

Development of Chitosan-Based Delivery Systems for Biomedical Applications

by

Nihal Olcay Doğan

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

Master of Science

In

Chemical and Biological Engineering



October 2019

Koç University
Graduate School of Sciences and Engineering

This is to certify that I have examined this copy of a master's thesis by

Nihal Olcay Doğan

and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
examining committee have been made.

Committee Members:

Assoc. Prof. Seda Kızılel (Advisor)

Assoc. Prof. Tuğba Bağcı Önder (Co-Advisor)

Prof. İhsan Solaroğlu

Asst. Prof. Erkan Şenses

Asst. Prof. Duygu Ekinici

Date: 24.10.2019



To my father,

ABSTRACT

Chitosan-based delivery systems have gained growing interest due to unique physicochemical properties of chitosan such as biocompatibility, biodegradability, low toxicity, low immunogenicity and cationic nature. Hence, these desirable characteristics of chitosan make it an advantageous biomaterial in wide range of biomedical applications including tissue engineering, wound healing, gene and drug delivery systems. This thesis investigates chitosan as nanoparticle-based gene delivery vehicles and potential drug carriers in biohybrid microrobotic design.

In the first part of this thesis, the impacts of three independent variables; PEG chain length, PEG concentration and nanoparticle concentration on transfection capabilities of plasmid DNA loaded and PEGylated chitosan nanoparticles were systematically investigated. First, various PEGylated chitosan polymers were synthesized using different PEG chain lengths (2, 5 and 10 kDa) and PEG concentrations (4, 8, 12, 15 and 20 μ moles of PEG per 25 mg of chitosan). Using altered PEGylated chitosan derivatives, tumor necrosis factor (TNF) α -related apoptosis-inducing ligand (TRAIL) inserted plasmid DNA and crosslinking agent, nanoparticles were synthesized. Synthesized nanoparticles were characterized with dynamic light scattering and scanning electron microscopy. Synthesized nanoparticles were incubated with HEK293-T cells and transfection efficiencies of nanoparticles were experimentally investigated either measuring green fluorescent protein expression or TRAIL protein quantification. With this experimental dataset, we developed an artificial neural network model using (i) PEG chain length, (ii) PEG concentration and (iii) nanoparticle concentration as input variables and green fluorescent expression as output variable. With the help of this computational model, transfection capabilities of nanoparticles with respect to different variables were systematically analyzed with less experimental effort. Overall, this chapter investigates the effect of PEG chain length, PEG concentration and nanoparticle concentration on transfection abilities of nanoparticles and uses artificial neural network as a computational tool to investigate the effect of each parameter on gene delivery studies.

In the second part of this thesis, we reported a novel biohybrid algal microswimmer design in which positively charged chitosan polyelectrolytes and superparamagnetic chitosan coated iron oxide nanoparticles attached to negatively charged membrane of *C.reinhardtii*. Most of the biohybrid designs in literature have limited coating yields, less than 10%. Herein, the novel biohybrid design that we reported has high coating yield which is more than 90%. In this chapter, first magnetic nanoparticles were attached to the membrane of microalgae only in presence of chitosan polyelectrolytes. Experimental conditions for cargo attachment were optimized without compromising the natural motility of *C.reinhardtii*. Attachment of cargos to the surface of *C.reinhardtii* were proved using fluorescence microscopy, scanning electron microscopy, energy-dispersive X-ray spectroscopy. Furthermore, microalgae retained its natural motility and light responsive behaviour with this novel design and this behaviour of biohybrid microswimmer could be used to actuate them in a controlled manner. Overall, this chapter reports a novel biohybrid algal microswimmer design which is promising for controlled drug delivery applications.

ÖZET

Kitosan bazlı dağıtım sistemleri, kitosanın biyouyumluluk, biyobozunurluk, düşük toksisite, düşük immünojeniklik ve katyonik yapısı gibi benzersiz fizikokimyasal özellikleri nedeniyle artan bir ilgi kazanmıştır. Kitosanın bu arzu edilen özellikleri onu doku mühendisliği, yara tedavisi, gen ve ilaç dağıtım sistemleri de dahil olmak üzere çeşitli biyomedikal uygulamalarda avantajlı bir biyomalzeme haline getirmiştir. Bu yüksek lisans tezi, kitosanı nanoparçacık bazlı gen dağıtım sistemi ve biyo-hibrid mikrorobotik tasarımda potansiyel ilaç taşıyıcısı olarak incelenmektedir.

Bu tezin ilk bölümünde, PEG zincir uzunluğu, PEG konsantrasyonu ve nanoparçacık konsantrasyonu olmak üzere üç bağımsız değişkenin plazmid DNA yüklü ve PEGile kitosan nanoparçacıkların transfeksiyon verimi üzerine olan etkileri sistematik olarak incelenmiştir. İlk olarak, farklı PEG zincir uzunlukları (2, 5 ve 10 kDa) ve PEG konsantrasyonları (25 mg kitosan başına 4, 8, 12, 15 ve 20 μ mol PEG) kullanılarak çeşitli PEGile chitosan polimerleri sentezlenmiştir. Farklı PEGile chitosan türevleri, tümör nekroz faktörü (TNF) α -aracılı apoptoz-indükleyen ligand (TRAIL) yerleştirilmiş plazmid DNA ve çapraz bağlama maddesi kullanılarak nanoparçacıklar sentezlenmiştir. Sentezlenen nanoparçacıklar, dinamik ışık saçılımı ve taramalı elektron mikroskobu ile karakterize edilmiştir. Sentezlenen nanoparçacıklar HEK293-T hücreleri ile inkübe edildikten sonra ya yeşil floresan protein ekspresyonu ya da TRAIL protein miktarı ölçülerek deneysel olarak araştırılmıştır. Bu deneysel veri setinde; (i) PEG zincir uzunluğu, (ii) PEG konsantrasyonu ve (iii) nanoparçacık konsantrasyonu giriş değişkenleri, yeşil floresan protein ekspresyonu ise çıkış değişkeni olarak kullanmış ve yapay bir sinir ağı modeli geliştirilmiştir. Bu hesaplama modeli yardımıyla, nanoparçacıkların farklı değişkenlere göre transfeksiyon verimleri daha az deneysel çaba ile sistematik olarak analiz edilmiştir. Genel olarak bu bölüm, PEG zincir uzunluğu, PEG konsantrasyonu ve nanoparçacık konsantrasyonunun nanoparçacıkların transfeksiyon verimi üzerindeki etkisini araştırmakta ve her bir parametrenin gen dağıtım çalışmaları

üzerindeki etkisini analiz etmek için bir hesaplama aracı olarak yapay sinir ağlarını kullanmaktadır.

Bu tezin ikinci bölümünde, *C.reinhardtii*'nin negatif yüklü membranına bağlanan, pozitif yüklü kitosan polielektrolitleri ve süper-paramanyetik kitosan kaplı demir oksit nanoparçacıklarını (SPIONP'ler) içeren yeni bir biyo-hibrid alg mikro yüzücü tasarımı rapor edilmiştir. Literatürdeki biyo-hibrid tasarımların çoğu, %10'dan daha az sınırlı kaplama verimliliğine sahiptir. Burada bildirdiğimiz yeni biyo-hibrid tasarımı ise %90'dan fazla kaplama verimine sahiptir. Bu bölümde, ilk önce manyetik nanoparçacıklar mikroalg zarına sadece kitosan polielektrolitleri varlığında bağlanabilmiştir. Kargo bağlantısı için gerekli olan deneysel koşullar, *C.reinhardtii*'nin doğal hareketliliğinden ödün vermeyecek şekilde optimize edilmiştir. Kargoların *C.reinhardtii* yüzeyine bağlanması floresan mikroskopi, taramalı elektron mikroskobu, enerji dağıtıcı X-ışını spektroskopisi kullanılarak kanıtlanmıştır. Ek olarak, kaplanmış mikro algler, bu yeni tasarım ile doğal hareketliliğini ve ışığa duyarlı davranışını korumuştur ve mikroalglerin bu davranışları onları kontrollü bir şekilde harekete geçirmek için kullanılabilir. Genel olarak bu bölüm, kontrollü ilaç teslimi uygulamaları için büyük bir umut vaat eden yeni bir biyo-hibrid alg mikro yüzücü tasarımını rapor etmektedir.

ACKNOWLEDGEMENTS

First, I would like to express my gratitude to my advisor, Assoc. Prof. Seda Kızılel for guiding, supporting and motivating me. It was a great pleasure for me to work with such a great scientist in this field. I have always admired her enthusiasm, ambition, immense knowledge and time management. I will take her work discipline as an example throughout my academic life.

I would like to thank my co-advisor, Assoc. Prof. Tuğba Bağcı-Önder for her helpful advices, suggestions, guidance and great knowledge. I also would like to thank her for allowing me to access her laboratories during my research.

I would like to express my special thanks to Prof. Metin Sitti for his sincere guidance on our project and for allowing me to access his laboratories.

In addition to my advisors, I would like to thank especially to the rest of my thesis committee members; Asst. Prof. Erkan Şenses, Prof. İhsan Solaroğlu and Asst. Prof. Duygu Ekinci, for their attention, insightful suggestions and precious time that they spent to evaluate my thesis.

I would like to thank to Uğur Bozüyük, Pelin Erkoç, Ahmet Cingöz and Fidan Şeker for their great help to my studies.

I would like to thank all group members Yasemin İnCEOğlu, Merve Yılmam, Burcu Beyaz, Rubab Batool and Muhammad Anwaar Nazeer. I would like to thank Yasemin İnCEOğlu and Burcu Beyaz for their wonderful friendship outside the lab. I am especially grateful to Merve Yılmam for being so kind and sweet to me. Thank you for your endless support whenever I needed. I will miss you so much. I would like to thank my best friend Harun Kulak for unforgettable memories that we shared since we met. I am so lucky to have such a wonderful friend who always cared, motivated and made me happy. Special thanks to Eren Kuşhan for all enjoyable times that we spent together.

To my wonderful family, my mother Zeynep Dođan, my dear sisters Elif Arzu Dođan and Zuhall Dođan, I appreciate you for your endless love, unconditional support and encouragements throughout my whole life. I love you with all my heart.

Most of all, I would like to dedicate this master thesis to my father who is with me forever. I know that you would be so proud of me, if you were in here.

Nihal Olcay Dođan

October 2019



TABLE OF CONTENTS

ABSTRACT	iv
ÖZET	vi
ACKNOWLEDGEMENTS.....	viii
LIST OF FIGURES.....	xiii
LIST OF TABLES.....	xvi
NOMENCLATURE	xvii
CHAPTER 1: INTRODUCTION	1
CHAPTER 2: LITERATURE REVIEW.....	3
2.1. Chitosan	3
2.2. Biomedical Applications of Chitosan.....	3
2.2.1. Tissue engineering	3
2.2.2. Wound healing.....	4
2.2.3. Gene and drug delivery systems	4
2.3. Biohybrid Microrobots for Drug Delivery Applications.....	8
CHAPTER 3: BIOADHESIVE PEGYLATED CHITOSAN NANOPARTICLES FOR TRAIL GENE DELIVERY.....	9
3.1. Introduction.....	9
3.2. Experimental	12
3.2.1. Materials.....	12
3.2.2. Cloning: Insertion of TRAIL gene into mammalian expression vector	13
3.2.3. Preparation of PEGylated chitosan polymer	14
3.2.4. Synthesis of TRAIL inserted pDNA encapsulated and PEGylated chitosan nanoparticles (CS-PEG-TRAIL pDNA NPs)	15
3.2.5. Encapsulation efficiency of CS-PEG-TRAIL pDNA NPs.....	15
3.2.6. Scanning electron microscopy (SEM) images and dynamic light scattering (DLS) measurements of CS-PEG-TRAIL pDNA NPs.....	15
3.2.7. Transfection of TRAIL pDNA using FuGENE transfection reagent	16

3.2.8. Cell viability	16
3.2.9. Transfection of CS-PEG-TRAIL pDNA NPs and measurement of TRAIL protein in cell culture media through enzyme-linked immunosorbent assay (ELISA)	17
3.2.10. Flow cytometry analysis	17
3.2.11. Neural network training	17
3.3. Results and Discussion	18
3.3.1. Selective toxicity of commercial TRAIL	18
3.3.2. Cloning: Insertion of TRAIL gene into mammalian expression vector	20
3.3.3. Preparation of PEGylated chitosan polymer	22
3.3.4. Encapsulation efficiency of CS-PEG-TRAIL pDNA NPs.....	23
3.3.5. Characterization of CS-PEG-TRAIL pDNA NPs	23
3.3.6. Transfection efficiencies of CS-PEG-TRAIL pDNA NPs.....	26
3.3.7. Effect of protamine sulfate on transfection efficiency.....	28
3.3.8. Cell viability experiments	29
3.3.9. Flow cytometry analysis	32
3.3.10. Characterization of TRAIL protein through ELISA.....	33
3.3.11. Incubation of cancer cells with medium collected from HEK293-T cells..	35
3.3.12. Neural network training	36
3.4. Conclusions and Future Outlook.....	40
CHAPTER 4: A NOVEL METHOD FOR FABRICATION OF BIOHYBRID ALGAL MICROSWIMMERS	43
4.1 Introduction.....	43
4.2. Experimental	45
4.2.1. Materials.....	45
4.2.2. Culture of <i>Chlamydomonas reinhardtii</i>	45
4.2.3. Fabrication of coated microalga-powered biohybrid microswimmers	45
4.2.4. Construction of the microfluidic channels	46
4.2.5. Scanning electron microscopy (SEM) imaging.....	46

4.2.6. Energy-dispersive X-ray spectroscopy	46
4.2.7. Fluorescence emission analysis	47
4.2.8. Swimming speed analysis	47
4.2.9. Light and magnetic field response	47
4.3. Results and Discussion	47
4.3.1. Growth curve of <i>C.reinhardtii</i>	48
4.3.2. Coating efficiency of <i>C.reinhardtii</i>	49
4.3.3. Fluorescence microscopy images of biohybrid algal microswimmers	50
4.3.4. SEM imaging of biohybrid algal microswimmers.....	51
4.3.5. EDX analysis	52
4.3.6. Fluorescence emission of biohybrid algal microswimmers	53
4.3.7. Swimming speed analysis	54
4.3.8. Light response of biohybrid algal microswimmers	56
4.3.9. Stability of microalgae coating.....	57
4.4. Conclusions and Future Outlook.....	59
CHAPTER 5: CONCLUSIONS.....	61
BIBLIOGRAPHY.....	62
APPENDIX A: Supplementary Information for Chapter 3	A-1
APPENDIX B: Supplementary Information for Chapter 4.....	B-1

LIST OF FIGURES

Figure 2. 1. Deacetylation reaction of chitin.	3
Figure 2. 2. Gene delivery through positively charged non-viral vectors.	5
Figure 2. 3. Representation of EPR effect, where certain sized molecules (nanoparticles, drugs and liposomes) have ability to accumulate in tumor tissues rather than normal tissues.....	6
Figure 2. 4. Key steps of PEGylated chitosan nanoparticle synthesis.	7
Figure 3. 1. Key steps of the study.	12
Figure 3. 2. Sequence and map of pIRES2-AcGFP1 mammalian expression vector. ..	14
Figure 3. 3. Selective toxicity of commercial TRAIL on HEK293-T and A-498 cells which were used as representative of normal and cancer cell lines, respectively. Microscopy images of cells were taken after overnight incubation with 200 ng/mL commercial TRAIL.....	19
Figure 3. 4. Viability of HEK293-T and A-498 cells after overnight incubation with 200 ng/mL commercial TRAIL.	20
Figure 3. 5. Transfection of HEK293-T cells with engineered pDNA using FuGENE transfection reagent. A) GFP expression of HEK293-T cells at t=0. B) GFP expression of HEK293-T cells after 24 h. C) GFP expression of HEK293-T cells after 48 h.....	21
Figure 3. 6. Microscopy images of HEK293-T and U87MG cells after incubation with fresh and TRAIL containing medium for 24 h.....	21
Figure 3. 7. Viability of HEK293-T and U87MG cells after incubation with TRAIL containing medium for 24 h.	22
Figure 3. 8. Synthesis of PEGylated chitosan.	22
Figure 3. 9. Characterization of 2, 5 and 10 kDa-20 μ mole PEG NPs. A) Size distributions of representative groups at pH 6.5. B) Zeta potential distributions of representative groups at pH 6.5. C) SEM images of representative groups.	26
Figure 3. 10. GFP expressions of HEK293-T and U87MG cells after transfecting with FuGENE-TRAIL pDNA complex or CS-PEG-TRAIL pDNA NPs.	27
Figure 3. 11. Condensation effect of protamine sulfate in gene delivery.	28

Figure 3. 12. GFP expression of HEK293-T and U87MG cells, where transfection was performed under different conditions.	29
Figure 3. 13. HEK293-T cells incubated with different doses of NPs for 24 h. A) Viability of HEK293-T cells with 2 kDa groups. B) Viability of HEK293-T cells with 5 kDa groups. C) Viability of HEK293-T cells with 10 kDa groups.	30
Figure 3. 14. Viability of HEK293-T cells after TRAIL protein was produced and secreted into cell culture medium. A) Viability of HEK293-T cells with 2 kDa groups. B) Viability of HEK293-T cells with 5 kDa groups. C) Viability of HEK293-T cells with 10 kDa groups.	31
Figure 3. 15. Correlation between GFP expression and TRAIL concentration.	34
Figure 3. 16. Schematic representation of the study.....	35
Figure 3. 17. Viability of HEK293-T and U87MG cells after incubation with medium collected from HEK293-T cells which transfected with CS-PEG-TRAIL pDNA NPs. 35	
Figure 3. 18. Correlation between predicted and observed GFP expression.	36
Figure 3. 19. Leave-p-out cross validation for p=3, 4, 5 and 6.	37
Figure 3. 20. Three dimensional plots of GFP expressing HEK293-T cells with respect to different variables. The graphs were obtained from ANN model. A) GFP expression of HEK293-T cells at constant PEG chain lengths. B) GFP expression of HEK293-T cells at constant PEG concentrations.	39
Figure 3. 21. The impact of PEG concentration on transfection efficiency of NPs.	40
Figure 4. 1. Fabrication of biohybrid algal microswimmers.....	48
Figure 4. 2. Growth curve of <i>C. reinhardtii</i> . All coating experiments were performed during the exponential growth stage of <i>C.reinhardtii</i> , i.e. at 100 h of the growth curve.	48
Figure 4. 3. Fluorescence microscopy images of biohybrid algal microswimmers. A) Microalgae coated with chitosan polymer. B) Microalgae coated with SPIONPs. C) Microalgae coated with chitosan-SPIONPs mixture. In all figures, green color represents chitosan polyelectrolytes, and red color represents SPIONPs.	50
Figure 4. 4. Fluorescence microscopy images of bare microalgae and biohybrid algal microswimmers. Green color represents chitosan polyelectrolytes, and red color represents SPIONPs.....	51

Figure 4. 5. SEM images of <i>C.reinhardtii</i> . A) Bare microalgae. B) Microalgae coated with chitosan polyelectrolytes. C) Microalgae coated with SPIONPs. D) Microalgae coated with chitosan-SPIONPs mixture.	52
Figure 4. 6. A) Emission of TAP medium, bare microalgae and biohybrid algal microswimmers at 528 nm (Excited at 485 nm). B) Emission of TAP medium, bare microalgae and biohybrid algal microswimmers at 680 nm (Excited at 540 nm).	54
Figure 4. 7. Swimming speed analysis of <i>C.reinhardtii</i> . A) Average speeds of bare microalgae and biohybrid algal microswimmers. B) Swimming trajectories of bare microalgae and biohybrid algal microswimmers.	55
Figure 4. 8. Light response of bare microalgae and biohybrid algal microswimmers. Light was applied on two different ends of the microchannel separately. Images were taken at 10 min illumination.	56
Figure 4. 9. Accumulation of bare microalgae and biohybrid algal microswimmers to the illuminated end of the channel.	57
Figure 4. 10. Fluorescence microscopy images of coated microalgae at different time intervals. Green color represents chitosan polymer, and red color represents SPIONPs.	58

LIST OF TABLES

Table 3. 1. Ingredients of FuGENE and TRAIL inserted pDNA mixtures.....	16
Table 3. 2. Encapsulation efficiencies of TRAIL inserted pDNA for altered NPs.	23
Table 3. 3. Experimental size and zeta potential values of NPs.....	24
Table 3. 4. GFP expression of cells transfected with altered CS-PEG-TRAIL pDNA NPs.	32
Table 3. 5. Characterization of TRAIL protein though ELISA.....	34
Table 3. 6. Pearson correlation coefficient, mean square and normalized mean square errors for leave-p-out cross validation technique.	37
Table 4. 1. EDX measurements demonstrating presence of iron atoms on the surface of biohybrid algal microswimmers.	53

NOMENCLATURE

PEG	Polyethylene glycol
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor α -related apoptosis-inducing ligand
DNA	Deoxyribonucleic acid
pDNA	Plasmid DNA
TPP	Polyanion tripolyphosphate
ANN	Artificial neural networks
NaOH	Sodium hydroxide
siRNA	Small interfering ribonucleic acid
mRNA	Messenger ribonucleic acid
EPR	Enhanced permeability and retention
NHS	N-hydroxysuccinimide
NPs	Nanoparticles
GFP	Green fluorescent protein
DMSO	Dimethyl sulfoxide
mPEG-SVA	Methoxypoly(ethylene glycol) succinimidyl valerate
HCl	Hydrochloric acid
DMEM	Dulbecco's modified Eagle medium
DPBS	Dulbecco' phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
CMV	Cytomegalovirus
PCR	Polymerase chain reaction
CS-PEG NPs	PEGylated chitosan NPs
CS-PEG-TRAIL pDNA NPs	PEGylated and TRAIL inserted pDNA encapsulated chitosan NPs
SEM	Scanning electron microscopy

DLS	Dynamic light scattering
PS	Protamine sulfate
ELISA	Enzyme-linked immunosorbent assay
MSE	Mean squared error
NMSE	Normalized mean squared error
R	Pearson correlation coefficient
R ²	Correlation coefficient
PEI	Polyethylenimine
<i>C. reinhardtii</i>	<i>Chlamydomonas reinhardtii</i>
PAH	Poly(allylamine hydrochloride)
PSS	Poly(sodium 4-styrenesulfonate)
SPIONPs	Superparamagnetic chitosan coated iron oxide nanoparticles
OD	Optical density
EDX	Energy-dispersive X-ray spectroscopy
DIC	Differential interference contrast
FITC	Fluorescein
TRITC	Tetramethylrhodamine
DOX	Doxorubicin

CHAPTER 1: INTRODUCTION

Biomaterials has been used in several different applications. Gene/drug delivery systems are the most popular areas that use biomaterials to treat some certain diseases. To date, several biomaterials have been used in different designs such as hydrogels, microspheres, micelles and nanoparticles. Among them, chitosan is one of the most suitable candidate for gene/drug delivery vehicles due to its unique properties such as biocompatibility¹, biodegradability², low toxicity³ and low immunogenicity⁴.

Currently, nanoparticle-based gene/drug delivery systems have gained enormous attention due to their excellent properties such as prolonged blood circulation half-life, increased cell membrane penetrability, high loading capacity and tendency to accumulate in tumor tissues rather than normal tissues.^{5, 6} On the other hand, biohybrid microswimmers in which living organisms are integrated with artificial structures, have gained growing interest to deliver cargos into specific tissues.^{7, 8} Chitosan is popular biomaterial for both of the delivery systems due to its attractive features.

In this thesis, we used chitosan in both nanoparticle-based gene/drug delivery systems and biohybrid microswimmers. In Chapter 2, chitosan and its biomedical applications were introduced. Chapter 3 is about the application of chitosan in nanoparticle-based gene delivery systems. Here, chitosan is conjugated with altered chain lengths and concentrations of polyethylene glycol (PEG) before the synthesis of nanoparticles to improve the poor solubility and poor stability of chitosan at physiological conditions. Next, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) inserted plasmid DNA (pDNA) encapsulated and PEGylated chitosan nanoparticles were synthesized by using tripolyphosphate (TPP) as a crosslinker. Furthermore, the effects of PEG chain length, PEG concentration and nanoparticle concentration on transfection capabilities of altered nanoparticles were investigated in detail. An artificial neural network (ANN) was also developed using experimental dataset

to analyze the impact of each parameter in a detailed way. In Chapter 4, the application of chitosan in biohybrid microswimmers was illustrated. A novel biohybrid algal microswimmer were designed in which *C.reinhardtii* were coated with chitosan polyelectrolytes and superparamagnetic chitosan coated iron oxide nanoparticles (SPIONPs). This novel design has excellent properties such as high coating yield by retaining the motility and functionality of microalgae which is commonly absent in other biohybrid microswimmer designs. Besides, this novel biohybrid design is very promising to be used in controlled drug release applications due to the advances in easiness to modify chemistry of chitosan.

CHAPTER 2: LITERATURE REVIEW

2.1. Chitosan

Chitin is the second most abundant polymer in worldwide found in cell walls of yeast or fungi and skeleton components of shrimps and crabs.⁹⁻¹¹ Chitosan is one of the most significant derivatives of chitin which is commonly extracted from shrimp shells due to the simplicity of process.^{11, 12} Chitosan could be obtained by partial deacetylation or enzymatic hydrolysis reactions.¹¹ In deacetylation reaction, acetyl groups of chitin are modified to amino groups in chitosan with the addition of sodium hydroxide (NaOH) at high temperatures. The new polymer in which the degree of deacetylation is obtained more than 50% is called as chitosan (Figure 2.1).¹⁰

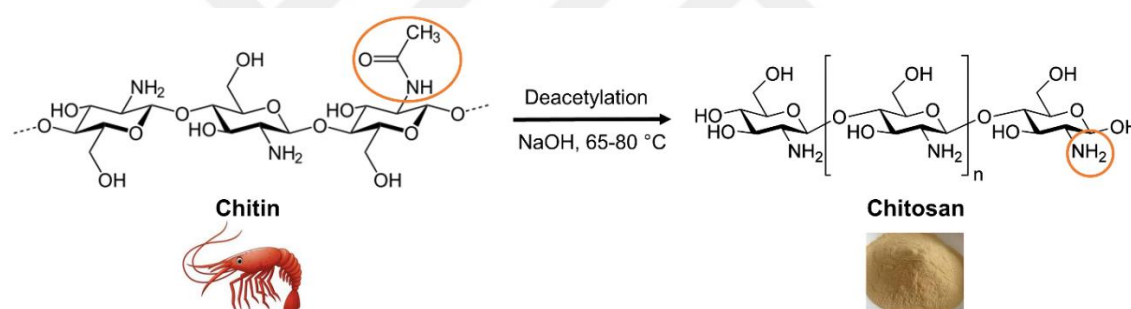


Figure 2. 1. Deacetylation reaction of chitin.

Chitosan has unique physicochemical properties including bioadhesivity¹³, biocompatibility¹, biodegradability² and non-toxicity³. Furthermore, it has also been reported that chitosan has antimicrobial^{14, 15}, anticancer¹⁶, anti-inflammatory¹⁶ and antioxidant¹⁷ features. All these desirable characteristics of chitosan make it a suitable candidate for wide range of biomedical applications including tissue engineering, wound healing, gene delivery and drug delivery systems.¹⁸⁻²²

2.2. Biomedical Applications of Chitosan

2.2.1. Tissue engineering

Tissue engineering is an interdisciplinary area which uses living cells, engineered scaffolds and suitable signal molecules to repair damaged parts of body and promote their functions.²³ Recently, various biodegradable polymers have been investigated as polymeric scaffolds for tissue engineering applications. Among them, chitosan has been found as a promising candidate due to its desirable characteristics to regenerate injured tissues.²⁴ The desirable properties of chitosan for tissue engineering applications can be listed as high surface area and tensile strength, porous structure, easiness of chemical modification and gel forming behaviour.¹¹ Commonly, this biomaterial is used in tissue engineering applications to repair skin, liver, blood vessel, bone and cartilage tissue analogs.²⁴

2.2.2. Wound healing

Biodegradability, biocompatibility, low immunogenicity and antimicrobial activity of chitosan makes it an advantageous biomaterial for wound healing applications.^{19, 25} In addition to these properties, chitosan accelerates healing procedure by inhibiting formation of scar, inducing fibroblast proliferation and supporting accumulation of collagen to wound site.²⁵

2.2.3. Gene and drug delivery systems

2.2.3.1. Gene delivery systems

Gene therapy a promising treatment which uses therapeutic genes to cure genetic diseases. With this therapy, therapeutic genes are introduced into specific target cells where production and secretion of gene products can occur.^{26, 27} However, the transfer of naked genes into specific target cells have some limitations for systemic administration due to the existence of serum nucleases in blood circulation and disability of negatively charged genes to pass through negatively charged cell membranes.²⁸ Therefore, many researches have directed to design ideal gene delivery vehicles.

To date, several variant systems were investigated to deliver foreign genetic materials into target cells. In general, gene carrying vectors can be classified as viral and non-viral vectors.²⁹ Recently, non-viral vectors have gained growing interest due to major disadvantages of viral vectors such as toxicity, low gene loading capacity and activation of host's immune system.³⁰ However, transfection capabilities of non-viral vectors are much lower than viral vectors.²⁹ Non-viral vectors can be classified as cationic lipids and polymers.³¹ Since cationic polymers are more advantageous due to their higher gene loading capacities and modification abilities, non-viral vectors commonly include pDNA molecules which are condensed into nanoparticles using cationic polymers.³¹ The mechanism of gene delivery by cationic polymers include the following steps: (i) interaction of cationic vehicles with anionic cell membrane, (ii) endocytosis of gene loaded cationic vehicles, (iii) release of deoxyribonucleic acid (DNA)/ small interfering ribonucleic acid (siRNA)/ messenger ribonucleic acid (mRNA) from endosomes, (iv) translocation of DNA molecules into nucleus for expression or binding of siRNA and mRNA to RISC and ribosome, respectively (Figure 2.2).²⁷

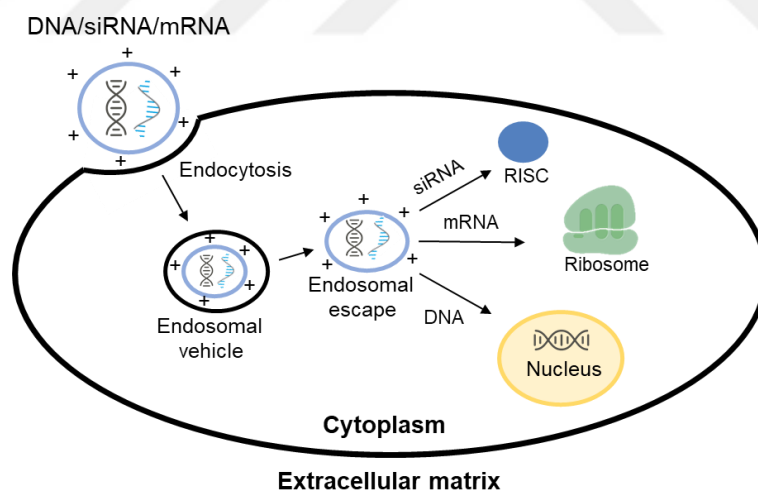


Figure 2. 2. Gene delivery through positively charged non-viral vectors.

Nanoparticle-based gene delivery systems

Recent advances in nanomedicine induces the development of nanoscale materials including nanocarriers, nanorobots and nanodrugs. Among all these nanoscale materials,

nanoparticle-based delivery systems have a great potential to improve the therapeutic efficacy of the system due to their unique properties such as prolonged blood circulation half-life, high loading capacity, increased cell membrane penetrability and low cellular toxicity.^{32, 33} Currently, nanoparticles have been started to use as gene delivery vehicles due to their tendency to accumulate in tumor tissues rather than normal tissues, which is called as enhanced permeability and retention (EPR) effect.^{34, 35} Figure 2.3 represents EPR effects of nanoparticles on tumor tissues.

Chitosan is one the most widely used biomaterial in nanoparticle-based gene delivery systems because of its excellent properties; biocompatibility, biodegradability, low toxicity, low immunogenicity and relatively higher positive charges.^{1-4, 36} However, chitosan-based gene delivery systems have lower transfection capabilities, poor solubility and poor stability at physiological conditions which restricts their clinical translations.³⁷

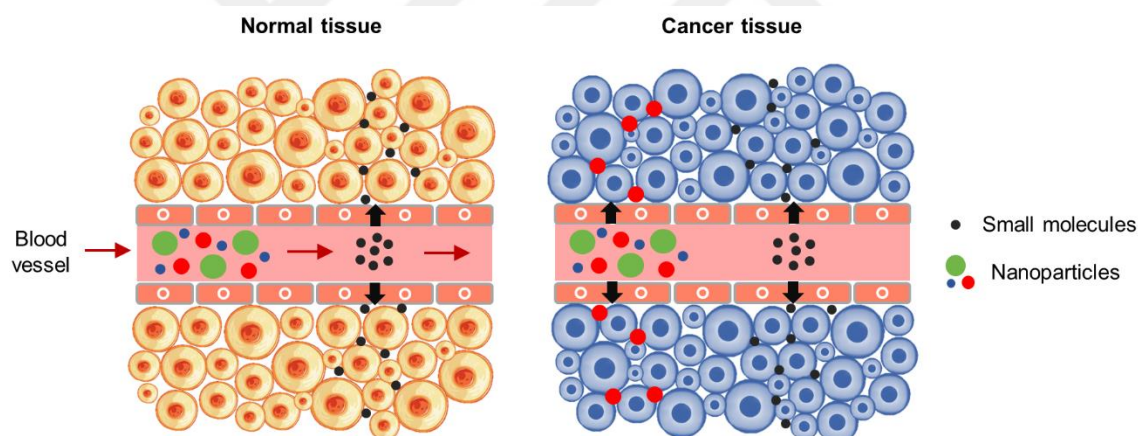


Figure 2. 3. Representation of EPR effect, where certain sized molecules (nanoparticles, drugs and liposomes) have ability to accumulate in tumor tissues rather than normal tissues.

Ionically Crosslinked Chitosan Nanoparticles as Gene Delivery Systems

Chitosan-based nanoparticles can be synthesized using multiple different techniques including ionotropic gelation^{38, 39}, microemulsion⁴⁰, emulsification solvent diffusion⁴¹ and reverse emulsion method⁴². Among these techniques, ionotropic gelation

is commonly used, because this reaction does not need organic solvents, complex steps or equipment. In this method, positively charged chitosan reacts with negatively charged TPP that results in synthesis of chitosan nanoparticles within short periods of time.^{38, 43, 44} However, unmodified chitosan has poor solubility and poor stability at physiological environments that restricts its use in biomedicine.^{37, 45} To overcome these challenges, PEGylation which is the conjugation of PEG to chitosan, contributes to formation of colloiddally stable chitosan nanoparticles at wide range of pH values.⁴⁶⁻⁴⁹

To date, various strategies have been utilized for the conjugation of PEG to chitosan such as hydroxyl group substitution and amino groups substitution. Among these strategies, N-hydroxysuccinimide (NHS)-amino coupling reaction is more common due to the simplicity of reaction. With this technique, amino groups of chitosan are functionalized with NHS groups of PEG in one step reaction at room temperature.⁴⁸ PEGylated chitosan nanoparticles could also be synthesized with ionotropic gelation method (Figure 2.4). PEG-modified chitosan nanoparticles have better properties, thus enhances usage of chitosan nanoparticles in biomedical applications.

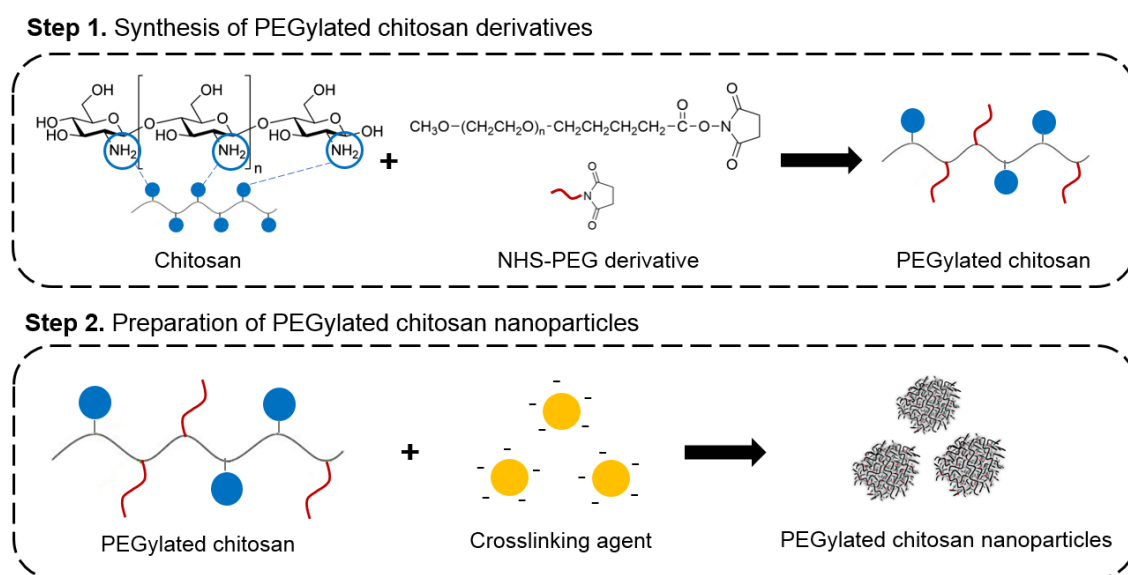


Figure 2. 4. Key steps of PEGylated chitosan nanoparticle synthesis.

2.2.3.2. Drug delivery systems

Chitosan has also been used in drug delivery systems in various forms including nanoparticles, hydrogels, microspheres, micelles and tablets.⁵⁰ It could be used in both injectable and implantable forms for oral, ocular and nasal routes.⁵¹ Chitosan is a suitable biomaterial for drug delivery systems due to its following properties: (i) Positively charged chitosan has muco-adhesive property due to the anionic nature of mucus layer. The electrostatic interactions between chitosan and mucus layer increase the residence time of chitosan-based drug delivery systems and therefore promotes therapeutic activity of chitosan carriers.⁵² (ii) Chitosan is a good candidate to achieve controlled release of anionic drugs.⁵³ (iii) The pH-dependency of chitosan in case of hydrogels increases its potential in drug delivery applications.⁵⁴ (iv) Chitosan has extended cell membrane permeability due to its cationic nature.⁵⁵

2.3. Biohybrid Microrobots for Drug Delivery Applications

Conventional drug delivery systems have some limitations in delivery of cargos such as inefficient distributions of therapeutics towards targeted pathological sites and requirement of repeated drug administrations.⁵⁶ Biohybrid microrobots in which living organisms are combined with artificial carriers have great potential to overcome these limitations due to their advances in actuation and sensing.⁵⁷

To this end, several microorganisms and drug carrying systems have been integrated to create novel biohybrid microrobots. Bacteria is the most widely used living organism in biohybrid microrobots despite its negative properties such as rapid growth rate at physiological conditions, pathogenicity and antibiotic resistancy.⁵⁸⁻⁶⁰ *Chlamydomonas reinhardtii*, eukaryotic unicellular green microalgae, has also been studied as a living organism for biohybrid microrobots. *C. reinhardtii* has a unique spherical shape with 5-10 μm diameter and high swimming speed around 100-200 $\mu\text{m}/\text{sec}$. Furthermore, it has an inherent guiding ability with visible light and has a natural autofluorescence behaviour.⁶⁰⁻⁶²

CHAPTER 3: BIOADHESIVE PEGYLATED CHITOSAN NANOPARTICLES FOR TRAIL GENE DELIVERY

3.1. Introduction

Nanomedicine plays an important role in the development of nanoscale materials such as nanocarriers, nanodrugs and nanorobots. Among all these materials, nanoparticles (NPs) have significant role in the delivery of medicines due to their specific properties such as prolonged systemic circulation half-time, high drug-loading capacity, increased cell membrane penetration, high drug-loading behaviour, and low toxicity.^{32, 33} Recently, NPs have been started to use as gene delivery vehicles, since they have abilities to protect DNA from degradation by serum nucleases, higher accumulation at tumor site and increased efficiency for delivery of cargos.^{36, 63, 64} Chitosan-based NPs have gained growing interest because of their desirable properties; biocompatibility, biodegradability, low toxicity and high-loading efficiency due to their positive charges.^{46, 65, 66}

Chitosan NPs can be prepared using multiple different techniques such as emulsification solvent diffusion, reverse emulsion, microemulsion and ionotropic gelation.³⁸⁻⁴² In ionotropic gelation, interaction between positively charged chitosan and negatively charged TPP induces the synthesis of chitosan NPs within a short time.^{43, 44} However, the usage of unmodified chitosan in biomedical applications has been limited due to its low solubility and low stability at physiological environments.^{37, 45} PEGylation, conjugation of PEG, is commonly used to eliminate these challenges of chitosan.⁴⁶⁻⁴⁹ Chemical attachment of PEG to chitosan can be utilized using various techniques. Among these strategies, NHS-amino coupling reaction, attachment of PEG to chitosan through NHS derivatives, is common due to its simplicity and lack of complex reactions.^{48, 67}

PEGylation of chitosan contributes to solubility and stability of chitosan NPs at physiological conditions. PEGylation also affects the transfection capabilities of chitosan

NPs. PEG chain length and PEG concentration are important parameters which play significant role on cellular internalization of NPs. In 2011, Walkey et al. investigated the impact of size and surface chemistry on serum protein adsorption of gold nanoparticles and their detection by macrophages. They observed that adsorption of serum proteins was related with size of nanoparticles and PEG density. Increasing PEG density was decreasing total serum protein adsorption.⁶⁸ In 2014, Cruje et al. examined the effect of PEG concentration and PEG chain length on cellular internalization of NPs. They observed that lower PEG concentrations and shorter PEG chain lengths resulted in higher cellular internalization and reduced nonspecific protein adsorption.⁶⁹ Pozzi et al. also investigated the impact of PEG chain length on internalization of lipid nanoparticles. They found that excessive PEGylation inhibited cellular uptake of nanoparticles and reduced the potential of delivery systems.⁷⁰ Recently, Yang et. al. examined the impact of PEGylation on transfection efficiency of chitosan/siRNA polyplexes. They implied that high PEG chain lengths and less PEG grafting rate was advantageous to improve the potential of chitosan/siRNA nanopcomplexes.⁷¹

ANN is a promising computational method which is used for complex and nonlinear systems.⁷² ANN model is established with experimental data, examines the impacts of independent parameters and predicts the effect of each critical variables on nanosystems.⁷³⁻⁷⁵ Using ANN approach, we have ability to synthesize nanosystems with desired properties using small experimental dataset with less experimental effort. In this chapter, the effect of PEG chain length, PEG concentration and NP concentration on transfection capabilities of gene loaded PEGylated chitosan NPs were investigated by using ANN. First, PEGylated chitosan polymers were synthesized using different PEG chain lengths (2, 5, 10 kDa) and PEG concentrations (4, 8, 12, 15, 20 μ mole per 25 mg of chitosan). Different PEG chain lengths were selected by proposing that (i) PEG chain lengths smaller than 2 kDa affects the stability of chitosan NPs in a negative way, (ii) PEG chain lengths higher than 10 kDa causes reduction in positive charges of chitosan which normally have a positive impact on the stability of chitosan NPs, (iii) 5 kDa PEG

chain length was selected as an intermediate value. On the other hand, different PEG concentrations were selected by suggesting that monodisperse NP solutions could not be obtained for smaller than 4 μ mole and higher than 20 μ mole PEG.

Since TRAIL is a protein that promotes apoptosis in cancer cells rather than healthy cell lines, we used TRAIL encoded pDNA as an example platform in the synthesis of gene carrying PEGylated chitosan nanoparticles.⁷⁶⁻⁸⁰ pDNA encapsulated and PEGylated chitosan NPs were prepared using different PEG chain lengths and PEG concentrations. After the synthesis of NPs, pH of nanoparticle solutions was arranged to 6.5 in order to increase the biomedical application of gene encapsulated chitosan NPs. Later, transfection efficiencies of altered groups of NPs were experimentally measured on HEK293-T cells. Using the experimental dataset, an ANN model was developed with three input variables; (i) PEG chain length, (ii) PEG concentration, (iii) NP concentration, and one output variable; number of green fluorescent protein (GFP) expressing cells. With this model, the impact of each independent variable on transfection capabilities of NPs were analyzed with less experimental effort in small periods of time. Overall, this chapter investigated the impact of PEG chain length, PEG concentration and NP concentration on transfection capabilities of pDNA encapsulated chitosan NPs and analyzed the effect of each parameter with the help of a developed ANN model. Key steps of this chapter are represented in Figure 3.1.

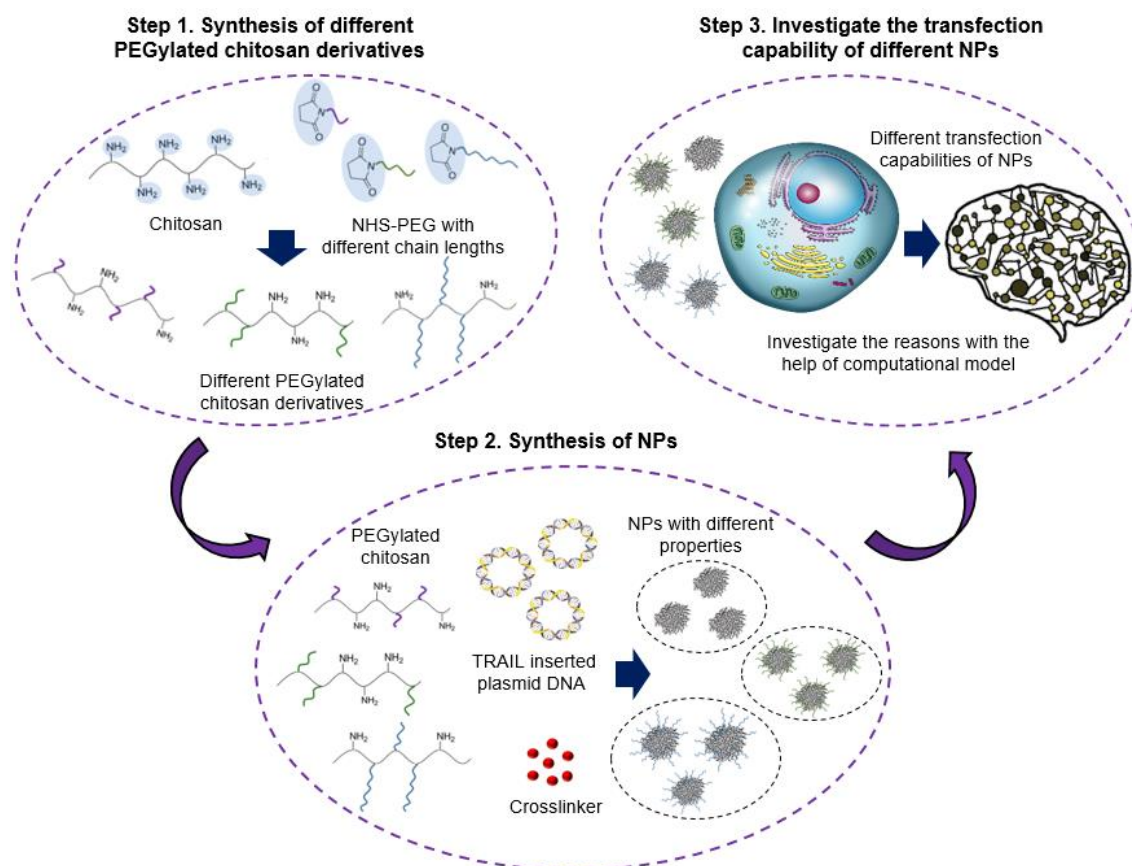


Figure 3. 1. Key steps of the study.

3.2. Experimental

3.2.1. Materials

Low molecular weight chitosan, sodium tripolyphosphate (TPP), anhydrous dimethyl sulfoxide (DMSO), dialysis membrane (14 kDa cut-off) and protamine sulfate were purchased from Sigma Aldrich (St. Louis, MO). 2, 5 and 10 kDa methoxypoly(ethylene glycol) succinimidyl valerate (mPEG-SVA) were obtained from Laysan Bio Inc. (Arab, AL). Acetic acid was obtained from Alfa Aesar (Ward Hill, MA). Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from Merck (Kenilworth, NJ). pIRES2-AcGFP1 mammalian expression vector was obtained from Clontech (Lot# 16041A). Restriction enzymes, Phusion® High-Fidelity DNA

polymerase, T4 DNA ligase, antarctic phosphatase were purchased from New England Biolabs (Ipswich, MA). Kanamycin was obtained from Gold Biotechnology (St Louis, MO). Commercial TRAIL was purchased from Enzo Life Sciences (Farmingdale, NY). FuGENE transfection reagent was obtained from Promega (Madison, WI). Dulbecco's modified eagle medium (DMEM), Dulbecco's phosphate buffered saline (DPBS), trypsin-ethylenediaminetetraacetic acid (EDTA) (0.05%), penicillin streptomycin and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY). CellTiter-Glo luminescent cell viability assay was purchased from Promega (Madison, WI). HEK293-T, U87MG and A-498 cell lines were obtained from ATCC (Manassas, VA). Ultrapure water was used for all experiments (18.2 MΩ).

3.2.2. Cloning: Insertion of TRAIL gene into mammalian expression vector

We used 5.3 kb pIRES2-AcGFP1 mammalian expression vector for cloning experiments in which human TRAIL was cloned into the vector to promote active synthesis of TRAIL protein (Figure 3.2).

Coding region of TRAIL and cytomegalovirus (CMV) promoter was amplified. We used the following forward and reverse primers for polymerase chain reaction (PCR) amplification:

Forward: 5'-ATTAGTCGACGGAGTTCCGCGTTACATAAC-3'

Reverse: 5'- CACCGGCCTTATTCCAAG -3'

After that, products obtained from PCR was purified, digested with BamHI and Sall restriction enzymes and ligated into pIRES2-AcGFP1 vector. Cloned vector was transformed into *E. coli* Oneshot® Stbl3 bacteria (ThermoFischer Scientific). Kanamycin selection was performed with 50 µg/ml final concentration. Isolation of pDNA from transformed bacteria was carried out using Macherey Nagel NucleoBond® Xtra plasmid purification kit (Bethlehem, PA).

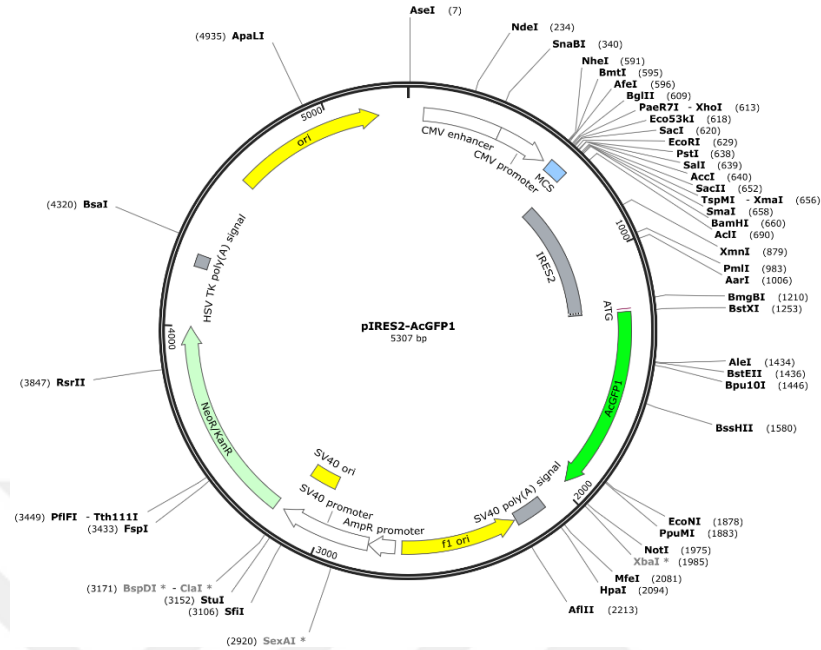


Figure 3. 2. Sequence and map of pIRES2-AcGFP1 mammalian expression vector.

3.2.3. Preparation of PEGylated chitosan polymer

We dissolved 25 mg of low molecular weight of chitosan in 2 mg/mL acetic acid solution for overnight with a final concentration of 0.5 mg/mL chitosan. pH of chitosan-acetic acid mixture was arranged to 6.0. Different chain lengths of mPEG-SVA (2, 5 and 10 kDa) was dissolved in DMSO at 50 mg/mL final concentration. mPEG-SVA solutions were added onto the chitosan-acetic acid mixtures at altered concentrations (4, 8, 12, 15, 20 μ mole per 25 mg of chitosan). Reaction was carried out for 48 h. Unbound chemicals were removed from reactions with dialysis. Dialysis were performed for 3 days against ultrapure water (Milli-Q system-18.2 M Ω). Then, reaction mixtures were lyophilized for 3 days. After lyophilization, resultant PEGylated chitosan polymers were dissolved in acetic acid solution (1 mg/mL) with a final concentration of 0.5 mg/mL chitosan. pH of PEGylated chitosan solutions was arranged to 4.8 using 6 M NaOH and stored them at -20 $^{\circ}$ C.

3.2.4. Synthesis of TRAIL inserted pDNA encapsulated and PEGylated chitosan nanoparticles (CS-PEG-TRAIL pDNA NPs)

First, 0.5 mg/mL TPP solution was prepared and pH of the solution was arranged to 2.5. The temperature of PEGylated chitosan polymer and TPP solution was adjusted to 37 °C and 4 °C, respectively. For a typical synthesis of CS-PEG-TRAIL pDNA NPs, 3 mL of PEGylated chitosan was stirred with 40 µg pDNA and 750 µL TPP solution for 1 h, at room temperature. Different NPs using altered PEG chain lengths and PEG concentrations were synthesized and altered NP groups were indicated based on their chain lengths and concentrations as follows; i.e. 5 kDa-20 µmole PEG.

3.2.5. Encapsulation efficiency of CS-PEG-TRAIL pDNA NPs

For encapsulation efficiency calculations, initial TRAIL inserted pDNA concentrations in different NP solutions were measured using NanoDrop Fluorospectrometer (Termo Scientific). Then, NP solutions were centrifuged at 13,3000 rpm for 30 min at 4 °C. Supernatants were taken and final TRAIL inserted pDNA concentrations were measured using NanoDrop. The ratio between initial and final TRAIL pDNA concentrations were taken as encapsulation efficiency.

3.2.6. Scanning electron microscopy (SEM) images and dynamic light scattering (DLS) measurements of CS-PEG-TRAIL pDNA NPs

Size and zeta potential values of altered groups of CS-PEG-TRAIL pDNA NPs were examined using DLS (Zetasizer, Malvern ZS). Size and zeta potential values of NPs were measured at two different pH values; 4.8 and 6.5. All measurements were performed in triplicates.

Morphologies and shapes of NPs were analyzed under SEM (Zeiss EVO LS15, Thornwood, NY). To remove salt molecules from NP solutions, first synthesized NPs were dialyzed with ultrapure water for overnight. Dialyzed solutions were put on silicon

wafers, air-dried for overnight and then coated with 10 nm gold. Images at different magnifications were taken using SEM.

3.2.7. Transfection of TRAIL pDNA using FuGENE transfection reagent

HEK293-T and U87MG cells (300,000 cells per well) were plated into 6-well plates and incubated for 24 h. FuGENE and TRAIL inserted pDNA mixtures were prepared as indicated in Table 3.1. Serum free & antibiotic free DMEM were used for the preparation of the mixtures. Next, TRAIL inserted pDNA mixture were added onto FuGENE mixture and incubated for 30 min at room temperature. Appropriate volumes from this solution were added onto cells, dropwise. Plates were shaken gently and incubated for overnight. After overnight incubation, FuGENE containing medium was removed and replaced with fresh DMEM (serum free) and waited for 48 h to allow the production of TRAIL and GFP proteins by transfected cells.

Table 3. 1. Ingredients of FuGENE and TRAIL inserted pDNA mixtures.

FuGENE Mixture	TRAIL inserted pDNA mixture
3 μ L FuGENE reagent	1500 ng DNA
97 μ L DMEM (-/-)	100 μ L DMEM

3.2.8. Cell viability

HEK293-T cells were seeded on 96-well plate and treated with altered concentrations of CS-PEG-TRAIL pDNA NPs in the presence of protamine sulfate (PS) for 1 day. Viabilities of cells were measured using Cell Titer-Glo reagent which was incubated with cells at 100 rpm, 25 °C for 15 min.

Toxicity of TRAIL protein secreted into the cell culture supernatants by transfected cells was also quantified. For this experiment, HEK293-T cells were incubated with altered concentrations of NPs for 1 day in the presence of PS. After that, media were changed with fresh one and waited for 48 h to allow the production and secretion of TRAIL protein in cell culture supernatants by transfected HEK293-T cells.

Cells were incubated with Cell Titer-Glo reagent for 15 min and luminescence was measured using a plate reader (BioTek's Synergy H1, Winooski, VT, USA). All cell viability experiments were performed in triplicates.

3.2.9. Transfection of CS-PEG-TRAIL pDNA NPs and measurement of TRAIL protein in cell culture media through enzyme-linked immunosorbent assay (ELISA)

HEK293-T cells were seeded on 6-well plates. pH of CS-PEG-TRAIL pDNA NPs were arranged to 6.5. NPs at different concentrations and 0.03 mg/mL PS were added onto each well and incubated for overnight. After that, NP including medium were aspirated and fresh medium were added onto each well. GFP expressions were observed 48 h after transfection using fluorescence microscopy.

TRAIL protein concentrations in cell culture supernatants were calculated using human TRAIL ELISA kit (Abcam, Cambridge, UK). HEK293-T cells were transfected as mentioned above. Cell culture media were collected 48 h after transfection. TRAIL concentration was measured at 450 nm using a plate reader (BioTek's Synergy H1, Winooski, VT, USA).

3.2.10. Flow cytometry analysis

HEK293-T cells were transfected with altered CS-PEG-TRAIL pDNA NPs. HEK293-T cells were washed with DPBS 48 h after transfection, detached from culture plate and collected into polypropylene tubes. The tubes were centrifuged at 1500 rpm for 5 min and then supernatants were removed. Cell pellets were dissolved in DPBS and filtered them through strainer snap cap of falcon tubes. The number of GFP expressing cells were measured using a flow cytometer (BD Accuri™ C6 Plus, San Jose, CA).

3.2.11. Neural network training

ANN model were set using three input variables; as PEG chain length, PEG concentration, NP concentration and one output variable; GFP expression. The dataset

(21) were divided as training (18)- test (3) and set the neural network. The quality of neural network was analyzed by calculating the correlation coefficient (R^2 , equation 1).

$$R^2 = 1 - \frac{\sum (y_i - \tilde{y})^2}{\sum (y_i - \bar{y})^2} \quad (1)$$

where y_i , $\tilde{y}$ and $\bar{y}$ are observed, predicted, and mean of the dependent variables, respectively. Table A.1 indicates parameters used to train network. Furthermore, we validated selected neural network using leave-p-out cross validation technique. With this technique, model was trained and validated in which p indicates number of left-out observations for validation. We calculated Pearson correlation coefficient, R, using the following equation (R, equation 2):

$$R = 1 - \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}} \quad (2)$$

where x , $\bar{x}$, y and $\bar{y}$ are observed, mean of the observed, predicted and mean of predicted values respectively. In this study, we selected p=3, 4, 5 and 6 for validation. R value was used to evaluate the quality of the model. We also calculated mean squared error (MSE) and normalized mean squared error (NMSE). We used MATLAB 2018a for neural network training.

3.3. Results and Discussion

3.3.1. Selective toxicity of commercial TRAIL

TRAIL is a protein that induces apoptosis in cancer cells rather than healthy cell lines. To indicate this behaviour of TRAIL, A-498 (human kidney carcinoma) and HEK293-T (human embryonic kidney) cells were used as cancer and normal cell lines, respectively. A-498 and HEK293-T cells were incubated with 200 ng/mL commercial TRAIL overnight. After this incubation time, most of the A-498 cells were detached from

cell culture plate, while HEK293-T cells were still maintaining their morphologies (Figure 3.3).

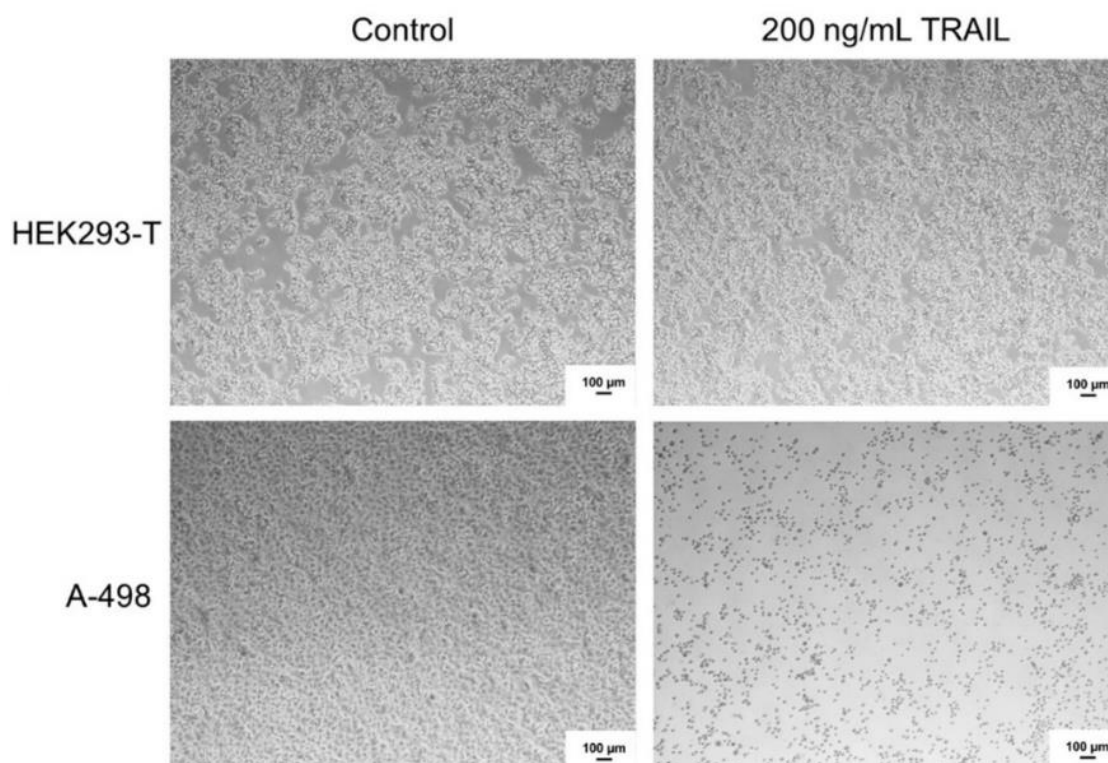


Figure 3. 3. Selective toxicity of commercial TRAIL on HEK293-T and A-498 cells which were used as representative of normal and cancer cell lines, respectively. Microscopy images of cells were taken after overnight incubation with 200 ng/mL commercial TRAIL.

Number of living cells after incubating them with commercial TRAIL for overnight were also quantified. HEK293-T cells showed 95% viability while this number was only around 2% for A-498 cells (Figure 3.4). This experiment suggested that TRAIL protein induces apoptosis in only cancer cells.

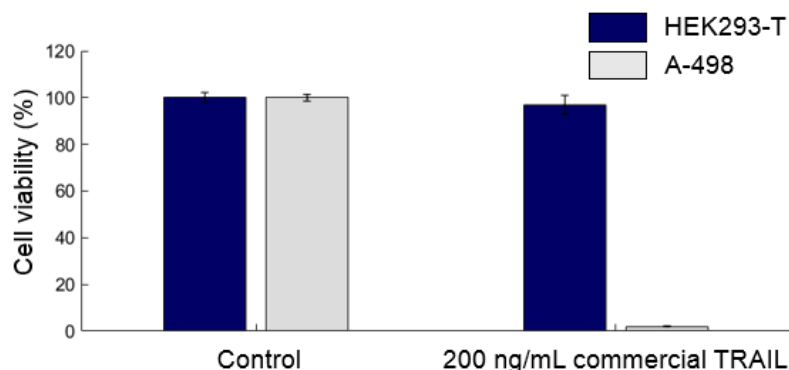


Figure 3. 4. Viability of HEK293-T and A-498 cells after overnight incubation with 200 ng/mL commercial TRAIL.

3.3.2. Cloning: Insertion of TRAIL gene into mammalian expression vector

For cloning experiments, a commercially available cloning vector, pIRES2-AcGFP1, was used. Here, this vector was selected due to its advantages: (i) Since small vectors results in higher transfection, 5.3 kb pIRES2-AcGFP1 was selected, (ii) pIRES2-AcGFP1 had internal ribosomal entry site (IRES) element. In eukaryotes, translation usually begins at 5' cap, so that only a single translation event occurs for each mRNA. However, IRES allows the initiation of translation from an internal region of mRNA, acts as another recruitment site, thereby resulting in co-expression of two different proteins from a single mRNA. pIRES2-AcGFP1 was originally encoding GFP and we inserted TRAIL gene into this vector. Thus, our new engineered pDNA was expressing both GFP and TRAIL proteins at the same time.

To demonstrate that the desired plasmid DNA was obtained after cloning steps, FuGENE transfection reagent was used to deliver engineered pDNA into HEK293-T cells. FuGENE is a commercially available, positively charged lipid-based transfection reagent. Due to its positive charges, this reagent acts as a linker between negatively charged DNA molecules and cell membranes which results in high transfection efficiencies. After transfecting HEK293-T cells with engineered pDNA using FuGENE reagent, GFP expression of cells were analyzed at 24 h and 48 h. This experiment implied

that HEK293-T cells produced GFP protein at both 24 h and 48 h of transfection (Figure 3.5). With this experiment, we ensured that our engineered pDNA was encoding GFP.

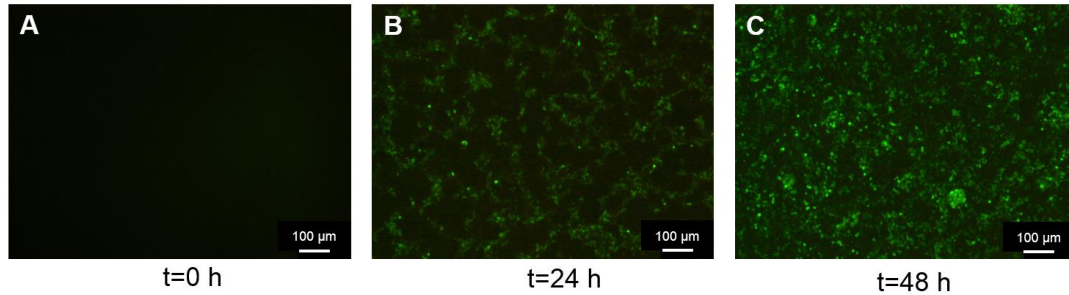


Figure 3. 5. Transfection of HEK293-T cells with engineered pDNA using FuGENE transfection reagent. A) GFP expression of HEK293-T cells at t=0. B) GFP expression of HEK293-T cells after 24 h. C) GFP expression of HEK293-T cells after 48 h.

Since TRAIL protein is secreted into cell culture medium after its production, medium was collected from transfected HEK293-T cells. The collected medium was added on both HEK293-T and U87MG cells. Here, HEK293-T and U87MG (human glioblastoma) cells were used as healthy and cancer cells, respectively. After incubating cells for 24 h, it was observed that most of U87MG cells were detached from the surface, while HEK293-T cells were maintaining their morphologies (Figure 3.6).

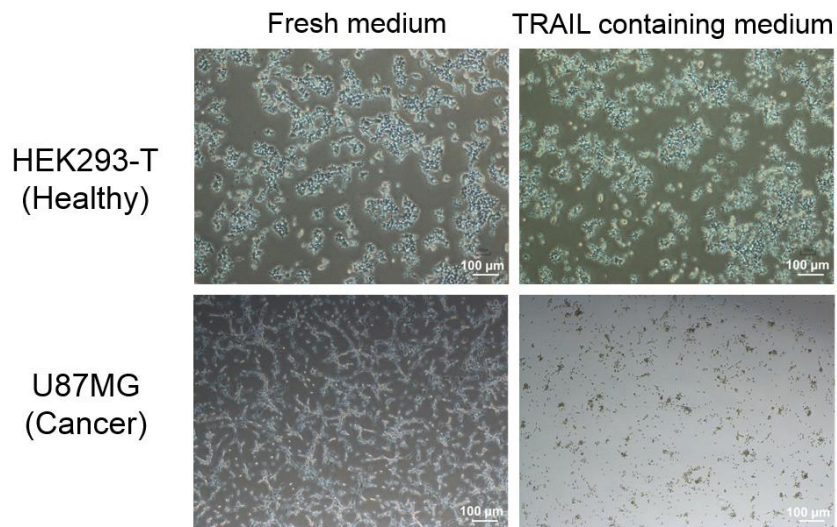


Figure 3. 6. Microscopy images of HEK293-T and U87MG cells after incubation with fresh and TRAIL containing medium for 24 h.

The number of living cells were also quantified using CellTiter-Glo reagent. Cell viabilities were obtained as 94% and 24% for HEK293-T and U87MG cells, respectively (Figure 3.7). This experiment suggested that our engineered pDNA was also encoding TRAIL. With these experiments, we demonstrated that gene cloning steps were performed successfully.

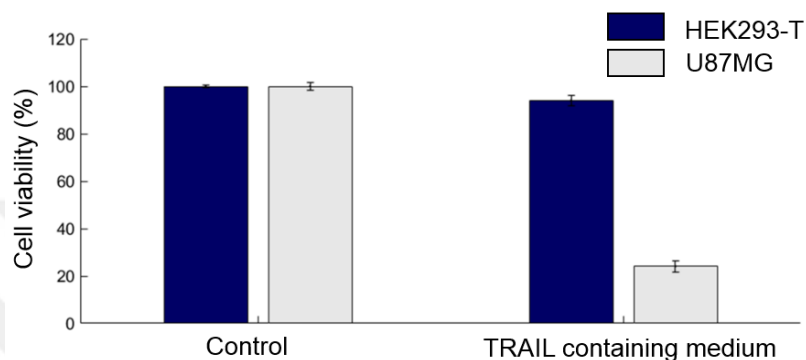


Figure 3. 7. Viability of HEK293-T and U87MG cells after incubation with TRAIL containing medium for 24 h.

3.3.3. Preparation of PEGylated chitosan polymer

Here, different PEGylated chitosan derivatives were synthesized using NHS-amine coupling chemistry in which amino groups of chitosan were reacted with NHS groups of mPEG-SVA (Figure 3.8).

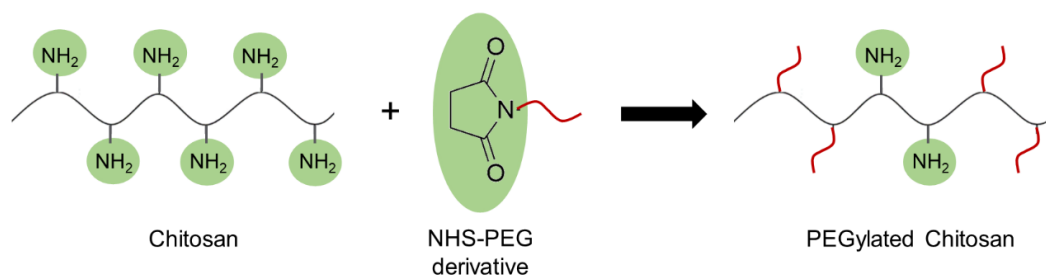


Figure 3. 8. Synthesis of PEGylated chitosan.

Since NHS groups of mPEG-SVA can react with both water and amino groups, NHS-water reaction restricts the conjugation of NHS groups of PEG to amino groups of

chitosan.⁸¹ mPEG-SVA has the highest hydrolysis half-life compared to other derivatives of PEG, therefore mPEG-SVA was used to reduce the competitive reaction between NHS-water and NHS-amino groups.⁸² Furthermore, to increase the efficiency of PEGylation reaction, the pH of chitosan solution was adjusted to 6.0 in which amine groups of chitosan reacted in a more effective way with NHS groups of PEG.

3.3.4. Encapsulation efficiency of CS-PEG-TRAIL pDNA NPs

Table 3.2 demonstrates TRAIL inserted pDNA encapsulation efficiencies for altered NPs.

Table 3. 2. Encapsulation efficiencies of TRAIL inserted pDNA for altered NPs.

Altered NPs	Encapsulation Efficiency
2 kDa-15 μ mole PEG	57%
2 kDa-20 μ mole PEG	64%
5 kDa-12 μ mole PEG	51%
5 kDa-20 μ mole PEG	62%
10 kDa-4 μ mole PEG	63%
10 kDa-8 μ mole PEG	48%

3.3.5. Characterization of CS-PEG-TRAIL pDNA NPs

NPs were synthesized using different PEGylated chitosan polymers, TRAIL inserted pDNA and TPP under rigorous stirring. After the preparation of NPs, pH of NP solutions was changed from 4.8 to 6.5. Since 6.0-7.4 is an applicable pH interval for biomedical applications, performing the experiments at a physiological pH improves the applicability of NPs in biomedicine.⁴⁶ Therefore, DLS measurements of NPs were carried out at both pH 4.8 and 6.5. In the literature, previous studies reported size and zeta potential values of chitosan NPs in only acidic conditions. However, acidic conditions do not represent the behaviour of chitosan NPs at physiologically relevant conditions because they lose their colloidal stability at physiological pH.

Size and zeta potential values of altered groups of NPs were represented in Table 3.3 and all raw data were provided in Figure A.1-44.

Table 3. 3. Experimental size and zeta potential values of NPs.

PEG MW (kDa)	PEG Mole* (μ mole)	pH	Size (nm)	Zeta Potential (mV)
2	12	4.8	97.64	28.17
2	12	6.5	94.85	9.68
2	15	4.8	101.11	26.00
2	15	6.5	91.42	11.20
2	20	4.8	94.10	28.07
2	20	6.5	93.19	10.77
5	4	4.8	92.94	29.07
5	4	6.5	98.22	9.25
5	12	4.8	92.35	22.33
5	12	6.5	90.14	7.36
5	15	4.8	98.70	21.40
5	15	6.5	95.70	7.28
5	20	4.8	91.95	19.40
5	20	6.5	96.06	6.34
10	4	4.8	91.35	26.63
10	4	6.5	89.52	7.34
10	8	4.8	93.10	22.07
10	8	6.5	92.33	5.70
10	12	4.8	83.47	20.17
10	12	6.5	84.28	5.04
10	20	4.8	82.44	13.40
10	20	6.5	91.84	3.20

* Number of PEG mole per 25 mg of chitosan.

All NP solutions showed similar size results at pH 4.8 and 6.5. On the other hand, zeta potential values of all NPs decreased when pH of the solutions were altered from 4.8 to 6.5. This result could be attributed to the deprotonation of amino groups of chitosan at

pH 6.5. The highest and lowest zeta potential values were obtained as 29.07 mV for 5 kDa-4 μ mole PEG at pH 4.8 and 3.20 mV for 10 kDa-20 μ mole PEG at pH 6.5, respectively. In addition, the highest and the lowest change in zeta potential values were obtained in 5 kDa-4 μ mole PEG and 10 kDa-20 μ mole PEG respectively when the pH of NP solutions were adjusted from 4.8 to 6.5.

From all NP solutions, 2 kDa-20 μ mole PEG, 5 kDa-20 μ mole PEG and 10 kDa-20 μ mole PEG were selected as representative groups. Figure 3.9 indicates size and zeta potential values of representative groups at pH 6.5. Sizes of 2, 5 and 10 kDa-20 μ mole PEG were measured as 93.19, 96.06 and 91.84 nm, respectively. We did not observe any significant change between size distributions of altered groups of NPs (Figure 3.9A). When sizes of NPs were compared with our previous study in which PEGylated chitosan NPs (CS-PEG NPs) were synthesized without pDNA, we observed that the sizes for the same PEG conditions were higher in this recent study, since (i) CS-PEG NPs did not include plasmid DNA and (ii) sizes of NPs were measured at pH 6.5 as different from our previous study in which all size measurements were performed at pH 6.0, because higher pH values results in higher hydrodynamic sizes.⁴⁶ Zeta potential values of 2, 5 and 10 kDa-20 μ mole PEG was obtained as 10.77, 6.34 and 3.20 mV, respectively. Based on these measurements, it was observed that higher PEG chain length resulted in lower zeta potential values for constant PEG concentration (Figure 3.9B). This result was consistent with our previous study, where we observed decline in zeta potential with higher PEG chain lengths.⁴⁶ Shape and morphology of NPs were also analyzed under SEM. The images demonstrated that sizes of NPs in SEM and DLS were consistent with each other (Figure 3.9C and Figure A.45-47).

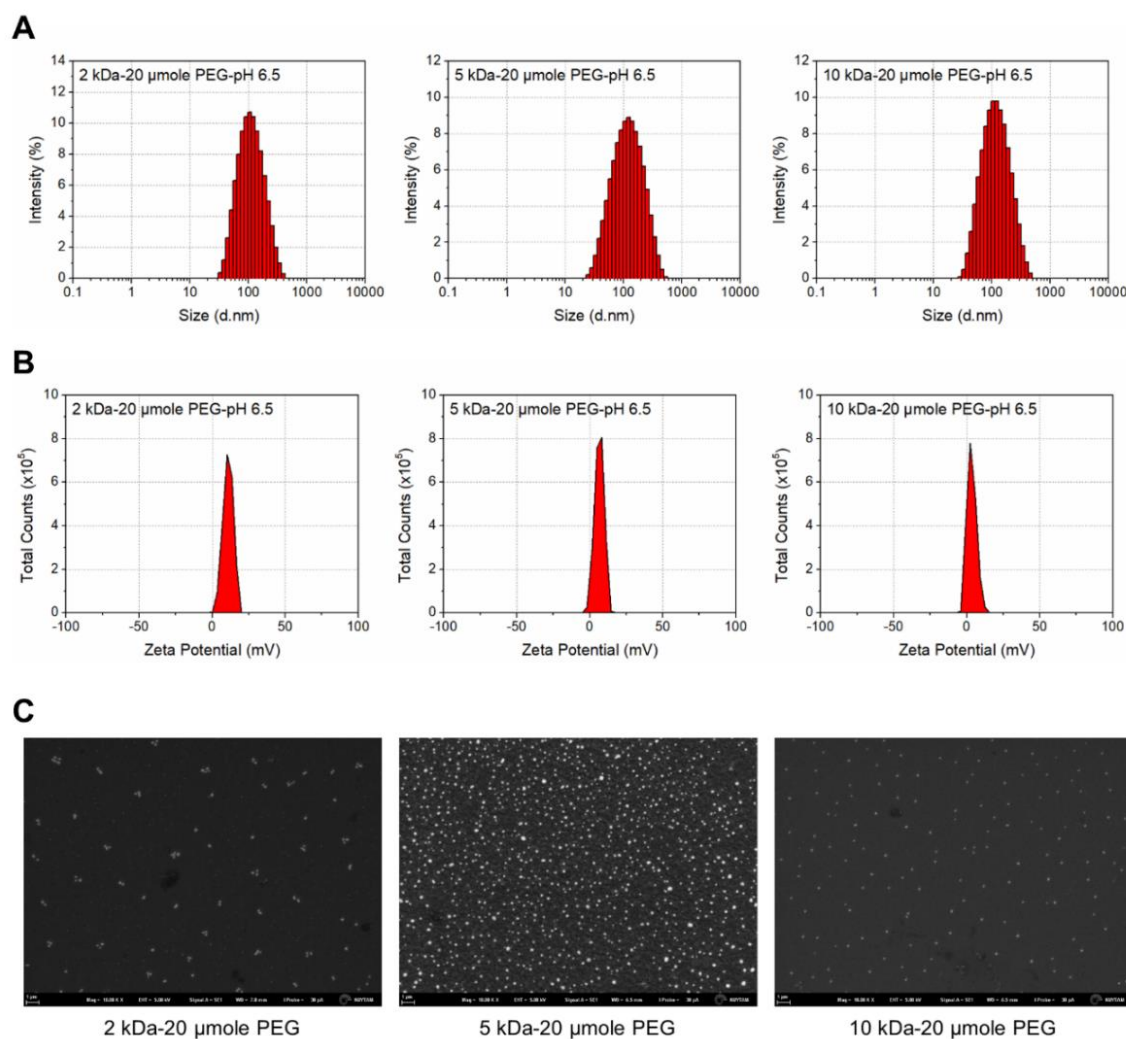


Figure 3. 9. Characterization of 2, 5 and 10 kDa-20 μ mole PEG NPs. A) Size distributions of representative groups at pH 6.5. B) Zeta potential distributions of representative groups at pH 6.5. C) SEM images of representative groups.

3.3.6. Transfection efficiencies of CS-PEG-TRAIL pDNA NPs

Since our cloned pDNA encodes both GFP and TRAIL genes separately, transfection efficiencies of CS-PEG-TRAIL pDNA NPs were first examined based on GFP expression.

After the synthesis of NPs, transfection capabilities of CS-PEG-TRAIL pDNA NPs were investigated in both HEK293-T and U87MG cells which were selected as healthy and cancer cells, respectively. Furthermore, transfection efficiency of FuGENE-TRAIL pDNA complex was measured as positive control of CS-PEG-TRAIL pDNA NPs. GFP expression of HEK293-T cells transfected with FuGENE reagent was obtained much higher than CS-PEG-TRAIL pDNA NPs. On the other hand, U87MG cells did not show any GFP expression for both FuGENE-TRAIL pDNA complex and CS-PEG-TRAIL pDNA NPs (Figure 3.10). This experiment suggested that transfection of U87MG cancer cells was challenging either using commercially available FuGENE or CS-PEG-TRAIL pDNA NP system. HEK293-T cells could be transfected with FuGENE; however, CS-PEG-TRAIL pDNA NPs were not effective to transfect those cell lines too. To contribute to the potential of CS-PEG-TRAIL pDNA NPs for transfection of HEK293-T cells, PS were added into our transfection medium and further experiments were carried out in the presence of PS.

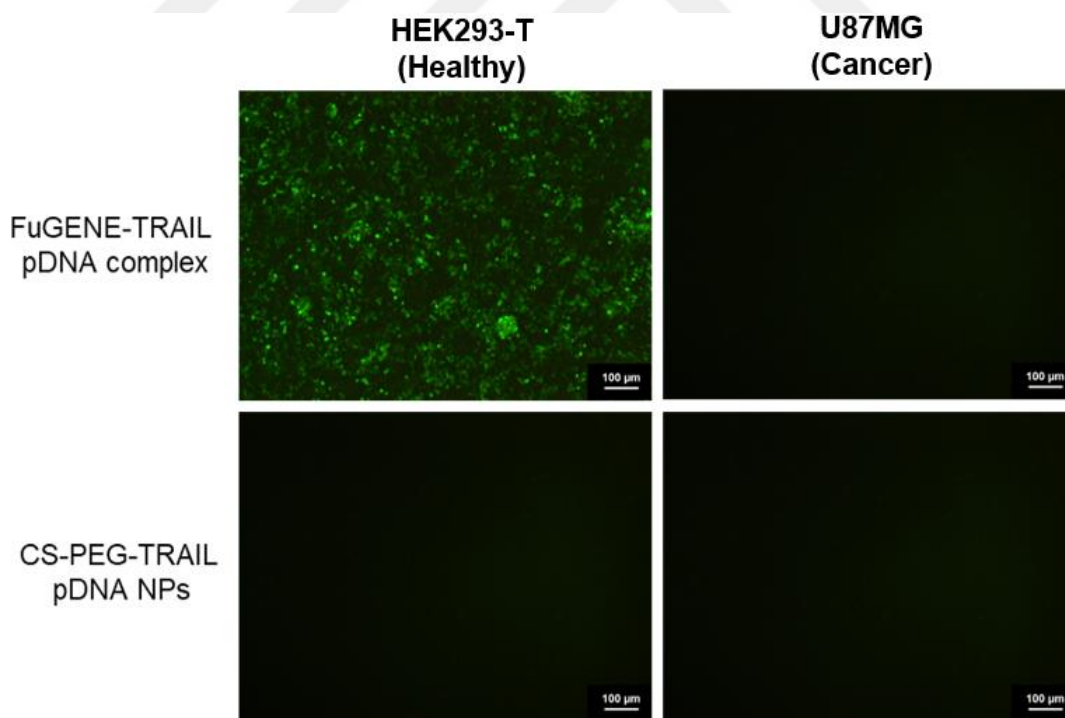


Figure 3. 10. GFP expressions of HEK293-T and U87MG cells after transfecting with FuGENE-TRAIL pDNA complex or CS-PEG-TRAIL pDNA NPs.

3.3.7. Effect of protamine sulfate on transfection efficiency

Protamine sulfate (PS) is a chemical which generally used in viral mediated delivery systems. Due to its positive charges, PS takes place in the condensation of negatively charged nucleic acids. Thus, it promotes the internalization of double-stranded DNA molecules into cells (Figure 3.11).^{83, 84}

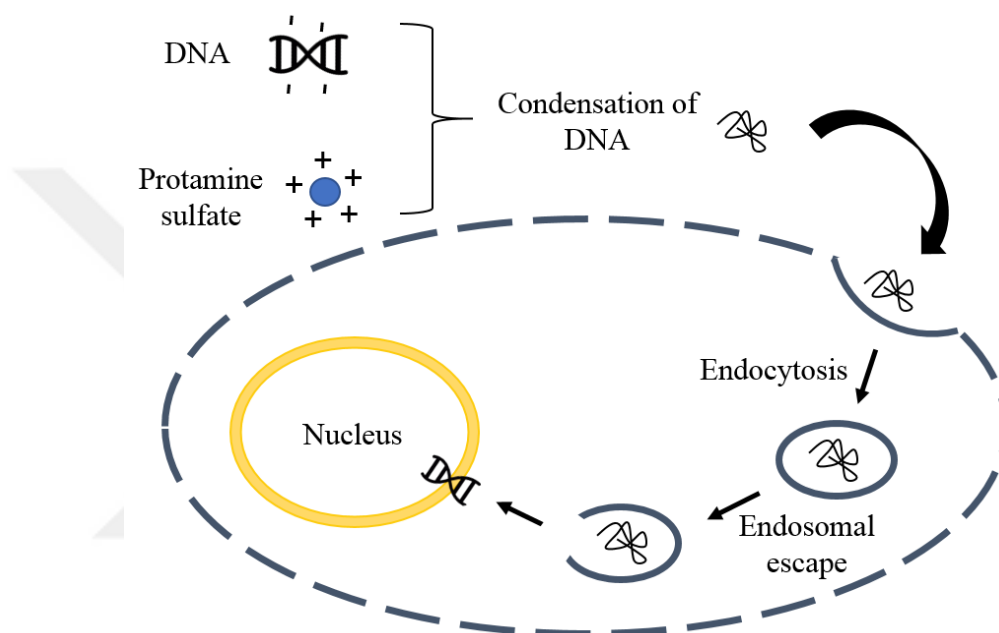


Figure 3. 11. Condensation effect of protamine sulfate in gene delivery.

Herein, PS were used to enhance cellular uptake of CS-PEG-TRAIL pDNA NPs. HEK293-T and U87MG cells were transfected with (i) naked TRAIL pDNA, (ii) naked TRAIL pDNA with PS (iii) CS-PEG-TRAIL pDNA NPs, and (iv) CS-PEG-TRAIL pDNA NPs with PS.

In HEK293-T cells, higher GFP expression were observed when cells were transfected with CS-PEG-TRAIL pDNA NPs in the presence of PS. Furthermore, it was observed that transfection efficiency of TRAIL pDNA was higher when they were encapsulated into PEGylated chitosan NPs compared to naked DNA. On the other hand, U87MG cells did not show any GFP expression for all conditions (Figure 3.12). Based

on this analysis, all following transfection and cell viability experiments were performed on HEK293-T cells using CS-PEG-TRAIL pDNA NPs with PS.

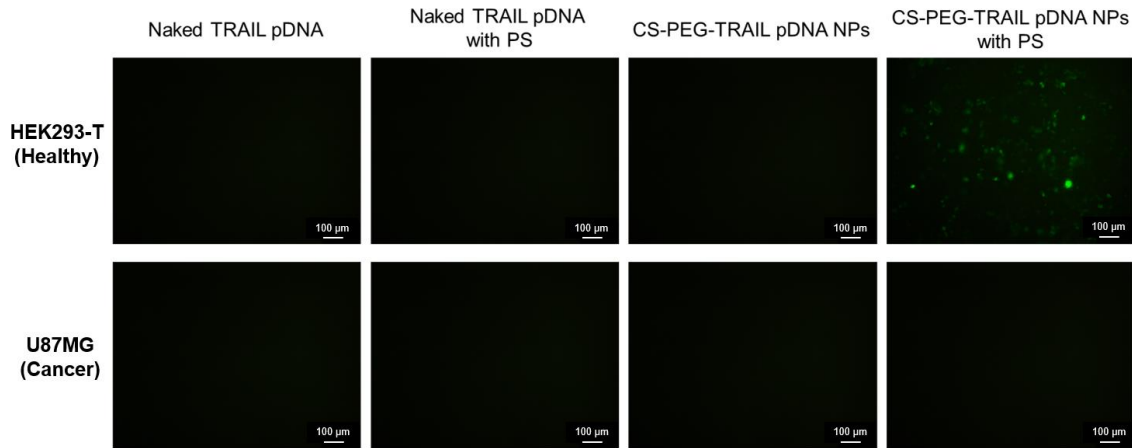


Figure 3. 12. GFP expression of HEK293-T and U87MG cells, where transfection was performed under different conditions.

3.3.8. Cell viability experiments

Cell viability assays were carried out using CellTiter-Glo reagent. HEK293-T cells were incubated with different doses of NPs (75, 150, 225 µg/mL) for 24 h in the presence of PS. HEK293-T indicated more than 70% cell viability for all groups and NP concentrations (Figure 3.13). Toxicity of TRAIL protein secreted into cell culture supernatants by transfected cells was also quantified. HEK293-T cells were treated with different doses of NPs (75, 150, 225 µg/mL) for overnight in the presence of PS. Then, NP containing medium was replaced with fresh medium and waited for 48 h to allow the production and secretion of TRAIL into cell culture medium. HEK293-T cells indicated more than 90% cell viability for all groups and NP concentrations (Figure 3.14).

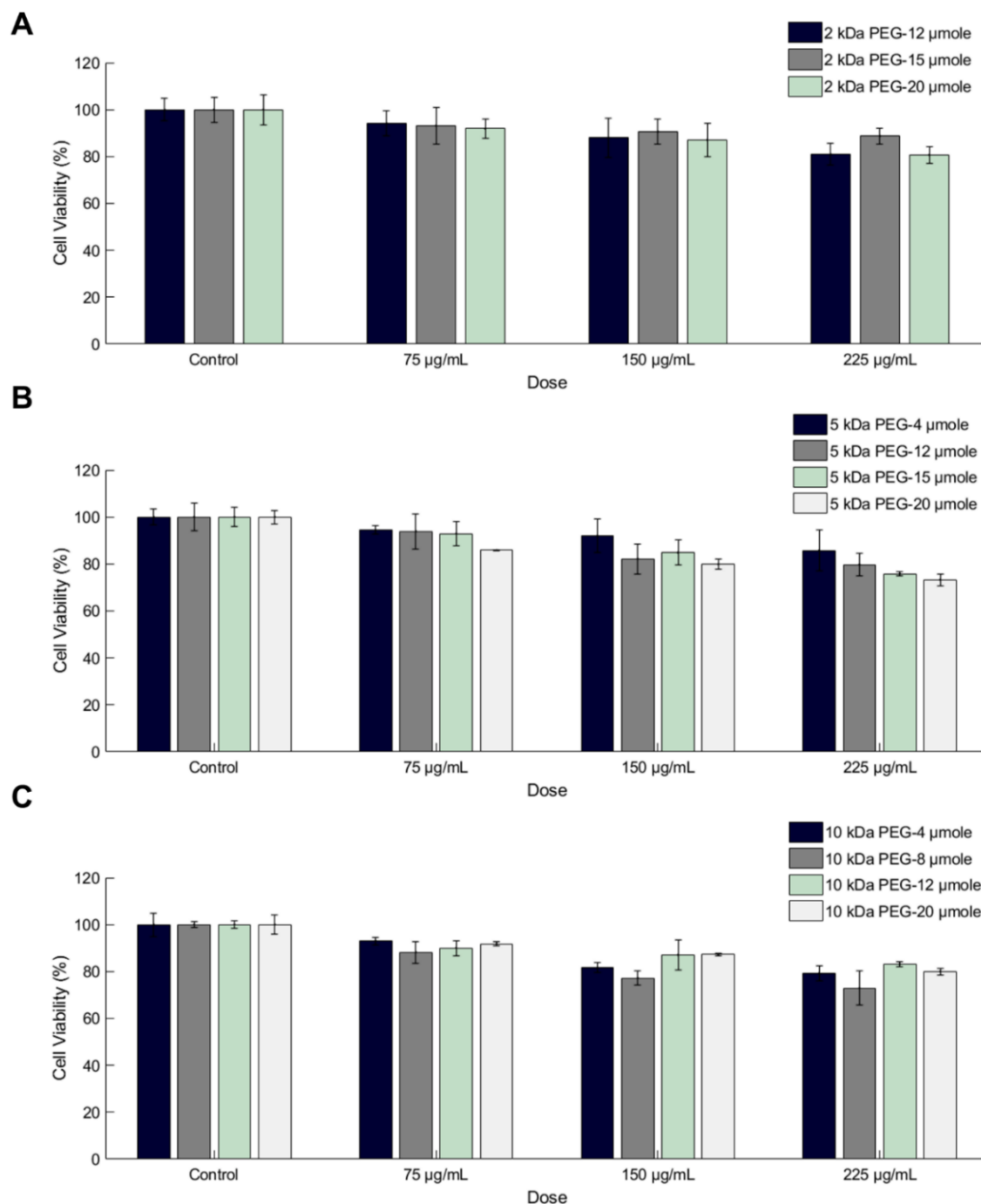


Figure 3. 13. HEK293-T cells incubated with different doses of NPs for 24 h. A) Viability of HEK293-T cells with 2 kDa groups. B) Viability of HEK293-T cells with 5 kDa groups. C) Viability of HEK293-T cells with 10 kDa groups.

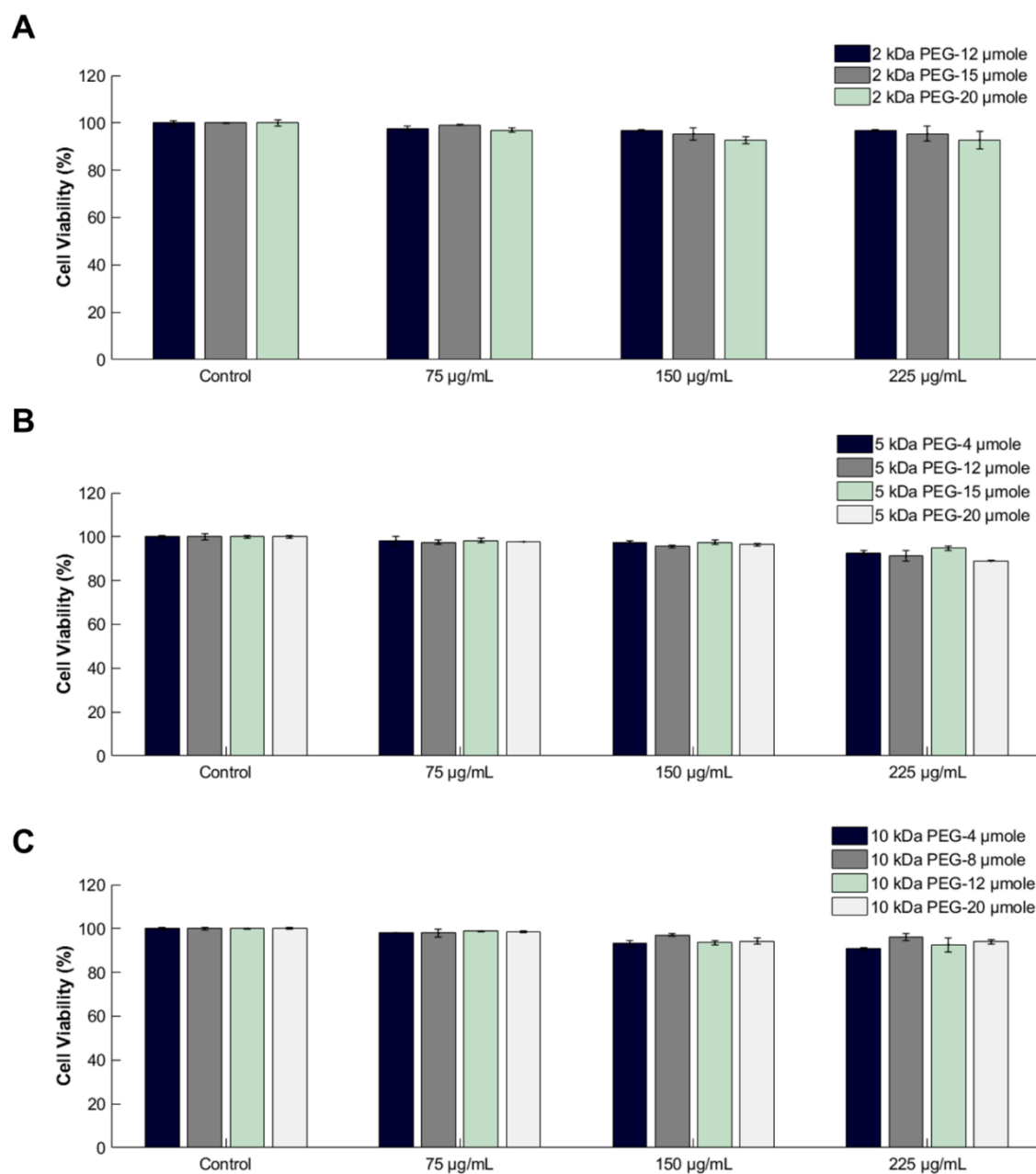


Figure 3. 14. Viability of HEK293-T cells after TRAIL protein was produced and secreted into cell culture medium. A) Viability of HEK293-T cells with 2 kDa groups. B) Viability of HEK293-T cells with 5 kDa groups. C) Viability of HEK293-T cells with 10 kDa groups.

3.3.9. Flow cytometry analysis

Flow cytometry is a technique that carries out cell sorting in a mixed cell population. With this technique, fluorescently labeled cells can be collected separately and percentage of different cell types can be quantified in a mixed cell population.⁸⁵

First, transfection capabilities of NPs were analyzed by measuring GFP expressions of HEK293-T cells transfected with altered groups of CS-PEG-TRAIL pDNA NPs. The maximum transfection efficiency was obtained in 10 kDa-8 μ mole PEG-100 μ g/mL NP group as 4.9% (Table 3.4).

Table 3. 4. GFP expression of cells transfected with altered CS-PEG-TRAIL pDNA NPs.

Sample Number	Input Parameters			Output Parameter
	PEG MW (kDa)	PEG Mole* (μ mole)	NP Concentration (μ g/mL)	GFP expressing cells (%)
1	2	12	20	1.1
2	2	12	80	2.2
3	2	15	60	2.4
4	2	15	100	4.4
5	2	20	20	0.9
6	2	20	60	3.2
7	5	4	20	1.0
8	5	4	50	1.1
9	5	12	10	0.3
10	5	12	70	3.5
11	5	15	70	4.3
12	5	20	20	0.4
13	5	20	30	0.6
14	5	20	100	4.6
15	10	4	80	2.6
16	10	4	100	2.7
17	10	8	40	2.4
18	10	8	100	4.9
19	10	12	10	0.4
20	10	12	50	1.3
21	10	20	50	0.7

* Number of PEG mole per 25 mg of chitosan.

Zeta potential and size are important parameters which affect internalization of NPs into cells. Commonly, it has been reported that NPs with higher zeta potential values have an increased tendency to interact with negatively charged cell membranes that results in increased cellular uptake.⁶⁷ However, our observations suggested that higher zeta potential did not lead to higher cellular uptake of NPs and so gene expressions (Table 3.3 and Table 3.4). Instead of zeta potential, we observed that PEG concentration and PEG chain length have more impact on internalization of NPs.

3.3.10. Characterization of TRAIL protein through ELISA

TRAIL concentration in cell culture medium was measured 48 h after transfection. Table 3.5 shows TRAIL concentrations for different NPs. Maximum amount of TRAIL was 47.69 ng/mL in 5 kDa-20 μ mole PEG-100 μ g/mL NP concentration condition.

When results obtained from ELISA with flow cytometry were compared, we observed that these different measurements have a good correlation with each other (Figure 3.15). Higher TRAIL concentrations were obtained for the groups that have higher GFP expressions. The correlation coefficient between flow cytometry and ELISA was measured as 0.87. This result proposed that quantifying number of GFP expressing cells with flow cytometry could be a reliable technique to track TRAIL concentration in cell culture supernatant. Therefore, further experiments were performed with data obtained from flow cytometry, where transfection efficiencies of NPs were measured by quantifying GFP expression.

Table 3. 5. Characterization of TRAIL protein though ELISA.

Sample Number	PEG MW (kDa)	PEG Mole* (μmole)	NP Concentration (μg/mL)	TRAIL concentration (ng/mL)
1	2	12	20	4.99
2	2	12	80	8.20
3	2	15	60	8.99
4	2	15	100	30.70
5	2	20	20	4.79
6	2	20	60	28.10
7	5	4	20	4.74
8	5	4	50	4.46
9	5	12	10	0.87
10	5	12	70	26.28
11	5	15	70	38.73
12	5	20	20	1.38
13	5	20	30	2.22
14	5	20	100	47.69
15	10	4	80	8.74
16	10	4	100	8.21
17	10	8	40	15.22
18	10	8	100	35.49
19	10	12	10	0.57
20	10	12	50	7.19
21	10	20	50	1.71

* Number of PEG mole per 25 mg of chitosan.

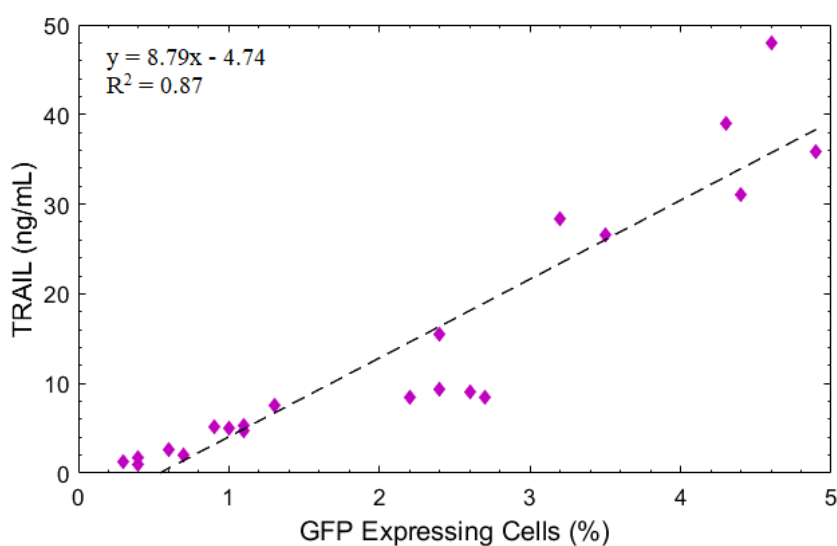


Figure 3. 15. Correlation between GFP expression and TRAIL concentration.

3.3.11. Incubation of cancer cells with medium collected from HEK293-T cells

The aim of this experiment was to measure the toxicity of TRAIL protein secreted by HEK293-T which transfected with CS-PEG-TRAIL pDNA NPs. First, HEK293-T cells were transfected with 5 kDa-15 μ mole PEG NPs. Medium were collected from transfected HEK293-T cells at 48 h of transfection and added onto both healthy and cancer cells. Schematic representation of this study is given in Figure 3.16.

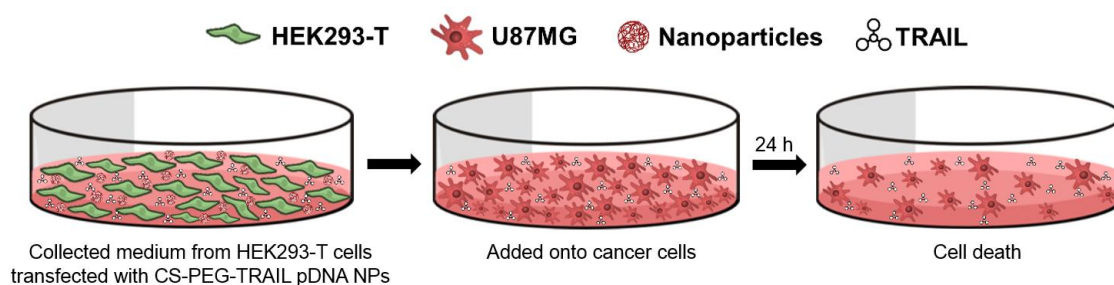


Figure 3. 16. Schematic representation of the study.

Viabilities of cells were measured at 24 h of incubation with medium collected from HEK293-T cells transfected with CS-PEG-TRAIL pDNA NPs. The number of living cells in U87MG cells was quantified as 50% (Figure 3.17).

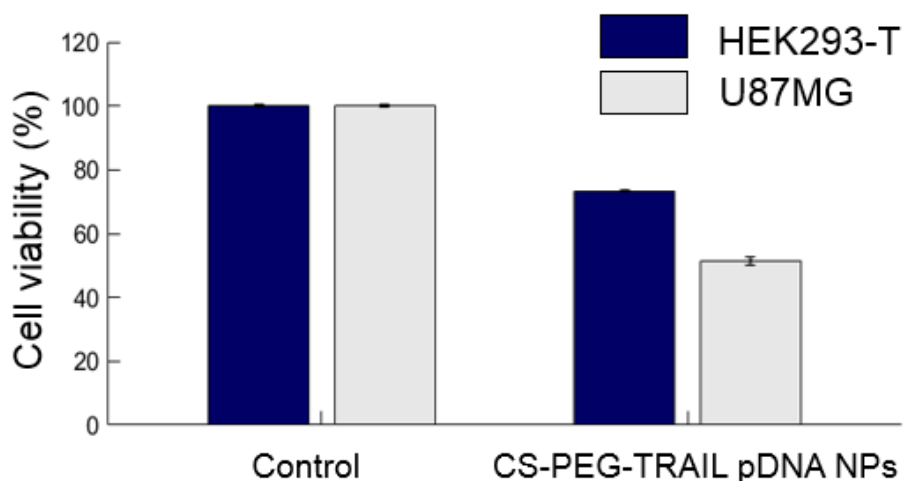


Figure 3. 17. Viability of HEK293-T and U87MG cells after incubation with medium collected from HEK293-T cells which transfected with CS-PEG-TRAIL pDNA NPs.

3.3.12. Neural network training

ANN is a computational method used for nonlinear and complex systems to investigate the effect of independent parameters on systems with minimum resources and time. Here, ANN model was used to predict the transfection efficiencies of altered NPs. Model was created using three input variables; (i) PEG chain length, (ii) PEG concentration, (iii) NP concentration and one output variable (i) number of GFP expressing cells. Data (21) used to set ANN model was given in Table 3.4 and this dataset were randomly divided into training (18) and test groups (3).

After creating neural network, we obtained R^2 values for the best predictive model as 0.9878 and 0.9769 for train and test data, respectively. These values showed that the model was reliable for prediction. Figure 3.18 indicates that observed and predicted GFP expressions were matching with each other along a 45° diagonal line.

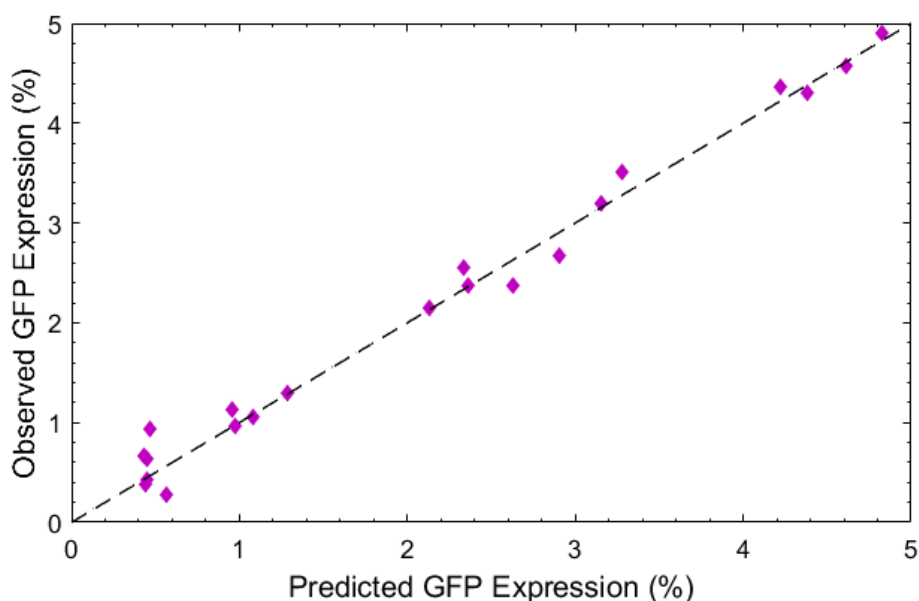


Figure 3. 18. Correlation between predicted and observed GFP expression.

Since we had a small dataset, we further validated the developed model using leave-p-out cross validation technique. We used 3, 4, 5 and 6 number of left-out observations (p) to evaluate the quality of the created model. Pearson correlation

coefficients were obtained higher than 0.9 for all p values. Mean square error and normalized mean square error slightly increased with increasing p value. The highest p value was obtained in p=3 as 0.9471 ± 0.1243 . With these results, we demonstrated that the developed model has a good quality and highly reliable for this particular system (Table 3.6 and Figure 3.19).

Table 3. 6. Pearson correlation coefficient, mean square and normalized mean square errors for leave-p-out cross validation technique.

	MSE	NMSE	Pearson Correlation Coefficient (R) for test data
3-hold	0.2833 ± 0.6277	0.0655 ± 0.0903	0.9471 ± 0.1243
4-hold	0.4091 ± 0.9005	0.0750 ± 0.1203	0.9392 ± 0.1144
5-hold	0.5165 ± 1.0948	0.0944 ± 0.1803	0.9273 ± 0.1176
6-hold	0.6107 ± 1.2628	0.1188 ± 0.1829	0.9096 ± 0.1303

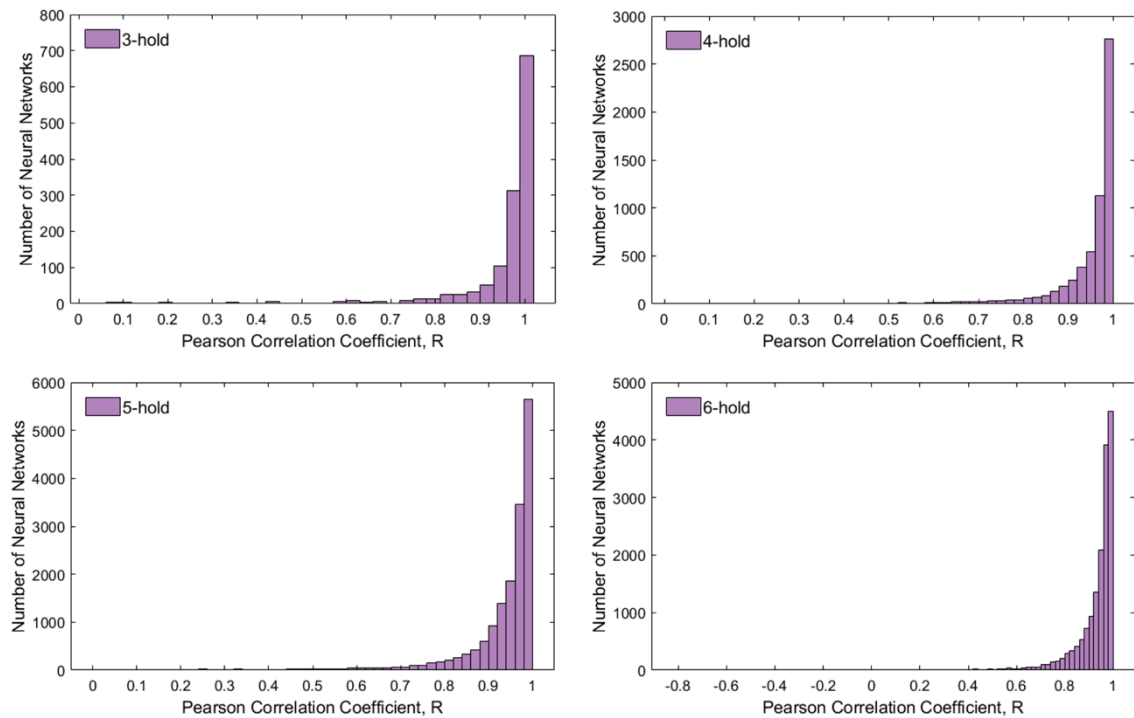


Figure 3. 19. Leave-p-out cross validation for p=3, 4, 5 and 6.

Based on the developed ANN model, GFP expression of cells were analyzed with respect to different variables. Figure 3.20 was obtained from ANN model. All groups in Figure 3.20 showed a similar trend; GFP expression increased with enhanced NP concentration.

Figure 3.20A indicates the number of GFP expressing cells at different NP and PEG concentrations. We observed that GFP expression increased with higher PEG concentration at constant NP amount for 2 and 5 kDa PEG chain lengths. On the other hand, we observed a threshold value for PEG concentration until which number of GFP expressing cells were increased and then showed a dramatic decrease at constant NP concentration.

Figure 3.20B indicates the percentage of GFP expressing cells at different NP concentrations and PEG chain lengths. It is known that higher PEG concentrations inhibit the adsorption of proteins onto the surface of NPs and therefore, reduces the recognition of them by immune system.⁶⁸ On the other hand, cancer cells refuse to uptake NPs with higher PEG concentrations. Due to this contradiction, higher PEG concentrations may either decrease the recognition of NPs by immune cells or reduce cellular uptake of NPs by cancer cells.⁶⁹ Herein, the minimum gene expression was observed in 4 μ mole PEG concentration. Excessive PEGylation has also a negative impact on transfection efficiency of NPs. Therefore, we usually observed lower gene expressions in 20 μ mole PEG compared to 12 μ mole PEG for the same PEG molecular weights and NP concentrations. Recently, Yang et al. reported that higher PEG chain lengths promotes cellular uptake of chitosan/siRNA complexes.⁷¹ Here, we also observed higher gene expressions with 10 kDa PEG chain length for 4 and 12 μ mole PEG concentrations. However, gene expression reduced with 10 kDa PEG in 20 μ mole PEG concentration. We explained this result due to the negative impact of excessive PEG concentration on cellular uptake of NPs.

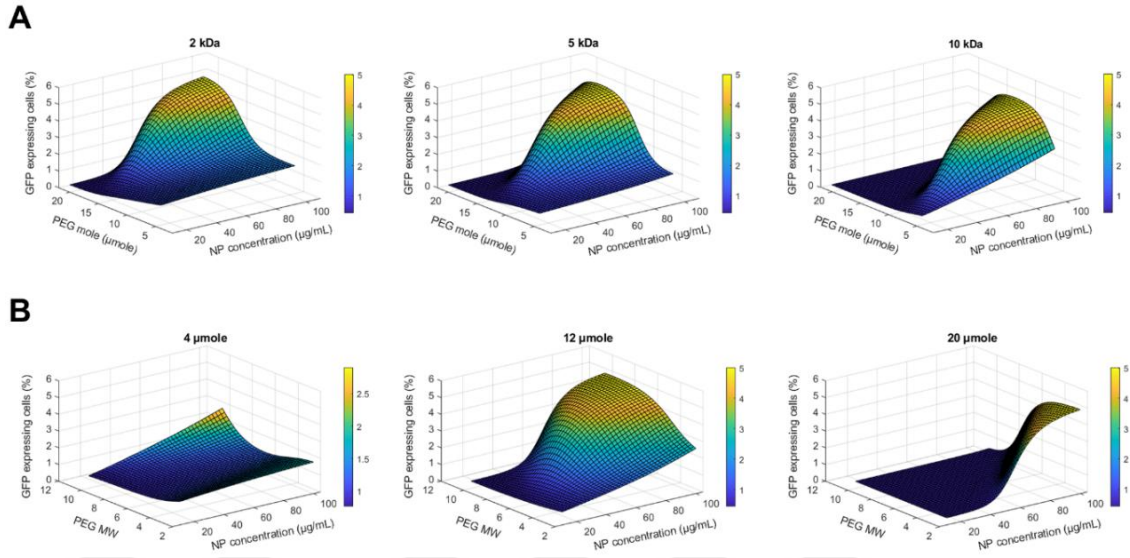


Figure 3. 20. Three dimensional plots of GFP expressing HEK293-T cells with respect to different variables. The graphs were obtained from ANN model. A) GFP expression of HEK293-T cells at constant PEG chain lengths. B) GFP expression of HEK293-T cells at constant PEG concentrations.

Figure 3.21 demonstrates the schematic representation of NPs prepared in different groups in terms of transfection efficiency, where transfection capabilities of NPs were increased with increasing PEG concentration. The only condition that is excluded from this observation is the highest PEG chain length and highest PEG concentration condition, which has the lowest amount of GFP expression characterized by flow cytometry. This could be associated with low internalization of NPs prepared with this particular condition by HEK293-T cells.

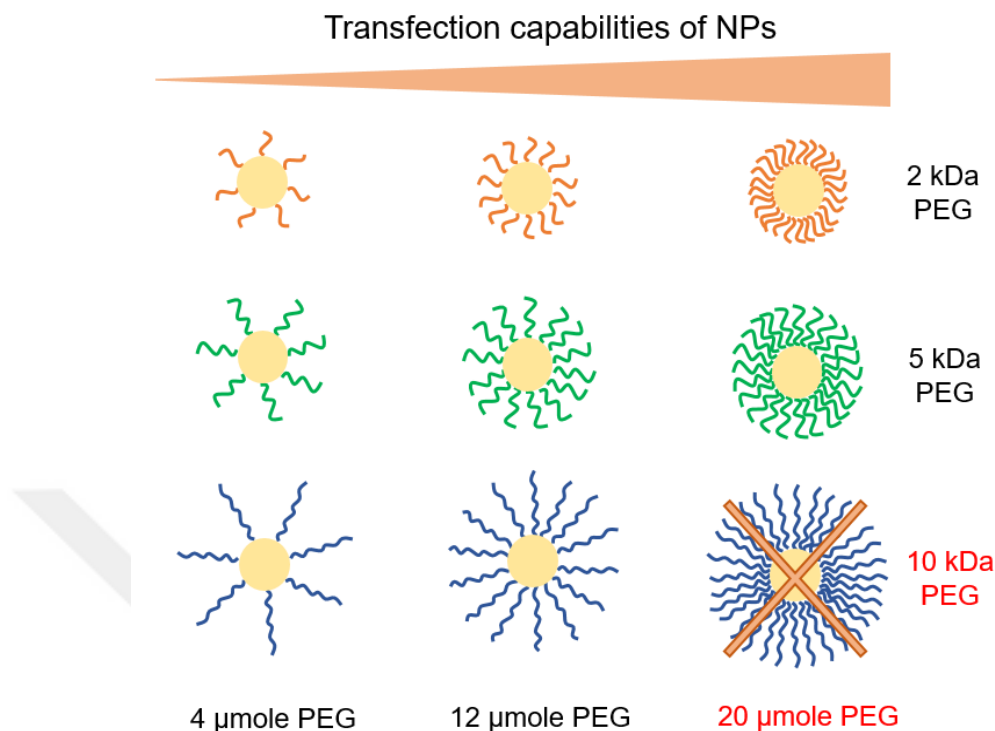


Figure 3. 21. The impact of PEG concentration on transfection efficiency of NPs.

3.4. Conclusions and Future Outlook

In this chapter, the impact of PEG chain length, PEG concentration and NP concentration on transfection efficiency of CS-PEG-TRAIL pDNA NPs were investigated. First, PEGylated chitosan polymers using different PEG chain lengths and PEG concentrations were prepared. After the synthesis of CS-PEG-TRAIL pDNA NPs, they experimentally characterized and an ANN model was developed using the experimental dataset. ANN model was including three input variables; (i) PEG chain length, (ii) PEG concentration, (iii) NP concentration and one output variable; (i) number of GFP expressing cells. With this model, we have ability to analyze the transfection capabilities of NPs with respect to different variables with less experimental effort. The results indicated that; (i) as concentration of NPs increased, higher gene expressions were observed, (ii) higher PEG concentrations promoted transfection efficiency of NPs, (iii)

however the combination of highest PEG chain length and highest PEG concentration had a negative impact on cellular internalization of NPs. Overall, this chapter shows the effect of PEG chain length, PEG concentration and NP concentration on transfection efficiency of CS-PEG-TRAIL pDNA NPs and utilizes ANN as a tool to analyze the impact of each significant parameter on gene delivery studies with less experimental effort.

Here, we observed that CS-PEG-TRAIL pDNA NPs had relatively poor efficacy compared to commercial transfection reagent. Therefore, it is particularly important to improve the efficiency of CS-PEG-TRAIL pDNA NPs in order to translate this system into clinics. To produce a protein by a cell with the help of NP transfection, first positively charged gene loaded NPs should interact with negatively charged cell membranes. NPs should be internalized by cells and escape from endosomes. After endosomal escape, cargo unpacking should occur, polymer backbone and DNA molecules should separate from each other. DNA molecules should also escape from degradation by nucleases in cytoplasm and translocate into nucleus of cells. In nucleus, DNA molecules are transcribed into mRNA and then this transcript is translated into protein. In this study, to find the problem in transfection of CS-PEG-TRAIL pDNA NP system, we can specifically track pDNA molecules by labeling them. First, to understand whether the problem is about internalization of NPs into cells, our engineered pDNA molecule and actin filaments of cells could be fluorescently labeled. After synthesis of labeled pDNA encapsulated PEGylated chitosan NPs, we could track fluorescence coming from labeled pDNA to understand the location of NPs. With this experiment, if we observe that most of labeled pDNA molecules are intersecting with labeled actin filaments, this will demonstrate that the problem is not about with internalization of NPs into cells. Second, nucleus of cells and pDNA could be labeled using different fluorescent dyes. Since pDNA molecules must enter the nucleus for protein production, with this experiment we could track the presence of pDNA in the nucleus and we could understand the reason of

transfection problem which could be about the translocation of pDNA molecules into cell nucleus after its internalization.

In addition to this proposal, we could improve the efficacy of CS-PEG-TRAIL pDNA NPs by adding polyethylenimine (PEI) into our system. PEI is a widely used synthetic polymer which effectively condense DNA due to its high positive charges and transfects broad range of cell lines with high efficiencies.⁸⁶ Furthermore, PEI has an ability to enhance endosomal escape through proton sponge effect, where influx of ions increases due to unprotonated amines of PEI, leads to osmotic swelling and rupture of endosomal membrane with release of PEI:DNA complexes into cytoplasm.⁸⁷ However, large positive charges of PEI make it highly toxic to cells.⁸⁸ Grafting PEI to PEG might lessen the toxicity of PEI and also the presence of PEG might increase colloidal stabilities of NPs. Here, by grafting chitosan with PEI and PEG, we can combine biocompatibility of chitosan with highly delivering efficiency of PEI and stabilization effects of PEG.

CHAPTER 4: A NOVEL METHOD FOR FABRICATION OF BIOHYBRID ALGAL MICROSWIMMERS

4.1 Introduction

Conventional drug delivery systems have limitations in their abilities to overcome biological barriers such as; (i) nonspecific distributions of medicines towards off-target body sites, (ii) the need for repeated drug administrations, (iii) inefficiency to transport therapeutics towards hard-to-reach tissues. These limitations are related to the deficiency of such drug delivery systems in steerability and active locomotion.^{8, 56} Over the past decade, biohybrid microrobots for biomedical applications have gained growing interest to overcome these challenges of conventional delivery systems. Biohybrid microrobots in which living organisms are integrated with artificial structures offer various advantages over conventional methods in controlled transportation to the inaccessible cavities of the human body and can be used in biomedical platforms for targeted drug delivery, disease monitoring and tissue engineering purposes.⁵⁷⁻⁶⁰

To date, multiple microorganisms and artificial structures have been combined to create unique biohybrid microrobots to address these limitations mentioned above.⁵⁸⁻⁶⁰ Among various living organisms, bacteria have been widely used despite its risks about to create pathogenicity, unavoidable rapid growth rate of bacteria in physiological environments and their antibiotic resistance.⁸⁹⁻⁹¹ Hence, the requirement to use slowly growing and more biocompatible microorganisms compared to bacteria has raised to develop novel biohybrid systems.^{57, 60}

Chlamydomonas reinhardtii (*C. reinhardtii*), unicellular photosynthetic green microalgae, has been explored in several previous studies either as a robotic template to image cells or as an actuator to utilize its mobility for the delivery of therapeutics. *C. reinhardtii* has a spherical shape with 5-10 μm diameter and high propulsion speed that

ranges between 100-200 $\mu\text{m}/\text{sec}$.⁶⁰⁻⁶² This organism is phototactic, can be steered by visible light, can harvest energy from surrounding environment and has natural autofluorescence.^{92,93} To this end, Weibel et al. demonstrated a method to attach micron-sized cargos to *C. reinhardtii*, applied phototaxis to steer the microorganism and utilized photochemistry to achieve controlled release of the cargo via UV exposure.⁹² Xie et al. used *Eudorina elegans* and *C. reinhardtii* as microrobots that could be precisely steered in zigzag or triangle trajectories based on their inherent phototaxis mechanism.⁹³ Meanwhile, Yan et al. fabricated a biotemplate composed of magnetic nanoparticle coated *Spirulina platensis*, a specific type of microalgae that has a helical shape, to develop a technique for imaging-guided therapy.⁵⁹ Recently, Yasa et al. utilized the natural motility function of the same microalgae strain to deliver polystyrene microparticles. Microparticles were coated with poly(allylamine hydrochloride) (PAH) and poly(sodium 4-styrenesulfonate) (PSS) prior to their attachment onto algae surface, which were then guided with magnetic field. The authors investigated the cytocompatibility of *C. reinhardtii* with mammalian cells.⁶⁰ Despite the advances in the field, uniform and high-yield coating of microalgae within natural polymers and magnetic nanoparticles without compromising the motility and functionality of algal microswimmers have not been explored to date.

In this chapter, a novel biohybrid algal microswimmer design were reported in which coating was achieved through electrostatic interactions between negatively charged cell membrane of *C. reinhardtii* and positively charged chitosan polymer-superparamagnetic chitosan coated iron oxide nanoparticles (SPIONPs) mixture. In literature, most of the biohybrid designs have limited manufacturing efficiencies which is around 10%. Here, our novel biohybrid design has high coating yield that is around 90%. Overall, this chapter reports a novel biohybrid algal microswimmer design which is promising for controlled drug delivery applications.

4.2. Experimental

4.2.1. Materials

Chlamydomonas reinhardtii (CC-125 wild type mt+) was obtained from Chlamydomonas Resource Center (St. Paul, MN). Chitosan Fluorescein (200-500 cP, 85% DDA, Excitation/Emission wavelength 494/518 nm) was received from Creative PEGworks (Chapel Hill, NC). Magnetic chitosan nanoparticles (Product name: nano-screenMAG-Chitosan, 200 nm size, Excitation/Emission wavelength 578/613 nm) was received from 'chemicell.com'. TAP medium was purchased from Thermo Fischer Scientific. Pluronic F-108 and hexamethyldisilazane were obtained from Sigma-Aldrich (St. Louis, MO).

4.2.2. Culture of *Chlamydomonas reinhardtii*

TAP medium, a standard maintenance medium, was used to grow *C. reinhardtii* and 12/12-hr light-dark cycle was provided (Philips MASTER TL-D 58W/840 Super 80 Weiss) in an orbital shaker at 150 rpm and room temperature.

4.2.3. Fabrication of coated microalga-powered biohybrid microswimmers

All coating experiments were performed when *C. reinhardtii* were in exponential growth stage (OD₆₈₀: 0.2-0.5). First, 500 µL microalgae found in exponential growth phase were collected and optical density of gathered cells were diluted to 0.2 at 680 nm. Later, microalgae solution was centrifuged at 300 rcf for 1.5 min and pellet was kept. 1.5 µL chitosan polymer from 0.5 mg/mL stock solution and 10 µL SPIONPs from 0.5 mg/mL stock solution were mixed in a microcentrifuge tube and volume of the solution was completed to 500 µL using TAP medium. This mixture was sonicated for a few seconds and added onto the microalgae pellet gently. For adhesion of chitosan polymer and SPIONPs to the membrane of microalgae, cell suspension was stirred in an orbital shaker for 5 min and then centrifuged at 300 rcf for 1.5 min. Collected microalgae pellet was dispersed in TAP medium for further use.

4.2.4. Construction of the microfluidic channels

Light response studies and swimming speed analysis were performed using 350 μm height and 2 mm width microfluidic channels. For the construction of these channels, double side adhesive films cut with a laser in a specific channel geometry. Constructed microfluidic channels were attached to cover glasses to simplify the observation of microalgae cells on microscope. Before using, microfluidic channels were treated with Pluronic F-108 for 30 min to inhibit the undesirable adhesion of microalgae cells on to the surface of channels.

4.2.5. Scanning electron microscopy (SEM) imaging

Coated and uncoated microalgae were placed on silicon wafers and incubated for 1 h to provide their attachments onto the surfaces. Cells were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) for 40 min at 4 °C and then rinsed using sodium cacodylate buffer (pH 7.4) for 3 times. Dehydration were performed using increasing concentrations of ethanol solutions (30%-50%-75%-90%-100%) for 10 min. Chemical dehydration were also performed using increasing concentrations of hexamethyldisilazane in ethanol solutions. Then, samples were air-dried at room temperature for overnight and coated with 10 nm gold using a sputter coater using Leica EM ACE600 sputter coater (Leica Microsystems, Wetzlar, Germany). Images were taken under Zeiss Ultra 550 Gemini scanning electron microscope (Carl Zeiss Inc., Oberkochen, Germany) with an accelerating voltage of 5 keV and an in-lens detector.

4.2.6. Energy-dispersive X-ray spectroscopy

For the elemental analysis, energy-dispersive X-ray spectroscopy (EDX) (Bruker, Billerica, MA) was performed on both coated and uncoated microalgae surfaces using an accelerating voltage of 15 keV.

4.2.7. Fluorescence emission analysis

After coating of microalgae with fluorescently-labeled chitosan polymer and SPIONPs, cells were centrifuged and rinsed twice to remove free polymer and nanoparticles from the solutions in order to eliminate the background coming from fluorescently-labeled chemicals. After that, pellets were dissolved in TAP medium. Fresh TAP medium, bare and coated microalgae were seeded on 96-well plate and fluorescence emission coming from these groups were analyzed at 485/528 nm and 540/680 nm wavelengths using a plate reader (BioTek's Synergy H1, Winooski, VT, USA).

4.2.8. Swimming speed analysis

Videos of coated and uncoated microalgae were captured when they were swimming in microfluidic channels. A code developed using MATLAB R2018a was used to analyze the swimming speeds and trajectories of bare and coated microalgae.

4.2.9. Light and magnetic field response

Light response analysis was performed for both bare and coated microalgae on microfluidic channels which had an axial length of 2 cm. Light was applied to one side of the channel for 10 min and moved to the opposite end of the channel. Images were always taken after applying the light source for 10 min.

4.3. Results and Discussion

In this chapter, our objective was to coat *C. reinhardtii* with chitosan and magnetic nanoparticles by retaining the motility and functionality of algae and to improve the coating yield. Schematics for fabrication of biohybrid algal microswimmers has been illustrated in Figure 4.1. For the fabrication of algal microswimmers, green and red fluorescently-labeled chitosan polyelectrolytes and SPIONPs were suspended in TAP medium. Then, microalgae cells were incubated with chitosan-SPIONPs mixture to

promote the coating of microalgae surfaces with positively charged chitosan-SPIONPs and negatively charged microalgae cell membranes, through electrostatic interactions.

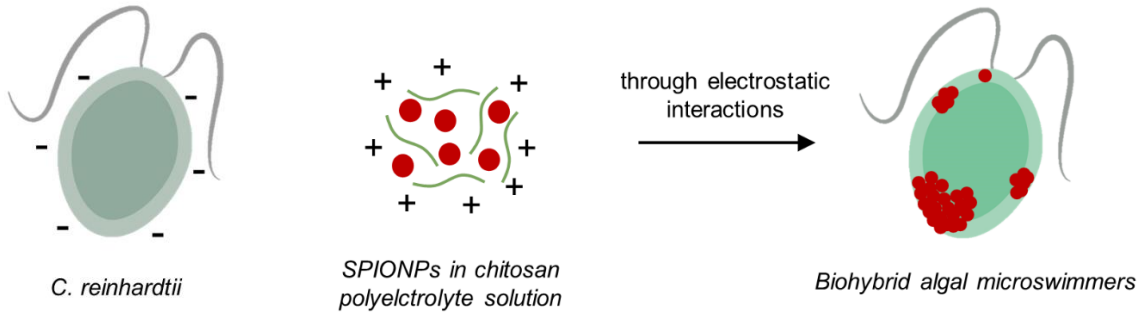


Figure 4. 1. Fabrication of biohybrid algal microswimmers.

4.3.1. Growth curve of *C.reinhardtii*

C.reinhardtii gives an autofluorescence at 680 nm due to its chlorophyll.⁹⁴ Therefore, growth curve of *C.reinhardtii* were obtained at 680 nm optical density (OD). All coating experiments were performed during the exponential growth stage of *C.reinhardtii* which ranged between 0.2-0.5 at OD₆₈₀ (Figure 4.2).

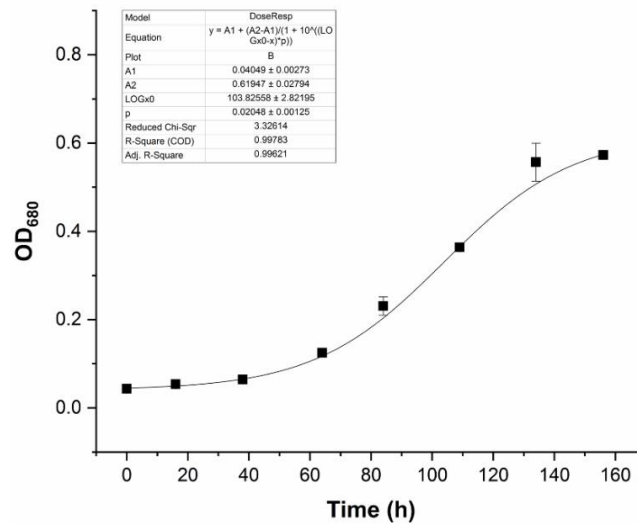


Figure 4. 2. Growth curve of *C. reinhardtii*. All coating experiments were performed during the exponential growth stage of *C.reinhardtii*, i.e. at 100 h of the growth curve.

4.3.2. Coating efficiency of *C.reinhardtii*

To characterize coating of microalgae surfaces, green and red fluorescently-labeled chitosan polymer and SPIONPs were used, respectively. First, coating efficiencies of three different groups were analyzed; (i) chitosan polymer, (ii) SPIONPs, and (iii) the mixture of chitosan-SPIONPs. After coating experiments, differential interference contrast (DIC), fluorescein (FITC) and tetramethylrhodamine (TRITC) channel images of algal microswimmers were taken and demonstrated in Figure 4.3. When microalgae were coated with chitosan polymer only, 98% coating efficiency was measured through counting the number of coated and total cells obtained from FITC and DIC channel images (Figure 4.3A). Non-uniform coating of microalgae was observed when SPIONPs were used only (Figure 4.3B). However, 95% coating efficiency was calculated on the surface of microalgae when SPIONPs were mixed with chitosan polyelectrolytes (Figure 4.3C). In the literature, most of biohybrid designs have limited coating yield which is less than 10%. Here, we achieved to coat microalgae cells with chitosan-SPIONPs mixture with 95% yield.

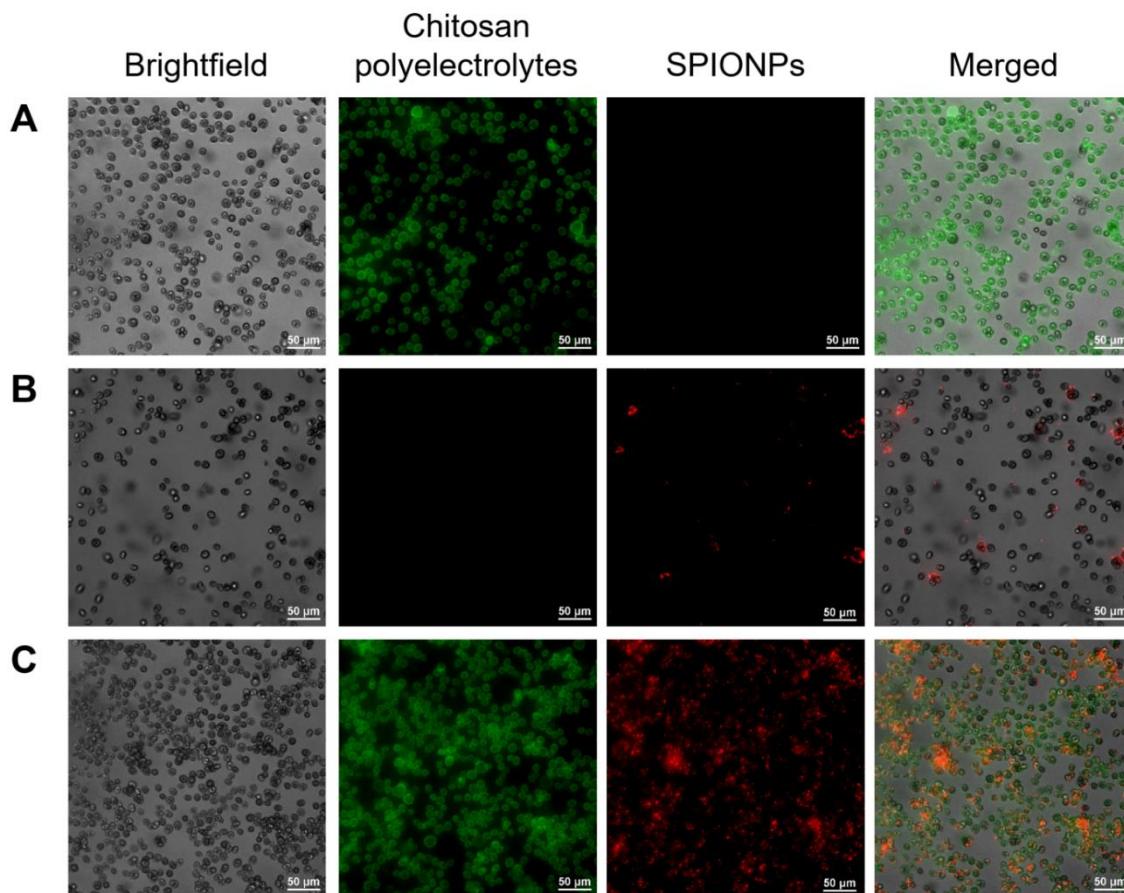


Figure 4. 3. Fluorescence microscopy images of biohybrid algal microswimmers. A) Microalgae coated with chitosan polymer. B) Microalgae coated with SPIONPs. C) Microalgae coated with chitosan-SPIONPs mixture. In all figures, green color represents chitosan polyelectrolytes, and red color represents SPIONPs.

4.3.3. Fluorescence microscopy images of biohybrid algal microswimmers

The immobilization of chitosan and SPIONPs on the surface of microalgae were further verified through fluorescent microscopy images. *C. reinhardtii* has a natural autofluorescence. Therefore, both bare microalgae and biohybrid algal microswimmers were analyzed under fluorescent microscopy. Images were confirmed that *C. reinhardtii* were uniformly coated with green fluorescent-labeled chitosan and red fluorescent-labeled SPIONPs (Figure 4.4). The limited red fluorescence coming from SPIONPs was

necessary for optimal motility of biohybrid algal microswimmers. Here, 0.01 mg/mL concentration of SPIONPs were used to coat microalgae cells and this amount was extremely critical to obtain maximum swimming speed for biohybrid algal microswimmers.

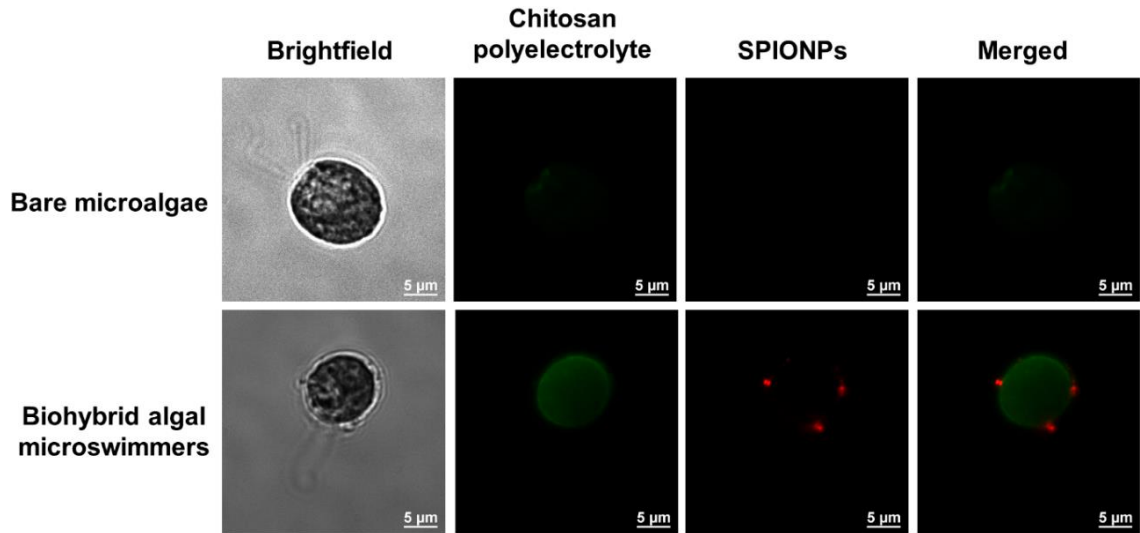


Figure 4. 4. Fluorescence microscopy images of bare microalgae and biohybrid algal microswimmers. Green color represents chitosan polyelectrolytes, and red color represents SPIONPs.

4.3.4. SEM imaging of biohybrid algal microswimmers

Furthermore, morphologies of coated and uncoated microalgae were analyzed under SEM. It was observed that SEM images of bare microalgae, microalgae coated with chitosan polymer or SPIONPs only were similar in their morphologies. On the other hand, successful deposition of SPIONPs onto the surface microalgae were obtained with algal microswimmers coated with chitosan-SPIONPs mixture. It was clearly observed that SPIONPs were attached to the surface of algal microswimmers, when we compared with bare microalgae (Figure 4.5 and Figure B.1).

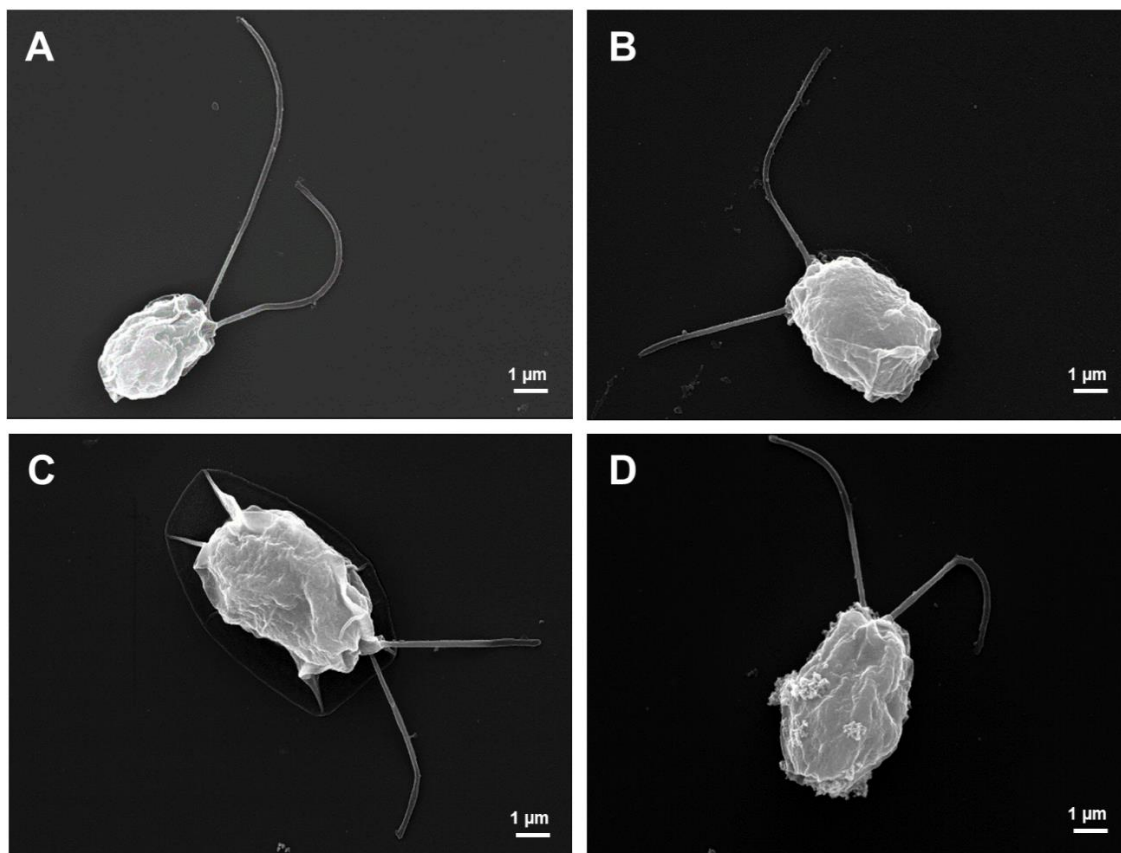


Figure 4. 5. SEM images of *C.reinhardtii*. A) Bare microalgae. B) Microalgae coated with chitosan polyelectrolytes. C) Microalgae coated with SPIONPs. D) Microalgae coated with chitosan-SPIONPs mixture.

4.3.5. EDX analysis

Deposition of SPIONPs onto the surface of microalgae was also supported with EDX measurements. EDX is a technique used to identify elemental composition of materials by analyzing X-rays emitted by the matter. Here, EDX confirmed the presence of iron mass as 5.39% for microalgae coated with chitosan-SPIONPs mixture which was not present in other groups (Table 4.1).

Table 4. 1. EDX measurements demonstrating presence of iron atoms on the surface of biohybrid algal microswimmers.

	Iron mass (%)	Others (%)
Bare microalgae	100.00	0.00
Microalgae coated with chitosan polyelectrolytes	100.00	0.00
Microalgae coated with SPIONPs	100.00	0.00
Microalgae coated mixture of chitosan-SPIONPs	5.39	94.61

4.3.6. Fluorescence emission of biohybrid algal microswimmers

Fluorescence emission coming from fresh TAP medium, bare microalgae and biohybrid algal microswimmers were also analyzed (Figure 4.6). To confirm the presence of chitosan polyelectrolytes on the surface of biohybrid algal microswimmers, 485 nm excitation and 528 nm emission wavelengths were utilized. Biohybrid algal microswimmers showed higher fluorescence emission at this wavelength compared to bare microalgae and fresh TAP medium (Figure 4.6A). On the other hand, to verify the presence of SPIONPs on the surface of biohybrid algal microswimmers, 540 nm excitation and 680 nm emission wavelengths were used. Similarly, biohybrid algal microswimmers showed higher fluorescence compared to bare microalgae and TAP medium at this wavelength (Figure 4.6B). The results obtained from microscopy images, EDX analysis and fluorescence emissions suggested that we achieved to fabricate biohybrid algal microswimmers, where *C. reinhardtii* coated with chitosan-SPIONPs mixture.

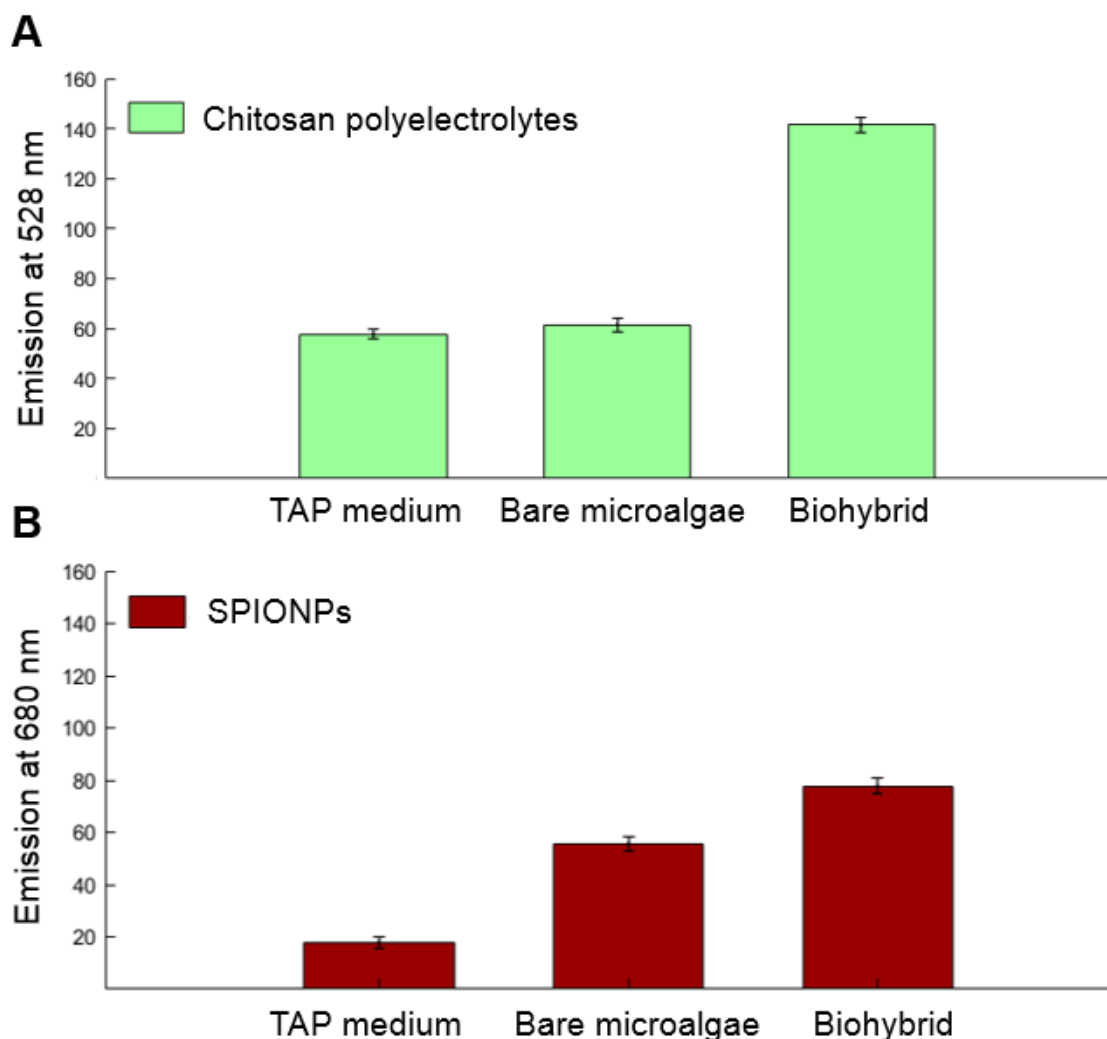


Figure 4. 6. A) Emission of TAP medium, bare microalgae and biohybrid algal microswimmers at 528 nm (Excited at 485 nm). B) Emission of TAP medium, bare microalgae and biohybrid algal microswimmers at 680 nm (Excited at 540 nm).

4.3.7. Swimming speed analysis

Next, swimming behaviour of biohybrid algal microswimmers were analyzed (Figure 4.7). Swimming speed analysis was performed in optimal growth medium of *C. reinhardtii*. Bare microalgae and biohybrid algal microswimmers were placed into microfluidic channels, videos were recorded and then analyzed using an in-house

MATLAB code. Gaussian distribution behaviour was observed in all groups. Mean speed values were obtained as 102.56 $\mu\text{m}/\text{sec}$ and 56.03 $\mu\text{m}/\text{sec}$ for bare microalgae and biohybrid algal microswimmers, respectively (Figure 4.7A). Swimming trajectories of bare microalgae and biohybrid algal microswimmers were also illustrated in Figure 4.7B. Both bare microalgae and biohybrid algal microswimmers showed similar 2D trajectories.

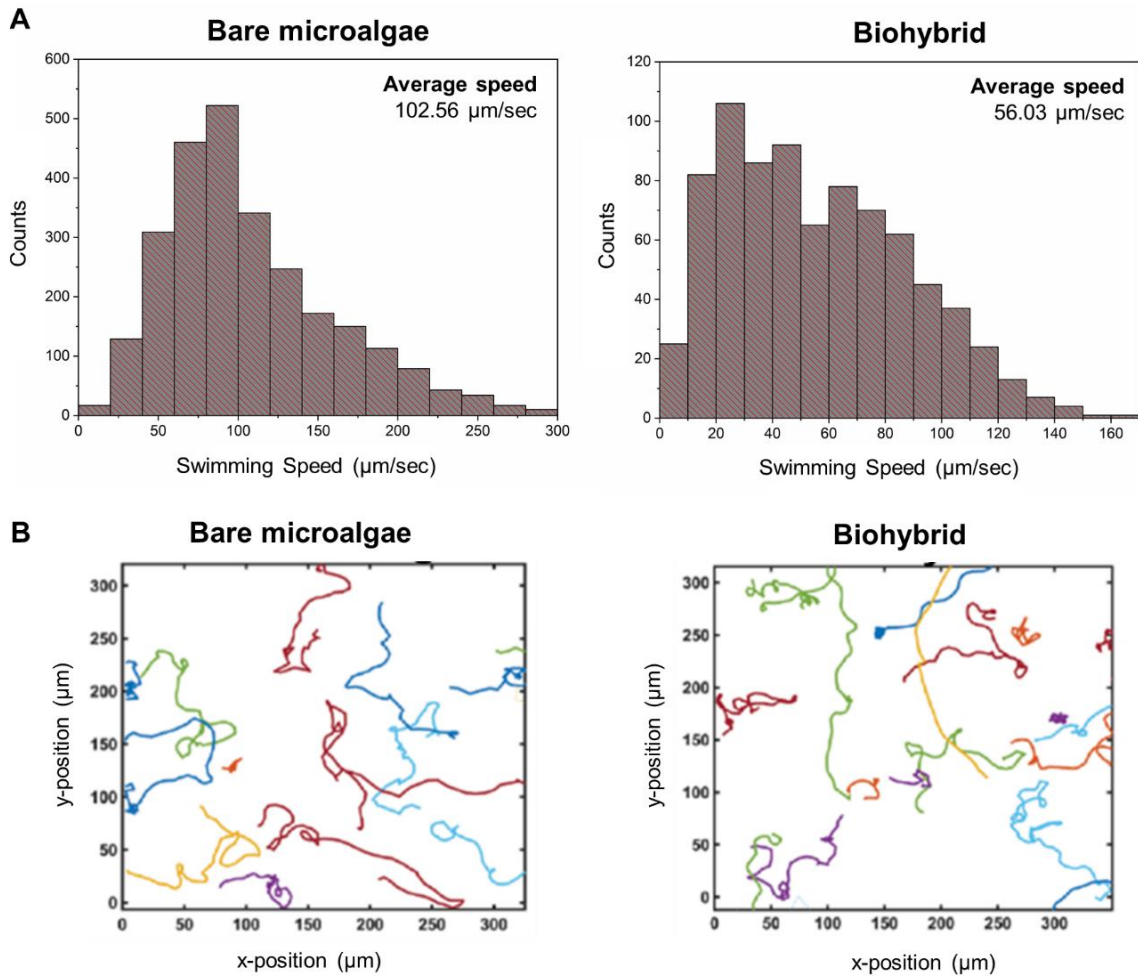


Figure 4. 7. Swimming speed analysis of *C.reinhardtii*. A) Average speeds of bare microalgae and biohybrid algal microswimmers. B) Swimming trajectories of bare microalgae and biohybrid algal microswimmers.

4.3.8. Light response of biohybrid algal microswimmers

Later, we were interested in whether coated microalgae were still responding light as a stimulus. Both bare microalgae and biohybrid algal microswimmers were feeded on microfluidic channels that has an axial length of 2 cm and monitored their migration ability towards light. First, light was applied on one end of the microchannel and they started to swim the illuminated area. When the light source was moved to the opposite end of the channel; they changed their swimming directions to the newly illuminated area. Images of bare microalgae and biohybrid algal microswimmers were demonstrated in Figure 4.8 at $t=0$, $t=10$ and $t=20$ min. Both bare microalgae and biohybrid algal microswimmers showed similar responses to light, they moved from dark side to the illuminated side of the channel (Figure 4.8).

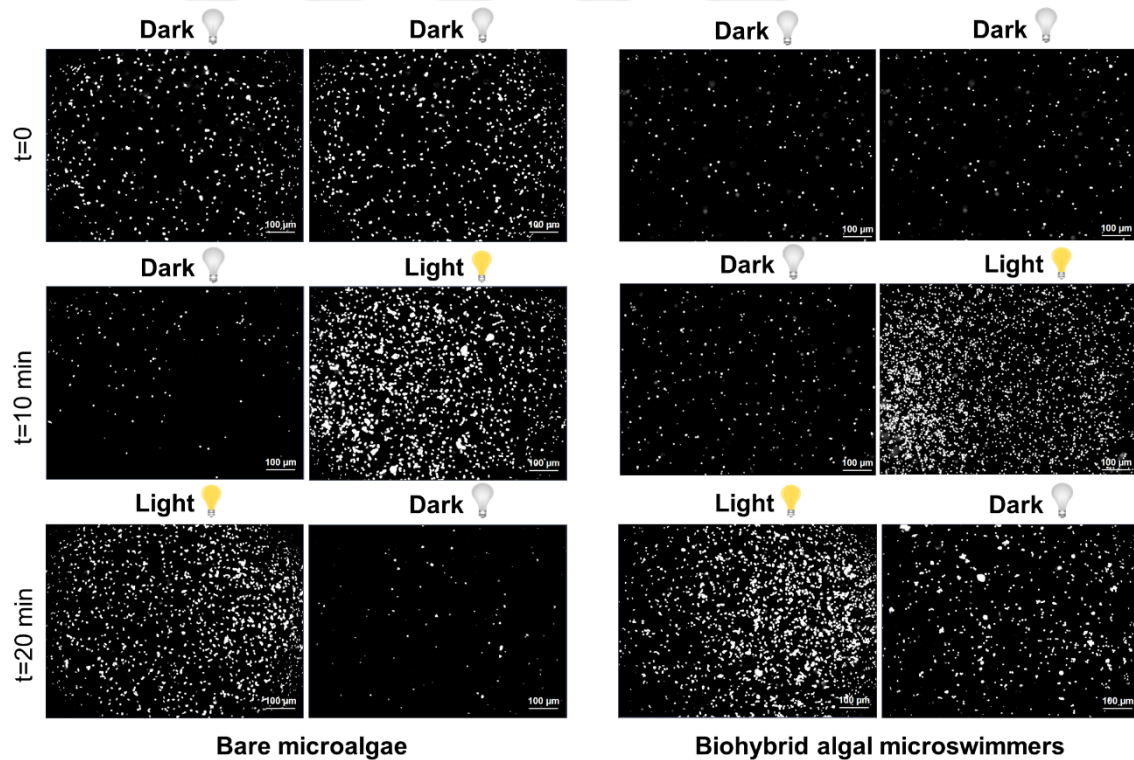


Figure 4. 8. Light response of bare microalgae and biohybrid algal microswimmers. Light was applied on two different ends of the microchannel separately. Images were taken at 10 min illumination.

The number of microalgae cells accumulated at illuminated side were quantified at $t=10$ min. Both bare microalgae and biohybrid algal microswimmers showed a great increase in the number of microalgae at illuminated sides even after 10 min (Figure 4.9). This experiment suggested that coating of microalgae with chitosan-SPIONPs mixture did not compromise the light response of *C.reinhardtii*.

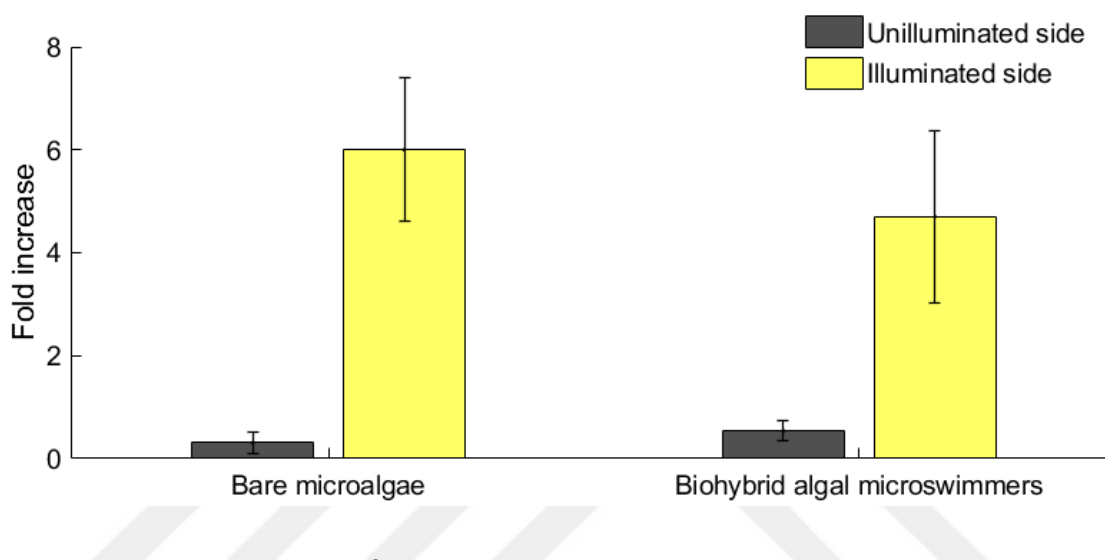


Figure 4. 9. Accumulation of bare microalgae and biohybrid algal microswimmers to the illuminated end of the channel.

4.3.9. Stability of microalgae coating

In addition, stability of coating on biohybrid algal microswimmers were further analyzed. The coating on algal microswimmers were checked at different time intervals. Even after 24 h, green and red fluorescence were visible which was an indication for the presence of both chitosan and SPIONPs on algal microswimmer surfaces. We also suggested that the reduced fluorescence at 24 h is due to the doubling time of *C. reinhardtii*, that is around 24 h (Figure 4.10).⁹⁵

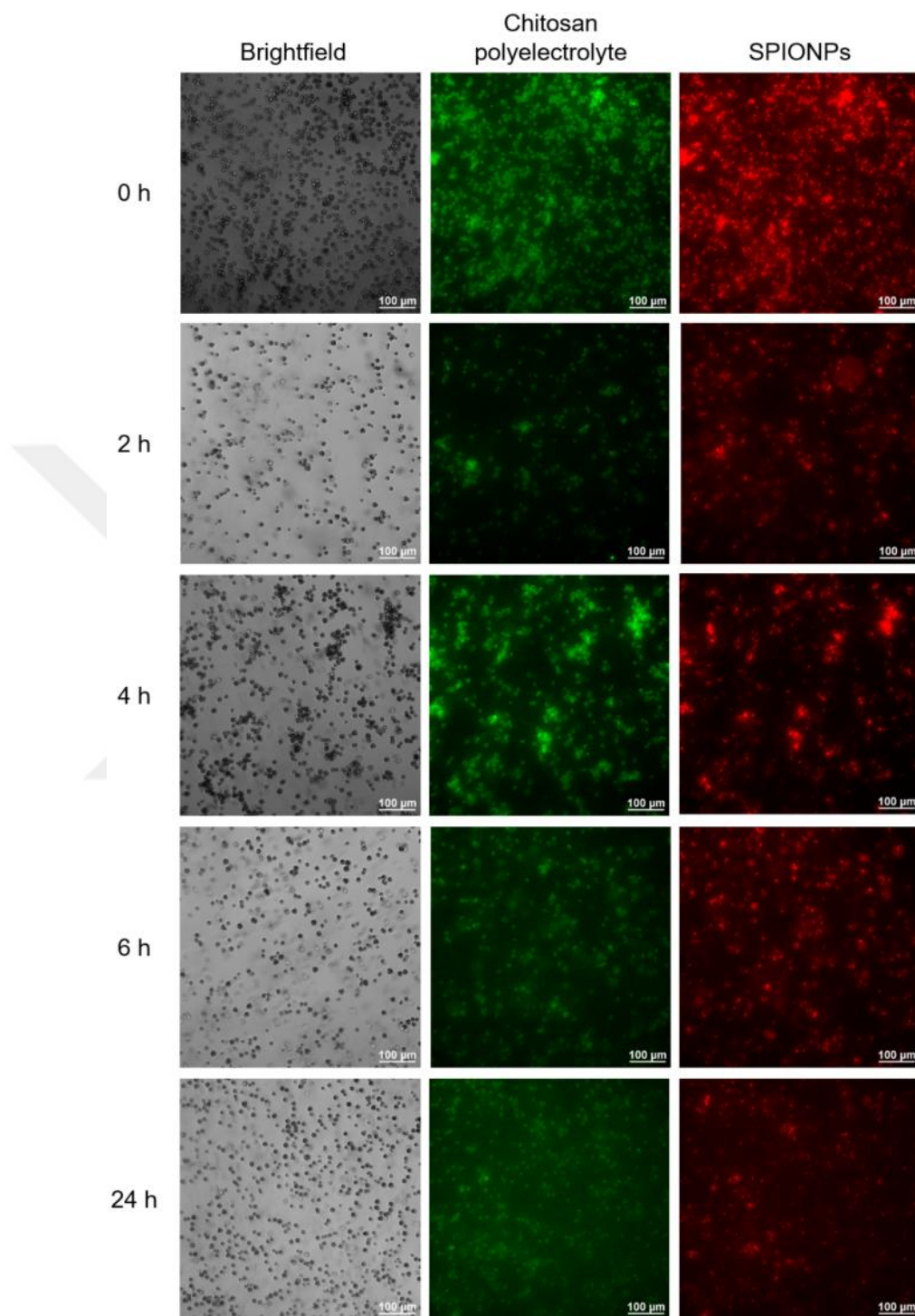


Figure 4. 10. Fluorescence microscopy images of coated microalgae at different time intervals. Green color represents chitosan polymer, and red color represents SPIONPs.

4.4. Conclusions and Future Outlook

Conventional drug delivery systems have limitations to overcome biological barriers due to; (i) nonspecific distributions of molecules towards off-target domains, (ii) the requirement of repeated drug administrations, (iii) difficulty in directing molecules towards hard-to-reach tissues. Recently, biohybrid microrobots in which living organisms attached to artificial structures have gained growing interest. Since biohybrid microrobots can be monitored and steered to the inaccessible cavities of human body, these systems have very important role for biomedical platforms especially for targeted drug delivery studies to overcome the disadvantages of conventional drug delivery systems.

Commonly, among various organism bacteria have been widely used in biohybrid microrobots. However, the risk to create pathogenicity, unavoidable growth rate of bacteria in physiological conditions lead to requirement to more biocompatible organisms such as microalgae. *C.reinhardtii* is a unicellular photosynthetic, phototactic green microalgae that has a high propulsion speed ranges between 100-200 $\mu\text{m}/\text{sec}$.

In this chapter, we reported a novel biohybrid algal microswimmer system in which positively charged chitosan polymer and SPIONPs attached to the negatively charged membrane of *C.reinhardtii* through electrostatic interactions. In the literature, most of the biohybrid microswimmer designs have low coating efficiency that is around 10%. However, the novel biohybrid microswimmer system that we reported in this chapter has high coating yield which is around 90%. Herein, first we indicated that SPIONPs could be attached to the surface of *C.reinhardtii* only in the existence of chitosan polyelectrolytes. We optimized experimental conditions without inhibiting the natural motility of *C.reinhardtii*. Furthermore, we showed that the novel biohybrid design had still light response behaviour and this ability of microalgae could be used to actuate them in a controlled manner. Overall, this novel biohybrid design is very promising and has a potential to be used in controlled drug release applications.

This novel biohybrid algal microswimmer design could be further improved as therapeutic delivery platform. Due to advances in chemistry of chitosan, SPIONPs could be attached with an anti-tumor drug, doxorubicin (DOX). Amino groups of SPIONPs could be functionalized with a photocleavable *o*-nitrobenzyl linker through NHS-amine reaction. Then, this complex could be conjugated to azide-modified DOX with the help of alkyne groups of photocleavable linker. DOX-conjugated SPIONPs could also be attached to the surface of microalgae in the presence of chitosan polyelectrolytes. Based on this chemistry, biohybrid algal microswimmers might have light-triggered DOX release mechanism at 365 nm. Since *C. reinhardtii* is cytocompatible with mammalian cells, this DOX-modified biohybrid algal microswimmers could be incubated with mammalian tumor cells and DOX could be released into cell culture supernatant to kill cancer cells. Furthermore, uptakes of DOX modified biohybrid algal microswimmers by macrophages could be investigated. Since microalgae would still have innate mobility with the addition of light and magnetic response behaviour, *in vivo* studies could be performed on these biohybrid microswimmers, where they could be actuated and steered into a specific location through either light or magnetic response to kill cancer tissue specifically.

CHAPTER 5: CONCLUSIONS

Biomaterial-based delivery systems have gained growing interest in treatment of several diseases. Some of these delivery systems were already commercialized and used as life-saving treatments for many patients. Unique properties of chitosan make it a suitable candidate as delivery vehicles which have wide range of applications from tissue engineering to water treatment. This thesis focuses on gene delivery and microrobotic applications of chitosan.

In the first part, plasmid DNA encapsulated and PEGylated chitosan NPs were synthesized using different PEG chain lengths and PEG concentrations. For the first time in the literature, ANN model was used to analyze PEG chain length, PEG concentration and NP concentration on transfection capabilities of CS-PEG-TRAIL pDNA NPs in detail. Here, PEGylation was used to promote poor solubility and poor stability of chitosan NPs at physiological conditions which enhances potential of chitosan-based nanocarriers in biomedical applications. However, the therapeutic efficiency of CS-PEG-TRAIL pDNA NPs should still be improved to enhance their cellular uptake.

In the second part of this thesis, we indicated that chitosan is also a promising candidate for microrobotic applications. Here, a novel biohybrid algal microswimmer design were reported in which *C.reinhardtii* were coated with chitosan polyelectrolytes and SPIONPs. This novel biohybrid algal microswimmers has excellent properties in terms of high coating yield that is commonly lack in other biohybrid designs found in the literature. Besides, the reported novel biohybrid design is very promising for controlled drug delivery applications due to the easiness of modifying chemistry chitosan.

BIBLIOGRAPHY

1. VandeVord, P. J.; Matthew, H. W.; DeSilva, S. P.; Mayton, L.; Wu, B.; Wooley, P. H., Evaluation of the biocompatibility of a chitosan scaffold in mice. *Journal of Biomedical Materials Research* **2002**, 59 (3), 585-590.
2. Kean, T.; Thanou, M., Biodegradation, biodistribution and toxicity of chitosan. *Advanced Drug Delivery Reviews* **2010**, 62 (1), 3-11.
3. Nagpal, K.; Singh, S. K.; Mishra, D. N., Chitosan nanoparticles: A promising system in novel drug delivery. *Chemical and Pharmaceutical Bulletin* **2010**, 58 (11), 1423-1430.
4. Jayakumar, R.; Menon, D.; Manzoor, K.; Nair, S.; Tamura, H., Biomedical applications of chitin and chitosan based nanomaterials—A short review. *Carbohydrate Polymers* **2010**, 82 (2), 227-232.
5. Singh, R.; Lillard Jr, J. W., Nanoparticle-based targeted drug delivery. *Experimental and Molecular Pathology* **2009**, 86 (3), 215-223.
6. Gelperina, S.; Kisich, K.; Iseman, M. D.; Heifets, L., The potential advantages of nanoparticle drug delivery systems in chemotherapy of tuberculosis. *American Journal of Respiratory and Critical Care Medicine* **2005**, 172 (12), 1487-1490.
7. Carlsen, R. W.; Sitti, M., Bio-hybrid cell-based actuators for microsystems. *Small* **2014**, 10 (19), 3831-3851.
8. Hosseinidoust, Z.; Mostaghaci, B.; Yasa, O.; Park, B.-W.; Singh, A. V.; Sitti, M., Bioengineered and biohybrid bacteria-based systems for drug delivery. *Advanced Drug Delivery Reviews* **2016**, 106, 27-44.
9. Min, B.-M.; Lee, S. W.; Lim, J. N.; You, Y.; Lee, T. S.; Kang, P. H.; Park, W. H., Chitin and chitosan nanofibers: Electrospinning of chitin and deacetylation of chitin nanofibers. *Polymer* **2004**, 45 (21), 7137-7142.
10. Rinaudo, M., Chitin and chitosan: Properties and applications. *Progress in Polymer Science* **2006**, 31 (7), 603-632.
11. Cheung, R. C. F.; Ng, T. B.; Wong, J. H.; Chan, W. Y., Chitosan: An update on potential biomedical and pharmaceutical applications. *Marine Drugs* **2015**, 13 (8), 5156-5186.

12. Younes, I.; Rinaudo, M., Chitin and chitosan preparation from marine sources. Structure, properties and applications. *Marine Drugs* **2015**, *13* (3), 1133-1174.
13. Lehr, C.-M.; Bouwstra, J. A.; Schacht, E. H.; Junginger, H. E., In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers. *International Journal of Pharmaceutics* **1992**, *78* (1-3), 43-48.
14. Zheng, L.-Y.; Zhu, J.-F., Study on antimicrobial activity of chitosan with different molecular weights. *Carbohydrate Polymers* **2003**, *54* (4), 527-530.
15. Rabea, E. I.; Badawy, M. E.-T.; Stevens, C. V.; Smagghe, G.; Steurbaut, W., Chitosan as antimicrobial agent: Applications and mode of action. *Biomacromolecules* **2003**, *4* (6), 1457-1465.
16. Azuma, K.; Osaki, T.; Minami, S.; Okamoto, Y., Anticancer and anti-inflammatory properties of chitin and chitosan oligosaccharides. *Journal of Functional Biomaterials* **2015**, *6* (1), 33-49.
17. Yen, M.-T.; Yang, J.-H.; Mau, J.-L., Antioxidant properties of chitosan from crab shells. *Carbohydrate Polymers* **2008**, *74* (4), 840-844.
18. Kumar, M. R.; Muzzarelli, R. A.; Muzzarelli, C.; Sashiwa, H.; Domb, A., Chitosan chemistry and pharmaceutical perspectives. *Chemical Reviews* **2004**, *104* (12), 6017-6084.
19. Croisier, F.; Jérôme, C., Chitosan-based biomaterials for tissue engineering. *European Polymer Journal* **2013**, *49* (4), 780-792.
20. Ueno, H.; Mori, T.; Fujinaga, T., Topical formulations and wound healing applications of chitosan. *Advanced Drug Delivery Reviews* **2001**, *52* (2), 105-115.
21. Erbacher, P.; Zou, S.; Bettinger, T.; Steffan, A.-M.; Remy, J.-S., Chitosan-based vector/DNA complexes for gene delivery: Biophysical characteristics and transfection ability. *Pharmaceutical Research* **1998**, *15* (9), 1332-1339.
22. Bhattarai, N.; Gunn, J.; Zhang, M., Chitosan-based hydrogels for controlled, localized drug delivery. *Advanced Drug Delivery Reviews* **2010**, *62* (1), 83-99.
23. Tabata, Y., Biomaterial technology for tissue engineering applications. *Journal of the Royal Society Interface* **2009**, *6* (3), S311-S324.
24. Kim, I.-Y.; Seo, S.-J.; Moon, H.-S.; Yoo, M.-K.; Park, I.-Y.; Kim, B.-C.; Cho, C.-S., Chitosan and its derivatives for tissue engineering applications. *Biotechnology Advances* **2008**, *26* (1), 1-21.

25. Ma, L.; Gao, C.; Mao, Z.; Zhou, J.; Shen, J.; Hu, X.; Han, C., Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering. *Biomaterials* **2003**, 24 (26), 4833-4841.
26. Santos-Carballal, B.; Fernández Fernández, E.; Goycoolea, F. M., Chitosan in non-viral gene delivery: Role of structure, characterization methods, and insights in cancer and rare diseases therapies. *Polymers* **2018**, 10 (4), 444.
27. Nayerossadat, N.; Maedeh, T.; Ali, P. A., Viral and nonviral delivery systems for gene delivery. *Advanced Biomedical Research* **2012**, 1.
28. Al-Dosari, M. S.; Gao, X., Nonviral gene delivery: principle, limitations, and recent progress. *The AAPS Journal* **2009**, 11 (4), 671-681.
29. Ramamoorth, M.; Narvekar, A., Non viral vectors in gene therapy-An overview. *Journal of Clinical and Diagnostic Research: JCDR* **2015**, 9 (1), GE01-GE06.
30. Seow, Y.; Wood, M. J., Biological gene delivery vehicles: Beyond viral vectors. *Molecular Therapy* **2009**, 17 (5), 767-777.
31. Guo, X.; Huang, L., Recent advances in nonviral vectors for gene delivery. *Accounts of Chemical Research* **2011**, 45 (7), 971-979.
32. Prabakaran, M., Chitosan-based nanoparticles for tumor-targeted drug delivery. *International Journal of Biological Macromolecules* **2015**, 72, 1313-1322.
33. Hoshyar, N.; Gray, S.; Han, H.; Bao, G., The effect of nanoparticle size on in vivo pharmacokinetics and cellular interaction. *Nanomedicine* **2016**, 11 (6), 673-692.
34. Peer, D.; Karp, J. M.; Hong, S.; Farokhzad, O. C.; Margalit, R.; Langer, R., Nanocarriers as an emerging platform for cancer therapy. *Nature Nanotechnology* **2007**, 2 (12), 751-760.
35. Greish, K., Enhanced permeability and retention (EPR) effect for anticancer nanomedicine drug targeting. *Cancer Nanotechnology* **2010**, 624, 25-37.
36. Jayakumar, R.; Chennazhi, K.; Muzzarelli, R.; Tamura, H.; Nair, S.; Selvamurugan, N., Chitosan conjugated DNA nanoparticles in gene therapy. *Carbohydrate Polymers* **2010**, 79 (1), 1-8.
37. Wang, Z.; Wang, Z.; Lu, W. W.; Zhen, W.; Yang, D.; Peng, S., Novel biomaterial strategies for controlled growth factor delivery for biomedical applications. *Npg Asia Materials* **2017**, 9 (10), 435.
38. Calvo, P.; Remunan-Lopez, C.; Vila-Jato, J. L.; Alonso, M., Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers. *Journal of Applied Polymer Science* **1997**, 63 (1), 125-132.

39. Divya, K.; Jisha, M., Chitosan nanoparticles preparation and applications. *Environmental Chemistry Letters* **2018**, *16* (1), 101-112.
40. Maitra, A.; Ghosh, P. K.; De, T. K.; Sahoo, S. K., Process for the preparation of highly monodispersed polymeric hydrophilic nanoparticles. Google Patents: 1999.
41. El-Shabouri, M., Positively charged nanoparticles for improving the oral bioavailability of cyclosporin-A. *International Journal of Pharmaceutics* **2002**, *249* (1-2), 101-108.
42. Brunel, F.; Véron, L.; David, L.; Domard, A.; Delair, T., A novel synthesis of chitosan nanoparticles in reverse emulsion. *Langmuir* **2008**, *24* (20), 11370-11377.
43. Bozuyuk, U.; Gokulu, I. S.; Dogan, N. O.; Kizilel, S., A novel method for PEGylation of chitosan nanoparticles through photopolymerization. *RSC Advances* **2019**, *9* (25), 14011-14015.
44. Lee, S.-T.; Mi, F.-L.; Shen, Y.-J.; Shyu, S.-S., Equilibrium and kinetic studies of copper (II) ion uptake by chitosan-tripolyphosphate chelating resin. *Polymer* **2001**, *42* (5), 1879-1892.
45. Sogias, I. A.; Khutoryanskiy, V. V.; Williams, A. C., Exploring the factors affecting the solubility of chitosan in water. *Macromolecular Chemistry and Physics* **2010**, *211* (4), 426-433.
46. Bozuyuk, U.; Dogan, N. O.; Kizilel, S., Deep insight into PEGylation of bioadhesive chitosan nanoparticles: Sensitivity study for the key parameters through artificial neural network model. *ACS Applied Materials & Interfaces* **2018**, *10* (40), 33945-33955.
47. Kim, Y.-J.; Chae, S. Y.; Jin, C.-H.; Sivasubramanian, M.; Son, S.; Choi, K. Y.; Jo, D.-G.; Kim, K.; Kwon, I. C.; Lee, K. C., Ionic complex systems based on hyaluronic acid and PEGylated TNF-related apoptosis-inducing ligand for treatment of rheumatoid arthritis. *Biomaterials* **2010**, *31* (34), 9057-9064.
48. Casettari, L.; Vllasaliu, D.; Castagnino, E.; Stolnik, S.; Howdle, S.; Illum, L., PEGylated chitosan derivatives: Synthesis, characterizations and pharmaceutical applications. *Progress in Polymer Science* **2012**, *37* (5), 659-685.
49. Malhotra, M.; Tomaro-Duchesneau, C.; Saha, S.; Kahouli, I.; Prakash, S., Development and characterization of chitosan-PEG-TAT nanoparticles for the intracellular delivery of siRNA. *International Journal of Nanomedicine* **2013**, *8*, 2041-2052.
50. Quiñones, J. P.; Peniche, H.; Peniche, C., Chitosan based self-assembled nanoparticles in drug delivery. *Polymers* **2018**, *10* (3), 235.

51. Cheung, R.; Ng, T.; Wong, J.; Chan, W., Chitosan: An update on potential biomedical and pharmaceutical applications. *Marine Drugs* **2015**, *13* (8), 5156-5186.
52. Ways, M.; Mohammed, T.; Lau, W. M.; Khutoryanskiy, V. V., Chitosan and its derivatives for application in mucoadhesive drug delivery systems. *Polymers* **2018**, *10* (3), 267.
53. Bernkop-Schnürch, A.; Dünnhaupt, S., Chitosan-based drug delivery systems. *European Journal of Pharmaceutics and Biopharmaceutics* **2012**, *81* (3), 463-469.
54. Gupta, S.; VYAS, S., Carbopol/chitosan based pH triggered in situ gelling system for ocular delivery of timolol maleate. *Scientia Pharmaceutica* **2010**, *78* (4), 959-976.
55. Schipper, N. G.; Olsson, S.; Hoogstraate, J. A.; deBoer, A. G.; Vårum, K. M.; Artursson, P., Chitosans as absorption enhancers for poorly absorbable drugs 2: Mechanism of absorption enhancement. *Pharmaceutical Research* **1997**, *14* (7), 923-929.
56. Erkoc, P.; Yasa, I. C.; Ceylan, H.; Yasa, O.; Alapan, Y.; Sitti, M., Mobile microrobots for active therapeutic delivery. *Advanced Therapeutics* **2019**, *2* (1), 1800064.
57. Alapan, Y.; Yasa, O.; Yigit, B.; Yasa, I. C.; Erkoc, P.; Sitti, M., Microrobotics and Microorganisms: Biohybrid Autonomous Cellular Robots. *Annual Review of Control, Robotics, and Autonomous Systems* **2019**, *2*, 205-230.
58. Han, J.-W.; Choi, Y. J.; Cho, S.; Zheng, S.; Ko, S. Y.; Park, J.-O.; Park, S., Active tumor-therapeutic liposomal bacteriobot combining a drug (paclitaxel)-encapsulated liposome with targeting bacteria (Salmonella Typhimurium). *Sensors and Actuators B: Chemical* **2016**, *224*, 217-224.
59. Yan, X.; Zhou, Q.; Vincent, M.; Deng, Y.; Yu, J.; Xu, J.; Xu, T.; Tang, T.; Bian, L.; Wang, Y.-X. J., Multifunctional biohybrid magnetite microrobots for imaging-guided therapy. *Science Robotics* **2017**, *2* (12), eaaq1155.
60. Yasa, O.; Erkoc, P.; Alapan, Y.; Sitti, M., Microalga-powered microswimmers toward active cargo delivery. *Advanced Materials* **2018**, *30* (45), 1804130.
61. Harris, E. H., Chlamydomonas as a model organism. *Annual Review of Plant Biology* **2001**, *52* (1), 363-406.
62. Jeanneret, R.; Pushkin, D. O.; Kantsler, V.; Polin, M., Entrainment dominates the interaction of microalgae with micron-sized objects. *Nature Communications* **2016**, *7*, 12518.
63. Corsi, K.; Chellat, F.; Yahia, L. H.; Fernandes, J. C., Mesenchymal stem cells, MG63 and HEK293 transfection using chitosan-DNA nanoparticles. *Biomaterials* **2003**, *24* (7), 1255-1264.

64. Perlstein, B.; Finniss, S. A.; Miller, C.; Okhrimenko, H.; Kazimirsky, G.; Cazacu, S.; Lee, H. K.; Lemke, N.; Brodie, S.; Umansky, F., TRAIL conjugated to nanoparticles exhibits increased anti-tumor activities in glioma cells and glioma stem cells in vitro and in vivo. *Neuro-oncology* **2012**, *15* (1), 29-40.
65. Onishi, H.; Machida, Y., Biodegradation and distribution of water-soluble chitosan in mice. *Biomaterials* **1999**, *20* (2), 175-182.
66. Tomihata, K.; Ikada, Y., In vitro and in vivo degradation of films of chitin and its deacetylated derivatives. *Biomaterials* **1997**, *18* (7), 567-575.
67. Kim, J. K.; Choi, S. H.; Kim, C. O.; Park, J. S.; Ahn, W. S.; Kim, C. K., Enhancement of polyethylene glycol (PEG)-modified cationic liposome-mediated gene deliveries: Effects on serum stability and transfection efficiency. *Journal of Pharmacy and Pharmacology* **2003**, *55* (4), 453-460.
68. Walkey, C. D.; Olsen, J. B.; Guo, H.; Emili, A.; Chan, W. C., Nanoparticle size and surface chemistry determine serum protein adsorption and macrophage uptake. *Journal of the American Chemical Society* **2012**, *134* (4), 2139-2147.
69. Cruje, C.; Chithrani, D., Polyethylene glycol density and length affects nanoparticle uptake by cancer cells. *Journal of Nanomedicine Research* **2014**, *1* (00006).
70. Pozzi, D.; Colapicchioni, V.; Caracciolo, G.; Piovesana, S.; Capriotti, A. L.; Palchetti, S.; De Grossi, S.; Riccioli, A.; Amenitsch, H.; Laganà, A., Effect of polyethyleneglycol (PEG) chain length on the bio-nano-interactions between PEGylated lipid nanoparticles and biological fluids: From nanostructure to uptake in cancer cells. *Nanoscale* **2014**, *6* (5), 2782-2792.
71. Yang, C.; Gao, S.; Dagnæs-Hansen, F.; Jakobsen, M.; Kjems, J., Impact of PEG chain length on the physical properties and bioactivity of PEGylated chitosan/siRNA nanoparticles in vitro and in vivo. *ACS Applied Materials & Interfaces* **2017**, *9* (14), 12203-12216.
72. Amani, A.; Mohammadyani, D., Artificial neural networks: applications in nanotechnology. In *Artificial Neural Networks-Application*, IntechOpen: 2011.
73. Amani, A.; York, P.; Chrystyn, H.; Clark, B. J., Factors affecting the stability of nanoemulsions—use of artificial neural networks. *Pharmaceutical Research* **2010**, *27* (1), 37-45.
74. Baharifar, H.; Amani, A., Cytotoxicity of chitosan/streptokinase nanoparticles as a function of size: An artificial neural networks study. *Nanomedicine: Nanotechnology, Biology and Medicine* **2016**, *12* (1), 171-180.

75. Hashad, R. A.; Ishak, R. A.; Fahmy, S.; Mansour, S.; Geneidi, A. S., Chitosan-tripolyphosphate nanoparticles: Optimization of formulation parameters for improving process yield at a novel pH using artificial neural networks. *International Journal of Biological Macromolecules* **2016**, *86*, 50-58.
76. Bagci-Onder, T.; Agarwal, A.; Flusberg, D.; Wanningen, S.; Sorger, P.; Shah, K., Real-time imaging of the dynamics of death receptors and therapeutics that overcome TRAIL resistance in tumors. *Oncogene* **2013**, *32* (23), 2818-2827.
77. Erkoç, P.; Cingöz, A.; Bagci-Onder, T.; Kizilel, S., Quinacrine Mediated Sensitization of Glioblastoma (GBM) Cells to TRAIL through MMP-Sensitive PEG Hydrogel Carriers. *Macromolecular Bioscience* **2017**, *17* (2), 1600267.
78. Kurt, I. C.; Sur, I.; Kaya, E.; Cingoz, A.; Kazancioglu, S.; Kahya, Z.; Toparlak, O. D.; Senbabaoglu, F.; Kaya, Z.; Ozyerli, E., KDM2B, an H3K36-specific demethylase, regulates apoptotic response of GBM cells to TRAIL. *Cell Death & Disease* **2017**, *8* (6), e2897.
79. Senbabaoglu, F.; Cingoz, A.; Kaya, E.; Kazancioglu, S.; Lack, N. A.; Acilan, C.; Bagci-Onder, T., Identification of Mitoxantrone as a TRAIL-sensitizing agent for Glioblastoma Multiforme. *Cancer Biology & Therapy* **2016**, *17* (5), 546-557.
80. Shao, J., Linear model selection by cross-validation. *Journal of the American Statistical Association* **1993**, *88* (422), 486-494.
81. Cline, G. W.; Hanna, S. B., Kinetics and mechanisms of the aminolysis of N-hydroxysuccinimide esters in aqueous buffers. *The Journal of Organic Chemistry* **1988**, *53* (15), 3583-3586.
82. Le, Y.; Scott, M. D., Immunocamouflage: the biophysical basis of immunoprotection by grafted methoxypoly (ethylene glycol)(mPEG). *Acta Biomaterialia* **2010**, *6* (7), 2631-2641.
83. Henner, W. D.; Kleber, I.; Benzinger, R., Transfection of Escherichia coli spheroplasts III. facilitation of transfection and stabilization of spheroplasts by different basic polymers. *Journal of Virology* **1973**, *12* (4), 741-747.
84. Morita, J.; Nishida, T.; Komano, T., Effect of polyethylene glycol on transfection of Escherichia coli spheroplasts with bacteriophage øX174 single-stranded and double-stranded DNAs. *Agricultural and Biological Chemistry* **1977**, *41* (3), 567-571.
85. Givan, A. L., Flow cytometry. *Methods in Molecular Biology: Flow Cytometry Protocols*, TSHaRG Hawley, Editor, Humana Press Inc **1992**, 1-31.

86. Sun, C.; Tang, T.; Uludağ, H.; Cuervo, J. E., Molecular dynamics simulations of DNA/PEI complexes: Effect of PEI branching and protonation state. *Biophysical Journal* **2011**, *100* (11), 2754-2763.
87. Benjaminsen, R. V.; Matthebjerg, M. A.; Henriksen, J. R.; Moghimi, S. M.; Andresen, T. L., The possible “proton sponge” effect of polyethylenimine (PEI) does not include change in lysosomal pH. *Molecular Therapy* **2013**, *21* (1), 149-157.
88. Kievit, F. M.; Veisheh, O.; Bhattarai, N.; Fang, C.; Gunn, J. W.; Lee, D.; Ellenbogen, R. G.; Olson, J. M.; Zhang, M., PEI–PEG–chitosan-copolymer-coated iron oxide nanoparticles for safe gene delivery: Synthesis, complexation, and transfection. *Advanced Functional Materials* **2009**, *19* (14), 2244-2251.
89. Martel, S., Bacterial microsystems and microrobots. *Biomedical Microdevices* **2012**, *14* (6), 1033-1045.
90. Park, S. J.; Park, S.-H.; Cho, S.; Kim, D.-M.; Lee, Y.; Ko, S. Y.; Hong, Y.; Choy, H. E.; Min, J.-J.; Park, J.-O., New paradigm for tumor theranostic methodology using bacteria-based microrobot. *Scientific Reports* **2013**, *3*, 3394.
91. Santomauro, G.; Singh, A. V.; Park, B. W.; Mohammadrahimi, M.; Erkoc, P.; Goering, E.; Schütz, G.; Sitti, M.; Bill, J., Incorporation of terbium into a microalga leads to magnetotactic swimmers. *Advanced Biosystems* **2018**, *2* (12), 1800039.
92. Weibel, D. B.; Garstecki, P.; Ryan, D.; DiLuzio, W. R.; Mayer, M.; Seto, J. E.; Whitesides, G. M., Microoxen: Microorganisms to move microscale loads. *Proceedings of the National Academy of Sciences* **2005**, *102* (34), 11963-11967.
93. Xie, S.; Jiao, N.; Tung, S.; Liu, L., Controlled regular locomotion of algae cell microrobots. *Biomedical Microdevices* **2016**, *18* (3), 47.
94. Rasala, B. A.; Barrera, D. J.; Ng, J.; Plucinak, T. M.; Rosenberg, J. N.; Weeks, D. P.; Oyler, G. A.; Peterson, T. C.; Haerizadeh, F.; Mayfield, S. P., Expanding the spectral palette of fluorescent proteins for the green microalga *Chlamydomonas reinhardtii*. *The Plant Journal* **2013**, *74* (4), 545-556.
95. Cross, F. R.; Umen, J. G., The *Chlamydomonas* cell cycle. *The Plant Journal* **2015**, *82* (3), 370-392.

APPENDIX A: Supplementary Information for Chapter 3

RAW DLS RESULTS FOR TEST AND TRAIN DATA

1) 2 kDa – 12 μ mole PEG – pH 4.8

a) Size

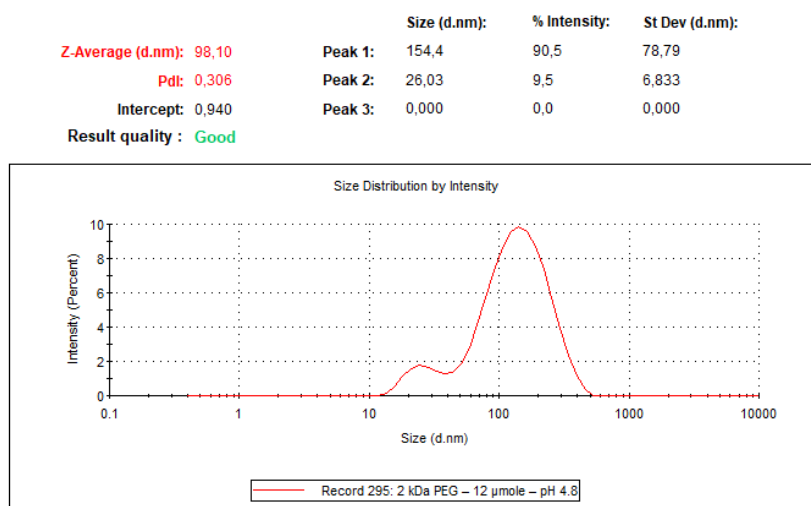


Figure A.1. Size of 2 kDa – 12 μ mole PEG – pH 4.8.

b) Zeta Potential

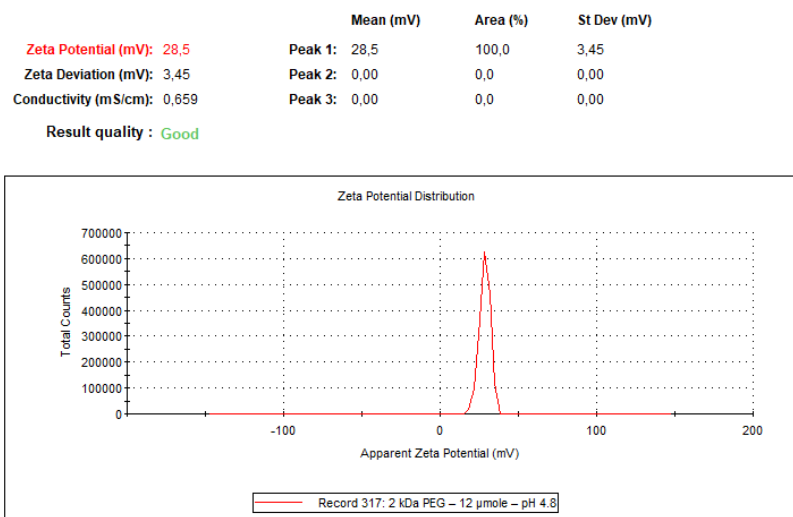


Figure A.2. Zeta potential of 2 kDa – 12 μ mole PEG – pH 4.8.

2) 2 kDa – 12 μ mole PEG – pH 6.5

a) Size

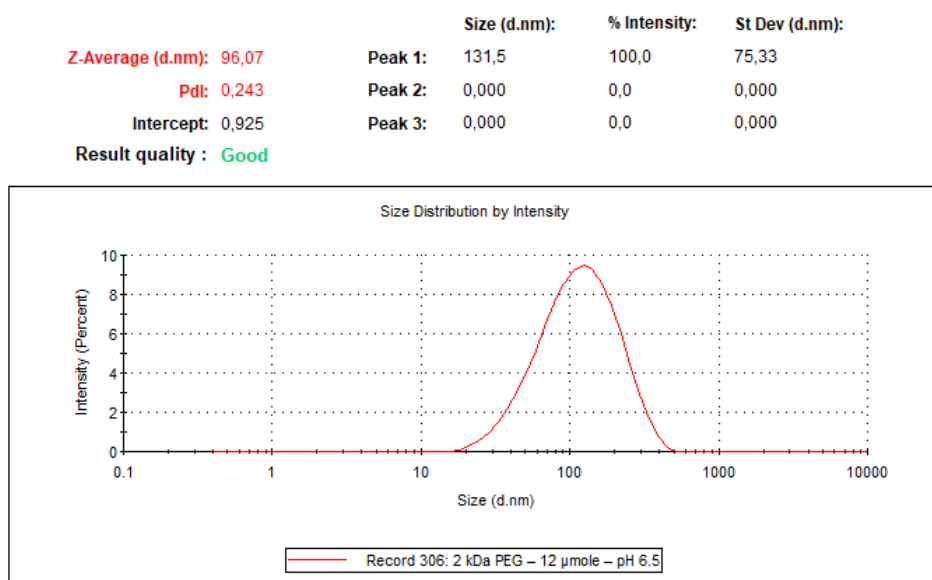


Figure A.3. Size of 2 kDa – 12 μ mole PEG – pH 6.5.

b) Zeta Potential

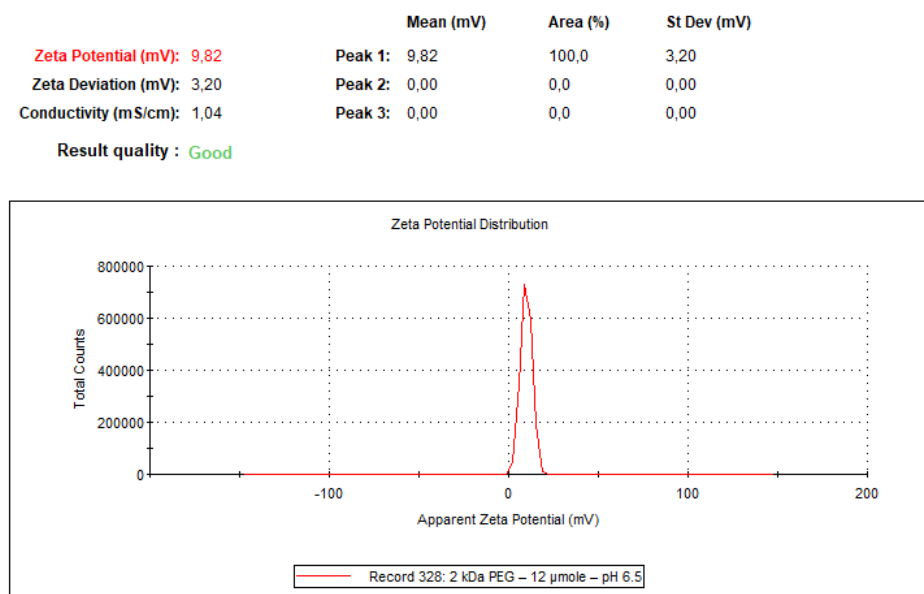


Figure A.4. Zeta potential of 2 kDa – 12 μ mole PEG – pH 6.5.

3) 2 kDa – 15 μ mole PEG – pH 4.8

a) Size

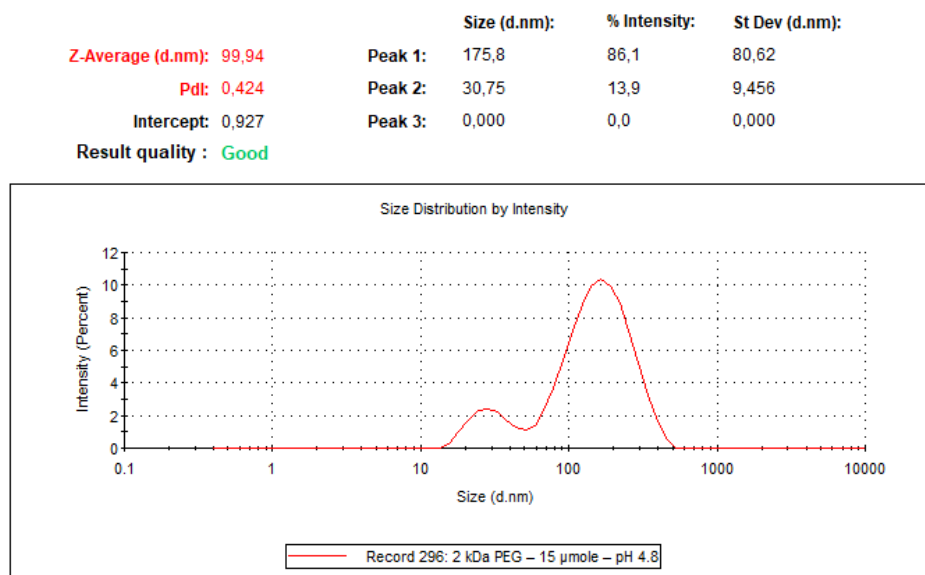


Figure A.5. Size of 2 kDa – 15 μ mole PEG – pH 4.8.

b) Zeta Potential

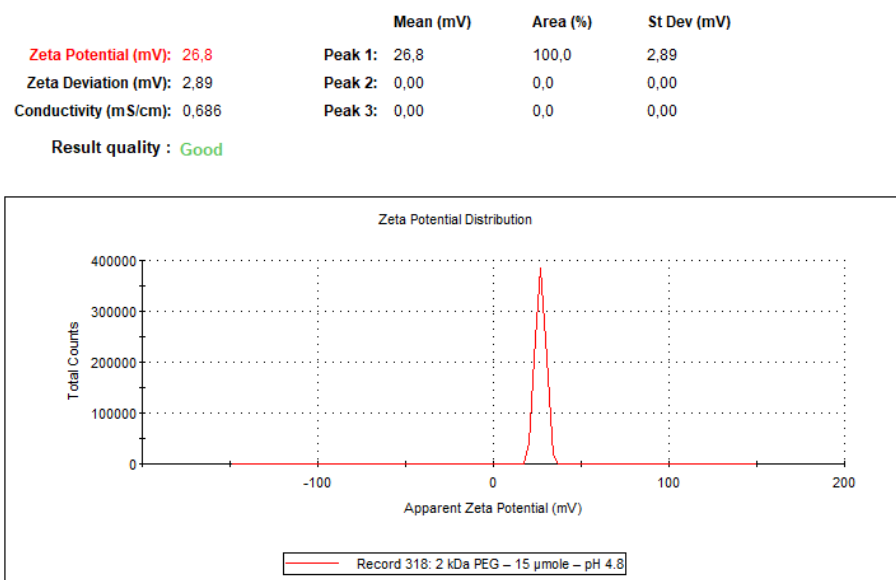


Figure A.6. Zeta potential of 2 kDa – 15 μ mole PEG – pH 4.8.

4) 2 kDa – 15 μ mole PEG – pH 6.5

a) Size

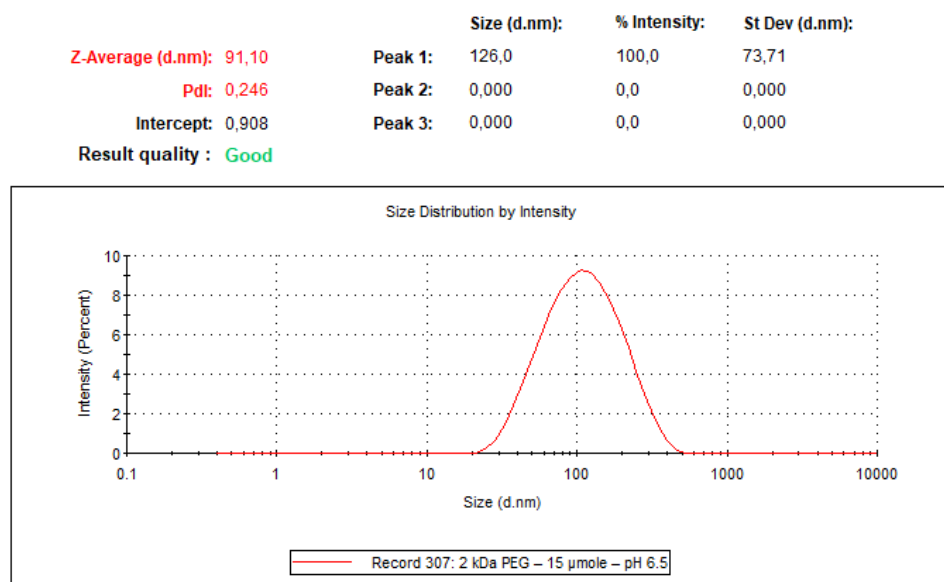


Figure A.7. Size of 2 kDa – 15 μ mole PEG – pH 6.5.

b) Zeta Potential

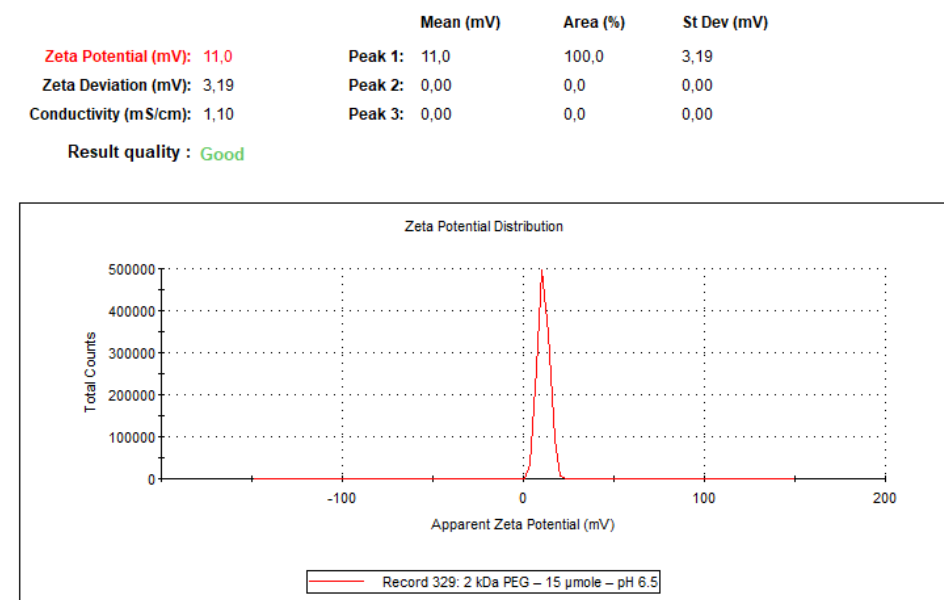


Figure A.8. Zeta potential of 2 kDa – 15 μ mole PEG – pH 6.5.

5) 2 kDa – 20 μ mole PEG – pH 4.8

a) Size

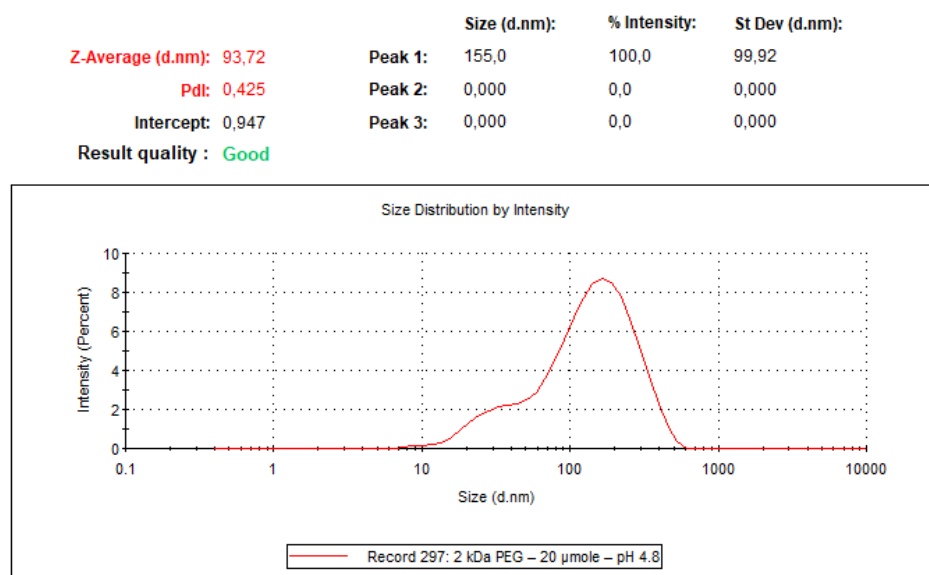


Figure A.9. Size of 2 kDa – 20 μ mole PEG – pH 4.8.

b) Zeta Potential

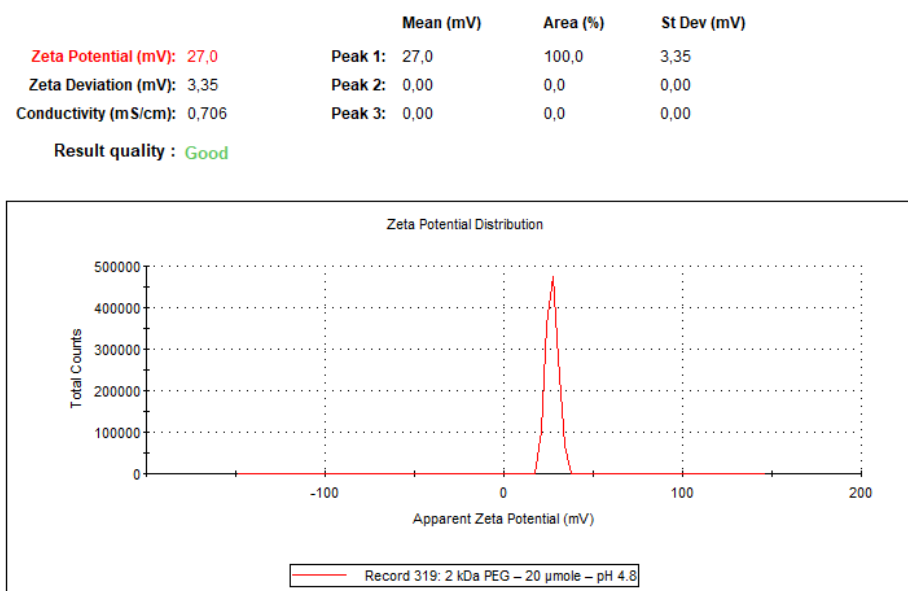


Figure A.10. Zeta potential of 2 kDa – 20 μ mole PEG – pH 4.8.

6) 2 kDa – 20 μ mole PEG – pH 6.5

a) Size

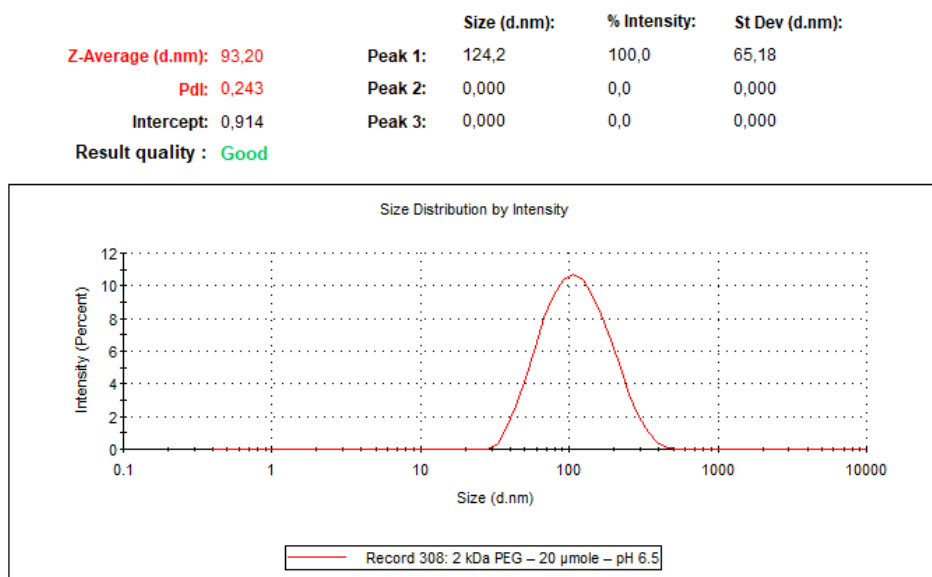


Figure A.11. Size of 2 kDa – 20 μ mole PEG – pH 6.5.

b) Zeta Potential

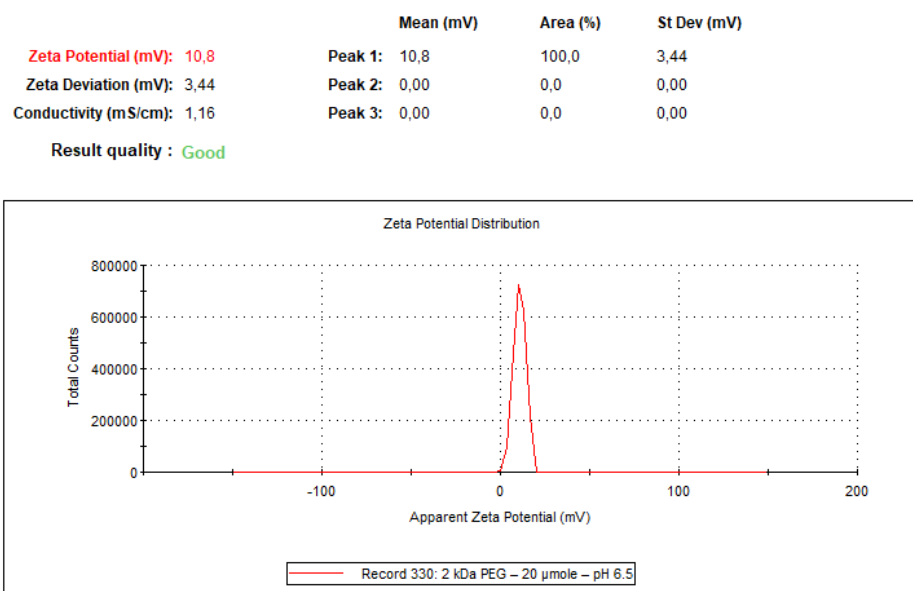


Figure A.12. Zeta potential of 2 kDa – 20 μ mole PEG – pH 6.5.

7) 5 kDa – 4 μ mole PEG – pH 4.8

a) Size

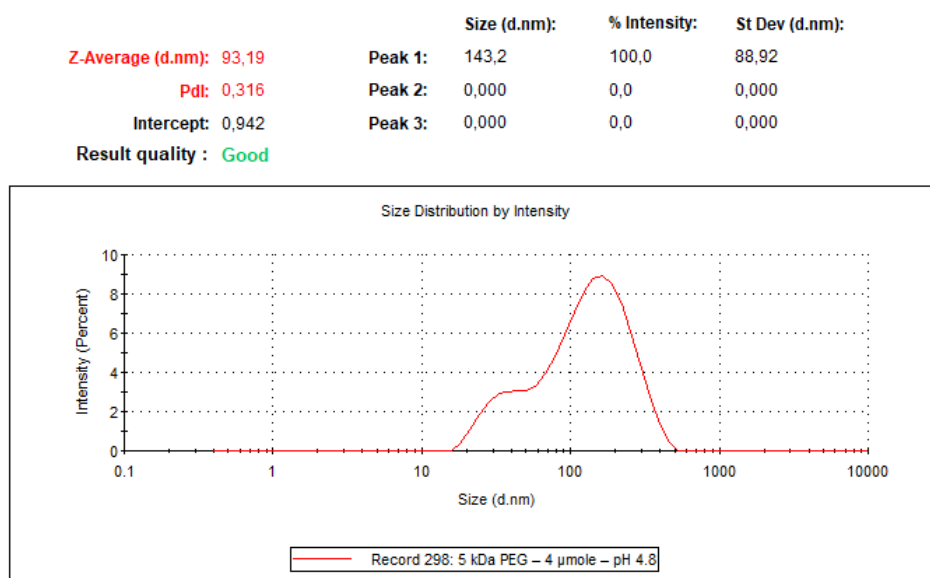


Figure A.13. Size of 5 kDa – 4 μ mole PEG – pH 4.8.

b) Zeta Potential

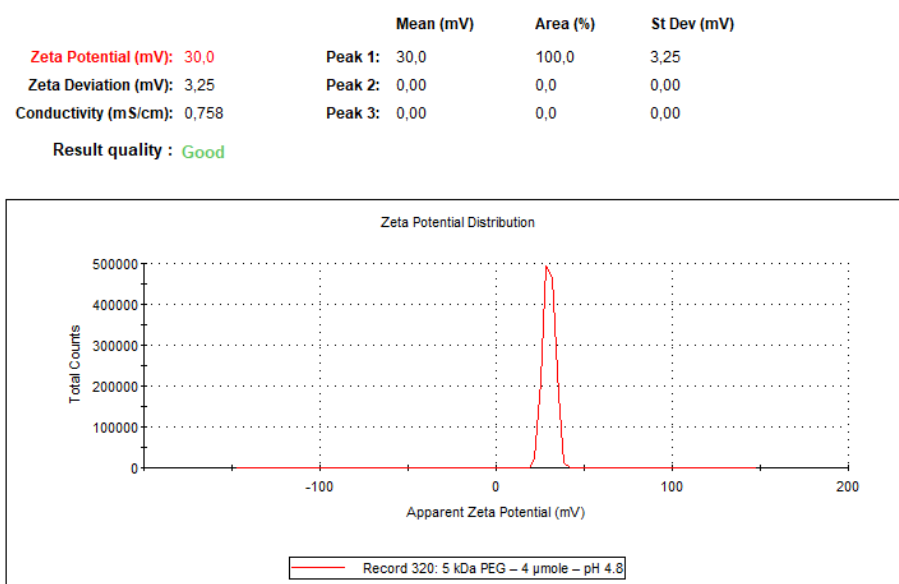


Figure A.14. Zeta potential of 5 kDa – 4 μ mole PEG – pH 4.8.

8) 5 kDa – 4 μ mole PEG – pH 6.5

a) Size

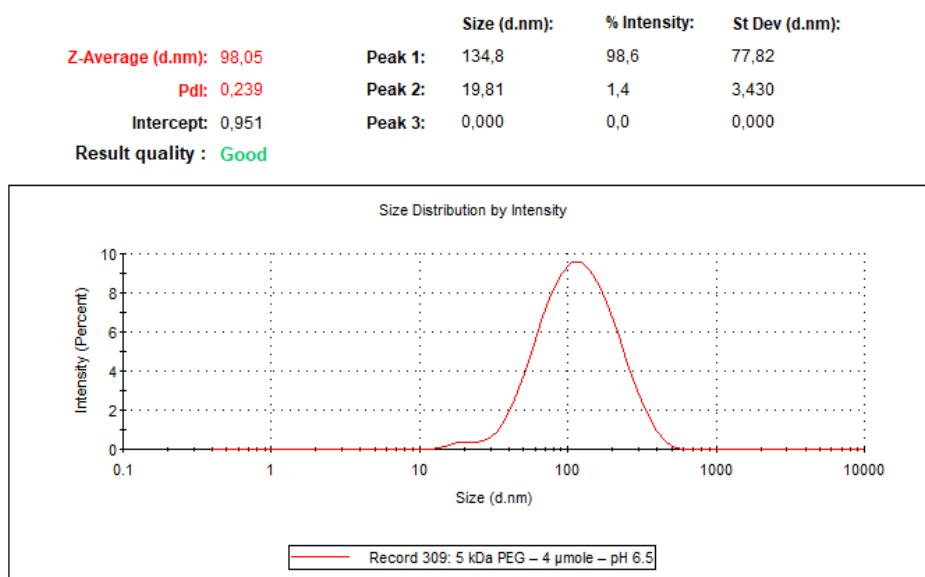


Figure A.15. Size of 5 kDa – 4 μ mole PEG – pH 6.5.

b) Zeta Potential

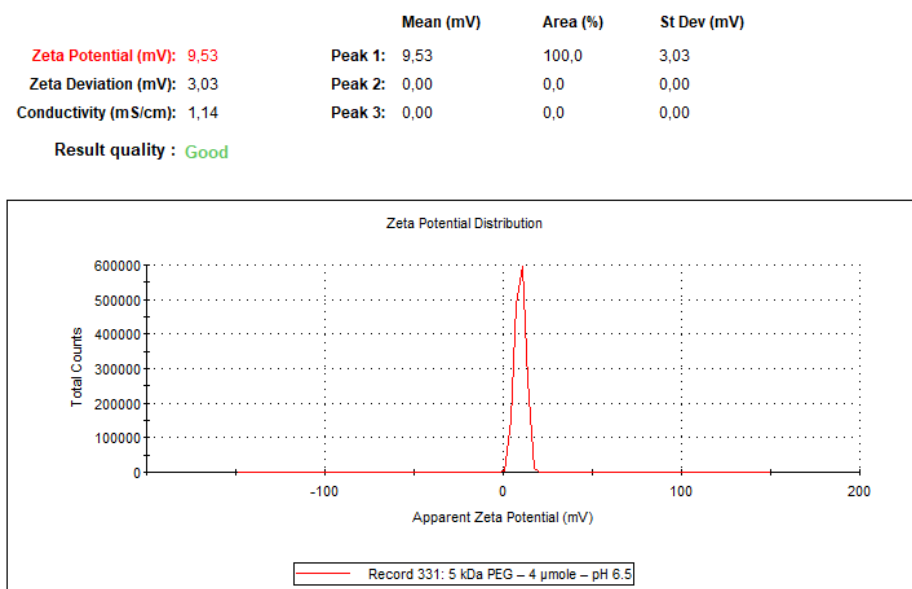


Figure A.16. Zeta potential of 5 kDa – 4 μ mole PEG – pH 6.5.

9) 5 kDa – 12 μ mole PEG – pH 4.8

a) Size

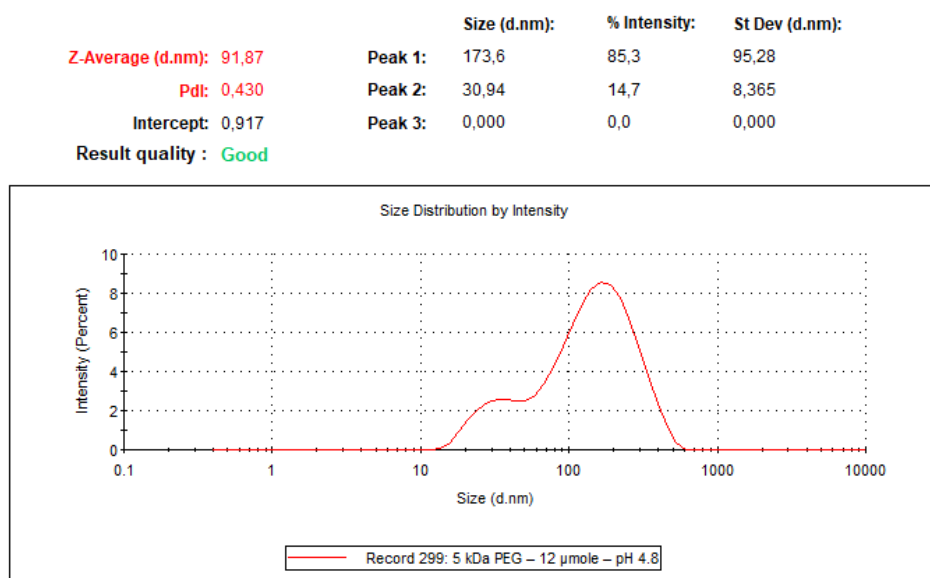


Figure A.17. Size of 5 kDa – 12 μ mole PEG – pH 4.8.

b) Zeta Potential

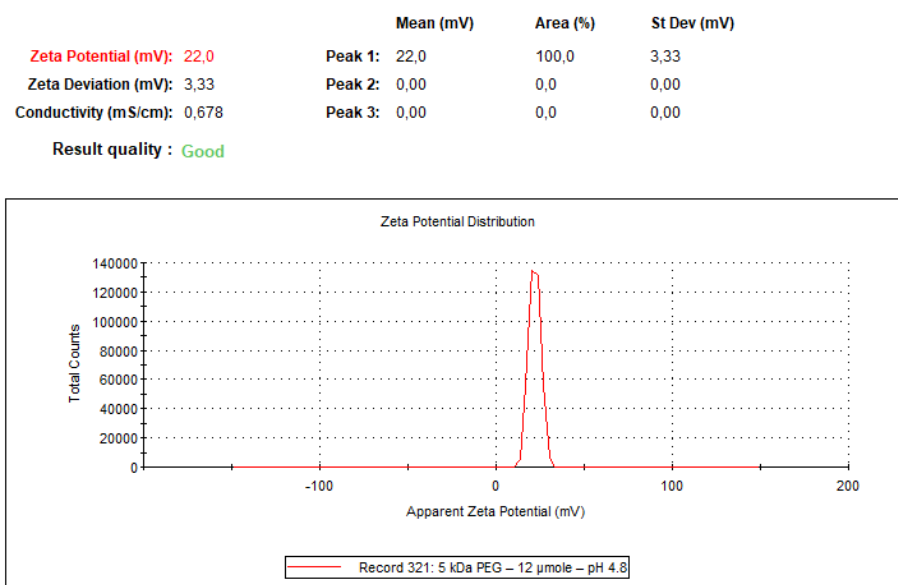


Figure A.18. Zeta potential of 5 kDa – 12 μ mole PEG – pH 4.8.

10) 5 kDa – 12 μ mole PEG – pH 6.5

a) Size

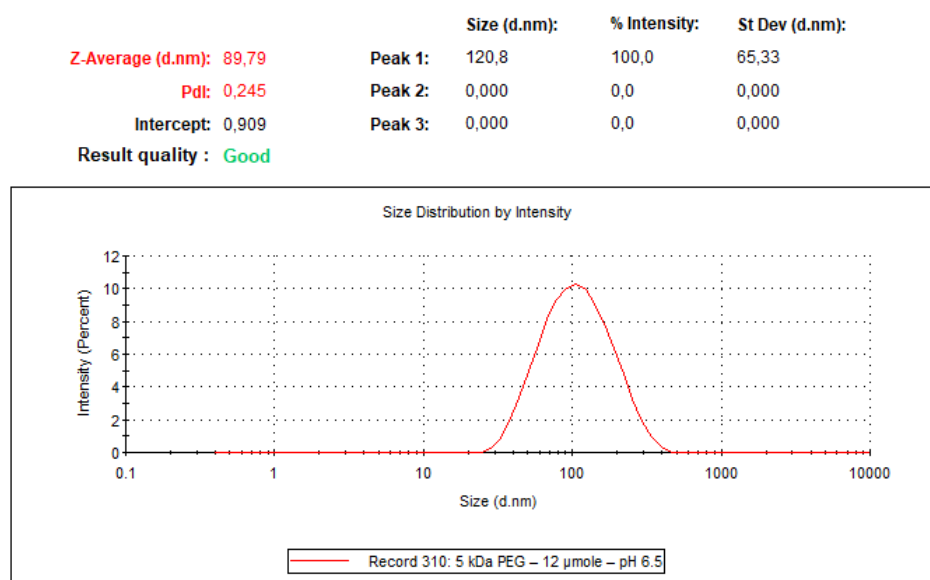


Figure A.19. Size of 5 kDa – 12 μ mole PEG – pH 6.5.

b) Zeta Potential

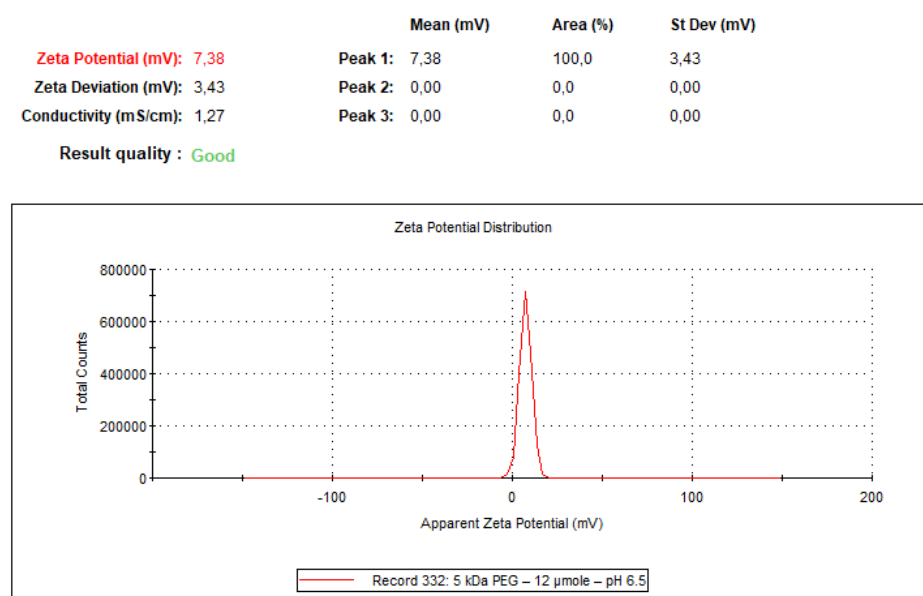


Figure A.20. Zeta potential of 5 kDa – 12 μ mole PEG – pH 6.5.

11) 5 kDa – 15 μ mole PEG – pH 4.8

a) Size

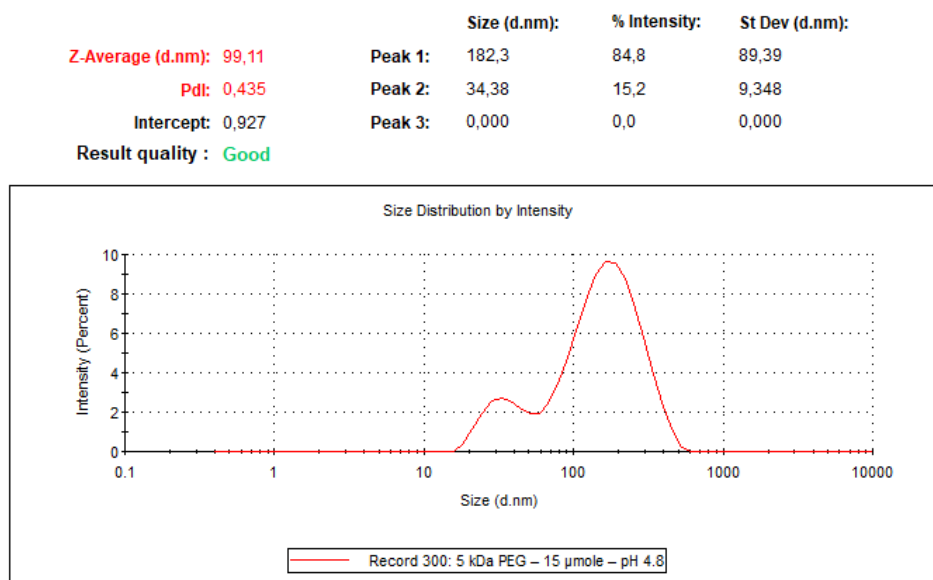


Figure A.21. Size of 5 kDa – 15 μ mole PEG – pH 4.8.

b) Zeta Potential

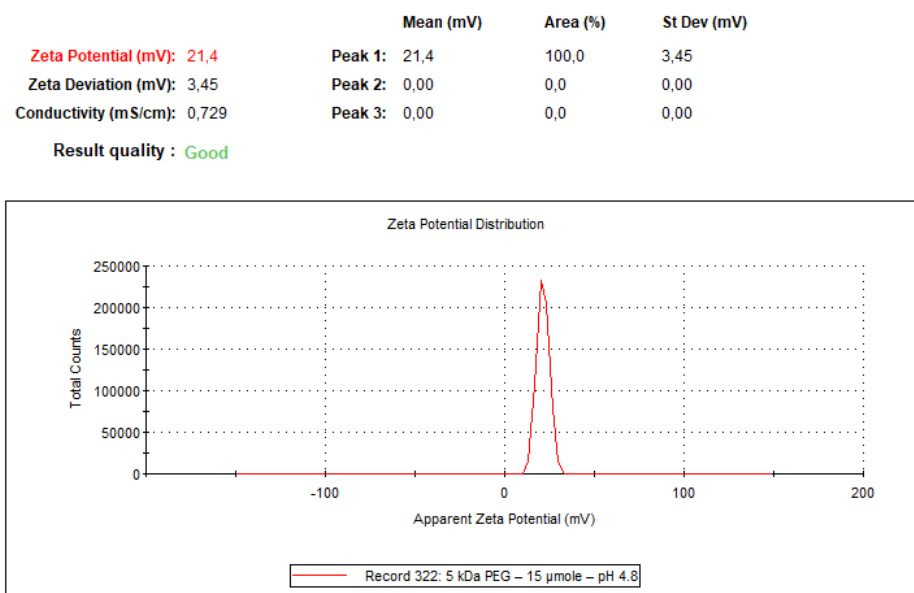


Figure A.22. Zeta potential of 5 kDa – 15 μ mole PEG – pH 4.8.

12) 5 kDa – 15 μ mole PEG – pH 6.5

a) Size

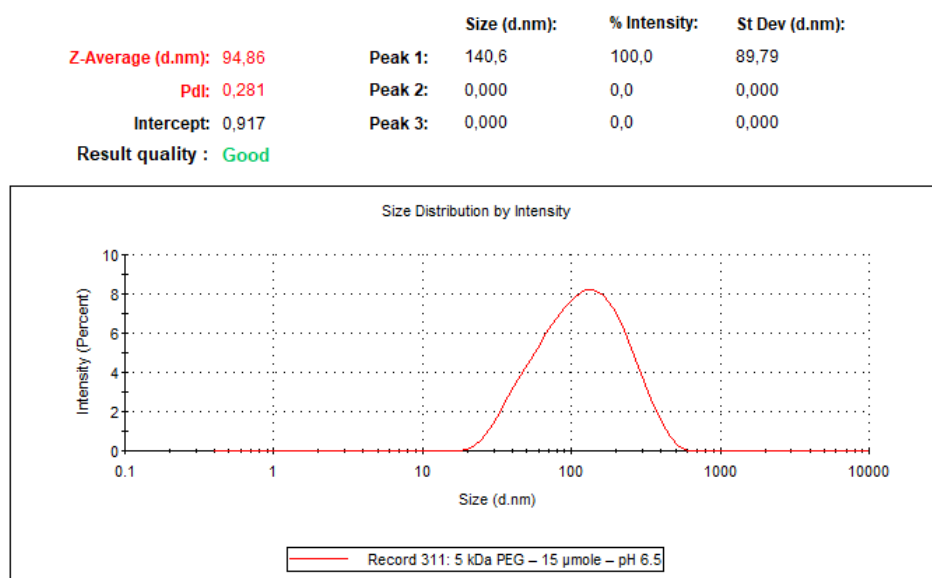


Figure A.23. Size of 5 kDa – 15 μ mole PEG – pH 6.5.

b) Zeta Potential

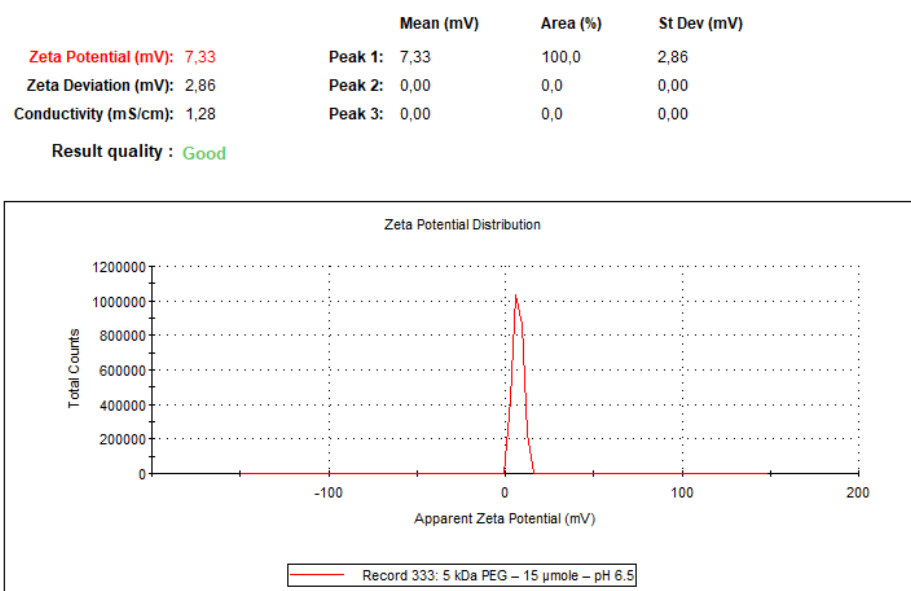


Figure A.24. Zeta potential of 5 kDa – 15 μ mole PEG – pH 6.5.

13) 5 kDa – 20 μ mole PEG – pH 4.8

a) Size

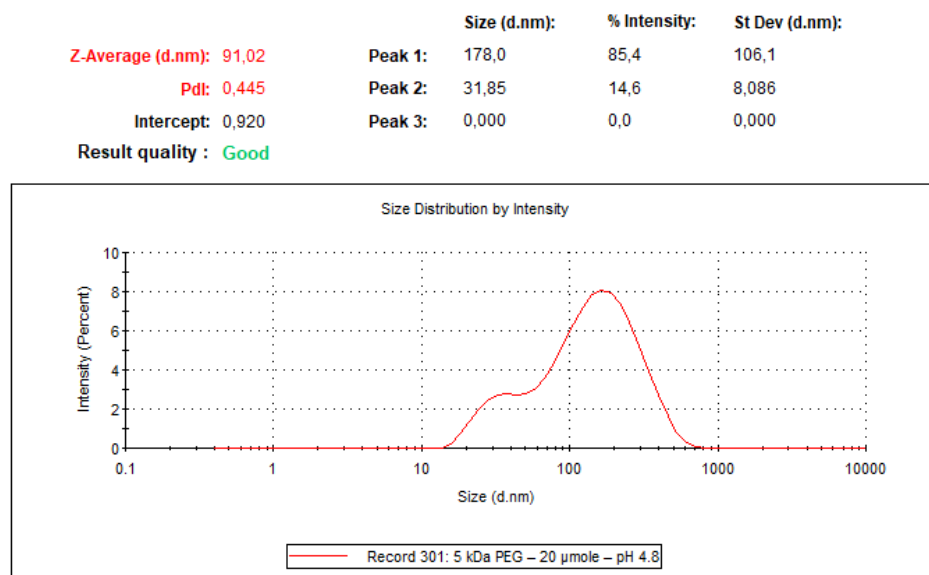


Figure A.25. Size of 5 kDa – 20 μ mole PEG – pH 4.8.

b) Zeta Potential

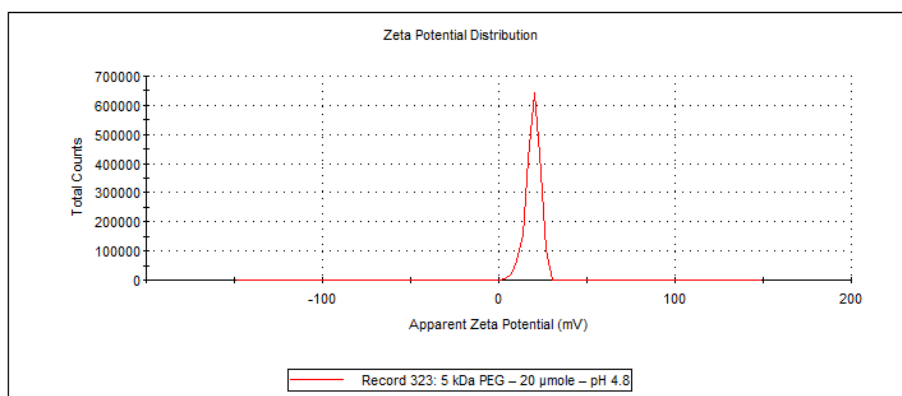
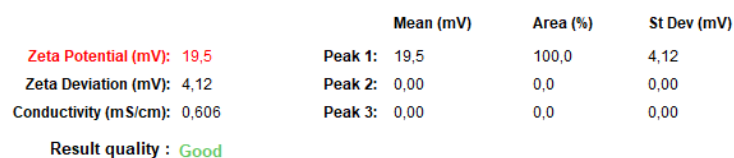


Figure A.26. Zeta potential of 5 kDa – 20 μ mole PEG – pH 4.8.

14) 5 kDa – 20 μ mole PEG – pH 6.5

a) Size

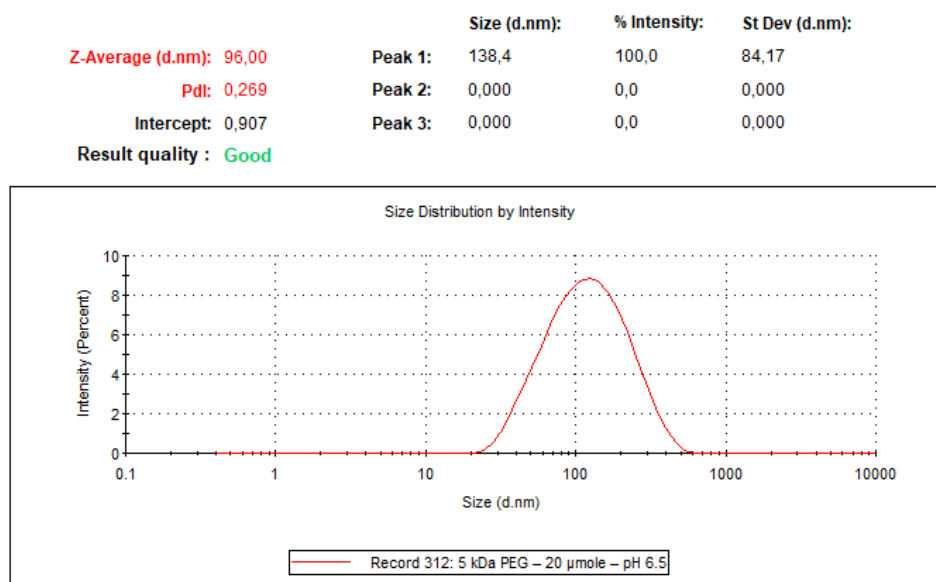


Figure A.27. Size of 5 kDa – 20 μ mole PEG – pH 6.5.

b) Zeta Potential

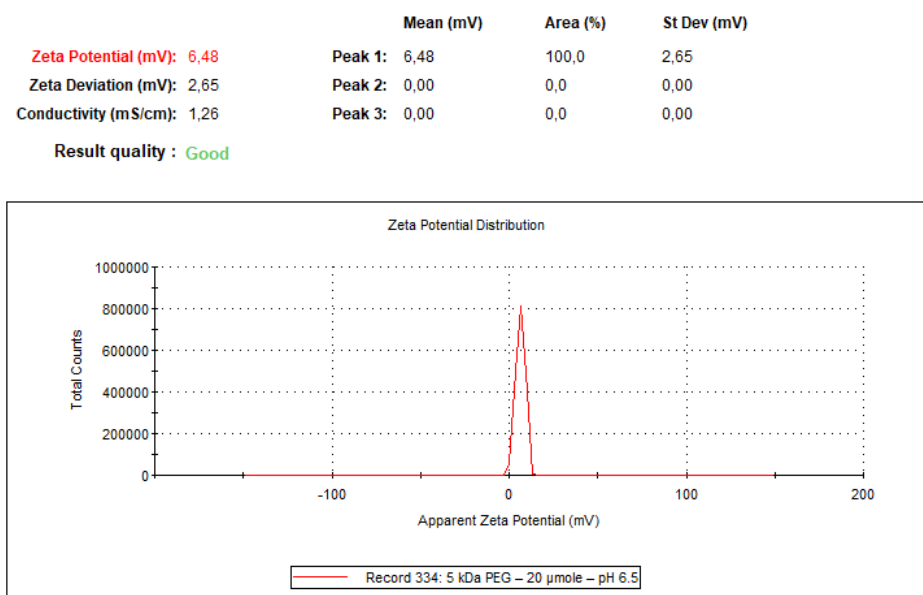


Figure A.28. Zeta potential of 5 kDa – 20 μ mole PEG – pH 6.5.

15) 10 kDa – 4 μ mole PEG – pH 4.8

a) Size

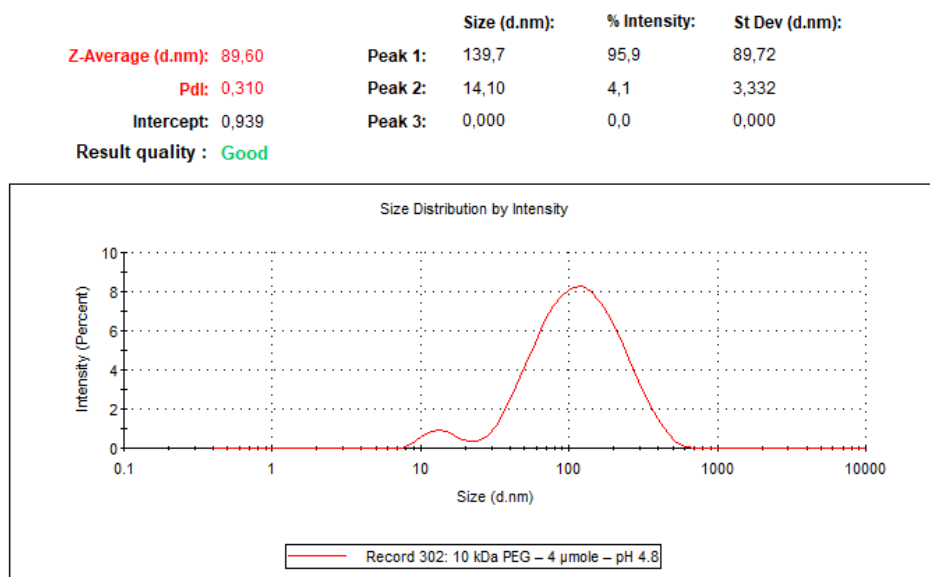


Figure A.29. Size of 10 kDa – 4 μ mole PEG – pH 4.8.

b) Zeta Potential

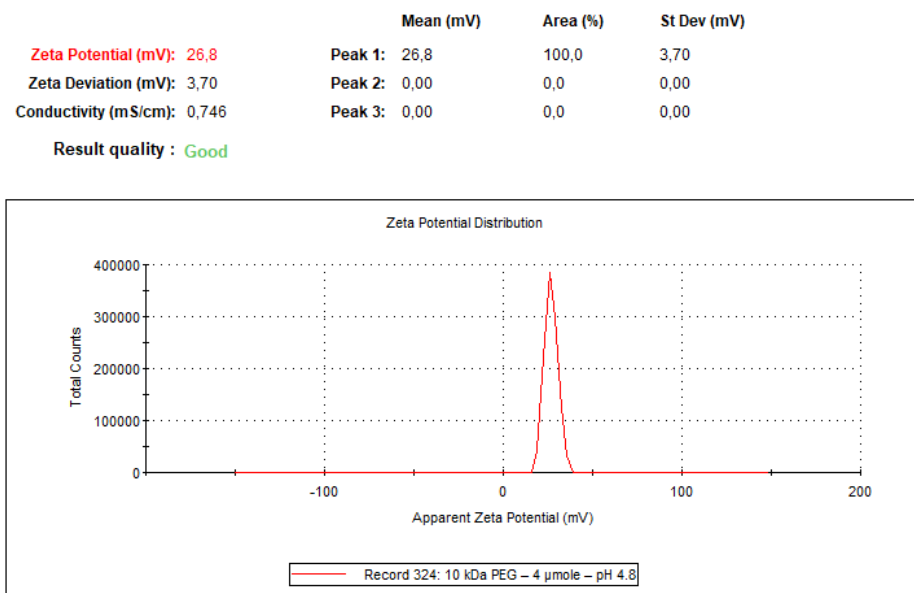


Figure A.30. Zeta potential of 10 kDa – 4 μ mole PEG – pH 4.8.

16) 10 kDa – 4 μ mole PEG – pH 6.5

a) Size

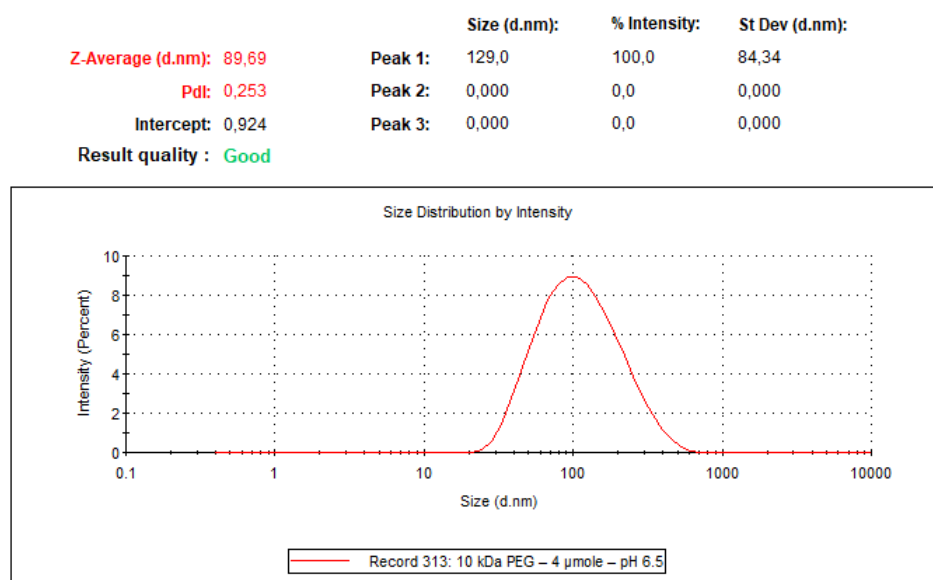


Figure A.31. Size of 10 kDa – 4 μ mole PEG – pH 6.5.

b) Zeta Potential

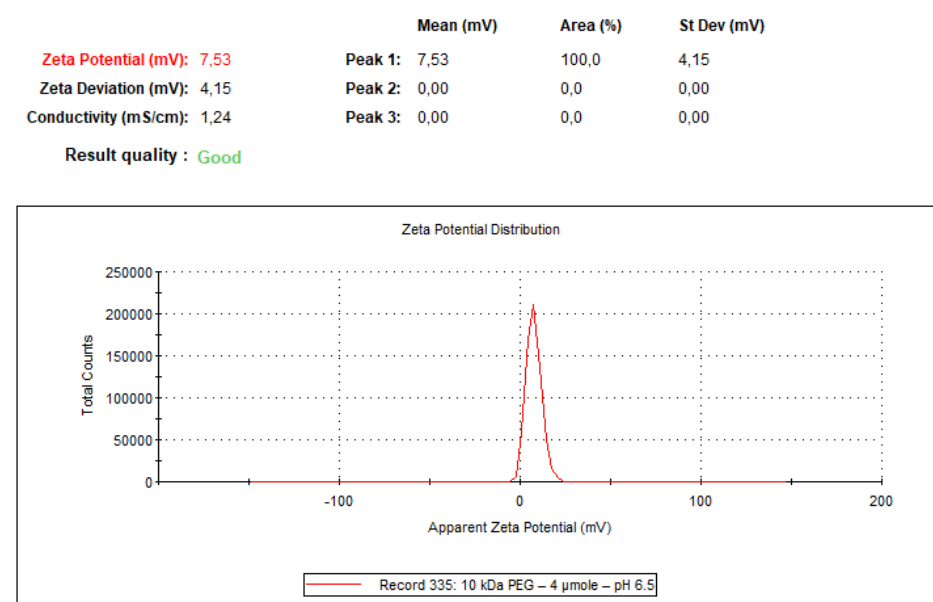


Figure A.32. Zeta potential of 10 kDa – 4 μ mole PEG – pH 6.5.

17) 10 kDa – 8 μ mole PEG – pH 4.8

a) Size

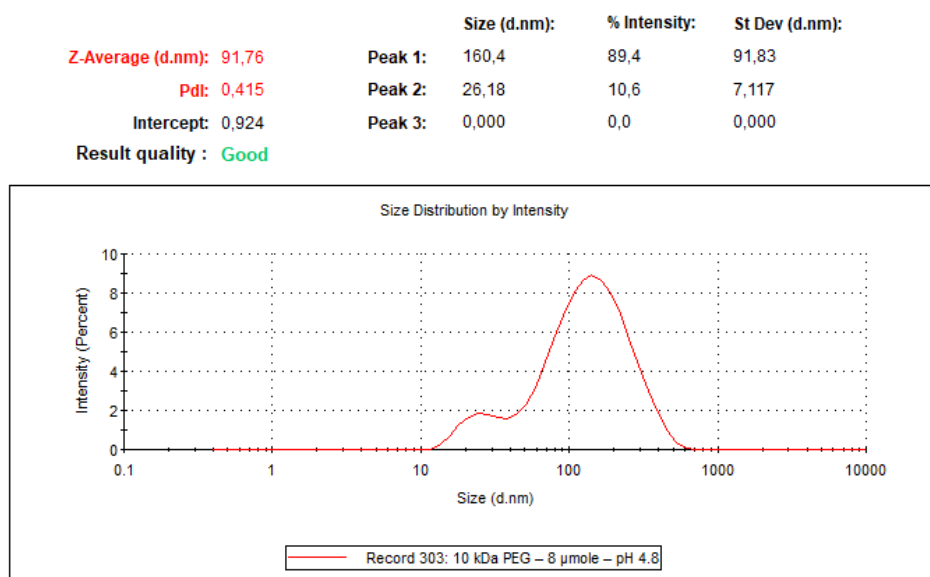


Figure A.33. Size of 10 kDa – 8 μ mole PEG – pH 4.8.

b) Zeta Potential

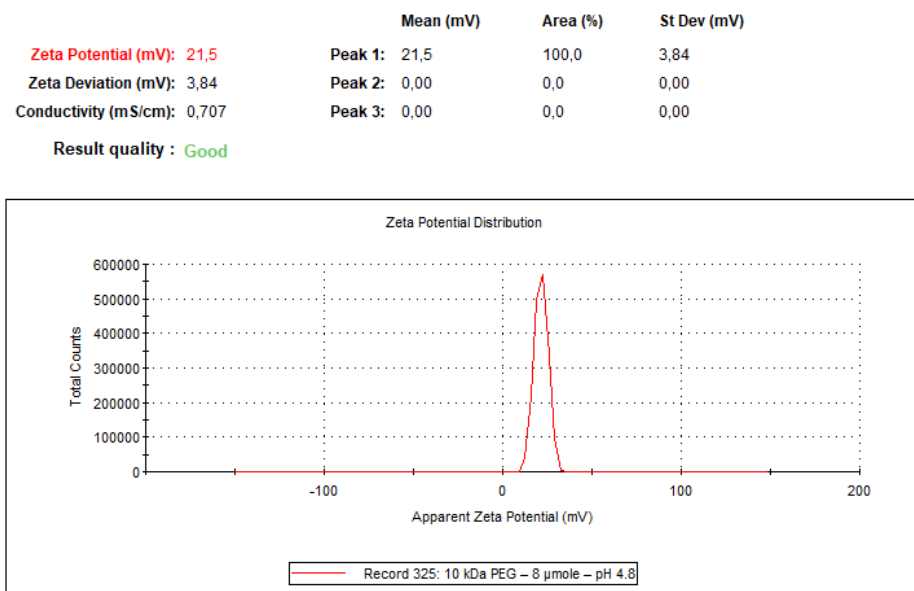


Figure A.34. Zeta potential of 10 kDa – 8 μ mole PEG – pH 4.8.

18) 10 kDa – 8 μ mole PEG – pH 6.5

a) Size

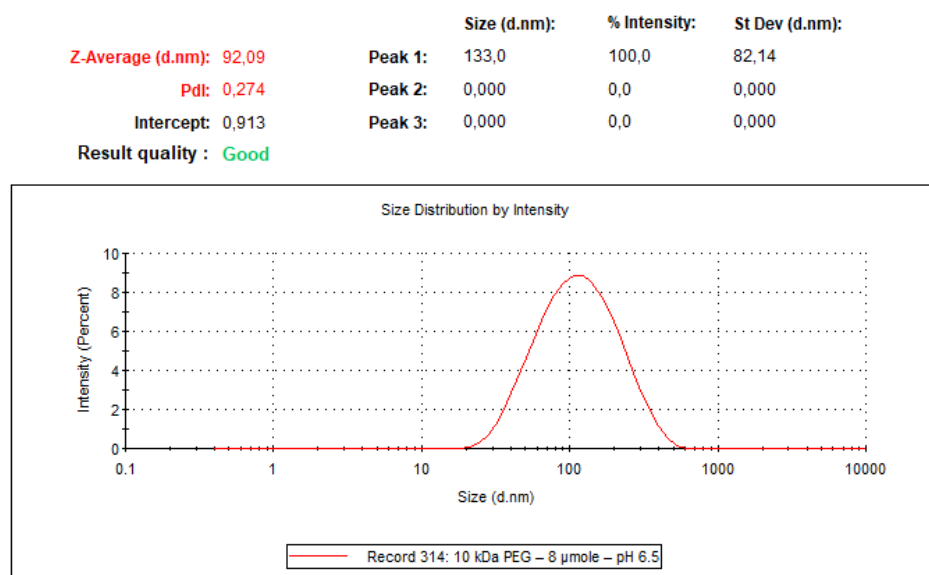


Figure A.35. Size of 10 kDa – 8 μ mole PEG – pH 6.5.

b) Zeta Potential

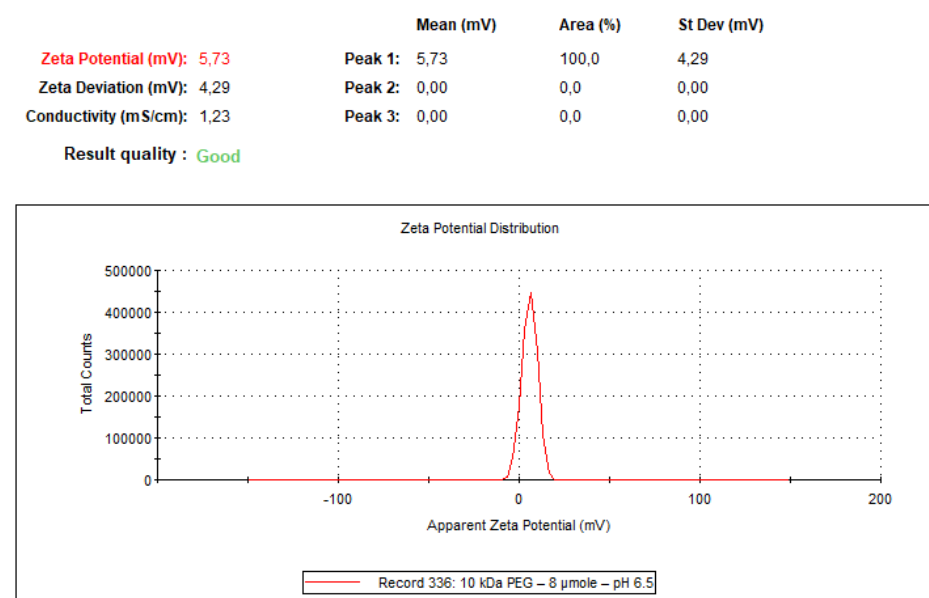


Figure A.36. Zeta potential of 10 kDa – 8 μ mole PEG – pH 6.5.

19) 10 kDa - 12 μ mole PEG – pH 4.8

a) Size

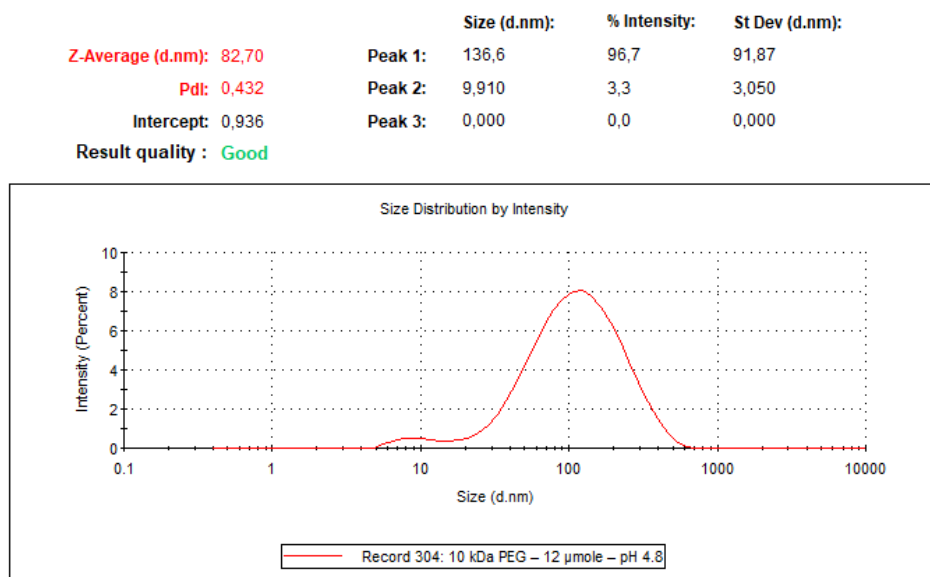


Figure A.37. Size of 10 kDa – 12 μ mole PEG – pH 4.8.

b) Zeta Potential

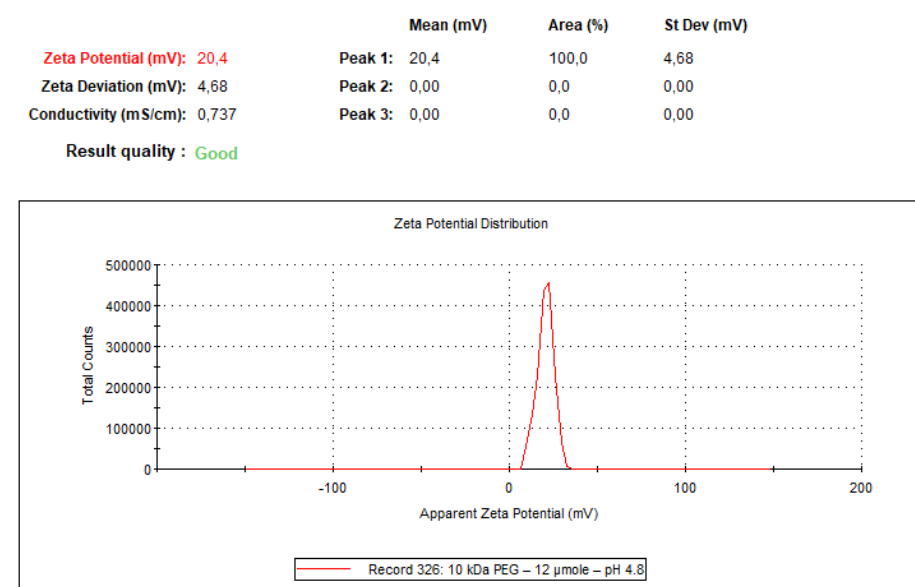


Figure A.38. Zeta potential of 10 kDa – 12 μ mole PEG – pH 4.8.

20) 10 kDa – 12 μ mole PEG – pH 6.5

a) Size

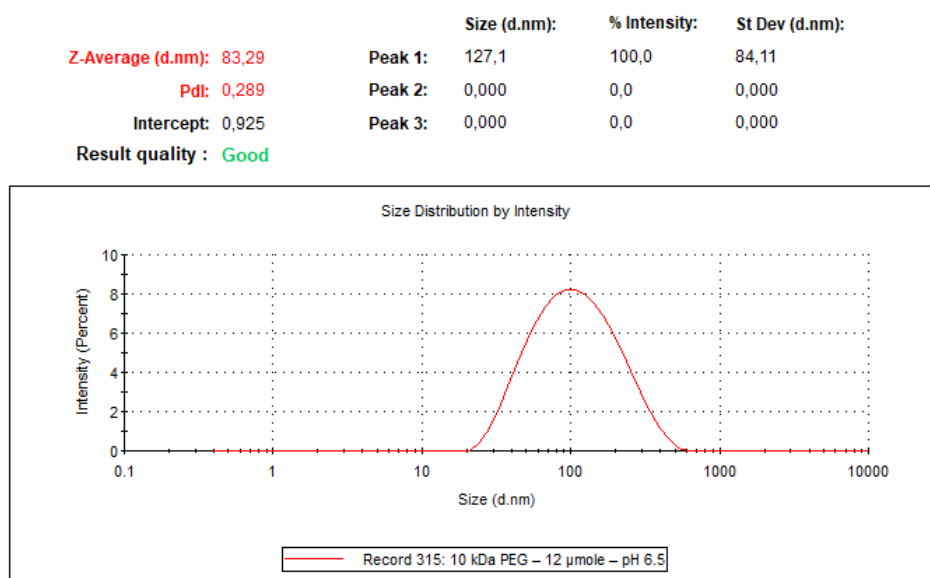


Figure A.39. Size of 10 kDa – 12 μ mole PEG – pH 6.5.

b) Zeta Potential

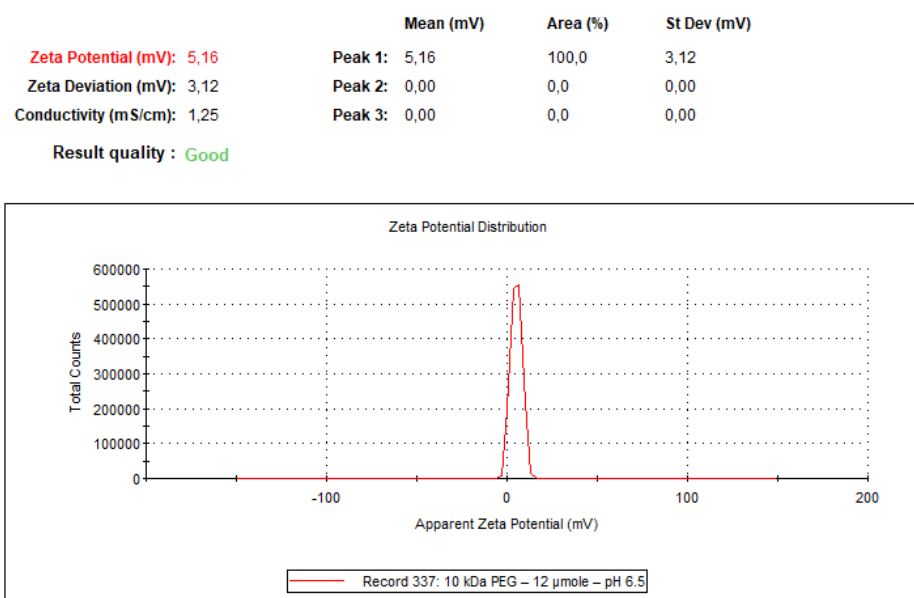


Figure A.40. Zeta potential of 10 kDa – 12 μ mole PEG – pH 6.5.

21) 10 kDa – 20 μ mole PEG – pH 4.8

a) Size

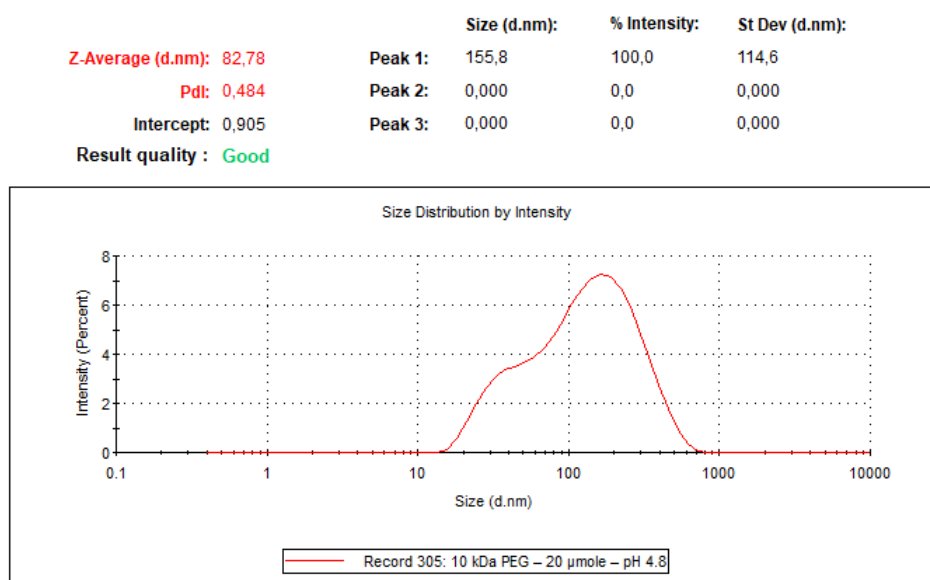


Figure A.41. Size of 10 kDa – 20 μ mole PEG – pH 4.8.

b) Zeta Potential

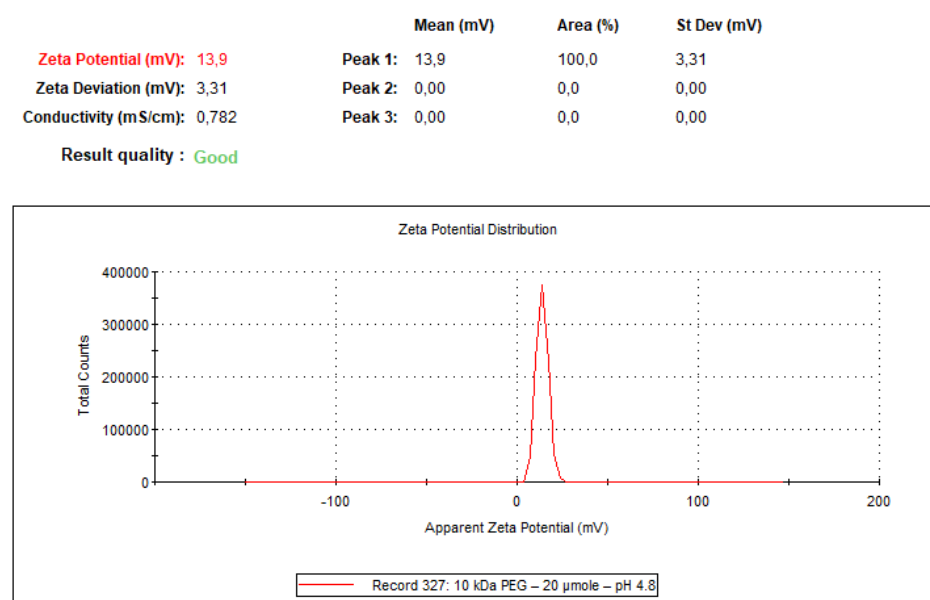


Figure A.42. Zeta potential of 10 kDa – 20 μ mole PEG – pH 4.8.

22) 10 kDa – 20 μ mole PEG – pH 6.5

a) Size

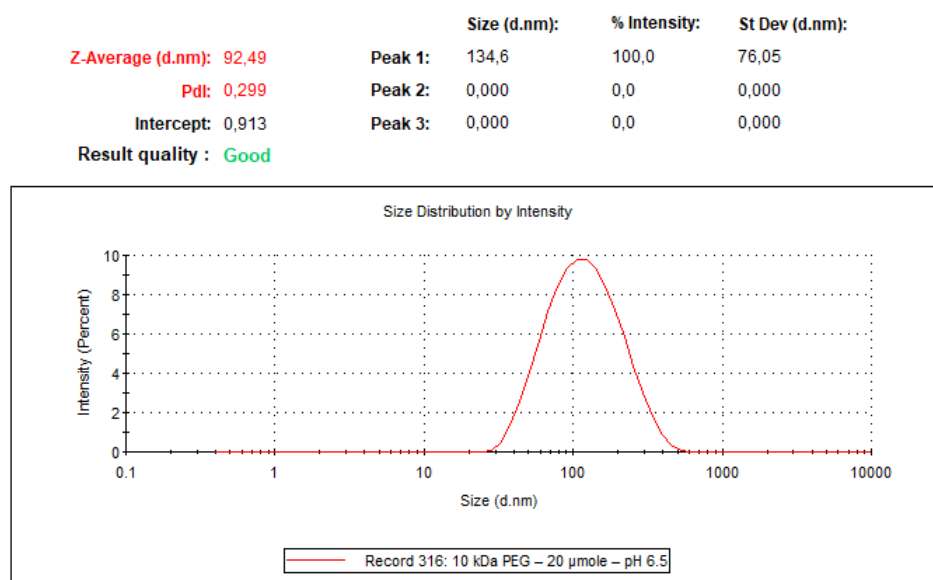


Figure A.43. Size of 10 kDa – 20 μ mole PEG – pH 6.5.

b) Zeta Potential

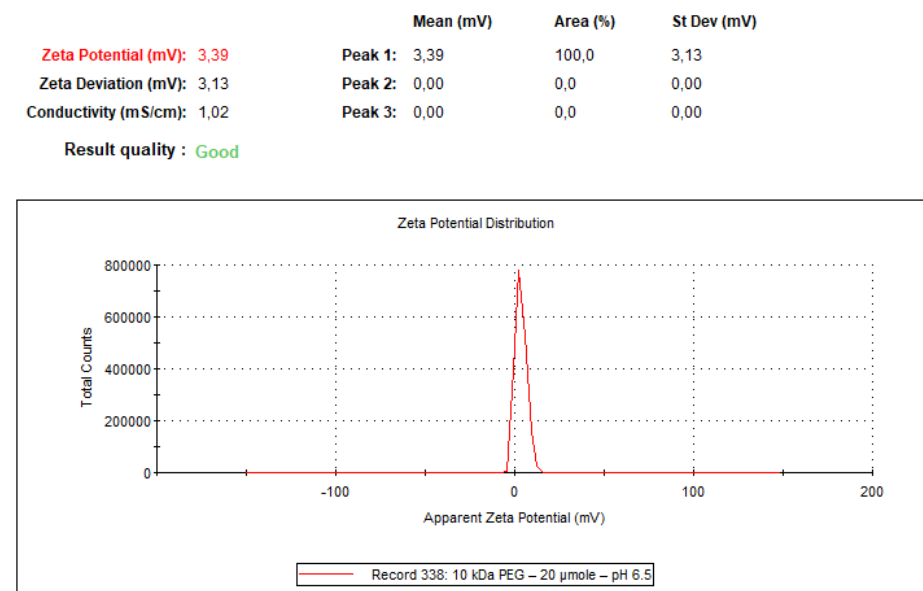


Figure A.44. Zeta potential of 10 kDa – 20 μ mole PEG – pH 6.5.

SEM EXPERIMENTS

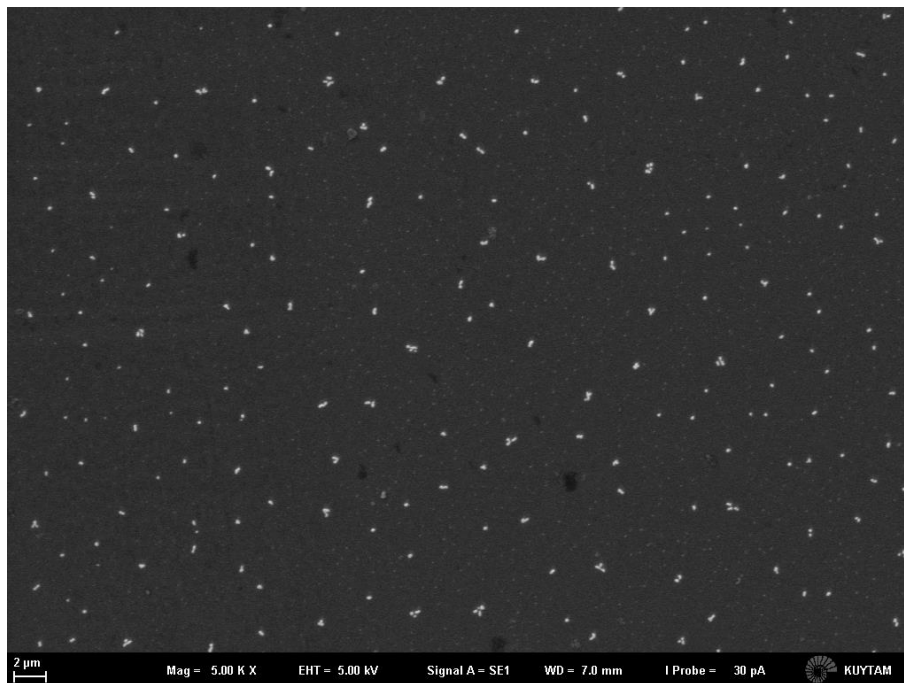


Figure A.45. SEM images of 2 kDa – 20 μmole PEG NPs.

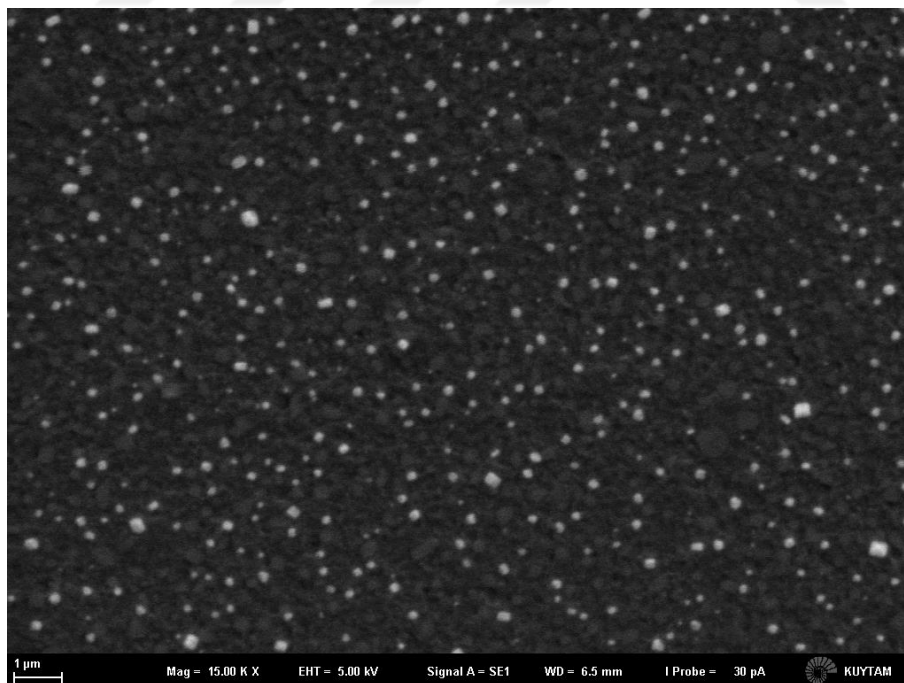


Figure A.46. SEM images of 5 kDa - 20 μmole PEG NPs.

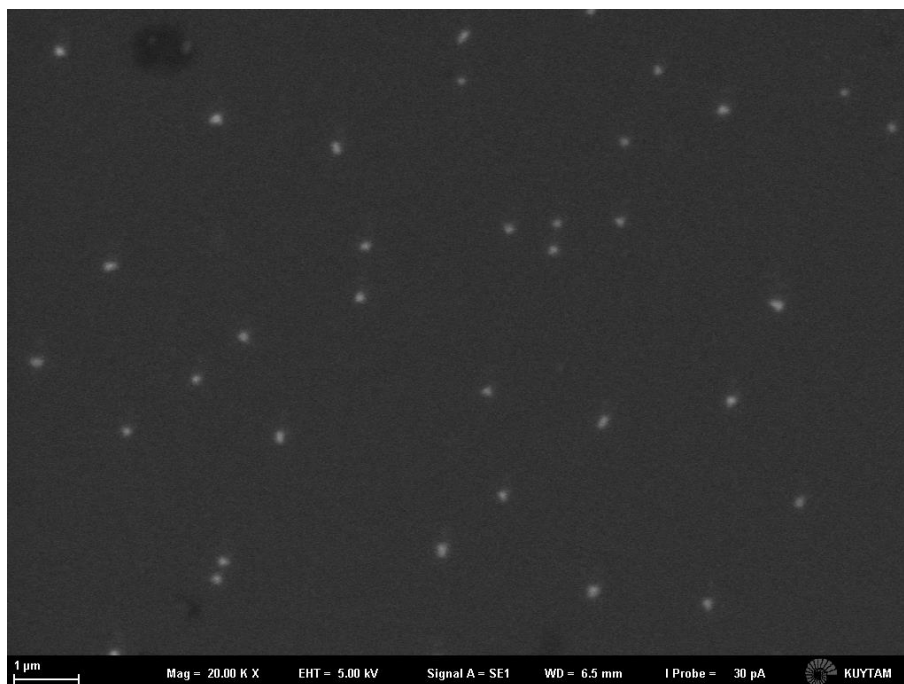


Figure A.47. SEM images of 10 kDa – 20 μ mole PEG NPs.

ANN TRAINING PARAMETERS

Table A.1. ANN training parameters.

Backpropagation type		Gradient descent with adaptive learning rate backpropagation
Network Structure	Number of hidden layers	3
	Number of nodes in hidden layer	3 2 2
Backpropagation parameters	Learning Rate	0.01
	Ratio to increase learning rate	1.05
	Ratio to decrease learning rate	0.7
Targets	Maximum Iterations	1000
	Minimum Performance Gradient	1e-5

APPENDIX B: Supplementary Information for Chapter 4

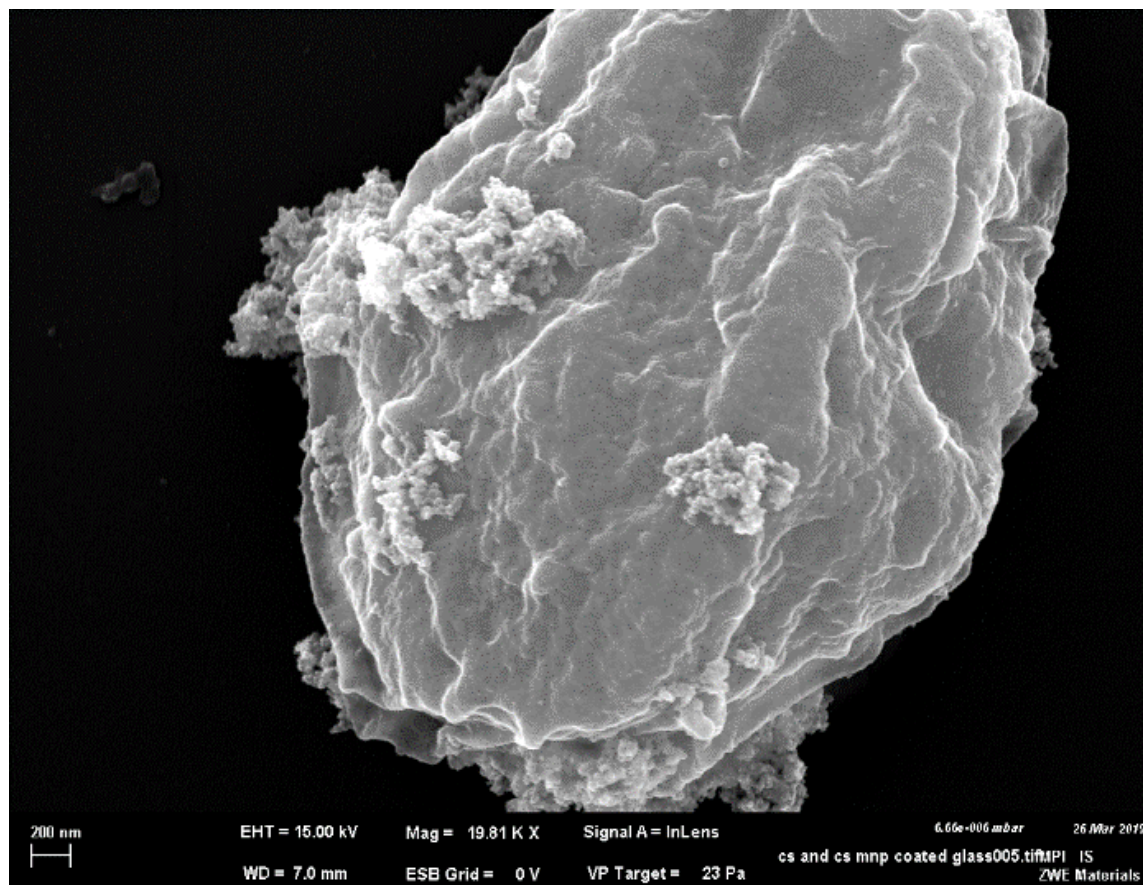


Figure B.1. SEM image of microalgae coated with chitosan-SPIONPs mixture.