

# Hemocompatibility of *N*-trimethyl chitosan chloride nanoparticles

L du Toit  
21075832  
(B.Sc)

Dissertation submitted in fulfillment of the requirements for the  
degree *Magister Scientiae* in Pharmaceutics at the  
Potchefstroom Campus of the North-West University

Supervisor: Prof LH du Plessis  
Co-Supervisor Prof JH Steenekamp

May 2014



**"The table of elements does not contain  
one of the most powerful elements that  
make up our world, and that is the  
element of surprise."**

**- *Lemony Snicket***



# Table of Contents

<b>Preface</b>	<b>i</b>
<b>Acknowledgements</b>	<b>iv</b>
<b>Abstract</b>	<b>v</b>
<b>Uittreksel</b>	<b>vii</b>
<b>List of Figures</b>	<b>ix</b>
<b>List of Tables</b>	<b>xiii</b>
<b>List of Abbreviations</b>	<b>xix</b>
<b>Chapter 1: Introduction and Aim of Study</b>	<b>1</b>
1.1 Introduction	2
1.2 References	5
<b>Chapter 2: Literature Study</b>	<b>9</b>
2.1 Drug delivery systems	10
2.2 Routes of administration	11
2.2.1 The oral route	11
2.2.2 The parenteral route	12
2.2.3 Other routes	13
2.3 New APIs and new excipients	13
2.3.1 Plant-derived polymers	14
2.3.2 Algae-derived polymers	14
2.3.3 Animal-derived polymers	15
2.4 Chitosan	15
2.4.1 Applications of chitosan	16
2.4.2 Chitosan modifications and derivatives	16
2.5 <i>N</i> -trimethyl chitosan chloride	17
2.5.1 Degree of quaternization	17
2.5.2 Applications of TMC	18

2.6	Nanoparticles	19
2.6.1	Characteristics and applications	20
2.7	Nanoparticle toxicity	21
2.8	Hemocompatibility	22
2.8.1	Hemolysis	22
2.8.2	Complement activation	23
2.8.3	Plasma protein interaction	26
2.9	Methods to improve hemocompatibility	27
2.10	Hemocompatibility of polymer nanoparticles	28
2.11	Conclusion	29
2.12	References	30
<b>Chapter 3: Article</b>		<b>39</b>
	Graphical abstract	40
	Abstract	41
	Keywords	41
1.	Introduction	41
2.	Materials and methods	44
2.1	Materials	44
2.2	Polymer synthesis and characterization	44
2.3	Synthesis of nanoparticles	46
2.4	Particle size distribution and zeta potential	46
2.5	Hemocompatibility analysis	46
2.6	Statistical analysis	49
3.	Results and discussion	49
3.1	Polymer synthesis and characterization	49
3.2	Concentration of synthesized nanoparticles	50
3.3	Particle size distribution and zeta potential	50
3.4	Hemocompatibility analysis	51
4.	Conclusion	57
5.	References	57

<b>Chapter 4: Conclusions and Future Prospects</b>	<b>61</b>
4.1 Conclusions and future prospects	62
4.2 References	65
<b>Annexure A: Materials, Methods and Results</b>	<b>66</b>
<b>Annexure B: Certificate of Analysis</b>	<b>100</b>
<b>Annexure C: Ethics Application</b>	<b>102</b>
<b>Annexure D: Statistical Data</b>	<b>104</b>

# Preface

This dissertation is submitted in article format, in accordance with the General Academic Rules (rule A.13.7.3) of the North-West University.

The Harvard referencing style is used in this dissertation, in accordance to the guide for authors of the International Journal of Pharmaceutics. The references in the text are sorted alphabetically, then chronologically. Multiple references from the same author in the same year are identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples of reference list entries:

Reference to a journal publication:

THANOU, M.M., KOTZÉ, A.F., SCHARRINGHAUSEN, T., LUEßEN, H.L., DE BOER, A.G., VERHOEF, J.C. & JUNGINGER, H.E. 2000a. Effect of degree of quaternization of *N*-trimethyl chitosan chloride for enhanced transport of hydrophilic compounds across intestinal Caco-2 cell monolayers. *J Control Release*, 64(1-3):15-25.

THANOU, M., VERHOEF, J.C., MARBACH, P. & JUNGINGER, H.E. 2000b. Intestinal absorption of octreotide: *N*-trimethyl chitosan chloride (TMC) ameliorates the permeability and absorption properties of the somatostatin analogue *in vitro* and *in vivo*. *J Pharm Sci*, 89(7):951-957.

Reference to a chapter in an edited book:

GARDNER, C.R. 1987. Drug delivery - where now? *In*: LLOYD-JONES, J.G. & JOHNSON, P., eds. *Drug Delivery Systems: Fundamentals and Techniques*, Chichester: Ellis Horwood. 11-31 p.

**Contributions of authors and consent for use**

L du Toit: Planning of study, synthesis of particles, conduction of experiments, data processing, interpretation of results and determination of conclusion, writing of dissertation

LH du Plessis: Study design and planning, provided funding for research, assistance and advice on experiments and biological data, assistance in data processing, interpretation of results and determination of conclusion, critical review of dissertation

JH Steenekamp: Assistance in and advice on synthesis and characterization of particles, data processing, interpretation of results and determination of conclusion, critical review of dissertation

---

Prof LH du Plessis\*

Prof JH Steenekamp\*

L du Toit\*

\*I declare that my role in the study as indicated above is representative of my actual contribution and that I hereby give my consent that it may be published as part of the dissertation of L. du Toit

This dissertation consists of four chapters and four annexures. Each chapter is followed by a list of references used.

- Chapter 1 introduces the study, as well as the aims thereof.
- Chapter 2 reviews the literature relevant to the study.
- Chapter 3 contains the International Journal of Pharmaceutics' guide for authors, as well as an article (not published).
- Chapter 4 provides a conclusion of the findings of the study, as well as recommendations and prospects for future studies.
- Annexure A gives a detailed account of the experimental methods used, as well as the results obtained.
- Annexure B provides the certificate of analysis of the ChitoClear® Chitosan used.
- Annexure C contains the ethics application.
- Annexure D contains all the raw statistical data.

# Acknowledgements

To my amazing supervisor and co-supervisor, **Prof Lissinda du Plessis** and **Prof Jan Steenekamp**, you complemented each other so well in leading this study. Thank you for your help and motivation and thank you for teaching me all I know about research. You were always available, giving advice and motivation freely and words cannot explain how much that meant to me.

Thanks to the **National Research Foundation** for their monetary support.

To my family, **Mada** and **Dada**, you did not always understand what I was talking about, but you always listened. Thank you so much for your love, support and prayers and for believing in me when I doubted myself. **Michelle**, thank you for giving me hope for the future and the unknown. You are the best sister anyone could have asked for.

My homies, **Karin** and **Jacques**, thank you for putting up with me for two years. It was not always easy, but it was a lot of fun! Good luck with your future endeavours. Karin, thank you for the pep talks and coffee breaks. They really meant a lot to me! Jacques, you are one of the best researchers I know. If you cannot find it, it does not exist. Thank you for helping me when I was in tears and felt like giving up. And remember that you SHOULD be able to beat him now! El-oh-el <little picture of a whale> #yolo

**Rohann**, your patience is astounding and for that, I cannot thank you enough. Thank you for drying my tears, always believing in me and giving me hope. You mean the world to me and I hope that I can give you the same love and support you so freely gave to me. It is definitely true. You do rock, and roll, all day long, Sweet Suzy.

**Rigard**, thank you for all your help and entertainment in the lab. Your chemistry knowledge is unparalleled and the formulation part of this dissertation would have been impossible without your help. By the way, what DO you get when you cross an owl with a bungee cord?

**Etienne**, I am very thankful for having a non-pharmacist friend during these past two years. Thank you for always being willing to help at a moment's notice and for teaching me so much.

To all my colleagues at the Department of Pharmaceutics, thank you for the times of work and play. Thank you for your words of encouragement. I could not have asked for better company.

Above all, I want to thank **God**, who gave me strength when I felt about to give up. You constantly comfort me, leading me to Your glorious destiny.

# Abstract

**Title:** Hemocompatibility of *N*-Trimethyl Chitosan Chloride Nanoparticles

Research on nanoparticles for pharmaceutical applications has become increasingly popular in recent years. *N*-trimethyl chitosan chloride (TMC) is a cationic polymer that can enhance absorption across mucosal surfaces. It has been explored as a nanoparticulate drug delivery system for the delivery of vaccines, vitamins, insulin and cancer medication. It has special interest for intravenous use, as it is soluble over a wide range of pH values. However, polycationic nanoparticles run a great risk for intravenous toxicity, as the positive surface charge allows easy electrostatic interactions with negatively charged blood components, such as red blood cells and plasma proteins. Additionally, the small size of the nanoparticles permits the binding of more proteins per mass, than larger particles do. These interactions can lead to extensive hemolysis, cell aggregation, complement activation, inflammation and fast clearance of the particles from the circulation. A decrease in the surface charge density can ameliorate these toxic interactions. Such a decrease is achieved by adding poly(ethylene) glycol (PEG) to the particle's formulation. PEG creates a steric shield around the particles, preventing a certain extent of interaction between the particles and the blood components.

To be able to use TMC nanoparticles as a successful drug delivery system, the hemocompatibility must first be determined, which was the aim of this study. The influence of particle size, concentration and the addition of PEG were also examined.

The extent of hemolysis and cell aggregation caused by the experimental groups (20% and 60% concentration small TMC nanoparticles, 20% larger TMC nanoparticles and 20% cross-linked PEG-TMC nanoparticles) were determined by incubating the groups with whole blood and/or blood components. Complement activation was determined with a Complement C3 Human enzyme-linked immunosorbent assay (ELISA) and plasma protein interactions were quantified through rapid equilibrium dialysis and a colorimetric assay.

It was determined that 60% concentration small TMC nanoparticles caused  $49.08 \pm 2.538\%$  hemolysis at the end of a 12-hour incubation period, significantly more than any other experimental group. This group had also caused mild aggregation of the white blood cells and platelets. This was the greatest extent of cell aggregation seen in any of the groups. No significant complement activation was seen by any of the experimental groups. Because of the cationic nature of the

particles, all groups had more than 50% of the initial particles in the sample bound to plasma proteins after a 4-hour incubation period. However, at  $90.68 \pm 0.828\%$ , the 60% small TMC nanoparticles had had significantly more interaction with the plasma proteins than the other groups.

Through the experimental measurements it was revealed that TMC nanoparticles had hemotoxic effects at high concentrations. The addition of PEG to the particle formulation stabilized the particles and decreased their zeta potential, but had no significant effect on improving hemocompatibility.

It was concluded that although further tests are needed, TMC nanoparticles seem to have potential as a successful intravenous carrier for high molecular weight active pharmaceutical ingredients.

**Keywords:** Hemocompatibility, *N*-trimethyl chitosan chloride, nanoparticles, poly(ethylene) glycol, hemolysis, aggregation, complement activation, plasma protein interaction

# Uittreksel

**Titel:** Bloedverenigbaarheid van *N*-Trimetiel Kitosaan Chloried Nanopartikels

Navorsing op nanopartikels vir farmaseutiese toepassings het in die afgelope paar jaar al meer populêr geword. *N*-trimetiel kitosaan chloried (TMC) is 'n kationiese polimeer wat absorpsie oor mukosale oppervlakte kan bevorder. Daar is na TMC in die vorm van 'n nanopartikel afleweringstelsel gekyk om onder andere vaksien, vitamien, insulien en kanker medikasie toe te dien. Dit het belang by intraveneuse toediening, siende dat TMC oor 'n wye reeks pH waardes oplosbaar is. Tog is daar die groot risiko dat polikationiese nanopartikels toksisiteit kan toon na intraveneuse toediening. Hulle positief gelaai oppervlakte laat maklike elektrostatiese interaksies met negatief gelaai bloedkomponente, soos rooibloedselle en plasma proteïene toe. Omdat die nanopartikels 'n groter oppervlakte tot volume verhouding het, kan hulle ook meer proteïene per massa bind as wat groter partikels kan. Interaksies met bloedkomponente kan lei tot uitermatige hemolise, sel-aggregasie, komplementaktivering, inflammasie en vinnige verwydering van die partikels vanuit die sirkulasie. Hierdie toksiese interaksies kan verminder word deur die oppervlaklading van die partikels te verlaag deur poli-etileen glikool (PEG) aan die partikel te heg. PEG veroorsaak 'n steriese hindernis om die partikels, wat die interaksies tussen die partikels en die bloedkomponente tot 'n mate voorkom.

Om TMC nanopartikels suksesvol in 'n afleweringstelsel te kan gebruik, moet die verenigbaarheid daarvan met die bloed eers bepaal word. Dit was dan ook die doel van hierdie studie. Die invloed van partikelgrootte, konsentrasie en die byvoeging van PEG is ook ondersoek.

Die mate waartoe die eksperimentele groepe (20% en 60% konsentrasie klein TMC nanopartikels, 20% groter TMC nanopartikels en 20% kruis-gekoppelde PEG-TMC nanopartikels) hemolise en sel-aggregasie veroorsaak het, is ondersoek deur die groepe saam met heel bloed en/of aparte bloedkomponente te inkubeer. Daar is vir komplementaktivering getoets met 'n komplement C3 menslike ensiem gekoppelde immunosorbenttoets (ELISA) en die interaksie met plasma proteïene is gekwantifiseer deur vinnige ewilibrum dialise en 'n kolorimetrisiese toets.

Dit is bepaal dat die 60% konsentrasie klein TMC nanopartikels teen die einde van die 12-uur inkubasie tydperk  $49.08 \pm 2.538\%$  hemolise veroorsaak het. Dit is beduidend hoër as enige van die ander eksperimentele groepe. Hierdie groep het ook ligte aggregasie van die witbloedselle en bloedplaatjies veroorsaak. Dit was die meeste aggregasie van al die groepe. Geen beduidende

komplement aktivering is by enige van die eksperimentele groepe waargeneem nie. As gevolg van die kationiese geaardheid van die partikels, het meer as 50% van die inisiële partikels van al die groepe aan die plasma proteïene gebind na 'n 4-uur inkubasie tydperk. Tog het die 60% klein TMC nanopartikels teen  $90.68 \pm 0.828\%$  beduidend meer interaksie met die plasma proteïene gehad.

Vanaf die eksperimentele waarnemings was dit duidelik dat TMC hemotoksiese effekte getoon het by hoë konsentrasies. Die byvoeging van PEG het die partikels meer stabiel gemaak en die zeta potensiaal daarvan verlaag, alhoewel dit nie 'n betekenisvolle effek gehad het op die verbetering van die bloedverenigbaarheid van die partikels nie.

Die gevolgtrekking is gemaak dat alhoewel verdere toetse nodig is, TMC nanopartikels potensiaal het as 'n suksesvolle intraveneuse draer van aktiewe farmaseutiese bestanddele met 'n hoë molekulêre gewig.

**Sleutelwoorde:** Bloedverenigbaarheid, *N*-trimetiel kitosaan chloried, nanopartikels, poli-etileen glikool, hemolise, aggregasie, komplement aktivering, plasma proteïen interaksie

# List of Figures

<b>Figure 2.1</b> –	Chemical structure of chitosan.	15
<b>Figure 2.2</b> –	Chemical structure of TMC, indicating the quaternized groups.	17
<b>Figure 2.3</b> –	Summary of the classical pathway of the complement cascade, starting when the complement C1 protein interacts with an antibody attached to a pathogen.	24
<b>Figure 2.4</b> –	Summary of the alternative pathway of complement activation, displaying its spontaneous start and self-sufficiency.	25
<b>Figure 2.5</b> –	Summary of the lectin pathway of complement activation. MBL = mannan-binding lectin, MASP = MBL-associated serine protease.	25
<b>Figure 1</b> –	Mean percentage hemolysis caused, with standard error of mean, at 1-, 6- and 12-hour intervals, as calculated for each of the experimental groups. For each bar n = 9.	52
<b>Figure 2</b> –	Mean C3 protein concentration with standard error of mean, as interpolated from the standard curve as activated by the different experimental groups and the control group. For each bar n = 9.	55
<b>Figure 3</b> –	Graphic representation of the mean percentage of particles of each experimental group bound to complement C3 proteins, along with the standard error of the mean values, as calculated from the standard curves of each particle type in glycerol. For each bar n = 9.	56
<b>Figure A.1</b> –	Mean values of the absorption measured, plotted against the corresponding TMC concentration to form a standard curve. Absorbance values were obtained using a UV spectrometer at 530 nm. Data was fitted with linear regression ( $r^2 = 0.9991$ , n = 25) for determination of unknown values.	73

- Figure A.2** – Standard curve of cross-linked PEG-TMC drawn from the mean values of the absorbance measured for each of the cross-linked PEG-TMC dilutions. Absorbance was measured with a BioTek microplate reader at 550 nm. Data was fit with linear regression to determine unknown PEG-TMC concentrations.  $r^2 = 0.9985$ ,  $n = 21$ . 74
- Figure A.3** – The log of the mean absorbance values (measured at 450 nm) plot against the log of the C3 protein concentrations, forming the standard C3 protein concentration curve for the complement activation experiment. Data was fit with a fourth-order polynomial regression line and used to determine the extent of complement activation caused by the experimental samples.  $r^2 = 0.9963$ ,  $n = 21$ . 78
- Figure A.4** – Standard curve of TMC nanoparticles in glycerol, drawn from different small TMC nanoparticle concentrations and the mean corresponding absorbance values, as measured with a BioTek microplate reader at 550 nm. Data was fit with linear regression for the interpolation of unknown concentration values found in the plasma protein interaction experiment.  $r^2 = 0.9974$ ,  $n = 63$ . 79
- Figure A.5** – Standard curve of larger TMC nanoparticles in glycerol. Absorbance values of different larger TMC nanoparticle dilutions were measured with a BioTek microplate reader at 550 nm and the mean values plot against the corresponding microparticle concentrations. The data was fit with linear regression for the interpolation of concentrations of absorbance values measured in the plasma protein interaction experiment.  $r^2 = 0.9967$ ,  $n = 63$ . 79
- Figure A.6** – Standard curve of cross-linked PEG-TMC nanoparticles in glycerol. Absorbance values of different cross-linked PEG-TMC nanoparticle dilutions were measured with a BioTek microplate reader at 550 nm and the mean values plot against the corresponding microparticle concentrations. The data was fit with linear regression for the interpolation of concentrations of absorbance values measured in the plasma protein interaction experiment.  $r^2 = 0.9967$ ,  $n = 63$ . 80
- Figure A.7** – NMR characterization of the synthesized TMC (DQ 60%, degree of *O*-methylation, 33%) 82

- Figure A.8** – Spectral scan of the absorbance of all the different TMC concentrations, as measured with a UV spectrometer, zoomed in to show the applicable absorbance area. The concentrations represented by the lines are (from the top): 0 µg/ml (control, CBR and PBS), 300 µg/ml (stock solution), 270 µg/ml, 240 µg/ml, 210 µg/ml, 180 µg/ml, 150 µg/ml, 120 µg/ml, 90 µg/ml, 60 µg/ml, 30 µg/ml and 3 µg/ml. 83
- Figure A.9** – Size distribution of the small TMC nanoparticles as measured with a Malvern Zetasizer ZEN 3600. The measured particle size is  $91.04 \pm 9.90$  nm. 84
- Figure A.10** – Countess® automated cell counter (Life technologies, Carlsbad, CA, USA) image of a representative blood sample diluted 200 times, resulting in a red blood cell count of  $8.6 \times 10^6$  cells per ml. 85
- Figure A.11** – Mean absorbance values, with standard error of mean, of the different experimental groups at one-, six- and twelve-hour intervals. Absorbance was measured with a BioTek microplate reader at 550 nm. For each bar  $n = 9$ . 86
- Figure A.12** – Mean percentage hemolysis caused, with standard error of mean, at one-, six- and twelve-hour intervals, as calculated for each of the experimental groups. For each bar  $n = 9$ . 86
- Figure A.13** – Light microscopy of positive control: blood components resuspended in saline after a 30-minute incubation with polyethyleneimine (Mw 25 000), displaying cell aggregation. A: Red blood cells. B: White blood cells. C: Blood platelets. D: Whole blood. 89
- Figure A.14** – Light microscopy of negative control: blood components after being incubated with normal saline for 30 minutes, displaying no cell aggregation. What looks to be clumped cells in picture B is crystals from the dye used. A: Red blood cells. B: White blood cells. C: Blood platelets. D: Whole blood. 90
- Figure A.15** – Light microscopy of blood components resuspended in saline showing cell aggregation caused by small TMC nanoparticles (20%) after a 30-minute incubation period. A: Red blood cells. B: White blood cells. C: Blood platelets. D: Whole blood. 91

- Figure A.16** – Light microscopy of blood components resuspended in saline after a 30-minute incubation period with small TMC nanoparticles (60%), showing cell aggregation caused. A: Red blood cells. B: White blood cells. C: Blood platelets. D: Whole blood. 93
- Figure A.17** – Light microscopy of blood components in saline after being incubated with larger TMC nanoparticles (20%) for 30 minutes, showing cell aggregation caused. A: Red blood cells. B: White blood cells. C: Blood platelets. D: Whole blood. 94
- Figure A.18** – Light microscopy of blood components in saline, after incubation with PEG-TMC nanoparticles (20%) for 30 minutes, showing cell aggregation caused. A: Red blood cells. B: White blood cells. C: Blood platelets. D: Whole blood. 95
- Figure A.19** – Mean C3 protein concentration with standard error of mean, as interpolated from the standard curve as activated by the different experimental groups and the control group. For each bar n = 9. 96
- Figure A.20** – Graphic representation of the mean absorbance values of each of the experimental and the control groups, as measured with a BioTek microplate reader at 550 nm, with standard error of mean. For each bar n = 9. 97
- Figure A.21** – Graphic representation of the mean percentage of particles of each experimental group bound to complement C3 proteins, along with the standard error of the mean values, as calculated from the standard curves of each particle type in glycerol. For each bar n = 9. 98

# List of Tables

<b>Table 1</b> – Summary of the size and zeta potential determination results, as measured with a Malvern Zetasizer ZEN 3600. The size and zeta potential results are expressed as an average $\pm$ standard deviation. The percentage relative standard deviation (%RSD) is an indication of the spread of the data.	50
<b>Table 2</b> – Summary of cell aggregation caused by the different experimental groups. (S) indicates small nanoparticles and (L) indicates larger nanoparticles. RBC = red blood cells and WBC = white blood cells.	53
<b>Table A.1</b> – Summary of the average measured particle sizes, the percentage relative standard deviation of each (%RSD) and the measured zeta potential of each of the particle groups.	85
<b>Table A.2</b> – Mean measured absorption values for the different experimental groups and positive and negative controls, with standard error of mean (SEM) over the course of the experiment.	87
<b>Table A.3</b> – Mean percentage of hemolysis caused, as calculated for the different experimental groups, with standard error of mean (SEM), over the course of the experiment.	87
<b>Table A.4</b> – Summary of cell aggregation caused by the different experimental groups.	96
<b>Table D.1</b> – Absorption of different TMC concentrations, as measured with a UV spectrometer at 530 nm.	105
<b>Table D.2</b> – Absorption of different PEG-TMC concentrations measured with a BioTek microplate reader at 550 nm.	105
<b>Table D.3</b> – Absorbance measured at 550 nm, after one hour of incubation with experimental samples.	105

<b>Table D.4</b> – Absorbance measured at 550 nm, after six hours of incubation with experimental samples.	106
<b>Table D.5</b> – Absorbance measured at 550 nm, after 12 hours of incubation with experimental samples.	106
<b>Table D.6</b> – Descriptive statistics of the absorbance of the different experimental groups after one hour of incubation.	107
<b>Table D.7</b> – One-way ANOVA of the absorbance of the different experimental groups after one hour of incubation, with Bonferroni post-test.	108
<b>Table D.8</b> – Descriptive statistics of the absorbance of the different experimental groups after six hours of incubation.	109
<b>Table D.9</b> – Repeated measures ANOVA of the absorbance of the different experimental groups after six hours of incubation, with Bonferroni post-test.	110
<b>Table D.10</b> – Descriptive statistics of the absorbance of the different experimental groups after 12 hours of incubation.	111
<b>Table D.11</b> – One-way ANOVA of the absorbance of the different experimental groups after 12 hours of incubation, with Bonferroni post-test.	112
<b>Table D.12</b> – Descriptive statistics of the absorption of the negative control group over the 12-hour span of the hemolysis experiment.	112
<b>Table D.13</b> – One-way ANOVA of the absorbance of the negative control group over 12 hours, with Bonferroni post-test.	113
<b>Table D.14</b> – Descriptive statistics of the absorption of the positive control group over the 12-hour span of the hemolysis experiment.	113
<b>Table D.15</b> – Repeated measures ANOVA of the absorbance of the positive control group over 12 hours, with Bonferroni post-test.	114
<b>Table D.16</b> – Descriptive statistics of the absorbance of the 20% concentration small TMC nanoparticle experimental group, throughout the experiment.	114
<b>Table D.17</b> – Repeated measures ANOVA of the absorbance of the 20% concentration small TMC nanoparticles experimental group, over 12 hours, with Bonferroni post-test.	115

<b>Table D.18</b> – Descriptive statistics of the absorbance of the 60% concentration small TMC nanoparticles experimental group, throughout the experiment.	115
<b>Table D.19</b> – One-way ANOVA of the absorbance of the 60% concentration small TMC nanoparticles experimental group, with Bonferroni post-test.	116
<b>Table D.20</b> – Descriptive statistics of the absorbance of the 20% concentration large TMC nanoparticle experimental group, throughout the experiment.	116
<b>Table D.21</b> – One-way ANOVA of the absorbance of the 20% concentration large TMC nanoparticle experimental group, with Bonferroni post-test.	117
<b>Table D.22</b> – Descriptive statistics of the absorbance of the 20% concentration cross-linked PEG-TMC nanoparticles experimental group, throughout the experiment.	117
<b>Table D.23</b> – Repeated measures ANOVA of the absorbance of the 20% concentration cross-linked PEG-TMC nanoparticles experimental group, over 12 hours, with Bonferroni post-test.	118
<b>Table D.24</b> – Percentage hemolysis calculated from absorbance after one hour of incubation with experimental samples.	118
<b>Table D.25</b> – Percentage hemolysis calculated from absorbance after six hours of incubation with experimental samples.	118
<b>Table D.26</b> – Percentage hemolysis calculated from absorbance after 12 hours of incubation with experimental samples.	119
<b>Table D.27</b> – Descriptive statistics of the percentage hemolysis caused by the different experimental groups after one hour of incubation.	119
<b>Table D.28</b> – One-way ANOVA of the percentage hemolysis caused by the different experimental groups after one hour, with Bonferroni post-test.	119
<b>Table D.29</b> – Descriptive statistics of the percentage hemolysis caused by the different experimental groups after six hours of incubation.	120
<b>Table D.30</b> – One-way ANOVA of the percentage hemolysis caused by the different experimental groups after six hours, with Bonferroni post-test.	120
<b>Table D.31</b> – Descriptive statistics of the percentage hemolysis caused by the different experimental groups after 12 hours of incubation.	121

<b>Table D.32</b> – One-way ANOVA of the percentage hemolysis caused by the different experimental groups after 12 hours, with Bonferroni post-test.	121
<b>Table D.33</b> – Descriptive statistics of the percentage hemolysis caused by the 20% concentration small TMC nanoparticles over the 12-hour span of the experiment.	122
<b>Table D.34</b> – One-way ANOVA of the percentage hemolysis caused by the 20% concentration small TMC nanoparticles.	122
<b>Table D.35</b> – Descriptive statistics of the percentage hemolysis caused by the 60% concentration small TMC nanoparticles over the 12-hour span of the experiment.	123
<b>Table D.36</b> – Repeated measures ANOVA of the percentage hemolysis caused by the 60% concentration small TMC nanoparticles.	123
<b>Table D.37</b> – Descriptive statistics of the percentage hemolysis caused by the 20% concentration large TMC nanoparticle over the span of the experiment.	124
<b>Table D.38</b> – One-way ANOVA of the percentage hemolysis caused by the 20% concentration of large TMC nanoparticles.	124
<b>Table D.39</b> – Descriptive statistics of the percentage hemolysis caused by the 20% concentration cross-linked PEG-TMC nanoparticles over the span of the experiment.	125
<b>Table D.40</b> – One-way ANOVA of the percentage hemolysis caused by the 20% concentration cross-linked PEG-TMC nanoparticles.	125
<b>Table D.41</b> – Absorption values of the C3 protein dilutions, measured with a BioTek microplate reader at 450 nm for drawing of a standard curve.	126
<b>Table D.42</b> – Log values of concentration and absorbance of the C3 protein dilutions for drawing of a standard curve.	126
<b>Table D.43</b> – Absorbance values of the different experimental groups measured with a BioTek microplate reader at 450 nm after completion of complement activation experiment.	126
<b>Table D.44</b> – Log values of measured absorbance values for interpolation from standard curve.	126

<b>Table D.45</b> – Log values of C3 protein concentrations, as interpolated from standard curve.	127
<b>Table D.46</b> – C3 protein concentrations of the different experimental groups as determined with a Complement C3 Human ELISA Kit.	127
<b>Table D.47</b> – Descriptive statistics of absorbance of the experimental formulations and the control group.	127
<b>Table D.48</b> – Repeated measures ANOVA of the absorbance of the experimental formulations and the control group, with Bonferroni post-test.	128
<b>Table D.49</b> – Descriptive statistics of the concentrations of the experimental formulations and the control group.	129
<b>Table D.50</b> – Repeated measures ANOVA of the concentrations of the experimental formulations and the control group, with Bonferroni post-test.	130
<b>Table D.51</b> – Absorption of different percentage concentrations of small TMC nanoparticles (in glycerol), as measured with a BioTek microplate reader at 550 nm, for the drawing of a standard curve.	131
<b>Table D.52</b> – Absorption of different percentage concentrations of large TMC nanoparticles (in glycerol), as measured with a BioTek microplate reader at 550 nm, for the drawing of a standard curve.	131
<b>Table D.53</b> – Absorbance of different percentage concentrations of cross-linked PEG-TMC nanoparticles (in glycerol), measured at 550 nm with a BioTek microplate reader, for the drawing of a standard curve.	131
<b>Table D.54</b> – Absorbance of the different experimental groups, as measured with a BioTek microplate reader at 550 nm.	131
<b>Table D.55</b> – Percentage of experimental particles unbound in plasma after incubation for four hours.	132
<b>Table D.56</b> – Percentage of experimental particles bound to plasma proteins after incubation for four hours.	132
<b>Table D.57</b> – Descriptive statistics of absorbance measured in plasma protein interaction experiment.	132

<b>Table D.58</b> – Repeated measured ANOVA of absorbance of the different experimental groups and the control, as measured in the plasma protein interaction experiment, with Bonferroni post-test.	133
<b>Table D.59</b> – Descriptive statistics of the percentage experimental particles bound to plasma proteins.	134
<b>Table D.60</b> – Repeated measures ANOVA of the percentage experimental particles bound to plasma proteins, with Bonferroni post-test.	134

# List of abbreviations

**ANOVA** – Analysis of variance

**API** – Active pharmaceutical ingredient

**CBR** – Cibacron Brilliant Red 3B-A

**DDS** – Drug delivery system

**DQ** – Degree of quaternization

**ELISA** – Enzyme-linked immunosorbent assay

**GI** – Gastrointestinal

**MASP** – MBL-associated serine protease

**MBL** – Mannan-binding lectin

**NMP** – 1-methyl-2-pyrrolidinone

**NMR** – Nuclear magnetic resonance

**PBS** – Phosphate buffered saline

**PEG** – Poly(ethylene) glycol

**PLGA** – poly lactic-co-glycolic acid

**RBC** – Red blood cells

**RED** – Rapid equilibrium dialysis

**ROS** – Reactive oxygen species

**RSD** – Relative standard deviation

**SEM** – Standard error of mean

**TMC** – *N*-trimethyl chitosan chloride

**TPP** – Tripolyphosphate

**WBC** – White blood cells