Chapter 4

Conclusions and Future Prospects

4.1 Conclusions and future prospects

The aim of this study was to determine the hemocompatibility of TMC in the form of nanoparticles, specifically looking at the extent that it causes hemolysis, cell aggregation, complement activation and the amount of plasma protein interaction it displays. The influence of particle size, concentration and the addition of PEG on the hemocompatibility of TMC nanoparticles concerning these factors were also examined, as particle size determines cellular uptake and concentration increases can exacerbate problems with hemocompatibility, whereas the addition of PEG can ameliorate them. The sizes of the small TMC nanoparticles and the cross-linked PEG-TMC nanoparticles are comparable, meaning that any differences in hemocompatibility between these groups can be attributed to the addition of PEG, with a fair amount of certainty. Although both the small and larger TMC nanoparticles fall well within the nanoparticle range (1-500 nm), the size difference should be great enough to observe the differences in hemocompatibility size alteration would make.

The extent of hemolysis caused by TMC nanoparticles was examined by incubating the experimental particle dispersions (20% concentration small TMC nanoparticles, 60% concentration small TMC nanoparticles, 20% larger TMC nanoparticles and 20% cross-linked PEG-TMC nanoparticles) with diluted whole blood for 12 hours. At 1-, 6- and 12-hour intervals, the absorbance of the samples were measured. Hemolysis is caused by a perturbation of the red blood cell membrane, which causes the hemoglobin to leak out (Dobrovolskaia *et al.*, 2008; Moreau *et al.*, 2000; Shelma & Sharma, 2011). Measuring the absorbance of the samples served as a way of quantifying the amount of free hemoglobin in the samples, i.e. the amount of hemolysis caused (Letchford *et al.*, 2009).

When the percentage hemolysis caused (in comparison with the positive and negative controls) was calculated, the effects of the experimental particle dispersions could be compared. Throughout the samples, a time-dependent increase in hemolysis was observed. All the samples had shown a significant increase in hemolysis from six to twelve hours.

The 60% concentration small TMC nanoparticles had caused significantly more hemolysis than the other experimental dispersions at each of the measured intervals, causing 49.08% hemolysis at the end of the 12-hour incubation period. None of the other experimental groups had caused any significant hemolysis compared to the negative control. This result is similar to the result of a study

done by 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).

None of the experimental groups caused any extensive cell aggregation. The 60% concentration small TMC nanoparticles had caused mild aggregation of the white blood cells and the platelets and the larger TMC nanoparticles caused mild aggregation when incubated with whole blood.

Platelets are extremely sensitive and interactions with foreign material in the blood can lead to aggregation (Cerda-Cristerna *et al.*, 2011). Concentration dependent activation and subsequent aggregation of platelets, as found in this study, has also been seen in studies on chitosan (Chou *et al.*, 2003; Okamoto *et al.*, 2003). Aggregation seen when the 60% small TMC nanoparticles were incubated with white blood cells is possibly due to the presence of platelets in the sample.

Coagulation factors are controlled by plasma proteins (Cerda-Cristerna *et al.*, 2011). The composition of the protein layer forming around a particle is, to a certain extent, responsible for the effect the particle has in the blood (Lynch & Dawson, 2008). Larger particles mean more opsonins, leading to better uptake by macrophages and more activation of coagulation factors (Cerda-Cristerna *et al.*, 2011; Schöll *et al.*, 2005), which explains why the larger TMC nanoparticles had caused mild aggregation when incubated with whole blood, but not with red blood cells alone.

Although all of the experimental groups activated the complement system to a certain extent, no one parameter caused more activation than the others, meaning no deduction could be made as to what influence size, concentration or the addition of PEG had on complement activation. It might be helpful to look at activation of more of the proteins in the complement cascade to be able to determine the influence of the parameters. Being part of the immune system, complement activation varies between individuals. For this reason, the use of more donors could help clarify complement activation of TMC nanoparticles.

Electrostatic plasma protein interactions are the basis of most toxic effects (Cerda-Cristerna *et al.*, 2011; Choksakulnimitr *et al.*, 1995). The plasma proteins interact with foreign material in the blood to protect other blood components from harmful interactions, but at the same time, it can lead to toxicity in the form of inflammation or coagulation (Cerda-Cristerna *et al.*, 2011; Dobrovolskaia *et al.*, 2009; Moreau *et al.*, 2002; Moreau *et al.*, 2000). As the proteins have a negative surface charge, they readily interact with polycations, such as the TMC nanoparticles (Moreau *et al.*, 2002). All of

the experimental groups had relatively extensive plasma protein interaction, but at 90.68%, the 60% small TMC nanoparticles had interacted most. PEG creates a steric shield around the particles, preventing a certain extent of interaction with plasma proteins, thereby decreasing possible toxicity (Cerda-Cristerna *et al.*, 2011; Gref *et al.*, 2000). A study examining the hemocompatibility of pullulan found that the extent of plasma protein interaction decreased with the addition of PEG (Rekha & Sharma, 2009). This effect was not seen in our study, however, suggesting that the addition of PEG did not have the desired effect on the hemocompatibility of TMC nanoparticles.

According to the experimental results, we can conclude that the toxicity of TMC nanoparticles is concentration dependent. Although the addition of PEG did not improve hemocompatibility of the particles as expected, it did seem to improve stability, as seen during the size distribution determination.

In future, gaps left by this study in future can be filled by using a wider concentration range when determining the hemocompatibility of TMC nanoparticles. Determining the maximum concentration at which the nanoparticles retain their hemocompatibility can have great value for clinical applications.

Another subject of interest worth exploring is the influence of TMC's degree of quaternization on not only its hemocompatibility, but also its cross-linking capabilities, determining whether it can cross-link with PEG or form nanoparticles through cross-linking with TPP.

The complement activation experiment's lack of specificity highlights the need for more specific tests of hemocompatibility. By identifying the specific interactions causing toxicity, the particles can be modified to make them more compatible with the blood components.

4.2 References

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