

Design of Biodegradable Hydrogels for the Development of *in vitro* models for Glioblastoma Multiforme

by

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**A Thesis Submitted to the
Graduate School of Sciences and Engineering
in Partial Fulfillment of the Requirements for
the Degree of**

Doctor of Philosophy

in

Biomedical Sciences and Engineering

Koc University

September 2017

Koc University

Graduate School of Sciences and Engineering

Koç University
Graduate School of Sciences and Engineering

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ABSTRACT

Glioblastoma multiforme (GBM) is the most frequent and malignant type of primary brain tumor. Even though GBM constitute only 1% of adult cancer, its malignant nature makes GBM the fourth highest reason of cancer-related deaths.

Numerous factors make the clinical treatment of GBM challenging. These factors include the resistance of tumors to conventional therapies such as surgery, radiation therapy and chemotherapy and difficulties in achieving the desired chemotherapeutic effect without side effects due to the selective permeability of blood–brain barrier (BBB). Therefore, new treatment options are needed for GBM patients.

Evading from the immune system of the patient and apoptosis are some of the acquired features of GBM cells that results in inefficient treatments. Hence, strategies that can reactivate apoptosis through the use of ligands interacting with receptors of extrinsic apoptosis pathway can be promising for clinical treatments. In this context, TNF-related apoptosis-inducing ligand (TRAIL) is a perfect candidate for GBM treatment since TRAIL can induce apoptosis particularly in cancer cells without damaging healthy cells. However, the efficacy of TRAIL is limited by the intrinsic and/or acquired resistance of tumor cell populations to the therapeutic substance. In our recent studies, we have combined the other anti-tumor therapeutics along with TRAIL to achieve a synergistic effect and successfully have overcome TRAIL resistance *in vitro*.

Another strategy to reach increased anti-cancer effect from therapeutics involves the design of biomaterial-based vehicles to deliver drug through the tumor. These vehicles can be modified to enhance their ability to deliver therapeutics to specific regions of the body in

response to an external stimulus. Biomaterials have also been used for designing brain-mimetic 3D platforms for GBM studies. The development of these *in vitro* models bridges the gap between *in vitro* 2D drug trials and *in vivo* animal studies. It is also promising for understanding the nature of GBM microenvironment to build efficient strategies for successful treatments.

In this thesis, we aimed to synthesize hydrogels from biodegradable materials and investigate their biocompatibility for GBM therapy, *in vitro*. First, we developed PEG hydrogel carriers for simultaneous delivery of the two molecules, Quinacrine (QC), a recently discovered TRAIL sensitizer drug, and TRAIL. Briefly, Matrix-metalloproteinase (MMP) degradable PEG hydrogel carriers were synthesized via visible-light-induced water-in-water emulsion polymerization. Critical biophysical properties of the hydrogels such as swelling, degradation, size and morphology were characterized. QC was loaded into the hydrogel carriers, and the effect of this drug on apoptosis of GBM was investigated through cell viability assay and apoptosis-related gene expression profile using quantitative real-time polymer chain reaction (qRT-PCR). The results suggested that these MMP-sensitive particles were cytocompatible and they are superior to promote TRAIL induced apoptosis in GBM cells when loaded with QC. Compared to the applications of QC and TRAIL alone, combination of QC-loaded PEG hydrogel and TRAIL demonstrated synergistic apoptosis inducing behavior. Furthermore, QC-loaded PEG hydrogels, but not QC or PEG-hydrogels alone, enhanced apoptosis as measured through expression of apoptosis-related genes.

In the second part of our work, we designed visible light-induced GelMA hydrogels to mimic 3D GBM microenvironment. For the first time in the literature we demonstrated a strategy that exploits visible-light-induced crosslinking of gelatin where reaction was carried

out in the absence of an additional crosslinker. Visible light-induced crosslinking permits the design of cancer microenvironment-mimetic system in a simple and inexpensive way without compromising the cellular DNA damage during the process. By using this approach, we accomplished the development of suspension and spheroid-based models for GBM to investigate cellular behavior and gene expression profiles within this 3D hydrogel network. Furthermore, mRNA expressions of malignancy and apoptosis-related genes, and sensitivity to an anti-cancer drug Digitoxigenin treatment were investigated in detail.

Within the scope of this thesis study, we developed a promising approach to improve the efficacy of chemotherapeutics and suggested a treatment for GBM via incorporation of QC and TRAIL into MMP-sensitive PEG nanocarriers. The photocurable gelatin is a promising candidate for clinical translation of cancer research through the analysis of malignant cancer cell behavior within an artificial 3D tumor microenvironment.

In summary, this thesis truly includes interdisciplinary aspects of material science, engineering and medicine such as the design of tumor microenvironment with biochemical and biophysical cues within scaffolds to address the clinical limitations of existing strategies, in particular for the treatment of GBM.

ÖZETÇE

Glioblastoma multiforme (GBM) beyin tümörünün en sık görülen ve kötü huylu türüdür. GBM yetişkin kanserlerinin sadece %1'ini oluştursa da, kötü huylu yapısı nedeniyle kanser sebebi ölümde dördüncü sırayı GBM teşkil eder.

Bir çok etken nedeniyle GBM'in klinik tedavisi zordur. Bu etkenler arasında ameliyat, radyoterapi ve kemoterapi gibi geleneksel tedavilere karşı direncin yanı sıra beyin kan bariyerinin (BBB) seçici geçirgen yapısından dolayı yan etkiler olmaksızın istenilen seviyede kemoterapötik etkiye ulaşamaması gösterilebilir. Bu nedenle GBM hastaları için yeni tedavi seçenekleri gereklidir.

GBM hücrelerinin sonradan edindiği, bağışıklık sisteminden ve apoptozdan kaçmak gibi özellikler, tedavilerin başarısızlıkla sonuçlanmasına neden olmaktadır. Dolayısı ile dış güdümlü apoptoz yolağının reseptörlerine bağlanan ligantların kullanılmasıyla apoptozu yeniden aktif hale getirebilen yöntemler klinik tedaviler için gelecek vaadedebilir. Bu kapsamda, sağlıklı hücrelere zarar vermeden kanserli hücrelerde apoptozu tetiklemesi bakımından Tümör-nekroz-faktör ilişkili apoptoz indükleyici ligand (TRAIL) mükemmel bir aday olarak karşımıza çıkmaktadır. Buna rağmen, TRAIL'in verimi tümör populasyonlarında var olan ve/veya sonradan kazanılan terapötik maddelere direnç nedeniyle kısıtlanmaktadır. Biz son çalışmalarımızda *in vitro* TRAIL direncini yenmek amacıyla sinerjistik etki yaratabilecek bilinen başka anti-kanser ilaçlarını TRAIL ile birlikte kullandık.

Terapötik moleküllerin anti-kanser etkisini arttırmak için bir başka yöntem de biyomalzeme temelli araçların ilaçların tümörün olduğu bölgeye doğru iletimini sağlamak için

kullanılmasıdır. Bu araçlar terapötik moleküller vücutta istenilen bölgede, dışarıdan bir uyarıcı sinyal varlığında salınacak şekilde geliştirilebilmektedirler.

Biyomalzemeler GBM çalışmaları için beyin ortamını taklit eden 3D platformlar tasarlamak için de kullanılmıştır. Bu *in vitro* modellerin geliştirilmesi, *in vitro* 2D ilaç denemeleri ile *in vivo* hayvan çalışmaları arasındaki boşluğu doldurmaktadır. Başarılı tedaviler için etkili stratejiler geliştirmek için GBM mikro-ortamının doğasını anlama konusunda da umut vaat ediyor.

Bu tezde, biyolojik olarak parçalanabilir malzemelerden hidrojel sentezlemeyi ve bu hidrojellerin GBM terapisi için biyolojik olarak uygunluğunu *in vitro* olarak araştırmayı amaçladık. Öncelikle, iki molekülün eşzamanlı olarak verilmesi için PEG hidrojel taşıyıcıları geliştirdik. Bu amaçla TRAIL ile birlikte son keşfedilen TRAIL sensitizörlerinden biri olan Kinakrin'i (QC) kullandık. Kısaca, Matriks metalloproteinaz (MMP) enzimi ile parçalanabilir PEG hidrojel taşıyıcılar, görünür ışık ile indüklenen su-içinde-su emülsiyon polimerizasyonu yoluyla sentezlenmiştir. Şişme, bozunma, boyut ve morfoloji gibi hidrojellerin kritik biyofiziksel özellikleri karakterize edildi. QC, hidrojel taşıyıcılarına yüklendi ve bu ilacın GBM'nin apoptozu üzerindeki etkisi, hücre canlılığı testi ve kantitatif gerçek zamanlı polimer zincir reaksiyonu (qRT-PCR) kullanılarak apoptozla ilişkili gen ekspresyon profili aracılığıyla araştırıldı. Sonuç olarak, bu MMP'ye duyarlı parçacıkların sitokompatible olduğu ve QC ile yüklendiklerinde GBM hücrelerinde TRAIL'in indüklediği apoptozu teşvik etmekten daha üstün olduğunu bulundu. QC ve TRAIL'in tek başına uygulamaları ile karşılaştırıldığında, QC yüklü PEG hidrojel ile TRAIL kombinasyonu sinerjik olarak apoptozu indükleyici davranış sergiledi. Buna ek olarak, apoptoz ile ilişkili genlerin ekspresyonlarının analizi ile QC yüklü

PEG hidrojellerinin, sadece QC veya sadece PEG-hidrojelleri kullanımına göre, apoptozu daha fazla arttırdığı bulundu.

Çalışmalarımızın ikinci bölümünde, 3D GBM mikro-çevresini taklit edebilmek için görünür ışık ile indüklenen jelatin-metakrilat (GelMA) hidrojelleri tasarladık. Literatürde ilk defa, reaksiyonun ilave bir çapraz bağlayıcı yokluğunda gerçekleştirildiği, görünür ışık ile indüklenen jelatin çapraz bağlamayı kullanan bir strateji ortaya koyduk. Görünür ışık ile indüklenen çapraz bağlanma, sentez süreci boyunca hücresel DNA hasarından ödün vermeksizin basit ve ucuz bir şekilde kanser mikro çevresi-taklidi sisteminin tasarımına izin verir. Bu yaklaşımı kullanarak, bu 3D hidrojel ağındaki hücresel davranış ve gen ekspresyon profillerini araştırmak için GBM hücrelerinden süspansiyon ve kürecik şeklinde modellerin geliştirilmesini başarmış olduk. Ayrıca malignite ve apoptoz ile ilişkili genlerin mRNA ekspresyonları ve Dijitoxigenin ilacının anti-GBM özellikleri detaylı olarak araştırılmıştır.

Genel olarak bu tez çalışması kapsamında, QC ve TRAIL'in MMP'ye duyarlı PEG nanotaşıyıcılarına dahil edilmesiyle kemoterapötiklerin etkinliğini artırmak için umut verici bir yaklaşım geliştirdik ve GBM için bir tedavi önerdik. Ayrıca, ışıkla indüklenen jelatinin, suni bir 3D tümör mikro-ortamı oluşturularak habis kanser hücresi davranışının analiz edilmesine olanak tanınmasıyla kanser araştırmalarının klinik çevirisi için umut verici bir aday olduğu ön görülmüştür.



Dedicated to my family

ACKNOWLEDGEMENTS

First of all, I would like to thank to my advisor, Assoc. Prof. Seda Kızılel for her guidance, great support, patience and encouragement throughout my PhD research studies. Her confidence in me, made me more productive. She also taught me how to survive in the competitive academic environment.

I would like to thank my co-advisor Assist. Prof. Tuğba Bağcı-Önder for her guidance and suggestions, positive perspective, sincerity and friendly attitude during my PhD.

I would like to express my gratitude to my thesis committee members Prof. Burak Erman, Prof. Nihan Nugay, and Assist. Prof. Tamer Önder for their attention, suggestions and time that they have devoted for the evaluation of my thesis.

I would like to thank to Koç University, Graduate School of Sciences and Engineering and The Scientific and Technological Research Council of Turkey for the funding.

I deeply appreciate the assistance, patience and friendship of Koç University Surface Technology Research Center (KUYTAM) team, Dr. Barış Yağcı and Cansu Yıldırım.

I would like to thank to current members and alumni of Seda Kızılel Lab. Dilem Ceren Oran and Günce Ezgi Cinay defined me the honest meaning of sisterhood, by helping me at the every step of my life and studies. I want to thank, Uğur Bozüyük and Birgül Akalpoğlu, I know them for 1 year but we shared nice scientific conversations and also enjoyable memories. My sincere thanks also go to my group-mates Tuğba Bal, Derya Aydın and Rubab Batool Syeda.

In addition, I would like to thank members of TBO lab; Ahmet Cingöz, Fidan Şeker, Tolga Lokumcu and Filiz Şenbabaoğlu deserve much credit for their help.

Caner Nazlı and Orkun Önal deserves a special thank for their valuable friendship and guiding me during the first years of my PhD.

My dear friend and colleague Sezen Vatansever should take a great amount of recognition for her support. We started PhD at the same term and we passed all examinations at the similar periods. She was my helpmate.

Also my lovely roommate and colleague Sinem Apaydın for her devoted support, understanding and invaluable friendship. She become a family member for me.

I deeply appreciate for the helps of Emine Büyükdurmuş and Elif Tüysüz. They guided us for whole 4 years by constructing bridges between the administration and graduate students.

I would like to express my sincere thanks to my family. I am very grateful to my mother Nihal Erkoç, my father Mustafa Erkoç and my brother Uğur Erkoç. I could not be what I am today without their endless and unconditional support, trust, encouragement, and love throughout my whole life.

Finally, I owe İbrahim Can Erik all my deepest thanks for his continuous and unconditional love, support, patience, motivation, trust and encouragement. He is my companion, we shared joy and sadness together during my PhD.

TABLE OF CONTENTS

TABLE OF CONTENTS.....	xii
LIST OF TABLES	xv
LIST OF FIGURES	xvi
LIST OF SCHEME.....	xix
1. INTRODUCTION.....	23
2. LITERATURE REVIEW.....	24
2.1. Overview of Glioblastoma Multiforme	24
2.2. Current Treatments	26
2.3. Alternative Strategies.....	27
2.3.1. Targeted Therapy.....	27
2.3.2. Apoptosis and TRAIL.....	28
2.3.3. Combinatory Drug Treatments	31
2.3.4. Biomaterials and GBM.....	32
2.3.4.1. Designing Hydrogels for GBM Therapy	33
3. QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS.....	45
3.1. Introduction	45
3.2. Materials and Methods	48
3.2.1. Materials	48
3.2.2. Synthesis of PEG-peptide hydrogel precursors.....	49
3.2.3. Characterization of Enzymatic Sensitivity	49
3.2.4. Synthesis of PEG Hydrogel Nanocarriers.....	50
3.2.5. Characterization of PEG Hydrogel Nanocarriers	50
3.2.6. Release profile of PEG hydrogel particles	51
3.2.7. Cytotoxicity Assays on U87MG Cells.....	53
3.2.8. Gene expression analysis	53
3.3. Results and Discussions	54

3.3.1.	Characterization of Protease Sensitive Hydrogel.....	54
3.3.2.	Synthesis of Targeted PEG Carriers	56
3.3.3.	QC Loading and Release Experiments	60
3.3.4.	Cell Culture Studies	62
3.3.5.	In vitro Cell Viability Assays	64
3.3.6.	Gene expression analysis	67
3.4.	Conclusions	71
4.	GELATIN METHACRYLATE HYDROGELS IN THE ABSENCE OF CROSSLINKER AS A 3D MICROENVIRONMENT FOR GLIOBLASTOMA MULTIFORME (GBM)	72
4.1.	Introduction	72
4.2.	Materials and Methods	74
4.2.1.	Materials	74
4.2.2.	Methacrylated Gelatin Synthesis	75
4.2.3.	Synthesis of Gelatin Hydrogels	76
4.2.4.	Characterization of Surface Morphology, Swelling, Gel Fraction, and Degradation	76
4.2.5.	Rheology Measurements.....	77
4.2.6.	Thermogravimetric Analysis.....	78
4.2.7.	Maintenance of Cells.....	78
4.2.8.	Cell Adhesion.....	78
4.2.9.	Cell Encapsulation	79
4.2.10.	Cell Viability	79
4.2.11.	The Effect of Chemo-tactic Agent and Anti-Cancer Drug Treatment.....	80
4.2.12.	Gene Expression Analysis.....	80
4.3.	Results and Discussion	83
4.3.1.	Synthesis and Characterization of GelMA Hydrogel Structure.....	83
4.3.2.	Effects of 2D and 3D GelMA Hydrogels on U373 Cell Viability	87
4.3.3.	Anti-cancer Drug Treatment	93
4.3.4.	Gene Expressions in U373 in GelMA Hydrogels.....	95
4.4.	Conclusion.....	100

5. CONCLUSIONS AND FUTURE DIRECTION	101
5.1. Publications, Conferences & Patents.....	104
A. Appendix A	107
B. Appendix B	113
C. Appendix C	115
BIBLIOGRAPHY	118
VITA.....	128



LIST OF TABLES

Table 3. 1. List of primers for Gene Expression Analysis	54
Table 4.1. List of Primers used for Gene Expression Analysis.	82
Table C. 1. Overview of Therapeutic Molecules	116



LIST OF FIGURES

Figure 3. 1. FESEM micrograph of PEG hydrogel particles. PEG hydrogel carriers were obtained in dextran 40kDa, lyophilized and image was taken with Zeiss Ultra Plus FE-SEM (Bruker) a) Freeze dried PEG particles with 8.96 KX magnification and 2 μ m scale bar. b) MMP sensitive PEG particles diluted in water and vacuum dried illustrated with 15KX magnification and 1 μ m scale bar.	56
Figure 3. 2. AFM micrographs of PEG hydrogel particles. Scans were obtained by Peak Force Quantitative Nanomechanical Property Mapping (QNM) analysis in air imaging mode illustrating a) height b) Log DMT (elasticity), c) adhesion, d) deformation profiles.....	57
Figure 3. 3. X-ray diffraction patterns. Dextran and PEGDA powders, and lyophilized MMP-sensitive PEG carriers, were shown with blue, green and red lines.	59
Figure 3. 4. Release profile of QC loaded PEG particles. a) QC release versus time from QC-loaded MMP-sensitive hydrogel carriers. PEG hydrogels (5 mg/ml) were mixed with QC with an initial concentration of 500 μ M. Total drug release was calculated from absorbance values of QC measured at 425 nm at pre-determined time points. b) The power-law equation coefficients for targeted carrier in the presence and absence of protease enzyme.....	61
Figure 3. 5. Overall expression pattern of MMP-2 in glioblastoma cell lines and representative fluorescent microscopy snapshots for cell behavior and survival with particles. a) qRT-PCR result showing that U87MG cells demonstrated higher level of MMP-2 mRNA expression compared to the other glioblastoma cells. b) Incubation of cells with PEG carriers. c) Untreated (control) mCherry expressing U87MG cells, d) cells, which were incubated with 2 mM RGDS incorporated 5 mg/ml of targeted carriers were captured with 10 X magnification on day 4.....	63
Figure 3. 6. Viability of U87MG cells with carriers and QC. 5 mg/ml gels and 1 μ M QC-loaded gels were suspended in DMEM and added on 96-well plates seeded with 5000 cells one day before. Viability of U87MG cells with carriers was measured 24 h after.....	65
Figure 3. 7. Viability of U87MG GBM cells with TRAIL and QC. Cells were treated with MMP-sensitive RGDS incorporated carriers a) loaded with QC and free TRAIL, b) loaded with TRAIL and free QC. Viability of untreated cells was used as control, single-agent treatments and dual treatments were used for comparison with nanocarrier-loaded treatments. One-way ANOVA followed by Tukey's multiple comparison test. *indicates statistical significance compared to the control group ($P < 0.05$).	66
Figure 3. 8. Overall expression pattern of 12 apoptotic genes evaluated in U87MG cells. a) The single-agent treatments were applied incubating cells with 1 μ M QC and 5 mg/ml MMP-sensitive RGDS containing PEG carriers (Gel) for 6 h. The same concentrations were used for QC-loaded MMP-sensitive RGDS containing PEG carriers (QC-Gel) treatment. b)	

mRNA expression levels of apoptotic genes were quantified from collected cell lysates by qRT-PCR, results were calculated with respect to GAPDH.....	68
Figure 4.1. Time sweep profiles of the elastic modulus (G') and viscous modulus (G''). Hydrogels prepared with A) 37 mM, B) 55.5 mM, C) 74 mM NVP concentrations, D) Table demonstrates loss and storage modulus's.	84
Figure 4. 2. TGA thermograms. A) Gelatin, B) GelMA, C) GelMA hydrogels, and D) Thermal degradation temperatures of gelatin powder, methacrylated gelatin (GelMA) and GelMA hydrogels.....	85
Figure 4.3. Physical properties of GelMA hydrogels. A) FESEM micrograph. Original magnification $\times 200$. Bar represents 100 μm . B) Swelling, C) Gel fraction, D) Degradation profile in the presence of collagenase type I (0.25 mg/ml).....	87
Figure 3. 4. Cytotoxicity Test for U373 Cells on GelMA surfaces at different time points. The cell viability for U373 cells seeded on 2D GelMA hydrogel disks were measured using a CTG assay. The data were normalized to the viability of cells seeded on plate as monolayer on day 1.....	88
Figure 4. 5. A) Schematic representation of spheroid preparation protocol by hanging drops for cell encapsulation. 20 μl drops of cell suspension (20,000 cells) are initially dropped on the covers of cell culture plates, and the inverted covers are incubated for 2 days. B-C) Fluorescence microscopy images of GFP labeled U373 cells laden in GelMA hydrogels on day 4 and day 8. Scale bars are 100 μm	90
Figure 4.6. Different trends in viability for U373 cells. Cells were seeded as sparsely and spheroids on TCPs (monolayer) and within 3D GelMA hydrogels. A-B) Representative images of Calcein AM (green) and EthD-1 (red) costaining of the U373 cells. C-D) Relative cell viability percentages obtained via CTG assay, normalized to viability of sparsely seeded cells on day 1 for either monolayer or 3D encapsulation conditions. Scale bars are 100 μm . Two-way ANOVA followed by Tukey's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).	92
Figure 4. 7. Effects of Digitoxigenin treatment on in vitro 3D models. Viability percentages of A) cells seeded on TCPs as sparsely and in spheroid forms (Normalized to sparsely seeded cells), B) cells encapsulated into 3D GelMA hydrogels as sparsely and in spheroid forms (Normalized to sparsely seeded cells) in the absence and presence of Dgx (2.5 μM). Decrease in viability percentages of sparsely seeded cells and cells in spheroid forms following Dgx treatment C) on TCPs, D) in 3D GelMA hydrogels were calculated relative to untreated cells for each condition. E) Fluorescence Microscopy Images of GFP labeled U373 cells and Dgx treated GFP labeled U373 cells laden in GelMA Hydrogels. Yellow circles show spheroid bodies. Scale bars are 100 μm . Two-way ANOVA followed by Tukey's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).	94
Figure 4.8. A) Schematic representation of four apoptotic genes (Bad, Puma, BCL-2, Caspase-3) in controlling the apoptosis on cancer cells. B-E) Bar graphs represent	

expressions of apoptotic genes evaluated in U373 cells mRNA expression levels of apoptotic genes were quantified on day 4 from collected lysates of cells seeded TCPs and in 3D GelMA hydrogels as sparsely and in spheroid forms by qRT-PCR, results were calculated with respect to the reference gene GAPDH and normalized to sparsely seeded cells for each condition. Data are expressed as mean $\pm$ error.	96
Figure 4.9. Bar graphs represent quantitative real-time PCR of invasion related MMP expressions evaluated in U373 cells. mRNA expression levels of apoptotic genes were quantified on day 4 from collected lysates of cells seeded on TCPs and in 3D GelMA hydrogels as sparsely and in spheroid forms by qRT-PCR, results were calculated with respect to the reference gene GAPDH and normalized to sparsely seeded cells for each condition. Data are expressed as mean $\pm$ error.	98
Figure A. 1. FTIR Spectra of MMP-sensitive peptide conjugation with acrylated PEG chains.	107
Figure A. 2. SEM image for MMP-sensitive millimeter-sized hydrogels. Image of the hydrogel captured with a 340 X magnification with scalebar of 100 μ m.....	108
Figure A. 3. Degradation behavior of MMP-sensitive hydrogels. Graph shows the increasing mass loss of hydrogels in 1 μ g/ml collagenase type-I solution for 1h at 37 $^{\circ}$ C.....	109
Figure A. 4. Size and zeta potential distribution of RGDS incorporated degradable PEG carriers measured by Zetasizer instrument.	110
Figure A. 5. BET analysis of hydrogel particles. Cumulative pore area (a) and cumulative pore volume (b) obtained from adsorption isotherms, and quantity of adsorbed N ₂ (c). The samples were degassed at 50 $^{\circ}$ C for 5 h under vacuum prior to analysis.	111
Figure A. 6. Laser Scanning Confocal Microscopy images of fixed U87MG cells (a) and BJ fibroblasts (b) after 1 h incubation with fluorescein o-acrylate labeled degradable RGDS containing carriers. Cellular uptake of carriers was quantified from 20 different images	112
Figure A. 7. Effects of TRAIL and QC on Human Dermal Fibroblast (BJ) Cells. Fibroblasts were treated with 1 μ M QC or/and 40 ng/ml TRAIL and viability measured 1 and 4 days after treatment using Cell Titer Glo Assay.	112
Figure B. 1. TNBSA Assay Calibration Curve.....	113
Figure B. 2. FTIR Spectra of Gelatin, Methacrylated Gelatin and synthesized Hydrogel	113
Figure C. 1. Chemical Structure of A) PEG, B) Dextran, and distribution of abundant aminoacids in gelatin structure	117

LIST OF SCHEME

Scheme 2. 1. Illustration of chromosomal and genetic alterations included in the formation of GBM. The relations between the genes, pathobiology and survival that lead to the genesis of primary (de novo) and secondary (progressive) GBMs [2]. Copyright (2017), with permission from Cold Spring Harbor Laboratory Press.	25
Scheme 2. 2. Targeting the growth factor signalling pathways in GBM. From reference [8] Copyright (2017), with permission from Elsevier.	28
Scheme 2. 3. Major elements of apoptosis pathways. © 2013 Goncharenko-Khaider N, Lane D, Matte I, Rancourt C, Piche A. Published in [22] under CC BY 3.0 license. Available from: http://dx.doi.org/10.5772/53380	30
Scheme 2. 4. Schematic demonstration of environment-sensitive drug delivery systems. Drugs comprising small molecules, peptides, therapeutic proteins and DNA, may be carried by delivery vehicles such as liposomes, micelles, magnetic nanoparticles, dendrimers and hydrogels, and release of them can be induced by stimuli such as irradiation, heat or pH. These intelligent delivery systems target various regions in cells and tissues as cell surface receptors, nucleus or organelles {Erkoc, 2015 #53}.	39
Scheme 2.5. Developments in experimental models of GBM.....	44
Scheme 3.1. Synthesis of peptide conjugates and targeted carriers via water-in-water emulsion photopolymerization. SVA group is removed from the structure in basic pH conditions and peptides bind to acrylate-PEG construct from their amino terminals and N groups of the side chains.....	55
Scheme 4.1. Representation of methacrylation and synthesis processes of gelatin hydrogels.	76

ABBREVIATIONS

PEG	Poly(ethylene glycol)
PEGDA	Poly(ethylene glycol) diacrylate
GelMA	Gelatin Methacrylate
Acr-PEG-SVA	Acrylate-PEG- Succinimidyl Valerate
TEA	Triethanolamine
NVP	1-vinyl-2-pyrrolidinone
MMP	Matrix metalloproteinase
DAPI	4',6-diamidino-2-phenylindole
GBM	Glioblastoma multiforme
BBB	Blood–brain barrier
TRAIL	TNF-related apoptosis-inducing ligand
TNF	Tumor necrosis factor
DR4	Death Receptor 4
DR5	Death Receptor 5
FADD	Fas-Associated protein with Death Domain
Bcl-2	B-cell leukemia 2
BH	Bcl-2 Homology Domain
Bcl-xL	B-cell lymphoma-extra large
Bad	Bcl-2-associated death promoter
Bid	BH3 interacting-domain death agonist
Mcl-1	Myeloid cell leukemia 1
PUMA	p53 upregulated modulator of apoptosis
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
HRK	Harakiri
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
QC	Quinacrine Dihydrochloride
DGX	Digitoxigenin
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
Eqn	Equation
EtOH	Ethanol
FBS	Fetal Bovine Serum
FTIR	Fourier Transform Infrared Spectra
GFP	Green Fluorescent Protein
RGDS	Arg-Gly-Asp-Ser
GGGPQGGQGK	Gly-Gly-Gly-Pro-Gln-Gly-Gly-Gln-Gly-Lys
IR	InfraRed
PBS	Phosphated Buffer Saline

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
FESEM	Field Emission Scanning Electron Microscopy
XRD	X-Ray Diffraction
DLS	Dynamic Light Scattering
qRT-PCR	Quantitative Real-time Polymer Chain Reaction



NOMENCLATURE

W_s	Weight at swollen (wet) state
W_d	Weight at dry state
Q	Volumetric swelling ratio
M_i	Initial amount of QC
M_f	Final amount of QC
k	Characteristic constants of hydrogel
n	Exponential coefficient
C_t	Concentration of QC in the buffer at time t
V	Volume of drug release buffer
V_s	Sample volume that is taken
M_∞	Total weight of QC released

Chapter 1

1. INTRODUCTION

Malignant glioblastoma multiforme (GBM) is one of the biggest problems encountered by the neuro-oncology community. Present treatments have not sufficient effects on patient survival and preventing the recurrence. Therefore, effective delivery of therapeutics to the glioblastoma tumors and comprehending the invasive nature of these tumors is critical [1].

The overall aim of this thesis was to design hydrogels for GBM therapy and investigate their therapeutic potentials *in vitro*. We have synthesized hydrogel-based carriers from PEG which can be used for the controlled delivery of chemotherapeutics based on the MMP activity for GBM treatment. We have also designed a 3D brain tumor model to help clarify the response of GBM cells to bioengineered material using GelMA hydrogels.

The outline of this thesis is as follows:

Chapter 2 provides relevant background and literature review on GBM tumors and biomaterials used for its treatment.

Chapter 3 covers the synthesis and characterization of MMP-sensitive PEG carriers and evaluation of synergistic effects of TRAIL and Quinacrine on glioblastoma viability.

Chapter 4 presents the the synthesis and characterization of GelMA hydrogels and investigation as a 3D microenvironment for GBM.

Chapter 5 summarizes the results of this dissertation and and discusses about the future work which can be applied with the proposed systems.

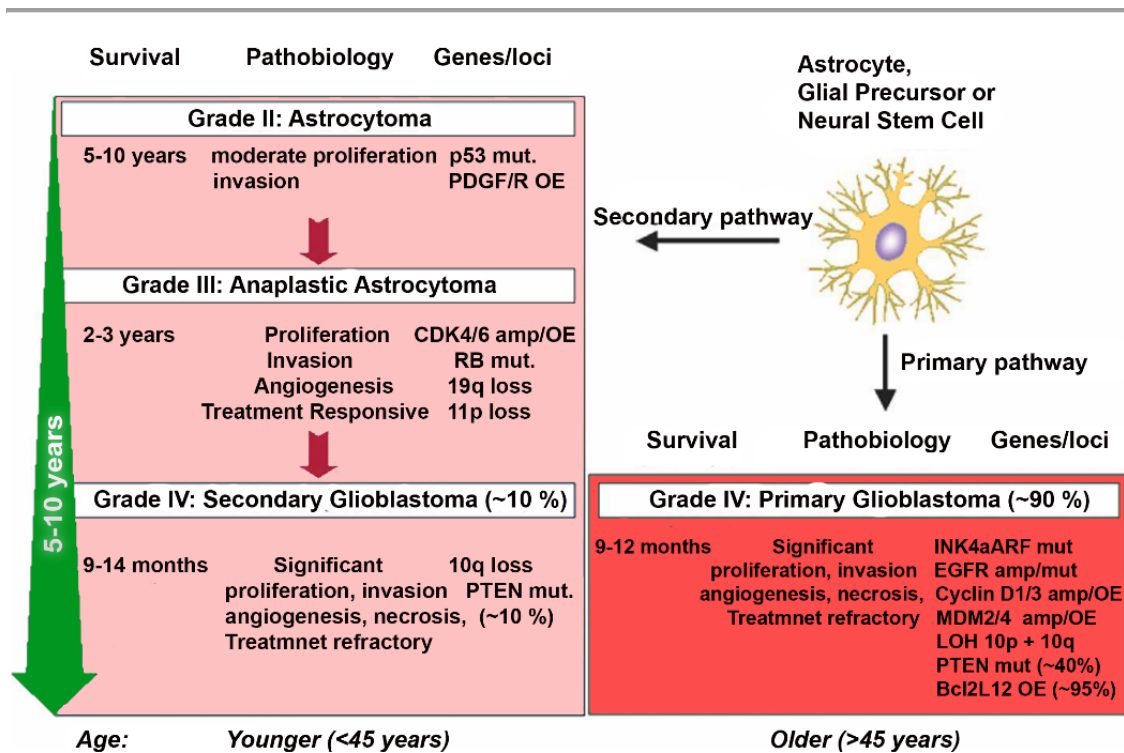
2. LITERATURE REVIEW

2.1. Overview of Glioblastoma Multiforme

Every year 6.4. per 100,000 individuals are diagnosed with brain tumors around the globe. Gliomas are the most commonly diagnosed brain and central nervous system (CNS) cancers [2].

Gliomas are histologically classified as astrocytomas, oligodendrogliomas, or oligoastrocytomas. Gliomas are also classified according to their malignancy degree, in the scale of I to IV. Grade IV tumors display the most aggressive features including angiogenesis and necrosis, and as they are refractory to radio and chemotherapy [3]. In fact, astrocytoma grade IV or glioblastoma multiforme (GBM) incidences make up approximately 70 % of all gliomas [4].

GBM tumors are highly heterogeneous regarding clinical presentation, pathology, genetic signature and response to the treatment as the term “multiforme” reflects. Although a small fraction of adult cancers are GBMs, their malignant nature makes them the fourth highest reason of cancer-related deaths [5].



Scheme 2. 1. Illustration of chromosomal and genetic alterations included in the formation of GBM. The relations between the genes, pathobiology and survival that lead to the genesis of primary (de novo) and secondary (progressive) GBMs [2]. Copyright (2017), with permission from Cold Spring Harbor Laboratory Press.

Recent studies presented that glioblastomas can also be sub-classified in gene expression profiles. The studies of The Cancer Genome Atlas Research Network categorized genetic alterations into three main pathways: retinoblastoma (RB) signaling, tumor protein 53 (TP53) signaling, and receptor tyrosine kinase signaling. Most of glioblastoma cases are in the classical subgroup, holding epidermal growth factor receptor (EGFR) mutations and deletion of cyclin-dependent kinase inhibitor 2A (CDKN2A). The mesenchymal subgroup resembles to classical glioblastomas but with usual deletions in neurofibromin 1 (NF1). The neural subgroup is the most poorly defined; however, the specific neural expression pattern is attributed to contamination of surrounding tissue. The proneural subgroup presented point mutations in isocitrate

dehydrogenase 1 and 2 (IDH1/IDH2), mutation and amplification of platelet derived growth factor receptor (PDGFRA) [6]. Indeed, the recent glioblastoma studies revealed that proneural tumors with IDH1 mutations and hypermethylation across the genome are the only subtype with enhanced survival [6, 7].

2.2. Current Treatments

In spite of the recent advances in surgical and medical techniques, the poor diagnosis for patients with GBM remains to be an issue. Current therapies including surgery, radiation therapy, and chemotherapy for GBM are deficient with a nearly worldwide recurrence [8].

Standard chemotherapies in GBMs are classified as systemic, local and convection-enhanced delivery (CED) approaches [9].

In 1996, for the first time, FDA approved the use of an alkylating agent loaded diffusion-based drug delivery system, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)-loaded polyanhydride, for direct transport of chemotherapeutics to brain tumors. In this system, drug-loaded implant was placed near to the tissue for producing the highest drug concentrations in the region most in need of treatment. The drug is released by diffusion near the implant, and reducing side effects to other tissues. The clinical product is named as Gliadel® [8, 10-13]. Implantation of a single polymer implant in the brain of an animal produces unprecedented concentrations of active BCNU within the tissue near the implant [8, 11, 12]. The drawback of Gliadel® system was the slow transport of large molecules such antibodies and small drug molecules which have higher diffusion rates are often rapidly removed by metabolism or capillary clearance [8].

In the early 1990s, development of convection-enhanced delivery (CED) system

offered solution for limited distribution volume problem related with diffusion-based delivery systems. In this prominent approach, a flow was applied through a cannula under constant or gradients of pressure to deliver therapeutic agents to the brain, in addition to concentration gradients. Using CEDs make it possible to distribute the drugs extensively in the brain, even large molecules, and they have low risk of neurologic injury when compared with implantable polymers. However effects of free drugs disappear soon after the infusion stops due to the short half-lives in the brain and inflammation adjacent to the catheter tract and at the catheter tip is the major source of concern [8, 14].

Besides the use of CED to deliver conventional chemotherapeutics directly into GBMs in clinical trials, CED is still under investigation to deliver new type of therapies including gene therapies, oligonucleotides, viral particles, nanoparticle and liposomal systems [14].

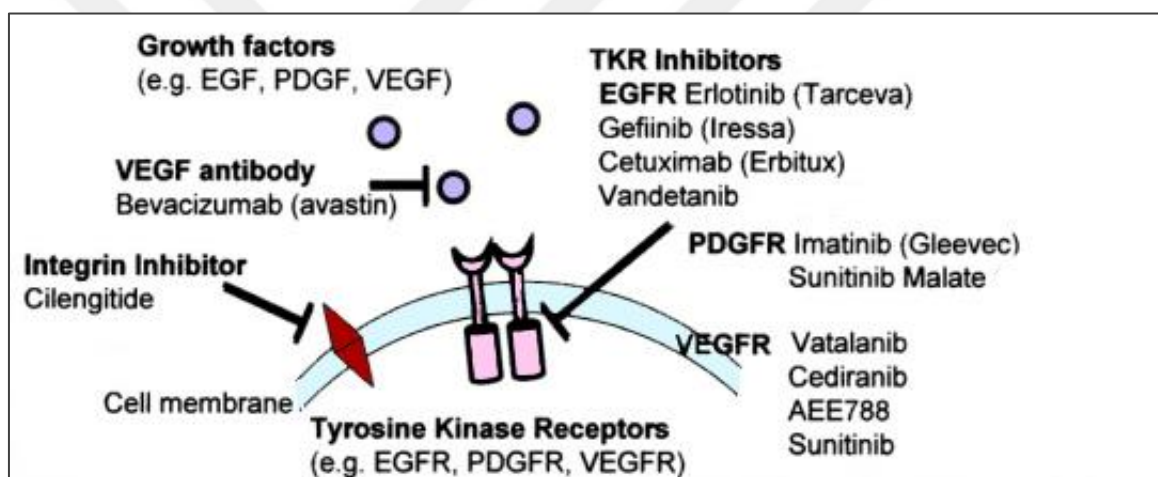
2.3. Alternative Strategies

Transport of drugs into the brain is the main problem in treatment for GBM. The blood-brain barrier (BBB) restricts the most systemic-delivery of molecules into the brain. Hence, alternative strategies have been investigated to obtain increased anti-cancer effect from therapeutics [7].

2.3.1. Targeted Therapy

The signaling pathways that are responsible for the malignant behavior of GBMs by inducing cell migration and tumor invasiveness can offer new molecular targeted therapy choices. Regulation of these pathways are usually sustained by amplification and/or overexpression of various tyrosine kinase-related growth factors, hormones [8].

Small drug molecules and large antibodies have been used to inhibit receptors of epidermal growth factor (EGF), platelet-derived growth factor (PDF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) (**Scheme 2.2.**). For example, Gefitinib and Erlotinib drugs were developed to target EGFR which has mutations leading to overexpression of it [8, 14]. Phase II trial of Gefitinib is well tolerated and has activity in patients with recurrent GBM [15]. Phase II trials of Erlotinib exhibit better radiographic responses on survival rates compared to gefitinib [16].



Scheme 2. 2. Targeting the growth factor signalling pathways in GBM. From reference [8] Copyright (2017), with permission from Elsevier.

2.3.2. Apoptosis and TRAIL

Evading apoptosis is some of the acquired features of GBM cells that results in inefficient treatments. Programmed cell death, i.e. apoptosis, plays a vital role in the developmental processes and maintaining of homeostasis in eucaryotes [17]. Apoptosis is controlled via two major pathways in mammalian cells; extrinsic and intrinsic pathways. Extrinsic pathway is facilitated by death receptors that are members of the tumor necrosis factor (TNF) receptor superfamily. Intrinsic pathway is activated by

CHAPTER 2: LITERATURE REVIEW

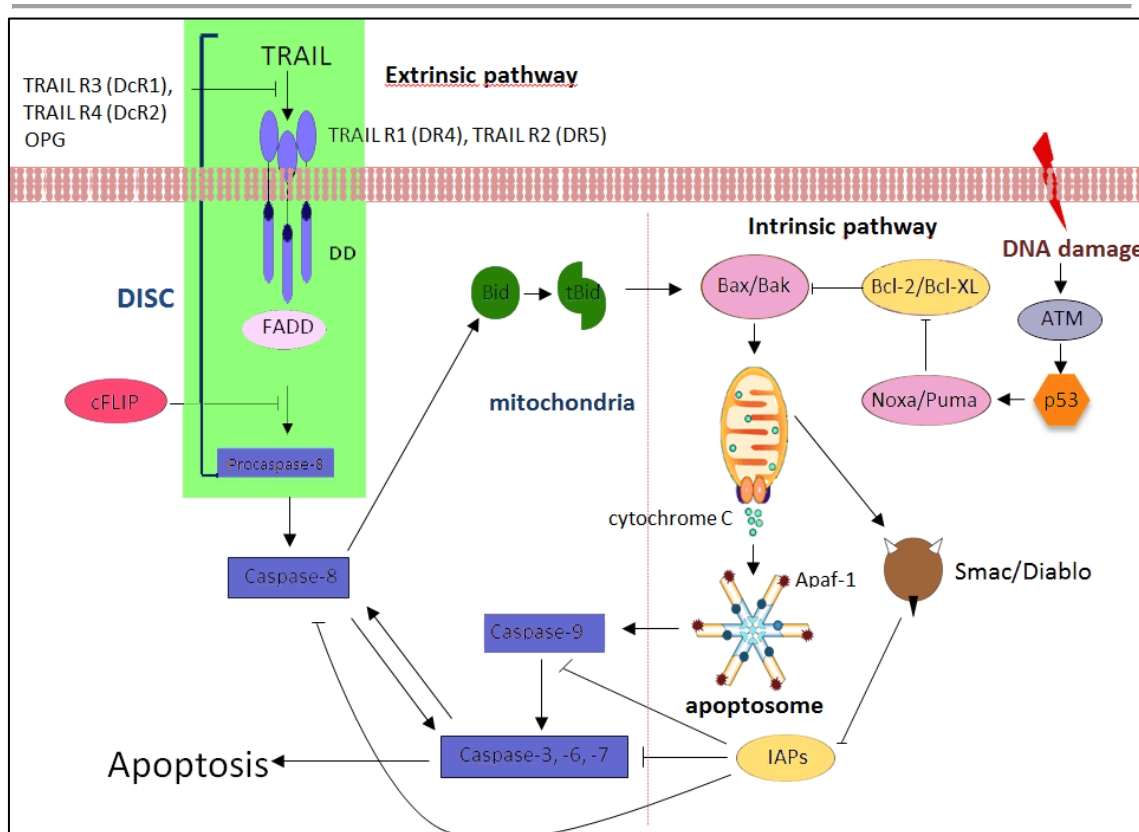
cellular stress and DNA damage, includes the activation of p53 and release of pro-apoptotic factors from mitochondria [18, 19].

Extrinsic pathway is induced from the external part of cell with the interaction of several ligands with death receptors (DRs). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is one of the pro-apoptotic ligands activating extrinsic programmed cell death pathway.

TRAIL was first discovered in 1990s by two separate groups, where they reported that it contains sequence homology with the extracellular domain of Fas-L and TNF [20, 21].

The gene codes the TRAIL is a 20 kb gene located on the 3th chromosome and consists of five exons and four introns [18, 20]. Human form of TRAIL is a type II transmembrane protein and composed of 281 amino acids and cleavage of its extracellular domain allows for a soluble form of TRAIL [18, 22].

TRAIL has ability to interact with four membrane-bound receptors from which only TRAIL-R1 and TRAIL-R2, also called as DR4 and DR5, have cytoplasmic death domains. Therefore, DR4 and DR5 can transduce the apoptosis signal to downstream molecules upon TRAIL binding. TRAIL-R3 and TRAIL-R4, known as decoy receptors, and one soluble TRAIL receptor osteoprotegerin (OPG) can not activate apoptosis due to the lack of cytoplasmic domains [18, 19].



Scheme 2. 3. Major elements of apoptosis pathways. © 2013 Goncharenko-Khaider N, Lane D, Matte I, Rancourt C, Piche A. Published in [22] under CC BY 3.0 license. Available from: <http://dx.doi.org/10.5772/53380>

TRAIL binding to DR4 and DR5 initiated to recruitment of the adaptor FADD and activation of initiator procaspase-8 and -10 that forms Death-inducing signaling Complex (DISC). Activation of caspase-8 and -10, lead to activation of effector caspase-3, -6 and -7, subsequently apoptosis [19].

There is a cross-talk between extrinsic and intrinsic pathways in TRAIL-induced apoptosis. Truncated version of BH3-interacting domain death agonist (tBid) engages the intrinsic apoptotic pathway by binding to Bcl-2 homologous antagonist killer (BAK) and Bcl-2-associated X protein (BAX) which cause oligomerization of these proteins and translocation to the mitochondrial outer membrane. This step is resulting to permeabilization of the mitochondrial outer membrane and release of pro-apoptotic

Cytochrome-c which forms a structure called apoptosome along with ATP and Apoptotic peptidase activating factor 1 (APAF-1). This complex is needed for caspase-9 activation and caspase-9 can activate effector caspase-3, -6 and -7 [18, 23].

Despite the fact that TRAIL can induce apoptosis particularly in cancer cells without damaging healthy cells due to the significantly high DR expressions on cancer cell surfaces, the efficacy of TRAIL is limited by the intrinsic and/or acquired resistance of tumor cells to the therapeutic substance. Thus, understanding the molecular mechanisms of TRAIL resistance, likewise finding new approaches to solve this problem for an effective therapy remains the keystone of many research studies [18]. Treatments with secondary therapeutics in addition to TRAIL is one of the possible solutions to overcome resistance [4].

2.3.3. Combinatory Drug Treatments

Considering the heterogeneity of GBM tumors, it is not surprising that single-drug treatments in clinical tests on patients are failing. Resistance to therapies GBM is usually resulted from the parallel activation of signaling pathways, such as activation of receptor tyrosine kinase receptors (RTKs). Targeting multiple binding sites by drug combinations and compensatory activation of alternative signaling pathways may be useful to overcome resistance problem [24].

In preclinical studies, TRAIL was shown to possess therapeutic potential when used in combination with chemo or radiotherapy [25]. Recently, it is reported that the small drug molecules Bortezomib, Mitoxantrone, Lanotoside C, Quinacrine, Digitoxigenin were capable of sensitizing GBM cells to TRAIL in drug screen experiments [4, 26-28].

2.3.4. Biomaterials and GBM

Several bioactive materials have been utilized for GBM chemotherapy [29].

Nanoparticles made of inorganic materials such as gold are widely preferred for delivering therapeutics. For example, an anticancer drug doxorubicin (DOX) was delivered by ultra small gold nanoparticles (5 nm) functionalized with a transactivator of transcription (TAT) peptide was more successful for the treatment of tumor than free form the drug in U87 mouse model [29, 30].

Liposomes are another type of delivery vehicles with sizes differ from nanometers to micrometers. These spherical structures contain hydrophilic cavities between lipid bilayers which can be loaded with hydrophilic drug molecules. In a recent *in vivo* GBM study, the combinatory treatment of TRAIL-liposomes and DOX-liposomes exhibited a higher anti-GBM effect than free drugs or drug-liposome complexes alone [31].

Polymers are also prominent candidates for GBM therapy considering their diverse structures and feasible functionality. Numerous bioactive materials have drawn the attentions of the scientists, including poly(ethylene glycol) (PEG), poly(lactic-co-glycolic acid) (PLGA), polyethylene imine (PEI) and so on [26, 32, 33].

Furthermore, natural polymers such as gelatin, chitosan, hyaluronic acid, alginate and their semi-synthetic methacrylated versions have been widely used for either delivering therapeutics in nano/micro particle form or investigating biomimetic GBM-microenvironment as 3D hydrogel networks [34-36].

2.3.4.1. Designing Hydrogels for GBM Therapy

2.3.4.1.1. Characteristics of Hydrogels

Conventional treatments are generally deficient in synchronizing the needed dose in therapeutic window and the actual drug concentration released from the dosage in the required time period. To fulfill this requirement, pharmaceutical scientists have focused on intelligent drug delivery systems in which the necessary quantity of active substance is made applicable at the desired time and region in the body. Hydrogels have come into prominence as a promising choice in this respect [1].

In 1960, Wichterle and Lim first proposed that hydrophilic gels can be used for biological uses [2]. This study paved the way for the development of hydrogels for biological purposes. In 1980s, scientists achieved to encapsulate cells and prepare matrices for wound dressings [1, 3].

Hydrogels are water-swollen, cross-linked polymeric networks, which can be made of natural or synthetic materials. Both natural and synthetic materials contain hydrophilic functional groups that provide high water absorption ability and they are resistant to dissolution due to their physical or chemical cross-links [4, 5].

Polymer chains can be physically cross-linked by various physicochemical interactions such as hydrophobic interactions, hydrogen bonding, charge condensation, and physical conditions like pH, ionic strength and temperature [6]. Calcium alginate is an example of physically cross-linked ionotropic hydrogels wherein a polyelectrolyte is combined with a multivalent ion of the opposite charge [3, 7]. Whereas physically cross-linked hydrogels are advantageous for not requiring chemical modifications or the addition of cross-linking moieties to form networks, all of the interactions that keep the structure as a monolith are reversible, and can be disrupted by changes in environmental

factors [3, 6]. For instance, a burst release of riboflavin was observed from PNIPAM-grafted hyaluronic acid gel that was formed *in vivo* [6].

Conversely, covalently cross-linked hydrogels are stable structures that prevent diffusion of the polymer away from the injection site. Besides, it is easy to control parameters such as gelation period, pore size, chemical functionalization and degradation time. Small molecules and polymers are generally used for chemical cross-linking processes. Unfortunately, small-molecule cross-linking agents such as peroxidases are potentially toxic, thus polymers whose reactive groups are pre-functionalized, are preferred to avoid this problem. However, this approach also has its limitation which is the necessity of polymer modification chemistry to prepare the functionalized pre-polymer [6]. One of those polymer modification reactions occurs between amines or thiols and vinyl groups to form amides or sulfides, called as Michael addition. This reaction is used to modify monomers of polysaccharides, collagen, PEG, PVA for polymeric reaction [3, 8, 9].

2.3.4.1.2. Techniques in preparation of Hydrogels

Hydrogels may be in the form of matrix, film, or microsphere which is determined by the method of polymerization used in the preparation procedure[37]. Both physical and chemical techniques have been utilized to create hydrogels. Chemically cross-linked hydrogels can be generated by various methods such as chemical reaction of complementary groups, radical polymerization, high energy irradiation and enzyme. Functional groups with complementary reactivity in polymer chains, such as an amine-carboxylic acid, can be used to create covalent bonds. Radiation-induced polymerization which uses gamma and electron beam can also be preferred to obtain hydrogels from a mixture of a functional acrylate and an appropriate cross-linker. In a recent study,

glutaminyl functionalized PEG and poly(lysine-co-phenylalanine) monomers were cross-linked with the addition of transglutaminase enzyme [38]. Another polymerization technique is radical polymerization that includes three main constituents: monomer, cross-linker and initiator [37]. Redox, thermal and photoinitiated mechanisms can be used to form radicals from initiator molecule to start a radical chain polymerization [39]. In the case of the initial reaction mixture are homogeneous, radical polymerizations can be accomplished as homogeneous processes, contrariwise initial heterogeneous conditions accompanies to heterogeneous processes. Homogeneous processes can be classified as bulk polymerization and solution polymerization, whereas emulsion and suspension polymerization are heterogeneous processes that are ascendant in solving thermal and viscosity problems [40]. Physical, chemical and mechanical features of hydrogels can be tuned depending on the selected polymerization technique. For example, dense hydrogels were obtained by the bulk radical-initiated polymerization of poly(2-hydroxyethyl methacrylate); however, by using the solution radical-initiated polymerization technique with water as a diluent, an interconnected porous hydrogel structure was obtained with the same monomer [41].

2.3.4.1.2.1. Bulk Polymerization

Bulk polymerization is a simple method which requires only a liquid monomer and an initiator in the absence of solvent. It has advantages like high reaction rate and yield, resulting in comparatively pure products. Nevertheless, viscosity of system rises quickly interfering with the quality of products in this method. Therefore, bulk polymerization processes have need of careful temperature control with a continuous stirring system to remove excessive heat of exothermic natured reactions [40].

2.3.4.1.2.2. Solution Polymerization

In solution polymerization, monomers are dissolved in a solvent of choice and then the reaction takes place. Overheating problem and difficulty of stirring due to high viscosity is handled with the addition of solvent as diluent in this system. However, if the solvent is one that is difficult to remove, its presence decreases the purity of the product [40].

2.3.4.1.2.3. Emulsion Polymerization

Emulsion polymerization is a dispersion polymerization method includes well distributed droplets around 0.5 -10 μm of monomers. It differs from suspension polymerization by the size and type of obtained particles[40]. This technique is commonly used to obtain waterborne resins with several colloidal and physicochemical features and advantageous for not requiring grinding to produce products in micro- and nano-sizes [37, 42]. Frequently, emulsification of the comparatively hydrophobic monomer occurs in water by an oil-in-water emulsifier in this free radical polymerization procedure, and then followed by initiation reaction [42]. A reverse process of this common oil-in-water (O/W) polymerization has been popular in recent years which is called as water-in-oil (W/O) process [37]. Emulsion polymerization provides a higher polymerization rate and ease of obtaining higher molecular weighted polymers by preventing bimolecular termination of free radicals in distinct particles. Transportation of monomer, free radicals and surfactant and diffusion of these substances among the continuous phase, monomer emulsions, primary reaction center and interface of two phases are the critical points that affect growth step of the particles [42].

2.3.4.1.2.3.1. Water-in-oil Polymerization

Limitation of moderately low monomer concentrations due to the high viscosity problem in solution polymerizations is overcome by using two-phase water-in-oil polymerization technique. An aqueous monomer solution is distributed in an organic continuous oil phase using an emulsifier and colloidal dispersion of water swollen polymer particles were obtained in oil by the free radical polymerization in this process [43].

Hydrophilic polymeric microparticles of natural monomers such as chitosan, starch, gelatin and silk fibroin[44], and synthetic polymer microparticles of acrylamide and PEG were produced by water-in-oil emulsion method [44, 45].

In a recent study, it was shown that microencapsulation of cells resulted in higher viability percentages comparing to bulk encapsulation, since it makes easier the diffusion of oxygen and nutrients. Also, cell-laden microparticles do not require invasive implantation operation, instead they can be delivered by a simple injection [45].

2.3.4.1.2.3.2. Water-in-water Polymerization

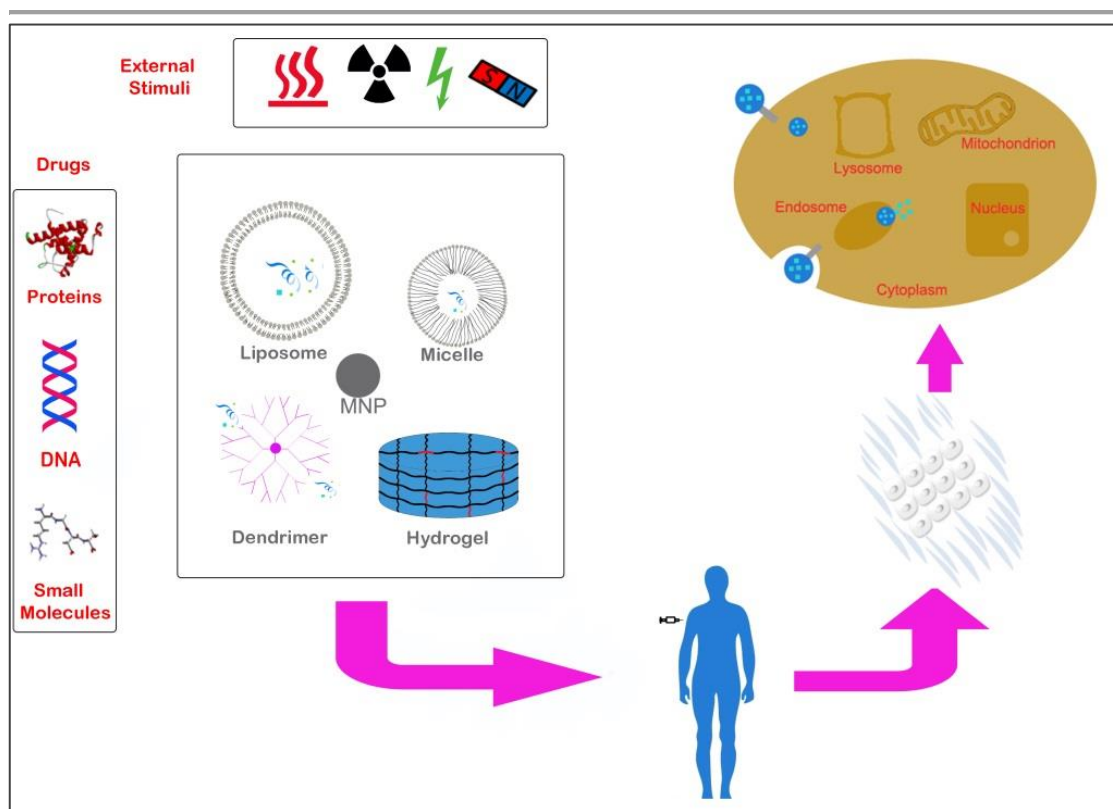
Hydrogel microspheres were produced by using water-in-oil emulsion polymerization method widely; however, organic solvents, shear forces and surfactants in the nature of this process can destroy stability of protein based therapeutics incorporated into the pre-polymer solution [46]. Recently, a water-in-water emulsion process was developed to synthesize hydrogel microspheres in the absence of organic solvents [47]. This technique leans on immiscibility of aqueous polymer combinations such as dextran, PEG, PVA, gelatin and starch. In 1986, Albertson found that above a certain concentration water-soluble polymers stay in solution as separated in two phases. The polymer in the dispersed phase can be cross-linked to obtain hydrogel microparticles

after emulsification process[48, 49]. In a study, hydrolytically and enzymatically degradable PEG microspheres were formed via thiol chemistry and UV light induced water-in-oil emulsion polymerization process [47].

2.3.4.1.3. Responsiveness to Environment

Controlled drug delivery is a hot prospect for the future of biomedical applications. This system is developed with intend of to overcome the inadequacies of conventional drug delivery routes. It is possible to deliver drugs at determined proportions in scheduled periods [50]. For the controlled delivery drug desorption should be settled in accordance with the sensitivity to the metabolic changes in the body [51].

Hydrogels are beneficial devices to protect drugs from the hostile environment of stomach in means of low pH and degradation enzymes. Additionally, they can be tailored to sense to the environmental parameters [50]. Those hydrogels can exhibit major changes in their scaffolds, consequently in their mechanical strength, pore sizes and swelling behavior in the presence of both internal and external stimuli [52]. Drug loading and release from hydrogels can be triggered by environmental stimuli such as temperature, pH, ions in solution, electric field and light (**Scheme 2.4.**) [53].



Scheme 2. 4. Schematic demonstration of environment-sensitive drug delivery systems. Drugs comprising small molecules, peptides, therapeutic proteins and DNA, may be carried by delivery vehicles such as liposomes, micelles, magnetic nanoparticles, dendrimers and hydrogels, and release of them can be induced by stimuli such as irradiation, heat or pH. These intelligent delivery systems target various regions in cells and tissues as cell surface receptors, nucleus or organelles [53].

Stimuli responsive hydrogels commonly exhibit reversible volume phase or gel–sol transitions in the presence of stimuli due their elastic deformability characteristics and they regain their original shape after the removal of stimuli [50, 54].

An appropriate controlled delivery system requires synergy between release kinetics of drug and pathological conditions. Depending on magnitude of signal resulted by disease sometimes multiple sensitive systems are needed that give response to more than one stimulus[51]. Temperature-sensitive poly(*N*-isopropylacrylamide) PNIPAAm and

pH-sensitive PAA containing composite hydrogels can be given as example of dual sensitive systems [55].

2.3.4.1.3.1. pH responsive hydrogels

Many regions in the body has significant pH differences such as stomach, gastrointestinal tract [56] and vagina [57]. Similarly, pH of normal tissues changes in disease conditions. For example, blood vessels in certain cardiovascular defects[58], tissue/wounds wherein inflammation is present [59]and the tumor microenvironment [60] have pH alterations from the healthy tissues. Thus, pH-responsive systems have found a place in recent studies.

Common feature of pH sensitive hydrogels is containing either acidic (e.g. carboxylic and sulfonic acids) or basic groups that receive or release protons as an answer to fluctuations in environmental pH. These hydrogel networks generally consist of ionizable functional groups and responsiveness is resulted from the electrostatic repulsion among charges present on the polymer chains. In the literature, hydrogels prepared with polyanions (PAA) cross-linked with azoaromatics used as colon specific drug delivery vehicles which show very low swelling and release profiles in stomach conditions [51].

2.3.4.1.3.2. Temperature Responsive Hydrogels

Several polymers display a temperature-sensitive phase transition property which results from the presence of hydrophobic groups. The polymers such as poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) with critical solution temperature (LCST) at around the body temperature, have been used extensively based on the sol–gel phase transition at the body temperature. Also, block copolymers of PEO–PPO, called as Pluronics, are commercially offered [51].

2.3.4.1.3.3. MMP responsive hydrogels

An ideal delivery vehicle should degrade in the duration of tissue regeneration process to facilitate no final foreign materials would stay [61]. For this purpose, natural materials are usually preferred where enzymes can cleave from either specific amino acid sequences or biologically relevant chemical structures. For example, collagen and the disaccharide chains of HA are degraded with matrix metalloproteinases (MMPs) and hyaluronidases, respectively [62].

Moreover, nondegradable hydrogels can be utilized to be degraded with the incorporation of mimetic peptide sequences into the polymeric networks. This method has been used widely to integrate MMP and plasmin sensitive peptides in both natural and synthetic polymers. As well, the peptide sequences can be tuned in their MMP-specific degradation spots [63].

The development of these hydrogels was firstly started with the use of MMP substrate site within the alpha chain of type I collagen (GPQGIAGQ) in the structures. It was shown that a single amino acid substitution (A/W) increased the activity. Then, several alterations in the peptide substrate sequence were applied to modulate the hydrogels to degrade faster or slower [61].

2.3.4.1.4. Hydrogels for Targeted Delivery

Cell-surface adhesion strength changes depending on the concentration of ECM proteins or anti-integrin antibodies adsorbed onto the surface. Three-dimensional hydrogel systems have been functionalized upon systematically varied quantities of ECM proteins. Since, integrins demonstrate higher tendencies to cluster and contact with the cytoskeleton by ligand binding, the use of the Arg-Gly-Asp (RGD) peptide motif derived from the cell-binding domain of fibronectin has been gained importance as the integrin

binding ligand. The RGD sequence has been presented to interact with $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins [37]. Smooth muscle cells seeded homogeneously in the RGD grafted PEG hydrogels during photopolymerization stayed viable, proliferated, and produced ECM proteins [38].

Another commonly studied peptide fragments is Ile-Lys-Val-Ala-Val (IKVAV), which is derived from laminin, is believed to have comparable biological effects to laminin. It has been shown to stimulate neuron adhesion, survival, migration and axon elongation *in vitro* and whereas decreasing glial scar formation *in vivo* [64]. For example, in a recent paper related to neural stem cells in the IKVAV peptide modified silk fibroin hydrogels showed that cell viability and neuronal differentiation capabilities were increased, that contributed to knowledge of IKVAV peptide [65].

A pentapeptide, Cys-Arg-Glu-Lys-Ala (CREKA), was found to have special affinity for fibrin [66]. This peptide is called as a tumor homing peptide, since it distinguishes fibrin-associated plasma proteins that are overexpressed in tumorous tissue but not in healthy tissue. Because of the protein leakage from the permeable vasculature of the tumor, the walls of tumor vessels and the interstitial space within tumors contains these fibrin-fibronectin multiplexes [67]. Conjugation of this peptide into the polyethylene glycol-polylactic acid nanoparticles stimulated glioblastoma-homing and prolonged NP retention at the tumor site [66].

2.3.4.1.5. Hydrogels as Tissue-Engineered 3D Matrixes for mimicking GBM Microenvironment

GBM progression is highly dependent on tumor-niche signalings including interactions of tumor cells with surrounding brain stroma cells as endothelial cells, pericytes, microglia, astrocytes and neurons. Creating a tumor vasculature, cell migration

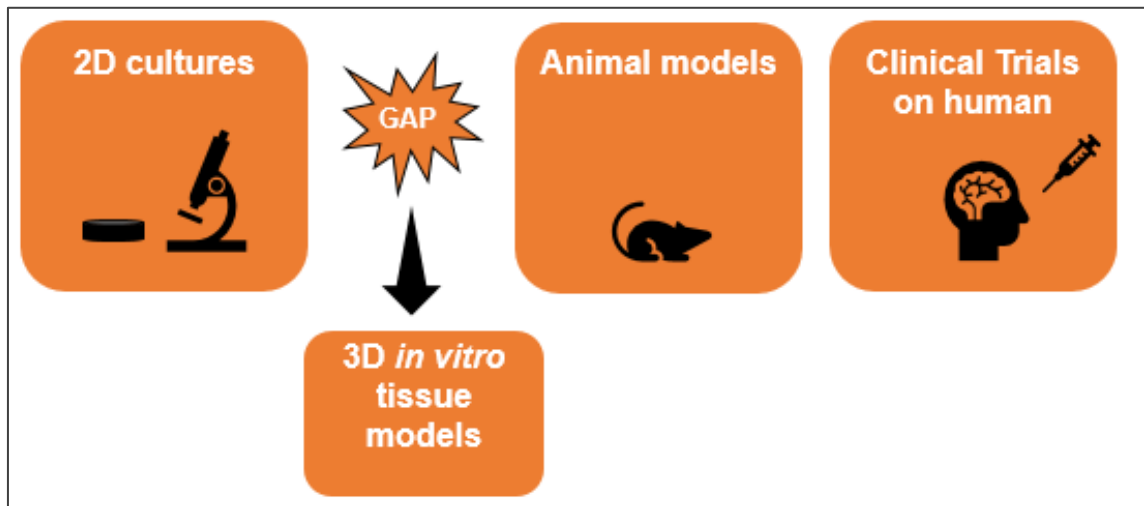
and invasion, resisting to hypoxic conditions are the main niches of GBMs that are connected to complex signaling pathways [68]. Previously in the literature, it was found that GBM cell behavior on 2D substrates is usually fail to reflect *in vivo* physiology. For instance, the punctate focal adhesions are not observed in 3D systems which are frequently detected in 2D cultures [69]. Considering the extreme complexity and potential significance of the microenvironment, development of three-dimensional (3D) *in vitro* models is strongly needed to improve understanding of microenvironmental regulation of GBM [70-72]. The development of these *in vitro* models bridges the gap between *in vitro* 2D drug trials and *in vivo* animal studies (**Scheme 2. 5.**).

ECM of GBM is composed of several natural polymers such as hyaluronic acid, collagen IV- V, fibronectin, and laminin [72]. Adhesion of cells to ECM scaffolds is regulated by a transmembrane protein family, integrins, which link the cell membrane with actin cytoskeleton and ECM components from opposite ends. Beside structural roles, integrin-linked signaling contributes to resistance of tumors to anti-cancer drugs [73]. GBM cells can also remodel ECM for opening space for their growth and improve migration through blood vessels. Proteolytic degradation of ECM by matrix metalloproteinases (MMP) is the main step of invasion in human gliomas [74]. Upregulation of MMPs in GBM patients have been shown to increase resistance to therapy [72].

In the last decade, researchers used these natural products to design materials to facilitate the analysis of GBM-niches [69, 70]. In addition to existing components of ECM, gelatin obtained by partial hydrolysis of collagen and synthetic polymers like poly(ethylene glycol) (PEG) are also preferred for preparing brain-mimetic hydrogels [75]. 3D networks synthesized from these materials can be used to effectively simulate

CHAPTER 2: LITERATURE REVIEW

cell invasion through native ECM, and the diffusion of nutrients and oxygen through tissue [69].



Scheme 2.5. Developments in experimental models of GBM.

Chapter 3

3. QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP- SENSITIVE PEG HYDROGEL CARRIERS

3.1. Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive type of primary brain tumor. Although GBMs constitute only 1% of adult cancers, these are considered as grade 4 due to the poor patient survival rate of 12 to 15 months [1, 5]. TRAIL, or apoptosis inducing ligand 2 (Apo2L), is a promising anti-tumor agent for GBM [71, 76]. TRAIL is a member of the TNF cytokine family secreted by mostly immune cells that can specifically induce apoptotic cell death in cancer cells rather than healthy cells [77]. This transmembrane protein, with 281 amino acids in its native human form, constitutes homo-trimers, which bind to death receptors, DR4 and DR5, on cell surface and oligomerize them to transduce the apoptotic signal [18, 78, 79]. The engagement of Fas-associated protein with death domain (FADD) activates extrinsic death pathway by recruiting pro-caspases 8 and 10, which transduce the apoptotic signal to effector caspase-3 upon activation. Another mechanism of action upon TRAIL binding to death receptors is the degradation of BH3-interacting domain death agonist (Bid) by activated initiator caspases. The truncated form of Bid (tBid) initiates intrinsic apoptosis pathway via diminishing mitochondrial membrane potential, which induces activation of

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

effector caspases and cell death through cytochrome c release [18, 80]. Subsequent increases occur in the levels of Bcl-2-associated X protein (BAX), Bcl-2 homologous antagonist killer (BAK), and BH3-only members such as PUMA, NOXA and HRK [80, 81].

TRAIL did not exhibit any negative effect on non-neoplastic tissue in early-phase clinical trials [25]. Despite significant success with TRAIL as a targeted cancer therapeutic, some malignant cancer types are either innately resistant or develop resistance to TRAIL-induced apoptosis [28, 82, 83]. Novel approaches are required to efficiently sensitize therapeutic resistant cells to TRAIL-induced apoptosis. In preclinical studies, TRAIL was shown to demonstrate therapeutic potential when used in combination with chemo or radiotherapy [25]. Recently, Senbabaoglu et al. reported that the small drug molecule Quinacrine (QC) was capable of sensitizing GBM cells to TRAIL in drug screen experiments [4, 76]. QC is an FDA-approved antimalarial drug, found to exhibit anti-cancer activity in recent reports [84-87]. Although its effects were shown in broad range of cancer cell types such as colon carcinoma, brain tumors, breast and prostate cancer, the anti-cancer mechanism of QC is complex [85, 86]. Various anti-cancer mechanisms were described for QC including autophagy, an evolutionary preserved lysosomal self-destruction process, and activation of apoptosis via transcriptional upregulation of proapoptotic markers p53, p21, Bax, PARP, caspase-3 and downregulation of Bcl-xL, NF- κ B [86, 88]. QC was also reported to have a promising blood-brain-barrier (BBB) penetration capability [87].

Another strategy to achieve an increased anti-cancer effect from therapeutics involves the use of sustained release systems. Hydrogels used for administration of

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

TRAIL were successful in increasing tumor responsiveness by maintaining the therapeutic level at tumor region, which provided long-term TRAIL delivery.[89, 90] Recently, in an *in vivo* xenograft mouse model, TRAIL was found to demonstrate increased antitumor effects when loaded into PEG cross-linked hyaluronic acid bulk hydrogels compared to its free therapeutic form in pancreatic cells [90]. Similarly, poly (lactic-co-glycolic acid) (PLGA)-based hydrolytically degradable synthetic microgels synthesized using double-emulsion solvent extraction, increased apoptotic effects of TRAIL significantly in human colon cancer cells [18, 89]. However, organic solvents and surfactants used during micro/nano gel synthesis process are not compatible with proteins or peptides, and may compromise the long-term therapeutic potential [91, 92]. Alternatively, hydrogel nano/micro particles can be synthesized based on water-in-water emulsion through phase separation of aqueous solutions of two different hydrophilic polymers, such as polyethylene glycol (PEG) and dextran (**Figure C.1.**), and these particles can be used for the delivery of therapeutics [91, 93].

Our goal here was to create novel sub-micron size hydrogel carriers for simultaneous delivery of the two anti-cancer molecules: QC and TRAIL. We used biocompatible synthetic polymer PEG functionalized with matrix-metalloproteinase (MMP)-sensitive ligand covalently cross-linked into PEG hydrogel network. Due to high amount of secretion of protease enzymes by GBM cells, particle degradation occurs and loaded drug can be released from the carrier [74, 94-96]. In addition, integrin-targeting RGDS domains were incorporated into the gel structure to promote adhesion of GBM cells onto drug-loaded particles [71, 94]. Targeted PEG carriers were synthesized via visible-light initiated radical cross-linking using water-in-water emulsion polymerization technique

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

and retrieved from noncross-linked dextran aqueous solution. Subsequently, QC release from the functionalized carriers was characterized and anti-tumor effect of QC in combination with TRAIL was evaluated in human U87MG cells via cytotoxicity assay and gene expression analysis of the apoptosis related genes *in vitro*. Results suggest that MMP-sensitive PEG hydrogel particles are promising to promote TRAIL induced cell death in GBM cells, when loaded with QC.

3.2. Materials and Methods

3.2.1. Materials

Acrylate PEG-Succinimidyl Valerate (Acryl-PEG-SVA, 3400 Da), Poly (ethylene glycol) diacrylate (PEGDA, MW: 10 kDa) was purchased from Laysan Bio (Arab, AL). The MMP-cleavable peptide (GGGPQGIWGQGK, 1141 Da) and integrin binding peptide (RGDS, 433 Da) were purchased from Elim Biopharmaceuticals (Hayward, CA). Triethanolamine (TEA), Amicon Ultra Centrifugal Filter (MWCO: 50 kDa), cellulose and nylon filters were purchased from Merck (Whitehouse Station, NJ). Collagenase type I was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), Penicillin/streptomycin, Phosphate Buffer Saline (PBS) were purchased from Gibco (Grand Island, NY). Soluble human recombinant, SuperKillerTRAIL™ was purchased from Enzo Life Sciences (Farmingdale, NY) and Quinacrine dihydrochloride dehydrate (508.92 Da) was supplied from Prestwick Chemical (Illkirch-Graffenstaden, France). CellTiter-Glo® Luminescent cell viability assay was purchased from Promega (Madison, WI, USA). NucleoSpin® RNA Macherey Nagel (North Rhine-Westphalia, Germany),

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

LightCycler 480 Multiwell Plate 96 and LightCycler 480 SYBR Green Master from Roche Diagnostics (Mannheim, Germany). All cell culture plates were from Corning (Tewksbury, MA). All other reagents, unless otherwise specified, were purchased from Sigma–Aldrich (St. Louis, MO).

3.2.2. Synthesis of PEG-peptide hydrogel precursors

MMP-degradable peptide sequence GGGPQG↓IWGQGK (1141 Da) and RGDS (433 Da) peptides were conjugated with acrylate-PEG-SVA (3400 Da) in 50 mM sodium bicarbonate buffer as described previously [95]. Briefly, acrylate-PEG-GGGPQGGQGK-PEG-acrylate and acrylate-PEG-RGDS conjugates were synthesized as a result of reaction of SVA with N-terminus of peptides and amine group on the side chain of lysine residue at the C-terminus of the degradable peptide. The final product was dialyzed (slide-a-lyzer dialysis cassette, MWCO: 7 kDa, Pierce, IL) against dH₂O to remove unreacted peptide and PEG. Next, the product was lyophilized and was stored at -20 °C. PEG-peptide conjugates in solid form were characterized by Fourier Transform Infrared Spectrometer (FTIR) (Thermo Scientific iS10 FTIR) to analyze the reaction yield with Attenuated Total Reflectance (ATR) crystal.

3.2.3. Characterization of Enzymatic Sensitivity

Degradability of GGGPQGIWGQGK peptide was tested by suspending cross-linked hydrogels which were synthesized with this sequence incorporated into the backbone of PEG-acrylate block polymers in 1 μM collagenase type-I (Worthington, 265U/mg) containing HEPES (0.1 M HEPES, 0.15 M NaCl, 5 mM CaCl₂, pH 7.4) buffer.

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

Degradation was evaluated by measuring the weight of disk shaped hydrogels (0.2 cm²) for 1 h with 5 min intervals.

3.2.4. Synthesis of PEG Hydrogel Nanocarriers

The hydrogel prepolymer solution was prepared with 5 % (w/v) of MMP-degradable acrylate-PEG-GGGPQGIWGQGK-PEG-acrylate, 225 mM triethanolamine (TEA, Merck), 37 mM 1-vinyl-2-pyrrolidinone (NVP, Sigma-Aldrich) and, a photoinitiator, 0.05 mM EosinY in dH₂O. The prepolymer solution was also prepared in the presence of acrylate-PEG-RGDS (2 mM) and 5 % (w/v) PEG diacrylate (PEGDA, Laysan Bio) (10 kDa) was used instead of acrylate-PEG-GGGPQGIWGQGK-PEG-acrylate for nondegradable hydrogel sets. For carrier synthesis PEG prepolymer solution was added into 40 % (w/v) dextran aqueous solution containing 0.22 M KCl and 10 mM sodium phosphate at pH 8 with 1:5 (PEG/Dextran) volumetric ratio and emulsified by continuous stirring for 45 minutes. The emulsions were exposed to 514 nm visible light at a flux of 2.5 mW/cm² using an argon ion laser (Coherent Inc., Santa Clara, CA) for 4 min. Unreacted reagents and non-crosslinked dextran phase were removed by washing the polymerized hydrogel particles using the Amicon Ultra centrifugal filter (Millipore, Merck).

3.2.5. Characterization of PEG Hydrogel Nanocarriers

Size and morphology of hydrogel carriers were characterized by Field Emission-Scanning Microscopy (FE-SEM) (Zeiss Ultra Plus, Bruker) and Atomic Force Microscope (AFM) (The Dimension Icon, Bruker, MA), and dynamic light scattering (DLS) (Zetasizer, Malvern ZS) was used to determine hydrodynamic radii of particles

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

suspended in dH₂O. The samples were coated with a thin layer of gold with 10 nm thickness for FE-SEM, and 1 to 5 kV acceleration voltage was used for imaging. In AFM measurements surface topology, and elastic modulus, adhesion, deformation maps of particles coated on glass slides were obtained by PeakForce QNM AFM modes together with a Nanoscope V controller and NanoScope Analysis (Bruker) software version 1.5. The standard RFESP (MPP 21100-10) AFM probes and the standard cantilevers (Veeco probes, USA, k=3 N/m, fo: 86-94 kHz) for operation in air were utilized for all measurements at room temperature.[97] The chemical compositions were analyzed by X-ray Diffraction (XRD) Spectroscopy (D2 PHASER, Bruker, MA).

3.2.6. Release profile of PEG hydrogel particles

QC was loaded into MMP-sensitive PEG carriers in order to investigate release profile. Briefly, 500 μ M of QC was suspended with 5 mg/ml of carriers for overnight with continuous shaking and unloaded drug molecules were removed by centrifugation in 2500 rpm for 10 min using centrifugal filters (Amicon, Milipore, US). The drug loading amount was quantified from the sample was taken from supernatant by UV-vis measurement in NanoDrop1000 and efficiency was calculated using **Eqn (3.1.)**. QC loaded degradable carriers were suspended in HEPES buffer in the presence and absence of 1 mg/ml of collagenase type I, in separate dialysis tubing membranes (MWCO: 14,000). The release experiment was allowed to continue for 3 days at 37°C with 90 rpm shaking. At specific time points, 1 ml of solution was collected from each sample and 1 ml of solution was replaced with fresh buffer. The samples were used to measure concentration of QC at each time point based on UV-vis absorption measurement at 425

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

nm [98]. Total amount of percent QC released were calculated using **Eqn (3.2.)** and **Eqn (3.3.)**.

$$QC \text{ Loading Efficiency} = \frac{(M_i - M_f)}{M_f} * 100 \quad (3.1.)$$

$$M_t = C_t * V + \sum C_{t-1} * V_s \quad (3.2.)$$

$$QC \text{ Release (\%)} = (M_t / M_\infty) * 100 \quad (3.3.)$$

where, C_t is the concentration of QC in the buffer at time t , V is the total volume of release buffer (10 ml) and V_s is the sample volume that is taken (1000 μ l) and M_∞ is the total weight of QC released during the experiment. After the release profiles of QC in the presence or absence of collagenase from MMP-sensitive RGDS modified PEG carriers were obtained, the results were fitted to the power-law correlation developed by Peppas et al. as was shown in **Eqn (3.4.)**.

$$M_t / M_\infty = k * t^n \quad (3.4.)$$

where k represents characteristic constants of hydrogel and n is the exponential coefficient, which describes the mode of transport mechanism.

Cell Culture Conditions: U87MG GBM cells were supplied from American Type Culture Collection and authenticated. Cells were maintained in DMEM supplemented with 10 % FBS, 2 mM L-glutamine, and 1 % Penicillin/Streptomycin. They were grown up to passages 8-22. Cells were maintained in a humidified environment at 37°C with 5% CO₂. For mCherry labeling, lentiviral vectors encoding mCherry was introduced to U87MG cells through transduction as described [99].

3.2.7. Cytotoxicity Assays on U87MG Cells

MMP-degradable PEG hydrogel carriers (5 mg/ml) functionalized with RGDS or without RGDS peptide, and nondegradable hydrogels with or without RGDS peptides were suspended in DMEM for 24 h, at 4°C on a rocking shaker. U87MG cells (5000 cells/well) were seeded to 96 well plates (black, Greiner Bio). One day after seeding, medium of cells was replaced with the medium containing hydrogel carriers. An ATP-based cell viability assay (CellTiter-Glo Luminescent Cell Viability Assay, Promega, WI, US) was applied to evaluate cytotoxicity of hydrogels on the next day and luminescence activity was measured with a luminometer (Biotek, Synergy H1). Results were normalized according to the untreated U87MG cells.

Subsequently, PEG hydrogel carriers were suspended in cell culture medium supplemented with 1 μ M of QC and added on confluent cells in a similar way (described above). QC loaded carriers were also added on cells in combination with 40 nM of TRAIL (SuperKillerTRAIL™, Enzo Life Sciences, US) to sensitize GBM cells to TRAIL-induced apoptosis.

3.2.8. Gene expression analysis

RNA was extracted by Macherey Nagel mini spin kit for RNA isolation and RNA concentration was measured by NanoDrop based on the absorbance value. 500 ng total RNA was used for cDNA synthesis. cDNA was synthesized by using M-MLV Reverse Transcriptase (Invitrogen). Quantitative RT-PCR was performed with 45 cycles using the following primer (**Table 3.1.**) pairs using Roche LightCycler:

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

Table 3. 1. List of primers for Gene Expression Analysis

Gene	Forward Primer	Reverse Primer
hGAPDH	AGCCACATCGCTCAGACAC	GCCAATACGACCAAATCC
MMP-2	GATACCCCTTTGACGGTAAGGA	CCTTCTCCCAAGGTCCATAGC
HRK	AGGTTGGTGAAAACCCTGTG	GCATTGGGGTGTCTGTTTCT
BCL-2	CAGGGCGATGTTGTCCACC	GGGGAGGATTGTGGCCTTC
BCL-xL	GGTCGCATTGTGGCCTTTTTC	TGCTGCATTGTTCCCATAGAG
DR4	ACCTTCAAGTTTGTCTCGTC	AACTCCCCAAAGGGCTATGT
DR5	AAGACCCTTGTGCTCGTTGT	AGGTGGACACAATCCCTCTG
Mcl-1	TGCTTCGGAACTGGACATCA	TAGCCACAAAGGCACCAAAAG
PUMA	GACCTCAACGCACAGTACGAG	AGGAGTCCCATGATGAGATTGT
NOXA	ACCAAGCCGGATTGTGCGATT	ACTTGCACTTGTTCTCTCGTGG
Caspase-3	CATGGAAGCGAATCAATGGACT	CTGTACCAGACCGAGATGTCA
Caspase-8	GGCGGAGGGTCGATCATCTAT	TGTCCAACTTTCCTTCTCCCA
FADD	GCTGGCTCGTCAGCTCAAA	ACTGTTGCGTTCTCCTTCTCT
Bid	CCTACCCTAGAGACATGGAGAAG	TTTCTGGCTAAGCTCCTCACG
All primers were designed using NCBI webpage.		

3.3. Results and Discussions

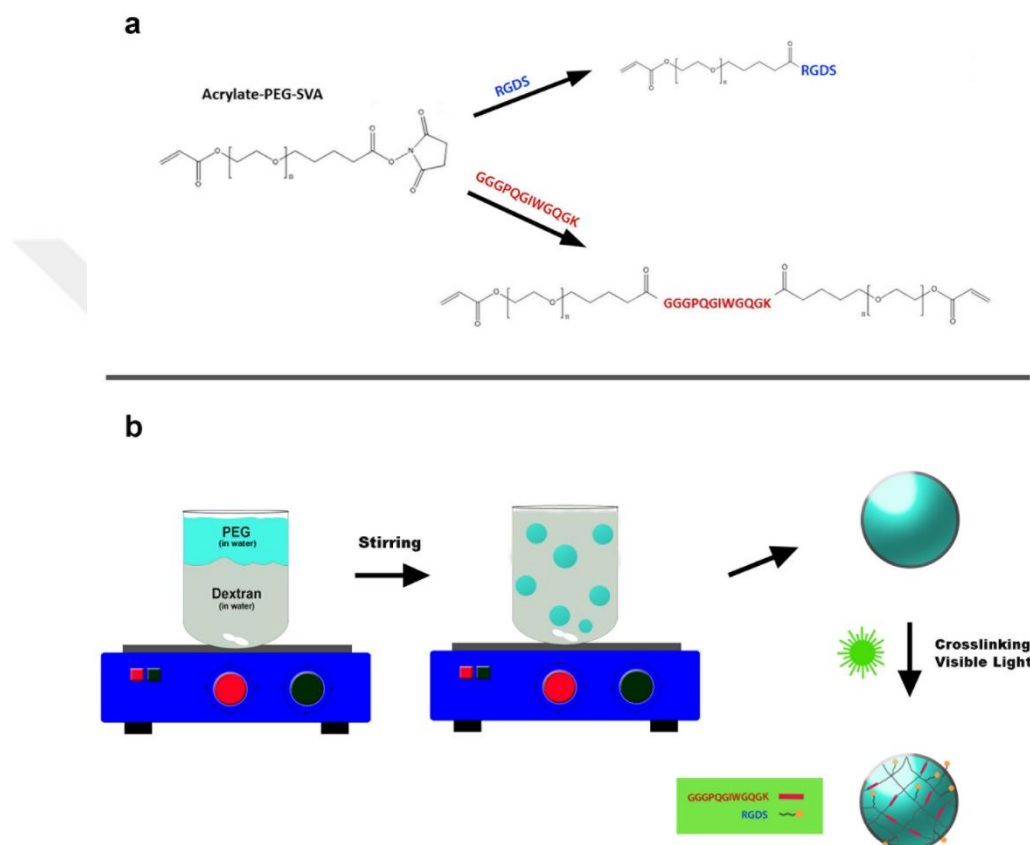
3.3.1. Characterization of Protease Sensitive Hydrogel

MMP-sensitive peptide sequence GGGPQG↓IWGQGK and integrin targeting ligand RGDS were selected to develop a targeted delivery vehicle for GBM therapy. Degradable peptide was covalently conjugated to PEG to obtain a monomer with diacrylate functionality. RGDS was incorporated to PEG from its N-terminus to create

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

PEG chain with free RGDS end that promoted integrin-dependent cell adhesion.[95]

Conjugation of these peptides with PEG was illustrated in **Scheme 3.1a**.



Scheme 3.1. Synthesis of peptide conjugates and targeted carriers via water-in-water emulsion photopolymerization. SVA group is removed from the structure in basic pH conditions and peptides bind to acrylate-PEG construct from their amino terminals and N groups of the side chains.

Conjugation of peptides to PEG was characterized by ATR-FTIR spectroscopy. FTIR spectra confirmed the presence of the functional groups of PEG-acrylate chains and amide bond formation with MMP-sensitive peptide (**Figure A.1.**). Enzymatic degradability of hydrogels was characterized through incubation of these gels with collagenase. Crosslinked hydrogels were synthesized at macroscopic scale and their

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

surface properties were monitored through Field Emission Scanning Microscopy (FESEM) (**Figure A.2.**). Hydrogels were then incubated in a buffer with 1 mg/ml collagenase, where complete degradation of those gels was observed in one hour (**Figure A.3.**). This result was consistent with the degradation kinetics of hydrogels prepared with same peptide using altered PEG and collagenase concentrations in earlier studies [96].

3.3.2. Synthesis of Targeted PEG Carriers

MMP-sensitive and RGDS functionalized hydrogel carriers were synthesized using visible light-induced water-in-water emulsion polymerization. The process of water-in-water emulsion photopolymerization was summarized in **Scheme 3.1b**. Emulsions of PEG polymer precursor were obtained in continuous dextran phase, the emulsions were then crosslinked with visible light induced photopolymerization and retrieved from non-crosslinked dextran.

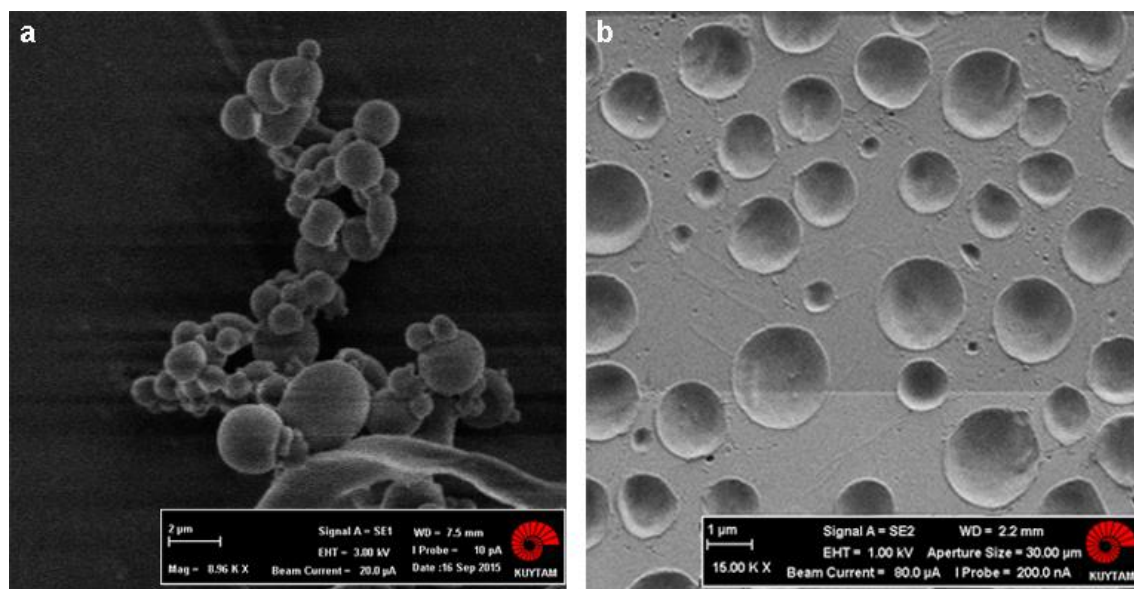


Figure 3. 1. FESEM micrograph of PEG hydrogel particles. PEG hydrogel carriers were obtained in dextran 40kDa, lyophilized and image was taken with Zeiss Ultra Plus FE-SEM (Bruker) a) Freeze

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

dried PEG particles with 8.96 KX magnification and 2 μm scale bar. b) MMP sensitive PEG particles diluted in water and vacuum dried illustrated with 15KX magnification and 1 μm scale bar.

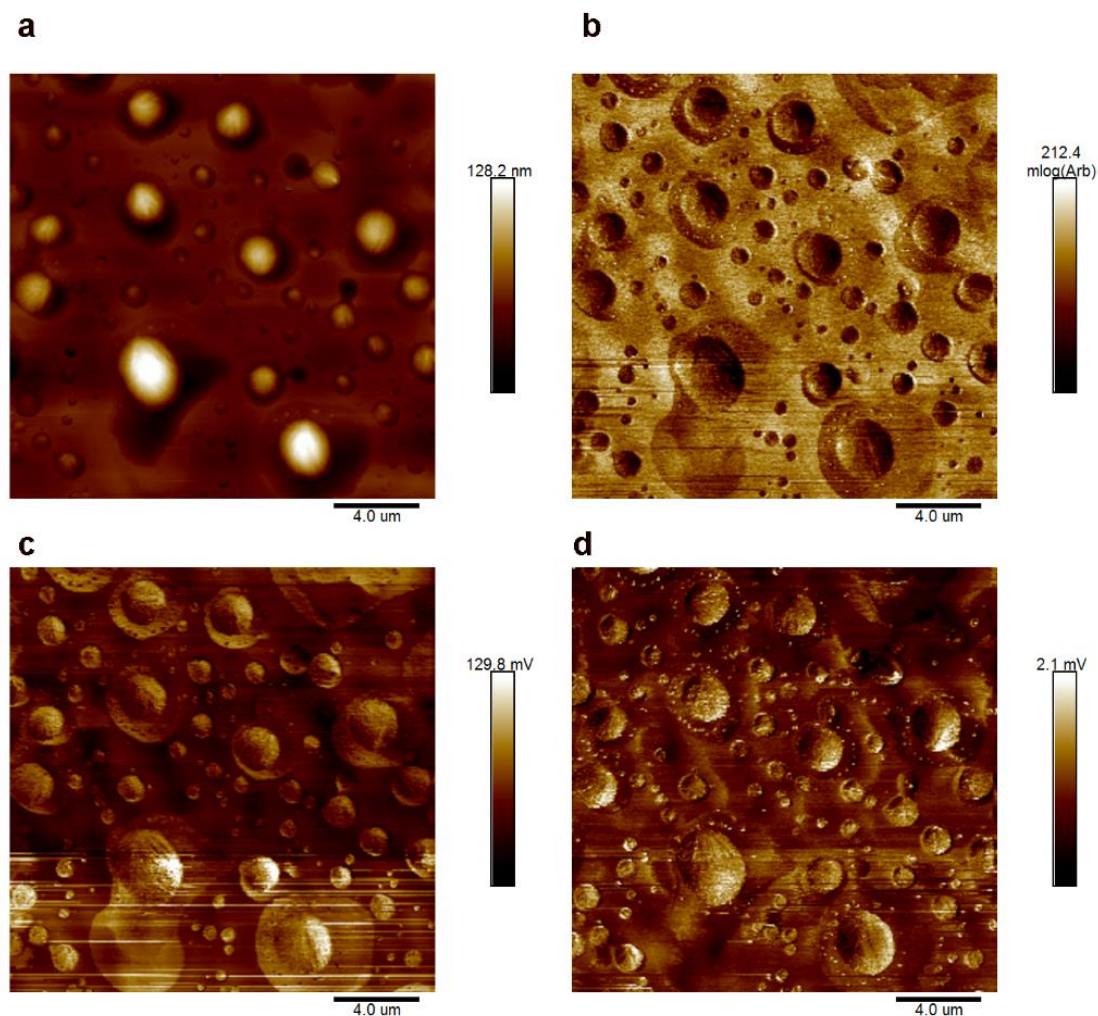


Figure 3. 2. AFM micrographs of PEG hydrogel particles. Scans were obtained by Peak Force Quantitative Nanomechanical Property Mapping (QNM) analysis in air imaging mode illustrating a) height b) Log DMT (elasticity), c) adhesion, d) deformation profiles

In previous studies, micron-size particles were synthesized via water-in-water emulsion polymerization, where morphological characterization was mainly based on optical light microscopy prior to freeze-drying process [91-93]. Here, we characterized size and morphology of hydrogel carriers both in wet and dry form using FESEM (**Figure**

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

3.1). Samples were either lyophilized and imaged in solid form (**Figure 3.1a**), or they were placed in liquid buffer and dried under vacuum before FE-SEM characterization (**Figure 3.1b**). FESEM images illustrate that the particles have spherical morphology with a size distribution of 200 nm to 2 μ m. AFM was also used for characterization of size and morphology of particles (**Figure 3.2a**).

Mechanical properties such as stiffness and adhesion properties of the gel particles were also analyzed with AFM force curve using force-volume technique. **Figure 3.2b** demonstrated elasticity, where logarithm of elastic modulus of the sample was quantified based on Derjaguin, Muller, Toropov model (DMT modulus) [100]. The stiffness range was defined in arbitrary (Arb) unit. Lower $\log$ (Arb) values of particles indicated that their elasticity was higher than glass surface. Previous studies showed that elastic modulus could be higher near the surface when cross-linking occurred for some polymers [101]. We also observed higher elastic modulus close to the particle surface (**Figure 3.2b**). Adhesion image of samples demonstrated relative adhesion interaction between the particles and the surface (**Figure 3.2c**). Deformation image illustrated a larger alterations around the edge compared to the center of particles (**Figure 3.2d**) [97, 101].

Further characterization of particle size was performed with dynamic light scattering (DLS) and Zetasizer Nano ZS, where hydrodynamic radius and polydispersity index (PDI) were evaluated. PDI was found as 0.473 indicating monomodal distribution. Hydrodynamic radius for particles was approximately measured as 20 nm for 78 %, 387 nm for 18 %, above and 1 μ m for the remaining particles (**Figure A.4a.**). Nanoparticles smaller than 100 nm were beyond the limits of imaging capacity of FESEM (**Figure 3.1**). FESEM results and DLS measurements suggested that size distribution could be reduced

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

down to nano-scale. Zeta potential distribution of carriers was found around -7.91 (Figure A.4b.). This slightly negative value is also consistent with our previous observation for iron oxide nanoparticles, which were coated with MMP-sensitive peptide incorporated PEG [95].

Pore properties of our carriers were characterized using an ASAP 2020 Surface Area Analyzer (Micromeritics Instrument, USA). Average pore size and BET (Brunauer, Emmet, Teller) surface area were measured as 18.98 nm and 0.2806 m²/g, respectively. Adsorption isotherms and N₂ adsorption capabilities were illustrated in Figure A.5.

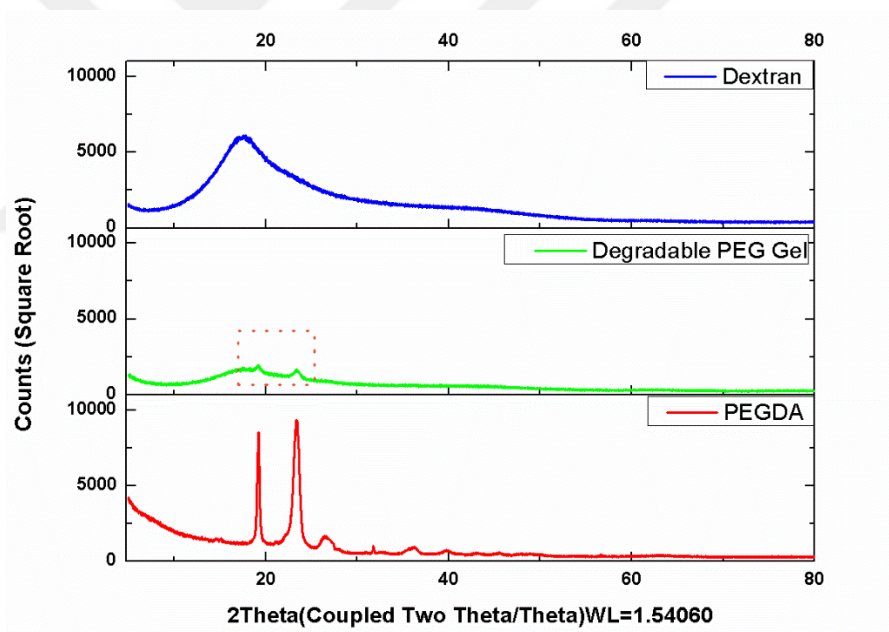


Figure 3. 3. X-ray diffraction patterns. Dextran and PEGDA powders, and lyophilized MMP-sensitive PEG carriers, were shown with blue, green and red lines.

The carriers were further characterized by X-Ray Diffraction (XRD) Spectroscopy, and the presence of PEG was confirmed by duplicate peak pattern, consistent with the degree of PEG crystallinity (Figure 3.3).

3.3.3. QC Loading and Release Experiments

The release profiles from QC-loaded PEG nanoparticles were investigated in HEPES buffer at 37 °C. To analyze the amount of loaded QC into particles, specific UV-vis absorption was measured at 425 nm [98]. QC was loaded into 5 mg of particles with an initial concentration of 500 μ M and the amount of loading was calculated as 51 % which corresponds to 12.75 mg of QC. The percentage could be improved by increasing amount of nanoparticles used for loading or decreasing initial QC concentration in the stock solution. BET analysis confirmed high surface area and nano-scale size of the pores for degradable and RGDS functional PEG particles, which suggested potential for drug loading into these structures (**Figure A5**). Figure 4a illustrates the amount of QC release in the presence and absence of collagenase (1 mg/ml).

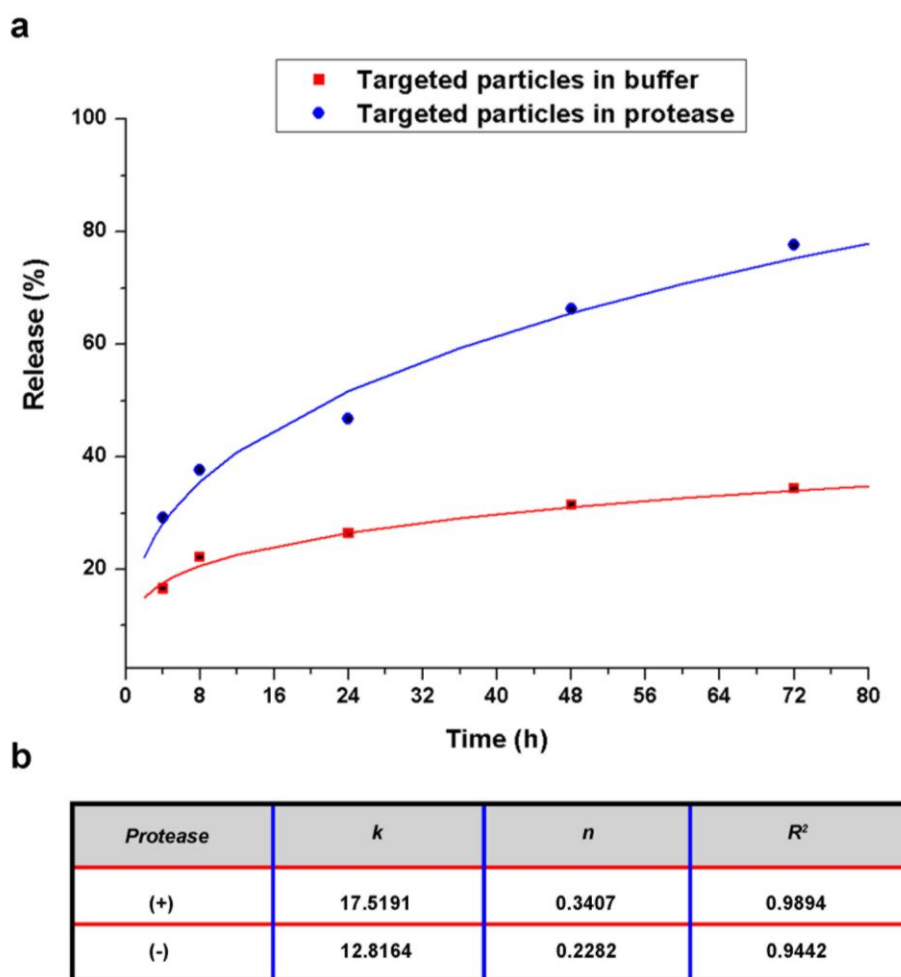


Figure 3. 4. Release profile of QC loaded PEG particles. a) QC release versus time from QC-loaded MMP-sensitive hydrogel carriers. PEG hydrogels (5 mg/ml) were mixed with QC with an initial concentration of 500 μ M. Total drug release was calculated from absorbance values of QC measured at 425 nm at pre-determined time points. b) The power-law equation coefficients for targeted carrier in the presence and absence of protease enzyme.

Figure 3.4a illustrates the amount of QC release in the presence and absence of collagenase (1 mg/ml). During the initial hours of experiment, fast release of QC was observed, which probably occurred due to the release of adsorbed QC on the surface of particles. QC release was improved when MMP-sensitive carriers were incubated with collagenase.

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

The release profile of QC from MMP-sensitive and RGDS functional PEG carriers was fitted to power-law correlation model as follows:

$$M_t / M_{\infty} = k * t^n \quad (3.4)$$

In the equation, k represents characteristic constants of hydrogel and n is the exponential coefficient, which describes the mode of transport mechanism. The value of n was calculated as 0.45 for Fickian diffusion and 0.89 for swelling-controlled diffusion [102, 103]. Model fitting results for our system were illustrated in **Figure 3.4b**, which suggested Fickian mode of diffusion of the drug from targeted carriers both in the presence and absence of collagenase.

3.3.4. Cell Culture Studies

Cell culture experiments were carried out with U87MG cells, as these cells demonstrated higher level of MMP-2 mRNA expression compared to the other GBM cells, as was characterized with qRT-PCR (**Figure 3.5a**).

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

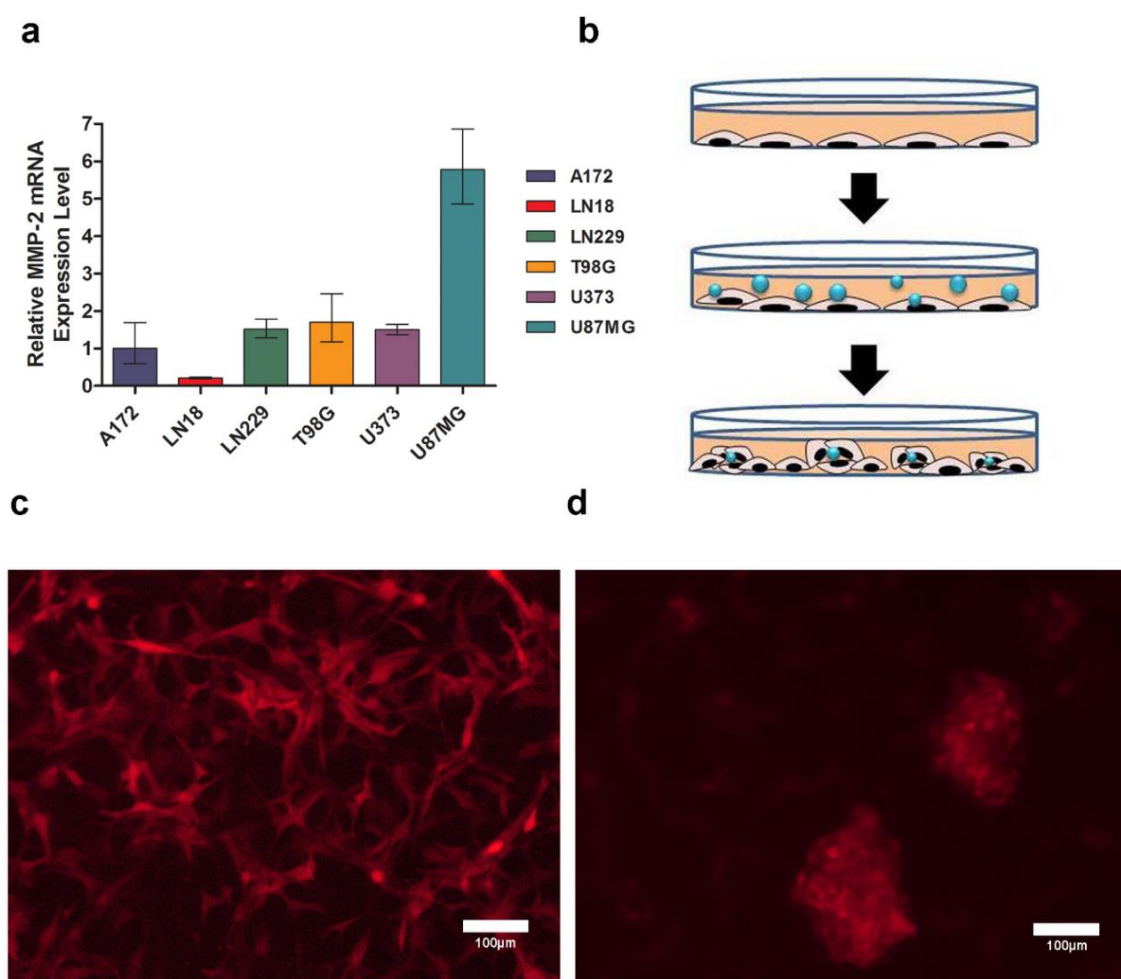


Figure 3. 5. Overall expression pattern of MMP-2 in glioblastoma cell lines and representative fluorescent microscopy snapshots for cell behavior and survival with particles. a) qRT-PCR result showing that U87MG cells demonstrated higher level of MMP-2 mRNA expression compared to the other glioblastoma cells. b) Incubation of cells with PEG carriers. c) Untreated (control) mCherry expressing U87MG cells, d) cells, which were incubated with 2 mM RGDS incorporated 5 mg/ml of targeted carriers were captured with 10 X magnification on day 4.

MMP-degradable and non-degradable carriers were suspended in cell culture medium for characterization of cytotoxicity. The particles were added on U87MG cell seeded plates in the presence or absence of drug (**Figure 3.5b**). Morphology and survival of mCherry-labelled U87MG cells were also evaluated with fluorescence microscopy

imaging. **Figure 3.5c** and **3.5d** demonstrate fluorescent images of untreated cells and cells incubated with MMP-degradable and RGDS functional carriers 4 days after treatment. Aggregation of cells in the presence of particles was observed, a phenotype not detected in control group which were not treated with particles. This aggregation was probably promoted due to the presence of RGDS sequence in particle carriers. This observation is also consistent with earlier reports, where RGDS promoted cell migration and attachment through integrins [29].

3.3.5. *In vitro* Cell Viability Assays

Cytotoxicity of carriers was tested prior to drug loading, where U87MG cells were treated with unloaded carriers with altered groups. Accordingly, carriers in altered groups were all found as biocompatible and did not compromise survival of U87MG cells (**Figure 3.6**). This result was expected due to the well-established biocompatibility of both PEG and dextran [104, 105].

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

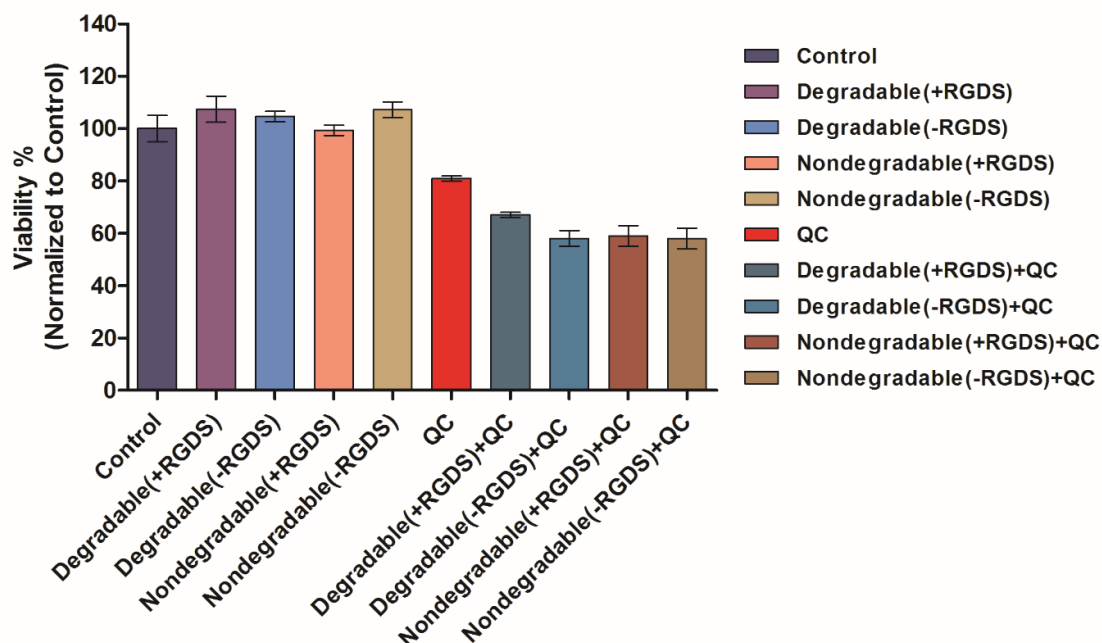


Figure 3. 6. Viability of U87MG cells with carriers and QC. 5 mg/ml gels and 1 μ M QC-loaded gels were suspended in DMEM and added on 96-well plates seeded with 5000 cells one day before. Viability of U87MG cells with carriers was measured 24 h after.

Cell viability was decreased to 80 % when the free form of QC was used at 1 μ M concentration. Approximately 20% decrease in cell survival was observed upon loading of the same concentration of QC into carriers. This result was expected due to the tendency of cell membrane to absorb positively charged nanoparticles or neutral polymers such as PEG [106].

Due to insignificant differences in viabilities of the cells treated with QC or QC-loaded hydrogel particles, we focused on degradable and RGDS functional particles. We investigated the targeting potential of the carriers for the subsequent experiments, where we used combination of QC and TRAIL to treat U87MG cells with our targeted delivery system. For these experiments, U87MG cells were treated with several combinations of

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

QC and TRAIL, and influences of therapeutics were tested both in the presence and absence of carriers (**Figure 3.7a**).

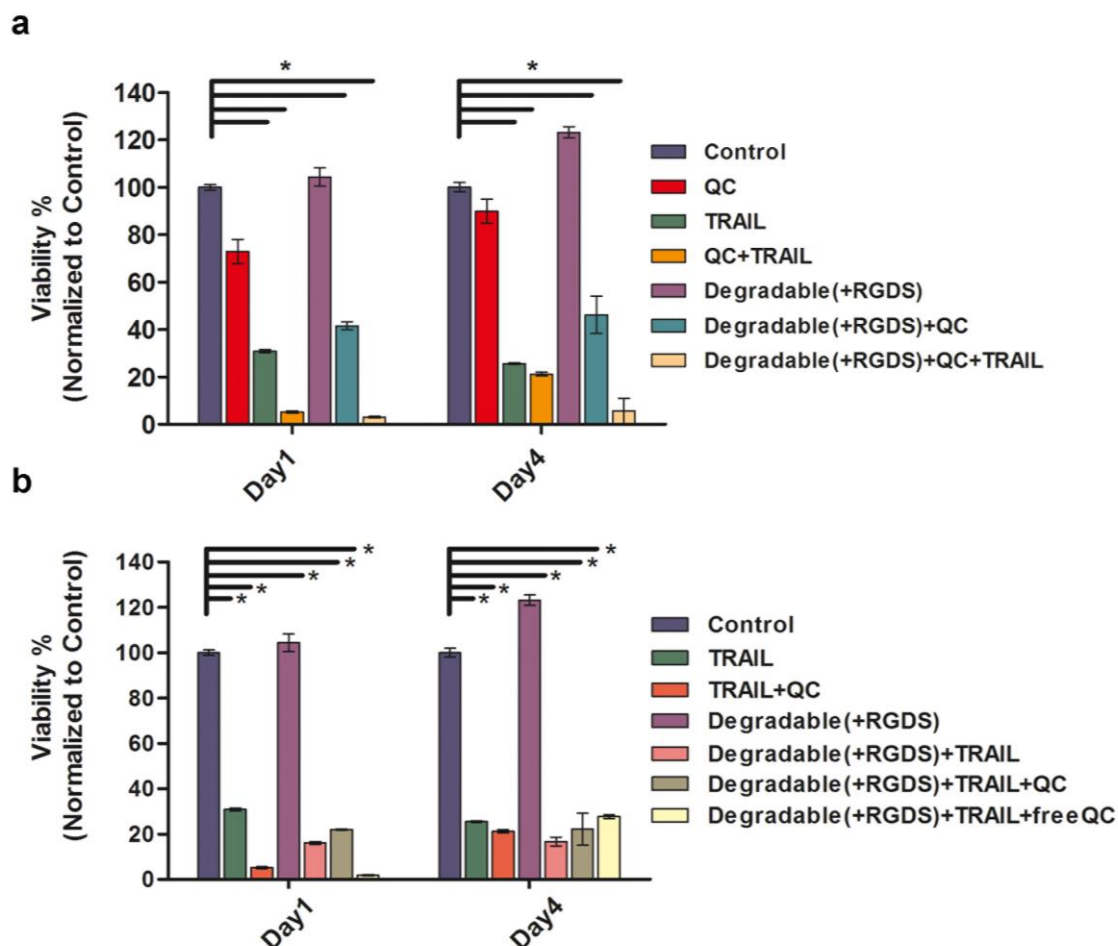


Figure 3. 7. Viability of U87MG GBM cells with TRAIL and QC. Cells were treated with MMP-sensitive RGDS incorporated carriers a) loaded with QC and free TRAIL, b) loaded with TRAIL and free QC. Viability of untreated cells was used as control, single-agent treatments and dual treatments were used for comparison with nanocarrier-loaded treatments. One-way ANOVA followed by Tukey's multiple comparison test. *indicates statistical significance compared to the control group ($P < 0.05$).

QC was loaded into carriers, where TRAIL was suspended in cell culture medium in these experiments. It is obvious from the results that combination of QC and TRAIL significantly improved antitumor effects compared to that of individual treatment. For

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

example, cell survival was significantly reduced with simultaneous incubation of cells with QC-loaded gels and free TRAIL. Additional experimental groups of particles for incubation of cells included TRAIL-loaded gels and free QC, and gels loaded with both TRAIL and QC (**Figure 3.7b**). The strongest synergistic apoptotic efficacy was obtained with QC-loaded gels and free TRAIL treatment. This could be due to the limited diffusion of large TRAIL protein (26 kDa) through crosslinked gel particles, when TRAIL was loaded into gel particles. We also examined the cytotoxicity of this system with healthy cells. Cells were treated with TRAIL and/or QC, and cell viability was detected on days 1 and 4 (**Figure A.7.**). Consequently, number of viable non-malignant cells was not markedly affected from TRAIL or gel treatments. Cellular uptakes of fluorescein o-acrylate labeled nanocarriers by U87MG and BJ fibroblast cells were also investigated via laser scanning confocal microscopy and uptake efficiency was further quantified as the ratio of FITC (green) to DAPI (blue) fluorescence intensity as was previously described [107]. Nanocarrier uptake percentage was 47 % higher in GBM cells compared to fibroblasts (**Figure A.6**).

3.3.6. Gene expression analysis in its free form.

To understand the possible cross-talk between TRAIL and QC, we performed qRT-PCR and analyzed mRNA expressions of apoptosis related genes. U87MG cells were treated with QC, QC-loaded and unloaded carriers as shown in **Figure 3.8a**. Following each treatment, cell lysates were collected, mRNAs were isolated and synthesized cDNAs were used for qRT-PCR analysis. It was measured from qRT-PCR that mRNA levels for indicated apoptotic genes were generally similar in untreated cells and carrier gel-treated

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

cells (**Figure 3.8b**). The gene expression results support the cytocompatibility of carriers observed with cytotoxicity assays earlier.

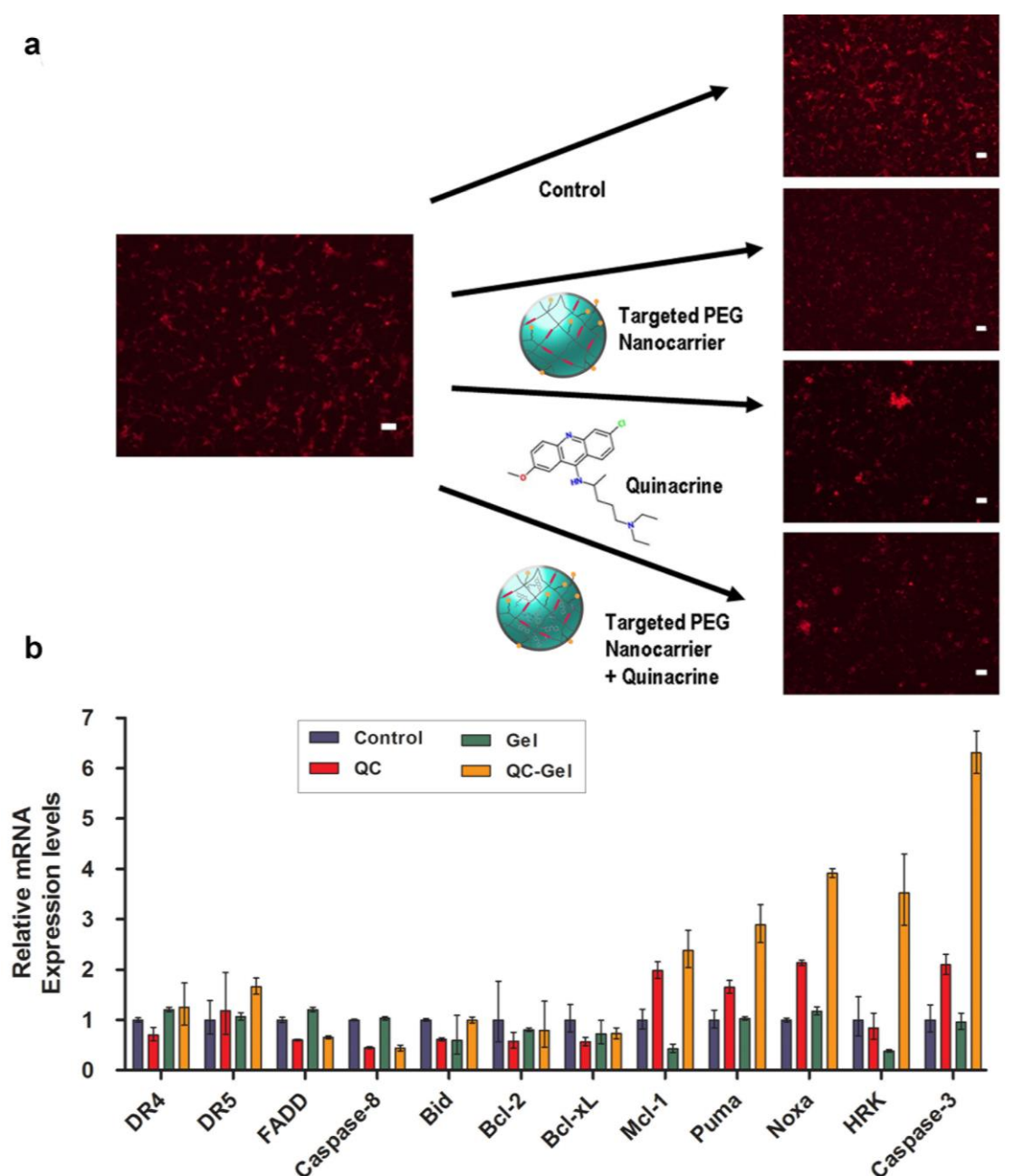


Figure 3. 8. Overall expression pattern of 12 apoptotic genes evaluated in U87MG cells. a) The single-agent treatments were applied incubating cells with 1 μ M QC and 5 mg/ml MMP-sensitive RGDS containing PEG carriers (Gel) for 6 h. The same concentrations were used for QC-loaded MMP-

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

sensitive RGDS containing PEG carriers (QC-Gel) treatment. b) mRNA expression levels of apoptotic genes were quantified from collected cell lysates by qRT-PCR, results were calculated with respect to GAPDH.

The synergistic cell death mechanism of TRAIL and QC could be evaluated by qRT-PCR analysis. TRAIL activates extrinsic apoptosis pathway through engagement with death receptors DR4 and DR5, which then activate caspase-8 that truncates BID to interact with the intrinsic death pathway elements BAX and BAK. These elements then release cytochrome *c* from mitochondria and BH3-only members like PUMA, NOXA and HRK [80, 81]. It is also possible for aggressive tumor cells to develop resistance to TRAIL as a result of increases in levels of Bcl-2, Bcl-xL and Mcl-1 which block BAX and BAK [81, 108]. Several studies have shown that upregulation of death receptors and downregulation of anti-apoptotic Bcl-2 family proteins expressions can be effective strategies for sensitizing resistant cells to TRAIL using combinatory delivery of TRAIL and chemotherapeutic agents [109].

To explore this possibility, we checked the influences of QC on both DR4 and DR5 expressions. We observed that mRNA level of DR5 gene was not affected from the unloaded carrier treatment; however, DR5 gene expression was slightly upregulated following QC only or QC-loaded carrier treatment, suggesting that receptor activation could be one possible reason for QC induced TRAIL sensitization. On the other hand, DR4 expression was not influenced by QC only or QC-loaded carrier treatments. NF- κ B blockage was reported to be responsible from the down-regulation of DR4 expression in a previous study and QC was shown to reduce NF- κ B expression [86, 110]. We also found that expressions of NF- κ B related genes p65 (RelA), RelB, p50 (NF- κ B1), and

CHAPTER 3: QUINACRINE MEDIATED SENSITIZATION OF GLIOBLASTOMA MULTIFORME (GBM) CELLS TO TRAIL THROUGH MMP-SENSITIVE PEG HYDROGEL CARRIERS

p100 (NF- κ B2) were decreased after QC treatment in U87MG cells. Hence this low level of NF- κ B related genes could be the reason for stationary DR4 result (data not shown). In addition, we observed 3 to 7-fold increases in Mcl-1, PUMA, NOXA and HRK genes when cells were treated with QC-loaded carriers. These increased levels were mainly resulted from QC, but the levels were significantly higher when cells were treated with QC-loaded carriers. Previously, it was found that PUMA had a tendency to bind all pro-survival proteins, where NOXA bound to Mcl-1 only, HRK bound to pro-survival Bcl-2 family members [111]. Bcl-2 and Bcl-xL levels decreased, consistent with the previous studies, while increased Mcl-1 level was not expected [86, 112]. Mcl-1 blockage is one of the mechanisms to prevent TRAIL resistance and QC was known to down-regulate Mcl-1 [18, 113]. Our results suggested that QC induced cell death was independent of down regulation of Mcl-1 and dependent on upregulation of DR5, PUMA, NOXA, HRK and caspase-3. These data were comparable with the results of Senbabaoglu et al., where a TRAIL sensitizer small molecule drug, Mitoxantrone, markedly upregulated mRNA expression of PUMA, NOXA and HRK pro-apoptotic genes, and the combination of TRAIL and Mitoxantrone treatment increased caspase-3 protein expression in U87MG cells [4]. In summary, QC-loaded carriers displayed the highest capacity to induce apoptosis related gene expression changes, as compared to QC or carrier treatment alone.

3.4. Conclusions

In this study, we developed an emulsion-based synthesis of biocompatible and enzymatically sensitive hydrogel particle carriers without the use of organic solvents. Selective targeting potential of ECM derived peptides into GBM cells was investigated. Loading of QC into MMP-sensitive particles improved the therapeutic efficacy of the drug compared to its direct application in its free form. The release of QC is not under control of any stimuli with these nanocarriers, and that the degradation of the carriers occurs simultaneously upon entry into the cells, as a result of MMP secretion by GBM cells. In addition, we observed that synergistic interactions between TRAIL and QC were enhanced when QC was loaded into gel particles. This synergistic treatment also resulted in stimulation of different cell-death pathways, which opens up the possibility of treating resistant GBM cells with dual targeting strategy. QC-loaded, degradable and biofunctional nanocarriers are promising for TRAIL-induced eradication of cancer cells.

Chapter 4

4. GELATIN METHACRYLATE HYDROGELS IN THE ABSENCE OF CROSSLINKER AS A 3D MICROENVIRONMENT FOR GLIOBLASTOMA MULTIFORME (GBM)

4.1. Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive form of brain tumors with poor survival rate of 12 to 15 months [4, 26]. Various mechanisms contributed to aggressiveness of GBM tumors. For example, detachment of cells from the natural basement membrane which guided to formation of 3D structures followed by communications of cells with each other and the surrounding cells [114]. This 3D interaction is the main reasons that leads to the failure of drug treatment tests in 2D systems. Understanding the nature of GBM microenvironment is critical to develop efficient strategies for successful treatments [29].

Natural biomaterials such as matrigel, fibrin, albumin, hyaluronan, chitosan, collagen, and gelatin have been attracting great attention for designing brain-mimetic 3D platforms for GBM studies due to their natural bioactivity [34, 35, 114-116].

Gelatin, obtained by the hydrolysis of collagen, is highly popular among the natural polymers for providing inherent cell attachment sites of the extra cellular matrix (ECM) such as arginine-glycine-aspartic acid (RGD) sequence that support cell proliferation, movement, and differentiation and for its biocompatibility and low antigenicity [117-119]. However, the main limitation of gelatin is its poor mechanical strength due to uncontrolled and rapid degradation behavior [119, 120]. Gelatin can form a hydrogel structure in water by non-covalent interactions below 30-35°C but this physical network

can easily be disturbed at higher temperatures which restricts their use at physiological temperatures [119].

To date, several chemicals (e.g. glutaraldehyde, genipin) were used as chemicals to crosslink gelatin [121]. Polymerization with these chemicals requires long crosslinking periods and careful washing steps to prevent cytotoxicity. In addition, they are mostly reactive towards amine groups which makes it difficult to encapsulate cells and conjugate additional proteins into hydrogel network structure to facilitate cell growth [122, 123].

Amine-containing groups on gelatin can also be modified by methacrylic anhydride. Methacrylate groups conjugated gelatin (GelMA) is a photo-crosslinkable and stable polymer at physiological temperatures.[118] This alternative polymerization strategy is useful to control the spatiotemporal cell culture microenvironment since it is fast and light is compatible with cells [122, 124]. Commonly, ultraviolet (UV) light polymerization systems were used to obtain GelMA hydrogels, where mechanical properties and potential of GelMA hydrogels as a three-dimensional tumor microenvironment are investigated [117, 118, 123, 125]. However, high intensity of UV light can damage cellular DNA, and commonly used UV photoinitiators like I2959, are oxygen sensitive, therefore air-tight protective barriers are needed which are not compatible with clinical use [126]. Recently, only several systems were reported with milder photo-crosslinking conditions using visible light to prepare GelMA hydrogels where an additional crosslinker was introduced in these systems [126]. For example, styrenated gelatin and furfuryl modified gelatin polymerized in the presence of photo-oxidative dyes, camphorquinone and Rose Bengal, respectively [127-130]. Nakayama et al. reported that eosin Y bound gelatin has the highest gelation efficacy among the xanthene dyes-derivatized gelatins and this hydrogel could adhere on damaged tissue of rat and stop

bleeding. However, the biodegradation rate and cytotoxicity of the gel degradation were not tested [131].

The system developed in this study has several benefits over existing GelMA polymerization methods compared to the reported UV polymerization systems. Since UV light is known to damage cell DNA, the visible light is innocuous for sustaining long term cell function [132, 133]. Previously several studies used visible light for photopolymerization of GelMA, where a crosslinker such as PEGDA and collagen were included [127, 130, 131, 133, 134]. In our work, for the first time in the literature we have not included an additional crosslinker or collagen into our prepolymer system and carried out extensive investigation of cellular behavior and gene expression profiles within this unique 3D hydrogel network.

Here, we developed a visible-light induced photopolymerization procedure for GelMA. Eosin Y was selected as a photoinitiator, triethanolamine (TEA) as an electron donor and N-vinyl-2-pyrrolidinone (NVP) as an accelerator. The polymerization kinetics of GelMA hydrogels were investigated using chemical, physical and mechanical characterization techniques. We found that cells in spheroid form and sparsely distributed in 3D GelMA hydrogel scaffolds exhibited altered cellular growth dynamics and gene expression levels in response to antitumor drug treatment.

4.2. Materials and Methods

4.2.1. Materials

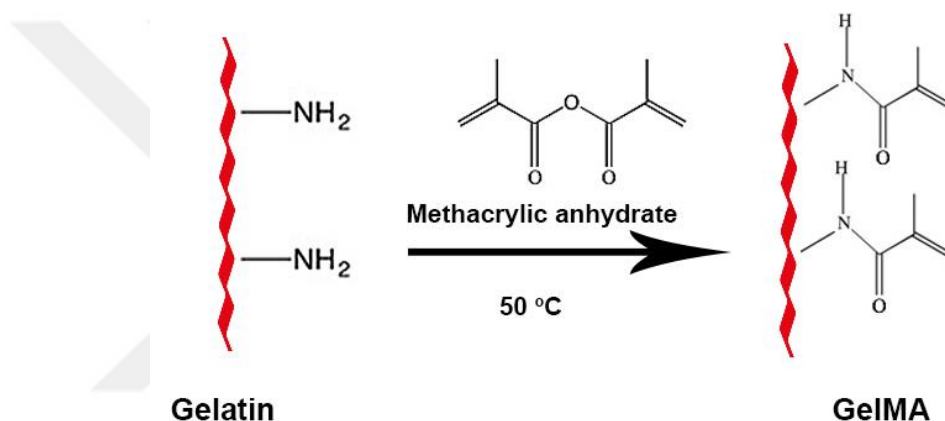
Gelatin Type B (Mw ~50 kDa, bloom value of 225 g and pI ~5), methacrylic anhydride (MA), eosin Y and N-vinyl-2-pyrrolidinone (NVP), L-glutamine (200 mM), trypsin-EDTA (25 %), were purchased from Sigma-Aldrich (St. Louis, MO).

Triethanolamine (TEA) was purchased from Merck. Collagenase type I was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Dulbecco's modified Eagle medium (DMEM, 41966), fetal bovine serum (FBS), penicillin/streptomycin, phosphate buffer saline (PBS) were purchased from Gibco (Grand Island, NY). Fluorescein o-acrylate was purchased from Sigma-Aldrich. CellTiter-Glo luminescent cell viability assay was purchased from Promega (Madison, WI, USA). 2,4,6-Trinitrobenzene Sulfonic Acid (TNBSA) and LIVE/DEAD® Cell Viability Assay were obtained from Thermo Fisher Scientific (Waltham, Mass). NucleoSpin® RNA Macherey Nagel (North Rhine-Westphalia, Germany), LightCycler 480 Multiwell Plate 96 and LightCycler 480 SYBR Green Master from Roche Diagnostics (Mannheim, Germany).

4.2.2. Methacrylated Gelatin Synthesis

GelMA conjugates were synthesized as described previously.[135, 136] Briefly, gelatin 10% (w/v) was dissolved in phosphate buffered saline (PBS, Amresco) at 50°C under stirring. A fraction of lysine residues reside on gelatin were modified via addition of 20 % (v/v) MA which was added dropwise and allowed to react for 1 h. To stop reaction, the mixture is 4-fold diluted with warm PBS and pH is set to 7.4. To remove methacrylic anhydride and impurities, GelMA was dialyzed against distilled water using 12–14 kDa cutoff dialysis tubing for 3 days. Purified GelMA was lyophilized and stored at -20°C. Degree of methacrylation was measured by 2,4,6-Trinitrobenzene Sulfonic Acid (TNBSA) assay according to the manufacturer's instructions. The functionalization degree was calculated by comparing the amount of remaining free amino groups in gelatin and GelMA [137, 138] Methacrylation processes of gelatin is illustrated in **Scheme 3.1**. Briefly, 200 µg/ml (w/v) of lyophilized GelMA and gelatin in several concentrations were dissolved at in 0.1 M sodium bicarbonate (pH 8.5). Then, 0.25 ml of

0.01% (w/v) TNBSA solution was added to 0.5 ml of each sample solution and samples were incubated at 37°C for 2 h. To stop the reaction 0.125 ml of 1 N HCl were added to each sample. Absorbance was determined at 335 nm using a plate reader (Biotek, Synergy H1). Free amine percentage was calculated as 67 % (**Figure B.1.**), this ratio was reported to be in optimal range in the respect of free lysine residues and mechanical properties [118, 135].



Scheme 4.1. Representation of methacrylation and synthesis processes of gelatin hydrogels.

4.2.3. Synthesis of Gelatin Hydrogels

GelMA conjugate was mixed with 0.05 mM eosin Y, 225 mM TEA, 74 mM NVP in PBS at pH 8. This prepolymer solution was placed in molds and exposed to argon ion laser for 5 min (100 mW, Coherent, Santa Clara, CA). Resulting hydrogels were in disk shape and had 0.2 cm² diameter.

4.2.4. Characterization of Surface Morphology, Swelling, Gel Fraction, and Degradation

Surface morphology and porosity of hydrogels was analyzed using field emission electron microscopy (FESEM) (Zeiss Ultra Plus, Bruker, MA) at 3 kV. For FESEM

characterization, lyophilized samples were attached on stubs as intact forms with double-sided carbon tape. Images were collected at the magnification of 200X.

For swelling experiments, GelMA hydrogels were placed in HEPES buffer and incubated at 37°C. Weight of the hydrogels were measured at indicated time points. The weight swelling ratio, Q , was calculated using the following equation:

$$Q = W_s/W_d \quad (4.1.)$$

where W_s is the weight of the swollen hydrogel and W_d is the initial weight of the dried hydrogel.

To calculate sol-gel ratio, hydrogels were put in tubes and dried to constant weight at 60°C. Then, the sol was extracted in dH₂O at 60°C for 1 day and washed in distilled water for three times. The remaining gel was dried to constant weight at 60°C. Gel fraction was determined gravimetrically and by using **Eqn (4.2.)**.

$$Gel\ Fraction\ (\%) = W_d/W_o \times 100 \quad (4.2.)$$

where W_o is the initial weight of dry gel and W_d is the weight of dry gel after extraction. Degradation behavior of GelMA hydrogels were evaluated with 0.25 mg/ml collagenase type I (Worthington Biochemical, Lakewood, NJ) prepared in HEPES buffer (0.1 M HEPES + 0.15 M NaCl + 5 mM CaCl₂ at pH 7.4). Briefly, swollen hydrogels were incubated in collagenase containing HEPES at 37 °C with shaking at 90 rpm and weight loss was determined over time by calculating the change in wet weight of the hydrogels normalized to equilibrium swelling measurements at time zero.

4.2.5. Rheology Measurements

Rheology measurements were carried out in time sweep mode with 10 Hz frequency, 1 % strain using a TA Instruments AR/DHR series rheometer (New Castle, DE) with UV

light attachment. GelMA hydrogel prepolymer solutions were prepared by the method outlined in above section.

300 μ L of gelatin hydrogels was loaded on the plate as a liquid, test geometry is lowered to 1000 μ m gap and exposed to UV light with 100 mW power. G' and G'' values were continuously monitored at 25 °C for 40 min. The time required for gelation was estimated by measuring the elastic modulus and the viscous modulus (G' and G'').

4.2.6. Thermogravimetric Analysis

Thermal resistance of hydrogels were examined by thermal gravimetric analysis (TGA) with a TA Q500 in open pans under nitrogen atmosphere at a gas flow rate of 60 ml/minute throughout the analysis and heated at a heating rate of 10°C/minute from 30 to 550°C. Samples of approximately 10 mg in powder form were placed in open platinum pans. The thermobalance was calibrated before measurements. Using TA universal analysis software degradation temperatures (T_D) were determined from the onsets of derivative weight % change versus temperature line.

4.2.7. Maintenance of Cells

U373 GBM cells were supplied and authenticated by American Tissue Type Culture Collection (ATCC). Cells were maintained in DMEM (Gibco) supplemented with 10 % FBS, and 1% Penicillin-Streptomycin (Gibco). Cells were maintained in a humidified environment at 37 °C with 5 % CO₂.

4.2.8. Cell Adhesion

For cell adhesion studies, disk shaped (2D) hydrogels with 0.2 cm² diameters were washed with PBS and incubated in DMEM for 12 h prior to cell seeding. Cells (cell number=8000) were seeded onto hydrogels placed in 24-well plates and covered with

DMEM. Media was changed every day. Cell viability was determined via Cell-Titer Glo (CTG) assay.

4.2.9. Cell Encapsulation

For the cell encapsulation studies, two different approaches were followed. In the first approach, U373 cells were trypsinized and resuspended in 10 % (w/v) GelMA prepolymer solution in PBS, sparsely. In the other experimental set, U373 spheroids were made by hanging drops containing 20,000 cells in 20 μ l medium. After 2 days, the spheroids were suspended in the solution of GelMA, similarly. Two million cells/ml (100,000 cells/ gel) final cell density was used for both conditions. The cell–prepolymer suspension was moved to UV-sterilized molds and exposed to visible light (514 nm) for 5 min. The mold was then removed, and the cell-laden hydrogel disks were placed in media. Media were refreshed every other day over the duration of experiment.

4.2.10. Cell Viability

Cell viability was determined via Cell-Titer Glo (CTG) and Live/Dead assays. CTG assay was applied as previously described.[139] ATP standard solutions with known concentrations were prepared in the cell culture medium. After CTG reagent addition, both ATP solutions and cell-laden hydrogels were incubated at 25 °C for 15 min and the luminescence was measured using a plate reader (Biotek, Synergy H1). ATP amounts of samples were calculated from measured luminescence values using ATP calibration curve. Triplicates of hydrogel samples were used for each condition.

For Live/Dead assay calcein AM (Invitrogen) and ethidium homodimer-1 (Invitrogen) dyes were used to stain viable or dead cells at days 1 and 4. Briefly at each time point, a mixture of reagents was prepared with final concentrations of 2 μ M calcein

AM and 4 μ M EthD-1. DMEM was removed from the wells which included cell seeded and cell-laden hydrogels. Following washing step with PBS, 500 μ l of reagent mixture was added onto the samples and incubated at RT for 30 min. Reagents were removed via washing with PBS and hydrogels were analyzed using a fluorescence microscope.

4.2.11. The Effect of Chemo-tactic Agent and Anti-Cancer Drug Treatment

Cells were encapsulated within hydrogels as described above, in sparse and spheroid forms. Bradykinin (BK) was applied to the cells (200 nM) as a chemo-tactic agent for 4 days. Viability, spheroid morphology and gene expressions of those cells were evaluated. Cells encapsulated into hydrogel were also treated with an anti-cancer drug, Digitoxigenin (2.5 μ M). Drug treatment started on day 1 of 3D culture and its effects were tested 48 h later using CTG.

4.2.12. Gene Expression Analysis

Total RNA was obtained from each sample as described, previously.[140] Briefly, hydrogels were transferred into 0.35 ml of lysis buffer containing 1% (v/v) β -mercaptoethanol in 2 ml tubes and disrupted by pipetting up and down several times. Cell-laden hydrogels were homogenized by snap-freezing in liquid nitrogen. Then, the samples were thawed at RT and homogenized manually, by carefully passing through a 19-gauge needle with a 5-ml syringe 15–20 times. The lysates were placed on spin columns and proceeded using Macherey-Nagel RNA isolation kit following manufacturer's instructions.

Total RNAs (350 ng) were reverse-transcribed using M-MLV Reverse Transcriptase (Invitrogen, USA). Relative gene expression levels were determined by using LightCycler® 480 SYBR Green I Master (Roche, Germany) and detection was performed

by LightCycler® 480 II (Roche, Germany). Gene expressions were determined using the 2- $\Delta\Delta C_t$ method, normalizing to housekeeping gene GAPDH. Oligo sequences for q-RT-PCR are listed in **Table 4.1**.



CHAPTER 4: GELATIN METHACRYLATE HYDROGELS IN THE ABSENCE OF CROSSLINKER AS A 3D MICROENVIRONMENT FOR GLIOBLASTOMA MULTIFORME (GBM)

Table 4.1. List of Primers used for Gene Expression Analysis.

Gene Name	Primer Sequence
<i>GAPDH</i>	Forward: AGCCACATCGCTCAGACAC Reverse: GCCAATACGACCAAATCC
<i>MMP-2</i>	Forward: GATACCCCTTTGACGGTAAGGA Reverse: CCTTCTCCCAAGGTCCATAGC
<i>MMP-3</i>	Forward: AGGCAAGACAGCAAGGCATA Reverse: GATATTTCTGAACAAGGTTTCATGC
<i>MMP-7</i>	Forward: CGAGGAGCTCATGGGGACTC Reverse: GACTGCTACCATCCGTCCAG
<i>MMP-9</i>	Forward: AGACCTGGGCAGATTCCAAC Reverse: CGGCAAGTCTTCCGAGTAGT
<i>MMP-14</i>	Forward: TCAACCCAGGACTACCTCCC Reverse: CAGCGCTCCTTGAAGACAAAC
<i>Caspase-3</i>	Forward: CATGGAAGCGAATCAATGGACT Reverse: CTGTACCAGACCGAGATGTCA
<i>Bad</i>	Forward: CCCAGAGTTTGAGCCGAGTG Reverse: CCCATCCCTTCGTCGTCCT
<i>Puma</i>	Forward: GACCTCAACGCACAGTACGAG Reverse: AGGAGTCCCATGATGAGATTGT
<i>Bcl-2</i>	Forward: CAGGGCGATGTTGTCCACC Reverse: GGGGAGGATTGTGGCCTTC

4.3. Results and Discussion

4.3.1. Synthesis and Characterization of GelMA Hydrogel Structure

GelMA hydrogels were prepared to mimic and replicate the microenvironment of GBM cell behavior *in vitro*. Methacrylate groups were incorporated into gelatin structure prior to hydrogel synthesis using similar methods published previously [118, 125, 141]. We prepared GelMA with 67 % free amine content measured by TNBSA assay (**Figure B.1.**) and further characterized by Fourier Transform Infrared (FTIR) Spectroscopy (**Figure B.2.**).

Crosslinking of a 10% (w/v) GelMA containing prepolymer was obtained using visible light photopolymerization.

The biomechanical properties of the ECM have great influence on cell viability, function and differentiation [141]. To characterize the mechanical properties, rheology experiments were carried out using a rheometer with UV attachment. Since eosin Y can be excited both by visible and UV light, we were able to polymerize the same prepolymer solution prepared for visible light curing for rheology measurements [142].

Storage modulus (G') quickly increased within 3 min and the increase was continued for 8 min for all samples. This time interval was in agreement with the reports illustrating rheological behavior of GelMA [143]. The sharp increase in G' for the hydrogels prepared with 74 mM NVP suggested the formation of denser crosslinked networks compared to those gels prepared with lower NVP concentrations.

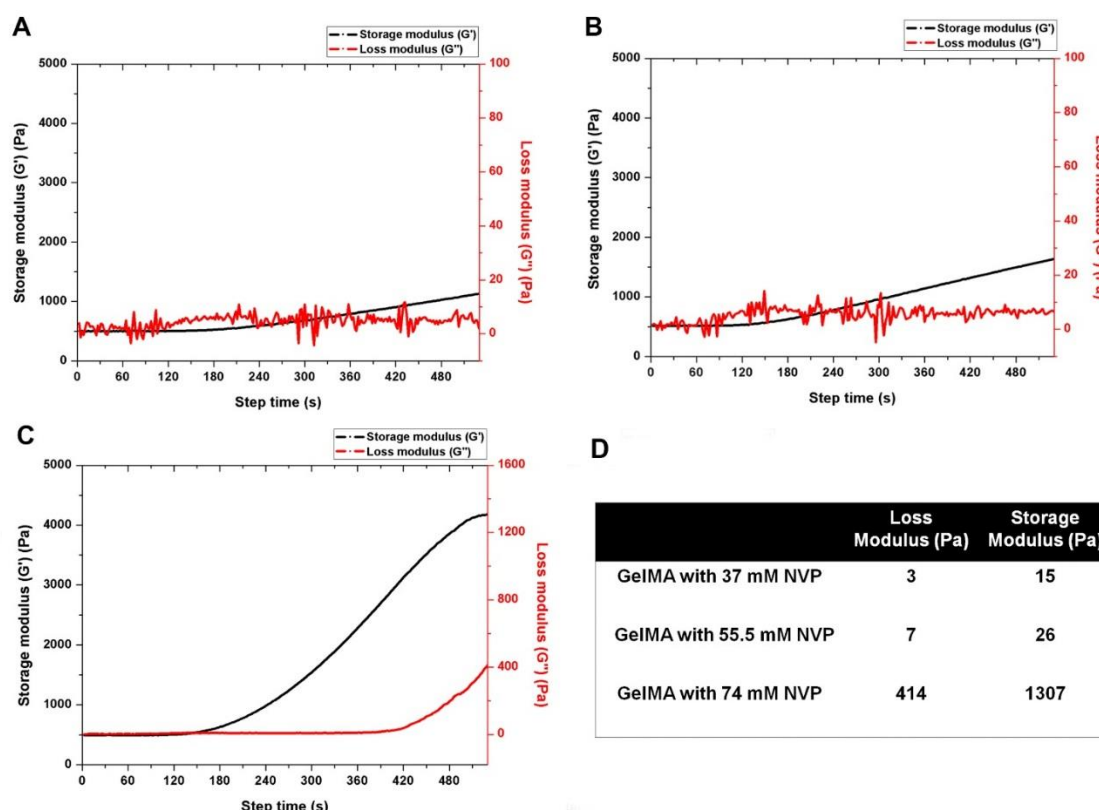


Figure 4.1. Time sweep profiles of the elastic modulus (G') and viscous modulus (G''). Hydrogels prepared with A) 37 mM, B) 55.5 mM, C) 74 mM NVP concentrations, D) Table demonstrates loss and storage modulus's.

We also conducted a TGA analysis to explore crosslinking efficiency and stability of hydrogels (**Figure 4.1**). Weight loss of about 10 % (wt/wt) was observed between 30 -200°C, where highest loss occurred below 120°C. This result was attributed to the loss of moisture below 120°C, where a weight loss of about 70 % (wt/wt) observed between 200- 550 °C was associated with protein degradation [144, 145]. The remaining residue was around 20 % at 550°C for all samples.

The temperature at which maximum decrease in mass occurs is known as extrapolated onset of degradation (T_D), where T_D was calculated from the first order derivative of TGA curves. These values were summarized in **Figure 4.2D**. As expected, T_D onset value of crosslinked gelatin was higher than that of Type B gelatin powder and

GelMA. The highest T_D observed for TEGDMA crosslinked hydrogel suggested the stability of these gels.

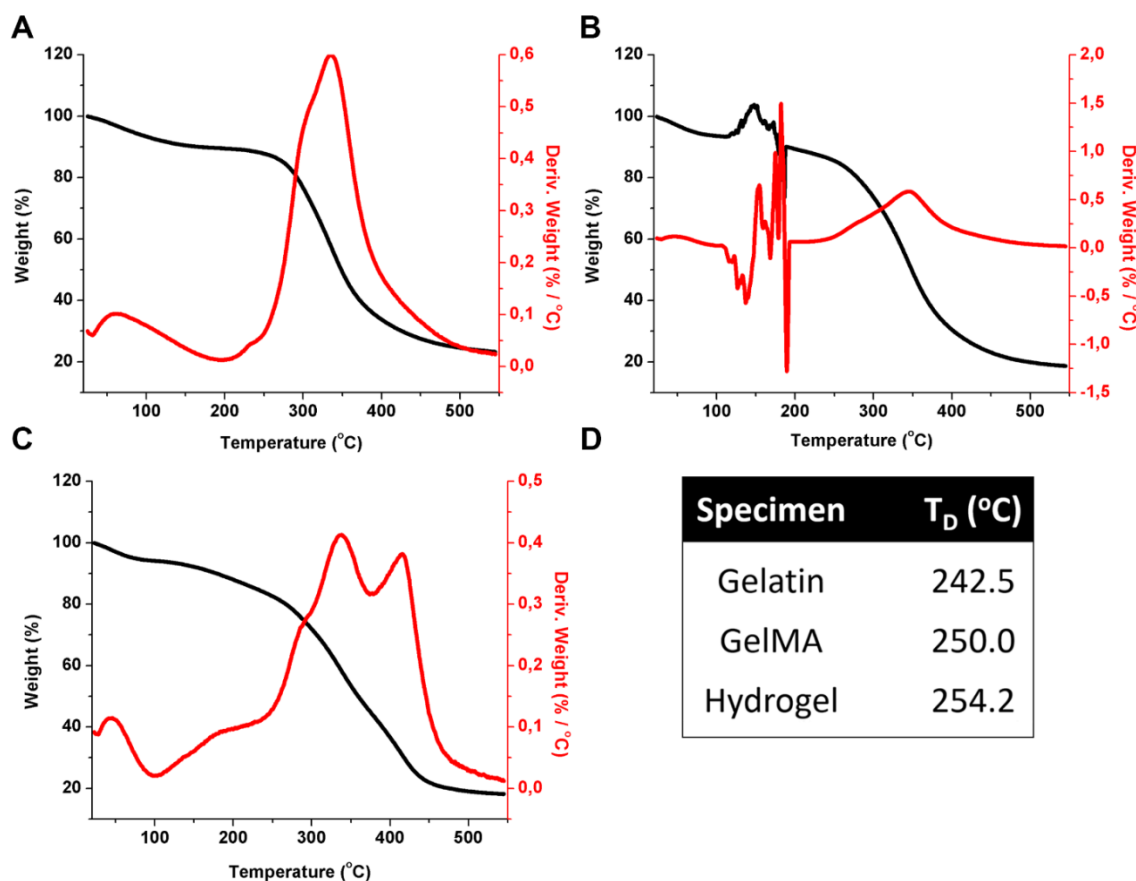


Figure 4. 2. TGA thermograms. A) Gelatin, B) GelMA, C) GelMA hydrogels, and D) Thermal degradation temperatures of gelatin powder, methacrylated gelatin (GelMA) and GelMA hydrogels.

The surface morphology of freeze-dried GelMA hydrogels was observed by FESEM. **Figure 4.3A** shows that crosslinked gelatin chains formed spongy networks. The network mesh size formed using 74 mM NVP was between 100-150 μm . This large mesh size is desirable to promote cell movement and proliferation [146].

Integrity of hydrogels were maintained at equilibrium swelling conditions which suggested the stability of crosslinked network at 37°C. Swelling ratios were in good

correlation with the literature, where the values ranged between 10-20 % for GelMA (10 % w/v) [147]. We found that swelling ratio was lowest for the highest NVP concentration, demonstrating the significance of NVP concentration on water storage capacity of the polymer.

Swelling, enzymatic degradation and sol-gel extraction studies were performed with altered NVP concentrations to study the crosslinking kinetics of GelMA hydrogels, while monomer, TEA and eosin Y concentrations were kept constant. For swelling experiments freeze-dried hydrogels were allowed to reach equilibrium through incubation in PBS at 37°C and the mass swelling ratio was calculated using **Eqn (4.1.)** from the wet weights measured at different time points (**Figure 4.3B**).

Sol-gel proportions of hydrogels were calculated using **Eqn (4.2.)**. Although, not a significant difference was observed among all groups tested, gel ratio of the samples prepared with 37 mM NVP had the lowest gel fraction compared to the hydrogels in other groups (**Figure 4.3C**).

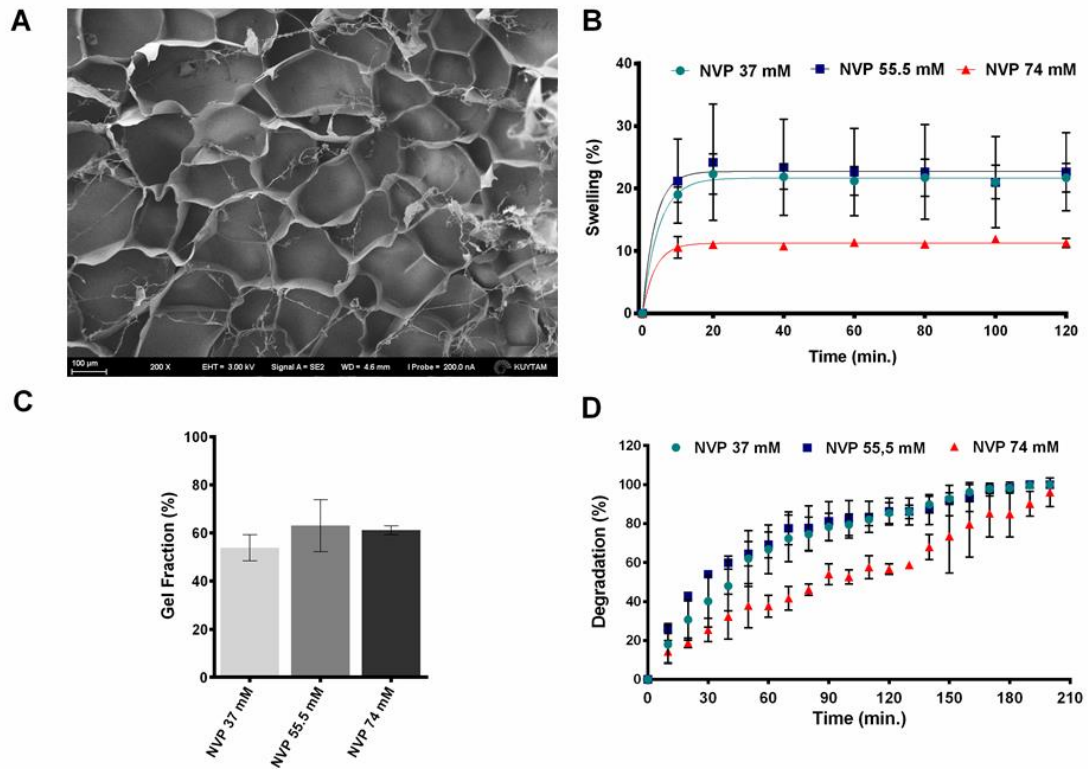


Figure 4.3. Physical properties of GelMA hydrogels. A) FESEM micrograph. Original magnification $\times 200$. Bar represents 100 μm . B) Swelling, C) Gel fraction, D) Degradation profile in the presence of collagenase type I (0.25 mg/ml).

Swollen hydrogels were tested for their degradation potential where collagenase type-I enzyme was used in the buffer medium to degrade hydrogel network. The decrease in weight of hydrogels in time were recorded and illustrated in **Figure 4.3D**. We observed extended duration of mass degradation ratio with increasing NVP concentration. The results in **Figure 4.3B-3D** are consistent with each other, suggesting higher crosslinking efficiencies of the system with increasing NVP concentration.

4.3.2. Effects of 2D and 3D GelMA Hydrogels on U373 Cell Viability

2D and 3D materials are preferred over tissue culture plates (TCP) in many *in vitro* research studies as TCP provide large surface area where proliferation of cells cultivated

in TCPs is terminated after cells reach confluency [148]. In this study, we tested the feasibility of GelMA hydrogels for U373 GBM cell culture in both 2D seeding and 3D encapsulation experiments. Prior to 3D cell-encapsulation experiments, the effects of using GelMA hydrogel as a cell culture material were investigated via seeding cells on disk-shaped 2D hydrogels. Cell viabilities were measured at day 1 and day 4 using CTG assay as was illustrated in **Figure 4.4**.

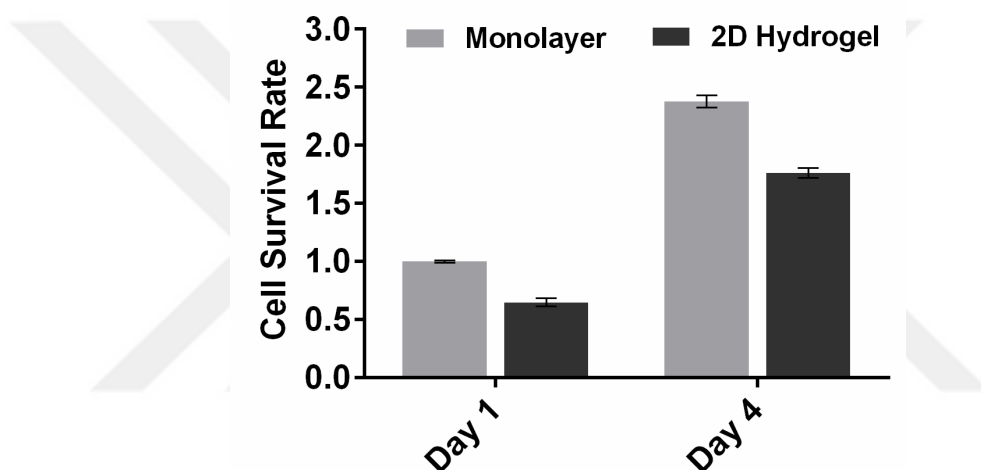


Figure 3. 4. Cytotoxicity Test for U373 Cells on GelMA surfaces at different time points. The cell viability for U373 cells seeded on 2D GelMA hydrogel disks were measured using a CTG assay. The data were normalized to the viability of cells seeded on plate as monolayer on day 1.

Cells seeded on 2D GelMA hydrogels demonstrated a slightly decreased confluency as compared to control cells seeded on TCP. The decrease could be due to non-adherent cells removed during washing steps. However, cells on GelMA surfaces demonstrated significantly improved confluency after 4 days, demonstrating 2-fold increase compared to day 1.

Once the biocompatibility of the hydrogels was confirmed, cell-encapsulation experiments were conducted. Since the ultimate goal of this study is to investigate

proliferation and invasiveness behavior of GBM cells within 3D hydrogel matrixes, the most crucial step was optimization of the photopolymerization conditions that will not compromise the survival of U373 cells. Optimal exposure time to visible light (514 nm) for encapsulation of cells within GelMA hydrogel network was found as 5 min. To assess further effects of 3D GelMA microenvironment on cellular fate, we designed two different models for the preparation of cells. Cells were either suspended in spheroid forms using hanging drop method or sparsely resuspended in prepolymer solution just before polymerization. Cell-laden hydrogels synthesized in these conditions were immediately moved into fresh DMEM and incubated in cell culture conditions.

The preparation steps to obtain cell spheroids using hanging drop method was demonstrated in **Figure 4.5A**. The gravitational force applied on cells in hanging drop suspended cell cultures triggered sedimentation to begin, and cells formed aggregate in 2 days [149]. **Figure 4.5B-5C** shows the representative fluorescence microscopy images of green fluorescence protein (GFP)-labeled cells within GelMA hydrogels. After incubation of GBM spheroids in GelMA hydrogels for 8 days protrusions from aggregate surface was observed within GelMA (**Figure 4.5B**).

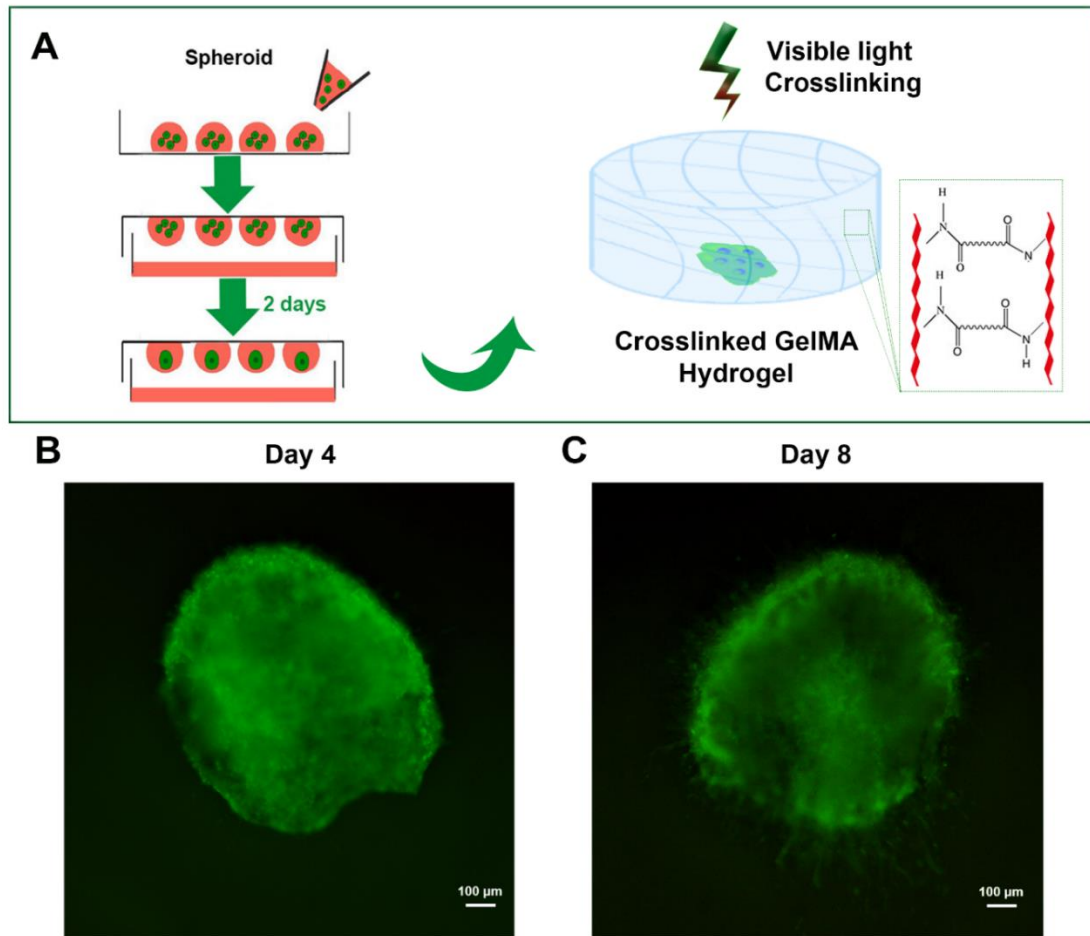


Figure 4. 5. A) Schematic representation of spheroid preparation protocol by hanging drops for cell encapsulation. 20 μ l drops of cell suspension (20,000 cells) are initially dropped on the covers of cell culture plates, and the inverted covers are incubated for 2 days. B-C) Fluorescence microscopy images of GFP labeled U373 cells laden in GelMA hydrogels on day 4 and day 8. Scale bars are 100 μ m.

After encapsulation experiments, cell viabilities were measured in the hydrogels at different time points. Cell viability was determined via live/dead and CTG assays and results were presented in **Figure 4.6**. High amount of green fluorescence intensities on the right panels of live/dead images (**Figure 4.6A-6B**) suggest that 3D environment enhances GBM cell viability. In addition, cells retained capacity to invade out of the recapitulating tumor model (**Figure 4.6A**). These results were also confirmed by CTG

assay as were demonstrated in **Figure 4.6C through 3.6F**. Even though initial cell densities were equal, sparsely seeded cells exhibited higher viability than the cells in spheroid form for both conditions (**Figure 4.6C-6D**). As reported, GBM tumors are highly heterogeneous and contains a necrotic core [150]. Thus, differences in cell viability could be due to the presence of death cells at the core of the spheroids and also due to the differences in the invasive capacity and phenotypes of these two models. Fold change in viability was higher for the cells in spheroid than the ones sparsely distributed either in monolayer or within 3D GelMA hydrogels. These results might be attributed to the phenomenon that the presence of support surface promote tumor growth and proliferation [150, 151].

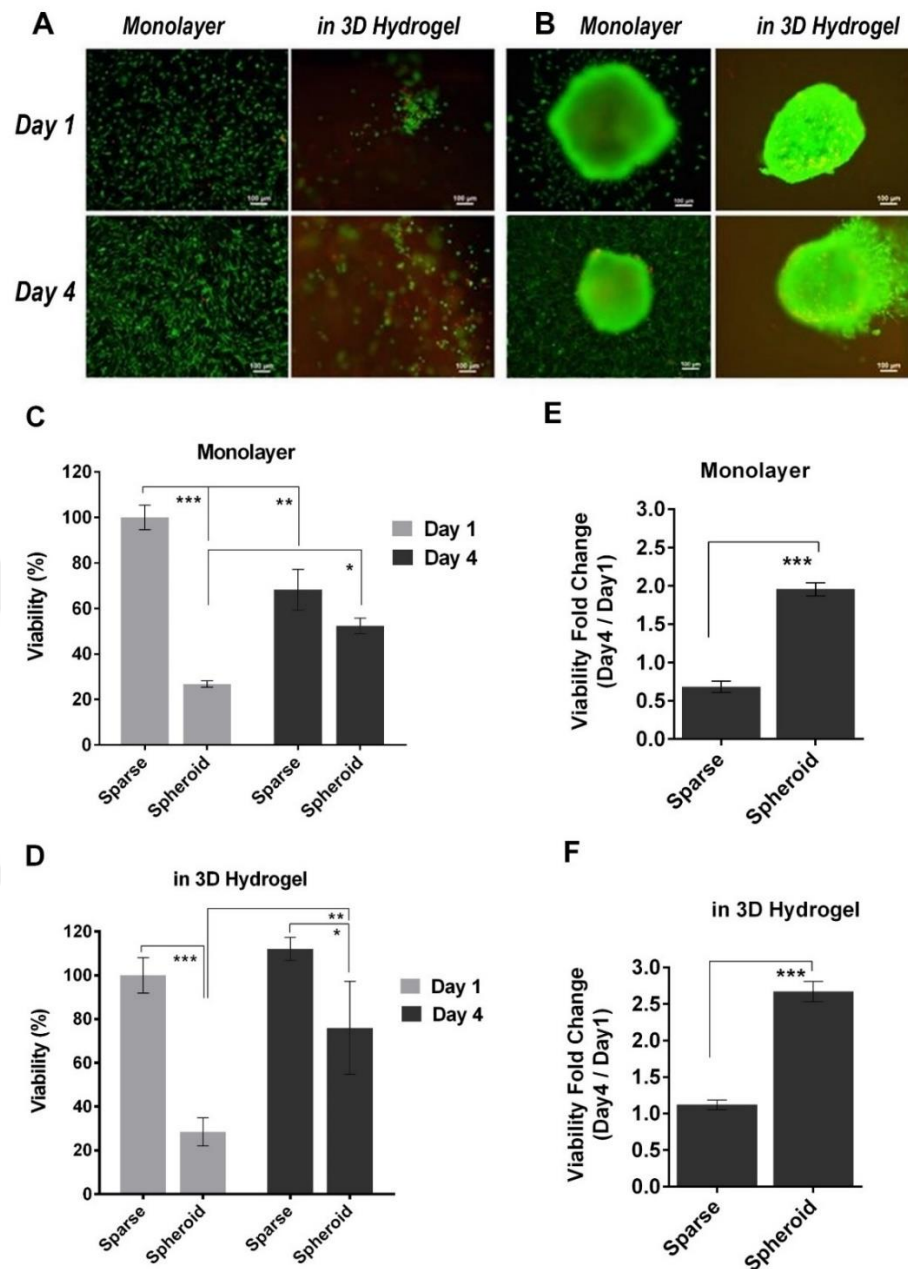


Figure 4.6. Different trends in viability for U373 cells. Cells were seeded as sparsely and spheroids on TCPs (monolayer) and within 3D GelMA hydrogels. A-B) Representative images of Calcein AM (green) and EthD-1 (red) costaining of the U373 cells. C-D) Relative cell viability percentages obtained via CTG assay, normalized to viability of sparsely seeded cells on day 1 for either monolayer or 3D encapsulation conditions. Scale bars are 100 μm . Two-way ANOVA followed by Tukey's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.3.3. Anti-cancer Drug Treatment

Next, we treated our *in vitro* tumor model with an anti-cancer drug, Digitoxigenin (Dgx). Dgx is in the family of cardiac glycosides, which is recently used as anti-cancer drug in GBM cells and lung cancer cells. [4, 152] We preferred to use this drug in our study due to its well-known effects on the expression of key regulators of metastatic mechanisms, MMPs. Previously, it was shown that treatment of lung cancer cells with Dgx decreased expression levels of MMP-2, MMP-9 and p-FAK . [152] According to our knowledge, Dgx is first time tried on U373 cells in this study. For these experiment, control cells on TCPs and spheroid-containing hydrogels were treated with Dgx, and alterations in cell viability in different models were measured as was demonstrated in **Figure 4.7A-7D**. Furthermore, decrease in spheroid radii of GFP-labeled U373 cells after Dgx treatment was illustrated in **Figure 4.7E**.

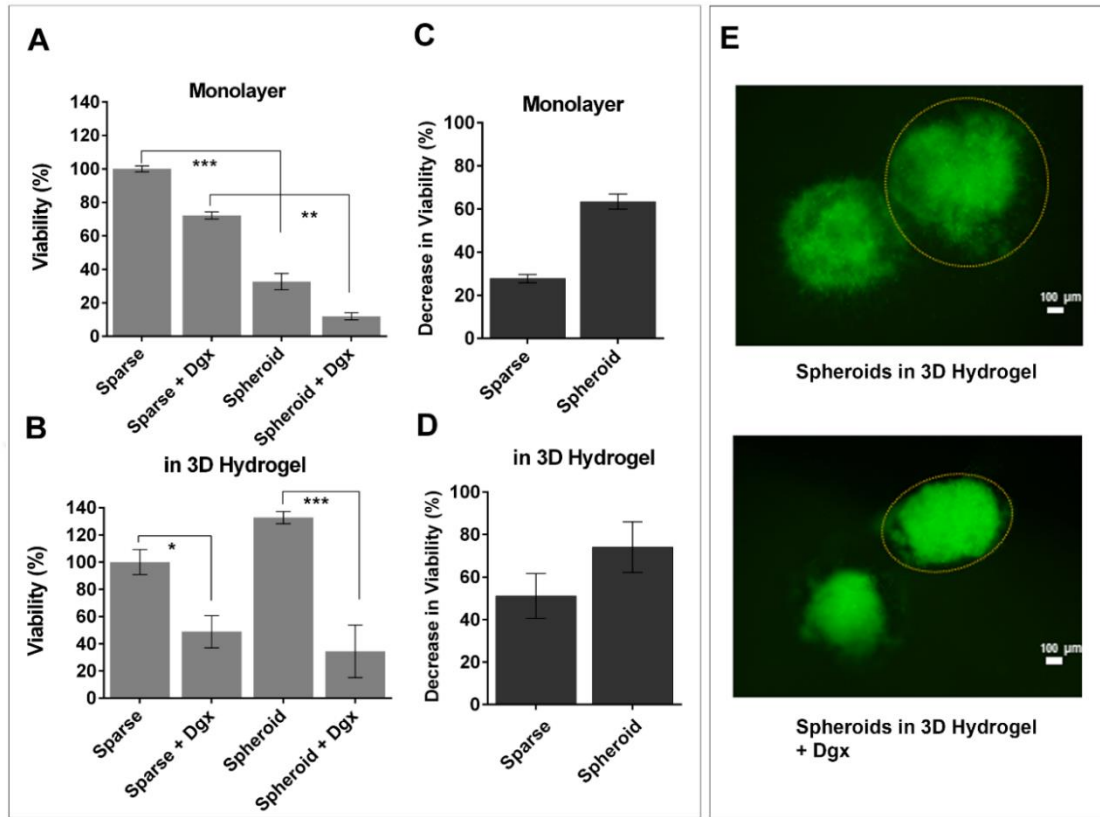


Figure 4. 7. Effects of Digitoxigenin treatment on *in vitro* 3D models. Viability percentages of A) cells seeded on TCPs as sparsely and in spheroid forms (Normalized to sparsely seeded cells), B) cells encapsulated into 3D GelMA hydrogels as sparsely and in spheroid forms (Normalized to sparsely seeded cells) in the absence and presence of Dgx (2.5 μ M). Decrease in viability percentages of sparsely seeded cells and cells in spheroid forms following Dgx treatment C) on TCPs, D) in 3D GelMA hydrogels were calculated relative to untreated cells for each condition. E) Fluorescence Microscopy Images of GFP labeled U373 cells and Dgx treated GFP labeled U373 cells laden in GelMA Hydrogels. Yellow circles show spheroid bodies. Scale bars are 100 μ m. Two-way ANOVA followed by Tukey's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 4.7 indicates that cells in spheroid form were more sensitive to drug compared to the ones that were sparsely seeded cells in both conditions. **Figure 4.7** also

demonstrates that while monolayer TCP compromised GBM spheroid viability, 3D gelatin hydrogel encapsulation system promoted GBM spheroid viability.

4.3.4. Gene Expressions in U373 in GelMA Hydrogels

The effects of Gelatin hydrogel microenvironment and cell encapsulation style (sparse or spheroid) on mRNA expressions in U373 cells were evaluated using qRT-PCR. Gene expression analysis was applied for candidate genes which were selected from both apoptosis pathway (**Figure 4.8**) and invasion related matrix-metalloproteinase (MMP) family (**Figure 4.9**).

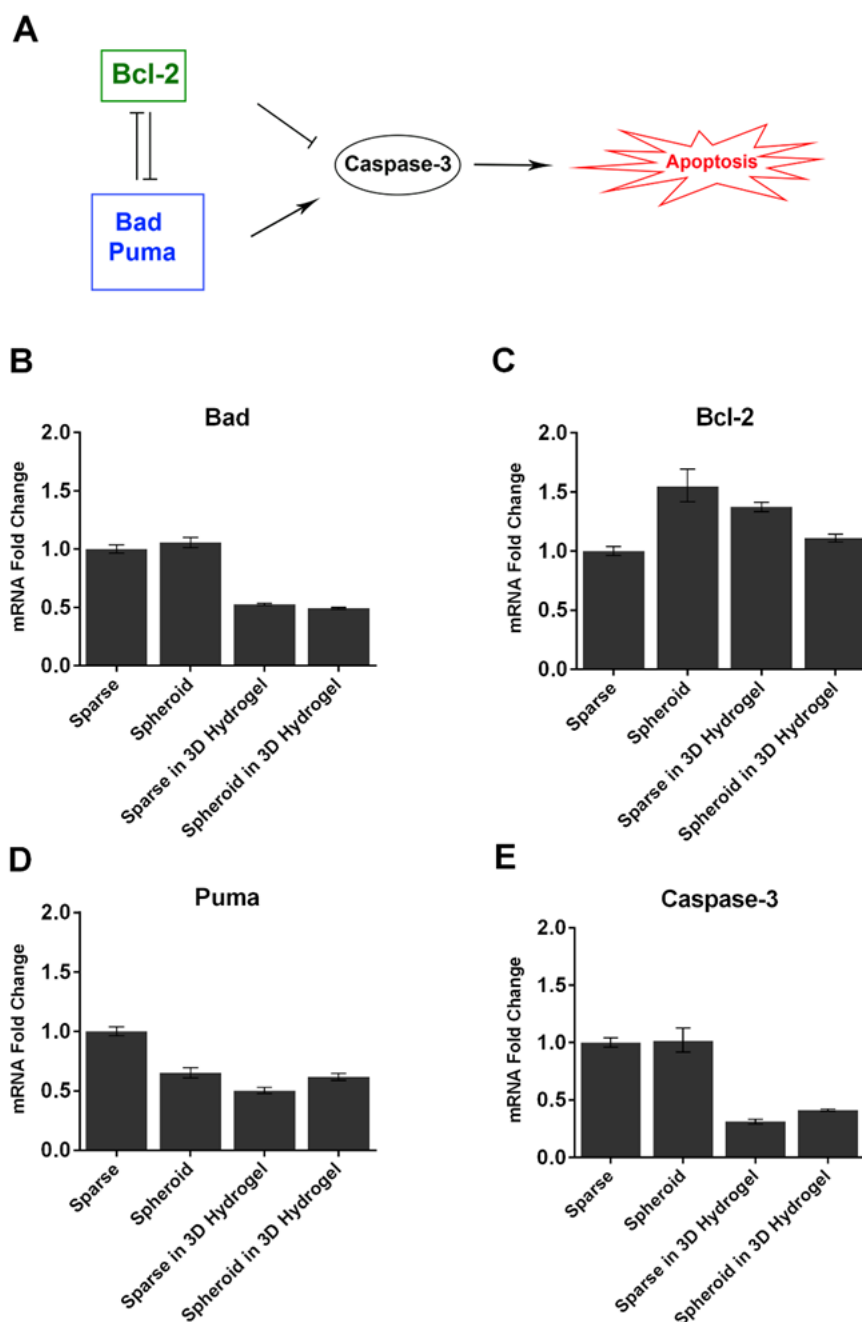


Figure 4.8. A) Schematic representation of four apoptotic genes (Bad, Puma, BCL-2, Caspase-3) in controlling the apoptosis on cancer cells. B-E) Bar graphs represent expressions of apoptotic genes evaluated in U373 cells mRNA expression levels of apoptotic genes were quantified on day 4 from collected lysates of cells seeded TCPs and in 3D GelMA hydrogels as sparsely and in spheroid forms by qRT-PCR, results were calculated with respect to the reference gene GAPDH and normalized to sparsely seeded cells for each condition. Data are expressed as mean $\pm$ error.

mRNA expression levels of apoptosis related Puma, Bad, Bcl-2 and Caspase-3 genes were shown in **Figure 4.8**.

The action mechanism of these genes in intrinsic apoptosis pathway can be described as follows: Bcl-2 protein is a member of anti-apoptotic Bcl-2 family, Puma is one of the Bcl-2-homology domain 3 (BH3)-only proteins that initiates apoptosis directly through the activation of Bax-Bak to release cytochrome c from mitochondria. Bad is a sensitizer BH3-only protein which dampens anti-apoptotic sequestering the BH3 domains of activator BH3-only function of Bcl-2 but cannot activate executioner Bax-Bak [26, 153]. Caspase-3 is essential for apoptotic chromatin condensation and DNA fragmentation during final steps of apoptosis [154].

The relation between these genes were roughly illustrated in **Figure 4.8A** where activation and inhibition of the pathway were illustrated with arrows and blind-end arrows, respectively. We observed significant decreases in mRNA expressions of apoptosis activating Bad, Puma and Caspase-3 genes, while Bcl-2 levels were remained high when cells were encapsulated into hydrogels. This result suggests that 3D encapsulation of GBM cells within gelatin either in sparse or spheroid form have positive effects on cell survival via downregulating the pro-apoptotic genes. Beside the differences in mRNA expressions resulted from 3D encapsulation, Bcl-2 and Puma levels were also altered in spheroid and sparsely seeded cells. Cells in spheroid form in 3D culture exhibited a higher level of Puma expression and lower level of Bcl-2 expression than sparsely seeded cells, suggesting that spheroids were more likely to go apoptosis. This result can be explained by the presence of a central necrotic core in GBM tumors [150].

In GBM cells increased expression levels of MMPs has been attributed to the malignant progression of the tumor [155]. Therefore, we analyzed the mRNA levels of MMP gene expressions in U373 cells in hydrogel microenvironment (**Figure 3.9**).

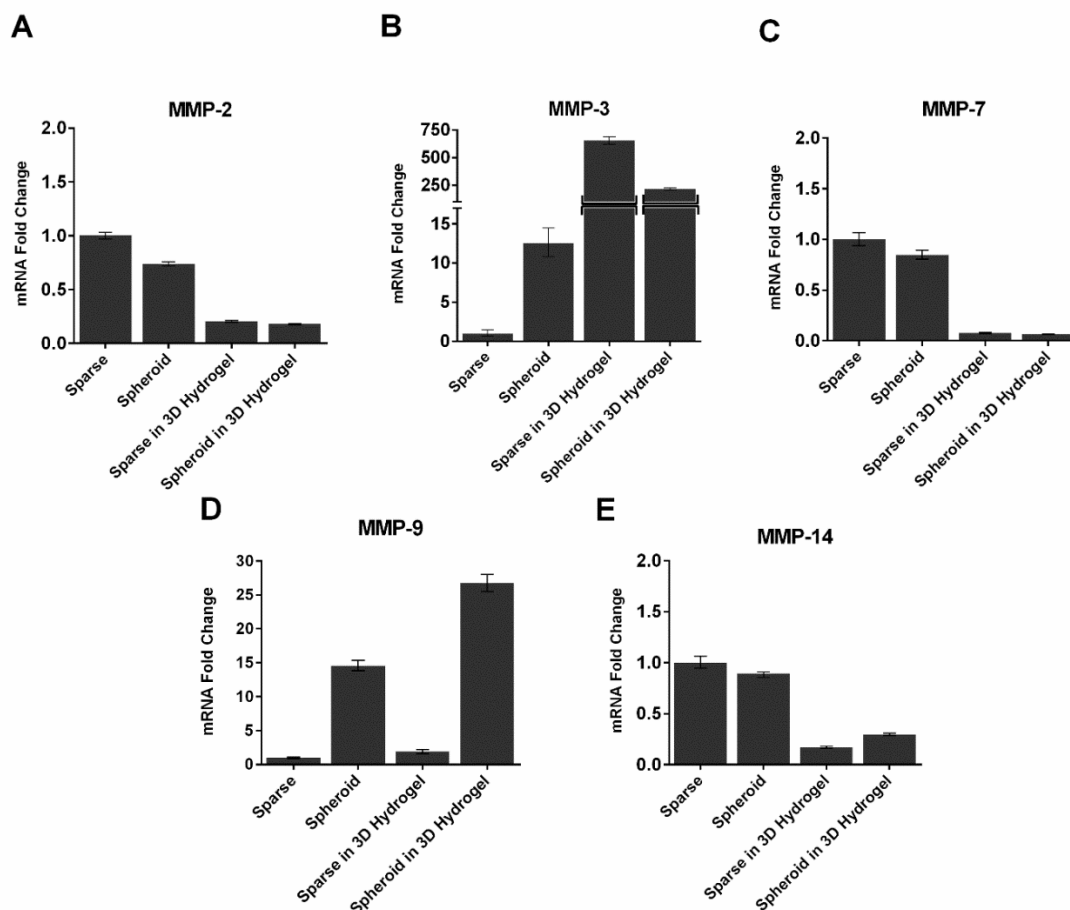


Figure 4.9. Bar graphs represent quantitative real-time PCR of invasion related MMP expressions evaluated in U373 cells. mRNA expression levels of apoptotic genes were quantified on day 4 from collected lysates of cells seeded on TCPs and in 3D GelMA hydrogels as sparsely and in spheroid forms by qRT-PCR, results were calculated with respect to the reference gene GAPDH and normalized to sparsely seeded cells for each condition. Data are expressed as mean $\pm$ error.

MMP-2 and MMP-9, known as gelatinases, are commonly analyzed in cell-laden GelMA hydrogel models. In addition, we also selected MMP-3, MMP-7, MMP-14 for the analysis, since these protease enzymes use gelatins as substrate [118, 156].

The results indicate that U373 cells are able to express gelatin-related MMP genes for ECM remodeling. The most striking result was the sharp increases in MMP-3 and MMP-9 expression in encapsulated cells. These two MMPs were also the most affected ones from spheroid morphology. Prominent level of MMP-9 expression could be associated with tumor invasiveness as was reported in previous literature [155]. One likely reason for the extended cell protrusions of spheroids displayed in **Figure 4.5** and **Figure 4.6** might be the upregulation of MMP-9.

The tremendous increase in MMP-3 levels together with MMP-9 might be explained by the regulation of cell motility and invasion via MMP-3 and MMP-9 gene expressions [157, 158].

On the other hand, expressions of MMP-2, MMP-7 and MMP-14 were downregulated in GelMA. Since, activation of pro-MMP-2 is dependent on MMP-14 and TIMP-2 interactions, we observed similar mRNA expression profiles for MMP-2 and MMP-14 (**Figure 4.9A, 9E**) [155].

These results are significant as the key role of specific MMP secretion on 3D tumor formation and proliferation was confirmed, which could make it possible to design MMP targeted drugs for the treatment of GBM.

The alterations in expressions of both apoptosis and migration-related genes in 2D versus 3D cultures propose that 3D models are more proper to mimic *in vivo* tumor progression.

4.4. Conclusion

GelMA hydrogels were photocrosslinked through visible light-induced polymerization and evaluated for its potential as an artificial 3D tumor microenvironment. This unique system is promising candidate for clinical translation of cancer research through the analysis of malignant cancer cell behavior. The novelties of this GelMA hydrogel system include its synthesis through visible light and in the absence of additional crosslinker. NVP was selected as an accelerator for this system and its influence on synthesis were analyzed using several characterization methods. Porosity and surface morphology was characterized using FESEM, chemical composition confirmed by FTIR spectral analysis, physical and mechanical stability was examined using TGA and rheology, favorable swelling and biodegradation kinetics were measured. Finally, cell encapsulation efficacy of these gels as a 3D ECM mimicking matrix was assessed by encapsulating U373, brain tumor cells. GelMA hydrogels synthesized with NVP were shown to be a promising model system for further biological assessment. Our gene expression analysis focuses on assessment of the effects 3D gel encapsulation and the comparison of spheroid and sparse morphology of cells. The present results demonstrate that 3D culture leads to differential tumor cell fates like proliferation and migration behavior.

Chapter 5

5. CONCLUSIONS AND FUTURE DIRECTION

In this thesis, we aimed to develop nanocarriers for the delivery of therapeutics and ECM-mimicking 3D hydrogel networks for GBM therapy.

To achieve this goal, in the first part, enzymatically responsive MMP-sensitive peptide incorporated PEG hydrogel carriers were synthesized via visible light-induced photopolymerization. The results of this study suggested that these MMP-sensitive PEG particles were cytocompatible and combination of QC-loaded PEG hydrogel and TRAIL demonstrated synergistic apoptosis-inducing behavior compared to the treatments of QC and TRAIL, as single agents. Furthermore, a detailed analysis was carried out on the expression profiles of apoptosis-related genes by qRT-PCR. The results indicated that Quinacrine-loaded PEG hydrogels, but not Quinacrine or PEG-hydrogels alone, changed the expression of apoptosis-related genes in favor of increased apoptosis. This system is promising to significantly improve the efficacy of chemotherapeutic cancer drugs and suggests treatment of GBM.

In the second part of the thesis, we utilized a simple approach to develop suspension and spheroid-based models for GBM to investigate cellular behavior and gene expression profiles within the 3D hydrogel network. The goal for this part was to design GBM microenvironment mimetic scaffolds such that GBM cells would remain viable and demonstrate realistic responses to anti-tumor drug treatments. Furthermore, sensitivity of this *in vitro* tumor model to an anti-cancer drug, was tested by treating with Digitoxigenin. The results indicate that GBM behaviors are highly dependent on dimensions of the matrix where they are located. Differential gene-expressions were

observed for both apoptosis and migration-related genes in 2D versus 3D cultures. The study describes a promising and adjustable system for mimicking the surrounding matrix of GBM cells and highlights the significance of developing such platforms for the clinical translation of GBM tumors.

As a conclusion, in this thesis, it has been shown first time in the literature that tumor cells can be sensitized to TRAIL with Quinacrine by using hydrogel micro/nano carriers. In addition to targeted drug delivery treatment of GBM cells, a detailed comparison study was provided for GBM tumors in sparse and spheroid morphology within 3D natural polymers. We have extended the knowledge about developing hydrogels functionalized with tumor-targeting peptides, *in vitro* testing of novel anti-cancer therapeutics including recombinant proteins, repositioning and combinatory treatment of small-molecule drugs. This study also study highlights the importance of developing biomimetic 3D platforms that can recapitulate regulatory elements of the complex tumor microenvironment, for exploring the etiology and early growth stages of GBM tumors. This work is important for providing a comprehensive interdisciplinary study of Chemistry, Material Science, and Molecular Biology.

For future applications, further detailed biochemical investigations of Quinacrine-loaded PEG particles against GBM are needed that will help to improve their chemotherapeutic potential against brain tumors. A molecular tracer can be incorporated into system to make it easier to validate these systems *in vivo*. In addition, these particles are tunable to pass through BBB so that they reach to the tumor site in the brain. Our gelatin-based 3D tumor model can be also developed further by incorporating ECM cues such as growth factors to illustrate their effects on invasion and migration potential of tumor spheroids. Finally, this thesis truly includes interdisciplinary aspects of material science, engineering and medicine such as the design of tumor microenvironment with

biochemical and biophysical cues within scaffolds and addresses the clinical limitations of cancer treatment to improve the efficacy of existing strategies, in particular for the treatment of GBM.



5.1. Publications, Conferences & Patents

Publications:

- Erkoc, P.; Seker, F.; Bagci-Onder T.; Kizilel S., “Visible Light-Induced Gelatin Methacrylate Hydrogels without Crosslinkers as a 3D Microenvironment for Glioblastoma Multiforme (GBM)” in preparation for submission.
- Erkoc, P.; Cingoz, A.; Onder, T. B.; Kizilel, S., Quinacrine Mediated Sensitization of Glioblastoma (GBM) Cells to TRAIL through MMP-Sensitive PEG Hydrogel Carriers. *Macromol Biosci* 2017, 17 (2). FEATURED as Frontispiece in this issue of *Macromolecular Bioscience*
- Cinay, G. E.; Erkoc, P.; Alipour, M.; Hashimoto, Y.; Sasaki, Y.; Akiyoshi, K.; Kizilel, S., Nanogel-Integrated pH-Responsive Composite Hydrogels for Controlled Drug Delivery. *ACS Biomaterials Science & Engineering* 2017, 3 (3), 370-380.
- Okur, A. C.; Erkoc, P.; Kizilel, S., Targeting cancer cells via tumor-homing peptide CREKA functional PEG nanoparticles. *Colloids and surfaces. B, Biointerfaces* 2016, 147, 191-200.
- Erkoc, P.; Cinay, G. E.; Kizilel, S., Recent Trends in Targeted Drug Delivery. Group, S., Ed. (S. Naskar): Dover, USA, 2015; pp.1-17.
- Guler, Z.; Erkoc, P.; Sarac, A. S., Electrochemical impedance spectroscopic study of single-stranded DNA-immobilized electroactive polypyrrole-coated electrospun poly(ϵ -caprolactone) nanofibers. *Materials Express* 2015, 5, 269-279(11).

Conferences:

- Erkoc P., Seker F., Bagci-Onder T., Kizilel S., “Visible Light-Induced Gelatin Methacrylate Hydrogels without Crosslinkers as a 3D Microenvironment for Glioblastoma Multiforme (GBM)”, BIOMED2017, Ankara, Turkey, 12/14-5/2017. Oral presentation
- Erkoc P., Okur A., Kizilel S., “Targeting Cancer Cells Via Tumor-Homing Peptide Creka Conjugated Peg Hydrogel Nanoparticles”, AIChE Annual Meeting, San Francisco, United States, 13/18-11/2016. Oral presentation
- Erkoc P., Aydin D., Kizilel S., “Water-in-Water Emulsion Based Synthesis of Hydrogel Nanospheres with Tunable Release Properties.”, San Francisco, United States, 13/18-11/2016. Oral presentation
- Erkoc P., Cingoz A., Bagci-Onder T., Kizilel S., “Quinacrine Mediated Sensitization of Glioblastoma (GBM) Cells to TRAIL through MMP-sensitive PEG Hydrogel Nanocarriers”, BMES Annual Meeting, Minneapolis, United States, 5/8-10/2016. Oral presentation
- Erkoc P., Cingoz A., Bagci-Onder T., Kizilel S., “Synthesis of Tumor-homing Peptide Functional PEG Hydrogel Nanocarriers for Targeting Cancer Cells”, Gordon Research Conference-Bioinspired Materials, Les Diablerets, Switzerland, 06/10-6/2016. Poster
- Erkoc P., Cingoz A., Bagci-Onder T., Kizilel S., “Delivery of Chemotherapeutic Drugs to Sensitize TRAIL Activity Against Glioblastoma Multiforme (GBM) Cells By Targeted MMP-Sensitive Peg-Hydrogel Microparticles”, AIChE Annual Meeting, Salt Lake City, UT, 11-11/2015. Poster

- Erkoc P., Cingoz A. Mohammadi M., Bagci-Onder T., Kizilel S."MMP-sensitive PEG hydrogel microspheres for targeted delivery of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) into Glioblastoma (GBM) Cells". Fourth International Symposium Frontiers in Polymer Science, Riva del Garda, ITALY, 20/22-6/2015. Poster



A. Appendix A

In **Figure A. 1.**, reaction of MMP-sensitive peptide ends with acr-PEG chains was confirmed with the formation of amide I-II peaks ($1690 - 1630 \text{ cm}^{-1}$) coming from peptide sequence and vanishing of -C=O ($1750 - 1735 \text{ cm}^{-1}$) stretching peaks of SVA. Functional groups of acrylate and PEG chains such as C=O stretching ($1740 - 1690 \text{ cm}^{-1}$), C=C stretching ($1680 - 1620 \text{ cm}^{-1}$), and peptide C-N stretching ($1080\text{-}1360 \text{ cm}^{-1}$) bands were also observed in the spectrum of the conjugate.

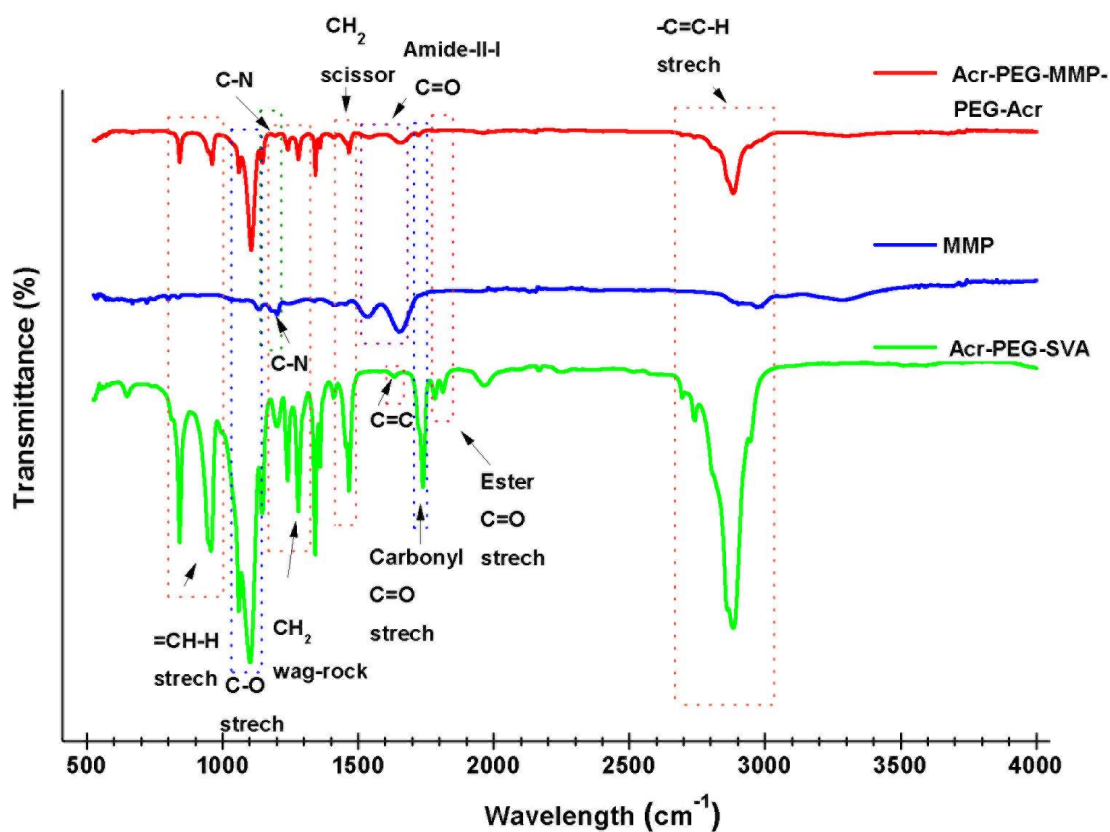


Figure A. 1. FTIR Spectra of MMP-sensitive peptide conjugation with acrylated PEG chains.

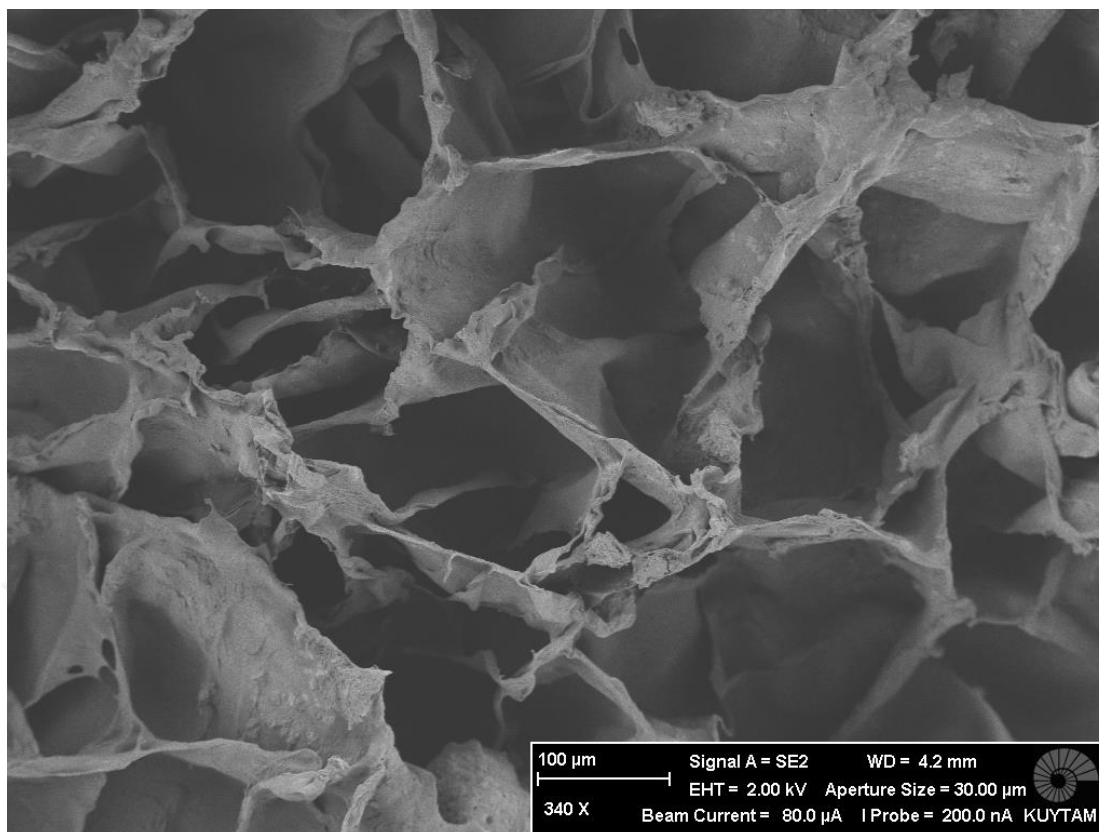


Figure A. 2. SEM image for MMP-sensitive millimeter-sized hydrogels. Image of the hydrogel captured with a 340 X magnification with scalebar of 100 μm.

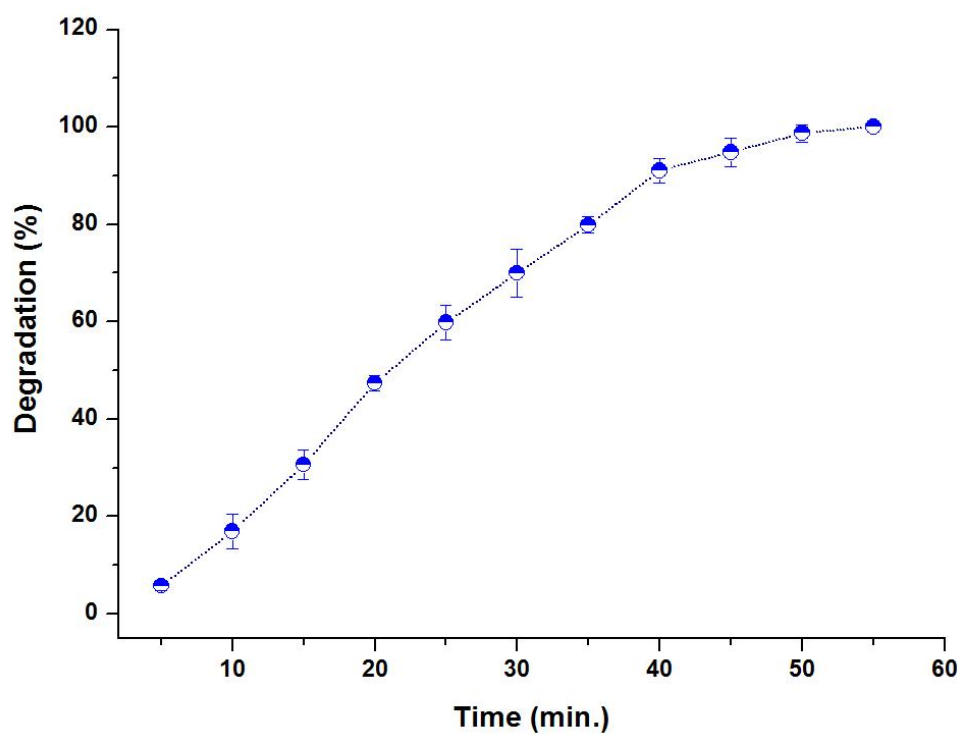


Figure A. 3. Degradation behavior of MMP-sensitive hydrogels. Graph shows the increasing mass loss of hydrogels in 1 $\mu\text{g}/\text{ml}$ collagenase type-I solution for 1h at 37 $^{\circ}\text{C}$.

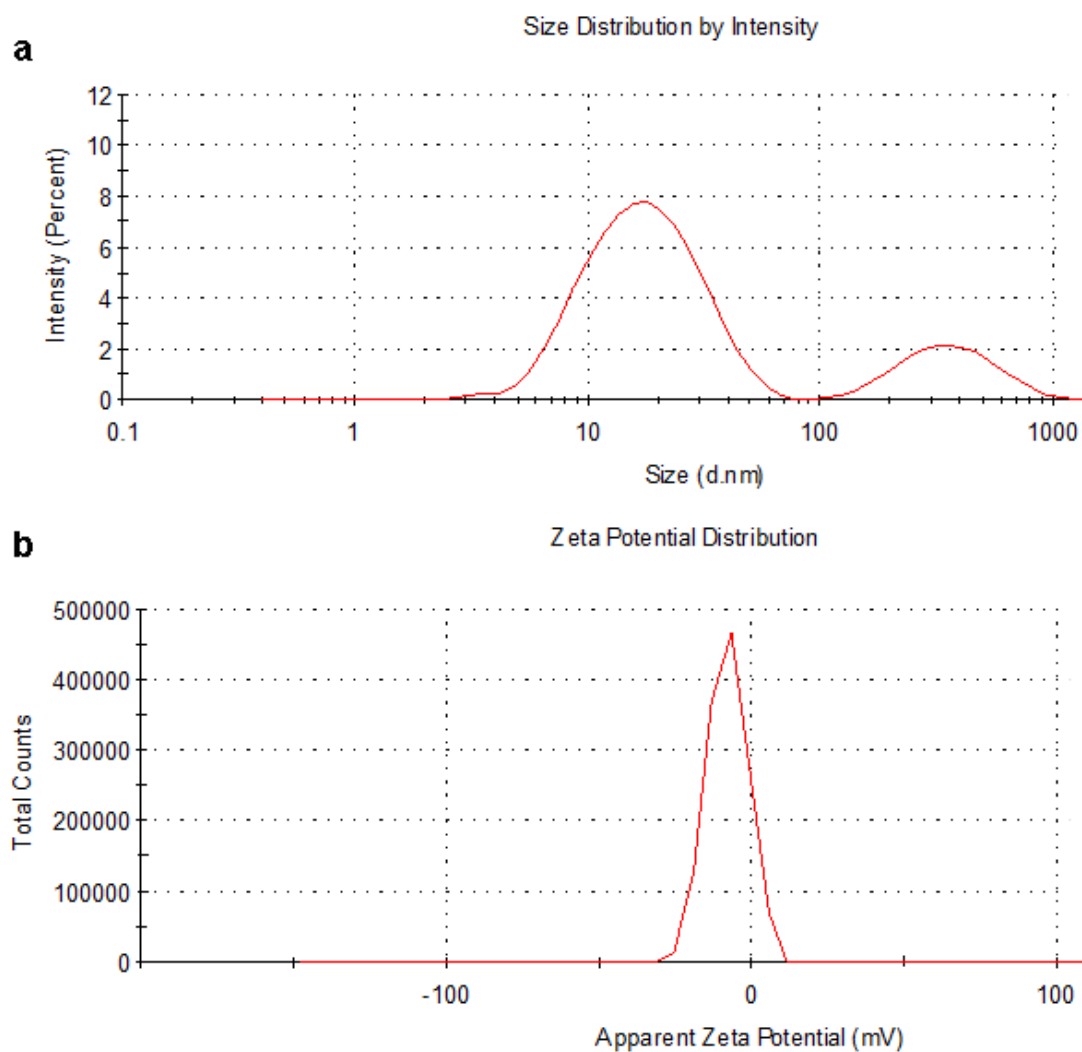


Figure A. 4. Size and zeta potential distribution of RGDS incorporated degradable PEG carriers measured by Zetasizer instrument.

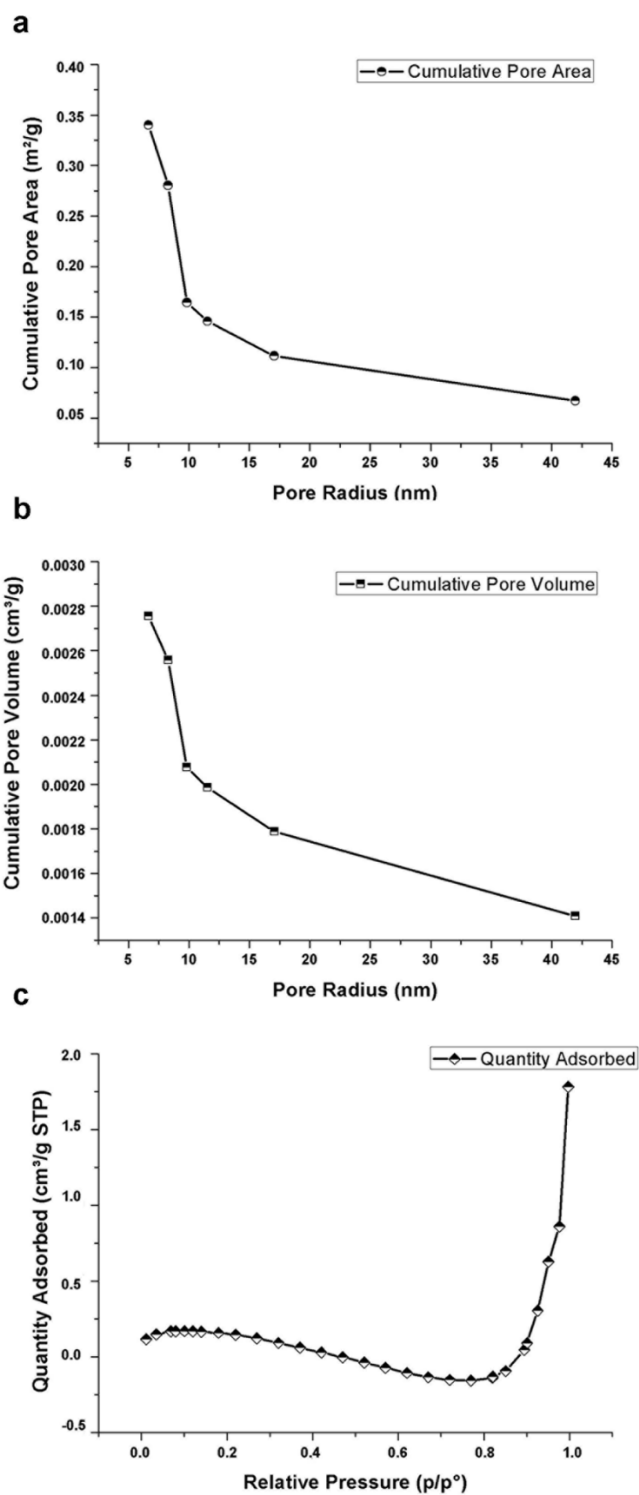


Figure A. 5. BET analysis of hydrogel particles. Cumulative pore area (a) and cumulative pore volume (b) obtained from adsorption isotherms, and quantity of adsorbed N_2 (c). The samples were degassed at 50°C for 5 h under vacuum prior to analysis.

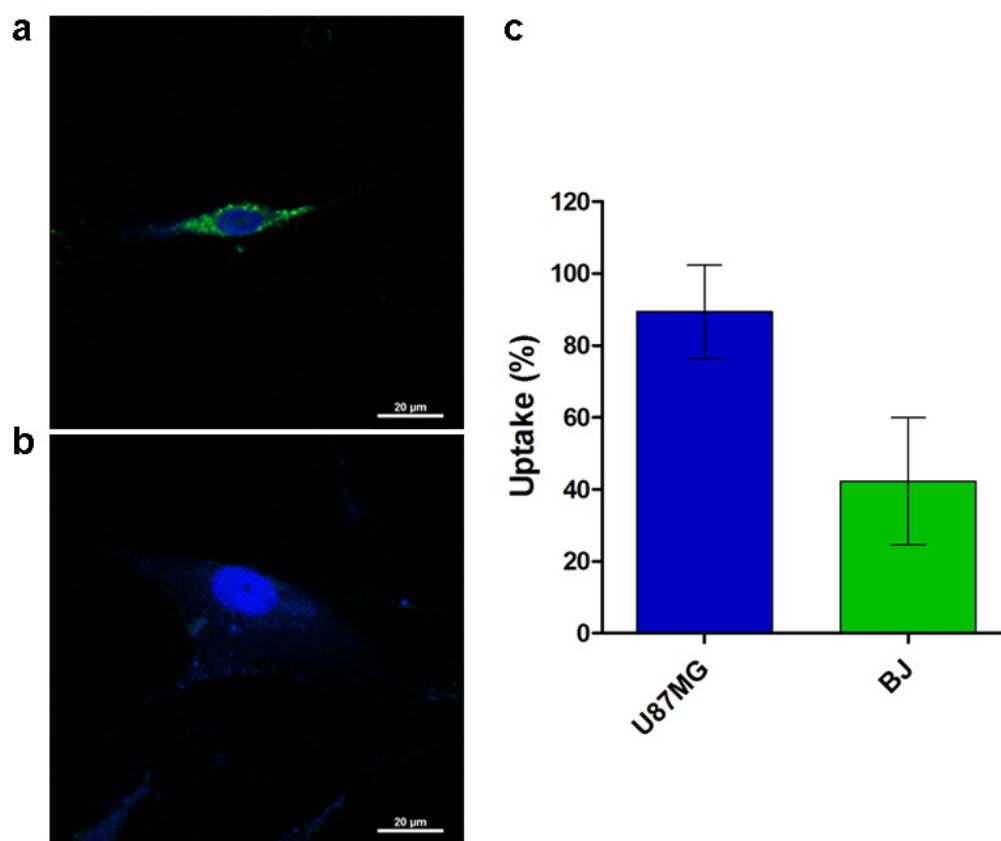


Figure A. 6. Laser Scanning Confocal Microscopy images of fixed U87MG cells (a) and BJ fibroblasts (b) after 1 h incubation with fluorescein o-acrylate labeled degradable RGDS containing carriers. Cellular uptake of carriers was quantified from 20 different images

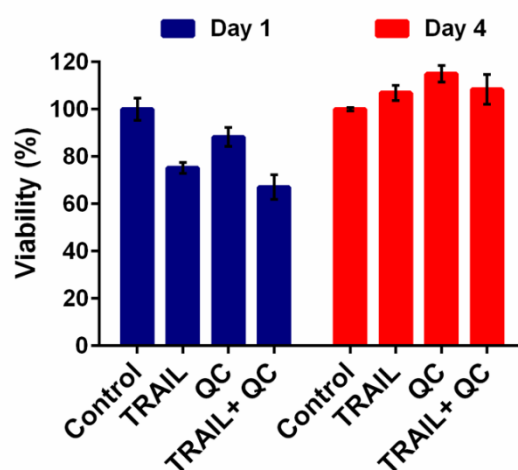


Figure A. 7. Effects of TRAIL and QC on Human Dermal Fibroblast (BJ) Cells. Fibroblasts were treated with 1 μM QC or/and 40 ng/ml TRAIL and viability measured 1 and 4 days after treatment using Cell Titer Glo Assay.

B. Appendix B

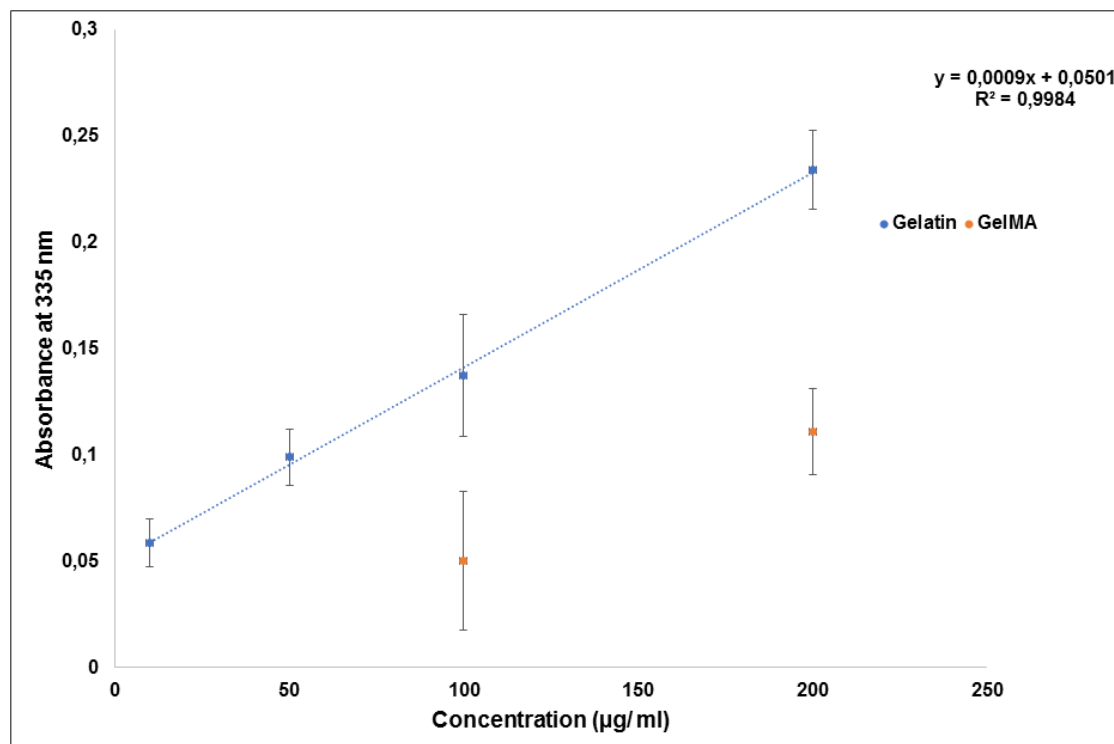


Figure B. 1. TNBSA Assay Calibration Curve

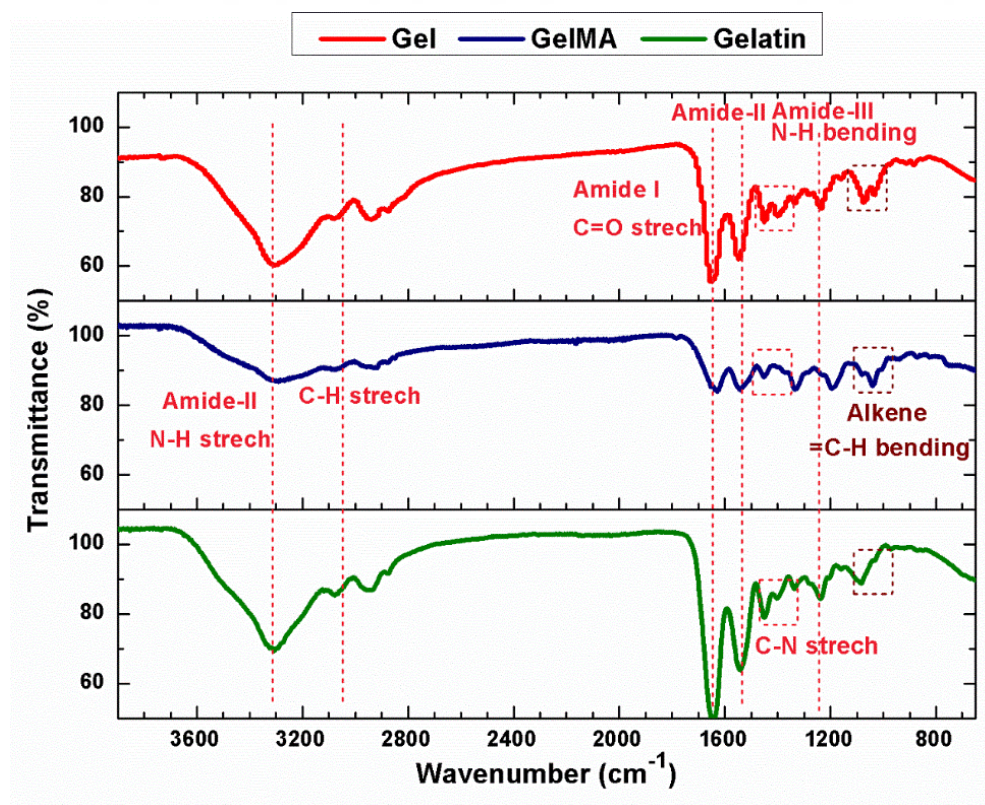


Figure B. 2. FTIR Spectra of Gelatin, Methacrylated Gelatin and synthesized Hydrogel

Nicolet iS10 model spectrometer (Thermo Scientific) with an attenuated total reflection (ATR) cell were used to collect IR spectra. Each IR spectrum was collected in air at room temperature.

FT-IR spectroscopy confirmed the methacrylation of gelatin (**Figure B.2.**). The characteristic peaks of gelatin spectrum were observed for hydrogels. The peaks observed at 3080 cm^{-1} and 3300 cm^{-1} were belong to C-H stretch and N-H stretch of amide II, at 1640 cm^{-1} , 1540 cm^{-1} and 1230 cm^{-1} resulted from N-H bending of amide I, amide II and amide III bonds. The peak at 1020 cm^{-1} in fingerprint region which was belong to the =C-H bending, was observable in the spectra of GelMA and hydrogel samples but not in gelatin.

In the FTIR spectra, the typical peak for C=C bond around is 1640 cm^{-1} , which are too close to the amide I stretching peaks. Therefore, we were not able to denote stretch of C=C [159-161].

C. Appendix C

Therapeutic molecules used in this thesis study were listed in **Table C.1**.

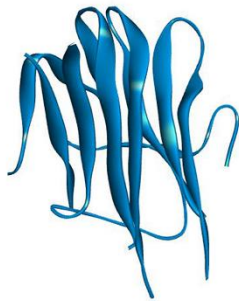
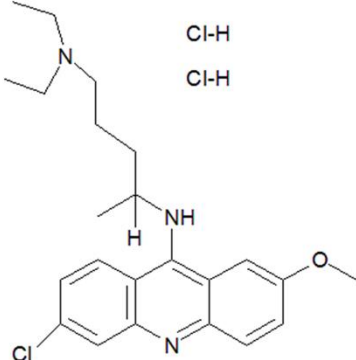
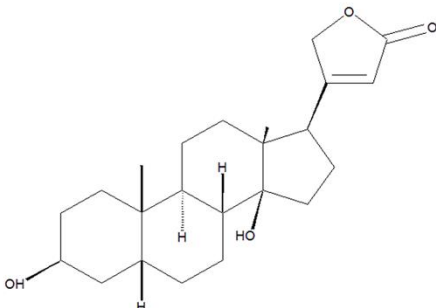
Ribbon diagram of human TRAIL protein monomer was drawn from crystal structure of 160 residues (between 121-281aa) of the protein named with “1D2Q” pdb id in RCSB databank [162].

In this study, soluble human SuperKillerTRAIL™ (Enzo, ~26kDa) was used. The residues between 95-281aa of this protein is fused to a His-tag and Killer™ linker peptide to be produced in *E.coli*. The Killer™ linker peptide improves the stability of oligomer via inserted mutation that allows the formation additional of disulfide bonds [163].

Quinacrine, Atabrine ® or with IUPAC name 4-N-(6-chloro-2-methoxyacridin-9-yl)-1-N,1-N-diethylpentane-1,4-diamine) is a heterocyclic compound consists of three rings with 508.92 Da molecular weight which is used in treatment of parasitic diseases like malaria, giardiasis and tapeworm infection [85].

Digitoxigenin is a small molecule drug with 374.53 Da molecular weight, used in the treatment of heart diseases like cardiac failure. As other members of cardiac glycosides, Digitoxigenin acts on the contraction of heart muscles via inhibition of Na⁺/K⁺ ATPase membrane pumps, and subsequent increase in intracellular Ca⁺⁺ ions [164]. Digitoxigenin is the aglycone of digitoxin, which is obtained by the change of glycosyl group of digitoxin with an hydrogen atom in the glycoside structure and digitoxin is extracted from a plant, purple foxglove [165].

Table C. 1. Overview of Therapeutic Molecules

<i>Name of Therapeutic</i>	<i>Therapeutic class</i>	<i>Structure</i>
TRAIL	Cytokine from Tumor necrosis factor (TNF) family proteins	
Quinacrine Dihydrochloride (QC)	Antihelmintic	
Digitoxigenin (Dgx)	Cardiac glycoside	

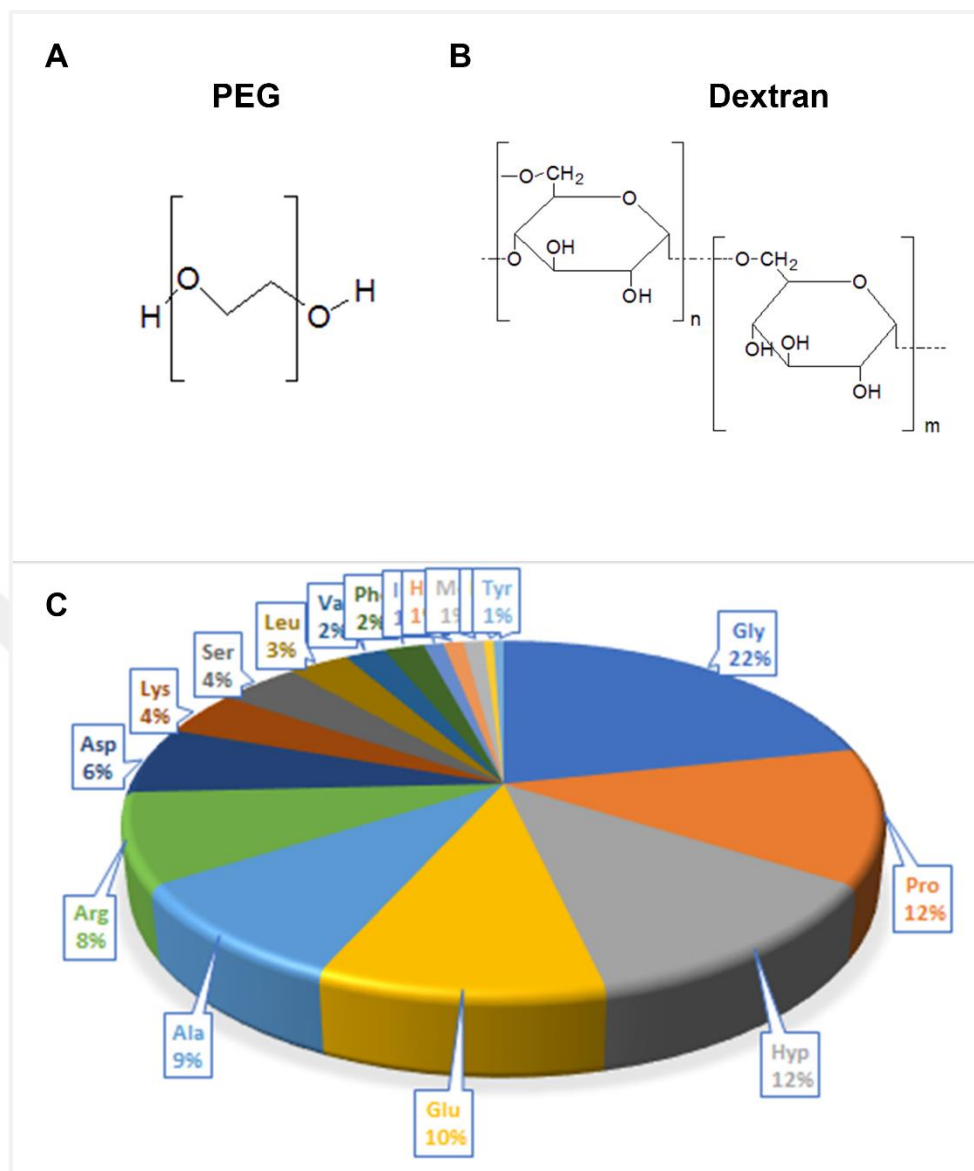


Figure C. 1. Chemical Structure of A) PEG, B) Dextran, and distribution of abundant aminoacids in gelatin structure

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