In the H-NMR spectrum of the LA3-MA, on the other side of the ester group, the m peak belongs to the CH₂ protons. The n, r, and k peaks (1,73 ppm) also represent the CH₂ protons in the disulfide-containing ring of the LA3-MA. Moreover, as expected, it is also observed from the integration value of this multiplet peak, the methyl protons in the methacrylic ester group are seen at 1,73 ppm. All in all, with this analysis, it was proved that the synthesized material is an LA3-MA drug molecule that contains 3 LA molecules and one reactive methacrylate group.

4.2. Nanogel Preparation and Characterization

4.2.1. Control Nanogel (C-NG) Preparation and Characterization

The procedure of nanogel preparation included several parameters: solvent type, temperature, amount of solvent (dilution effect), photo-initiator: polymer ([I]: [P]) ratio, and polymer length. First of all, in order to confirm which type of parameters are the most suitable for nanogel, the polymer of PEG-dimethacrylate (PEG-DiMA, Mwt:750 gmol⁻¹) was used.

Firstly, to optimize which type of solvent was going to be used for the nanogel preparation was detected. Thus, The PEG₇₅₀-DiMA polymer was subjected to a radical reaction in DMF and 10 mM PBS, respectively. The size of the obtained nanogel was measured by a dynamic light scattering (DLS) instrument. When the size measurements that were taken at the 5th minute of the reaction were observed, it was seen that large aggregates started to form (Figure 16.A).

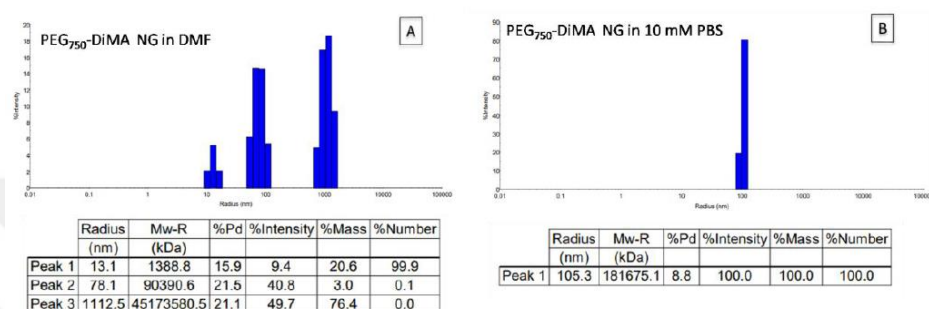


Figure 16. Effect of solvent type on nanogel (NG) preparation. (A) DMF; (B) 10mM PBS.

In the reaction of 10mM PBS, as seen in Figure 16.B, the nano-scaled and monomodal particle was obtained under the same conditions. According to this result, 10 mM PBS was chosen as a solvent for further nanogel preparations.

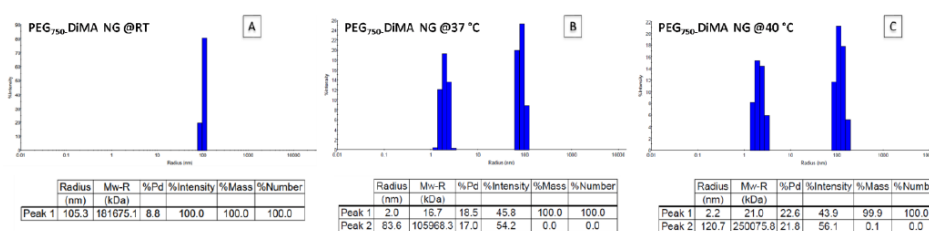


Figure 17. Effect of temperature on nanogel (NG) preparation (A) RT; (B) 37 °C (C) 40 °C.

For the temperature optimization, the nanogels were prepared at three different temperatures: RT (20-22 °C), 37 °C (body temperature), and 40 °C (hyperthermia) with the same procedure above in 10 mM PBS, separately. It was found by DLS

measurement that when the temperature increased, the size of the nanogels became bigger, the polydispersity value increased and branching began to occur. Therefore, RT was decided to be the most suitable temperature for further nanogel preparation.

Table 1. Effect of [I]: [P] ratio on nanogel (NG) preparation (A) 1:50 (B) 1:100 (C) 1:150 (D) 1:200.

A				B			
Time	Radius (nm)	PDI (%)	Intensity (%)	Time	Radius (nm)	PDI (%)	Intensity (%)
5'	119.3	22	100	5'	105.3	8.8	100
10'	101.2	15	98.9	10'	141	29	100
15'	113.1	39.5	100	15'	113	17	100
20'	111	18.1	100	20'	173.2	48	100
25'	101.8	13.6	95.5	25'	131.8	29	96.5
30'	126.4	47.1	73.4	30'	132.6	22	100
60'	152.1	25.5	88.2	60'	113	8	100

C				D			
Time	Radius (nm)	PDI (%)	Intensity (%)	Time	Radius (nm)	PDI (%)	Intensity (%)
5'	148.8	17	100	5'	163.5	23	100
10'	149.9	19	93.4	10'	141.2	26	93.5
15'	103.5	34	78.8	15'	155.1	30	98.9
20'	98.7	11.8	72.2	20'	146.3	28.7	94.5
25'	119.4	15	87.2	25'	109.4	30	98
30'	160.2	38.6	89.6	30'	140.8	23.2	82.2
60'	134.4	29.3	86.7	60'	156.1	11.9	100

In addition to that, both [I]: [P] ratio and reaction time were optimized for the nanogel preparation. First of all, the different [I]: [P] ratios (1:50, 1:100, 1:150, and 1:200) were tested. The samples were taken at different times and the sizes were measured using the DLS instrument. It is seen in Table 1., for 1:100 (moles), the size and % PDI increased as the reaction time increased. Considering the all given data (Table 1.) at different reaction times, it was observed that the most optimum-sized nanogel with the lowest % PDI and the highest density was at the 20th minute of the reaction for the [I]: [P] ratios of 1:50 and 1:100. All in all, the 20th minute of the reaction was decided to be the reaction time parameter for nanogel preparation. It was also seen that there were some nanogels above desired sizes (70-100 nm). Therefore, it was decided to continue with the ratios of 1:50 and 1:100 and with their size reduction optimizations.

Table 2. Effect of dilution on nanogel (NG) preparation (A) PEG750 -DIMA 1]:50 (A) PEG750 -DIMA 1:100 for different time intervals.

A			
Time	2 mL	4 mL	8 mL
	[Radius/ PDI (%) / Intensity (%)]	[Radius/ PDI (%) / Intensity (%)]	[Radius/ PDI (%) / Intensity (%)]
5'	54.1/ 3.6/ 100	70.7/ 8.8/ 100	107.3/ 16.9/ 52.2
10'	76.1/ 11.9/ 100	80.6/ 10.7/ 100	58.4/ 19/ 100
15'	86/ 8/ 100	74.6/ 35.2/ 96.2	62.1/ 19/ 61
20'	89.2/ 8.2/ 100	79.7/ 18.1/ 96.5	76.9/ 11.9/ 100
30'	94.6/ 11.7/ 100	76.8/ 11.9/ 98.4	68.5/ 18.8/ 97.1

B			
Time	2 mL	4 mL	8 mL
	[Radius/ PDI (%) / Intensity (%)]	[Radius/ PDI (%) / Intensity (%)]	[Radius/ PDI (%) / Intensity (%)]
5'	72.1/ 19.8/ 98.3	68.5/ 5.6/ 98.6	61.1/ 16.5/ 98.0
10'	65.5/ 8.7/ 100	59.6/ 11.9/ 100	98.6/ 37.1/ 91.2
15'	81/ 10.4/ 100	57.3/ 10.7/ 100	59.6/ 23.8/ 100
20'	84.8/ 6.5/ 100	61.2/ 11.8/ 100	66.4/ 12/ 98.3
30'	78.6/ 11.6/ 100	68.9/ 17.6/ 96.4	174.2/ 5.3/ 49

In this optimization which is the amount of solvent (dilution effect), the amount of solvent doubled its initial reaction solvent amount and became 4 mL, and quadrupled (8 mL). In Table 2., the size and related data are shown depending on the amount of solvent for different time intervals. It is observed that at the 20th minute, the smallest size was achieved for all dilutions, and as the amount of solvent increased from 2 mL to 8 mL, the sizes of the nanogels decreased for almost all time points. Therefore, 8 mL of PBS was selected as the most optimum amount of solvent for the nanogel preparation.

Table 3. Effect of centrifugation method on nanogel size.

Conditions	Radius (nm)	PDI (%)	Intensity (%)
Before centrifuge	89.7	42.9	92
After centrifuge	66.4	12	98.3

For size reduction optimizations, after the nanogel reaction, the centrifuge was used to remove aggregates and reduce high PDI values which are above ~20%. The nanogel solution was centrifuged at 6000 rpm for 10 minutes. The size and related data

of nanogels were measured before and after centrifugation and it was seen that the centrifugation method was an efficient way to get rid of aggregates and reduce the size and PDI value (Table 3.).

Table 4. Effect of polymer molecular weight and dilution on nanogel size.

PEG-DiMA	1:50/ 4 mL	1:50/ 8 mL
Mwt (kDa)	[Radius/ PDI (%) / Intensity (%)]	[Radius/ PDI (%) / Intensity (%)]
2	128.9/ 15/ 100	107.9/ 17.5/ 94.6
6	98.3/ 11.8/ 95.9	98/ 36.5/ 98.5
10	88.5/ 21/ 86.2	98.8/ 43.9/ 97.4
PEG-DiMA	1:100/ 4 mL	1:100/ 8 mL
Mwt (kDa)	[Radius/ PDI (%) / Intensity (%)]	[Radius/ PDI (%) / Intensity (%)]
2	132.8/ 17.5/ 94.2	95.2/ 11.9/ 92.1
6	100.3/ 24.1/ 96.7	87.5/ 36.4/ 97.3
10	98.6/ 13.3/ 89.1	102/ 28.8/ 96.9

After all optimization studies, the nanogels were decided to be prepared at 1:50 and 1:100 ratios, at RT and in 8 mL of 10 mM PBS in 20'. It was also found that centrifugation was a beneficial procedure to remove aggregates and reduce % PDI. After these optimizations, the polymer of PEG-DiMA (Mwt: 2 kDa, 6 kDa, and 10 kDa) was tested both using 4 mL and 8 mL of 10 mM PBS (Table 4.).

The dilution effect was observed in the 2 kDa PEG-DiMA for both [I]: [P] ratios of 1:50 and 1:100. For the 10 kDa PEG-DiMA, while the effect of dilution was seen especially in size for 1:100, the size of the nanogels which had the ratio of 1: 50 increased. For the 6 kDa PEG-DiMA, the dilution effect was shown at the ratio of 1:100, whereas there was no change in the ratio of 1:50. However, in both the ratios of 1:50 and 1:100, the PDI values increased. Therefore, the centrifugation method was applied after further nanogel preparation.

Table 5. Size analysis of nanogels after centrifugation.

PEG-DiMA	1:50	1:100
Mwt (kDa)	[Radius/ PDI (%) / Intensity (%)]	[Radius/ PDI (%) / Intensity (%)]
2	97/ 21.6/ 97.8	122.7/ 28.4/ 100
6	81.2/ 11.3/ 97.6	92.2/ 19.9/ 95.3
10	75.2/ 14.9/ 96.1	75.8/ 12.8/ 88.7

As seen in Table 5., after centrifugation, there was a significant decrease in size for the nanogels that were obtained with only 6 kDa and 10 kDa PEG-DiMA polymers. However, for the nanogels that were prepared with 2 kDa PEG-DiMA polymer, the size increased for both the ratios of 1:50 and 1:100. Therefore, the 6 kDa and 10 kDa PEG-DiMA polymers were chosen to prepare nanogels for further experiments.

Table 6. Effect of centrifugation and filtration on nanogel size.**A**

PEG-DiMA	Before Centrifuge	After Centrifuge	After Filtration
Mwt (kDa)	[Radius/ PDI (%) / Intensity (%)]	[Radius/ PDI (%) / Intensity (%)]	[Radius/ PDI (%) / Intensity (%)]
6	100.1/ 38.4/ 96.9	86.2/ 22.4/ 97.4	42.8/ 4.4/ 90.5
10	116.2/ 44/ 97.2	149.6/ 80.4/ 96.9	65.3/ 33.9/ 86.6

B

PEG-DiMA	Before Centrifuge	After Centrifuge	After Filtration
Mwt (kDa)	[Radius/ PDI (%) / Intensity (%)]	[Radius/ PDI (%) / Intensity (%)]	[Radius/ PDI (%) / Intensity (%)]
6	94.7/ 26.5/ 94.5	99.1/ 50.2/ 98.4	52.1/ 25.2/ 91.8
10	110.9/ 17.2/ 91.5	106.1/ 36.5/ 93.7	59.5/ 22.9/ 85.6

For further size reduction procedures, in addition to centrifugation, the filtration method was also used (Table 6.). The filtration was conducted using regenerated cellulose (RC) filters (0,22 μ m). The size of the nanogels was measured before centrifuge or filtration and after centrifuge and after filtration. When the sizes were compared, for both 1:50 and 1:100 ratios and both 6 kDa and 10 kDa polymers, the sizes of the nanogels were much smaller after filtration. In addition to their sizes, the PDI values were also much smaller, while the intensity of the nanogels was quite high (>85 %). Furthermore, after the centrifugation method, there was only a decrease in sizes and PDI value when the nanogels were prepared with the ratio of 1:50 and 6 kDa PEG-DiMA polymer. For 10 kDa PEG-DiMA, in general, the size of the nanogels was

much larger and their PDI values were higher for both 1:50 and 1:100 ratios. All in all, when the results were compared, the most suitable nanogel (size: 85,6, PDI: 4,4%, intensity: 90,5%) was with a ratio of 1:50, and the polymer of 6 kDa PEG-DiMA. These parameters were chosen to prepare drug-loaded nanogels.

After all optimizations, the prepared drug-free nanogels or control nanogels (C-NG) were characterized in terms of physical (size and surface charge), chemical (functional group analysis and proton diversity), morphological (SEM and TEM) properties and stability profiles in different temperatures.

Below is the DLS analysis of the nanogel, which was determined to be the most suitable after the optimized procedures (Figure 18.).

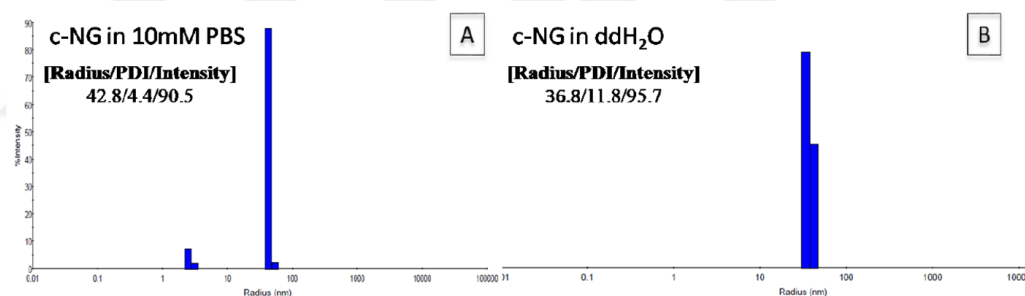


Figure 18. The size of the control nanogel with the most suitable properties.

The surface charge of the C-NG (-8,1 mV) was obtained by using the LitesizerTM 500 (Anton-Paar) instrument (Figure 19.).

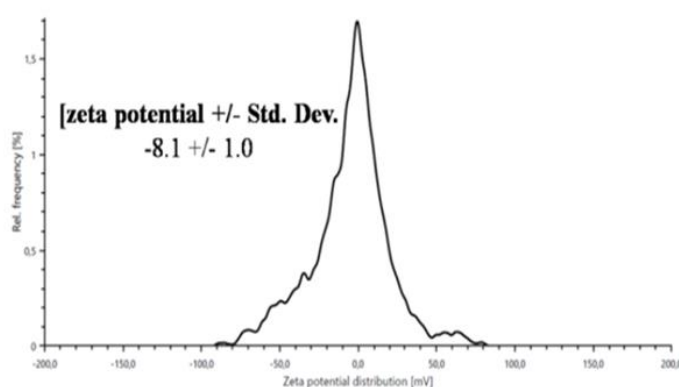


Figure 19. The surface charge (zeta potential) of the control nanogel (C-NG).

For the morphological analysis, Scanning Electron Microscopy (SEM-Quanta650) and Transmissive Electron Microscopy (TEM-Talos L120C) were used. The size of the C-NG was measured and whether there was an aggregate or not was also examined. Figure 20.A-B shows the SEM images of C-NG in both PBS and ddH₂O. It is observed that the morphology of the C-NG was smooth and round structure and was highly dispersed which did not lead to any aggregates. In addition, the SEM images might have shown the smaller size of C-NG compared to obtained DLS measurements.

The C-NG was also visualized with a TEM (Figure 20.C). The C-NG samples were also prepared in ddH₂O. In this TEM image, it is also seen that the C-NG morphology was round, smooth, and nano-sized. Furthermore, in comparison with the previous DLS measurements, it can be said that smaller particles are seen and the C-NGs were dispersed homogeneously.

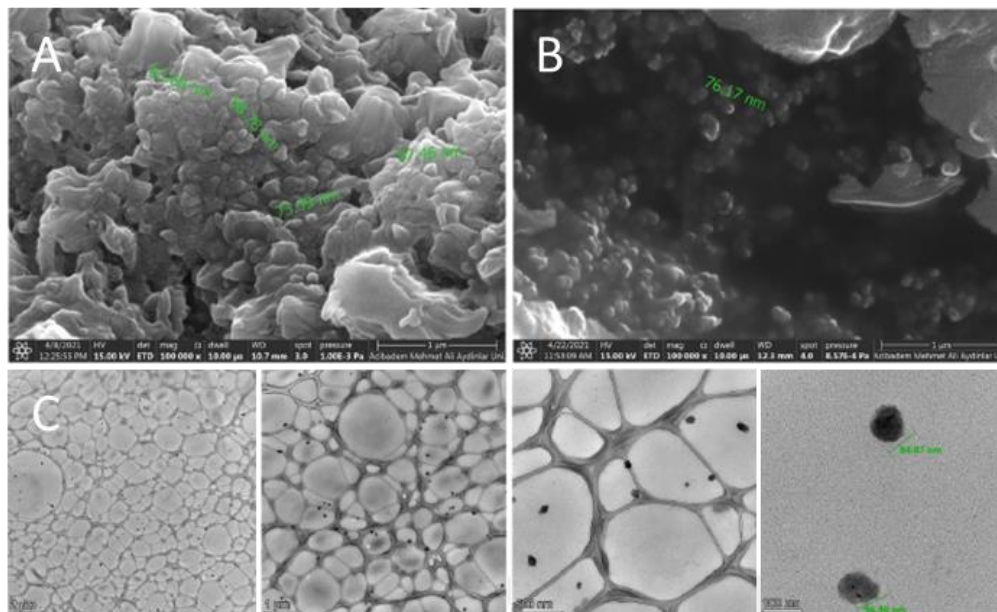


Figure 20. SEM images of C-NG prepared in (A) 10 mM PBS & 1:50 and (B) ddH₂O & 1:100 and (C) TEM images of C-NG at different scale values.

4.2.2.1. The Stability Test of The Control Nanogel (C-NG)

For the stability test, the control nanogel which was made of 6 kDa PEG-DiMA polymer (1:50 and 1:100 ratio) was selected. The stability of this control nanogel which was drug-free had been investigated in specific physiological environments (e.g., inflammation and healthy-environment mimicking) at two different temperatures (RT and 37 °C; body temperature) for over 20 days. The control nanogels which were prepared and optimized as given in earlier procedures were put into 10 mM PBS buffer solution (pH:7.4) for mimicking blood and healthy tissues in the body and acetate buffer solution (pH:5.5) for mimicking the inflammation environment in the body. The samples were taken at specific time points and their size was measured by the DLS instrument.

According to the measured results (Figure 21.), the control nanogels (1:50 and 1:100) were stable and their size did not change for at least two weeks in 10 mM PBS

and acetate buffer solutions at RT. However, the C-NG swelled slightly more when the temperature was at 37 °C for both PBS and acetate buffer solutions and swelled more in the acidic environment (in acetate buffer, pH:5.5) at the same temperature.

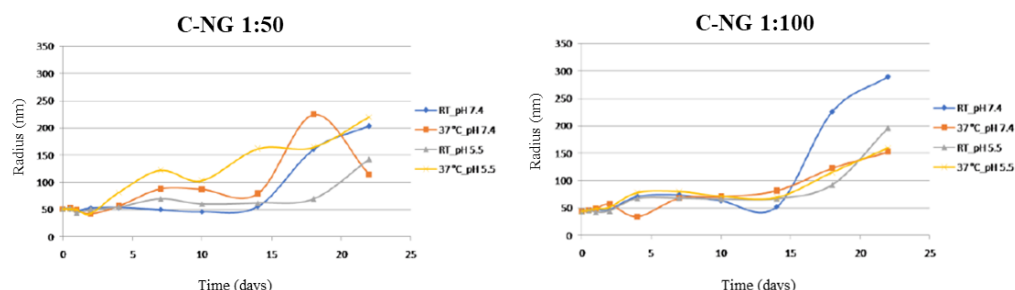


Figure 21. Stability test of control nanogels (C-NG).

For 1:50 C-NG, an increase in size change was observed and the swelling of the nanogels was more at 37 °C than RT for both 10 mM PBS and acetate buffer solutions. While 1:50 C-NG was quite stable for almost two weeks and there was nearly no change in size, especially at RT in 10 mM PBS buffer, the 1:100 C-NG swelled after two weeks at RT in both 10 mM PBS and acetate buffer solutions. Thus, 1:50 C-NG was selected for further drug-loading experiments.

4.2.2. Drug-Loaded Nanogel Preparation

After all optimization, the control nanogel which was made of 6 kDa PEG-DiMA polymer (1:50 [I]: [P] ratio, in 20', in 8 mL of 10 mM PBS buffer and at RT) was used to prepare three different ratios (% by mass) of LA3-MA containing nanogels. The nanogels contained 5, 10, and 20% of LA3-MA. After preparation, the 5% LA3-MA containing nanogel (NG-LA5), 10% LA3-MA containing nanogel (NG-LA10), and 20% LA3-MA containing nanogel (NG-LA20) were subjected to the filtering process for size reduction, and aggregate elimination using 0,22 μm RC filter (for once). However, this filtering was not sufficient for getting rid of low molecular weight

impurities and free drug monomer molecules. Therefore, the “Amicon® Ultra-15 Centrifugal Filter Unit” was used to eliminate low molecular weight impurities and free LA3-MA monomers from drug-loaded nanogels (five times in a row).

After the Amicon filter was used, some recurring aggregates were observed. Therefore, a 0,22 µm RC filter was again used to get rid of the aggregates. This filtration process was repeated 1, 5, and 10 times for the optimization of the LA3-MA containing nanogel.

Table 7. The size measurement of drug-loaded nanogels based on the LA3-MA ratio.

LA3-MA ratio (%)	Radius (nm)	PDI (%)	Intensity (%)
0	46.3	23.6	100
5	43.5	37.5	100
10	31.9	49.3	100
20	52.8	27.2	91.9

Compared to 1 filtration, the samples that were filtered 5 times with a 0,22 µm RC filter showed a decrease in both the size and PDI values. Furthermore, when 10 filtrations were compared to 5 filtrations, the size, and PDI values did not change. Therefore, after the Amicon ultrafiltration, it was decided to filter the nanogel solutions 5 times.

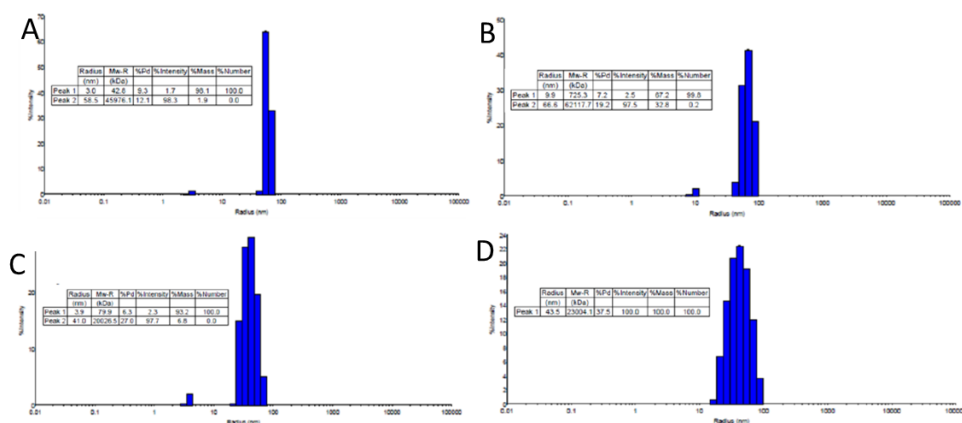


Figure 22. NG-LA5, the nanogel containing 5% LA3-MA (A) after the first filtration, (B) after ultrafiltration 5 times, (C) after filtration 5 times, and (D) after 5 minutes of sonication.

In addition to filtration, in order to reduce aggregation and increase dispersity, sonication was found to be effective. The samples were sonicated for 5, 10, and 20 minutes, respectively and it was investigated that while 5 minutes of sonication was effective in reducing the size and aggregates, 10 or 20 minutes of sonication led to swelling and an increase in the size of the nanogels. All in all, the optimization method for LA3-MA containing nanogels was concluded to be 1 filtration (0,22 μm RC filter), 5 times Amicon ultrafiltration, 5 times filtration (0,22 μm) and 5 minutes sonication, respectively.

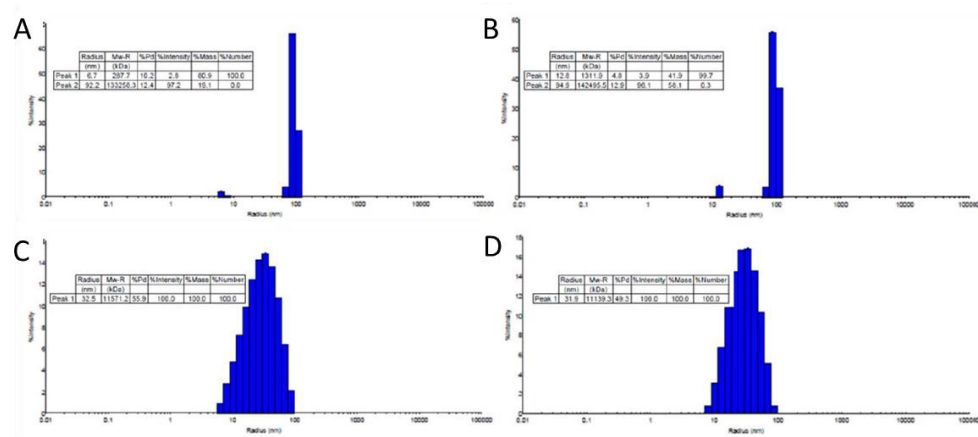


Figure 23. NG-LA10, the nanogel containing 10% LA3-MA (A) after the first filtration, (B) after ultrafiltration 5 times, (C) after filtration 5 times, and (D) after 5 minutes of sonication.

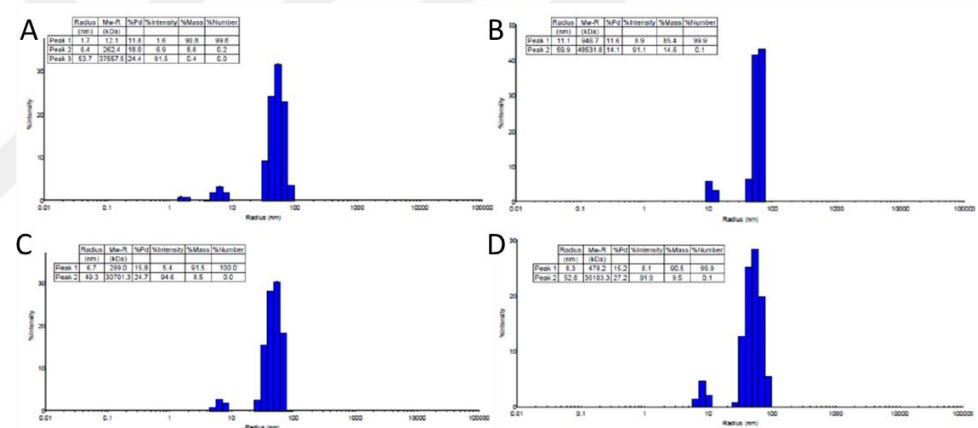


Figure 24. NG-LA20, the nanogel containing 20% LA3-MA (A) after the first filtration, (B) after ultrafiltration 5 times, (C) after filtration 5 times, and (D) after 5 minutes of sonication.

The surface charge (zeta potential) of the drug-loaded nanogels was measured (Table 8.). As seen in Figure 25., while the LA3-MA amount increased, there was a shift through positive value. As the amount of LA3-MA increased, the change in shift also increased (from C-NG to NG-LA20).

Table 8. The surface charge (zeta potential) results of the drug-loaded nanogels based on the LA3-MA ratio.

LA3-MA ratio (%)	Mean zeta potential (mV)	+/- Standard deviation (mV)	Electrophoretic mobility ($\mu\text{m}^2\text{cm/Vs}$)
0	-1.4	2.4	-0.1124
5	0	0.2	-0.0007
10	0.6	1	-0.0455
20	2	0.7	0.1533

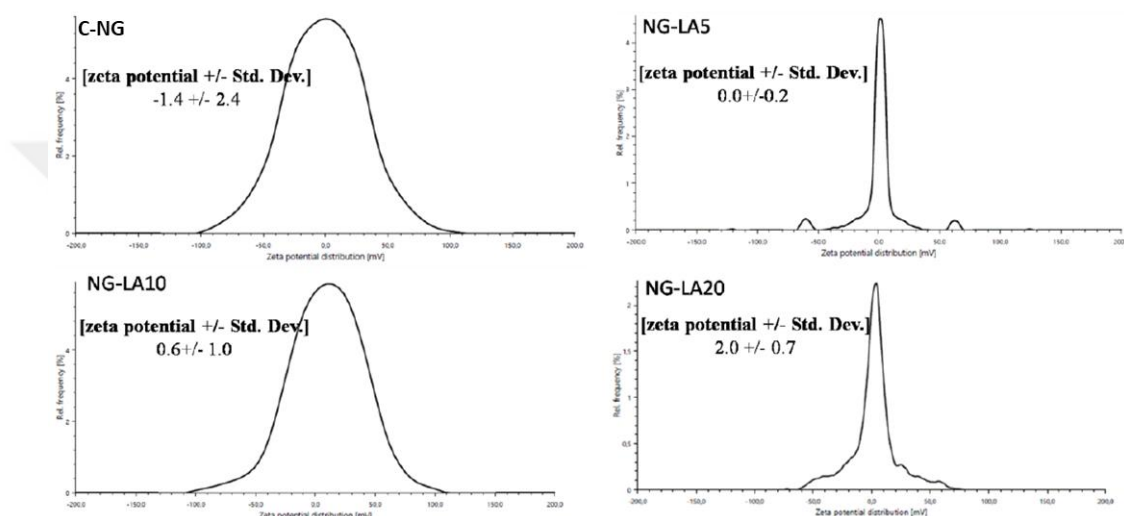


Figure 25. The surface charge (zeta potential) results of the control nanogel (C-NG) and drug-loaded nanogels based on the LA3-MA ratios.

4.2.2.1. Determination Of Drug Release Kinetics from Drug-Loaded Nanogels

To determine the amount of drug loaded into LA3-MA containing nanogels, the LC-MSMS was used. First of all, NG-LA5, NG-LA10, and NG-LA20 were treated with esterase enzyme at 37 °C at 200 rpm for 24 hours to release LA3-MA drug molecules from the nanogel structure. From the given calibration curve, the percentage of the drug-loading capacity of the nanogels was calculated. In addition to that, the drug loading efficiency was calculated (loaded drug amount/the amount of NG-LA obtained). It was investigated that NG-LA20 showed the highest drug-loading

efficiency and total drug amount compared to other drug-loaded nanogels (NG-LA5 and NG-LA10), Table 9. However, when the sizes of the drug-loaded nanogels were compared, it was found that NG-LA20's size (105,6 nm) was bigger than NG-LA5's (87 nm) and NG-LA10's (63,8 nm). Moreover, the PDI value of NG-LA10 was higher than the other drug-loaded nanogels. Therefore, all three drug-loaded nanogels were subjected to a drug-release experiment in two different conditions.

Table 9. Total drug amount analysis results of drug-loaded nanogels.

Amount of PEG6K-DiMA added (mg)	Ratio of LA3-MA added (%)	Amount of LA3-MA added (mg)	Amount of LA3-MA loaded (mg)	The amount of NG-LA obtained (mg)	Drug loading capacity (%)	Drug loading efficiency (%)
27	5	1.42	0.72	18.6	3.7	50.39
27	10	3	2.33	19.8	10.51	77.53
27	20	6.75	6.17	21.5	22.31	91.47

In the drug-release graph (Figure 26.), for NG-LA5, it is observed that the LA3-MA molecule was released at a high rate in the first hours of the experiment. Furthermore, since the NG-LA5 had the lowest total drug amount, it is determined that nearly the total amount of the drug was released almost in the first two days. For NG-LA10, it is seen that the LA3-MA molecules were released in a slow manner. Also, the total amount of the drug from NG-LA10 was released in about a week. In final, it was detected that NG-LA20 showed a much slower and more continuous (sustained) drug release profile than the other drug-loaded nanogels for up to almost two weeks. All in all, when the release profile was compared, NG-LA20 was decided to be used for further experiments due to its being close to the target range which was around 100 nm, and the highest total drug amount (6,17 mg).

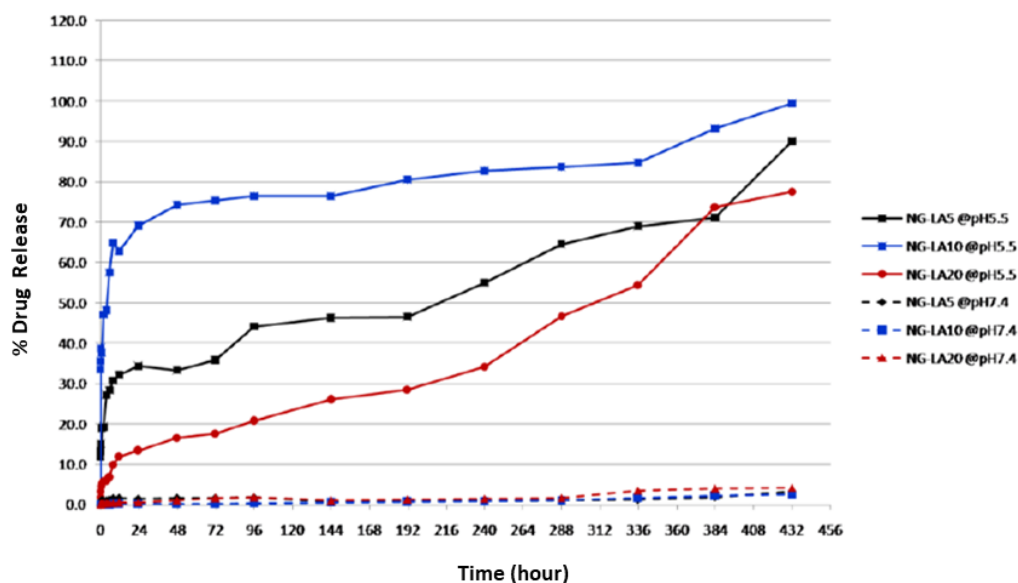


Figure 26. Time-dependent drug release profiles of drug-loaded nanogels.

According to the release test results shown in Figure Serum, it was observed that NG-LA20 released only 4% of the total drug in PBS (pH: 7.4) at the end of the 16th day, and overall, there was no significant change in its release profile. On the other hand, when the esterase enzyme was administered, the drug release increased by around 46% till the 10th day.

In an acidic environment (in acetate buffer pH: 5.5), it was seen that 8% of the total drug was released quickly on the first day. In addition, the drug release increased day by day and showed a controlled manner. Furthermore, it was investigated that the esterase enzyme in this pH significantly increased the release of the total drug by approximately 95% at the end of one week.

When the drug release in serum was examined (Figure 27.), approximately 80% of the total drug was released at the end of the first week, but the measurements made from the first week were fluctuating. As a result of these release studies, it has been shown that NG-LA20 are sensitive to both pH and esterase enzyme and release the

drug according to different factors in the environment, thus allowing the controlled drug release of the prepared nanogels.

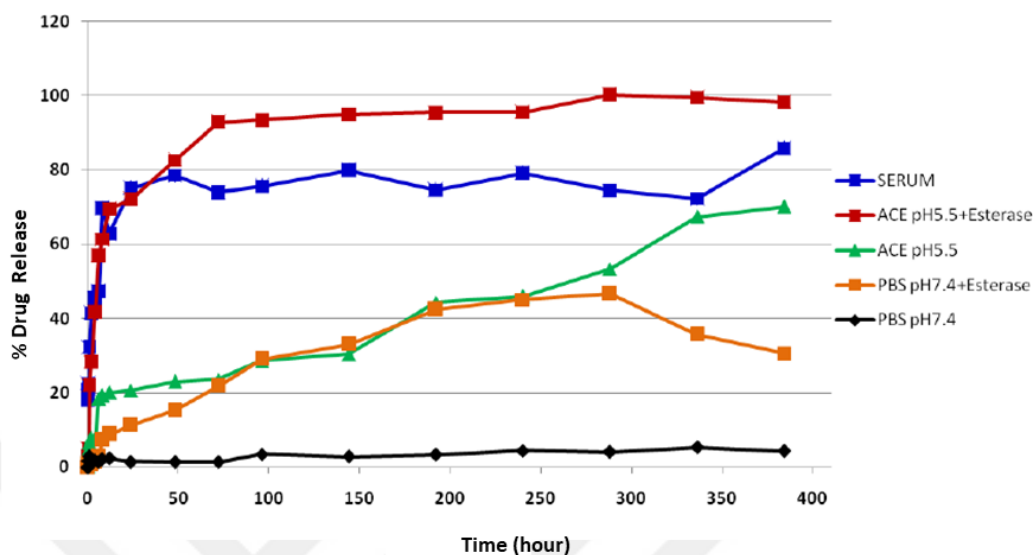


Figure 27. Time-dependent drug release profiles of NG-LA20 in serum, Acetate buffer (ACE), and PBS.

4.2.3. Functionalized Nanogel Preparation and Characterization

The prepared nanogels were conjugated to Cy-5 fluorescent dye for nanogels to be visible for cellular experiments. For this Cy-5 conjugation, the surface of the nanogels was functionalized with amide bonds that are not hydrolyzed and unbreakable in the physiological environment. Due to this functionalization, 2-aminoethyl methacrylate (AE-MA) which has free amino and reactive methacrylate groups was used for radical gelation reaction. After the synthesis of the amine-functionalized nanogels (NG- NH₂), the amine functional group was detected by FT-IR and ¹H NMR and the size and charge were measured by DLS and Litesizer, respectively. Then, to functionalize the synthesized NG- NH₂, the fluorescent dye (Cy-5) and antibody molecule (VCAM-1) were conjugated with the amidation reaction.

4.2.3.1. Amine Containing Nanogel (NG-NH₂) Preparation and Characterization

For the preparation of NG- NH₂, AE-MA monomer was in different mole ratios (1%, 2%, and 4% moles) depending on the amount of polymer. Firstly, PEG-DiMA (Mwt: 750 Da) was used to prepare AE-MA-containing nanogels, and to get rid of aggregates and reduce the size, the NG- NH₂ was subjected to centrifugation (Table 10.). When the sizes of the nanogels (1%, 2% AE-MA, and 4% AE-MA) were compared, 2% AE-MA had the lowest size and PDI values. Therefore, it was decided to continue with this amount of AE-MA (Table 10.).

Table 10. The effect of the centrifugation method on the size of AE-MA containing nanogels.

Conditions	1% AE-MA	2% AE-MA	4% AE-MA
	[Radius/ PDI (%) / Intensity (%)]	[Radius/ PDI (%) / Intensity (%)]	[Radius/ PDI (%) / Intensity (%)]
Before centrifuge	91.4/ 45/ 98	65.2/ 16.3/ 97.9	236.7/ 16.4/ 100
After centrifuge	79.7/ 25.4/ 97.4	69.9/ 16/ 98.8	86.6/ 17.5/ 98.1

After the most suitable amine mole was decided, PEG-DiMA polymers (2, 6, and 10 kDa) were tested for the preparation of amine-containing nanogels. For all polymers, 2% AE-MA was prepared for both in 1:50 and 1:100 ratios ([I]: [P]). The aggregates were observed after the reaction and then, the centrifugation method was used to get rid of the aggregates. Table 11. shows the size measurements before and after centrifugation for both 1:50 and 1:100 ratios. According to the results, 2 kDa PEG-DiMA polymer showed large size, low density, and aggregates. Therefore, it was decided to continue with 6 kDa and 10 kDa PEG-DiMA polymer with 2% AE-MA.

Table 11. The effect of the centrifugation method on the size of 2% AE-MA containing nanogels.

PEG-DIMA Mwt (kDa)	[I]:[P]= 1:50	
	Before Centrifuge	After Centrifuge
	[Radius/ PDI (%) / Intensity (%)]	[Radius/ PDI (%) / Intensity (%)]
2	125/ 12.5/ 97.7	121.4/ 17.6/ 98.3
6	99.3/ 43.7/ 98.2	87.1/ 4.7/ 97.2
10	118.5/ 44.6/ 96.8	112.8/ 48.6/ 96.8
PEG-DIMA Mwt (kDa)	[I]:[P]= 1:100	
	Before Centrifuge	After Centrifuge
	[Radius/ PDI (%) / Intensity (%)]	[Radius/ PDI (%) / Intensity (%)]
2	118.8/ 14.4/ 86.8	103.7/ 9.9/ 73.2
6	113.2/ 47/ 98.2	104.9/ 21.3/ 96.2
10	136.2/ 50.6/ 96.9	96.8/ 40.8/ 89

The 2% AE-MA containing nanogels which were made of 6 kDa and 10 kDa PEG-DiMA polymer were optimized again by 0,22 μm filters to reduce their size for both 1:50 and 1:100 ratios. The results in Table 12. show that the filtration method was more effective than the centrifugation corresponding to their size and PDI values. Even though the PDI values seem relatively high after filtration, it was seen that the aggregates were removed, when the size distribution graph was examined. In conclusion, 2% AE-MA containing nanogels which were made of 6 kDa PEG-DiMA polymer with a ratio of 1: 50 were chosen to continue with the further experiments.

Table 12. The effect of centrifugation and filtration method on the size of 2% AE-MA containing nanogels.

[I]:[P]= 1:50			
PEG-DIMA Mwt (kDa)	Before Centrifuge [Radius/ PDI (%) / Intensity (%)]	After Centrifuge [Radius/ PDI (%) / Intensity (%)]	After Filtration [Radius/ PDI (%) / Intensity (%)]
6	110.6/ 34.6/ 87.7	115.6/ 20.7/ 92.8	55.5/ 19.4/ 87.1
10	169.1/ 87.4/ 95.4	96.7/ 13/ 90.3	64.3/ 24.5/ 84.2

[I]:[P]= 1:100			
PEG-DIMA Mwt (kDa)	Before Centrifuge [Radius/ PDI (%) / Intensity (%)]	After Centrifuge [Radius/ PDI (%) / Intensity (%)]	After Filtration [Radius/ PDI (%) / Intensity (%)]
6	108.1/ 38.4/ 96.6	64.6/ 9.7/ 98.5	42.5/ 27.2/ 79.9
10	96.2/ 26/ 63.8	98.3/ 11.8/ 90.5	72.7/ 34.7/ 89.3

For the 2% AE-MA containing 6 kDa PEG-DiMA nanogels, the high molecular weight impurities with a radius of around 2-4 nm appeared in high concentrations.

Figure 28.A shows the size measurement right after the crosslinking reaction which was quite high (264 nm) in diameter. After it was filtered once by 0,22 μm RC filters (Figure 28.B) and it showed that aggregates were removed and the size of the nanogel was decreased to 100,2 nm in diameter.

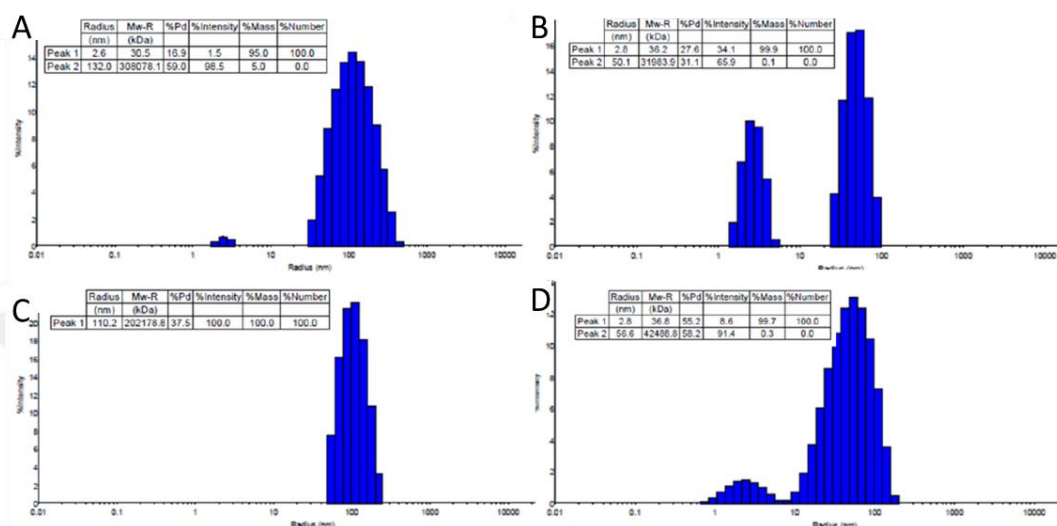


Figure 28. Size measurement results of amine nanogel (NG-NH₂) containing 2% AE-MA monomer. (A) first measurement after gelation, (B) measurement after filtration once, (C) measurement after purification with ultrafiltration tube 5 times, (D) measurement after filtration.

Although the size of the amine-containing nanogel was suitable, there were still unwanted impurities with a density of 34,1%, molecular weight of 36,2 kDa, and size of 5,6 nm. In order to remove these small impurities, the “Amicon® Ultra-15 Centrifugal Filter Unit (Mwt cut-off:50 kDa)” was used. The nanogel solution was centrifuged five times at 4000 rpm for 3 minutes. After centrifugation, the size of the nanogel was measured and it was observed that all small impurities were eliminated (Figure 28.C). However, the size of the nanogels was increased. To reduce the size, the nanogels once again were filtered by a 0,22 μm filter. It was observed that nanogels were dispersed better and the density was reduced from 34,1% to 8,6% and the size of the nanogel was reduced to its targeted range (Figure 28.D). After all, the optimization

steps were concluded into three steps: filtering once with 0,22 μm , centrifugation 5 times, and filtering once again with a 0,22 μm filter.

After all the purification and optimization of amine-containing nanogel which is mentioned above, the surface charge of the 2% AE-MA containing 6 kDa PEG-DiMA nanogels was measured by LitesizerTM 500.

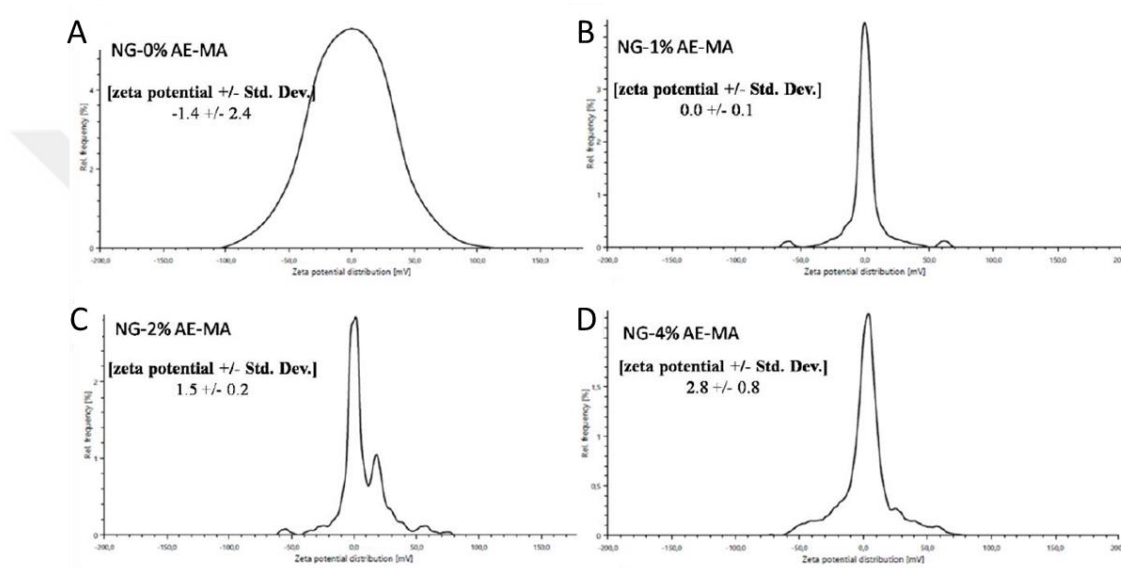


Figure 29. Surface charge measurements of the amine-containing nanogels.

According to the results given in Figure 29., it is observed that the surface charges of the amine-containing nanogels increased. In addition, as the amount of AE-MA increased, it shifted in the positive direction in correlation with the amount of AE-MA monomer. This increasing profile indicated that the AE-MA monomer was successfully incorporated into the nanogel structure.

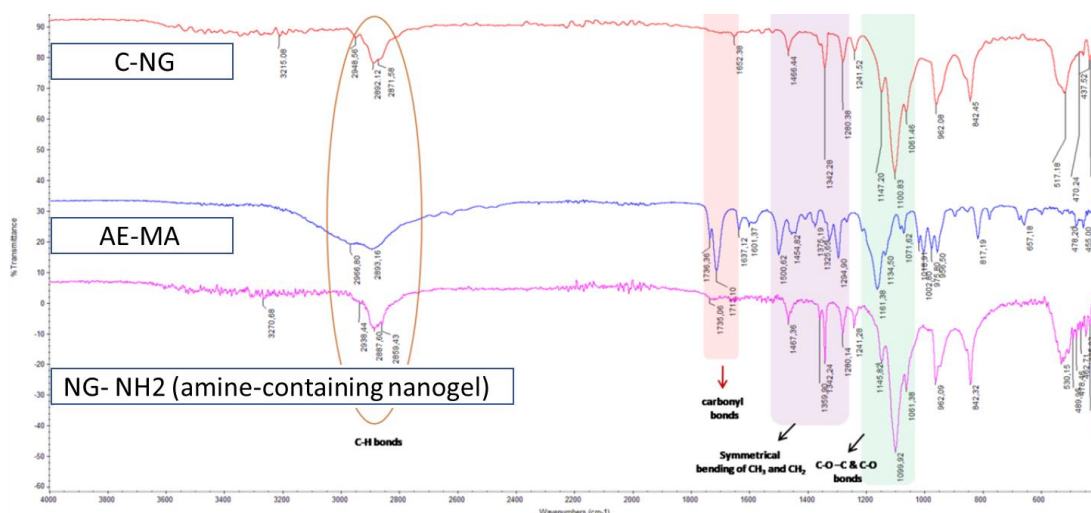


Figure 30. The FT-IR spectrum of NG-NH₂, in comparison with C-NG and AE-MA FT-IR spectra.

In order to confirm the AE-MA monomer integration into nanogel structure, FT-IR spectroscopy was used (Figure 30.). When the NG-NH₂ spectrum was compared to pure AE-MA monomer, the stretching peaks of C-O-C bonds (1145 cm⁻¹), ester carbonyl (1736 cm⁻¹), and amide were overlapped and indicated that the nanogel was amine functionalized. In addition to that, the increase in the width of the C-H bonds (around 2950 cm⁻¹) compared to C-NG demonstrated the added labile protons which were from the -NH₂ functional group to the nanogel structure.

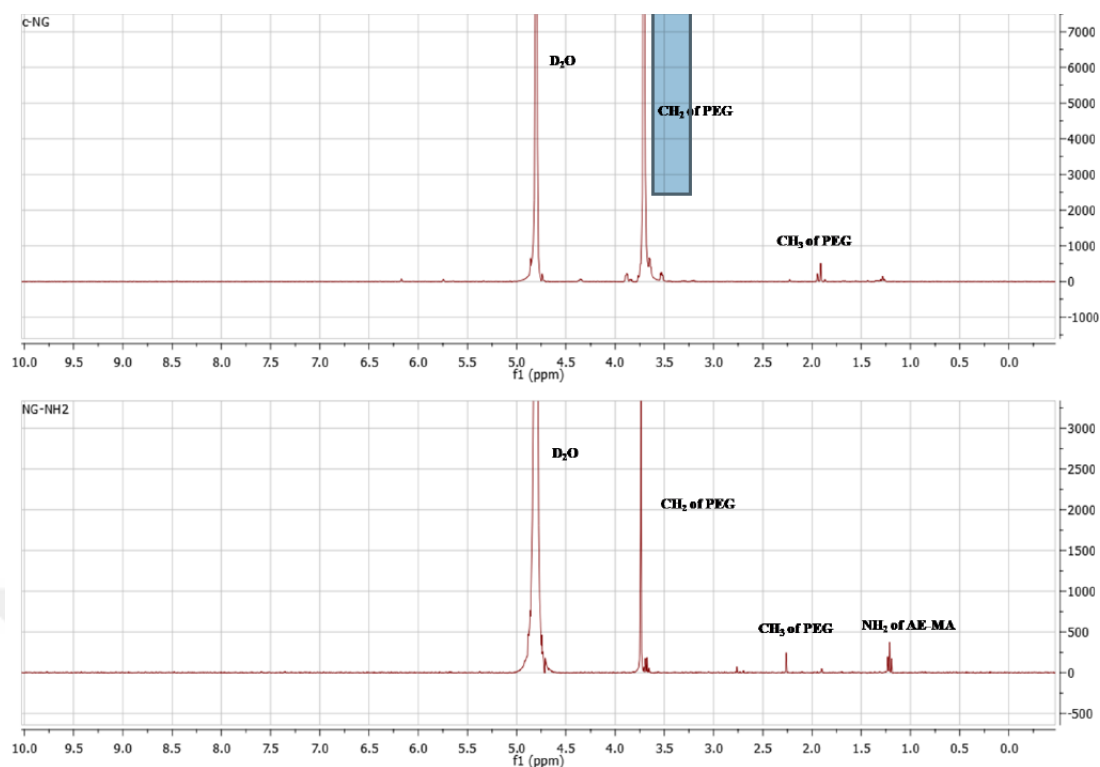


Figure 31. Comparison of ^1H NMR spectra of control nanogel (NG-C) and amine-containing nanogel (NG-NH₂).

The presence of an amine functional group was also investigated using ^1H NMR spectroscopy. When the ^1H NMR spectra of C-NG and NG-NH₂ were compared (Figure 31.), the multiplet peak at 1,24 ppm represented the protons that belonged to the primary amine ($-\text{NH}_2$) group in the structure of the nanogel. In addition, the singlet peak at around 2,7 ppm represented methyl $-\text{CH}_3$ protons of the methacrylate groups in both the AE-MA monomer and PEG-DiMA polymer. In final, the peak at 3,6 ppm represented the $-\text{O}-\text{CH}_2$ protons that belonged to the structure of PEG polymer and could be corresponded to the amine functional group. All in all, it was shown that the nanogel contained amine functional groups in its structure.

4.2.3.2. Cy5 Fluorescent Dye-Bounded Nanogel (NG-Cy5) Preparation and Characterization

Cy5 fluorescent dye was conjugated to the surface of the NG-NH₂ nanogel. Cy5 fluorescent dye-bounded nanogel (NG-Cy5) was characterized both physically (e.g., morphologically, TEM) and chemically. In addition, the amount of Cy5 fluorescent dye that was bounded on the surface of the nanogel was quantitatively measured by LC-MSMS instrument.

In order to get rid of impurities and excess molecules (e.g., Cy5), the optimized procedure of NG-NH₂ was used for NG-Cy5 purification. According to the given size measurement of the NG-NH₂ (Figure 32.A), the both higher size of the nanogel and the aggregates were seen after Cy-5 binding to NG-NH₂ (Figure 32.B). The NG-Cy5 was centrifuged with the “Amicon® Ultra-15 Centrifugal Filter Unit” and it was observed that small molecules and the aggregates were eliminated (Figure 32.C). However, it was investigated that the size of the NG-Cy5 increased. Therefore, the NG-Cy5 was filtered with 0,22 µm RC filters and an around 91 nm sized of NG-Cy5 was obtained (Figure 32.D).

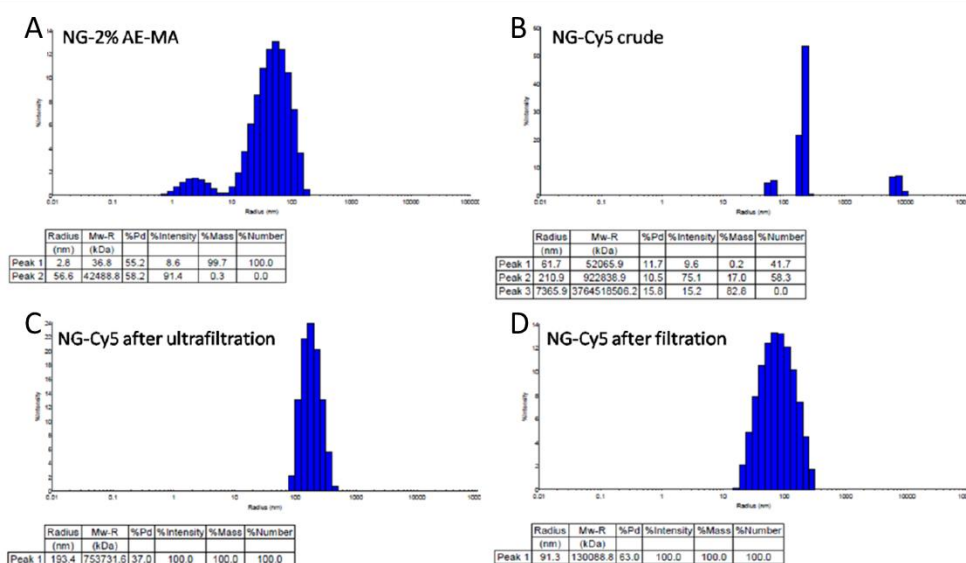


Figure 32. Comparative size measurements of the NG-Cy5.

To determine the Cy5 conjugation to NG-NH₂, the surface charge (zeta potential) of the NG-Cy5 was measured by LitesizerTM 500. As observed in Figure 33.A-B, the surface charge of the nanogel was shifted from positive to negative values when the amine-containing nanogel was functionalized with Cy5 fluorescent dye (from 1.5 ± 0.2 to -4.1 ± 0.5). It proved that the Cy5 conjugated onto the surface of the NG-NH₂ successfully.

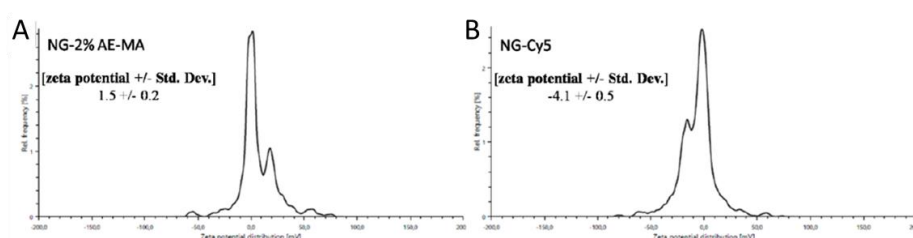


Figure 33. Surface charge measurements of the Cy5- bounded nanogels (NG-Cy5) in comparison with the 2% amine-containing nanogel (NG- 2% AE-MA).

It is seen in Figure 34., the FT-IR spectrum of the NG-Cy5 was compared with the starting materials of fluorescent dye Cy5 and the amine-containing nanogel (NG-NH₂). It was observed from the very prominent chemical functional groups, bending

and stretching peaks of the interatomic bonds especially, at the bending peaks of aromatic groups ($600\text{--}700\text{ cm}^{-1}$) in both the Cy5 structure and NG-Cy5 indicated that Cy5 was conjugated to the nanogel successfully.

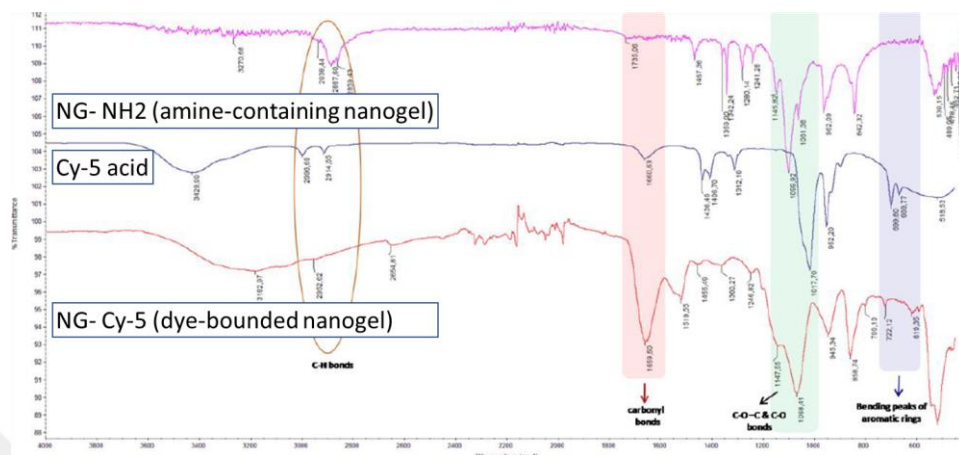


Figure 34. The FT-IR spectrum of NG-Cy5, in comparison with Cy5 acid and NG-NH₂ FT-IR spectra.

As seen in Figure 35., in the ^1H NMR spectrum of the NG-Cy5, the fluorescent dye molecule, Cy5, was chemically bonded to the surface of the NG-NH₂ by amide bonds. The ^1H NMR spectrum of the NG-Cy5 was compared to the spectrum of the initial starting material NG-NH₂. The peaks of the $-\text{CH}_2$ (3,65 ppm) and $-\text{CH}_3$ (2,75 ppm) protons of the PEG polymer came from the structure of the nanogel. The peaks at around 1,24 ppm which came from the protons of the amine group of the NG-NH₂ decreased after Cy5 was conjugated to the nanogel. Furthermore, the low intensity of the peaks at around 7 ppm (aromatic protons, Ar-H) of the NG-Cy5 was thought to come from the aromatic rings in the chemical structure of the Cy5 molecule.

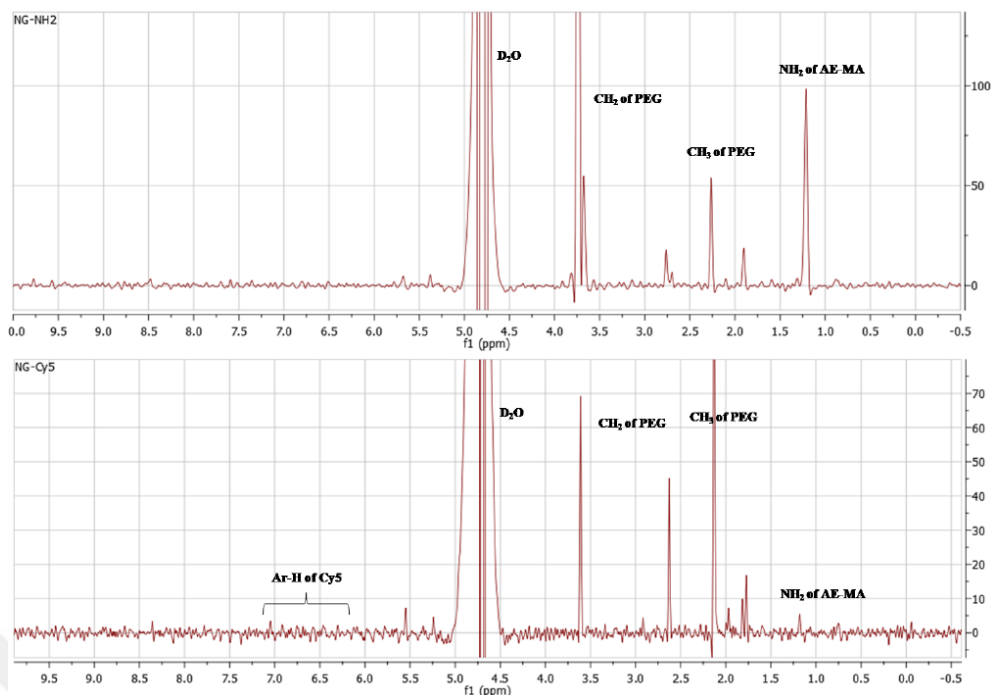


Figure 35. Comparison of ^1H NMR spectra of amine-containing nanogel (NG-NH₂) and Cy5-bounded nanogel (NG-Cy5) in D₂O.

The morphological analysis of the NG-Cy5 was performed using a TEM instrument (Talos L120C). As seen in Figure 36., the NG-Cy5 had a spherical shape and the inner (core) part of the nanogel was filled. Also, the diameter of the nanogels was found to be 80 ± 10 nm. In addition, it was observed that NG-Cy5 was dispersed homogeneously.

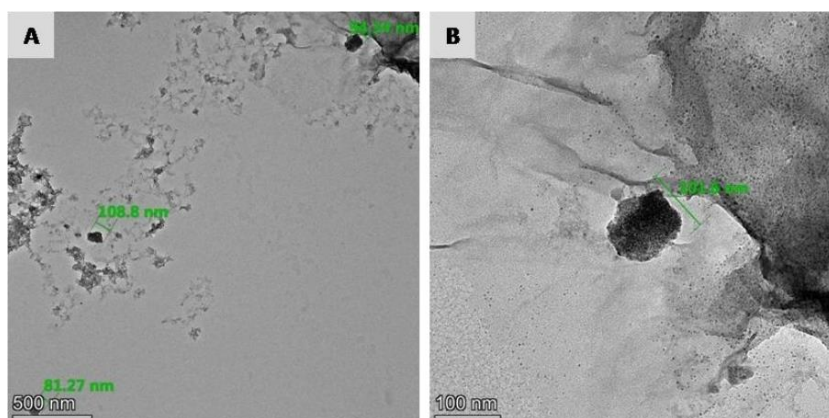


Figure 36. TEM images of NG-Cy5.

In order to determine the amount of dye attached to the surface of the nanogels, the LC-MSMS instrument (the MRM method) was used. The calculation of the dye concentration was made by using a pre-plotted calibration curve of the Cy5 fluorescent dye molecule. As a result, it was found that there was 400 µg/ mL dye in the NG-Cy5 solution.

As seen in Figure 37., the size of the drug-loaded and Cy5-bounded nanogels (NG-LA20-Cy5) had a diameter of 298,6 nm in DLS analysis Figure (37.D), and 157,3 nm in diameter in TEM analysis (Figure 37.C). In the SEM analysis, it was seen that the nanogels were quite round and had smooth morphology, did not form aggregation, and had diameters between 100-150 nm (Figure 37.A-B). In addition, the zeta potential (surface charge) of the NG-LA20-Cy5 was found $-35,9 \pm 8,28$ mV.

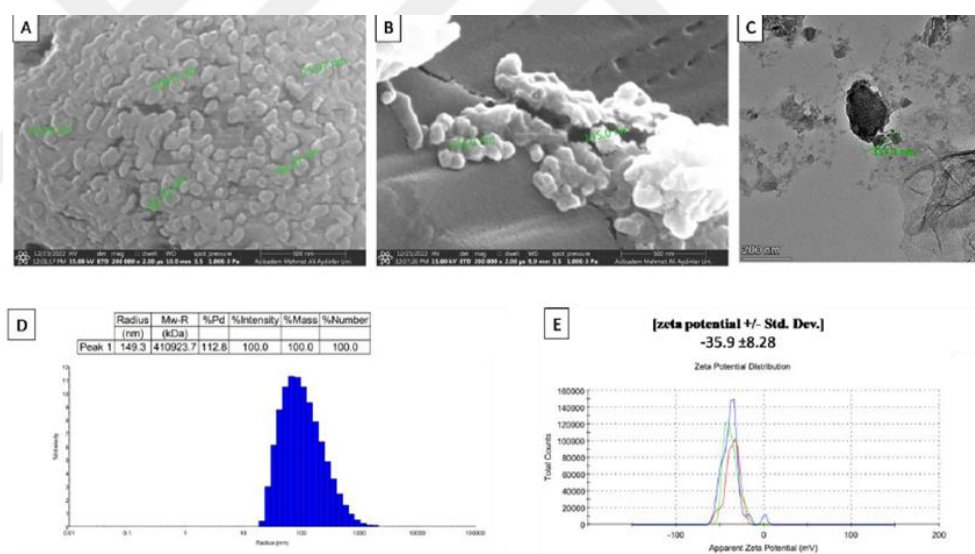


Figure 37. Characterization details for drug-loaded and Cy5-bounded nanogel NG-20LA-Cy5. (A, B) SEM images; (C) TEM image; (D) dimensional analysis; (E) surface charge analysis.

To evaluate the results, it was investigated that the obtained NG-20LA-Cy5 had a drug loading efficiency of 75,77%, which is lower than expected when compared to the NG-LA20 which was prepared without AE-MA (91,47%). On the other hand, the amount of Cy5 was calculated to be 598,4 µg/mL in these nanogels, which is higher than the LA-free and Cy5 bounded nanogels (400 µg/mL) itself (Table 13.).

Table 13. The drug-loading details of the drug-loaded and Cy5-bounded nanogel (NG-LA20-Cy5).

Sample Name	Amount of PEG6K-DlMA added (mg)	Ratio of LA3-MA added (%)	Amount of LA3-MA added (mg)	Amount of LA3-MA loaded (mg)	The amount of NG-LA obtained (mg)	Drug loading capacity (%)	Drug loading efficiency (%)
NG-LA20-Cy5	27	20	6.75	5.11	15.8	24.45	75.77

4.2.3.3. Anti-Vcam1 Antibody Bounded Nanogel (NG-Cy5-ANT and NG-LA20-Cy5-ANT) Preparation and Characterization

After NG-Cy5 was prepared and optimized, the anti-VCAM1 antibody was conjugated onto remaining free amine groups (all amine groups: remaining free amine groups, 1: 0,6 moles) which were on the surface of the nanogels. After the antibody was conjugated, the optimization steps (centrifugation five times with the Amicon filter units, and filtering once with the 0,22 µm RC filter) were applied to the antibody and Cy5-bounded nanogels (NG-Cy5-ANT). Right after the antibody conjugation, both very large particles around 800 nm in diameter and small particles around 20 nm in diameter were observed (Figure 38.B). In order to get rid of the impurities (e.g., free antibodies) and reduce the size, the centrifugation method was performed and it was seen that small-sized impurities were eliminated (Figure 38.C) However, there were still some aggregates with a radius of 650 nm. Once again, the nanogels were filtered using 0,22 µm RC filters, and although the size of the nanogels decreased in the range of the expected size, some aggregates were still seen (Figure 38.D).

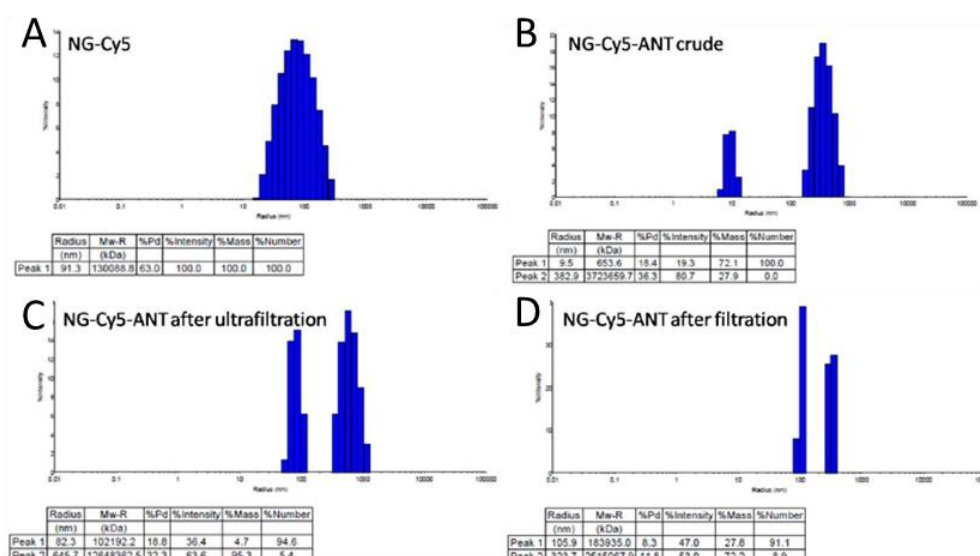


Figure 38. Comparative size measurements of the NG-Cy5-ANT.

The fluorescent and antibody conjugation of NG-Cy5-ANT could be also shown by surface charge analysis. As seen in Figure 39., it was investigated that the surface charge (zeta potential) of the NG-Cy5 was negative and it was shifted through positive values by antibody conjugation (from -4.1 ± 0.5 to 0.8 ± 1.2). All in all, this surface charge indicated the surface modification (antibody conjugation) on NG-Cy5.

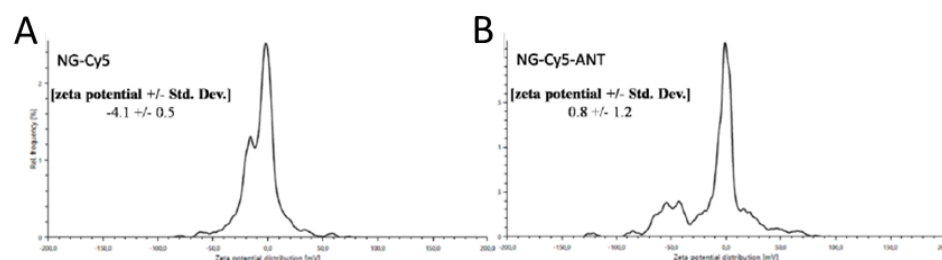


Figure 39. Surface charge measurements of the Anti-VCAM1 antibody and Cy5-bounded nanogels (NG-Cy5-ANT) in comparison with the Cy5-bounded nanogels (NG-Cy5).

The presence of the antibody in the structure of the nanogels was demonstrated using FT-IR spectroscopy. In the FT-IR spectra of the initial starting materials anti-VCAM1 antibody and NG-Cy5 and NG-Cy5-ANT were seen in Figure 40. that NG-

Cy5-ANT consisted of both the aromatic peaks of Cy5 fluorescent dye molecule (around 600-700 cm^{-1}) and the peaks that came from the structure of the nanogel itself (C-O-C between 1150-1050 cm^{-1} , ester carbonyl at around 1670 cm^{-1} and C-H at around 2950 cm^{-1}). In addition, the common secondary amide carbonyl peaks that were observed in both the antibody and the NG-Cy5-ANT at around 1622-1634 cm^{-1} indicated that the antibody was conjugated to NG-Cy5 successfully.

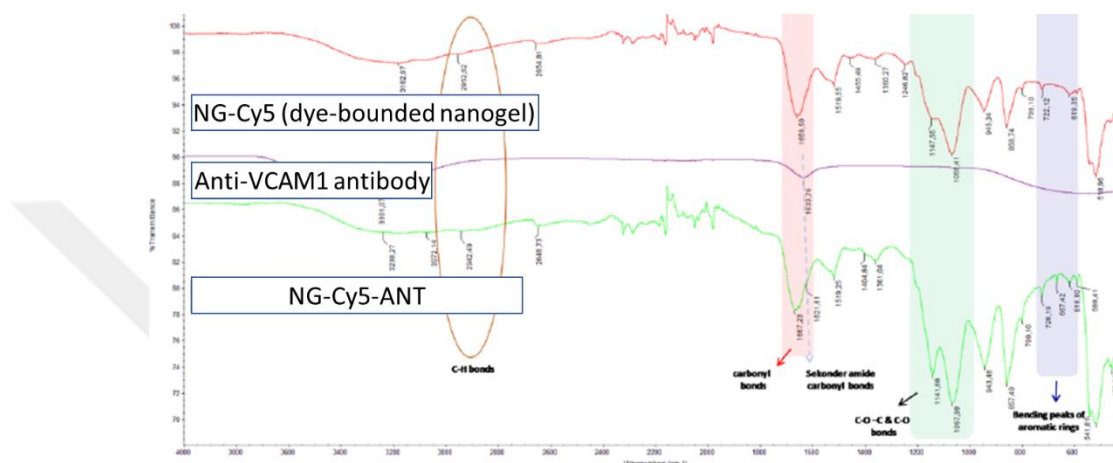


Figure 40. The FT-IR spectrum of NG-Cy5-ANT, in comparison with Anti-VCAM1 antibody and NG-Cy5 FT-IR spectra.

In order to confirm antibody conjugation to NG-Cy5, the chemical structure of the NG-Cy5-ANT was examined using ^1H NMR spectroscopy. As observed in Figure 41., when compared to NG-Cy5, the peaks of $-\text{CH}_2$ and $-\text{CH}_3$ which came from the structure of PEG polymer were also observed in the structure of NG-Cy5-ANT at the expected ppm. Furthermore, the peaks of $-\text{NH}_2$ groups which were at 1,24 ppm only appeared in the ^1H NMR spectrum of NG-Cy5 but not in NG-Cy5-ANT, which indicates that all free amine groups were saturated with anti-VCAM1 antibody. Also, the singlet peak that appeared at 8,45 ppm (amide-NH protons) only in the ^1H NMR spectrum of NG-Cy5-ANT may also indicate that the antibody was conjugated to the NG-Cy5 successfully. On the other hand, the small peaks at around 7,0 ppm which belong to the protons of Cy5's aromatic rings showed that NG-Cy5-ANT had fluorescent dye conjugation as well. All in all, it was observed that both dye and antibody conjugation was performed successfully.

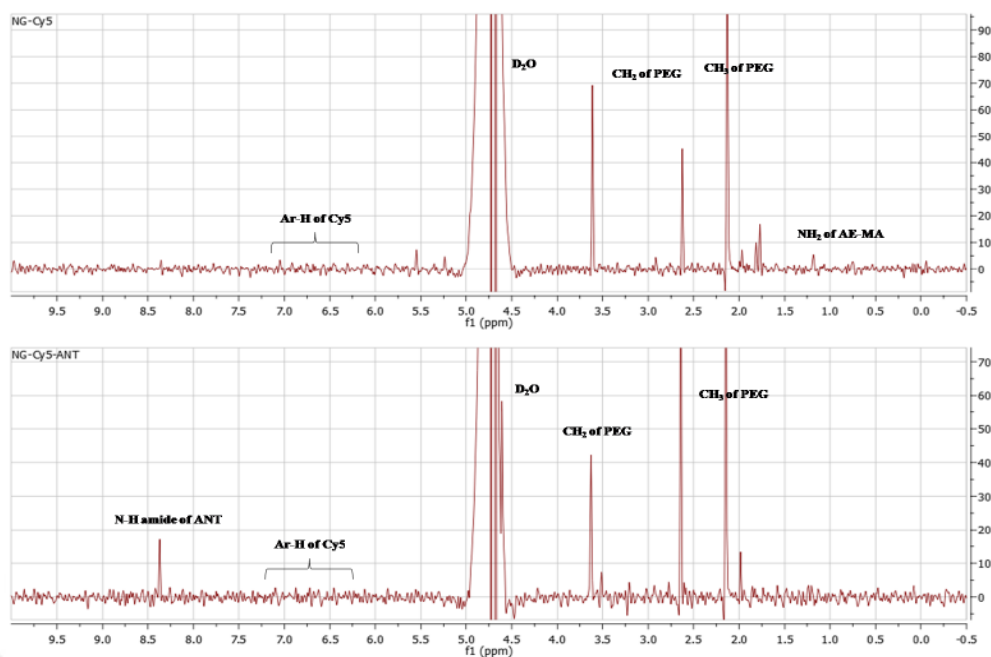


Figure 41. Comparison of ¹H NMR spectra of Cy-5-bounded nanogel (NG-Cy5) and Anti-VCAM1 antibody and Cy5- bounded nanogels (NG-Cy5-ANT) in D₂O.

The morphological analysis of the NG-Cy5-ANT was performed using a TEM instrument (Talos L120C). As seen in Figure 42., although there were nano-sized spherical nanogels, there were very large aggregates at around 1-2 μ m diameter in size as well. It is seen that the nanogels whose sizes were between 120-140 nm were compatible with the size measurement analysis that was performed by using the DLS instrument.

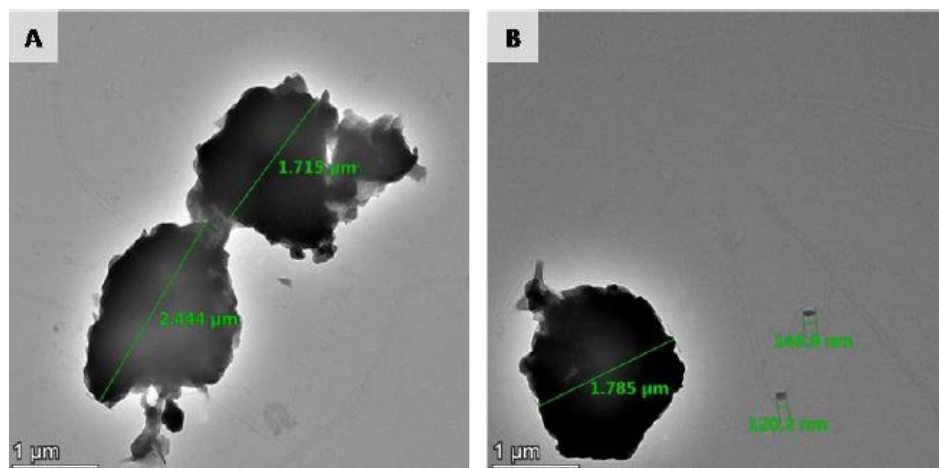


Figure 42. TEM images of NG-Cy5-ANT.

Antibody amount was calculated according to the pre-plotted graph of BSA standard at 480 nm by nanodrop. The amount of anti-VCAM1 antibody that was conjugated to nanogel was found to be 0,573 $\mu\text{g/mL}$.

The same optimized procedure that was performed for NG-Cy5-ANT, except 10 minutes of sonication steps, was applied to drug-loaded and antibody and Cy5-bounded nanogels (NG-LA20-Cy5-ANT). After centrifugation with the Amicon filter units, NG-LA20-Cy5-ANT was sonicated for 10 minutes and then filtered once with the 0,22 μm RC filters. It is seen in Figure 43. that the size of the NG-LA20-Cy5-ANT was 316,6 nm in diameter (DLS measurement) and 198,6 nm in diameter (Figure 43.C). When the surface charge of the NG-LA20-Cy5-ANT was compared to NG-LA20-Cy5, the surface charge increased from -35,6 mV to -30,3 mV. For the morphological analysis of the NG-LA20-Cy5-ANT, as seen in the SEM and TEM images (Figure 43.A-C), the size of the NG-LA20-Cy5-ANT was between 200-300 nm in diameter and had good dispersion. Therefore, it was decided that NG-LA20-Cy5-ANT was a sufficient candidate for in vitro biological assays.

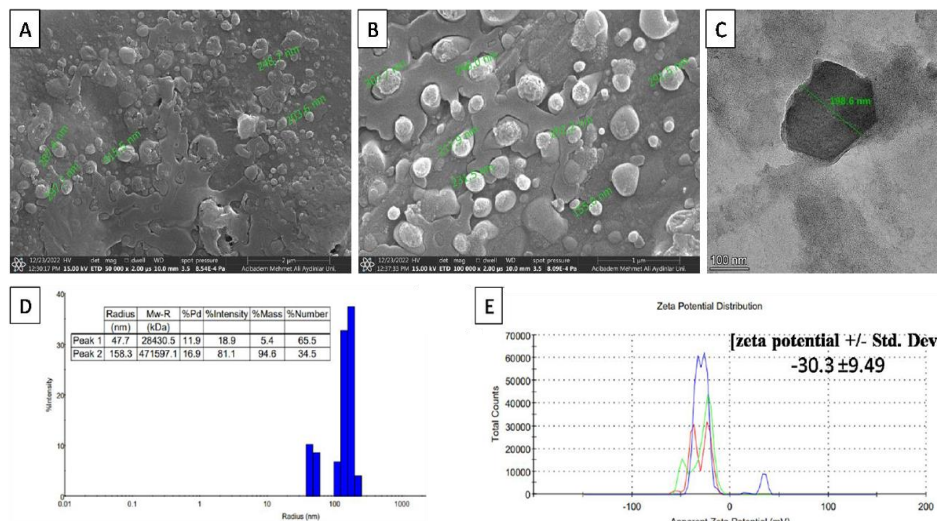


Figure 43. Characterization details for drug-loaded, anti-VCAM1 antibody and Cy5-bounded nanogels (NG-LA20-Cy5-ANT) (A, B) SEM images; (C) TEM image; (D) dimensional analysis; (E) surface charge analysis.

4.3. In Vitro Biological Assays

4.3.1. Development of Atherosclerosis Disease Model in In Vitro Cell Culture

The in vitro modeling of atherosclerosis disease was modeled using Human Umbilical Vein Endothelial Cells (HUVEC). The HUVEC were activated with bacterial lipopolysaccharides (LPS), a bacterial endotoxin, to create an inflammatory environment. For this inflammation model, 10×10^3 cells/well were seeded on a 96-well plate, and different concentrations of LPS (10 ng/ mL, 50 ng/ mL, and 100 ng/ mL) were applied for 6 hours at 5% CO₂ and 37 °C. To observe the change in inflammation, the most prominent inflammation biomarker, Interleukin 6 (IL-6) was quantified by the ELISA kit.

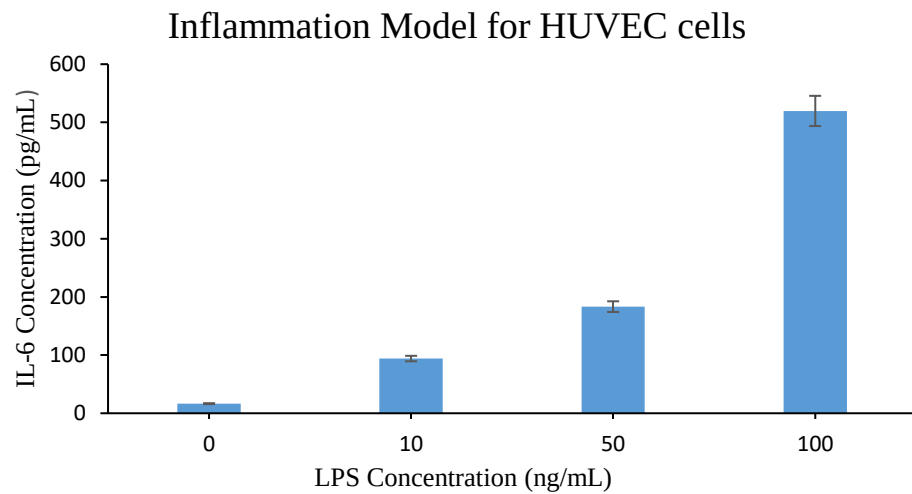


Figure 44. Change in the amount of IL-6 protein produced by HUVEC cells depending on the LPS concentration.

As seen in Figure 44., in general, in direct proportion to LPS concentration, the amount of IL-6 increased. First of all, even though the cells were not treated with LPS, still showed a very low (16,5 pg/mL) concentration of IL-6. When 50 and 100 ng/mL of LPS were applied to HUVEC, the concentrations of IL-6 were found to be 94 and 519,7 pg/mL, respectively. Based on these given results, it was decided to continue the next experiments with 100 ng/mL LPS.

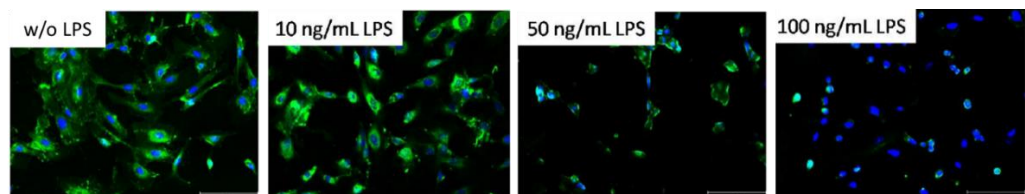


Figure 45. An increasing LPS concentration on HUVEC's morphology.

It can also be seen in Figure 45., depending on the dosage applied to HUVEC, there were morphological changes. It was observed that, as the LPS concentration increased, the HUVEC's cell membrane was disrupted and took much less space compared to healthy HUVEC (w/o LPS).

When the morphological changes were examined with the IL-6 concentration measurements (Figure 44. and 45.), there was a direct correlation depending on the LPS concentration and cell membrane disruption.

4.3.2. Evaluation of In Vitro Cytotoxicity of Drug-Loaded Nanogels

The cytotoxicity of all prepared nanogels was performed using the CCK-8 assay kit for normal human skin fibroblasts, and HUVEC cell lines. To compare the cytotoxicity of the prepared drug-loaded and targeted nanogels (NG-LA20-Cy5 and NG-LA20-Cy5-ANT), drug-free or control nanogel (NG-C) and free drug molecules (free LA) were used as control groups. The prepared nanogels were administered to the normal skin fibroblasts, and then, to the HUVEC cells.

As seen in Figure 46., the control nanogel (NG-C), especially at 10,50 $\mu\text{g}/\text{mL}$ and lower concentrations (dilutions up to 0,04 $\mu\text{g}/\text{mL}$), did not have any significant cytotoxic effect on skin fibroblasts. In addition to NG-C, all prepared nanogels did not show any cytotoxicity at all.

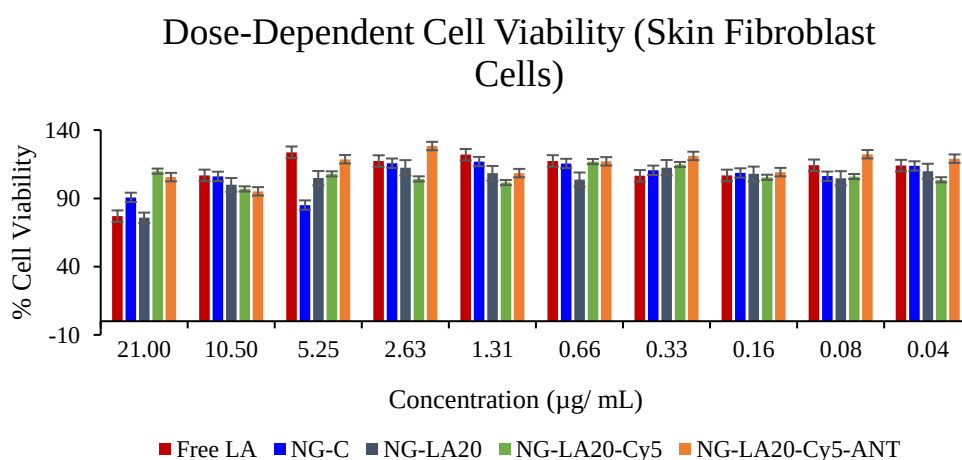


Figure 46. Cytotoxicity test on normal human skin fibroblast cells.

Although the prepared drug-loaded and non-loaded nanogels did not show cytotoxic effects against fibroblast cells, it was also investigated for the HUVEC cells which were used for modeling atherosclerotic environment.

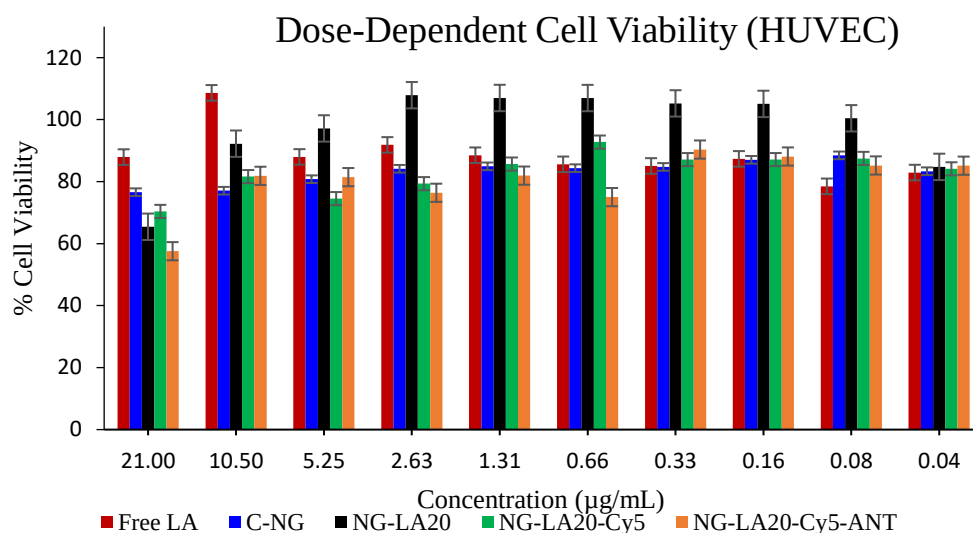


Figure 47. Cytotoxicity test on HUVEC cells.

It is seen in Figure 47., no cytotoxic effect was observed for the serial dilution groups, the concentration of the LA changed from 10,5 µg/mL to 0,041 µg/mL for LA-containing nanogel groups, and the percentage of the HUVEC cell viability was mostly around 80 and above. This indicated that all prepared nanogels did not show any cytotoxic effects on HUVEC cells.

4.3.3. Evaluation of In Vitro Metabolic Activities of Drug-Loaded Nanogels

4.3.3.1. IL-6 Assay

The developed inflammation model (Figure 44.), LPS treatment on HUVEC cells, was used to test free lipoic acid and drug-loaded nanogels' effectiveness in reducing IL-6 amount. The IL-6 amount was measured using an ELISA kit.

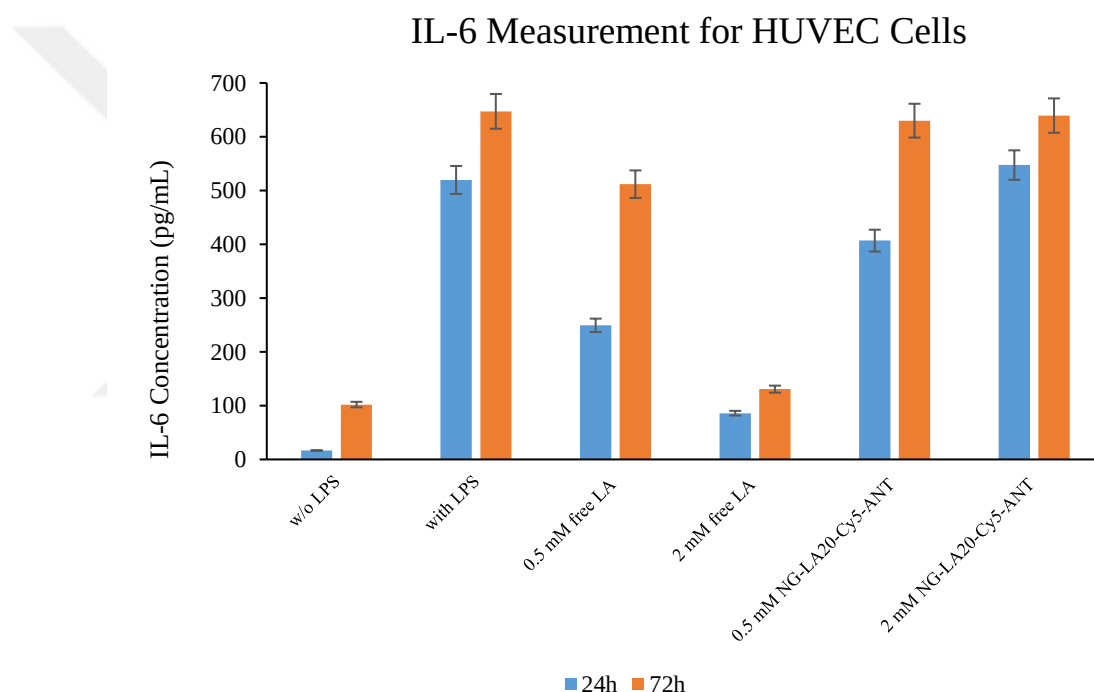


Figure 48. IL-6 measurement on HUVEC cells.

As seen in Figure 48., when the control groups were compared, there was a significant increase in the amount of IL-6 produced in the control group with LPS for both 24 h and 72 h. This indicated that LPS led to an inflammation of HUVEC cells. In addition, even after 72 h of treatment, the increase in the concentration of IL-6 showed the inflammation was still preserved. For the control group without the LPS treatment, it was a 6,2-fold increase in IL-6 concentration from 24 h to 72 h. For the

control group that was treated with LPS, the IL-6 concentration increased by 1,25 times as the incubation time increased from 24 h to 72 h. When both with and without LPS groups were compared at 24 h and 72 h separately, the IL-6 concentration increased 31 times for 24 h and 6 times for 72 h of incubation.

The HUVEC cells were LPS-activated for free LA and LA-loaded nanogel (NG-LA20-Cy5-ANT) treatment. 0,5 mM and 2 mM of free LA and NG-LA20-Cy5-ANT were used for 24 h and 72 h treatment. For the 0,5 mM free LA groups, it was seen that the IL-6 concentration decreased by 52% (for 24 h) and 21% (for 72 h) compared to the control group (with LPS). Furthermore, for the 2 mM free LA groups showed that the IL-6 concentration decreased by 83,4% (for 24 h) and 80% (for 72 h) compared to the control group that was LPS activated.

For both 0,5 mM and 2 mM of LA-containing nanogels, NG-LA20-Cy5-ANT, the IL-6 concentration was more after 72 h treatment than 24 h treatment. For 0,5 mM LA-containing nanogels, while there was a decrease of 22% in IL-6 concentration after 24 h, a decrease of 2,7% was seen after 72 h of treatment. In addition, in 2 mM LA-containing nanogels, there was an increase of 5% in IL-6 concentration after 24 h, however, a decrease of 1,2 % was observed after 72 h of treatment. Based on the obtained results, when the IL-6 concentrations were examined, it was observed that LA-loaded nanogels were less effective than the free LA.

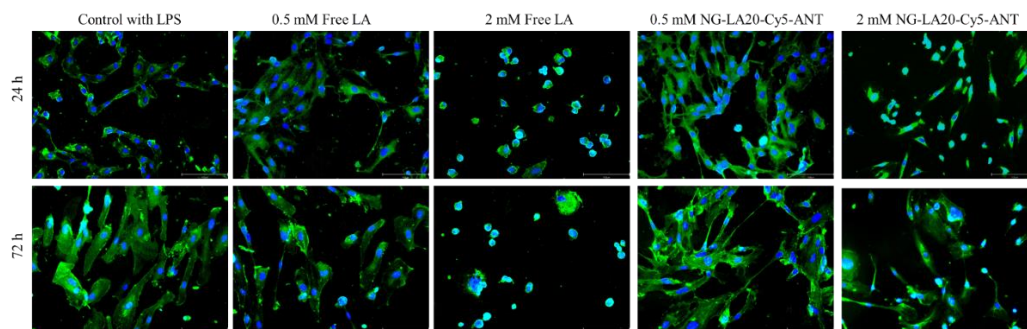


Figure 49. The morphological changes of HUVEC cells when free LA molecule and LA-containing nanogels (NG-LA20-Cy5-ANT) were applied.

The morphological features of the HUVEC cells were observed with fluorescent microscopy (Figure 49.). When free LA and LA-containing nanogels (0,5 and 2 mM) were compared, it was observed that LA-containing nanogels improved the cell morphology. LPS-activated HUVEC cells treated with free LA (2 mM) remained round after both 24 and 72 hours, while the cells treated with LA-containing nanogels were seen to spread and adhered like healthy HUVEC cells for both concentrations and time points. Thus, there was a conflict between the IL-6 ELISA test results and obtained fluorescent images.

4.3.3.2.ROS Assay

The free radicals, which are also known as reactive oxygen species in the inflammatory environment were measured. The effect of drug-loaded and targeted nanogels (NG-LA20-Cy5-ANT) on ROS production was investigated and compared with the free LA molecules' effect on ROS production. The 0,5 mM and 2 mM of free LA and (NG-LA20-Cy5-ANT) were tested on HUVEC cells.

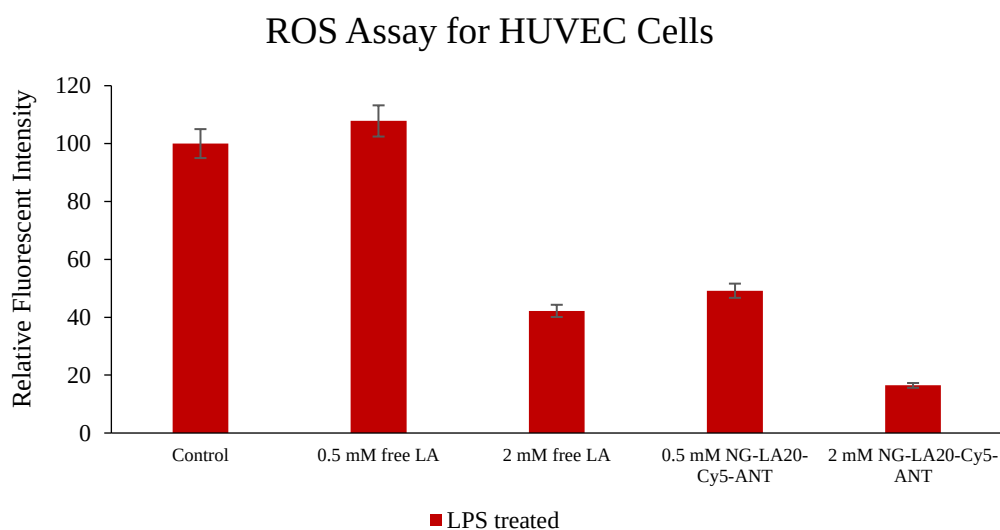


Figure 50. ROS measurements on HUVEC cells.

As seen in Figure 50., it was observed that LA which was free or in the nanogel decreased the amount of ROS. Furthermore, it was seen that compared to its free form, the LA-loaded nanogel was more effective. For the nanogel group that contained 0,5 mM LA, the amount of ROS was reduced 2,2 times more than the free LA group, whereas the ROS decreased 2,6 times more in the 2 mM LA containing nanogel group than the 2 mM free LA group. In addition, when the drug amount was increased from 0,5 mM to 2 mM (4 times), the effect of LA on ROS was almost 3 times more effective. Moreover, compared to the control group, it was observed that when the low dosage of free LA was applied, there was no effect on ROS. However, LA-loaded nanogel groups had a very significant effect on reducing ROS production. This indicated that LA molecules in their nanogel form were more efficient in reducing ROS production.

4.3.3.3.Lipid Peroxidation (MDA) Assay

To determine the amount of oxidized lipids in the inflammation environment, the lipid peroxidation assay, MDA assay, was used. For the MDA assay, the amount of

malondialdehyde (MDA) which is one of the final products of oxidized lipids (degraded due to oxidative stress in the environment after cells are treated with free drug or drug-containing nanogels) was measured at 532 nm by a microplate reader (Biotek).

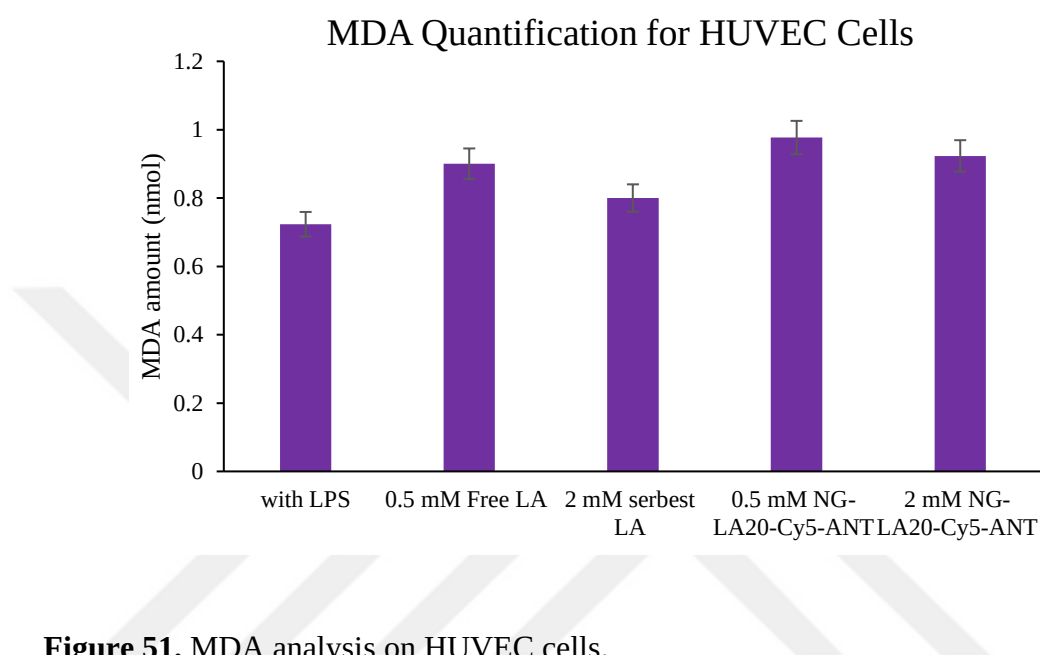


Figure 51. MDA analysis on HUVEC cells.

As seen in Figure 51., using a pre-plotted standard curve of the MDA, the different concentrations of free LA molecules (0,5 mM and 2 mM) and NG-LA20-Cy5-ANT (0,5 mM and 2 mM) tested for MDA analysis. It is observed that as the amount of drug increased in both free drug and drug-loaded nanogel groups, there was a decrease in the amount of MDA produced. Overall, the drug was effective but was not sufficient enough.

5. DISCUSSION AND CONCLUSION

First of all, the drug-containing monomer (LA3-MA) which was covalently bonded to nanogels was synthesized in two steps for this project. For the first step, a

reactive methacrylate group was added to the structure of this monomer which allowed a reaction with PEG-diMA polymers by radical reaction under UV light at 365 nm and formed drug-loaded nanogels. Then, for the second step, three drug molecules (LA) were attached to the structure of the drug monomer via ester bonds that acted as an effective anti-oxidant agent more than free LA itself because of its multivalent effect (125).

After the first step of drug-containing monomer synthesis (Penta-MA synthesis), the purification and characterization of the synthesized Penta-MA molecule were performed by prep-HPLC first (Figure 8.). As known from the literature, the substances generally absorb at a UV wavelength of 254 nm and the methacrylic ester group gives a maximum signal at 214 nm (126,127). Therefore, these nanometers were used for the characterization methods. All in all, as a result of the Penta-MA synthesis, as seen in (Figures 9 -11.) a methacrylic group attached to one hydroxyl group in the pentaerythritol structure with an ester bond was added successfully.

For the second step of the drug-containing monomer synthesis (LA3-MA synthesis), three LA molecules from their carboxylic acid groups were bounded to the 3 primary hydroxyl groups of the Penta-MA molecule via ester bonds (Figure 7.). As seen in Figure (12-15.), the purification and characterization of the synthesized LA3-MA molecule were performed and indicated that LA3-MA was synthesized successfully. As known from the controlled-release literature, the slow-breaking ester bonds are hydrolyzed in a specific psychological environment (e.g., acidic) leading to a slow and controlled release of drugs (128). Therefore, the drug-linked monomer (LA3-MA) synthesized in this project had the characteristics that can both increase the effectiveness of the drug due to its multivalent feature and can be released in a controlled and slow manner due to its selectively breakable ester bonds.

In this project, the prepared nanogels were made of mainly PEG polymer which is highly water-soluble due to its hydrophilic structure. The modified linear PEG chains which contain reactive methacrylate groups at both ends were crosslinked to form nanogels using photo-initiator Irgacure under the wavelength of 365 nm UV light (129).

During the reaction, although the polymers tend to form a bulk gel, the nano-sized and crosslinked polymers so called nanogels can be achieved when the polymer concentration is reduced and in a diluted environment. In this project, to prevent the bulk formation and obtain nano-sized particles, controlled dilution, different temperatures, different photo-initiator to polymer ratios, and different molecular weights of polymers were tested for creating nanogels.

First of all, the solvent type was decided to form nanogels. Therefore, PEG₇₅₀-DiMA polymer was subjected to a radical reaction in 2mL of DMF and 10 mM PBS solvent at 1200 rpm for 5 min. separately. When the size measurements were compared, very large aggregates were observed in the DMF solvent (Figure 16.A). This can be attributed to the fact that DMF is a polar aprotic solvent that probably caused the radicals to move fast and delay the saturation with hydrogen, and in the end, this may have caused an uncontrolled and faster reaction (130). Furthermore, when the reaction was in 10 mM PBS which mimics the physiological environment of the body and is more biocompatible the monomodal and nano-sized nanogels were obtained (131). Thus, 10 mM PBS was decided to continue with for the nanogel preparation (Figure 16.B).

After the solvent type was decided, the optimum temperature was selected for the nanogel preparation. As seen in Figure 17. the most ideal temperature was RT. For this reason, it was decided to continue with RT. After that, the [I]: [P] ratio and reaction time were decided. Considering the data given in Table 1.A. and B., it is seen that the

most ideal nanogels which was with the highest density and the lowest PDI% value in the 20th minute of the reaction for the ratio of 1:50 and 1:100 ([I]: [P]).

For controlled dilution, as seen in Table 2., 8 mL of the 10 mM PBS demonstrated the most suitable solvent amount for both ratios of 1:50 and 1:100. Then, in order to lower the PDI value and decrease aggregates, centrifugation was performed. All in all, it was observed that this method was successful enough for the optimization of the nanogels.

Although the nanogels that were made of PEG₇₅₀-DiMA were optimized above, the different molecular weights (2 kDa, 6 kDa, and 10 kDa) of PEG-DiMA were tested within the scope of this project. Since the PEG chains will be released when the nanogel is broken down by breaking the ester bonds that are located in methacrylate groups of the polymer, the nanogel that does not exceed 10 kDa can be easily cleared by renal systems (132). Therefore, it can be said that the nanogels prepared for this project is biodegradable.

As shown in Table 4., the nanogels that were made of the polymer of PEG-DiMA (Mwt: 2 kDa, 6 kDa, and 10 kDa) were prepared both using 4 mL and 8 mL of 10 mM PBS and with the ratios of 1:50 and 1:100, separately. The effect of both dilution and [I]: [P] was observed. For the 10 kDa PEG-DiMA, while the size increased in the ratio of 1:50, the effect of dilution was observed in the 1:100 ratio. This may indicate that PEG polymers were swollen in diluted solution due to their hydrophilic nature (133). Also, when the results were considered (Table 5-6.), both 6 kDa and 10 kDa PEG-DiMA polymers were chosen over 2 kDa PEG-DiMA due to the effectiveness of the centrifugation method on size reduction.

As observed in Table 5-6., in order to reduce size and eliminate aggregates, filtration, and centrifugation methods were performed. When the results were compared, it was investigated that only 6 kDa PEG-DiMA polymer with the ratio of

1:50 in 8 mL of 10 mM PBS at 20 min. of reaction was the most ideal nanogel depending on their size and PDI value (with a diameter of 85,6 nm, with a PDI value of 4,4%, and a high density of 90,5%). As stated in the literature, nanoparticles with a diameter of 70-100 nm were set as a success criterion for atherosclerotic damaged tissue (134). In addition, the PDI value was in the optimal range (below than 20%) as mentioned in the literature (135) .

After all optimizations, the drug-free or C-NG which was prepared with 6 kDa PEG-DiMA polymer with the ratio of 1:50 in 8 mL of 10 mM PBS at 20 min. of reaction was visualized by SEM and TEM. As seen in Figure 20.A-B, the smaller size nanogels were seen compared to the measurements of the DLS (Figure 18.), it is thought that the of the nanogels was caused by its placement on the carbon band that eventually caused loss of the water from their structure. This is a common situation in the literature for all nanogels with a high water-holding capacity which is displayed under high vacuum (136). Furthermore, when the results compared (Figure 18. and Figure 20.C), the much smaller particles were seen in TEM images. As stated also in the literature that the particles shrank under high vacuum due to the evaporation of water when the nanogels were dried for the TEM procedure (136).

The stability of this C-NG was investigated under the conditions that model physiological environments against time (131). In this context, the nanogels were prepared in pH: 7.4 10mM PBS buffer solution (modeling a healthy environment) and pH: 5.5 acetate buffer solution (modeling an inflammatory environment) at different temperatures (RT and 37 °C), separately.

According to the obtained results for both 1:50 and 1:100 C-NG (Figure 21.), while their size did not change and they were stable for at least two weeks in both 10 mM PBS and acetate buffer solutions at RT, both C-NG swelled a bit more when the temperature was at 37 °C. This swelling was considered normal due to the hydrophilic

structure and potential high water-holding capacity of the nanogels, moreover, this increase in size also is observed for many nanogels in the literature (62). Considering the effect of pH value on the dimensions of control nanogels incubated at the same temperature, it can be seen that nanogels swell more in acidic environments. This can be attributed to the protonation of oxygen atoms in the PEG structure at pH: 5.5 and the ability to interact with more water molecules (80).

For the drug-containing nanogels, 3 different LA3-MA ratios (5, 10, and 20%, % by mass) were prepared. As observed in Figures 22-24., the nanogels were optimized for reducing size and PDI, removing large aggregates, and also, for small particle elimination. According to the results that were obtained (Figures 22-24.), the prepared drug-loaded nanogels were filtered once with 0,22 μm RC filters, then, centrifuged five times with as “Amicon® Ultra-15 Centrifugal Filter Unit, was used to remove low molecular weight impurities from drug-loaded nanogels. In this process, molecules with molecular weights smaller than the membrane cut-off value (30 kDa) were allowed to go down with the centrifugal force, and high molecular weight molecules (polymers or nanoparticles) remained in the membrane above (137). After centrifugation, 5 times filtration was performed again with 0,22 μm filters.

In addition, it has been shown in the literature that sonication is an effective method of dispersing the aggregates that may form (138). Therefore, after centrifugation, 5 minutes of sonication was found to be effective in reducing the size and decreasing density of the aggregates.

The amount of drug that was loaded into nanogels (NG-LA5, NG-LA10 and NG-LA20) was determined by LC-MS/MS. It is stated from the literature that the esterase enzyme can break the 1 μmol of the ester bond in one minute at 25 °C (139). Therefore, drug-loaded nanogels were treated with esterase enzyme at 37 °C for 24 hours (5 times the total amount of μmol of the ester bond in the prepared nanogel solution).

According to the time-dependent drug release profiles of NG-LA5, NG-LA10, and NG-LA20 (Figure 26.), as also stated in the literature that drug release was much more in an acidic environment as expected (131). All in all, NG-LA20 was selected due to having the highest total drug amount (6,17 mg) and the most optimum size (100 nm).

Another time-dependent drug-release profile of NG-LA20 was also performed in the serum-containing environment (Figure 27.). Depending on the drug release in PBS without esterase (4%) and with esterase (around 46%), the drug release did not only dependent on hydrolysis but also the ester bonds of the drug that can be broken with the enzyme and released into the environment (139). When the drug release in serum was examined, it was seen that there was a fluctuation. It was thought that released drug molecules may have been adsorbed on enzymes or proteins in the environment (140). According to drug release in an environment that had esterase decreased after 10 days (in the last two measurements). It was also assumed the possibility that the drug may have been adsorbed to the enzyme (141). All in all, the drug-release profile of NG-LA20 was found to be controlled in different environments (e.g., serum and esterase).

After the most drug-efficient nanogel was prepared, the surface of the nanogel was functionalized with AE-MA first to make nanogels bind to Cy-5 fluorescent dye and anti-VCAM1 antibody. According to the obtained results (Table 10-12.), the most ideal nanogel was 2% AE-MA-containing 6 kDa PEG-DiMA nanogels. As observed in Figure 28., after centrifugation was performed five times, while the small particles were eliminated, there was an increase in the average size of the nanogels. This may be caused by either centrifugation which may have caused nanogels to come together because of their amine groups forming hydrogen bonds or the nanogel's globular shape that may have stuck in Amicon tubes. All in all, after purification, optimization, and characterization methods were performed (Figure 29-31.), it was proved that nanogels were functionalized with the AE-MA successfully.

After amine-containing nanogels were created, Cy5 fluorescent dye was conjugated with NHS/EDC chemistry for further in vitro cellular experiments. After the reaction, the prepared and the most suitable NG-Cy5 which was 91 nm diameter in size was optimized into three steps: filtering once with 0,22 μm RC filter, then, centrifugation with the Amicon tubes five times, and filtering once with 0,22 μm RC filter, respectively (Figure 32.). In order to prove Cy5 conjugation onto the NG-NH₂, zeta potential analysis was done. As seen in Figure 33., the charge of the particle was shifted from positive to negative. When the chemical structure of the Cy5 molecule was examined, it was observed that it had two aromatic rings and a small chain with an electron conjugation between them. In addition, there are methyl groups that donate electrons to these aromatic groups. Therefore, it is thought that the total surface charge value of the NG-NH₂ after the Cy5 bonding decreased due to the dense electron clouds that brought this partial negative charge. The amine presence was also proved with ¹H NMR (Figure 35.) and FT-IR (Figure 34.) analysis. Lastly, the morphological analysis was performed using TEM and it was found that the smaller nanogels were seen compared to DLS measurements. This is thought that the particles shrank under high vacuum due to the evaporation of water when the nanogels were dried on the Cu grid (136).

For the drug-loaded and Cy5-bounded nanogel (NG-LA20-Cy5), As a result of the evaluation of the obtained results with the relevant calibration curves (Table 13.), it was observed that the obtained dye-bound nanogels had a drug loading efficiency of 75,77%, which was lower than expected when compared to the drug-loaded nanogels (NG-LA20) prepared without adding amine monomers (91,47%). However, it is in the acceptable range according to the drug loading efficiencies in the literature (142). On the other hand, the amount of Cy5 obtained was calculated to be 598,4 $\mu\text{g/mL}$ in these NG-LA20-Cy5, which was higher than the NG-LA20 nanogels (400 $\mu\text{g/mL}$). Compared to other nanogels (e.g., NG-LA20), the decrease in surface charges of NG-LA20-Cy5 can be attributed to the aromatic groups and degree of conjugation (i.e., the number of double bonds in its structure) of the excess Cy5 dye. Furthermore, it can be also attributed to loaded LA molecules.

It is known from the literature that in atherosclerosis, a high amount of VCAM1 receptors is found because of the inflammation in the vascular area (143). Within the aim of this project, the previously synthesized NG-Cy5 which had remaining free amine groups was used for anti-VCAM1 antibody functionalization. After the preparation of NG-Cy5-ANT, the nanogels were purified and optimized into two steps (centrifugation five times with the Amicon filter units, and filtering once with the 0, 22 μ m RC filter). As seen in Figure 38., it is thought that the particles at around 20 nm may have belonged to structures of antibody dimers or tetramers (144). In addition, it is assumed that NG-Cy5-ANT came together and became large aggregates. This may be caused by protein-protein interactions (Figure 42.) (144). The antibody presence was proved successfully with ^1H zeta (Figure 39.), FT-IR (Figure 40.), and NMR (Figure 41.) analysis.

Compared to NG-Cy5-ANT, the drug-loaded version of the nanogel which is NG-LA20-Cy5-ANT was observed in the most optimal size range of around 200-300 nm in diameter (Figure 43.A-C.). While NG-Cy5-ANT showed large aggregates around 800 nm, NG-LA20-Cy5-ANT did not show any large aggregates. This is most probably caused by the only optimization procedure (10 min sonication) differed in NG-LA20-Cy5-ANT. It is also known from the literature that sonication is a very compelling method for eliminating aggregates (138). All in all, NG-LA20-Cy5-ANT was the most ideal candidate for *in vitro* assays.

First of all, in order to test NG-LA20-Cy5-ANT in the atherosclerotic environment, an inflammatory environment was mimicked by LPS treatment on HUVEC cells. As stated in the literature that LPS is a kind of lipopolysaccharide derived from the outer shell of Gram-negative bacteria, leading an inflammation, therefore, it was used for the *in vitro* modeling of atherosclerosis disease in this project (145).

For this reason, different concentrations of LPS (10 ng/ mL, 50 ng/ mL, and 100 ng/ mL) were applied to HUVEC cells for 6 h incubation. In order to maximize the number of HUVEC cells in these wells and to ensure that the number of cells in the experiment is close to the number of cells seed in the well, it was decided to continue with a concentration of 10×10^4 cells/mL, where no significant decrease in cell viability values was observed (146). Therefore, the number of HUVEC cells that were seeded on 96-well plates was decided to be 10×10^3 cells per well.

As seen in Figures 44., as the concentration of LPS increased (from 10 ng/mL to 100 ng/mL), the amount of IL-6 which is one of the prominent inflammation biomarkers increased from 94,02 pg/mL to 519,64 pg/mL, respectively (146,147). Also, when there was no treatment with LPS, a very low (16,5 pg/mL) IL-6 presence was detected in the cells. In addition, as also observed in Figure 45., there was a corresponding degree of cell membrane disruption or damage, when the LPS concentration increased. Within the scope of this project, to evaluate the effectiveness of the synthesized NG-LA20-Cy5-ANT, the 100 ng/mL of LPS on 10×10^4 cells/mL HUVEC cells was decided to be studied.

The cytotoxicity of the prepared NG-LA20-Cy5-ANT for both normal human skin fibroblasts and HUVEC cells (human umbilical vein endothelial cells) was analyzed using CCK-8 assay. The CCK-8 assay kit, which is a frequently used colorimetric assay in the literature, allows the comparative analysis of proliferation profiles depending on the growth rate of cells. This kit is based on the conversion of a tetrazolium salt called WST-8 into a metabolically active substance by the dehydrogenase enzyme of living cells. Spectrophotometric analysis of this colored salt is made to determine the viability of cells cultivated in plates (148).

The cytotoxicity experiments were performed primarily on normal human skin fibroblast cells. Fibroblast cells are a type of cell that is most common in the human

body (149). Therefore, any material that will have a toxic effect on this cell type cannot be injected or implanted into the cell. Since the nanogel solutions prepared in this project (in the future stages) are planned to be administered by intravenous injection in vivo and clinical studies, it was decided that the fibroblast type to be used in the first stage would be skin fibroblasts.

As seen in Figure 46., all the concentrations that were applied to nanogel groups did not show any cytotoxic effect on normal human skin fibroblasts. Furthermore, the least cell viability percentage was observed at around 75% which was far distant from half maximal inhibitory concentration (IC₅₀). In order to determine the IC₅₀ value which is a drug concentration that inhibits half of the cell population of the prepared nanogel groups, there is a need for drug-dosage adjustment (150).

In addition, since HUVEC cells were the main cell line used in atherosclerosis disease modeling, the cytotoxic activity of the applied nanogel groups was important. As also investigated in normal human skin fibroblast, all applied nanogel groups in all concentrations did not show any cytotoxicity to HUVEC cells, and cell viability percentage was mostly around 80% and above (except for the 21 µg/ mL of NG-LA20-Cy5-ANT which was around 57 %). All in all, these results indicate that all nanogel formulations did not show any cytotoxic effects for normal human skin fibroblasts and HUVEC cells. Moreover, drug-dosage adjustment is needed for IC₅₀ value determination.

To evaluate the anti-inflammatory effect of prepared NG-LA20-Cy5-ANT on a developed 2D in vitro atherosclerosis disease model, the ELISA kit which provides IL-6 measurement was used. Both free-LA molecules and NG-LA20-Cy5-ANT were applied to the HUVEC cells for 24 h and 72 h in two different concentrations (0,5 mM and 2 mM). According to obtained results (Figure 48.), when control groups (with or without LPS-treated cells) were compared in both 24 h and 72 h, LPS-activated

HUVEC cells indeed induced the inflammation pathway and led to IL-6 production (147). As the incubation time increased from 24 h to 72 h, a 6.2-fold increase of IL-6 in the control group (without LPS treatment) was observed and it is indicated that the cells kept in the incubator started to become inflamed due to natural or environmental effects such as nutritional deficiency or stress (151). For overall projection, while free-LA molecules decreased the IL-6 concentration in both two concentrations and two-time points, only the 2 mM of NG-LA20-Cy5-ANT could not decrease the IL-6 concentration for both 24 h and 72 h. On the contrary, there were significant differences in the morphology of LPS-activated HUVEC cells when the free-LA and NG-LA20-Cy5-ANT were applied (Figure 49.). These results indicated that NG-LA20-Cy5-ANT was much more effective according to their corresponding morphological features. All in all, this contradiction may be eliminated by repetition of the experiment or the IL-6 amount can be examined at the gene level. The IL-6 mRNA encodes the IL-6 protein and accumulates in the cytoplasm (152). Therefore, the protein expression may be decreased in cells treated with the free-LA molecules but perhaps the amounts of IL-6 mRNA in the cytoplasm of cells are greater than that of cells treated with nanogels containing the same amount of LA molecules.

The ROS molecules cause the oxidation of LDL molecules in arteries and in the end form lipid plaques which are also called atherosclerosis (103). Within the scope of this project, the LA molecule which is a kind of antioxidant was expected to inhibit ROS production and eventually slow atherosclerosis progression (153). Therefore, the free-LA molecules and NG-LA20-Cy5-ANT were tested on the developed 2D in vitro inflammation model. To determine the change in the amount of ROS production, DCFDA/H2DCFDA Cellular ROS Detection Test kit was used. As seen in Figure 50., except for the 0,5 mM free-LA molecule, the other groups reduced the ROS production successfully. In addition, when the NG-LA20-Cy5-ANT was compared to free-LA molecule groups, it showed a significant decrease in ROS production. This is most probably caused by targeting moiety which is the anti-VCAM1 antibody of the drug-loaded nanogel. This proves that targeting features of the nanogel was efficient for the intended the reducing of ROS production.

One of the important analyses of determining the effect of the NG-LA20-Cy5-ANT on a developed 2D in vitro inflammation model was lipid peroxidation assay. In this assay, the amount of oxidized lipid was determined by detecting the amount of final product malondialdehyde (MDA). As seen in the Figure 51., compared to the control group (with LPS), the free-LA molecule and NG-LA20-Cy5-ANT were not sufficient enough to lower the MDA production, however, there was a direct correlation of decrease in each group when the concentration of LA increased from 0,5 mM to 2 mM. It is thought that the effect of these free-LA molecules and NG-LA20-Cy5-ANT on the inflammation model was not enough because of the dosage of the LA.

All in all, in this thesis, it is aimed to develop targeted drug delivery system to diminish oxidative stress, inflammation and oxidation of the lipids, related to atherosclerosis. The novel antioxidant (α -lipoic acid)-containing drug molecules (LA3-MA) were successfully synthesized and covalently conjugated to PEG-DiMA nanogels. By this conjugation strategy (e.g., ester bonds), the LA3-MA molecules were released slow and in a sustained manner in only at the pH of 5.5 which mimicked the inflammatory environment in atherosclerosis. First of all, the nanogels were functionalized with Cy5 fluorescent dye for upcoming in vitro cellular uptake assays. Then, to target the atherosclerotic region, anti-VCAM1 antibodies were conjugated on the surface of the nanogel with EDC/NHS chemistry and this conjugation was expected to target only the region of atherosclerosis. To test out the synthesized nanogels, 2D inflammation model was developed with the HUVEC cells successfully. Thereafter, the different concentrations of drug-loaded nanogels were tested on normal skin fibroblast and HUVEC cells and it was found to be non-cytotoxic for both cell lines at the all tried concentrations. It was seen from the ROS, IL-6 and MDA assays that synthesized nanogels were generally more effective than free drug molecules, however, there was a need for repetition due to the applied concentrations. Because of this, the further in vitro experiments with higher concentrations of drug molecules are still in progress.

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7. APPENDICES

Appendix 1: Determination of Total Drug Amounts of Drug-Loaded Nanogels

In order to determine the amount of drug molecule, LC-MSMS analysis was performed and calibration curve of LA was plotted. First of all, Lipoic acid solution was prepared at a concentration of 2500 ppb and given to the device at a flow of 0,5 mL/ min for 1 minute with stationary phase C18 prep-HPLC column and mobile phase 50% ACN: %50 H₂O. The mass peak and distribution of the free drug molecule was analyzed in the MS2Scan/ MS2SIM modes. The expected theoretical molecular weight of 206,33 gmol⁻¹ was found to be 205,1 gmol⁻¹ in mass analysis.

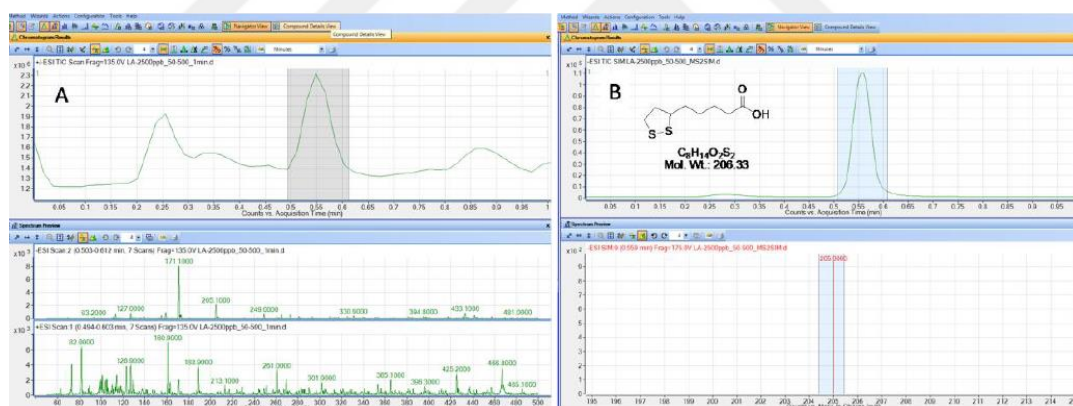


Figure 52. The LC-MSMS analysis of free drug molecule (LA).

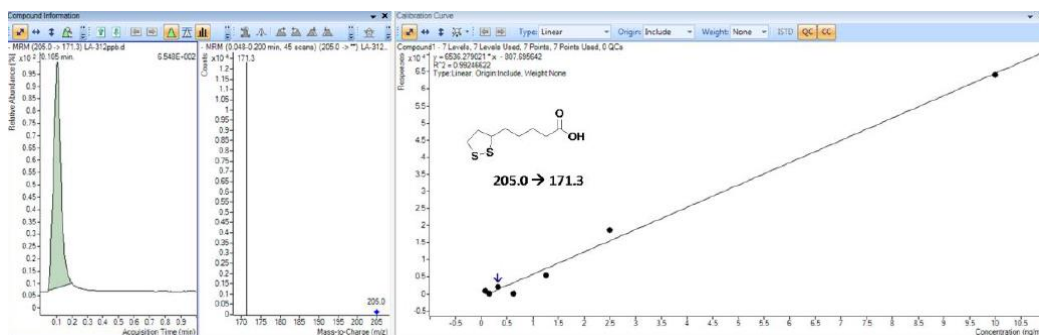


Figure 53. The calibration curve of free drug molecule (LA) plotted by LC-MSMS.

Appendix 2: Determination of Cy5 Concentration

First of all, the most suitable device method was created for the free Cy5 molecule, and then a special calibration curve was drawn for Cy5 using this method. For this purpose, C18 prep-HPLC column was used as the stationary phase and 50%ACN: %50 H₂O solution was used as the mobile phase in the LC-MS/MS device. Formic acid, a weak acid of 0,05% by volume, was added to these solutions in order to ionize the molecules and to capture and analyze them in mass detectors. The method in which this mobile phase mixture was passed through the column at a flow rate of 0,5 mL/min per minute was set for a total of 1 minute.

The mass peak and distribution of the dye molecule was analyzed in the MS2Scan scanning analysis. In MS2Scan scanning mode, the MRM method was created, including the peaks of 300,8 gmol⁻¹ and 365,0 gmol⁻¹, which are seen at high intensity in the positive scanning part.

After introducing the Cy5 dye molecule to the LC-MS/MS device and creating a suitable method for this molecule, a calibration curve was drawn using this method in order to quantify it. For this, Cy5 solutions at different concentrations were prepared by serial dilution method starting from 0,3 mg/mL with a ratio of 1:1. These samples

were analyzed using the method prepared from low to high concentration. Using the instrument's software for quantitative analysis, a very reliable ($R^2 > 0.989$) calibration curve was drawn by matching the corresponding signals with the concentration values of these solutions.

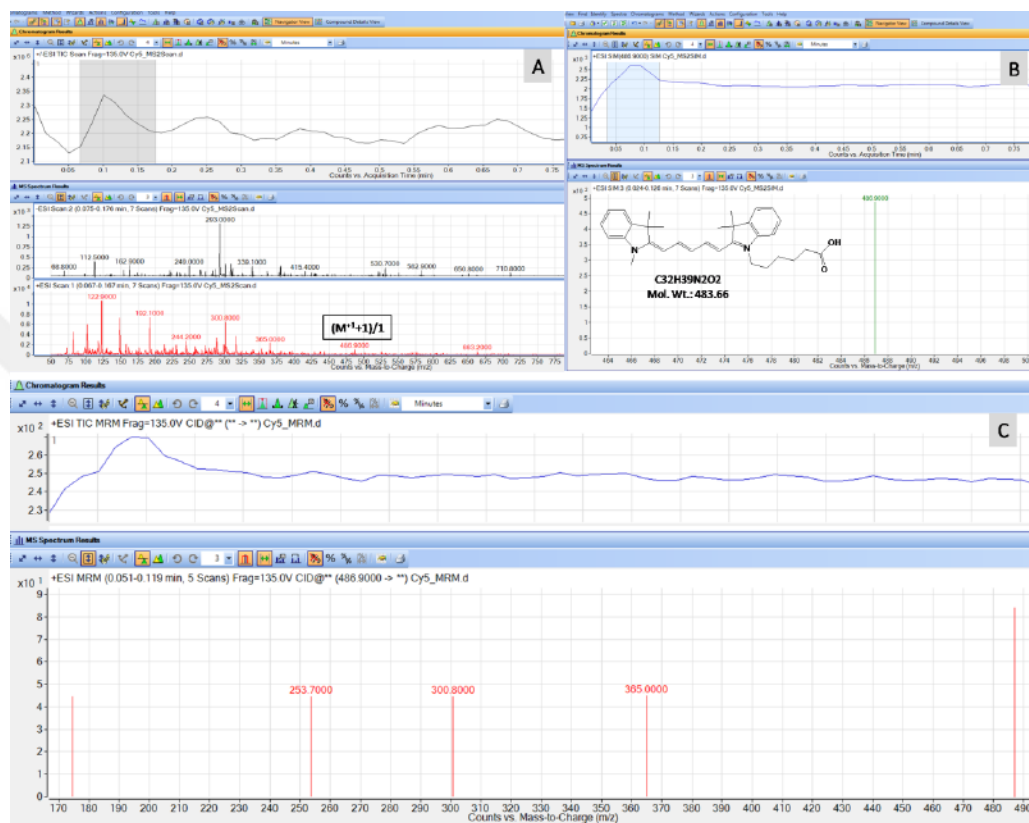


Figure 54. The LC-MSMS analysis of Cy5 fluorescent dye molecule.

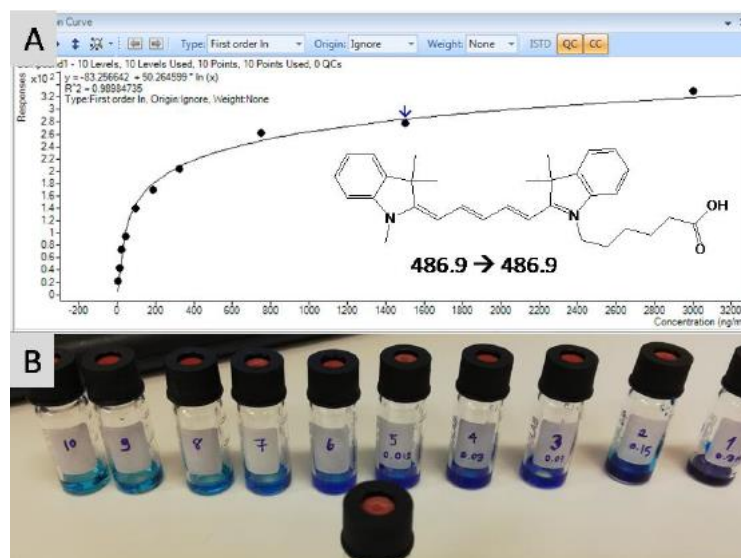


Figure 55. (A) Calibration curve of Cy5 fluorescent dye molecule plotted by LC-MS/MS and (B) the photograph of Cy5 solutions at different concentrations prepared for the calibration curve.

Appendix 3: BSA Calibration Curve for Determining the Amount of Antibody on Nanogel Surface

In order to determine the amount of anti-VCAM1 antibody on the surface of the nanogels, the bovine serum albumin (BSA) was used as a standard. BSA solutions at different concentrations were prepared by serial dilution method starting from 2 mg/mL with a ratio of 1:1. These samples were analyzed using the nanodrop (Thermo Scientific) at Ex: 280 nm.

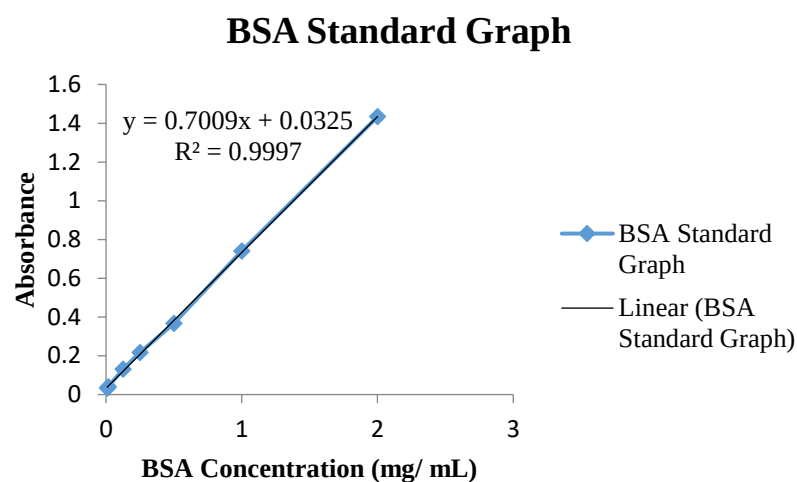


Figure 56. The BSA standard graph plotted by using nanodrop at 280 nm.

Appendix 4: IL-6 Concentration Determination

In order to determine the amount of IL-6, standard IL-6 solution was used. IL-6 solutions at different concentrations were prepared by serial dilution method starting from 400 pg/mL with a ratio of 1:1. These samples were analyzed using the microplate reader (Biotek) at Ex: 450/550 nm.

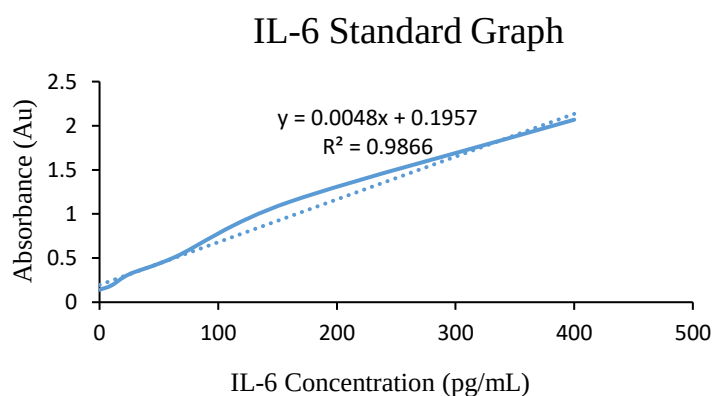


Figure 57. Calibration curve prepared for the IL-6 Human ELISA kit.

Appendix 5: MDA Concentration Determination

In order to determine the amount of MDA, standard MDA solution was used. MDA solutions at different concentrations were prepared by serial dilution method starting from 2 mM. These samples were analyzed using the Varioskan (Thermo Fisher) at absorbance (A) 532 nm.

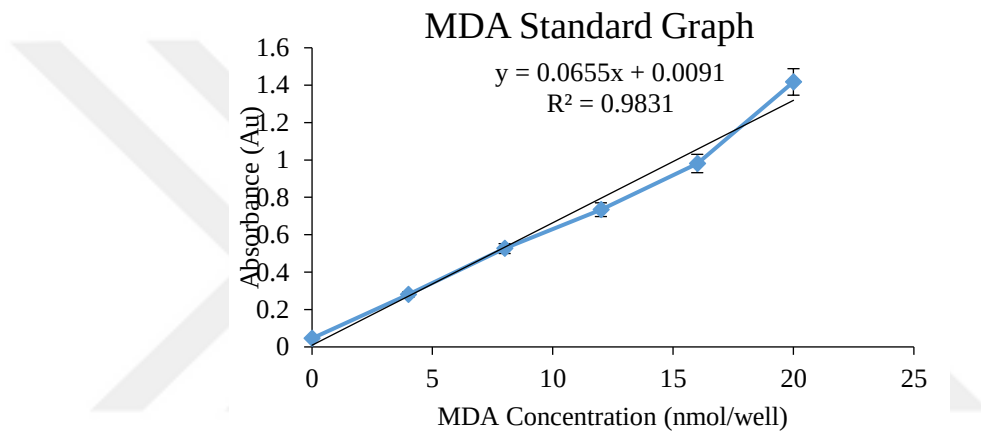


Figure 58. Calibration curve prepared for the MDA assay.

Appendix 6: Ethical Authorization of the Research

This study was approved by Acıbadem Mehmet Ali Aydınlar University Medical Research Ethics Committee (ATADEK) (Ethics Committee decision no. ATADEK 2023-03/58).

8. CURRICULUM VITAE

