Chapter 3

Article

Chapter 3 will give a short background on the literature, as well as providing the methods and results of the conducted study. These results will be discussed and prospects for future studies suggested. For ease of reading and for presentation in this dissertation, the Figures and Tables are incorporated within the text and not presented separately as required by the International Journal of Pharmaceutics. Sources are cited in the Harvard referencing style, according to the guide for authors of the International Journal of Pharmaceutics. To view the guide, please visit http://www.elsevier.com/journals/international-journal-of-pharmaceutics/0378-5173/guide-for-authors.

Title page

Hemocompatibility of *N*-Trimethyl Chitosan Chloride Nanoparticles

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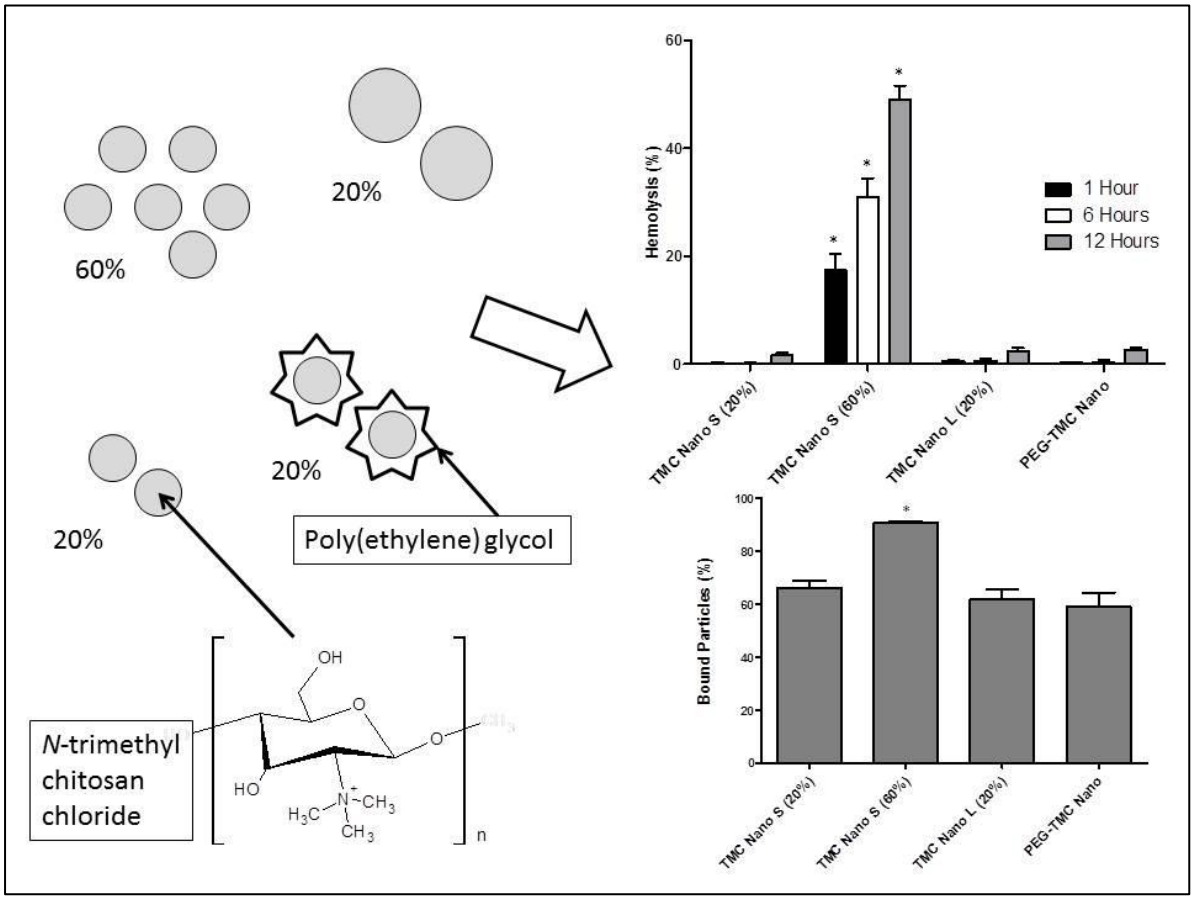
Graphical Abstract



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Abstract

N-trimethyl chitosan chloride (TMC) is a cationic, water-soluble derivative of chitosan, used as an intravenous drug delivery system. Its cytotoxicity has been extensively studied, but there is a lack of information about the hemocompatibility. This study investigated the hemocompatibility of TMC nanoparticles by examining the influence of particle size, concentration and the addition of poly(ethylene) glycol (PEG). The extent of hemolysis caused by the particles was investigated by determining the amount of free hemoglobin in the samples after incubation. Cell aggregation was observed through light microscopy. An enzyme-linked immunosorbent assay was used to determine complement activation, and the plasma protein interactions were quantified through rapid equilibrium dialysis and a subsequent colorimetric assay. The addition of PEG lowered the nanoparticle zeta potential and decreased their extent of agglomeration. The 60% concentration TMC nanoparticles caused the most hemolysis (49%) after 12 hours ($p<0.05$). This group also caused the most cell aggregation and plasma protein interaction (90.68%, $p<0.05$). All the groups caused complement activation, with no parameter having a more prominent effect than the others do.

We thus conclude that concentration, rather than particle size has the biggest influence on the hemocompatibility of TMC nanoparticles and that PEG is needed to increase stability.

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

1. Introduction

Nanoparticles have been a subject of increasing interest over the last few decades. They are already used in many commercial products, including certain foods, cosmetics, clothing and medical equipment, and other applications are still being explored (Sonia & Sharma, 2011; Wani *et al.*, 2011). Polymeric nanoparticles have piqued special interest in the pharmaceutical industry (Sonia & Sharma, 2011). These nanoparticles have shown great potential for the delivery of high molecular weight active pharmaceutical ingredients (APIs[b]), such as proteins or peptides (Sadeghi *et al.*, 2008). They are also an attractive carrier option for intravenous API administration, as they can easily move through the blood capillaries and tend to be stable in biological environments (Dobrovolskaia *et al.*, 2008; Hans & Lowman, 2002).

[b] Abbreviations: ANOVA– Analysis of variance, API – active pharmaceutical ingredient, DQ – degree of quaternization, ELISA – enzyme-linked immunosorbent assay, NMP – 1-methyl-2-pyrrolidinone, NMR – nuclear magnetic resonance, PBS – phosphate buffered saline, PEG – poly(ethylene) glycol, PLGA – poly lactic-co-glycolic acid, RED – rapid equilibrium dialysis, RSD – relative standard deviation, TMC – *N*-trimethyl chitosan chloride

One polymer that has been explored for use as a nanoparticulate drug delivery system in recent years is *N*-trimethyl chitosan chloride (TMC). It is the partially quaternized derivative of chitosan, which is made from one of the most abundant natural polymers, chitin (Malafaya *et al.*, 2007; Polnok *et al.*, 2004). TMC has many characteristics that make it an attractive prospect for use in the administration of vaccines, vitamins, insulin and cancer medication, especially intravenously (De Britto *et al.*, 2012; Du Plessis *et al.*, 2010; Li *et al.*, 2012; Sayin *et al.*, 2008). It has excellent loading capacity for peptide and protein APIs (Amidi *et al.*, 2006; Geisberger *et al.*, 2013). TMC is characteristically mucoadhesive and cationic and because of these properties it can enhance absorption across mucosal surfaces, by opening of tight junctions (Geisberger *et al.*, 2013; Sandri *et al.*, 2005; Thanou *et al.*, 2000). One of the characteristics that make TMC especially useful in drug delivery formulations is its solubility over a wide range of pH values (Polnok *et al.*, 2004).

However, the risk when using TMC nanoparticles intravenously is two-fold. The size of the nanoparticles, as well as the cationic surface charge increases the risk of toxicity (Wani *et al.*, 2011). Once nanoparticles enter the blood stream, they are met by numerous red blood cells, proteins and immune cells, and interactions with these blood components can have deleterious consequences (Dobrovolskaia *et al.*, 2008). Negatively charged plasma proteins readily interact with cationic nanoparticles. This interaction neutralizes the nanoparticle charge, preventing potentially harmful interactions it might have with other blood components or cells, e.g. hemolysis (Cerda-Cristerna *et al.*, 2011; Dobrovolskaia *et al.*, 2008). However, this interaction can also have less than ideal consequences, as it can elicit an immune response or cause activation of the coagulation factors (Cerda-Cristerna *et al.*, 2011; Dobrovolskaia *et al.*, 2009). Interactions with the proteins of the complement system can activate a cascade that results in the removal of the particles from circulation (Janeway Jr *et al.*, 2001). The complement system assists the immune system by non-specifically recognizing and removing foreign matter from the body (Bertholon *et al.*, 2006; Dobrovolskaia *et al.*, 2008). The alternative pathway is one of the first lines of defence of the body, as it does not rely on antibodies for activation. Instead, the alternative pathway is activated by the spontaneous hydrolysis of the C3 protein into C3a and C3b. C3b binds to the surface of the foreign particles in the body, allowing phagocytic cells to recognize the particles and remove them from circulation (Janeway Jr *et al.*, 2001).

The nature of the interactions between nanoparticles and blood components is dependent on the surface properties of the particles, as well as the characteristics of the polymer from which they are

made (Mailänder & Landfester, 2009; Thasneem *et al.*, 2011). The capability of a particle to interact safely with blood components is called its hemocompatibility. There are various methods to improve the hemocompatibility of a particle. One of these methods involves the addition of poly(ethylene) glycol (PEG) to the particle formulation (Dobrovolskaia *et al.*, 2008). PEG is a charge neutral polymer and by binding it to the surface of a nanoparticle, it will cause a decrease in the zeta potential of the particle (Casettari *et al.*, 2012; Geisberger *et al.*, 2013; Gref *et al.*, 2000). By doing this, PEG creates a "steric shield" around the particles, lessening their potentially deleterious interactions with the surrounding blood components (Cerda-Cristerna *et al.*, 2011; Dobrovolskaia *et al.*, 2008).

Although polymers have been a subject of interest for quite some time, studies determining the hemocompatibility of polymers, especially in the form of nanoparticles are a relatively new occurrence. Polymers whose hemocompatibility have been tested include pullulan, poly lactic-co-glycolic acid (PLGA) and chitosan. Pullulan is a fungal polysaccharide with the ability to bind to liver cells, giving it potential for use in gene delivery applications. It was determined that the hemocompatibility of this polymer was linked to its zeta potential, as cationic pullulan with the lowest zeta potential of their formulations displayed the best hemocompatibility (Rekha & Sharma, 2009). PLGA, a polymer explored for use in nuclear targeting or the delivery of immunosuppressant medication, displayed good hemocompatibility. Its hemocompatibility could further be improved, however, by making certain surface modifications, such as adding glucosamine or mucin to the PLGA particles. These modifications lead to a decrease in plasma protein interactions, thereby preventing platelet and complement activation, improving hemocompatibility (Italia *et al.*, 2007; Thasneem *et al.*, 2013a; Thasneem *et al.*, 2013b).

Chitosan is an appealing prospective for many pharmaceutical applications, due to its biocompatible and biodegradable properties (Chua *et al.*, 2012; Sieval *et al.*, 1998). However, its hemocompatibility is poor. Many modifications have been tested, with some success, to improve the hemocompatibility of chitosan. These modifications include binding of PEG to the surface of chitosan particles or developing derivatives (Luangtana-anan *et al.*, 2010; Shelma & Sharma, 2011; Smitha *et al.*, 2014). One of the most popular chitosan derivatives is TMC (Polnok *et al.*, 2004). It has been shown to be non-toxic by various studies (Amidi *et al.*, 2006; Du Plessis *et al.*, 2010; Thanou *et al.*, 2001), but, to our knowledge, no hemocompatibility studies have been performed on TMC nanoparticles. This study aims to fill this void by determining the hemocompatibility of TMC nanoparticles, including the influence surface modification has on this hemocompatibility.

To investigate the hemocompatibility of TMC, different concentrations nanoparticles, different size nanoparticles and particles with or without the addition of PEG was incubated with whole blood or blood components for specific amounts of time. Samples were analysed after incubation to determine the amount of hemolysis, cell aggregation or complement activation via the alternative pathway the particles caused, as well as the extent of their interaction with the plasma proteins. These data were used to determine the influence of concentration, particle size and the addition of PEG to the particle formulation on hemocompatibility.

2. Materials and methods

2.1 Materials

ChitoClear® Chitosan was purchased from Primex Ehf (Siglufjörður, Iceland). Methyl iodide, 1-methyl-2-pyrrolidinone, sodium hydroxide, poly(ethylene) glycol (Mw 200), tripolyphosphate, histopaque, polyethyleneimine, normal saline and cibacron brilliant red 3B-A were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium iodide, ethanol, diethylether, formic acid, formaldehyde, tween 80 and glycerol were purchased from ACE (Camden Park, Australia). Deuterium oxide was purchased from Merck (Darmstadt, Germany), hydrochloric acid from Saarchem (Honeydew, Gauteng, South Africa) and phosphate-buffered saline from Invitrogen (Carlsbad, CA, USA).

Single-use RED plates with inserts were purchased from Thermo Scientific (Waltham, MA, USA), while Complement C3 Human ELISA kits were purchased from Abcam (Cambridge, England, United Kingdom). BD Vacutainer® blood collection tubes were purchased from Lasec South Africa (Pty) Ltd (Ndabeni, Cape Town, South Africa).

2.2 Polymer synthesis and characterization

TMC was synthesized through three-step reductive methylation of chitosan, as described by Sieval *et al.* (1998). For the first step, chitosan was dissolved in 1-methyl-2-pyrrolidinone (NMP) and the solution heated to 60 °C with continuous stirring. Sodium iodide (NaI), methyl iodide (CH_3I) and 15% sodium hydroxide (NaOH) was added. The mixture was kept stirring at 60 °C for 60 minutes after which the polymer was precipitated with absolute ethanol. The precipitated polymer was filtered and washed with ethanol and diethyl ether.

The polymer was dissolved in NMP and the process repeated for the second step of the reductive methylation process. For the third step, the polymer was dissolved in NMP and the solution heated to 60 °C. NaI, CH_3I and 15% NaOH added and the mixture stirred at 60 °C for 30 minutes, after which additional NaOH pellets and CH_3I was added. The mixture was left for 60 minutes at 60 °C, with continued stirring. The final TMC polymer was precipitated and filtered with ethanol and diethylether, and left in a flow cabinet to dry. The dry powder was subsequently stored in an airtight container.

PEG was cross-linked to some of the synthesized TMC using an adaptation of a method described by Kulkarni *et al*. (2005). TMC was dissolved in formic acid. An excess amount of liquid PEG (Mw 200) was added, to ensure that the maximum amount of Schiff's bases was formed and that no polymer strings developed. The mixture was stirred for 15 minutes and formaldehyde was added. The mixture was stirred for an additional 60 minutes, after which a 10% NaOH solution was added to stop the reaction and recover the cross-linked PEG-TMC.

The cross-linked polymer was precipitated with absolute ethanol and washed in the same way as the synthesized TMC. The cross-linked PEG-TMC was left in a flow cabinet to completely dry, after which it was stored in an airtight container.

Samples of the synthesized polymer were sent for ^{1}H- and ^{13}C-NMR characterizations. Using the following formula (equation 1), the ^{1}H-NMR measurement was used to calculate the degree of quaternization of the synthesized TMC:

$$DQ(\%) = \left[\left(\int TM \Big/ \int H\right) \times \frac{1}{9}\right] \times 100 \qquad [1]$$

In the equation, *DQ (%)* is the degree of quaternization expressed as a percentage, *∫TM* represents the integral of the trimethyl amino group peak at 3.3 ppm and *∫H* represents the integral of the ^{1}H peaks at 4.7 – 5.7ppm.

2.3 Synthesis of nanoparticles

Nanoparticles of the synthesized TMC and the cross-linked PEG-TMC were made based on the ionic gelation method described by Amidi *et al*. (2006). A solution of polymer (TMC or cross-linked PEG-TMC) and Tween 80 was prepared in 300 ml distilled water. This solution was sonicated until the components were completely dissolved. Meanwhile, a separate solution of tripolyphosphate (TPP) was prepared and the pH adjusted to 8 with a 0.1 M HCl solution. While continually agitating the polymer solution by sonication, 108 ml of the TPP solution was added drop-wise. Sonication was continued for about an hour after the TPP solution had been added to ensure maximum cross-linking between the polymer and the TPP. Slight opalescence of the final suspension indicated the presence of nanoparticles. (Amidi *et al.*, 2006)

To remove the formed nanoparticles from the final suspension, 25 ml aliquots were transferred to 50 ml tubes, on top of a 3 ml layer of glycerol. The tubes were then centrifuged at 5500 rpm for 30 minutes at 5 °C. The supernatant of each tube was discarded and the glycerol (containing the nanoparticles) collected for use in the hemocompatibility experiments.

To determine the influence of size on hemocompatibility, larger nanoparticles were synthesized in the same manner as described above, except that the solution was agitated by magnetic stirring, rather than sonication.

The concentrations of the particles collected in the glycerol were determined with a colorimetric assay as described by Van der Merwe (2004a).

2.4 Particle size distribution and zeta potential

Particle size distribution and zeta potential were measured using a Malvern Zetasizer ZEN 3600. Samples (1 ml) of the different particle groups were diluted with deionised water to a volume of 10 ml. Particle size distribution was measured in cuvettes, while zeta potential measurements were conducted in folded capillary cells.

2.5 Hemocompatibility analysis

The nanoparticles (in glycerol) were diluted with PBS (pH 7.4) to give four different experimental dispersions; a 20% concentration small TMC nanoparticle dispersion, a 60% concentration small TMC nanoparticle dispersion, a 20% concentration larger TMC nanoparticle dispersion and a 20% concentration cross-linked PEG-TMC nanoparticle dispersion. These experimental dispersions were freshly

prepared before each experiment. Each experiment was conducted in triplicate and repeated three times for statistical accuracy.

A registered nurse drew blood from a healthy, unmedicated male donor, into lithium heparin and trisodium citrate BD Vacutainer® blood collection tubes (ethics application number: NWU-00025-09-S5).

2.5.1 Hemolysis

The method for determining the hemolytic properties of the experimental dispersions was based on a method described by Letchford *et al*. (2009), wherein the amount of free hemoglobin, a representation of the extent of hemolysis, is determined by measuring absorbance at 540 nm.

Whole blood was diluted with PBS (pH 7.4) to obtain an approximate red blood cell concentration of 1 x 10^8 cells/ml (Letchford *et al.*, 2009). The diluted whole blood was incubated with the experimental dispersions in a 1:1 ratio, on a plate shaker (250 rpm) for 12 hours at 37 °C. Positive and negative controls, consisting of distilled water and PBS respectively, were also incubated with diluted whole blood in a 1:1 ratio. Samples were prepared and divided in three separate 1.5 ml microcentrifuge tubes, representing 1-, 6- and 12-hour intervals. At these time intervals the appropriate tubes were centrifuged at 2 000 rpm for 10 minutes to separate the cells and the plasma containing the free hemoglobin. After centrifugation, 200 µl of the plasma was extracted and placed in a 96-well plate. The absorbance of each sample was measured at 550 ± 35 nm and the percentage hemolysis caused calculated with the equation (equation 2) seen in Letchford's article (2009).

$$\%Hemolysis = \frac{(Abs_{sample} - Abs_{spontaneous})}{Abs_{100\%\ hemolysis}} \times 100 \qquad [2]$$

The calculated percentage hemolysis data were used for statistical analyses.

2.5.2 Cell aggregation

The extent of cell aggregation, if any, caused by the experimental samples was determined with a method presented by Shelma & Sharma (2011). Whole blood collected in trisodium citrate tubes were separated by centrifugation into red blood cells (clumped at the bottom), white blood cells (small cloudy layer in the middle) and platelet-rich plasma (straw-coloured layer at the top) using

histopaque. The mentioned layers were carefully separated and placed in microcentrifuge tubes and the clear plasma layer above the red blood cells discarded.

The experimental samples were incubated with the different blood components (red blood cells, white blood cells and platelet rich plasma), as well as whole blood, for 30 minutes at 37 °C. Poly-ethyleneimine (Mw 25 000) was incubated with the blood components and whole blood as the positive cell aggregation control, whereas normal saline served as the negative control. Samples were briefly vortexed before incubation, to ensure thorough mixing. After incubation, the samples were resuspended in saline to make wet mounted slides, which were then examined through light microscopy.

2.5.3 Complement activation

Complement activation caused was measured using a 96-well Complement C3 Human ELISA kit. Blood drawn in trisodium citrate tubes were centrifuged at 2 000 rpm for 10 minutes, separating the blood plasma from the cells. The plasma was recovered and placed in a microcentrifuge tube and the cells discarded. The experimental dispersions and blood plasma were combined in a 1:1 ratio to form the experimental samples. Plasma and PBS served as a negative control. The ELISA kit protocol (Abcam, 2012) was followed to determine the complement activation and to draw a standard curve. Absorbance values were measured at 450 nm. For the standard curve, the log of the absorbance (y-axis) was plotted against the log of the corresponding concentration (x-axis). A fourth-order polynomial regression line was fitted to the data for the interpolation of the experimental absorbance values.

2.5.4 Plasma protein interaction

Plasma protein interactions of the experimental solutions were quantified with the use of a Single-use Rapid Equilibrium Dialysis (RED) plate and a subsequent colorimetric assay (Thermo Scientific, 2007; Van der Merwe *et al.*, 2004b). Blood drawn in lithium heparin tubes were centrifuged at 2 000 rpm for 10 minutes and the plasma collected in microcentrifuge tubes. Experimental samples consisted of 200 µl plasma and experimental dispersion (1:1), placed in the red sample chamber on the RED plate. Plasma and PBS (1:1) was used as a negative control. The corresponding white chambers were filled with 350 µl PBS. The plate was covered with sealing tape and incubated at 37 °C on a plate shaker (250 rpm) for four hours.

After the incubation period had been completed, 50 µl aliquots were drawn from the applicable red and white chambers and transferred to a 96-well plate, where 100 µl cibacron brilliant red 3B-A solution (75 µg/ml) was added. The absorbance at 550 nm was read and the resulting values were fitted to standard nanoparticle concentration curves (small TMC, larger TMC and cross-linked PEG-TMC) to determine the amount of unbound particles in the samples. PBS absorbance values were used for background subtraction.

2.6 Statistical analysis

Data were analysed with GraphPad Prism 5, calculating descriptive statistics, determining the differences between samples with the use of Analysis of Variance (ANOVA) and quantifying the correlations between the different experimental parameters. GraphPad Prism 5 was also used to draw standard curves, column graphs and line graphs. Standard curves were fitted with linear regression or fourth-order polynomial regression (in the complement activation experiment) for the interpolation of unknown experimental values. Colorimetric assay data was firstly analysed with GEN5 Software, after which statistical analysis were done with GraphPad Prism 5.

3. Results and discussion

3.1 Polymer synthesis and characterization

TMC was synthesized through the reductive methylation of chitosan. In this process, amine groups in chemical structure of chitosan were trimethylated. The degree of quaternization (DQ) is a measure of the percentage of the amine groups that were trimethylated, and thus a measure of the positive charge of the TMC (Hamman *et al.*, 2003). Studies have also found that the DQ of TMC influences its mucoadhesive properties (Sandri *et al.*, 2005; Snyman *et al.*, 2003). Higher DQ means a more positively charged molecule and therefore increased interaction with cell membranes for the opening of tight junctions (Hamman *et al.*, 2003). In their study, Chen *et al.* (2007) observed that higher DQ TMC was able to produce smaller particles with increased zeta potential.

The ^{1}H NMR spectrometry results were used to calculate the 60% DQ of the synthesized TMC. The degree of *O*-methylation was also calculated and was found to be 33% (results not shown). The high degree of quaternization means good mucoadhesive properties and tight junction opening. The *O*-methylation is sufficiently low, thereby not negatively affecting the solubility of the synthesized polymer (Polnok *et al.*, 2004).

3.2 Concentration of synthesized nanoparticles

Absorbance of different TMC and cross-linked PEG-TMC concentrations were measured and standard curves drawn. The curves were fitted with linear regression, to be able to calculate unknown values. The absorbance of the small TMC nanoparticles, the larger TMC nanoparticles and the cross-linked PEG-TMC nanoparticles (all in glycerol) were measured at the same wavelength as the standard curves. These absorbance values were fitted to the appropriate curves and the concentration determined. As colorimetric analyses measure the amount of TMC or PEG-TMC present in the particles and not the particle concentrations themselves, the determined concentrations were used as an indirect way of determining the concentration of the synthesized particles in the glycerol. The small TMC nanoparticles in glycerol had a calculated TMC concentration of 1789.90 µg/ml and the larger TMC nanoparticles a TMC concentration of 1909.00 µg/ml. The cross-linked PEG-TMC nanoparticles in glycerol had a calculated PEG-TMC concentration of 1344.50 µg/ml.

These concentration values allowed us to compare the results of the different hemocompatibility experiments.

3.3 Particle size distribution and zeta potential

The size and zeta potential of the small and larger TMC nanoparticles and the cross-linked PEG-TMC nanoparticles were measured with a Malvern Zetasizer ZEN 3600. Determining the size of the different particles allowed investigation of the influence of size difference on the hemocompatibility of the nanoparticles. To be able to compare the particle sizes more accurately, the percentage relative standard deviation (%RSD) was also calculated.

Zeta potential is a measure of charge density on the particle surface. It is postulated that the addition of PEG will lower the zeta potential of the particles (Gref *et al.*, 2000), thereby possibly improving the hemocompatibility profile. The size and zeta potential determination results are summarized in Table 1.

Table 1 – 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 ± standard deviation. The percentage relative standard deviation (%RSD) is an indication of the spread of the data.

	Average size (nm)	%RSD	Zeta potential (mV)
Small TMC nanoparticles	122.78 ± 45.28	36.88	18.98 ± 2.91
Larger TMC nanoparticles	243.05 ± 7.19	7.19	20.38 ± 1.62
PEG-TMC Nanoparticles	124.75 ± 6.15	4.93	12.32 ± 2.02

The small TMC nanoparticles and the cross-linked PEG-TMC nanoparticles had comparable average particle sizes. The small TMC nanoparticles, however, had a much larger standard deviation and %RSD than the cross-linked PEG-TMC nanoparticles. Particle size and zeta potential similar to the small TMC nanoparticles were found by Wang *et al.* (2010), when they synthesized nanoparticles from conjugated TMC-PLGA. Before the particle size determination in this study, the particle-containing glycerol was sonicated to disperse the particles evenly and to ensure that no agglomeration of particles affected readings. The small TMC nanoparticles seemed unstable, as they agglomerated soon after sonication, causing increased sized readings. The cross-linked PEG-TMC nanoparticles, however, did not display the same behaviour, remaining stable throughout the measurements. The explanation for the observation is presented in the zeta potential results. Addition of PEG to the formulation had caused a decrease in zeta potential from 18.98 ± 2.91 mV to 12.32 ± 2.02 mV, an observation also made by Gref *et al.* (2000) in their study. A decrease in zeta potential would cause a decrease in the reactivity of the particle, thus causing increased stability and less particle agglomeration (Cerda-Cristerna *et al.*, 2011; Dobrovolskaia *et al.*, 2008).

Amidi *et al.* (2006) synthesized TMC nanoparticles, which had comparable size (254 ± 9 nm) and zeta potential (20 ± 2 mV) to the larger TMC nanoparticles in this study. Although the preparation method was the same, their DQ was much lower (25%). The TMC particles synthesized in a study by Chen *et al.* (2007) also had sizes comparable to the larger TMC nanoparticles (240 ± 10 nm), but had lower zeta potential values at 16.8 ± 0.6 mV. Again the method of particle synthesis was the same, but they too had a much lower DQ (33%). As the DQ is a measure of the positive charge of the particle (Hamman *et al.*, 2003), our higher DQ might explain why we measured higher zeta potential.

3.4 Hemocompatibility analysis

3.4.1 Hemolysis

Upon entering the blood, injected particles will most likely first encounter and interact with red blood cells. Electrostatic interactions between the red blood cells and the nanoparticles can cause perturbation of the membrane, thereby causing hemolysis (Dobrovolskaia *et al.*, 2008). In this study, we investigated the amount of hemolysis caused by the different experimental groups, by incubating these groups with diluted whole blood for specific amounts of time, after which the absorbance was measured to determine the amount of hemolysis caused. Figure 1 depicts the mean percentage hemolysis caused after 1-, 6- and 12-hours of incubation with the different experimental groups.

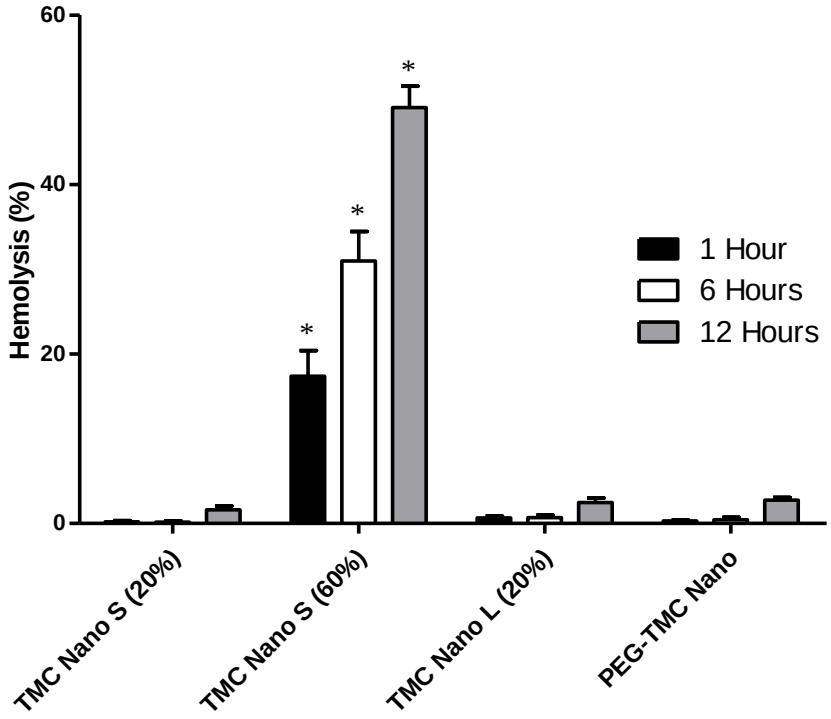

Figure 1 – Mean percentage hemolysis caused, with standard error of mean, at 1-, 6- and 12-hour intervals, as calculated for each of the experimental groups. For each bar n = 9.
* Statistical significance compared to other experimental groups in the same time interval.

The only group to cause significant hemolysis ($p<0.05$) in comparison to the other experimental groups was the 60% concentration small TMC nanoparticles. This group had caused significant hemolysis at the 1-, 6- and 12-hour intervals, although its absorbance was continually significantly lower than the positive control. After 12 hours, the 60% concentration small TMC nanoparticle solution had caused a considerable percentage of hemolysis (49.08 ± 2.54%), whereas the 20% concentration small TMC nanoparticle solution had only caused 1.63 ± 0.44%. The 20% concentration larger TMC nanoparticle solution had caused 2.45 ± 0.54% and the cross-linked PEG-TMC nanoparticle solution, 2.75 ± 0.33%.

In their study, Mayer *et al.* (2009) observed that smaller cationic particles caused more hemolysis than larger particles did. However, this was not observed in our study, possibly because the zeta potential difference between the small and larger TMC nanoparticles was not as great as was seen between the smaller and larger particles in Mayer *et al.*'s study. The extent of hemolysis a particle causes is more dependent on the characteristics of the polymer from which it is made (Moreau *et al.*, 2002). The result of this study is, however, similar to the study of Wang *et al.* (2008). They found that an increase in the concentration of chitosan microspheres resulted in an increase in hemolysis (Wang *et al.*, 2008).

3.4.2 Cell aggregation

Incubation of the experimental groups with separate blood components, as well as whole blood allowed investigation of not only the aggregation caused by the groups, but also of the protective effect of plasma protein binding. After incubation, the aggregation caused to each blood component and the whole blood was observed through light microscopy. The extent of aggregation caused is summarized in Table 2.

Table 2 – 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.

	Control		TMC Nanoparticles (S)		TMC Nano-particles (L)	PEG-TMC nanoparticles
	Positive	Negative	20%	60%		
RBC	++++	--	--	+	--	--
WBC	++++	--	--	++	+	--
Platelets	++++	--	--	++	+	+
Whole blood	++++	--	+	+	++	--

Although none of the experimental groups caused any excessive cell aggregation, the 60% concentration small TMC nanoparticles was the only group to cause slight to mild aggregation of all the blood components and whole blood. The larger TMC nanoparticles were second to last in the hemocompatibility line, causing mild aggregation of the whole blood and slight aggregation of the white blood cells and platelets. It did not cause any aggregation of the red blood cells in the 30-minute incubation period, however.

Platelets are extremely sensitive, easily reacting with foreign material in the blood stream, such as nanoparticles. Disturbance of the surface charge of these or any other blood cells can lead to aggregation (Cerda-Cristerna *et al.*, 2011). Studies have shown that chitosan causes increased platelet aggregation with increased concentration (Chou *et al.*, 2003; Okamoto *et al.*, 2003). As TMC is a derivative of chitosan, it can be expected to see a similar result in this study. Although very little aggregation was seen, the 60% concentration small TMC nanoparticles caused the most aggregation of all the experimental groups. This group caused mild aggregation of the platelets, as well as the white blood cells. The white blood cell sample possibly contained some platelets, causing increased concentration.

Particles approximately 200 nm in size, such as the larger TMC nanoparticles, are readily phagocytised by macrophages in the blood stream, causing activation of coagulation factors (Cerda-Cristerna *et al.*, 2011; Schöll *et al.*, 2005). This explains why the larger TMC nanoparticles displayed the most cell aggregatory effects when incubated with whole blood, where all of the components for

coagulation activation are present and why no effect was seen when incubated with red blood cells alone. While determining the influence of size on hemocompatibility, Mayer *et al.* (2009) found very little plasma coagulation, as we did. They surmised that the cationic charge of the particles they had used was insufficient to disturb the surface charge of the platelets (Mayer *et al.*, 2009).

3.4.3 Complement activation

As this study was conducted *in vitro*, the best course of action to determine the ability of particles to activate the complement system was to look at the alternative pathway of complement activation. Unlike the other activation pathways, the alternative pathway does not need antibody interaction to start the complement cascade. A Complement C3 Human ELISA was used to determine complement activation by the different experimental groups. This kit measures the amount of C3 protein present in the samples. C3 is central to all of the pathways, as they all create C3 convertase, which cleaves C3 into its activated forms, C3a and C3b. The less C3 protein present, the more complement activation had taken place (Abcam, 2012; Janeway Jr *et al.*, 2001). The results from the assay are depicted in Figure 2.

All the experimental groups had significantly lower C3 protein concentrations after incubation than the negative control group. This suggests that all of the experimental groups caused complement activation to a certain extent. There were no statistical differences in the C3 protein levels between the experimental groups, however, and thus no deduction could be made as to what influence size, concentration or addition of PEG had on complement activation. Krajewski *et al*. (2013) found that the complement activation caused by silver nanoparticles increased with an increase in concentration. However, as this study does not make use of inorganic material nanoparticles, the result could be quite different. Performing a more specific analysis, looking at more of the proteins in the cascade might be helpful in this regard. Complement is a big part of non-specific immunity (Bertholon *et al.*, 2006). However, it still varies between individuals. Using more donors could also help to shed light on the complement activation of TMC nanoparticles.

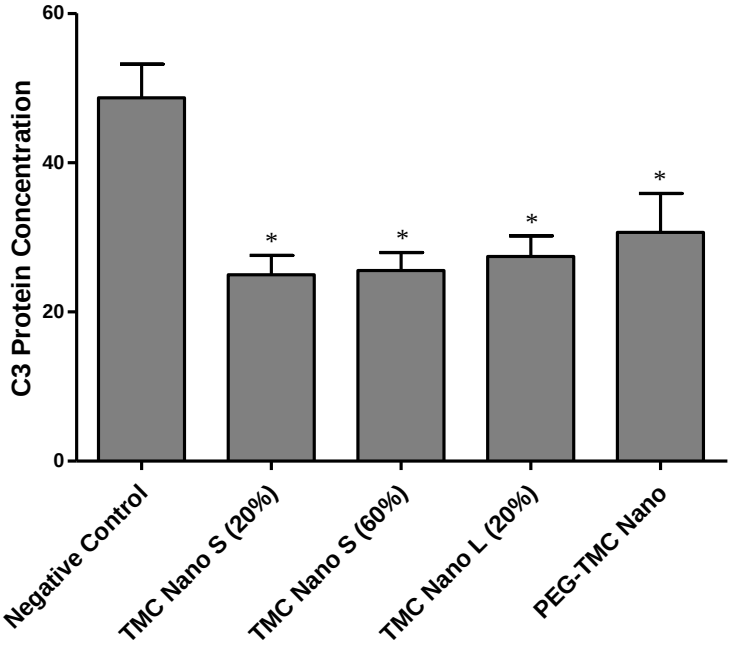

Figure 2 – Mean C3 protein concentration with standard error of mean, as interpolated from the standard curve as activated by the different experimental groups and the control group. For each bar n = 9.
* Statistical significance compared to control group

3.4.4 Plasma protein interaction

Plasma protein interactions were quantified by incubating the experimental groups with blood plasma for four hours. After this time, the amount of free particles in the sample was determined using a colorimetric assay and standard concentration curves of each of the experimental particle groups in glycerol. The percentage of the initial particles in the sample bound to plasma proteins was then determined. As plasma proteins influence inflammation and coagulation factors, extensive interaction of particles would be a sign of low hemocompatibility and possibly have toxic consequences (Cerda-Cristerna *et al.*, 2011; Dobrovolskaia *et al.*, 2009). The percentages of experimental particles bound to plasma proteins are shown in Figure 3.

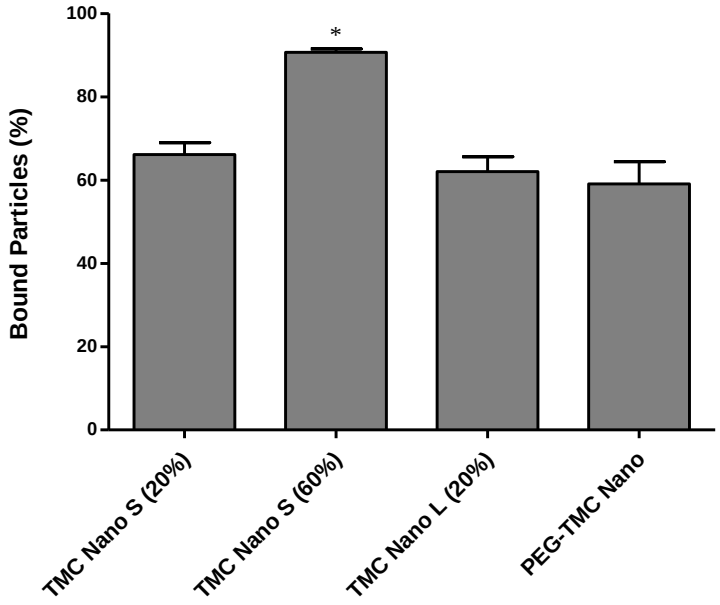

Figure 3 – Graphic representation of the mean percentage of particles of each experimental group bound to complement C3 proteins, along with the standard error of the mean values, as calculated from the standard curves of each particle type in glycerol. For each bar n = 9.

* Statistical significance compared to the values of the other experimental groups.

At 90.68 ± 0.83%, the 60% concentration small TMC nanoparticles had significantly more interaction with plasma proteins than any of the other experimental groups had. No particles or APIs were present in the control group and thus it could only be used in the comparison of absorption values, where it showed significance compared to the 60% small TMC nanoparticles (results not shown).

Because of the cationic nature of TMC, it is expected that the nanoparticles will readily interact with the negatively charged plasma proteins (Thanou *et al.*, 2000). Rekha & Sharma (2009) investigated the hemocompatibility of pullulan, a cationic polymer. They found that the extent of plasma protein binding to the polymer was proportional to the surface charge density. Therefore, when they added PEG to the formulation, the extent of plasma protein interaction decreased. Although the cross-linked PEG-TMC nanoparticles had the lowest percentage plasma protein interaction (59.08 ± 5.31%), the difference between it and the 20% small TMC nanoparticles (62.04 ± 3.57%) was not significant, suggesting that the addition of PEG did not have the desired effect on hemocompatibility.

4. Conclusion

It was though that size would have the biggest influence on the hemocompatibility of TMC particles, as previous studies have found that particles size was one of the main characteristics influencing interactions with the blood components and that smaller particles were more prone to toxicity than their larger counterparts were (Mayer *et al.*, 2009; Soppimath *et al.*, 2001; Wani *et al.*, 2011). However, the experimental and statistical data have shown that concentration, rather than particle size has the biggest influence on the hemocompatibility of TMC nanoparticles. This result was also seen in literature in studies pertaining to chitosan (Chou *et al.*, 2003; Okamoto *et al.*, 2003; Wang *et al.*, 2008).

Studies have found that the addition of PEG resulted in improved hemocompatibility (Koziara *et al.*, 2005; Rekha & Sharma, 2009). This effect was not observed in this study. It was found, however, that the TMC nanoparticles seemed unstable and reactive during the size determination. The formulations containing PEG displayed much better stability and did not tend to agglomerate as much. Therefore, we can conclude that PEG needs to be used in the formulation of TMC nanoparticles, to add stability, although another method of incorporating the PEG in the formulation should be explored to get the additional hemocompatibility-improving benefit.

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