Hemocompatibility of *N*-trimethyl chitosan chloride nanoparticles

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(B.Sc)

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"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

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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., LUEβEN, 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.

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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.

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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 ± 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 ± 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 oppervlaktes kan bevorder. Daar is na TMC in die vorm van 'n nanopartikel afleweringsisteem gekyk om onder andere vaksiene, vitamiene, 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 gelaaide oppervlaktes laat maklike elektrostatiese interaksies met negatief gelaaide bloed komponente, 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 bloed komponente kan lei tot uitermatige hemolise, sel-aggregasie, komplement aktivering, inflammasie en vinnige verwydering van die partikels vanuit die sirkulasie. Hierdie toksiese interaksies kan verminder word de ur die oppervlakslading 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 bloed komponente tot 'n mate voorkom.

Om TMC nanopartikels suksesvol in 'n afleweringsisteem 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 partikel grootte, 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 bloed komponente te inkubeer. Daar is vir komplement aktivering getoets met 'n komplement C3 menslike ensiem gekoppelde immunosorbent toets (ELISA) en die interaksie met plasma proteïene is gekwantifiseer deur vinnige ekwilibrium dialise en 'n kolorimetriese toets.

Dit is bepaal dat die 60% konsentrasie klein TMC nanopartikels teen die einde van die 12-uur inkubasie tydperk 49.08 ± 2.538% hemolise veroorsaak het. Dit is beduidend hoër as enige van die ander eksperimentele groepe. Hieridie groep het ook ligte aggregasie van die witbloedselle en bloed plaatjies 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 ± 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

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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