Annexure A

Materials, Methods and Results

A.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).

A.2 Methods

A.2.1 Synthesis of *N*-Trimethyl chitosan chloride

N-trimethyl chitosan chloride (TMC) was synthesized through three-step reductive methylation of chitosan (Sieval *et al.*, 1998; Snyman *et al.*, 2003). The first step of this method involved putting 4 g of chitosan and 160 ml 1-methyl-2-pyrrolidinone (NMP) (as solvent) in a two-neck round flask. A stirring magnet was added and a thermometer secured in one of the openings. The flask was then suspended in water, on a hot plate with a stirrer. The mixture was heated to 60 °C under magnetic stirring. When the right temperature was reached, 9.6 g of sodium iodide (NaI), 23 ml of methyl iodide (CH_3I) and 22 ml of a 15% sodium hydroxide (NaOH) solution was added and a Liebig condenser secured on top of the flask. The flask was kept at 60 °C for 60 min, with continued stirring. After the 60 min had passed, the flask was taken out of the water and the Liebig condenser, the thermostat and the stirring magnet removed. Absolute ethanol was added to the flask with occasional stirring for thorough mixture, to precipitate the polymer. The flask filled with the absolute ethanol was left overnight, to ensure complete precipitation.

The filtration of the polymer took place in a fume hood. A vacuum pump connected to a flask with a porcelain filter cup secured to the top was set up. Each time, a small volume of the contents of the flask was added to the filter cup, to which more absolute ethanol was added. The solution was

stirred to verify complete precipitation of the polymer before the vacuum pump was turned on to remove the remaining liquid. Care was taken not to clog the filter. The flask was rinsed with ethanol to retrieve all of the polymer. When the entire content of the flask has been added to the filter cup, the polymer was washed with diethyl ether, until the liquid flow-through ran clear.

For the second step of the method, the filtered and washed polymer was returned to the round flask and another 160 ml of NMP was added. The rest of the step proceeded exactly like the first.

In the third step of the method, the filtered and washed polymer was returned, once again, to the round flask and 160 ml of NMP added. As in the previous steps, a stirring magnet was added to the flask and a thermostat secured in one of the flask's openings. The flask was again suspended in water on a hot plate, under the magnetic stirring and the mixture heated to 60 °C. Upon reaching the correct temperature, 9.6 g NaI, 14 ml CH_3I and 22 ml 15% NaOH was added to the flask and a Liebig condenser placed in the second flask opening. The flask was kept at 60 °C with continuous stirring, but in this step, only for 30 min, after which an additional 1.2 g of NaOH pellets and 5 ml CH_3I was added. The mixture was subsequently left for 60 min at 60 °C, with continued stirring.

Finally, the produced polymer was precipitated with absolute ethanol, left overnight, and filtered and washed as described above. The washed polymer was left in the flow cabinet for two days to allow the remaining diethyl ether to evaporate and the polymer to completely dry. The formed TMC was then stored in airtight containers for later use. A sample of the synthesized TMC was dissolved in D_2O and was sent for ^{1}H and ^{13}C NMR characterization.

A.2.2 Poly(ethylene) glycol – TMC cross-linking

It was determined that the best and most consistent method of incorporating poly(ethylene) glycol (PEG) into the particles used in the experiments, would be to cross-link it to the TMC before the particles were even made. For this, an adaptation of a method described by Kulkarni *et al*. (2005) was used.

The procedure took place in a fume hood, where a magnetic stirrer was set up. TMC (1 g) was dissolved in 10 ml of formic acid in a 100 ml beaker with mild magnetic stirring, as to avoid splashing, while hastening the dissolving process. Once all of the TMC was dissolved and a viscous solution was formed, 10 ml of liquid PEG (Mw 200) was added. The large excess of PEG used ensured that the maximum amount of Schiff's bases was formed and that no polymer strings developed. The mixture was stirred for 15 minutes, after which 1 ml of formaldehyde was added. This solution was then left

to stir for 60 min. The reaction was stopped after 60 min and the cross-linked PEG-TMC recovered by filling the beaker with 10% NaOH, while still stirring slowly (Kulkarni *et al.*, 2005). The PEG-TMC was then precipitated and washed with ethanol and diethyl ether, using the same set-up as was used when filtering the TMC polymer. The formed PEG-TMC was left in the flow cabinet for two days to allow it to thoroughly dry, after which it was stored in an airtight container.

A sample of the formed PEG-TMC was dissolved in D_2O and sent for NMR characterization (^{1}H and ^{13}C).

A.2.3 NMR characterization of polymers

The synthesized polymers were characterized by nuclear magnetic resonance spectroscopy. The samples were sent for ^{1}H- and ^{13}C-NMR characterizations. Polymer samples (45 mg) were dissolved in 1 ml D_2O and filtered through cotton wool into a NMR tube, ensuring that only completely dissolved polymer was eventually present in the tube.

A 600 MHz DMX Brucker apparatus (Karlsruhe, Germany) was used to measure the solutions. The ^{1}H-NMR measurement was used to calculate the synthesized TMC's degree of quaternization, with the following equation:

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

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.

A.2.4 Synthesis of TMC nanoparticles and PEG-coated nanoparticles

The method for synthesis of TMC nanoparticles was based on the method proposed by Amidi *et al*. (2006).

For the first attempt, a solution of 2 mg/ml TMC and 1% (w/v) Tween 80 was prepared in 5 ml of water. Magnetic stirring was employed to assist in the dissolving process. Meanwhile, a separate 1 mg/ml solution of tripolyphosphate (TPP) was prepared and the pH adjusted to 8 with a 0.1 M HCl solution. While continuously stirring, 1.8 ml of the TPP solution was added drop-wise to the TMC

solution, using a micropipette, to create the particles in suspension. After the TPP and the TMC had cross-linked to form the TMC particles, 1.5 ml aliquots of the suspension was placed on a drop of glycerol (± 10 µl) in a 2 ml microcentrifuge tube. The tubes were centrifuged at 7 750 rpm for 15 minutes. The idea was to discard the supernatant and to resuspend the pellet, but the particles were trapped in the glycerol and we did not have access to a small enough filter to be able to remove them from it. Light microscopy confirmed the presence of small particles in the glycerol.

The aim of the following attempts was to synthesize and isolate the TMC nanoparticles without trapping them in glycerol. The second attempt started with a solution of 2 mg/ml TMC and 1% (w/v) Tween 80 in 5 ml of water. Again, a 1 mg/ml TPP solution was made and the pH adjusted to 8 with a 0.1 M HCl solution. With continuous magnetic stirring, the TPP solution was added drop-wise to the TMC solution, using a micropipette. This time, the particle-containing suspension was washed with methanol. The suspension was transferred to a 15 ml falcon tube, along with methanol in a 1:1 ratio. After careful mixing, the tube was centrifuges at 5 000 rpm for 15 minutes. The supernatant was carefully removed and the pellet resuspended in water. This suspension was placed in a -80 °C freezer overnight and then freeze-dried. Light microscopy showed that the synthesized particles agglomerated extensively and that the unreacted TPP had formed large crystals that could potentially damage cells.

The method was next adjusted to exclude the Tween 80 from the formulation. Without washing, the particle-containing solution was placed in a -80 °C freezer overnight and then freeze-dried. However, without the Tween 80, the TMC and the TPP had failed to cross-link and no particles had formed.

Based on a method proposed by Sadeghi *et al*. (2008) a fourth attempt at making TMC nanoparticles was made. TMC (1 mg/ml) was dissolved in water and the pH was adjusted to 5.2 with a 0.01 M NaOH solution. A 1 mg/ml TPP solution was also prepared and added drop-wise to the TMC solution in a 1:4 ratio, whilst continuously stirring with a magnet. The resulting mixture was centrifuged at 14 000 rpm for 30 minutes. The idea was to carefully remove the supernatant and freeze-dry the pellet, but no pellet formed, even after centrifuging for 30 minutes at 25 000 rpm. Thus, this method was laid to rest and we continued adjusting the method of Amidi *et al*. (2006).

For the next method adjustment a 2 mg/ml TMC and 1% (w/v) Tween 80 solution was prepared in water. A separate 1 mg/ml TPP solution was prepared and the pH adjusted to 8. The TMC solution was agitated with an ultrasonic processor UP100H (Hielscher Ultrasonics GmbH, Teltow, Germany), while the TPP solution was added drop-wise using a Watson-Marlow 205S peristaltic pump (Watson-Marlow Pumps, Falmouth, Cornwall, UK) at 2 rpm. The resultant mixture was washed with

methanol, as described before and the pellet resuspended in a very small amount of water. This suspension was then centrifuged again at 14 000 rpm for 30 minutes. The acquired pellet was placed in a -80 °C freezer overnight and then freeze-dried. Light microscopy again showed the presence of large TPP crystals.

To try to remove the TPP crystals, the TPP was filtered before the drop-wise addition to the TMC solution. It was unsuccessful, however, and the TPP crystals were still present at the end of the method.

For the seventh attempt, a slightly larger volume was used. 2 mg/ml TMC and 1% (w/v) Tween 80 were dissolved in 25 ml of water. The 1 mg/ml TPP solution was prepared as before and was not filtered. The pH 8 TPP solution was added drop-wise to the TMC solution, with continuous magnetic stirring. The resultant suspension was washed with methanol, the pellet resuspended in a very small volume of water and this suspension centrifuged at 14 000 rpm for 30 minutes, as before. The acquired pellet was placed in a -80 °C freezer overnight and then freeze-dried. Apart from the TPP crystals, sheets of unreacted Tween 80 were also seen through light microscopy. The synthesized particles seemed to be stuck to the Tween 80 sheets.

To counteract the unreacted Tween 80 sheets, the concentrations of the TMC and the TPP were increased to 10 mg/ml and 5 mg/ml respectively. The method was repeated as before. No improvement was seen and it was decided that the centrifugation of the particles into the glycerol was needed to remove any unreacted reagents.

The first method was repeated in a slightly larger volume (20 ml of water), as the 5 ml was deemed too small for comfortable use. After the particles were centrifuged into the glycerol layer, the watery layer was removed and discarded. We attempted to remove the particles from the glycerol with the use of dialysis tubes (pore size 3.5-5 kD, Sigma-Aldrich, St. Louis, MO, USA). The outer tube was filled with 20 ml absolute ethanol. Ethanol was added to the particle-containing glycerol until the volume reached 750 µl and this mixture was then placed in the inner dialysis tube. The dialysis tubes were placed in a 30 °C water-bath for 15 minutes. After this incubation period, the contents of the inner dialysis tubes were collected and the tubes rinsed with water to remove any particles still left over. The contents of the inner dialysis tube was placed in a -80 °C freezer overnight and then freeze-dried. Inspection of the freeze-dried particulate matter revealed that dialysis had not removed any of the glycerol.

After many discussions, it was decided that the glycerol should not have a significant effect on the experiments, as glycerol does not affect cells negatively. We then set out to increase the volume of the synthesis to save time and resources. Eventually the volume was deemed large enough (300 ml

water) and the TMC and cross-linked PEG-TMC nanoparticles were synthesized as follows, based on the method described by Amidi *et al*. (2006), with modifications.

The synthesis of the PEG cross-linked and uncross-linked nanoparticles started with a solution of 2 mg/ml TMC (or cross-linked PEG-TMC) and 1% (w/v) Tween 80 prepared in 300 ml distilled water. The solution was sonicated with an ultrasonic processor UP100H at 100% amplitude, until the components were completely dissolved. A separate solution of tripolyphosphate (TPP) (1 mg/ml) was prepared and the pH adjusted to 8 with a 0.1 M HCl solution. TPP solution (108 ml) was added to the TMC and Tween 80 solution with the use of a Watson-Marlow 205S peristaltic pump at 2 rpm, while the ultrasonic processor UP100H agitated the solution at ambient temperature. A pipet and needle were used to drip the TPP solution close to source of agitation, to ensure thorough dispersion of the droplets, thereby creating smaller particles. When the TPP solution's drop-wise addition started, a five-hour timer was set. This provided time for the TPP solution to be added, plus extra time for sonication to ensure that maximum cross-linking between the TMC (or cross-linked PEG-TMC) and the TPP occurred. The Tween 80 served as a surfactant during the reaction.

After sonication had been completed, the formed nanoparticle suspension was concentrated and purified of any unreacted reagents through centrifugation. Aliquots of the suspension (25 ml) 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 experiments. One repetition of the described process provided enough PEG cross-linked particles, whereas the synthesis of the nanoparticles without PEG was repeated eleven times to ensure sufficient particles for experimental purposes. (Amidi *et al.*, 2006)

For comparison of size influence on hemocompatibility, larger nanoparticles were synthesized in the same manner as described above, except that the solution was agitated with the use of a magnetic stirrer, rather than sonification.

A.2.5 Concentration of particles in glycerol

The concentration of the synthesized TMC particles in the glycerol after centrifugation was determined with a SPECCORD® 200 PLUS UV Visible Spectrophotometers (Analytik Jena AG, Jena, Germany). A 150 µg/ml solution of Cibacron Brilliant Red 3B-A (CBR) was prepared in a 500 ml

volumetric flask, using distilled water. In a 250 ml volumetric flask, 225 mg of TMC was dissolved in 50 ml distilled water. The flask was then filled with phosphate buffered saline (PBS, pH 7.4). This TMC solution was diluted with the CBR solution in a 1:2 ratio, to form the stock solution with resulting concentration of 300 µg/ml.

Dilutions of the stock solution were made with PBS, to obtain the following TMC concentrations: 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. Absorption of these dilutions, along with the stock solution was measured from 200 nm to 900 nm. PBS was used as a reference sample. Peak absorbance (530 nm) values were plotted against concentration values to form a standard curve.

The process was repeated three times to ensure accuracy. Mean values were used to draw a final standard curve. Data was fit with linear regression and the equation of this line used to determine the concentration of the synthesized TMC particles in the glycerol (Figure A.1).

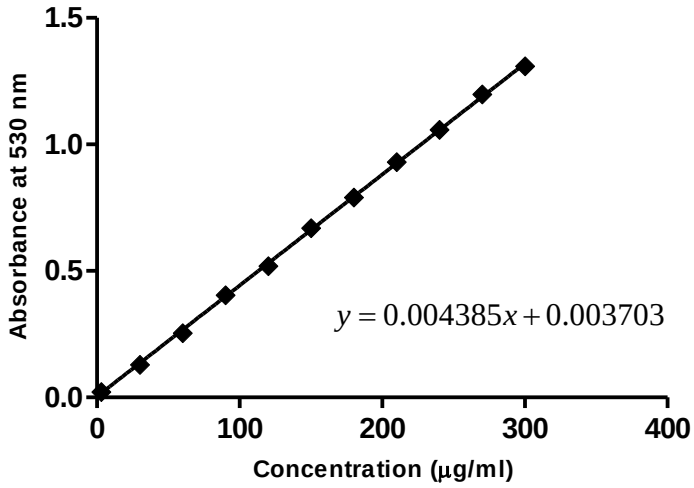


Figure A.1 – Mean values of the absorption measured, plotted against the corresponding TMC concentration to form a standard curve. Absorbance values were obtained using a UV spectrometer at 530 nm. Data was fitted with linear regression (r^2 = 0.9991, n = 25) for determination of unknown values.

The concentration of the PEG-TMC particles in glycerol was determined using a BioTek ELX800 absorbance microplate reader (BioTek, Winooski, VT, USA). A stock solution of PEG-TMC was prepared in the same way as the TMC stock solution; a 900 µg/ml PEG-TMC solution (made with water and PBS), diluted 1:2 with a 150 µg/ml CBR solution, to give a final concentration of 300 µg/ml. Triplicate dilutions of the stock solution were made with PBS in a 96-well plate, in a 1:1 ratio to give the following PEG-TMC concentrations: 150 µg/ml, 75 µg/ml, 37.5 µg/ml, 18.75 µg/ml, 9.375 µg/ml and 4.6875 µg/ml. PBS (with no CBR) was used as background subtraction. Absorbance of the dilutions, as well as the stock solution and PBS was measured at 550 nm, using a BioTek

microplate reader. The mean absorbance values were calculated and a standard curve drawn. Data was fit with linear regression and the equation of the line used for determination of the concentration of PEG-TMC particles in glycerol (Figure A.2).

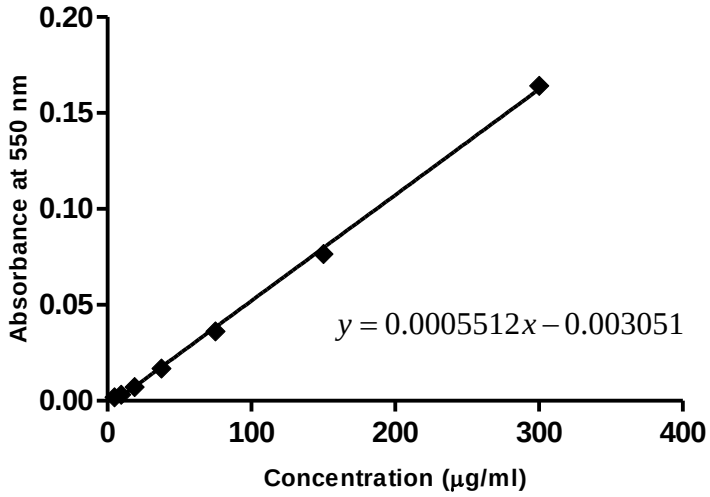


Figure A.2 – Standard curve of cross-linked PEG-TMC drawn from the mean values of the absorbance measured for each of the cross-linked PEG-TMC dilutions. Absorbance was measured with a BioTek microplate reader at 550 nm. Data was fit with linear regression to determine unknown PEG-TMC concentrations. $r^2 = 0.9985$, n = 21.

A.2.6 Determination of particle size distribution and zeta potential

Both particle size and zeta potential measurements commenced by diluting 1 ml of sample (small TMC nanoparticles, larger TMC nanoparticles or cross-linked PEG-TMC nanoparticles) with deionised water to a volume of 10 ml.

For determination of the particle size distribution, 1 ml of the diluted sample was added to a disposable polystyrene cuvette and placed in the holding chamber of the Malvern Zetasizer ZEN 3600 (Malvern Instruments, Malvern, UK). The cuvette was left for two minutes, to allow the temperature to reach equilibrium, after which the particle size analysis was performed. Each particle size measurement consisted of at least 14 runs. Two measurements were performed for each of the particle samples and the average calculated to determine the particle size.

To determine the zeta potential, 1 ml of the diluted sample was added to a folded capillary cell as per the manufacturer's instructions and placed in the holding chamber of the Malvern Zetasizer ZEN 3600. Again, two minutes were allowed for the temperature to reach equilibrium, after which the zeta potential measurement was performed. Each zeta potential measurement consisted of at least 14 runs. Four measurements were performed for each of the particle samples.

The results of the particles size distribution and the zeta potential determination were obtained using Zetasizer software (Malvern Instruments, Malvern, UK).

A.2.7 Collection of blood samples and preparation of experimental samples

This study was approved by the Ethics Committee of the North-West University (application number NWU-0025-09-S5). A registered nurse drew blood from a healthy, unmedicated male donor, into trisodium citrate and lithium heparin BD Vacutainer® tubes (Lasec South Africa (Pty) Ltd, Ndabeni, Cape Town, South Africa). The blood samples were stored at 5 °C for up to five days, after which they were discarded as biological waste.

Experimental samples were freshly prepared before each experiment in 1.5 ml microcentrifuge tubes. Particles were diluted with PBS (pH 7.4) to give four different experimental solutions; a 20% concentration small TMC nanoparticle solution (TMC Nano S (20%)), a 60% concentration small TMC nanoparticle solution (TMC Nano S (60%)), a 20% concentration larger TMC nanoparticle solution (TMC Nano L (20%)) and a 20% concentration cross-linked PEG-TMC nanoparticle solution (PEG-TMC Nano).

A.2.8 Hemolysis

The method for determining the hemolytic properties of the experimental samples was based on a method described by Letchford *et al.* (2009), wherein the amount of hemolysis caused by a substance is determined by measuring absorbance at 540 nm, an indication of the amount of free hemoglobin in a sample. Even though whole blood was used for the experiment, the red blood cell count was determined with the use of a Countess® automated cell counter (Life technologies, Carlsbad, CA, USA). The whole blood was then diluted with PBS to give an approximate red blood cell concentration of $1x10^8$ cells/ml (Letchford *et al.*, 2009). The diluted whole blood was incubated with the experimental solutions in a 1:1 ratio, on a Stuart Microtitre SSM5 plate shaker (Bibby Scientific Limited, Staffordshire, United Kingdom) (250 rpm) at 37 °C. Positive and negative controls, consisting of distilled water and PBS respectively, were also incubated with the diluted blood in a 1:1 ratio. Absorbance was measured at 1-, 6- and 12-hour intervals. Samples were divided in separate 1.5 ml microcentrifuge tubes for each time interval. This allowed centrifugation (2000 rpm for 10 minutes) of the tubes before measurements, to ensure that no cells interfered with the measured

absorbance. After centrifugation of the tubes, 200 µl of the supernatant was extracted and placed in a 96-well plate and the absorbance measured with a BioTek microplate reader at 550 ± 35 nm.

The percentage hemolysis caused by each of the experimental groups was calculated with the following equation, as seen in Lecthford's article (2009):

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

These data were then used to calculate the analysis of variance (ANOVA), determining any significant difference between the experimental groups for each time interval and for each experimental group over the span of the experiment.

A.2.9 Cell aggregation

The extent of cell aggregation, if any, caused by the experimental samples was determined with a method of Shelma & Sharma (2011). Whole, citrated blood was separated into red blood cells, white blood cells and platelet-rich plasma using histopaque. The whole blood was carefully placed on top of a 3 ml layer of histopaque in two 15 ml falcon tubes, in a 1:1 ratio. The tubes were then centrifuged for 10 minutes at 2 000 rpm to separate the blood components: straw coloured platelet-rich plasma in the top layer, a small cloudy white blood cell layer in the middle, a layer of clear plasma (which was discarded) underneath the white blood cells and clumped red blood cells at the bottom of the tube. The components were carefully separated and placed in 1.5 ml microcentrifuge tubes.

For the experiment, 100 µl of experimental sample was incubated with 100 µl of each of the separate blood components, as well as 100 µl of whole blood for 30 minutes at 37 °C on a Stuart Microtitre SSM5 plate shaker (250 rpm). Polyethyleneimine (Mw 25 000) was incubated with the blood components as a positive control for cell aggregation, whereas normal saline served as a negative control. After incubation, the different blood components and the whole blood samples were resuspended in saline, to make wet mounted slides. These slides were subsequently examined with a Nikon Eclipse Ti-E Inverted Microscope (Nikon Corporation, Tokyo, Japan) and photos taken using Nikon EZC1 confocal microscope software (Nikon Corporation, Tokyo, Japan) for comparison (Shelma & Sharma, 2011).

A.2.10 Complement activation

Complement activation caused by the experimental samples was determined by measuring the amount of C3 protein present in blood plasma after incubation with the experimental particles. This was accomplished using a 96-well Complement C3 Human ELISA kit (Abcam, Cambridge, England, United Kingdom). Plasma plus PBS (1:1) served as a control in this experiment.

Before the experiment, all reagents of the Complement C3 Human ELISA kit were diluted and prepared as described in the kit's instruction booklet and brought to room temperature. A standard curve was drawn by diluting the given Complement C3 Standard with Diluent using two fold serial dilution. The first standard point was made up of the reconstituted Complement C3 Standard (30 µg/ml). For the first dilution, 1 part of the reconstituted standard (30 µg/ml) and 1 part Diluent was combined, to give a 15 µg/ml concentration. Part of this solution was then used with more Diluent in a 1:1 ratio, to create a third standard point, with a 7.5 µg/ml concentration. This method was repeated to additional standard points with concentrations of 3.75 µg/ml, 1.88 µg/ml, 0.94 µg/ml and 0.47 µg/ml. For the final standard point Diluent with no C3 Standard present was used, giving a 0 µg/ml concentration.

The experiment was started by centrifuging 2 ml of whole, citrated blood at 2 000 rpm for 10 minutes, to separate the blood plasma from the cells. After centrifugation, the plasma was removed and placed in a 1.5 ml microcentrifuge tube and the cells discarded. Six microplate strips were placed on the plate frame (three for experimental purposes and three for the standard curve, both in triplicate) and the rest of the strips were returned to the foil pouch to minimize water vapour exposure.

Standard (25 µl of the different dilutions) or sample (12.5 µl plasma plus 12.5 µl experimental sample) were added to the applicable wells, after which 25 µl Biotinylated Complement C3 was added to each of these wells. The wells were tightly covered with sealing tape and incubated for two hours at room temperature.

After incubation, the microplate strips were washed five times with Wash Buffer. Each time, 200 µl of Wash Buffer was added to every well containing samples. The plate was then inverted to decant the liquid and tapped about five times on an absorbent paper towel to remove all of the liquid. When washing had been completed, 50 µl of Streptavidin-Peroxidase Conjugate was added to each of the wells that had contained standard or sample. The plate was again covered with sealing tape and incubated at room temperature for 30 minutes. The microplate strips were then washed with Wash Buffer another five times.

Chromogen Substrate (50 µl) was added to each applicable well and the plate incubated for about 10 minutes, to allow the blue colour to develop. When the optimal blue colour had developed, 50 µl of Stop Solution was added to the wells, causing the colour to change from blue to yellow. The absorbance of the wells was immediately read with a BioTek microplate reader at 450 nm.

To draw the standard curve (Figure A.3), the average absorbance of the three wells of each concentration was calculated. The log of these values (y-axis) was plot against the log of the corresponding concentration (x-axis). A fourth-order polynomial regression line was fitted through the standard curve, to allow interpolation of the unknown values of the experiment. (Abcam, 2012)

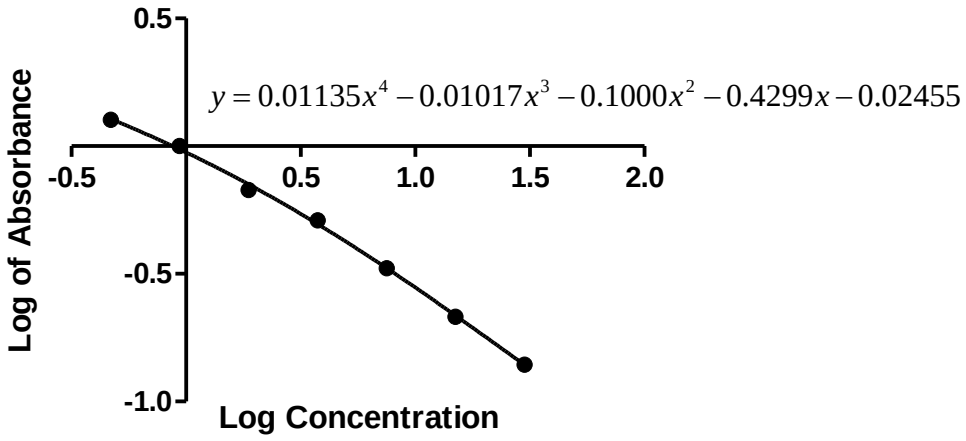


Figure A.3 – The log of the mean absorbance values (measured at 450 nm) plot against the log of the C3 protein concentrations, forming the standard C3 protein concentration curve for the complement activation experiment. Data was fit with a fourth-order polynomial regression line and used to determine the extent of complement activation caused by the experimental samples. $r^2 = 0.9963$, n = 21.

The log of each of the average experimental absorbances was interpolated on the standard curve. Reversing the log of the interpolated values provided the concentration of C3 present in each of the experimental samples. Column graphs were drawn to assess visually the variance between the experimental groups. Variance between the complement C3 protein concentrations of experimental samples and the controls were statistically analysed (ANOVA) with GraphPad Prism 5.

A.2.11 Plasma protein interaction

For this experiment, standard curves were drawn by making dilutions of the experimental particles (Figure A.4, A.5 and A.6). A 90% solution of each of the TMC nanoparticles, TMC microparticles and

PEG-TMC nanoparticles was made. From this, a 1:2 dilution was made with 75 µg/ml Cibacron Brilliant Red 3B-A (CBR), to form the stock solutions. In a 96-well plate, triplicate 1:1 dilutions were made with PBS (pH 7.4) to give the following concentrations: 30% (the stock solution), 15%, 7.5%, 3.75%, 1.88%, 0.94% and 0.47%. A 0% standard point, obtained with PBS with no CBR or experimental particles added, was used for background subtraction. The absorbance of each of the wells was read at 550 nm with a BioTek microplate reader, after which a standard curve (concentration on the x-axis and absorbance on the y-axis) was drawn. Linear regression was used to fit a straight line through the data for interpolation of unknown experimental values.

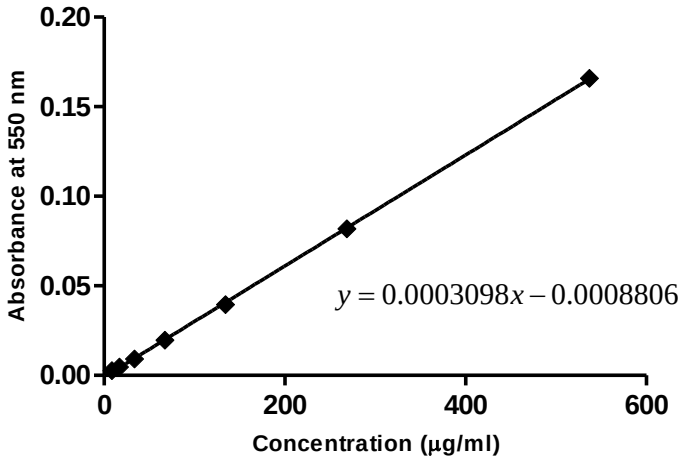


Figure A.4 – Standard curve of TMC nanoparticles in glycerol, drawn from different small TMC nanoparticle concentrations and the mean corresponding absorbance values, as measured with a BioTek microplate reader at 550 nm. Data was fit with linear regression for the interpolation of unknown concentration values found in the plasma protein interaction experiment. $r^2 = 0.9974$, n = 63.

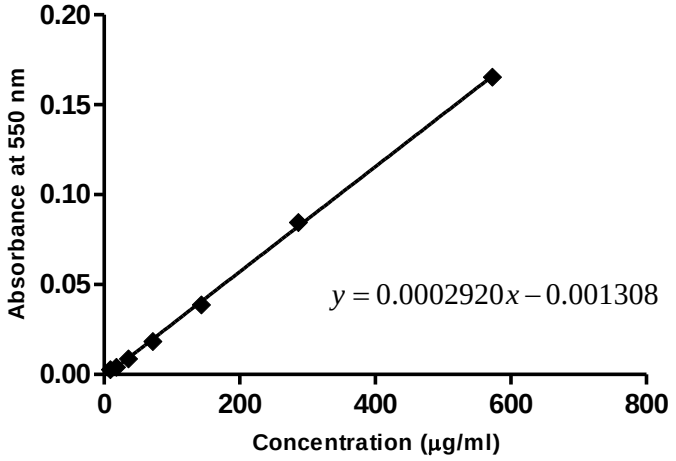


Figure A.5 – Standard curve of larger TMC nanoparticles in glycerol. Absorbance values of different larger TMC nano-particle dilutions were measured with a BioTek microplate reader at 550 nm and the mean values plot against the corresponding microparticle concentrations. The data was fit with linear regression for the interpolation of concentrations of absorbance values measured in the plasma protein interaction experiment. $r^2 = 0.9967$, n = 63.

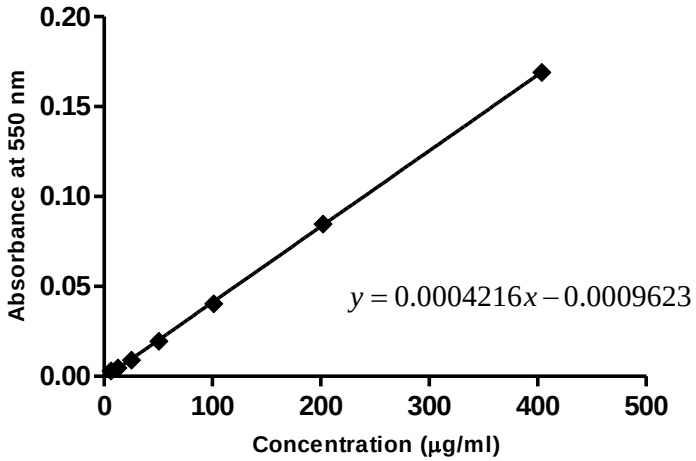


Figure A.6 – Standard curve of cross-linked PEG-TMC nanoparticles in glycerol. Absorbance values of different cross-linked PEG-TMC nanoparticle dilutions were measured with a BioTek microplate reader at 550 nm and the mean values plot against the corresponding microparticle concentrations. The data was 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.

To measure the amount of plasma protein interaction of the different experimental samples, a Single-use Rapid Equilibrium Dialysis (RED) plate with inserts (Waltham, MA, USA) and a subsequent colorimetric assay was used. Blood drawn into heparin tubes were centrifuged at 2 000 rpm for 10 minutes and the plasma collected in separate 1.5 ml microcentrifuge tubes.

Plasma and experimental samples were combined (150 µl of each) in marked 1.5 ml microcentrifuge tubes. Plasma and PBS were combined to form a negative control.

From each of the marked 1.5 ml microcentrifuge tubes, 200 µl was drawn and placed in separate red sample chambers on the RED plate. The corresponding white chambers were filled with 350 µl PBS. The plate was covered with sealing tape and incubated at 37 °C for four hours (minimum) on a Stuart Microtitre SSM5 plate shaker (250 rpm) (Thermo Scientific, 2007).

After the incubation period had been completed, the liquid was drawn from the various red and white chambers on the RED plate and was placed in corresponding empty, marked 1.5 ml microcentrifuge tubes. An aliquot (50 µl) of each of these samples was placed in a separate well on a 96-well plate, where 100 µl of a 75 µg/ml CBR solution was added. The absorbance at 550 nm was read with a BioTek microplate reader and the resulting values fit on the standard curve to determine concentration of particles unbound in the solutions (Van der Merwe *et al.*, 2004). The amount of particles not bound to plasma proteins was calculated as the percentage of the initial particles in the sample seen with the colorimetric assay, using Microsoft Excel. From these data, the percentages of the initial particles bound to the plasma proteins (and thus a measure of plasma protein interaction)

were determined. Statistical significance of the plasma protein interaction between the experimental groups was determined with Analysis of Variance (ANOVA), using GraphPad Prism 5.

A.2.12 Statistical analysis

Calculations were done and tables drawn with Microsoft Excel. Data were analysed with GraphPad Prism 5, calculating descriptive statistics and determining the differences between samples with the use of Analysis of Variance (ANOVA). GraphPad Prism 5 was also used to draw standard curves, column graphs and line graphs. Standard curves were fit 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.

A.3 Results

A.3.1 NMR characterization

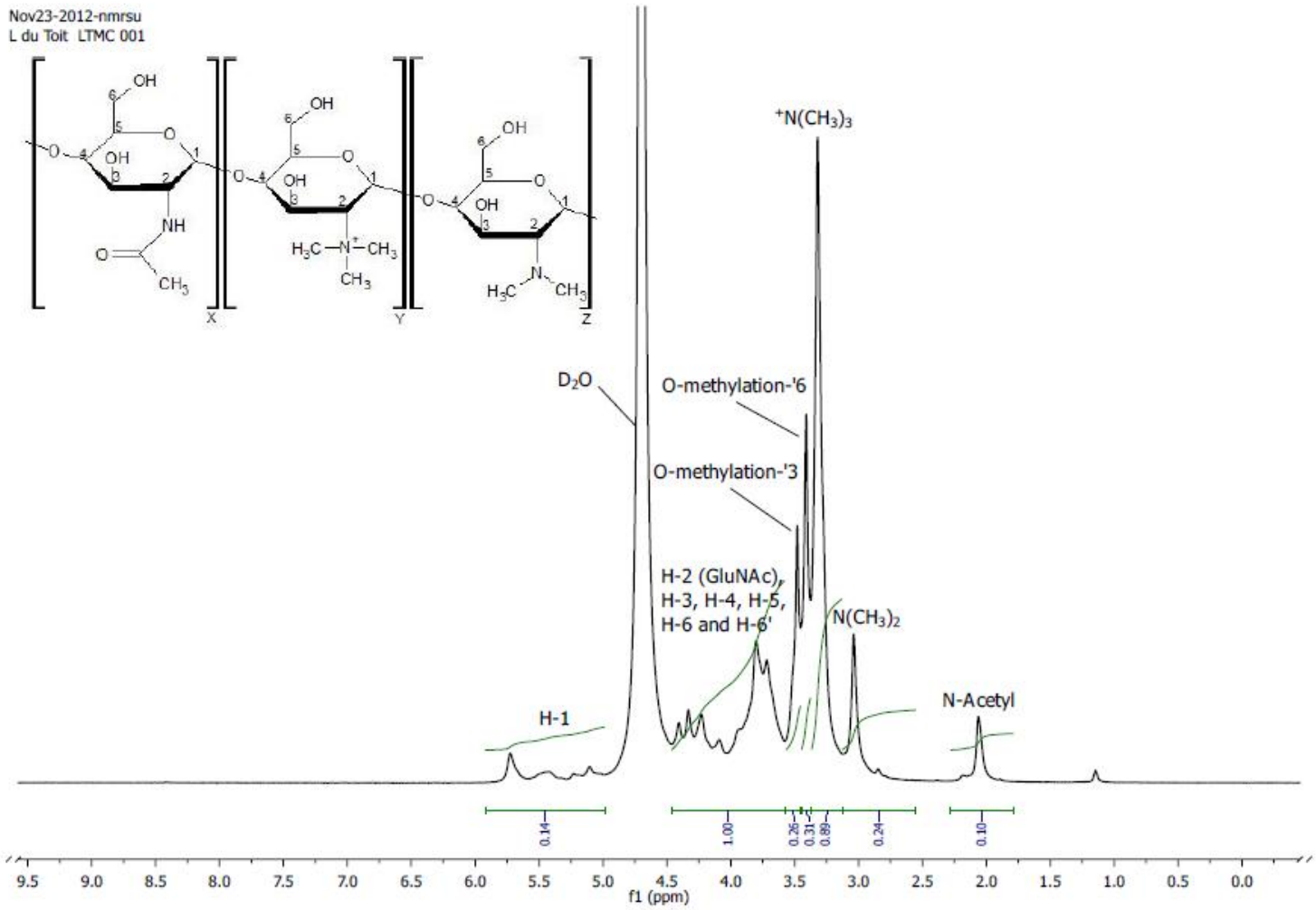


Figure A.7 – NMR characterization of the synthesized TMC (DQ 60%, degree of *O*-methylation, 33%)

A.3.2 Particle concentration determination

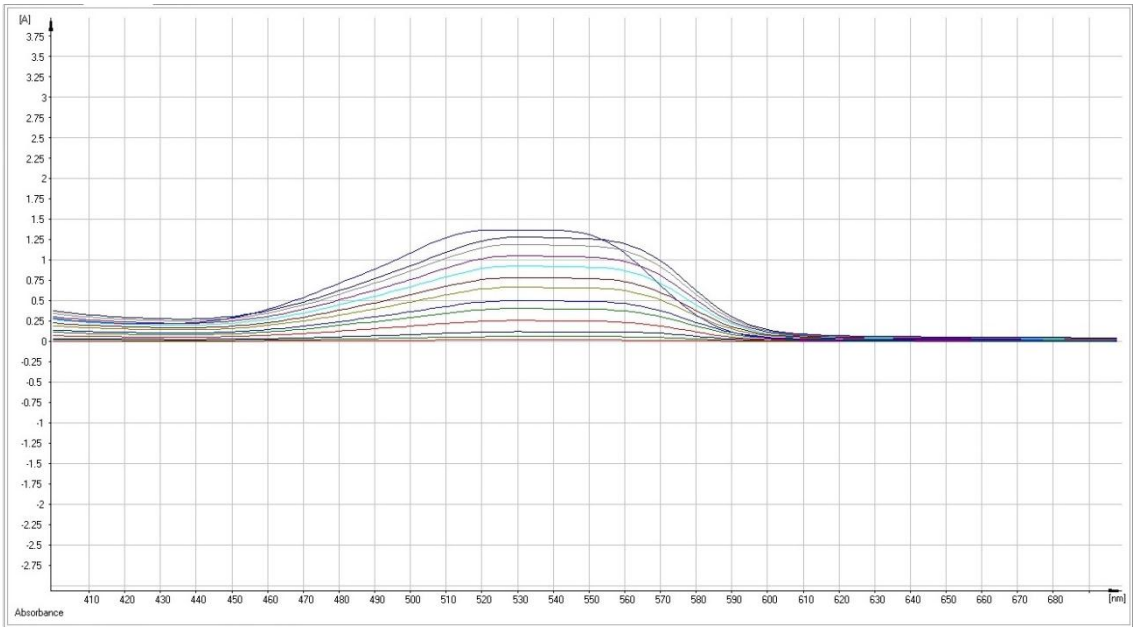


Figure A.8 – Spectral scan of the absorbance of all the different TMC concentrations, as measured with a UV spectrometer, zoomed in to show the applicable absorbance area. The concentrations represented by the lines are (from the top): 0 µg/ml (control, CBR and PBS), 300 µg/ml (stock solution), 270 µg/ml, 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.

Absorbance values of different dilutions of TMC and cross-linked PEG-TMC were measured with a UV spectrometer (Figure A.8) and a BioTek microplate reader, and used to draw standard curves. These standard concentrations curves, seen in Figure A.1 and Figure A.2, were then used to determine the concentrations of the TMC nanoparticles (small and larger) and the cross-linked PEG-TMC nanoparticles in glycerol, where they had been collected. The small TMC nanoparticles in glycerol were found to have a concentration of 1789.90 µg/ml and the larger TMC nanoparticles a concentration of 1909.00 µg/ml. The cross-linked PEG-TMC nanoparticles in glycerol's concentration were determined at 1344.50 µg/ml. These values were used for concentration curves and calculation of concentrations in the experiments.

A.3.3 Particle size distribution and zeta potential

Particle size distribution and zeta potential were measured with a Malvern Zetasizer ZEN 3600. As the Zetasizer's results are an average of at least 14 readings, the measurement was only repeated

twice for size determination. An example of the size distribution results is shown in Figure A.9. The zeta potential measurement, however, was repeated four times for accuracy.

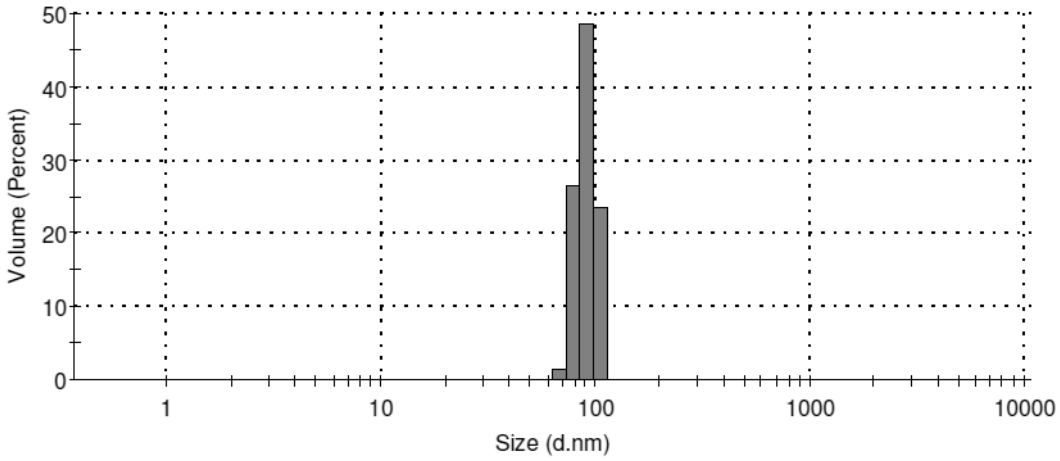


Figure A.9 – Size distribution of the small TMC nanoparticles as measured with a Malvern Zetasizer ZEN 3600. The measured particle size is 91.04 ± 9.90 nm.

The results of the size distribution and zeta potential determination are summarized in Table A.1. The small TMC nanoparticles (122.78 ± 45.28 nm) and the cross-linked PEG-TMC nanoparticles (124.75 ± 6.15 nm) had comparable average sizes. The percentage relative standard deviation (%RSD) of the small TMC nanoparticles (36.88%) is much higher than the %RSD of the cross-linked PEG-TMC nanoparticles (4.93%). This indicates instability of the small TMC nanoparticles, as was seen during the measurement. Small particle sizes of the small TMC nanoparticles were measured directly after sonication, but with subsequent readings, the measurements became larger and larger, as the particles seemed to agglomerate. The cross-linked PEG-TMC nanoparticles, however, did not display this apparent agglomeration and were more stable over all.

The larger TMC nanoparticles were quite stable at 243.05 ± 7.19 nm, with a %RSD of 7.19%. Therefore, although the size difference between the TMC nanoparticles seems small, it is large enough to give added stability to the particles.

As predicted, the addition of PEG to the nanoparticle formulation lowered the zeta potential from 18.98 ± 2.91 mV to 12.32 ± 2.02 mV.

Table A.1 – 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.

	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

A.3.4 Hemolysis

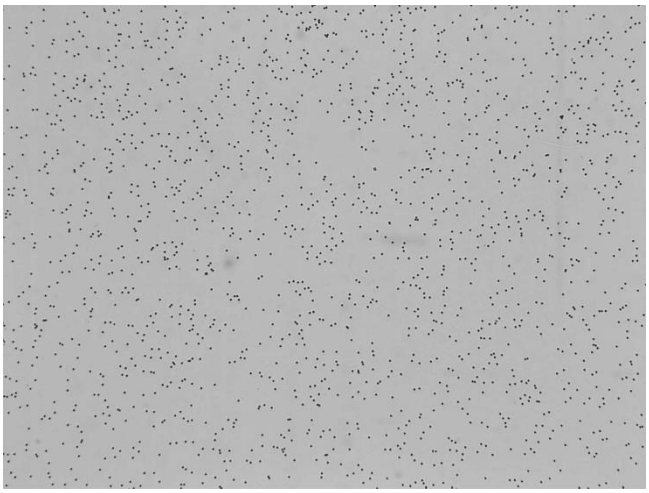


Figure A.10 – Countess® automated cell counter (Life technologies, Carlsbad, CA, USA) image of a representative blood sample diluted 200 times, resulting in a red blood cell count of 8.6 x 10^6 cells per ml.

According to literature (Letchford *et al.*, 2009) an approximate red blood cell concentration of 1 x 10^8 cells/ml was to be used for the determination of hemolysis caused by the experimental samples. To determine the dilution factor needed for this, the red blood cell count was determined with a Countess® automated cell counter. Different blood dilutions were made with PBS, most of which had a red blood cell count above the accurate range of the cell counter. When the representative sample was diluted 200 times (Figure A.10), an accurate cell count of 8.6 x 10^6 cells/ml as obtained. This cell count was then used to calculate the 17.2 x dilution factor used to produce the suggested 1 x 10^8 cells/ml red blood cell concentration.

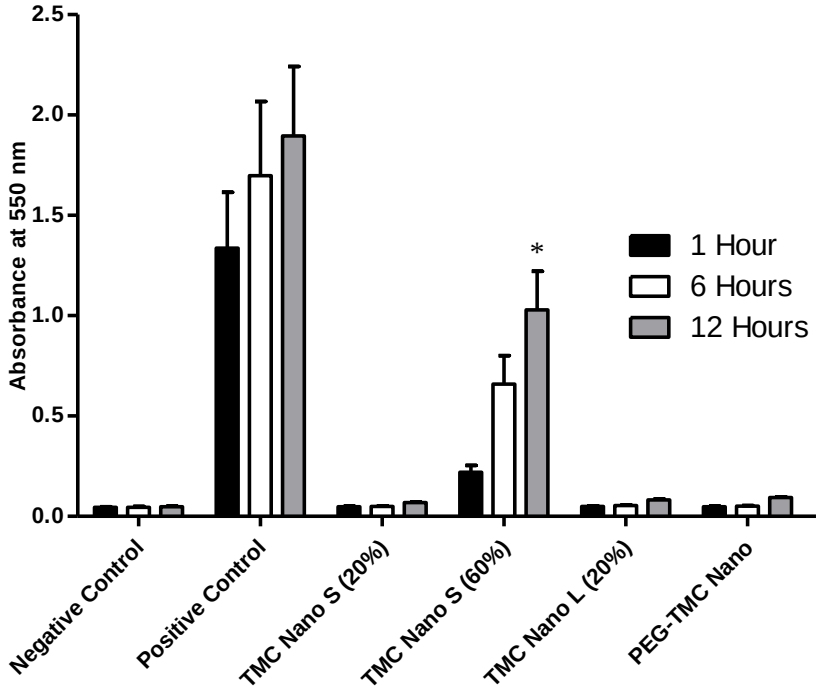


Figure A.11 – Mean absorbance values, with standard error of mean, of the different experimental groups at one-, six- and twelve-hour intervals. Absorbance was measured with a BioTek microplate reader at 550 nm. For each bar n = 9.
* Statistical significance compared to other experimental groups in the same time-interval.

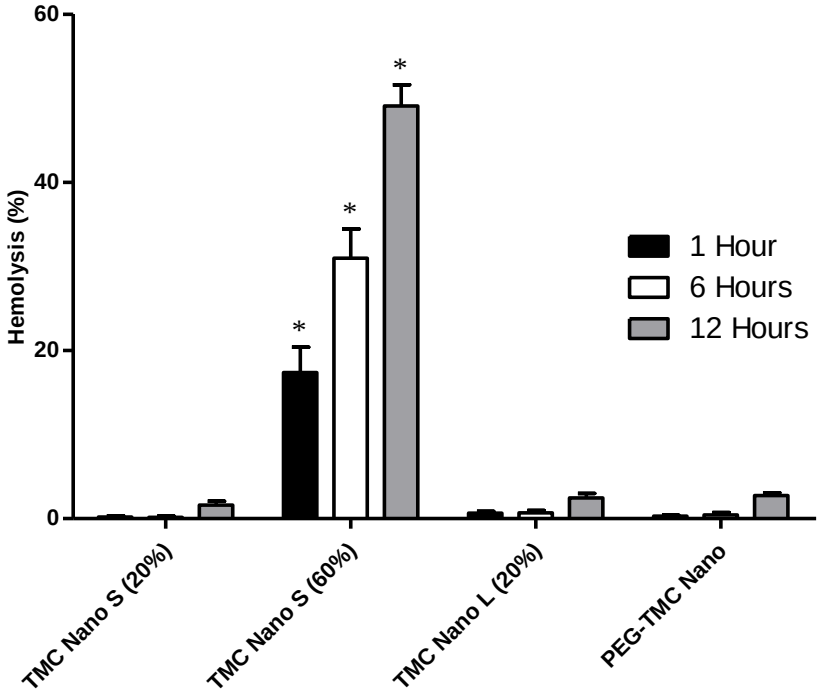


Figure A.12 – Mean percentage hemolysis caused, with standard error of mean, at one-, six- and twelve-hour intervals, as calculated for each of the experimental groups. For each bar n = 9.
* Statistical significance compared to other experimental groups in the same time interval.

The absorbance values measured at the different time intervals of the hemolysis experiment (as seen in Figure A.11 and Table A.2) represent the amount of free hemoglobin present in the samples. This, in turn, is a measure of the amount of hemolysis caused, as the broken red blood cells become perforated and the hemoglobin leaks out into the surrounding plasma. By using the formula mentioned in the methods section, the measured absorbance values were converted to determine the percentage hemolysis caused by each of the experimental groups (Figure A.12 and Table A.3), as this gives a much clearer picture of the hemolysis the groups caused, compared to the other groups.

Table A.2 – 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.
* Statistical significance compared to other experimental groups at the same time-interval.

	Negative Control	± SEM	**Positive Control**	± SEM	**TMC Nano S (20%)**	± SEM
1h	0.05	0.004	1.34	0.278	0.05	0.002
6h	0.05	0.003	1.70	0.370	0.05	0.002
12h	0.05	0.003	1.90	0.346	0.07	0.002
	TMC Nano S (60%)	± SEM	**TMC Nano L (20%)**	± SEM	**PEG-TMC Nano**	± SEM
1h	0.22	0.035	0.05	0.003	0.05	0.003
6h	0.66	0.142	0.05	0.003	0.05	0.002
12h	1.03*	0.192	0.08	0.004	0.09	0.004

Table A.3 – Mean percentage of hemolysis caused, as calculated for the different experimental groups, with standard error of mean (SEM), over the course of the experiment.
* Statistical significance when compared to other experimental groups at the same time-interval.

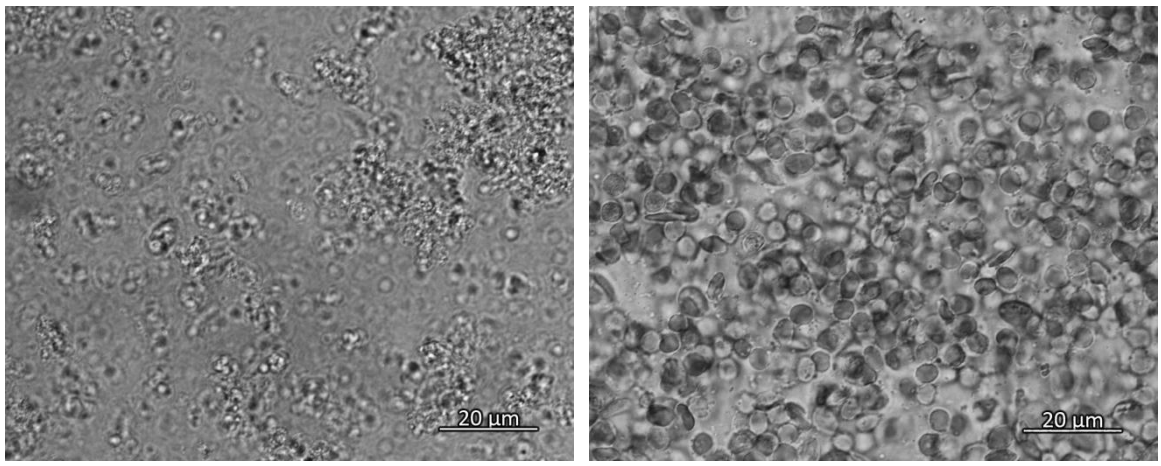
	TMC Nano S (20%)	± SEM	**TMC Nano S (60%)**	± SEM	**TMC Nano L (20%)**	± SEM	**PEG-TMC Nano**	± SEM
1h	0.21	0.116	17.40*	3.003	0.66	0.197	0.32	0.084
6h	0.16	0.134	30.97*	3.497	0.68	0.312	0.45	0.265
12h	1.63	0.439	49.08*	2.538	2.45	0.536	2.75	0.333

Clear trends were seen when looking at the results of both the measured absorbance values (Table A.2) and the calculated percentage hemolysis (Table A.3) of the different experimental groups. A statistically significant ($p<0.05$) increase in the percentage hemolysis caused, was seen between the 6-hour and the 12-hour intervals for all of the experimental groups. This trend was seen in the measured absorbance values of most of the experimental samples, except for the 60% small TMC nanoparticle group.

Of the measured absorbance values (Figure A.11 and Table A.2), only the 60% small TMC nanoparticle group displayed statistical significance. At the 12-hour interval, this group had significantly higher absorbance values (1.03 ± 0.192) than all the other experimental groups and the negative control group, but its absorbance was still significantly lower than the positive control group's absorbance (1.90 ± 0.346). The percentage hemolysis caused by the 60% small TMC nanoparticle group, however, was already significantly higher than the other experimental groups from the first hour (17.40 ± 3.003%). After 12 hours, the 60% concentration small TMC nanoparticle solution had caused considerable hemolysis (49.08 ± 2.538%), whereas the 20% concentration small TMC nanoparticle solution had only caused 1.63 ± 0.439%, the 20% concentration larger TMC nanoparticle solution only 2.45 ± 0.536% and the cross-linked PEG-TMC nanoparticle solution, 2.75 ± 0.333%. At all the time intervals, the 20% small TMC nanoparticle group consistently had the lowest percentage of hemolysis caused, followed closely by the PEG-TMC nanoparticle- and the 20% larger TMC nanoparticle groups.

A.3.5 Cell aggregation

A



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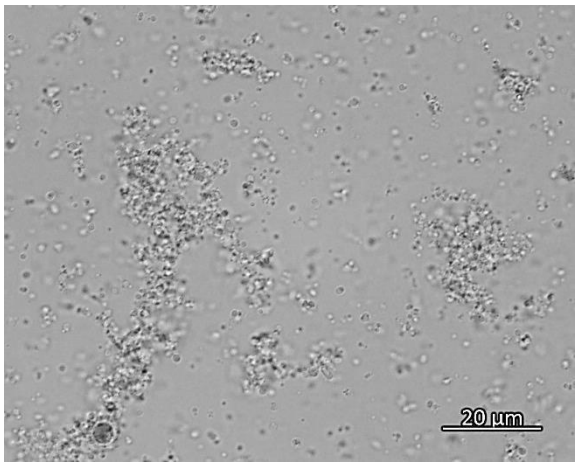


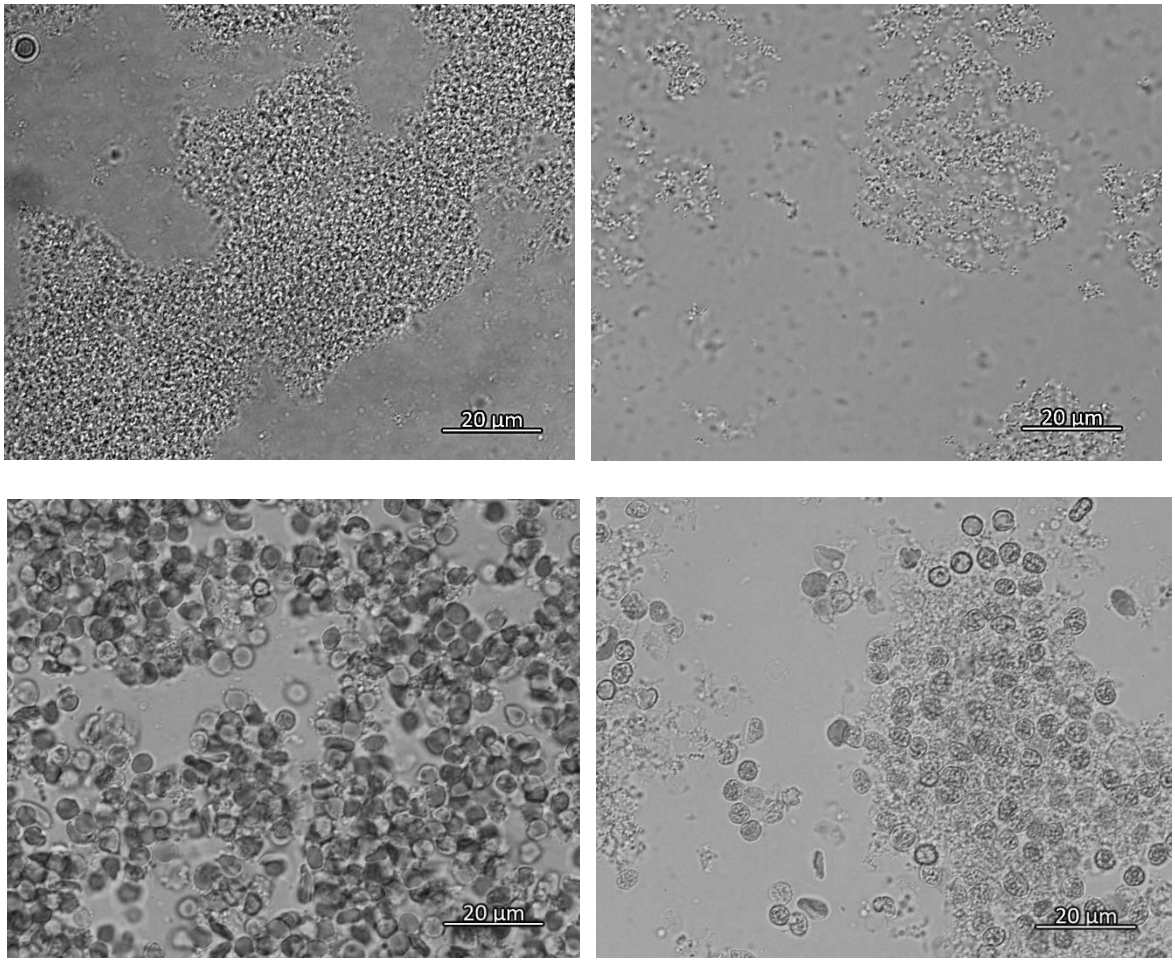
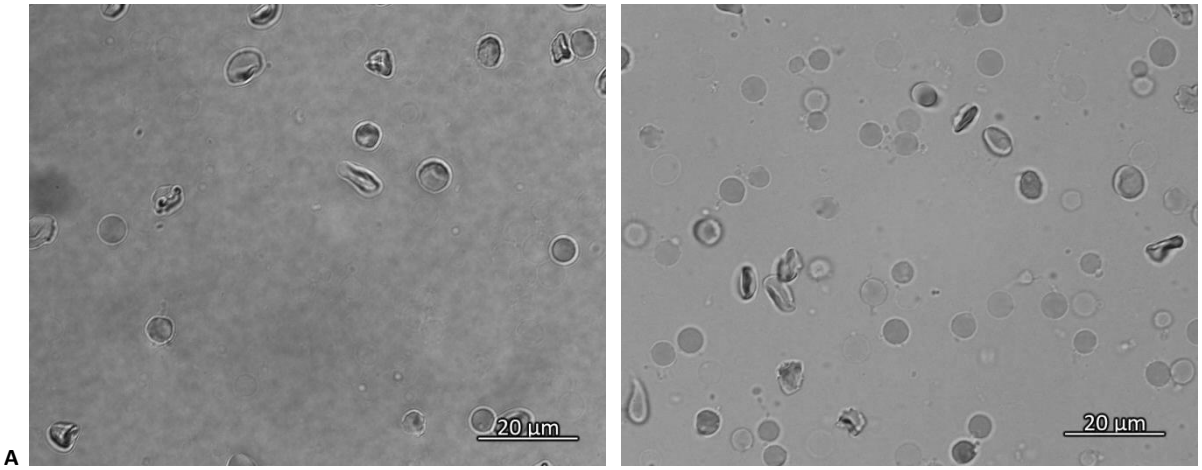


Figure A.13 – Light microscopy of positive control: blood components resuspended in saline after a 30-minute incubation with polyethyleneimine (Mw 25 000), displaying cell aggregation. **A**: Red blood cells. **B**: White blood cells. **C**: Blood platelets. **D**: Whole blood.



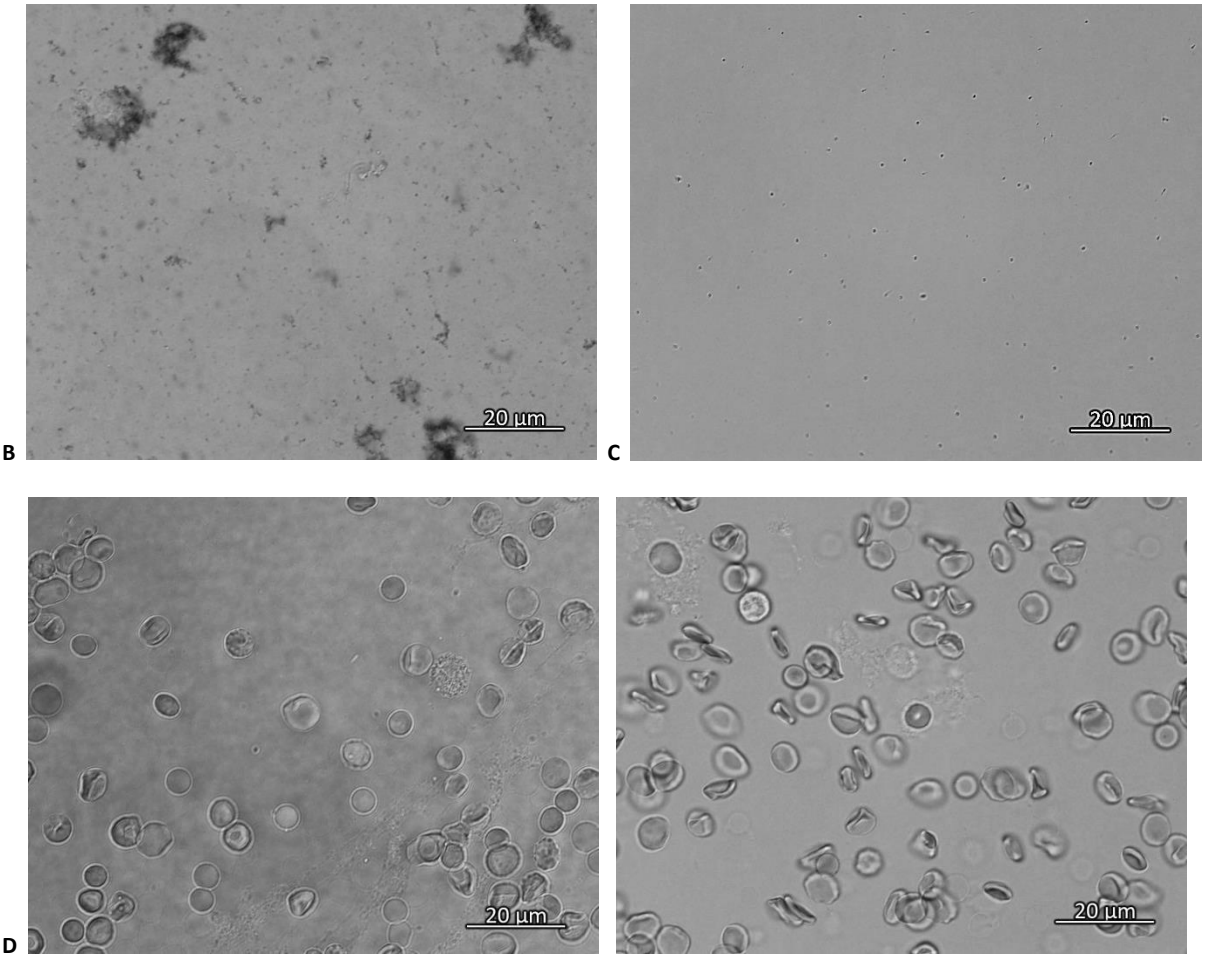
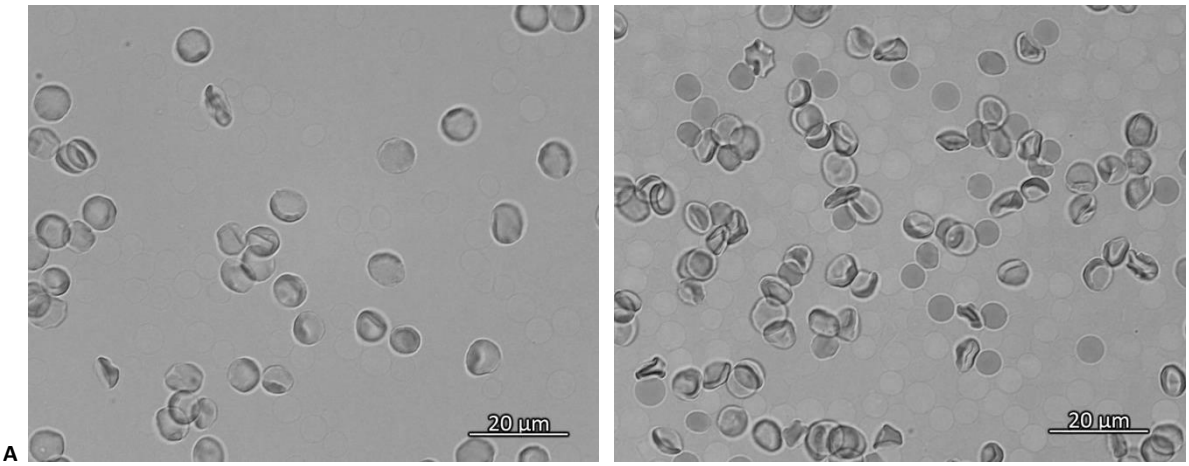


Figure A.14 – Light microscopy of negative control: blood components after being incubated with normal saline for 30 minutes, displaying no cell aggregation. What looks to be clumped cells in picture B is crystals from the dye used. **A**: Red blood cells. **B**: White blood cells. **C**: Blood platelets. **D**: Whole blood.



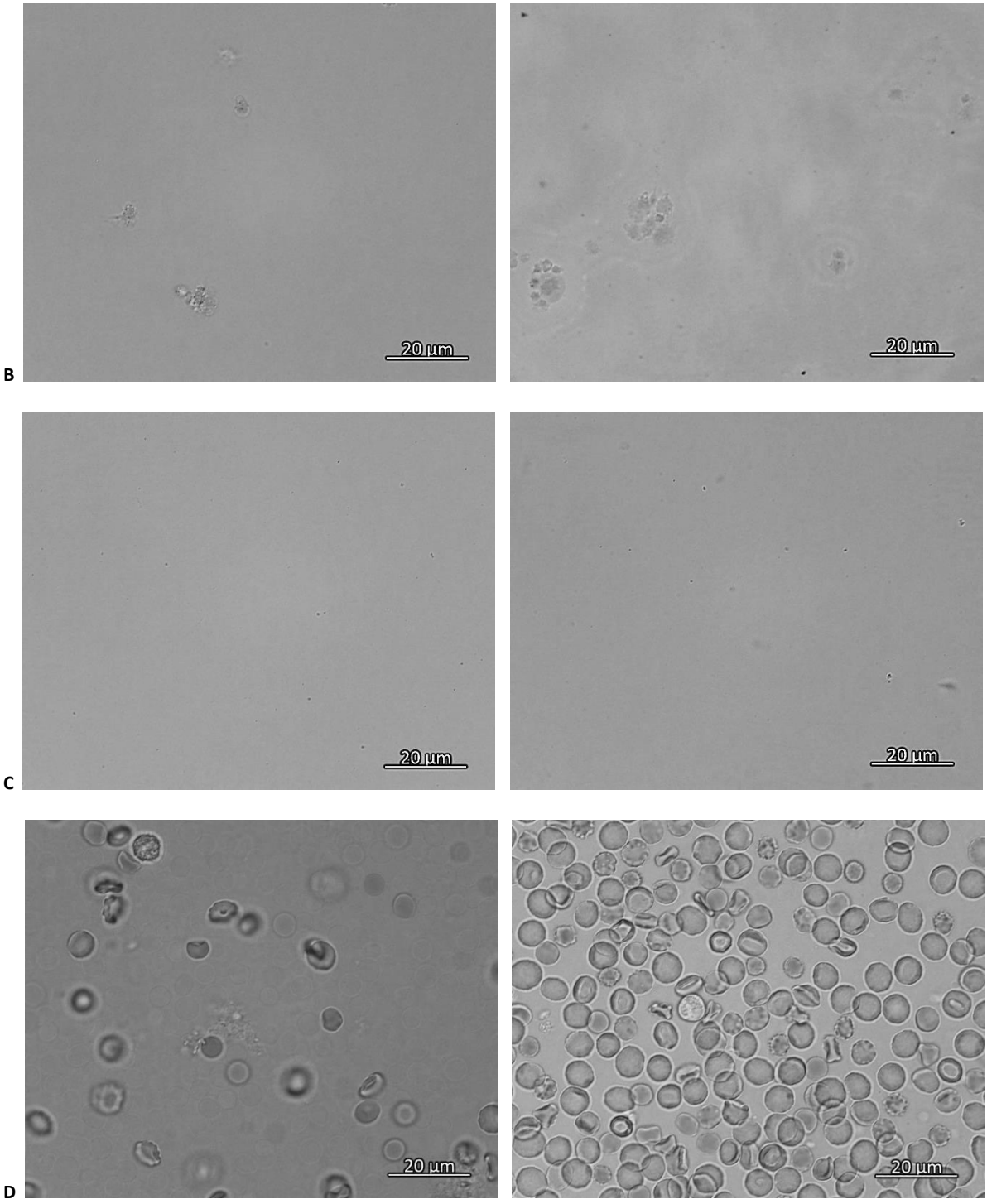
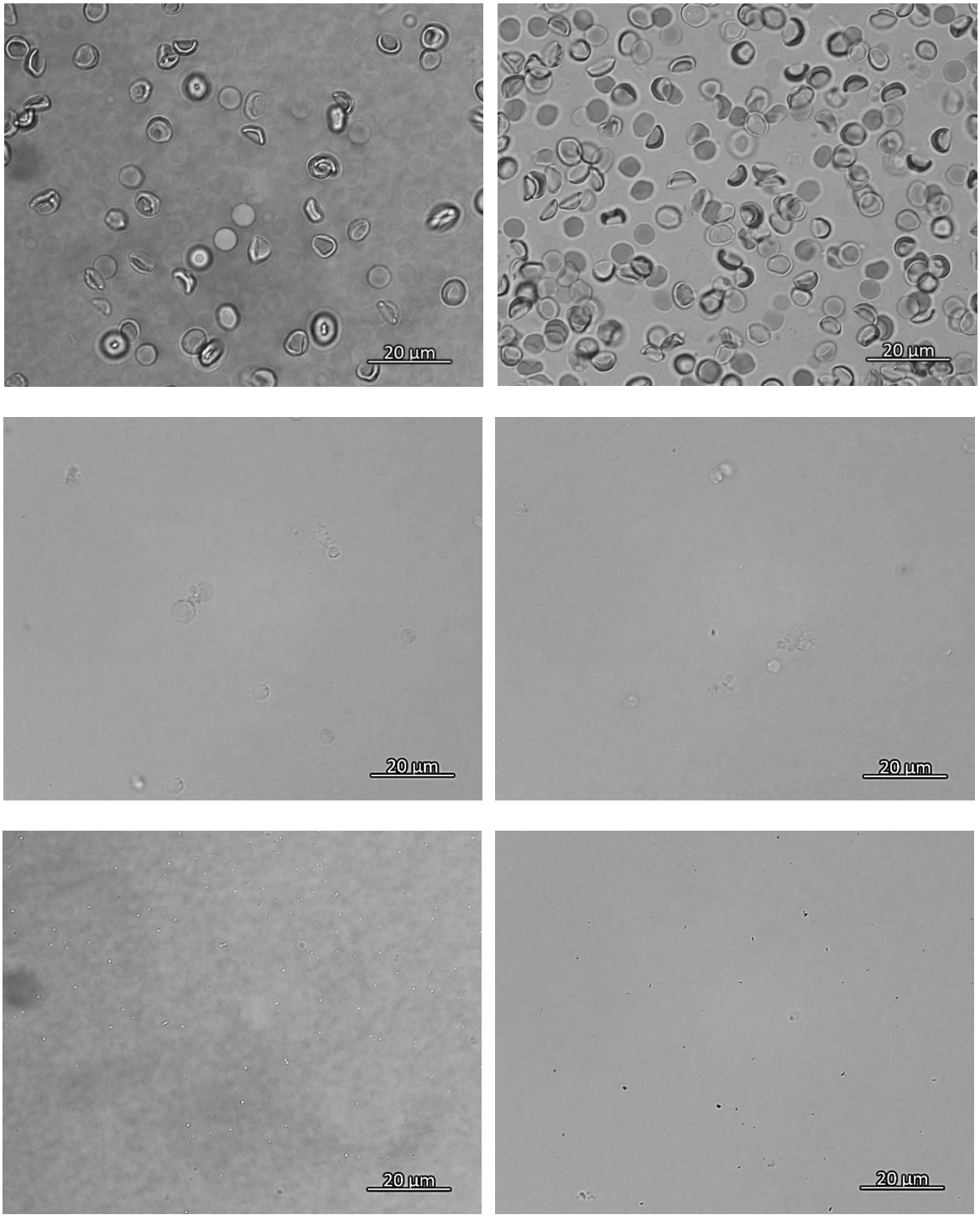
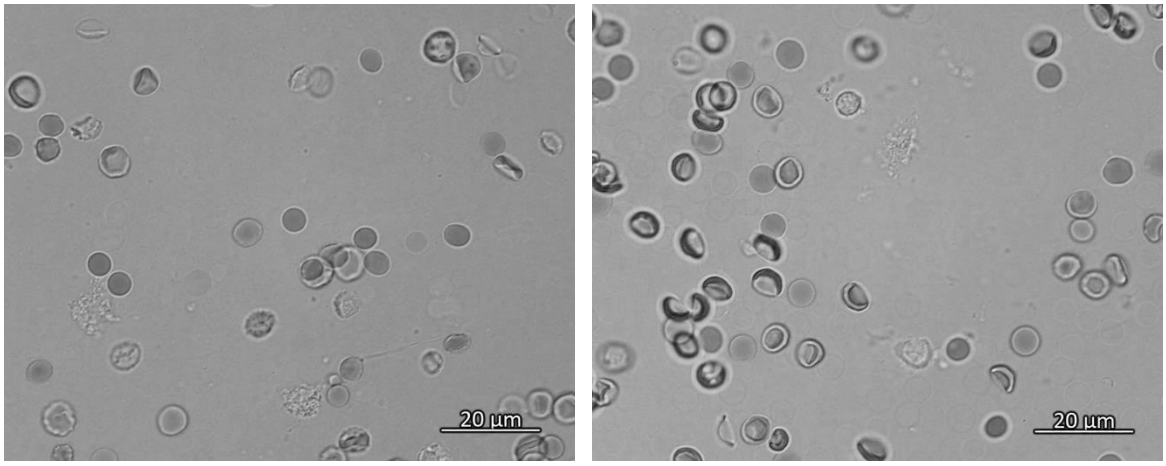


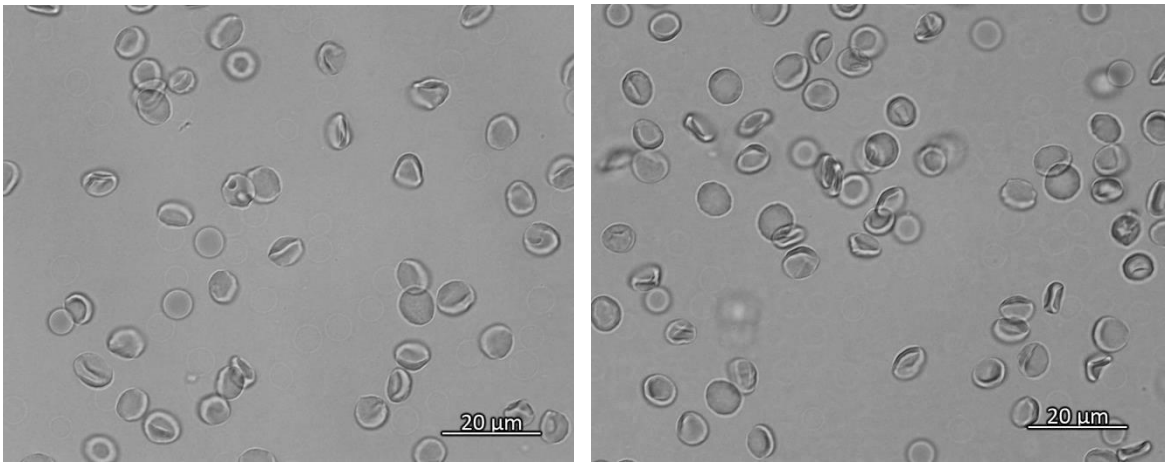
Figure A.15 – Light microscopy of blood components resuspended in saline showing cell aggregation caused by small TMC nanoparticles (20%) after a 30-minute incubation period. **A**: Red blood cells. **B**: White blood cells. **C**: Blood platelets. **D**: Whole blood.


A
20 µm
20 µm
B
20 µm
20 µm
C
20 µm
20 µm

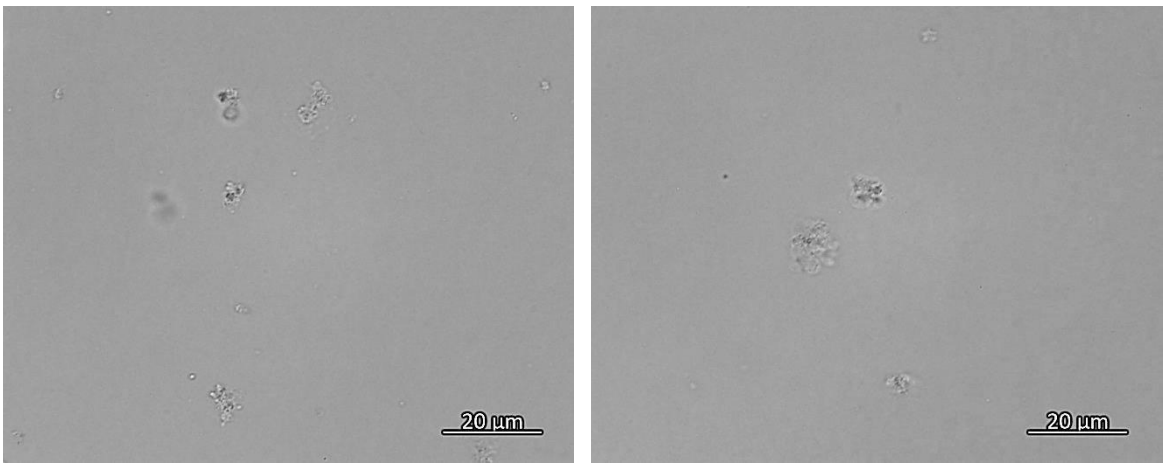


D

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.



A



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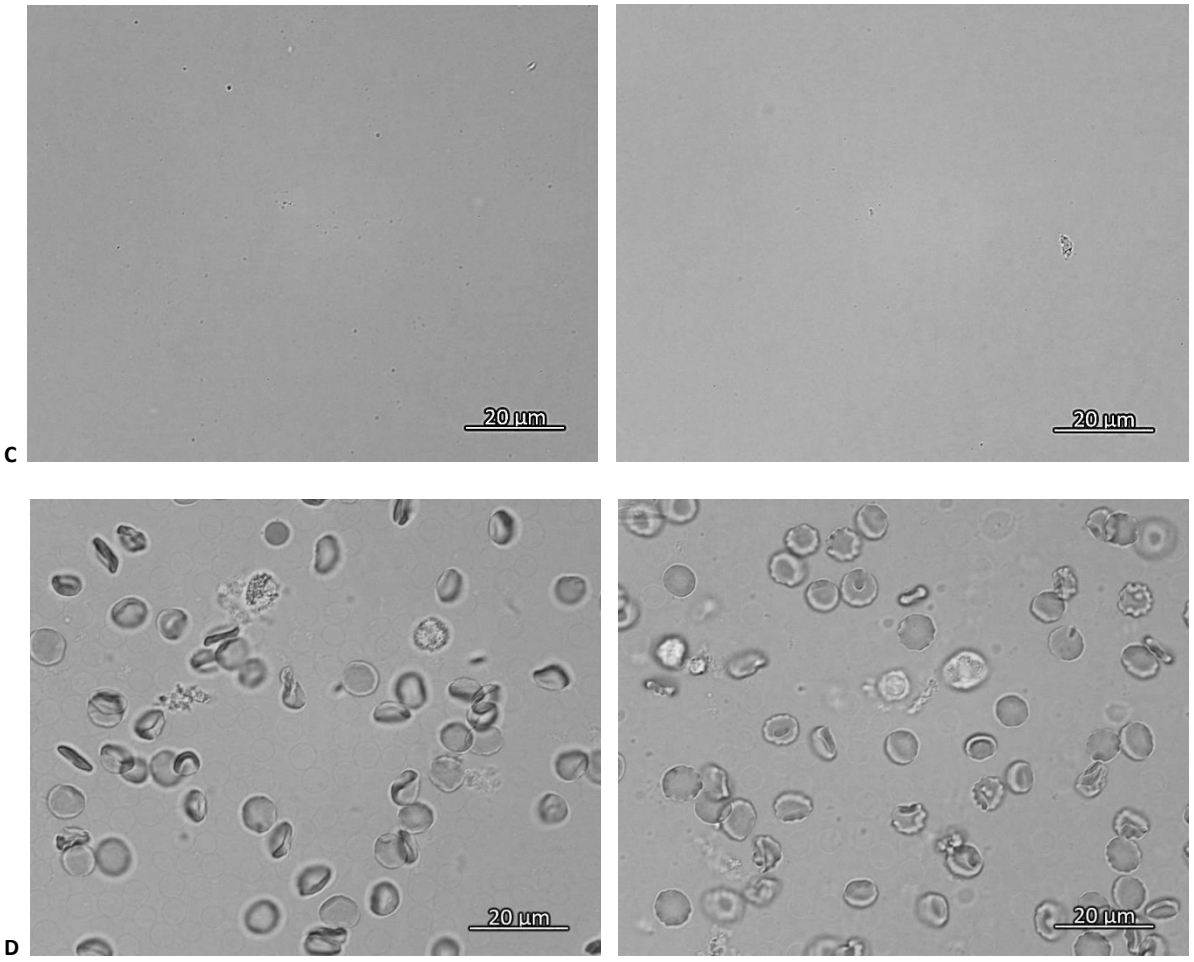
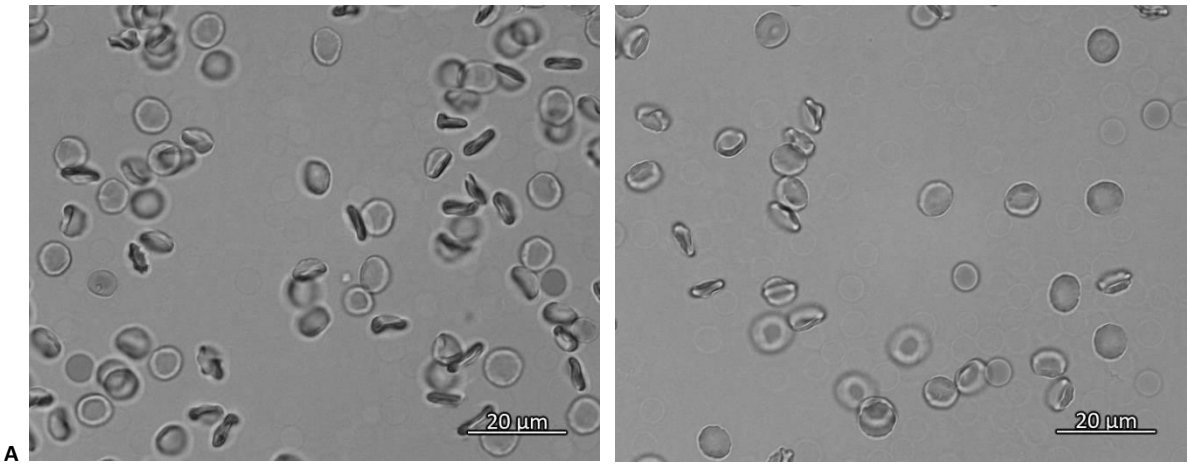


Figure A.17 – Light microscopy of blood components in saline after being incubated with larger TMC nanoparticles (20%) for 30 minutes, showing cell aggregation caused. **A**: Red blood cells. **B**: White blood cells. **C**: Blood platelets. **D**: Whole blood.



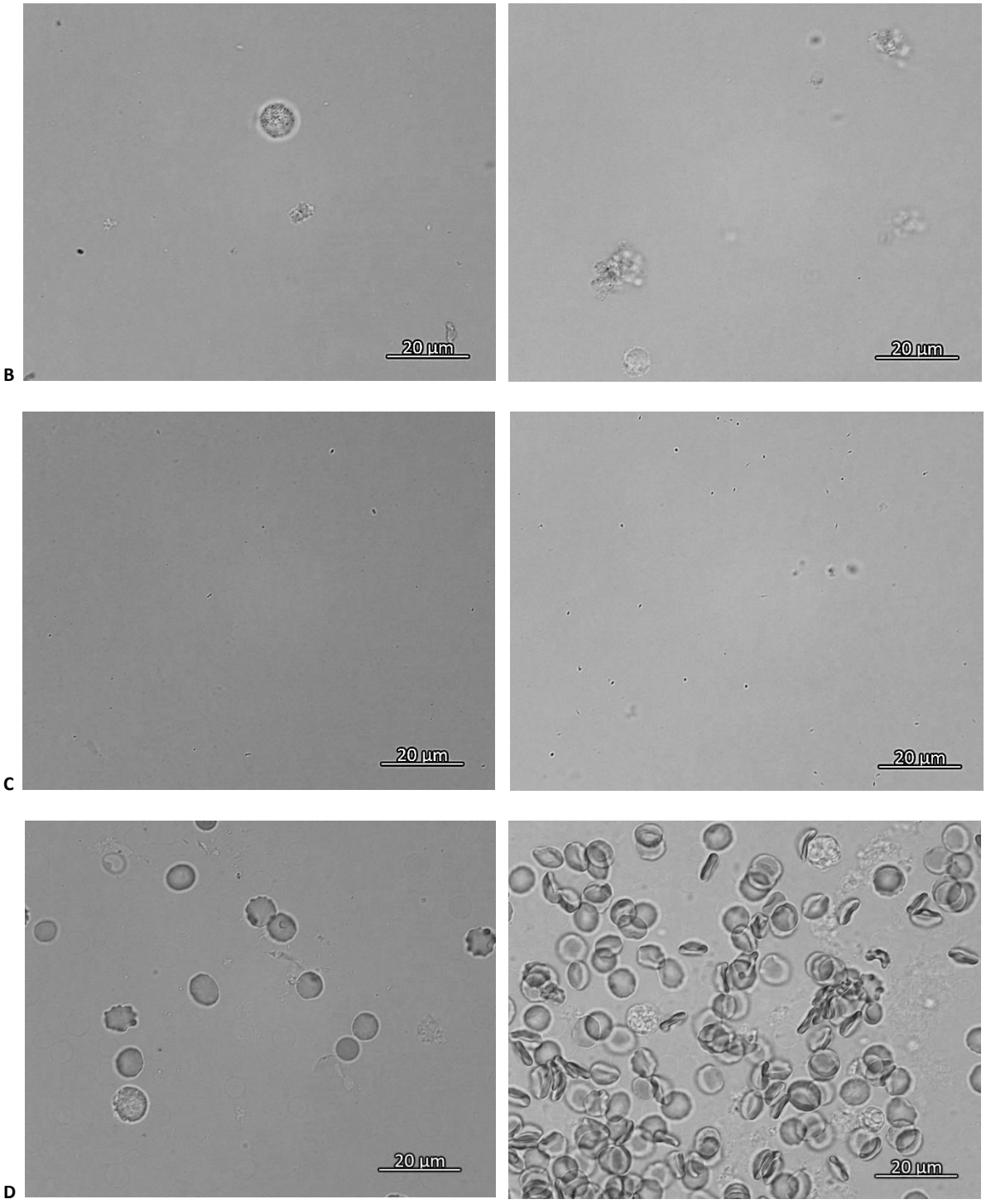


Figure A.18 – Light microscopy of blood components in saline, after incubation with PEG-TMC nanoparticles (20%) for 30 minutes, showing cell aggregation caused. **A**: Red blood cells. **B**: White blood cells. **C**: Blood platelets. **D**: Whole blood.

The positive control, polyethyleneimine (Mw 25 000), caused extensive cell aggregation of all the separate blood components, as well as whole blood, as seen in Figure A.13. As expected, blood components incubated with saline (negative control), showed no cell aggregation (Figure A.14). The 20% small TMC nanoparticle- (Figure A.15) and the PEG-TMC nanoparticle groups (Figure A.18)

caused almost no cell aggregation of the different blood components, although slight aggregation of whole blood was seen after incubation with 20% small TMC nanoparticles and slight aggregation of platelets was caused by the PEG-TMC nanoparticles. Of the experimental samples, 60% small TMC nanoparticles (Figure A.16) caused the most cell aggregation, even though in itself, the aggregation caused was at most only mild. The 60% small TMC nanoparticles caused mild aggregation when incubated with white blood cells or platelets, but also caused light aggregation of red blood cells and whole blood. Larger TMC nanoparticles (Figure A.17) caused no aggregation to light aggregation of the separate blood components, but caused mild aggregation when incubated with whole blood.

The extent of cell aggregation caused by each of the experimental formulations is summarized and compared to positive and negative controls in Table A.4.

Table A.4 – Summary of cell aggregation caused by the different experimental groups.

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

A.3.6 Complement activation

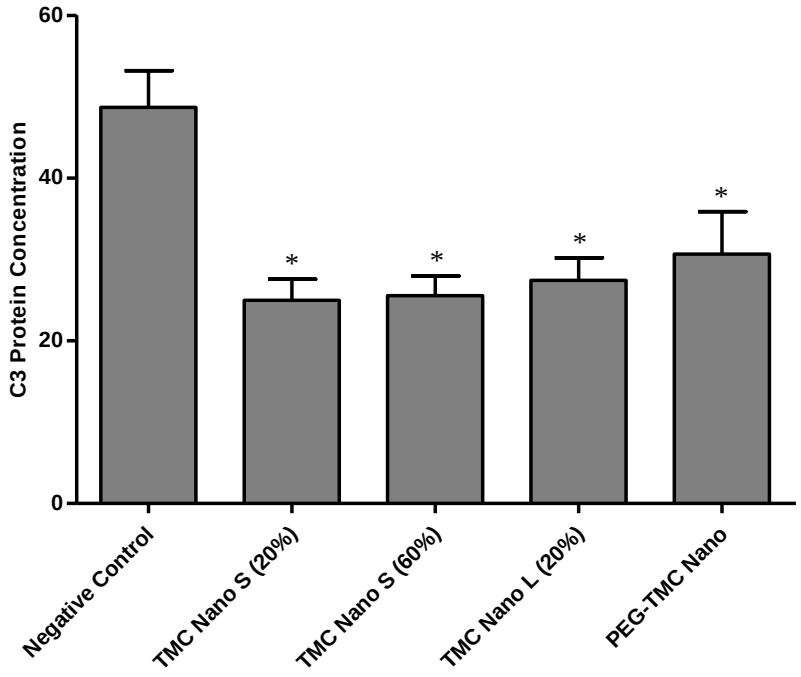


Figure A.19 – Mean C3 protein concentration with standard error of mean, as interpolated from the standard curve as activated by the different experimental groups and the control group. For each bar n = 9.

* Statistical significance compared to control group

The standard C3 protein concentration curve (Figure A.3) was used to determine the amount of C3 protein left over by each of the experimental samples after incubation. All of the experimental groups displayed significantly lower complement C3 protein concentration values compared to the control group, as seen in Figure A.19. This means that all the experimental groups caused complement activation to a certain extent. There was no significant difference in activation between the experimental groups, however.

A.3.7 Plasma protein interaction

After the experimental samples had been incubated with plasma in the rapid equilibrium dialysis (RED) plate for four hours, a colorimetric assay was performed. The concentration of the particles in the samples were determined by interpolation from the standard concentration curves that were drawn for each of the particle types in glycerol (Figure A.4, Figure A.5 and Figure A.6), using the absorbance values measure with a BioTek microplate reader at 550 nm (Figure A.20). These concentration values represented the experimental particles that had not bound to the plasma proteins during the incubation period and as such, they were used to calculate the percentage of particles that had bound to the plasma proteins. These data are shown in Figure A.21.

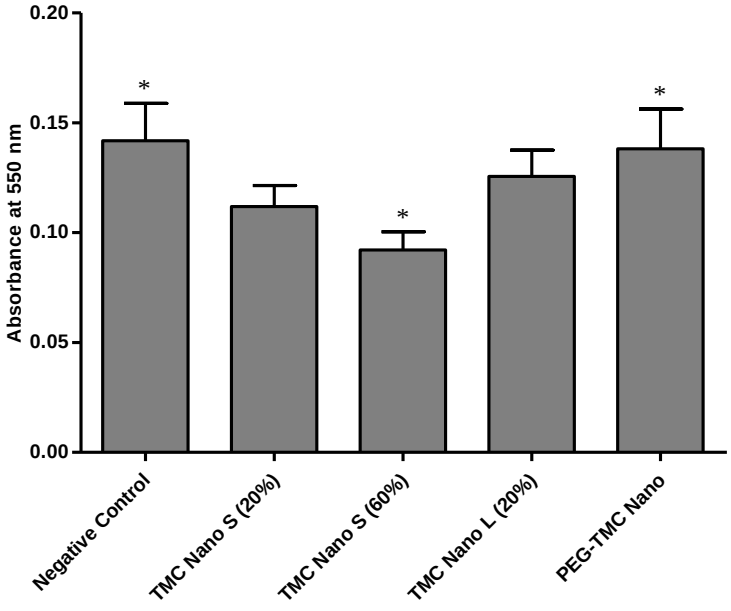


Figure A.20 – Graphic representation of the mean absorbance values of each of the experimental and the control groups, as measured with a BioTek microplate reader at 550 nm, with standard error of mean. For each bar n = 9.
* Statistical significance between the TMC Nano S (60%) group and both the negative control and the PEG-TMC Nano groups.

The measured absorbance values (Figure A.20) showed that at 0.09 ± 0.008, the 60% concentration small TMC nanoparticle group had a significantly lower absorbance that both the control group (0.14 ± 0.017), which consisted of only plasma and PBS, and the cross-linked PEG-TMC nanoparticle group (0.14 ± 0.018).

The calculations of the percentage of experimental particles bound to plasma proteins (mean values and standard error of mean shown in Figure A.21) showed that at 90.68 ± 0.828%, the 60% concentration small TMC nanoparticle group had had the most interaction with plasma proteins of all the experimental groups. This group's interaction was significantly higher than the interactions of the other experimental groups.

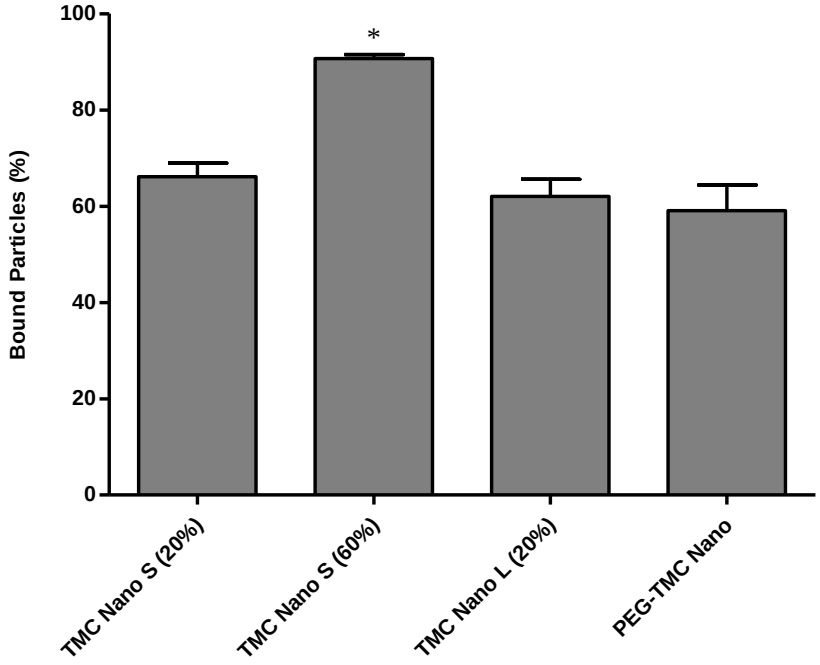


Figure A.21 – Graphic representation of the mean percentage of particles of each experimental group bound to complement C3 proteins, along with the standard error of the mean values, as calculated from the standard curves of each particle type in glycerol. For each bar n = 9.
* Statistical significance compared to the values of the other experimental groups.

The extent of plasma protein interaction of the control group could not be determined in the same way as the experimental groups, as there was no API involved. However, the relation of the absorbance values suggests that the plasma protein interaction of the 20% small TMC nanoparticles (66.10 ± 2.878%), the 20% larger TMC nanoparticles (62.04 ± 3.570%) and the cross-linked PEG-TMC nanoparticles (59.08 ± 5.306%) was not statistically significant.

A.4 References

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Annexure B

Certificate of Analysis of Chitosan

primex

Certificate of analysis

Reanalysis 22.07.2010

Product name	ChitoClear® (Chitosan)
Source	*Pandalus borealis*
Generic name	β(1→4) D-glucosamine / N-acetyl-D-glucosamine
Lot number	TM2832
Revision number	Rev. 03
Date of manufacture	5.1.2007
Retest date	5.1.2010
Extended esp. Date	5.1.2011

Manufactured by:
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Oskarsgata 7
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Iceland
Ph: +354 460 6900
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PARAMETER	TEST METHOD	RESULTS	Comment
Dry Matter Content	CP-001	86,9 %	
Ash	CP-002	0,4 %	
Turbidity	CP-003	126 NTU	
Viscosity	CP-004 (1% chitosan)	1066 cP (mPa·s)	
Solubility	CP-006	99,5 %	
Degree of Deacetylation	CP-010 (Colloidal titration)	92 %	
Sieve Analysis	CP-008	100% through 18 mesh	
Tap Density	CP-009	0,3 g/cc	
Appearance	CP-007	White powder	
Taste and odor	CP-007	No taste or smell	
Microbial:			*detection limit:*
Aerobic plate count	NMKL 86	<1000 cfu/g	10 cfu/g
Yeast and mold	NMKL 98	<100 cfu/g	10 cfu/g
Coliform bacteria	ISO 4831	absent	10 cfu/g
E. coli	ISO 7251	absent	0,3 cfu/g
Salmonella	NMKL 71	absent	Neg.
Toxic Heavy Metals:			
Arsenic	SW-846 6020 ICP/MS	none detected	1,0 ppm
Cadmium	SW-846 6020 ICP/MS	none detected	0,20 ppm
Lead	SW-846 6020 ICP/MS	<1,0ppm	0,10 ppm
Mercury	SW-846 7471 CVAA	none detected	0,01 ppm

MBR – Master Batch Record
CP – Primex Standard Test Methods
NMKL - Nordic Committee on Food Analysis
ISO – International Organization for Standardization
ICP/MS – Inductively Coupled Plasma Mass Spectrometry
CVAA – Cold Vapor Atomic Absorption

Reported By: Vala Arnadottir
Name: Vala Arnadottir
Title: Laboratory Manager
Date: 22. 7. 2010.

Methods of analysis available upon request

NOTE: *Lack of colored logo indicates this document is a copy and is not controlled*

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Annexure C

Ethics Application

Annexure C

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Research Ethics Committee

North-West University

Potchefstroom Campus

Box116

26 June 2009

Dear Mrs Halgryn

ETHICS APPLICATION: NWU-00025-09-S5

TOXICITY OF ANTI-MALARIAL DRUGS IN COMBINATION WITH PHEROID™ TECHNOLOGY

The application in general is in compliance with current and relevant ethical codes. We therefore approve ethics application **NWU-0025-09-S5.**

Yours sincerely

PROFJ DU PLESSIS
DIRECTOR

Annexure D

Statistical Data

D.1 Determination of concentration of particles in glycerol

Table D.1 – Absorption of different TMC concentrations, as measured with a UV spectrometer at 530 nm.

Concentration (mg/ml)	Absorbance			Average
300	1.2832	1.3380	1.3073	1.3095
270	1.1910	-	1.2058	1.1984
240	1.0525	1.0643	-	1.0584
210	0.9255	-	0.9369	0.9312
180	0.7851	0.7959	-	0.7905
150	0.6649	-	0.6727	0.6688
120	0.4998	0.5384	-	0.5191
90	0.4024	0.4027	0.4096	0.4049
60	0.2545	-	-	0.2545
30	0.1185	0.1333	0.1360	0.1293
3	0.0166	0.0196	0.0276	0.0213

Table D.2 – Absorption of different PEG-TMC concentrations measured with a BioTek microplate reader at 550 nm.

Concentration (mg/ml)	Absorbance			Average
300	0.1653	0.1663	0.1613	0.1643
150	0.0763	0.0763	0.0773	0.0766
75	0.0343	0.0383	0.0363	0.0363
37.5	0.0173	0.0163	0.0173	0.0170
18.75	0.0073	0.0073	0.0073	0.0073
9.375	0.0033	0.0033	0.0033	0.0033
4.6875	0.0013	0.0023	0.0023	0.0020

D.2 Hemolysis

Table D.3 – Absorbance measured at 550 nm, after one hour of incubation with experimental samples.

	Absorbance									Average
Negative Control	0.058	0.057	0.057	0.036	0.038	0.038	0.036	*0.688**	0.038	0.0448
Positive Control	0.216	0.344	0.132	1.865	1.992	1.986	1.880	1.823	1.794	1.3369
TMC Nano S (20%)	0.056	0.057	0.058	0.045	0.044	0.044	0.045	0.046	0.043	0.0487
TMC Nano S (60%)	0.135	0.110	0.117	0.219	0.218	0.245	0.270	0.449	0.215	0.2198
TMC Nano L (20%)	0.062	0.059	0.060	0.041	0.042	0.045	0.042	0.046	0.046	0.0492
PEG-TMC Nano	0.058	0.057	0.059	0.042	0.043	0.050	0.041	0.042	0.043	0.0483

* outlier value, excluded from calculations

Table D.4 – Absorbance measured at 550 nm, after six hours of incubation with experimental samples.

	Absorbance									Average
Negative Control	0.060	0.057	0.057	0.040	0.038	0.043	0.039	0.039	0.039	0.0458
Positive Control	0.154	0.447	0.091	2.408	2.510	2.534	2.117	2.478	2.535	1.6971
TMC Nano S (20%)	0.056	0.059	0.059	0.044	0.044	0.046	0.044	0.047	0.048	0.0497
TMC Nano S (60%)	0.097	0.118	0.089	0.839	0.938	0.796	1.057	1.031	0.968	0.6592
TMC Nano L (20%)	0.058	0.059	0.065	0.046	0.046	0.045	0.045	0.060	0.060	0.0538
PEG-TMC Nano	0.056	0.060	0.063	0.044	0.047	0.049	0.048	0.045	0.051	0.0514

Table D.5 – Absorbance measured at 550 nm, after 12 hours of incubation with experimental samples.

	Absorbance									Average
Negative Control	0.057	0.060	0.062	0.043	0.042	0.042	0.041	0.044	0.043	0.0482
Positive Control	0.673	0.317	0.574	2.575	2.678	2.661	2.542	2.646	2.401	1.8963
TMC Nano S (20%)	0.078	0.067	0.082	0.063	0.070	0.072	0.063	0.062	0.067	0.0693
TMC Nano S (60%)	0.254	0.318	0.264	1.583	1.576	1.368	1.268	1.183	1.444	1.0287
TMC Nano L (20%)	0.075	0.090	0.081	0.082	0.082	0.072	0.069	0.107	0.081	0.0821
PEG-TMC Nano	0.073	0.086	0.079	0.092	0.108	0.105	0.098	0.101	0.099	0.0934

Table D.6 – Descriptive statistics of the absorbance of the different experimental groups after one hour of incubation.

	Negative Control	Positive Control	TMC Nano S (20%)	TMC Nano S (60%)	TMC Nano L (20%)	PEG-TMC Nano
Number of values	8	9	9	9	9	9
Minimum	0.036	0.132	0.043	0.11	0.041	0.041
25% Percentile	0.0365	0.28	0.044	0.126	0.042	0.042
Median	0.038	1.823	0.045	0.218	0.046	0.043
75% Percentile	0.057	1.933	0.0565	0.2575	0.0595	0.0575
Maximum	0.058	1.992	0.058	0.449	0.062	0.059
Mean	0.04475	1.337	0.04867	0.2198	0.04922	0.04833
Std. Deviation	0.01046	0.8339	0.006325	0.1034	0.008555	0.007714
Std. Error	0.003697	0.278	0.002108	0.03447	0.002852	0.002571
Lower 95% CI of mean	0.03601	0.6959	0.04381	0.1403	0.04265	0.0424
Upper 95% CI of mean	0.05349	1.978	0.05353	0.2993	0.0558	0.05426
D'Agostino & Pearson omnibus normality test						
K2	4.718	3.543	3.298	6.185	2.916	3.987
P value	0.0945	0.1701	0.1922	0.0454	0.2327	0.1362
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	No	Yes	Yes
P value summary	ns	ns	ns	*	ns	ns
Skewness	0.623	-0.849	0.8153	1.333	0.7241	0.5486
Kurtosis	-2.21	-1.645	-1.609	2.637	-1.58	-1.919
Sum	0.358	12.03	0.438	1.978	0.443	0.435

Table D.7 – One-way ANOVA of the absorbance of the different experimental groups after one hour of incubation, with Bonferroni post-test.

P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	19.89				
R squared	0.6791				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	227.2				
P value	P<0.0001				
P value summary	***				
Do the variances differ signif. (P < 0.05)	Yes				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	11.96	5	2.392		
Residual (within columns)	5.651	47	0.1202		
Total	17.61	52			
Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
Negative Control vs Positive Control	-1.292	7.669	Yes	***	-1.813 to -0.7710
Negative Control vs TMC Nano S (20%)	-0.003917	0.02325	No	ns	-0.5250 to 0.5172
Negative Control vs TMC Nano S (60%)	-0.175	1.039	No	ns	-0.6961 to 0.3461
Negative Control vs TMC Nano L (20%)	-0.004472	0.02654	No	ns	-0.5256 to 0.5166
Negative Control vs PEG-TMC Nano	-0.003583	0.02127	No	ns	-0.5247 to 0.5175
Positive Control vs TMC Nano S (20%)	1.288	7.881	Yes	***	0.7827 to 1.794
Positive Control vs TMC Nano S (60%)	1.117	6.834	Yes	***	0.6116 to 1.623
Positive Control vs TMC Nano L (20%)	1.288	7.877	Yes	***	0.7821 to 1.793
Positive Control vs PEG-TMC Nano	1.289	7.883	Yes	***	0.7830 to 1.794
TMC Nano S (20%) vs TMC Nano S (60%)	-0.1711	1.047	No	ns	-0.6766 to 0.3344
TMC Nano S (20%) vs TMC Nano L (20%)	-0.0005556	0.003399	No	ns	-0.5061 to 0.5050
TMC Nano S (20%) vs PEG-TMC Nano	0.0003333	0.002039	No	ns	-0.5052 to 0.5059
TMC Nano S (60%) vs TMC Nano L (20%)	0.1706	1.043	No	ns	-0.3350 to 0.6761
TMC Nano S (60%) vs PEG-TMC Nano	0.1714	1.049	No	ns	-0.3341 to 0.6770
TMC Nano L (20%) vs PEG-TMC Nano	0.0008889	0.005438	No	ns	-0.5046 to 0.5064

Table D.8 – Descriptive statistics of the absorbance of the different experimental groups after six hours of incubation.

	Negative Control	Positive Control	TMC Nano S (20%)	TMC Nano S (60%)	TMC Nano L (20%)	PEG-TMC Nano
Number of values	9	9	9	9	9	9
Minimum	0.038	0.091	0.044	0.089	0.045	0.044
25% Percentile	0.039	0.3005	0.044	0.1075	0.0455	0.046
Median	0.04	2.408	0.047	0.839	0.058	0.049
75% Percentile	0.057	2.522	0.0575	0.9995	0.06	0.058
Maximum	0.06	2.535	0.059	1.057	0.065	0.063
Mean	0.04578	1.697	0.04967	0.6592	0.05378	0.05144
Std. Deviation	0.009311	1.111	0.006461	0.4264	0.00809	0.006729
Std. Error	0.003104	0.3704	0.002154	0.1421	0.002697	0.002243
Lower 95% CI of mean	0.03862	0.843	0.0447	0.3314	0.04756	0.04627
Upper 95% CI of mean	0.05293	2.551	0.05463	0.987	0.06	0.05662
D'Agostino & Pearson omnibus normality test						
K2	3.204	3.381	2.666	3.448	4.688	1.391
P value	0.2015	0.1845	0.2636	0.1783	0.0959	0.4989
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes	Yes
P value summary	ns	ns	ns	ns	ns	ns
Skewness	0.8132	-0.8375	0.7515	-0.721	-0.0549	0.7597
Kurtosis	-1.585	-1.612	-1.48	-1.719	-2.125	-0.7847
Sum	0.412	15.27	0.447	5.933	0.484	0.463

Table D.9 – Repeated measures ANOVA of the absorbance of the different experimental groups after six hours of incubation, with Bonferroni post-test.

P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	19.3				
R squared	0.707				
Was the pairing significantly effective?					
R squared	0.09532				
F	1.798				
P value	0.1062				
P value summary	ns				
Is there significant matching? (P < 0.05)	No				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	20.12	5	4.023		
Individual (between rows)	2.998	8	0.3747		
Residual (random)	8.337	40	0.2084		
Total	31.45	53			
Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
Negative Control vs Positive Control	-1.651	7.673	Yes	***	-2.323 to -0.9795
Negative Control vs TMC Nano S (20%)	-0.003889	0.01807	No	ns	-0.6757 to 0.6680
Negative Control vs TMC Nano S (60%)	-0.6134	2.85	No	ns	-1.285 to 0.05841
Negative Control vs TMC Nano L (20%)	-0.008	0.03717	No	ns	-0.6799 to 0.6639
Negative Control vs PEG-TMC Nano	-0.005667	0.02633	No	ns	-0.6775 to 0.6662
Positive Control vs TMC Nano S (20%)	1.647	7.655	Yes	***	0.9756 to 2.319
Positive Control vs TMC Nano S (60%)	1.038	4.823	Yes	***	0.3660 to 1.710
Positive Control vs TMC Nano L (20%)	1.643	7.636	Yes	***	0.9715 to 2.315
Positive Control vs PEG-TMC Nano	1.646	7.647	Yes	***	0.9738 to 2.318
TMC Nano S (20%) vs TMC Nano S (60%)	-0.6096	2.832	No	ns	-1.281 to 0.06229
TMC Nano S (20%) vs TMC Nano L (20%)	-0.004111	0.0191	No	ns	-0.6760 to 0.6677
TMC Nano S (20%) vs PEG-TMC Nano	-0.001778	0.008261	No	ns	-0.6736 to 0.6701
TMC Nano S (60%) vs TMC Nano L (20%)	0.6054	2.813	No	ns	-0.06641 to 1.277
TMC Nano S (60%) vs PEG-TMC Nano	0.6078	2.824	No	ns	-0.06407 to 1.280
TMC Nano L (20%)vs PEG-TMC Nano	0.002333	0.01084	No	ns	-0.6695 to 0.6742

Table D.10 – Descriptive statistics of the absorbance of the different experimental groups after 12 hours of incubation.

	Negative Control	Positive Control	TMC Nano S (20%)	TMC Nano S (60%)	TMC Nano L (20%)	PEG-TMC Nano
Number of values	9	9	9	9	9	9
Minimum	0.041	0.317	0.062	0.254	0.069	0.073
25% Percentile	0.042	0.6235	0.063	0.291	0.0735	0.0825
Median	0.043	2.542	0.067	1.268	0.081	0.098
75% Percentile	0.0585	2.654	0.075	1.51	0.086	0.103
Maximum	0.062	2.678	0.082	1.583	0.107	0.108
Mean	0.04822	1.896	0.06933	1.029	0.08211	0.09344
Std. Deviation	0.008715	1.039	0.006964	0.5772	0.01123	0.01193
Std. Error	0.002905	0.3462	0.002321	0.1924	0.003743	0.003976
Lower 95% CI of mean	0.04152	1.098	0.06398	0.585	0.07348	0.08428
Upper 95% CI of mean	0.05492	2.695	0.07469	1.472	0.09074	0.1026
D'Agostino & Pearson omnibus normality test						
K2	2.858	3.349	1.429	3.262	6.523	1.003
P value	0.2395	0.1874	0.4893	0.1957	0.0383	0.6055
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	No	Yes
P value summary	ns	ns	ns	ns	*	ns
Skewness	0.9002	-0.8645	0.8444	-0.6756	1.391	-0.6298
Kurtosis	-1.379	-1.579	-0.2894	-1.705	2.673	-0.7517
Sum	0.434	17.07	0.624	9.258	0.739	0.841

Table D.11 – One-way ANOVA of the absorbance of the different experimental groups after 12 hours of incubation, with Bonferroni post-test.

P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	22.57				
R squared	0.7015				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	225.8				
P value	P<0.0001				
P value summary	***				
Do the variances differ signif. (P < 0.05)	Yes				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	26.56	5	5.311		
Residual (within columns)	11.3	48	0.2354		
Total	37.86	53			
Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
Negative Control vs Positive Control	-1.848	8.081	Yes	***	-2.555 to -1.142
Negative Control vs TMC Nano S (20%)	-0.02111	0.09231	No	ns	-0.7276 to 0.6854
Negative Control vs TMC Nano S (60%)	-0.9804	4.287	Yes	**	-1.687 to -0.2739
Negative Control vs TMC Nano L (20%)	-0.03389	0.1482	No	ns	-0.7404 to 0.6726
Negative Control vs PEG-TMC Nano	-0.04522	0.1977	No	ns	-0.7517 to 0.6613
Positive Control vs TMC Nano S (20%)	1.827	7.989	Yes	***	1.120 to 2.534
Positive Control vs TMC Nano S (60%)	0.8677	3.794	Yes	**	0.1611 to 1.574
Positive Control vs TMC Nano L (20%)	1.814	7.933	Yes	***	1.108 to 2.521
Positive Control vs PEG-TMC Nano	1.803	7.883	Yes	***	1.096 to 2.509
TMC Nano S (20%) vs TMC Nano S (60%)	-0.9593	4.195	Yes	**	-1.666 to -0.2528
TMC Nano S (20%) vs TMC Nano L (20%)	-0.01278	0.05587	No	ns	-0.7193 to 0.6937
TMC Nano S (20%) vs PEG-TMC Nano	-0.02411	0.1054	No	ns	-0.7306 to 0.6824
TMC Nano S (60%) vs TMC Nano L (20%)	0.9466	4.139	Yes	**	0.2400 to 1.653
TMC Nano S (60%) vs PEG-TMC Nano	0.9352	4.089	Yes	**	0.2287 to 1.642
TMC Nano L (20%) vs PEG-TMC Nano	-0.01133	0.04955	No	ns	-0.7179 to 0.6952

Table D.12 – Descriptive statistics of the absorption of the negative control group over the 12-hour span of the hemolysis experiment.

	1h	**6h**	**12h**
Number of values	8	9	9
Minimum	0.036	0.038	0.041
25% Percentile	0.0365	0.039	0.042
Median	0.038	0.04	0.043
75% Percentile	0.057	0.057	0.0585
Maximum	0.058	0.06	0.062
Mean	0.04475	0.04578	0.04822
Std. Deviation	0.01046	0.009311	0.008715

Std. Error	0.003697	0.003104	0.002905
Lower 95% CI of mean	0.03601	0.03862	0.04152
Upper 95% CI of mean	0.05349	0.05293	0.05492
D'Agostino & Pearson omnibus normality test			
K2	4.718	3.204	2.858
P value	0.0945	0.2015	0.2395
Passed normality test (alpha=0.05)?	Yes	Yes	Yes
P value summary	ns	ns	ns
Skewness	0.623	0.8132	0.9002
Kurtosis	-2.21	-1.585	-1.379
Sum	0.358	0.412	0.434

Table D.13 – One-way ANOVA of the absorbance of the negative control group over 12 hours, with Bonferroni post-test.

P value	0.7396				
P value summary	ns				
Are means signif. different? (P < 0.05)	No				
Number of groups	3				
F	0.3057				
R squared	0.02589				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	0.2424				
P value	0.8859				
P value summary	ns				
Do the variances differ signif. (P < 0.05)	No				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	5.493E-05	2	0.00002746		
Residual (within columns)	0.002067	23	0.00008985		
Total	0.002122	25			
Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
1h vs 6h	-0.001028	0.2231	No	ns	-0.01292 to 0.01086
1h vs 12h	-0.003472	0.7538	No	ns	-0.01536 to 0.008420
6h vs 12h	-0.002444	0.547	No	ns	-0.01398 to 0.009093

Table D.14 – Descriptive statistics of the absorption of the positive control group over the 12-hour span of the hemolysis experiment.

	1h	**6h**	**12h**
Number of values	9	9	9
Minimum	0.132	0.091	0.317
25% Percentile	0.28	0.3005	0.6235
Median	1.823	2.408	2.542
75% Percentile	1.933	2.522	2.654
Maximum	1.992	2.535	2.678
Mean	1.337	1.697	1.896
Std. Deviation	0.8339	1.111	1.039
Std. Error	0.278	0.3704	0.3462
Lower 95% CI of mean	0.6959	0.843	1.098
Upper 95% CI of mean	1.978	2.551	2.695

D'Agostino & Pearson omnibus normality test			
K2	3.543	3.381	3.349
P value	0.1701	0.1845	0.1874
Passed normality test (alpha=0.05)?	Yes	Yes	Yes
P value summary	ns	ns	ns
Skewness	-0.849	-0.8375	-0.8645
Kurtosis	-1.645	-1.612	-1.579
Sum	12.03	15.27	17.07

Table D.15 – Repeated measures ANOVA of the absorbance of the positive control group over 12 hours, with Bonferroni post-test.

P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	20.31				
R squared	0.7175				
Was the pairing significantly effective?					
R squared	0.9209				
F	82.46				
P value	P<0.0001				
P value summary	***				
Is there significant matching? (P < 0.05)	Yes				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	1.447	2	0.7236		
Individual (between rows)	23.5	8	2.938		
Residual (random)	0.57	16	0.03562		
Total	25.52	26			
Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
1h vs 6h	-0.3602	4.049	Yes	**	-0.5980 to -0.1224
1h vs 12h	-0.5594	6.288	Yes	***	-0.7973 to -0.3216
6h vs 12h	-0.1992	2.239	No	ns	-0.4370 to 0.03860

Table D.16 – Descriptive statistics of the absorbance of the 20% concentration small TMC nanoparticle experimental group, throughout the experiment.

	1h	**6h**	**12h**
Number of values	9	9	9
Minimum	0.043	0.044	0.062
25% Percentile	0.044	0.044	0.063
Median	0.045	0.047	0.067
75% Percentile	0.0565	0.0575	0.075
Maximum	0.058	0.059	0.082
Mean	0.04867	0.04967	0.06933
Std. Deviation	0.006325	0.006461	0.006964
Std. Error	0.002108	0.002154	0.002321
Lower 95% CI of mean	0.04381	0.0447	0.06398
Upper 95% CI of mean	0.05353	0.05463	0.07469
D'Agostino & Pearson omnibus normality test			

K2	3.298	2.666	1.429
P value	0.1922	0.2636	0.4893
Passed normality test (alpha=0.05)?	Yes	Yes	Yes
P value summary	ns	ns	ns
Skewness	0.8153	0.7515	0.8444
Kurtosis	-1.609	-1.48	-0.2894
Sum	0.438	0.447	0.624

Table D.17 – Repeated measures ANOVA of the absorbance of the 20% concentration small TMC nanoparticles experimental group, over 12 hours, with Bonferroni post-test.

P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	108.7				
R squared	0.9314				
Was the pairing significantly effective?					
R squared	0.2472				
F	9.578				
P value	P<0.0001				
P value summary	***				
Is there significant matching? (P < 0.05)	Yes				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	0.002445	2	0.001222		
Individual (between rows)	0.000862	8	0.0001078		
Residual (random)	0.00018	16	0.00001125		
Total	0.003487	26			
Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
1h vs 6h	-0.001	0.6325	No	ns	-0.005226 to 0.003226
1h vs 12h	-0.02067	13.07	Yes	***	-0.02489 to -0.01644
6h vs 12h	-0.01967	12.44	Yes	***	-0.02389 to -0.01544

Table D.18 – Descriptive statistics of the absorbance of the 60% concentration small TMC nanoparticles experimental group, throughout the experiment.

	1h	**6h**	**12h**
Number of values	9	9	9
Minimum	0.11	0.089	0.254
25% Percentile	0.126	0.1075	0.291
Median	0.218	0.839	1.268
75% Percentile	0.2575	0.9995	1.51
Maximum	0.449	1.057	1.583
Mean	0.2198	0.6592	1.029
Std. Deviation	0.1034	0.4264	0.5772
Std. Error	0.03447	0.1421	0.1924
Lower 95% CI of mean	0.1403	0.3314	0.585
Upper 95% CI of mean	0.2993	0.987	1.472

D'Agostino & Pearson omnibus normality test			
K2	6.185	3.448	3.262
P value	0.0454	0.1783	0.1957
Passed normality test (alpha=0.05)?	No	Yes	Yes
P value summary	*	ns	ns
Skewness	1.333	-0.721	-0.6756
Kurtosis	2.637	-1.719	-1.705
Sum	1.978	5.933	9.258

Table D.19 – One-way ANOVA of the absorbance of the 60% concentration small TMC nanoparticles experimental group, with Bonferroni post-test.

P value	0.0017				
P value summary	**				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	8.422				
R squared	0.4124				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	16.05				
P value	0.0003				
P value summary	***				
Do the variances differ signif. (P < 0.05)	Yes				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	2.952	2	1.476		
Residual (within columns)	4.206	24	0.1752		
Total	7.157	26			
Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
1h vs 6h	-0.4394	2.227	No	ns	-0.9473 to 0.06843
1h vs 12h	-0.8089	4.099	Yes	**	-1.317 to -0.3010
6h vs 12h	-0.3694	1.872	No	ns	-0.8773 to 0.1384

Table D.20 – Descriptive statistics of the absorbance of the 20% concentration large TMC nanoparticle experimental group, throughout the experiment.

	1h	**6h**	**12h**
Number of values	9	9	9
Minimum	0.041	0.045	0.069
25% Percentile	0.042	0.0455	0.0735
Median	0.046	0.058	0.081
75% Percentile	0.0595	0.06	0.086
Maximum	0.062	0.065	0.107
Mean	0.04922	0.05378	0.08211
Std. Deviation	0.008555	0.00809	0.01123
Std. Error	0.002852	0.002697	0.003743
Lower 95% CI of mean	0.04265	0.04756	0.07348
Upper 95% CI of mean	0.0558	0.06	0.09074
D'Agostino & Pearson omnibus normality test			

K2	2.916	4.688	6.523
P value	0.2327	0.0959	0.0383
Passed normality test (alpha=0.05)?	Yes	Yes	No
P value summary	ns	ns	*
Skewness	0.7241	-0.0549	1.391
Kurtosis	-1.58	-2.125	2.673
Sum	0.443	0.484	0.739

Table D.21 – One-way ANOVA of the absorbance of the 20% concentration large TMC nanoparticle experimental group, with Bonferroni post-test.

P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	32.38				
R squared	0.7296				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	0.978				
P value	0.6133				
P value summary	ns				
Do the variances differ signif. (P < 0.05)	No				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	0.005716	2	0.002858		
Residual (within columns)	0.002118	24	0.00008825		
Total	0.007834	26			
Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
1h vs 6h	-0.004556	1.029	No	ns	-0.01595 to 0.006842
1h vs 12h	-0.03289	7.427	Yes	***	-0.04429 to -0.02149
6h vs 12h	-0.02833	6.398	Yes	***	-0.03973 to -0.01694

Table D.22 – Descriptive statistics of the absorbance of the 20% concentration cross-linked PEG-TMC nanoparticles experimental group, throughout the experiment.

	1h	**6h**	**12h**
Number of values	9	9	9
Minimum	0.041	0.044	0.073
25% Percentile	0.042	0.046	0.0825
Median	0.043	0.049	0.098
75% Percentile	0.0575	0.058	0.103
Maximum	0.059	0.063	0.108
Mean	0.04833	0.05144	0.09344
Std. Deviation	0.007714	0.006729	0.01193
Std. Error	0.002571	0.002243	0.003976
Lower 95% CI of mean	0.0424	0.04627	0.08428
Upper 95% CI of mean	0.05426	0.05662	0.1026
D'Agostino & Pearson omnibus normality test			
K2	3.987	1.391	1.003

P value	0.1362	0.4989	0.6055
Passed normality test (alpha=0.05)?	Yes	Yes	Yes
P value summary	ns	ns	ns
Skewness	0.5486	0.7597	-0.6298
Kurtosis	-1.919	-0.7847	-0.7517
Sum	0.435	0.463	0.841

Table D.23 – Repeated measures ANOVA of the absorbance of the 20% concentration cross-linked PEG-TMC nanoparticles experimental group, over 12 hours, with Bonferroni post-test.

P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	51.76				
R squared	0.8661				
Was the pairing significantly effective?					
R squared	0.01571				
F	0.2384				
P value	0.977				
P value summary	ns				
Is there significant matching? (P < 0.05)	No				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	0.01143	2	0.005713		
Individual (between rows)	0.0002105	8	0.00002631		
Residual (random)	0.001766	16	0.0001104		
Total	0.0134	26			
Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
1h vs 6h	-0.003111	0.6282	No	ns	-0.01635 to 0.01013
1h vs 12h	-0.04511	9.109	Yes	***	-0.05835 to -0.03187
6h vs 12h	-0.042	8.481	Yes	***	-0.05524 to -0.02876

Table D.24 – Percentage hemolysis calculated from absorbance after one hour of incubation with experimental samples.

	Percentage hemolysis calculated									**Average**
TMC Nano S (20%)	-0.5780	-0.1450	0.2890	0.3936	0.3423	0.3423	0.4366	0.4912	0.3275	0.2110
TMC Nano S (60%)	33.6710	22.8320	25.8670	9.3274	9.2761	10.6623	12.7160	22.4850	9.7144	17.3946
TMC Nano L (20%)	2.0230	0.7230	1.1560	0.1883	0.2396	0.3936	0.2729	0.4912	0.4912	0.6643
PEG-TMC Nano	0.2890	-0.1450	0.7230	0.2396	0.2909	0.6504	0.2183	0.2729	0.3275	0.3185

Table D.25 – Percentage hemolysis calculated from absorbance after six hours of incubation with experimental samples.

	Percentage hemolysis calculated									**Average**
TMC Nano S (20%)	-0.8670	0.4340	0.4340	0.1476	0.1476	0.2281	0.2104	0.3366	0.3787	0.1611
TMC Nano S (60%)	16.9080	26.0120	13.4390	32.1524	36.1380	30.4214	42.8331	41.7391	39.0884	30.9701
TMC Nano L (20%)	0.0000	0.4340	3.0350	0.2281	0.2281	0.1879	0.2525	0.8836	0.8836	0.6814
PEG-TMC Nano	-0.8670	0.8670	2.1680	0.1476	0.2684	0.3489	0.3787	0.2525	0.5049	0.4521

Table D.26 – Percentage hemolysis calculated from absorbance after 12 hours of incubation with experimental samples.

	Percentage hemolysis calculated									Average
TMC Nano S (20%)	3.5170	1.4070	4.2840	0.7830	1.0490	1.1250	0.8040	0.7640	0.9620	1.6328
TMC Nano S (60%)	37.2760	49.5520	39.1940	58.4030	58.1370	50.2530	48.4390	45.0780	55.3960	49.0809
TMC Nano L (20%)	2.9410	5.8180	4.0920	1.5040	1.5040	1.1250	1.0410	2.5430	1.5150	2.4537
PEG-TMC Nano	2.5580	5.0510	3.7080	1.8830	2.4890	2.3760	2.1870	2.3060	2.2270	2.7539

Table D.27 – Descriptive statistics of the percentage hemolysis caused by the different experimental groups after one hour of incubation.

	TMC Nano S (20%)	TMC Nano S (60%)	TMC Nano L (20%)	PEG-TMC Nano
Number of values	9	9	9	9
Minimum	-0.578	9.276	0.1883	-0.145
25% Percentile	0.072	9.521	0.2562	0.229
Median	0.3423	12.72	0.4912	0.289
75% Percentile	0.4151	24.35	0.9395	0.4889
Maximum	0.4912	33.67	2.023	0.723
Mean	0.211	17.39	0.6643	0.3185
Std. Deviation	0.3475	9.01	0.59	0.2521
Std. Error	0.1158	3.003	0.1967	0.08405
Lower 95% CI of mean	-0.05607	10.47	0.2108	0.1247
Upper 95% CI of mean	0.4782	24.32	1.118	0.5123
D'Agostino & Pearson omnibus normality test				
K2	9.766	1.352	9.981	0.801
P value	0.0076	0.5087	0.0068	0.67
Passed normality test (alpha=0.05)?	No	Yes	No	Yes
P value summary	**	ns	**	ns
Skewness	-1.878	0.7235	1.841	0.01516
Kurtosis	3.039	-0.8527	3.345	1.074
Sum	1.899	156.6	5.979	2.867

Table D.28 – One-way ANOVA of the percentage hemolysis caused by the different experimental groups after one hour, with Bonferroni post-test.

P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	4		
F	31.84		
R squared	0.749		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)	103.4		
P value	P<0.0001		
P value summary	***		
Do the variances differ signif. (P < 0.05)	Yes		
ANOVA Table	**SS**	**df**	**MS**
Treatment (between columns)	1951	3	650.3
Residual (within columns)	653.6	32	20.43
Total	2605	35	

Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
TMC Nano S (20%) vs TMC Nano S (60%)	-17.18	8.065	Yes	***	-23.18 to -11.19
TMC Nano S (20%) vs TMC Nano L (20%)	-0.4533	0.2127	No	ns	-6.445 to 5.538
TMC Nano S (20%) vs PEG-TMC Nano	-0.1075	0.05044	No	ns	-6.099 to 5.884
TMC Nano S (60%) vs TMC Nano L (20%)	16.73	7.853	Yes	***	10.74 to 22.72
TMC Nano S (60%) vs PEG-TMC Nano	17.08	8.015	Yes	***	11.08 to 23.07
TMC Nano L (20%) vs PEG-TMC Nano	0.3458	0.1623	No	ns	-5.646 to 6.338

Table D.29 – Descriptive statistics of the percentage hemolysis caused by the different experimental groups after six hours of incubation.

	TMC Nano S (20%)	TMC Nano S (60%)	TMC Nano L (20%)	PEG-TMC Nano
Number of values	9	9	9	9
Minimum	-0.867	13.44	0	-0.867
25% Percentile	0.1476	21.46	0.208	0.2
Median	0.2281	32.15	0.2525	0.3489
75% Percentile	0.4063	40.41	0.8836	0.686
Maximum	0.434	42.83	3.035	2.168
Mean	0.1611	30.97	0.6814	0.4521
Std. Deviation	0.4018	10.49	0.9344	0.7939
Std. Error	0.1339	3.497	0.3115	0.2646
Lower 95% CI of mean	-0.1477	22.91	-0.03684	-0.1581
Upper 95% CI of mean	0.4699	39.04	1.4	1.062
D'Agostino & Pearson omnibus normality test				
K2	20	1.071	18.16	5.354
P value	P<0.0001	0.5855	0.0001	0.0688
Passed normality test (alpha=0.05)?	No	Yes	No	Yes
P value summary	***	ns	***	ns
Skewness	-2.552	-0.6537	2.43	0.8945
Kurtosis	7.061	-0.7592	6.35	3.345
Sum	1.45	278.7	6.133	4.069

Table D.30 – One-way ANOVA of the percentage hemolysis caused by the different experimental groups after six hours, with Bonferroni post-test.

P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	4		
F	75.12		
R squared	0.8757		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)	83.95		
P value	P<0.0001		
P value summary	***		
Do the variances differ signif. (P < 0.05)	Yes		
ANOVA Table	SS	df	MS
Treatment (between columns)	6296	3	2099
Residual (within columns)	894	32	27.94

Total	7190	35			
Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
TMC Nano S (20%) vs TMC Nano S (60%)	-30.81	12.36	Yes	***	-37.82 to -23.80
TMC Nano S (20%) vs TMC Nano L (20%)	-0.5203	0.2088	No	ns	-7.528 to 6.487
TMC Nano S (20%) vs PEG-TMC Nano	-0.291	0.1168	No	ns	-7.298 to 6.716
TMC Nano S (60%) vs TMC Nano L (20%)	30.29	12.16	Yes	***	23.28 to 37.30
TMC Nano S (60%) vs PEG-TMC Nano	30.52	12.25	Yes	***	23.51 to 37.53
TMC Nano L (20%) vs PEG-TMC Nano	0.2293	0.09203	No	ns	-6.778 to 7.237

Table D.31 – Descriptive statistics of the percentage hemolysis caused by the different experimental groups after 12 hours of incubation.

	TMC Nano S (20%)	**TMC Nano S (60%)**	**TMC Nano L (20%)**	**PEG-TMC Nano**
Number of values	9	9	9	9
Minimum	0.764	37.28	1.041	1.883
25% Percentile	0.7935	42.14	1.315	2.207
Median	1.049	49.55	1.515	2.376
75% Percentile	2.462	56.77	3.517	3.133
Maximum	4.284	58.4	5.818	5.051
Mean	1.633	49.08	2.454	2.754
Std. Deviation	1.315	7.615	1.608	0.9998
Std. Error	0.4385	2.538	0.5361	0.3333
Lower 95% CI of mean	0.6217	43.23	1.217	1.985
Upper 95% CI of mean	2.644	54.93	3.69	3.522
D'Agostino & Pearson omnibus normality test				
K2	5.773	0.6561	4.466	10.35
P value	0.0558	0.7203	0.1072	0.0057
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	No
P value summary	ns	ns	ns	**
Skewness	1.613	-0.3164	1.348	1.903
Kurtosis	1.151	-0.9791	1.228	3.336
Sum	14.7	441.7	22.08	24.79

Table D.32 – One-way ANOVA of the percentage hemolysis caused by the different experimental groups after 12 hours, with Bonferroni post-test.

P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	4		
F	311.5		
R squared	0.9669		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)	41.73		
P value	P<0.0001		
P value summary	***		
Do the variances differ signif. (P < 0.05)	Yes		
ANOVA Table	**SS**	**df**	**MS**
Treatment (between columns)	14790	3	4930

Residual (within columns)	506.5	32	15.83		
Total	15300	35			
Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
TMC Nano S (20%) vs TMC Nano S (60%)	-47.45	25.3	Yes	***	-52.72 to -42.17
TMC Nano S (20%) vs TMC Nano L (20%)	-0.8209	0.4377	No	ns	-6.095 to 4.453
TMC Nano S (20%) vs PEG-TMC Nano	-1.121	0.5978	No	ns	-6.395 to 4.153
TMC Nano S (60%) vs TMC Nano L (20%)	46.63	24.86	Yes	***	41.35 to 51.90
TMC Nano S (60%) vs PEG-TMC Nano	46.33	24.7	Yes	***	41.05 to 51.60
TMC Nano L (20%) vs PEG-TMC Nano	-0.3002	0.1601	No	ns	-5.574 to 4.974

Table D.33 – Descriptive statistics of the percentage hemolysis caused by the 20% concentration small TMC nanoparticles over the 12-hour span of the experiment.

	1h	**6h**	**12h**
Number of values	9	9	9
Minimum	-0.578	-0.867	0.764
25% Percentile	0.072	0.1476	0.7935
Median	0.3423	0.2281	1.049
75% Percentile	0.4151	0.4063	2.462
Maximum	0.4912	0.434	4.284
Mean	0.211	0.1611	1.633
Std. Deviation	0.3475	0.4018	1.315
Std. Error	0.1158	0.1339	0.4385
Lower 95% CI of mean	-0.05607	-0.1477	0.6217
Upper 95% CI of mean	0.4782	0.4699	2.644
D'Agostino & Pearson omnibus normality test			
K2	9.766	20	5.773
P value	0.0076	P<0.0001	0.0558
Passed normality test (alpha=0.05)?	No	No	Yes
P value summary	**	***	ns
Skewness	-1.878	-2.552	1.613
Kurtosis	3.039	7.061	1.151
Sum	1.899	1.45	14.7

Table D.34 – One-way ANOVA of the percentage hemolysis caused by the 20% concentration small TMC nanoparticles.

P value	0.001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	9.368		
R squared	0.4384		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)	16.61		
P value	0.0002		
P value summary	***		
Do the variances differ signif. (P < 0.05)	Yes		
ANOVA Table	**SS**	**df**	**MS**

Treatment (between columns)	12.57	2	6.284		
Residual (within columns)	16.1	24	0.6708		
Total	28.67	26			
Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
1h vs 6h	0.04994	0.1293	No	ns	-0.9438 to 1.044
1h vs 12h	-1.422	3.682	Yes	**	-2.415 to -0.4280
6h vs 12h	-1.472	3.812	Yes	**	-2.465 to -0.4780

Table D.35 – Descriptive statistics of the percentage hemolysis caused by the 60% concentration small TMC nanoparticles over the 12-hour span of the experiment.

	1h	**6h**	**12h**
Number of values	9	9	9
Minimum	9.276	13.44	37.28
25% Percentile	9.521	21.46	42.14
Median	12.72	32.15	49.55
75% Percentile	24.35	40.41	56.77
Maximum	33.67	42.83	58.4
Mean	17.39	30.97	49.08
Std. Deviation	9.01	10.49	7.615
Std. Error	3.003	3.497	2.538
Lower 95% CI of mean	10.47	22.91	43.23
Upper 95% CI of mean	24.32	39.04	54.93
D'Agostino & Pearson omnibus normality test			
K2	1.352	1.071	0.6561
P value	0.5087	0.5855	0.7203
Passed normality test (alpha=0.05)?	Yes	Yes	Yes
P value summary	ns	ns	ns
Skewness	0.7235	-0.6537	-0.3164
Kurtosis	-0.8527	-0.7592	-0.9791
Sum	156.6	278.7	441.7

Table D.36 – Repeated measures ANOVA of the percentage hemolysis caused by the 60% concentration small TMC nanoparticles.

P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	20.91		
R squared	0.7233		
Was the pairing significantly effective?			
R squared	0.03879		
F	0.2917		
P value	0.9587		
P value summary	ns		
Is there significant matching? (P < 0.05)	No		
ANOVA Table	**SS**	**df**	**MS**
Treatment (between columns)	4549	2	2274

Individual (between rows)	253.8	8	31.72		
Residual (random)	1740	16	108.8		
Total	6543	26			
Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
1h vs 6h	-13.58	2.761	Yes	*	-26.72 to -0.4344
1h vs 12h	-31.69	6.445	Yes	***	-44.83 to -18.55
6h vs 12h	-18.11	3.684	Yes	**	-31.25 to -4.970

Table D.37 – Descriptive statistics of the percentage hemolysis caused by the 20% concentration large TMC nanoparticle over the span of the experiment.

	1h	**6h**	**12h**
Number of values	9	9	9
Minimum	0.1883	0	1.041
25% Percentile	0.2562	0.208	1.315
Median	0.4912	0.2525	1.515
75% Percentile	0.9395	0.8836	3.517
Maximum	2.023	3.035	5.818
Mean	0.6643	0.6814	2.454
Std. Deviation	0.59	0.9344	1.608
Std. Error	0.1967	0.3115	0.5361
Lower 95% CI of mean	0.2108	-0.03684	1.217
Upper 95% CI of mean	1.118	1.4	3.69
D'Agostino & Pearson omnibus normality test			
K2	9.981	18.16	4.466
P value	0.0068	0.0001	0.1072
Passed normality test (alpha=0.05)?	No	No	Yes
P value summary	**	***	ns
Skewness	1.841	2.43	1.348
Kurtosis	3.345	6.35	1.228
Sum	5.979	6.133	22.08

Table D.38 – One-way ANOVA of the percentage hemolysis caused by the 20% concentration of large TMC nanoparticles.

P value	0.003				
P value summary	**				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	7.496				
R squared	0.3845				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	7.246				
P value	0.0267				
P value summary	*				
Do the variances differ signif. (P < 0.05)	Yes				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	19.03	2	9.514		
Residual (within columns)	30.46	24	1.269		

Total	49.49	26			
Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
1h vs 6h	-0.01711	0.03222	No	ns	-1.384 to 1.350
1h vs 12h	-1.789	3.369	Yes	**	-3.156 to -0.4225
6h vs 12h	-1.772	3.337	Yes	**	-3.139 to -0.4054

Table D.39 – Descriptive statistics of the percentage hemolysis caused by the 20% concentration cross-linked PEG-TMC nanoparticles over the span of the experiment.

	1h	**6h**	**12h**
Number of values	9	9	9
Minimum	-0.145	-0.867	1.883
25% Percentile	0.229	0.2	2.207
Median	0.289	0.3489	2.376
75% Percentile	0.4889	0.686	3.133
Maximum	0.723	2.168	5.051
Mean	0.3185	0.4521	2.754
Std. Deviation	0.2521	0.7939	0.9998
Std. Error	0.08405	0.2646	0.3333
Lower 95% CI of mean	0.1247	-0.1581	1.985
Upper 95% CI of mean	0.5123	1.062	3.522
D'Agostino & Pearson omnibus normality test			
K2	0.801	5.354	10.35
P value	0.67	0.0688	0.0057
Passed normality test (alpha=0.05)?	Yes	Yes	No
P value summary	ns	ns	**
Skewness	0.01516	0.8945	1.903
Kurtosis	1.074	3.345	3.336
Sum	2.867	4.069	24.79

Table D.40 – One-way ANOVA of the percentage hemolysis caused by the 20% concentration cross-linked PEG-TMC nanoparticles.

P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	29.89				
R squared	0.7135				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	11.38				
P value	0.0034				
P value summary	**				
Do the variances differ signif. (P < 0.05)	Yes				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	33.74	2	16.87		
Residual (within columns)	13.55	24	0.5644		
Total	47.29	26			
Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**

1h vs 6h	-0.1336	0.3772	No	ns	-1.045 to 0.7779
1h vs 12h	-2.435	6.876	Yes	***	-3.347 to -1.524
6h vs 12h	-2.302	6.499	Yes	***	-3.213 to -1.390

D.4 Complement activation

Table D.41 – Absorption values of the C3 protein dilutions, measured with a BioTek microplate reader at 450 nm for drawing of a standard curve.

Concentration (µg/ml)	Absorbance			Average
30	0.144	0.133	0.142	0.1397
15	0.220	0.215	0.210	0.2150
7.5	0.340	0.342	0.317	0.3330
3.75	0.532	0.500	0.507	0.5130
1.875	0.730	0.633	0.661	0.6747
0.938	0.976	1.066	0.965	1.0023
0.469	1.281	1.230	1.298	1.2697
0	1.782	1.801	1.898	1.8270

Table D.42 – Log values of concentration and absorbance of the C3 protein dilutions for drawing of a standard curve.

Log-Concentration	Log-Absorbance			Average
1.4771	-0.8416	-0.8761	-0.8477	-0.8552
1.1761	-0.6576	-0.6676	-0.6778	-0.6676
0.8751	-0.4685	-0.4660	-0.4989	-0.4778
0.5740	-0.2741	-0.3010	-0.2950	-0.2900
0.2730	-0.1367	-0.1986	-0.1798	-0.1717
-0.0278	-0.0106	0.0278	-0.0155	0.0006
-0.3288	0.1075	0.0899	0.1133	0.1036

Table D.43 – Absorbance values of the different experimental groups measured with a BioTek microplate reader at 450 nm after completion of complement activation experiment.

	Absorbance									Average
TMC Nano S (20%)	0.219	0.21	0.231	0.133	0.147	0.135	0.139	0.139	0.141	0.1660
TMC Nano S (60%)	0.197	0.177	0.218	0.126	0.135	0.146	0.178	0.133	0.139	0.1610
TMC Nano L (20%)	0.209	0.209	0.193	0.126	0.139	0.128	0.135	0.127	0.134	0.1556
PEG-TMC Nano	0.194	0.24	0.212	0.128	0.121	0.144	0.085	0.127	0.138	0.1543
Control	0.137	0.142	0.132	0.09	0.092	0.09	0.095	0.092	0.091	0.1068

Table D.44 – Log values of measured absorbance values for interpolation from standard curve.

	Log - absorbance									Average
TMC Nano S (20%)	-0.6596	-0.6778	-0.6364	-0.8761	-0.8327	-0.8697	-0.8570	-0.8570	-0.8508	-0.7908
TMC Nano S (60%)	-0.7055	-0.7520	-0.6615	-0.8996	-0.8697	-0.8356	-0.7496	-0.8761	-0.8570	-0.8008
TMC Nano L (20%)	-0.6799	-0.6799	-0.7144	-0.8996	-0.8570	-0.8928	-0.8697	-0.8962	-0.8729	-0.8180
PEG-TMC Nano	-0.7122	-0.6198	-0.6737	-0.8928	-0.9172	-0.8416	-1.0706	-0.8962	-0.8601	-0.8316
Control	-0.8633	-0.8477	-0.8794	-1.0458	-1.0362	-1.0458	-1.0223	-1.0362	-1.0410	-0.9797

Table D.45 – Log values of C3 protein concentrations, as interpolated from standard curve.

	Interpolated values (Log-concentration)									Average
TMC Nano S (20%)	1.1701	1.1988	1.1334	1.5074	1.4401	1.4974	1.4777	1.4777	1.4681	1.3745
TMC Nano S (60%)	1.2424	1.3149	1.1732	1.5438	1.4974	1.4447	1.3111	1.5074	1.4777	1.3903
TMC Nano L (20%)	1.2020	1.2020	1.2563	1.5438	1.4777	1.5332	1.4974	1.5385	1.5024	1.4170
PEG-TMC Nano	1.2528	1.1069	1.1923	1.5332	1.5711	1.4539	1.8127	1.5385	1.4826	1.4382
Control	1.4875	1.4633	1.5125	1.7730	1.7578	1.7730	1.7357	1.7578	1.7653	1.6695

Table D.46 – C3 protein concentrations of the different experimental groups as determined with a Complement C3 Human ELISA Kit.

	C3 protein concentrations (µg/ml)									Average
TMC Nano S (20%)	14.7929	15.8047	13.5944	32.1661	27.5470	31.4307	30.0408	30.0408	29.3834	24.9779
TMC Nano S (60%)	17.4726	20.6505	14.9002	34.9791	31.4307	27.8397	20.4702	32.1661	30.0408	25.5500
TMC Nano L (20%)	15.9239	15.9239	18.0426	34.9791	30.0408	34.1349	31.4307	34.5527	31.7949	27.4248
PEG-TMC Nano	17.8974	12.7923	15.5706	34.1349	37.2492	28.4408	64.9678	34.5527	30.3786	30.6649
Control	30.7227	29.0636	32.5445	59.2895	57.2514	59.2895	54.4104	57.2514	58.2552	48.6754

Table D.47 – Descriptive statistics of absorbance of the experimental formulations and the control group.

	Negative Control	TMC Nano S (20%)	TMC Nano S (60%)	TMC Nano L (20%)	PEG-TMC Nano
Number of values	9	9	9	9	9
Minimum	0.09	0.133	0.126	0.126	0.085
25% Percentile	0.0905	0.137	0.134	0.1275	0.124
Median	0.092	0.141	0.146	0.135	0.138
75% Percentile	0.1345	0.2145	0.1875	0.201	0.203
Maximum	0.142	0.231	0.218	0.209	0.24
Mean	0.1068	0.166	0.161	0.1556	0.1543
Std. Deviation	0.02285	0.04102	0.03257	0.03661	0.04996
Std. Error	0.007617	0.01367	0.01086	0.0122	0.01665
Lower 95% CI of mean	0.08921	0.1345	0.136	0.1274	0.1159
Upper 95% CI of mean	0.1243	0.1975	0.186	0.1837	0.1927
D'Agostino & Pearson omnibus normality test					
K2	3.208	2.963	1.314	3.023	0.822
P value	0.2011	0.2273	0.5184	0.2206	0.663
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes
P value summary	ns	ns	ns	ns	ns
Skewness	0.8873	0.8871	0.6721	0.8662	0.6036
Kurtosis	-1.513	-1.433	-0.9413	-1.477	-0.5799
Sum	0.961	1.494	1.449	1.4	1.389

Table D.48 – Repeated measures ANOVA of the absorbance of the experimental formulations and the control group, with Bonferroni post-test.

P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	5				
F	18.28				
R squared	0.6956				
Was the pairing significantly effective?					
R squared	0.6177				
F	21.23				
P value	P<0.0001				
P value summary	***				
Is there significant matching? (P < 0.05)	Yes				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	0.02058	4	0.005145		
Individual (between rows)	0.04781	8	0.005977		
Residual (random)	0.009007	32	0.0002815		
Total	0.0774	44			
Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
Negative Control vs TMC Nano S (20%)	-0.05922	7.488	Yes	***	-0.08307 to -0.03538
Negative Control vs TMC Nano S (60%)	-0.05422	6.856	Yes	***	-0.07807 to -0.03038
Negative Control vs TMC Nano L (20%)	-0.04878	6.168	Yes	***	-0.07262 to -0.02493
Negative Control vs PEG-TMC Nano	-0.04756	6.013	Yes	***	-0.07140 to -0.02371
TMC Nano S (20%) vs TMC Nano S (60%)	0.005	0.6322	No	ns	-0.01884 to 0.02884
TMC Nano S (20%) vs TMC Nano L (20%)	0.01044	1.321	No	ns	-0.01340 to 0.03429
TMC Nano S (20%) vs PEG-TMC Nano	0.01167	1.475	No	ns	-0.01218 to 0.03551
TMC Nano S (60%) vs TMC Nano L (20%)	0.005444	0.6884	No	ns	-0.01840 to 0.02929
TMC Nano S (60%) vs PEG-TMC Nano	0.006667	0.843	No	ns	-0.01718 to 0.03051
TMC Nano L (20%) vs PEG-TMC Nano	0.001222	0.1545	No	ns	-0.02262 to 0.02507

Table D.49 – Descriptive statistics of the concentrations of the experimental formulations and the control group.

	Negative Control	TMC Nano S (20%)	TMC Nano S (60%)	TMC Nano L (20%)	PEG-TMC Nano
Number of values	9	9	9	9	9
Minimum	29.06	13.59	14.9	15.92	12.79
25% Percentile	31.63	15.3	18.97	16.98	16.73
Median	57.25	29.38	27.84	31.43	30.38
75% Percentile	58.77	30.74	31.8	34.34	35.9
Maximum	59.29	32.17	34.98	34.98	64.97
Mean	48.68	24.98	25.55	27.42	30.66
Std. Deviation	13.53	7.811	7.256	8.272	15.66
Std. Error	4.51	2.604	2.419	2.757	5.221
Lower 95% CI of mean	38.28	18.97	19.97	21.07	18.63
Upper 95% CI of mean	59.07	30.98	31.13	33.78	42.7
D'Agostino & Pearson omnibus normality test					
K2	3.508	3.281	2.388	3.179	5.402
P value	0.1731	0.1939	0.303	0.204	0.0671
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes
P value summary	ns	ns	ns	ns	ns
Skewness	-0.8312	-0.7762	-0.2313	-0.7402	1.234
Kurtosis	-1.651	-1.637	-1.69	-1.639	2.405
Sum	438.1	224.8	229.9	246.8	276

Table D.50 – Repeated measures ANOVA of the concentrations of the experimental formulations and the control group, with Bonferroni post-test.

P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	5				
F	20.93				
R squared	0.7234				
Was the pairing significantly effective?					
R squared	0.4217				
F	10.55				
P value	P<0.0001				
P value summary	***				
Is there significant matching? (P < 0.05)	Yes				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	3512	4	878		
Individual (between rows)	3541	8	442.6		
Residual (random)	1343	32	41.96		
Total	8395	44			
Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
Negative Control vs TMC Nano S (20%)	23.7	7.761	Yes	***	14.49 to 32.90
Negative Control vs TMC Nano S (60%)	23.13	7.573	Yes	***	13.92 to 32.33
Negative Control vs TMC Nano L (20%)	21.25	6.959	Yes	***	12.04 to 30.46
Negative Control vs PEG-TMC Nano	18.01	5.898	Yes	***	8.804 to 27.22
TMC Nano S (20%) vs TMC Nano S (60%)	-0.5721	0.1874	No	ns	-9.778 to 8.634
TMC Nano S (20%) vs TMC Nano L (20%)	-2.447	0.8014	No	ns	-11.65 to 6.759
TMC Nano S (20%) vs PEG-TMC Nano	-5.687	1.862	No	ns	-14.89 to 3.519
TMC Nano S (60%) vs TMC Nano L (20%)	-1.875	0.614	No	ns	-11.08 to 7.331
TMC Nano S (60%) vs PEG-TMC Nano	-5.115	1.675	No	ns	-14.32 to 4.091
TMC Nano L (20%) vs PEG-TMC Nano	-3.24	1.061	No	ns	-12.45 to 5.966

D.5 Plasma protein interaction

Table D.51 – 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.

% concentration	Absorbance									Average
30	0.1659	0.1689	0.1699	0.1589	0.1699	0.1679	0.1599	0.1679	0.1639	0.1659
15	0.0809	0.0759	0.0799	0.0839	0.0799	0.0879	0.0799	0.0829	0.0859	0.0819
7.5	0.0379	0.0389	0.0379	0.0409	0.0429	0.0299	0.0429	0.0449	0.0399	0.0396
3.75	0.0169	0.0179	0.0179	0.0199	0.0199	0.0249	0.0219	0.0199	0.0189	0.0198
1.875	0.0079	0.0089	0.0079	0.0089	0.0099	0.0109	0.0099	0.0099	0.0089	0.0093
0.9375	0.0039	0.0039	0.0039	0.0039	0.0039	0.0059	0.0059	0.0059	0.0049	0.0047
0.46875	0.0019	0.0029	0.0029	0.0009	0.0019	0.0029	0.0029	0.0039	0.0039	0.0027

Table D.52 – 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.

% concentration	Absorbance									Average
30	0.1629	0.1699	0.1639	0.1769	0.1649	0.1619	0.1599	0.1709	0.1569	0.1654
15	0.0789	0.0779	0.0809	0.0919	0.0849	0.0849	0.0839	0.0899	0.0879	0.0846
7.5	0.0379	0.0369	0.0379	0.0379	0.0389	0.0389	0.0419	0.0369	0.0409	0.0387
3.75	0.0179	0.0169	0.0169	0.0199	0.0189	0.0179	0.0189	0.0199	0.0189	0.0185
1.875	0.0089	0.0069	0.0109	0.0079	0.0089	0.0079	0.0089	0.0099	0.0089	0.0088
0.9375	0.0039	0.0039	0.0049	0.0039	0.0039	0.0039	0.0039	0.0039	0.0039	0.0040
0.46875	0.0029	0.0039	0.0039	0.0019	0.0029	0.0029	0.0019	0.0019	0.0019	0.0027

Table D.53 – 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.

% concentration	Absorbance									Average
30	0.1639	0.1609	0.1609	0.1649	0.1759	0.1759	0.1659	0.1759	0.1779	0.1691
15	0.0799	0.0889	0.0869	0.0849	0.0869	0.0749	0.0859	0.0849	0.0889	0.0847
7.5	0.0399	0.0389	0.0379	0.0409	0.0379	0.0419	0.0419	0.0429	0.0409	0.0404
3.75	0.0199	0.0189	0.0199	0.0189	0.0169	0.0199	0.0219	0.0199	0.0199	0.0196
1.875	0.0089	0.0089	0.0089	0.0089	0.0079	0.0099	0.0099	0.0099	0.0089	0.0091
0.9375	0.0049	0.0049	0.0049	0.0039	0.0049	0.0059	0.0049	0.0039	0.0039	0.0047
0.46875	0.0019	0.0039	0.0029	0.0019	0.0029	0.0029	0.0029	0.0049	0.0029	0.0030

Table D.54 – Absorbance of the different experimental groups, as measured with a BioTek microplate reader at 550 nm.

	Absorbance									Average
Negative Control	0.130	0.184	0.233	0.070	0.086	0.103	0.168	0.149	0.154	0.1419
TMC Nano S (20%)	0.119	0.115	0.101	0.075	0.092	0.074	0.136	0.140	0.155	0.1119
TMC Nano S (60%)	0.094	0.092	0.093	0.052	0.067	0.076	0.126	0.126	0.103	0.0921
TMC Nano L (20%)	0.157	0.153	0.171	0.070	0.087	0.090	0.150	0.130	0.123	0.1257
PEG-TMC Nano	0.207	0.206	0.202	0.068	0.082	0.100	0.119	0.130	0.130	0.1382

Table D.55 – Percentage of experimental particles unbound in plasma after incubation for four hours.

	Percentage Unbound									Average
TMC Nano S (20%)	36.0338	34.8315	30.6234	22.8083	27.9181	22.5077	41.1437	42.3460	46.8547	33.8963
TMC Nano S (60%)	9.5064	9.3060	9.4062	5.2983	6.8012	7.7029	12.7126	12.7126	10.4082	9.3172
TMC Nano L (20%)	47.3308	46.1348	51.5165	21.3196	26.4022	27.2991	45.2379	39.2583	37.1655	37.9627
PEG-TMC Nano	61.1480	60.8539	59.6778	20.2773	24.3937	29.6863	35.2730	38.5074	38.5074	40.9250

Table D.56 – Percentage of experimental particles bound to plasma proteins after incubation for four hours.

	Percentage Bound									Average
TMC Nano S (20%)	63.9662	65.1685	69.3766	77.1917	72.0819	77.4923	58.8563	57.6540	53.1453	66.1037
TMC Nano S (60%)	90.4936	90.6940	90.5938	94.7017	93.1988	92.2971	87.2874	87.2874	89.5918	90.6828
TMC Nano L (20%)	52.6692	53.8652	48.4835	78.6804	73.5978	72.7009	54.7621	60.7417	62.8345	62.0373
PEG-TMC Nano	38.8521	39.1461	40.3222	79.7228	75.6063	70.3137	64.7270	61.4927	61.4927	59.0750

Table D.57 – Descriptive statistics of absorbance measured in plasma protein interaction experiment.

	Control	TMC Nano S (20%)	TMC Nano S (60%)	TMC Nano L (20%)	PEG-TMC Nano
Number of values	9	9	9	9	9
Minimum	0.07	0.074	0.052	0.07	0.068
25% Percentile	0.0945	0.0835	0.0715	0.0885	0.091
Median	0.149	0.115	0.093	0.13	0.13
75% Percentile	0.176	0.138	0.1145	0.155	0.204
Maximum	0.233	0.155	0.126	0.171	0.207
Mean	0.1419	0.1119	0.09211	0.1257	0.1382
Std. Deviation	0.05111	0.02872	0.0248	0.03583	0.05414
Std. Error	0.01704	0.009575	0.008265	0.01194	0.01805
Lower 95% CI of mean	0.1026	0.08981	0.07305	0.09813	0.09661
Upper 95% CI of mean	0.1812	0.134	0.1112	0.1532	0.1798
D'Agostino & Pearson omnibus normality test					
K2	0.1782	0.8789	0.06577	1.617	1.988
P value	0.9147	0.6444	0.9676	0.4455	0.3702
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes
P value summary	ns	ns	ns	ns	ns
Skewness	0.2941	0.02032	-0.05706	-0.4084	0.312
Kurtosis	-0.2023	-1.226	-0.529	-1.392	-1.559
Sum	1.277	1.007	0.829	1.131	1.244

Table D.58 – 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.

P value	0.0017				
P value summary	**				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	5				
F	5.535				
R squared	0.4089				
Was the pairing significantly effective?					
R squared	0.5477				
F	8.194				
P value	P<0.0001				
P value summary	***				
Is there significant matching? (P < 0.05)	Yes				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	0.01501	4	0.003752		
Individual (between rows)	0.04444	8	0.005555		
Residual (random)	0.02169	32	0.0006779		
Total	0.08115	44			
Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
Control vs TMC Nano S (20%)	0.03	2.444	No	ns	-0.007006 to 0.06701
Control vs TMC Nano S (60%)	0.04978	4.055	Yes	**	0.01277 to 0.08678
Control vs TMC Nano L (20%)	0.01622	1.322	No	ns	-0.02078 to 0.05323
Control vs PEG-TMC Nano	0.003667	0.2987	No	ns	-0.03334 to 0.04067
TMC Nano S (20%) vs TMC Nano S (60%)	0.01978	1.611	No	ns	-0.01723 to 0.05678
TMC Nano S (20%) vs TMC Nano L (20%)	-0.01378	1.123	No	ns	-0.05078 to 0.02323
TMC Nano S (20%) vs PEG-TMC Nano	-0.02633	2.145	No	ns	-0.06334 to 0.01067
TMC Nano S (60%) vs TMC Nano L (20%)	-0.03356	2.734	No	ns	-0.07056 to 0.003450
TMC Nano S (60%) vs PEG-TMC Nano	-0.04611	3.757	Yes	**	-0.08312 to -0.009105
TMC Nano L (20%) vs PEG-TMC Nano	-0.01256	1.023	No	ns	-0.04956 to 0.02445

Table D.59 – Descriptive statistics of the percentage experimental particles bound to plasma proteins.

	TMC Nano S (20%)	TMC Nano S (60%)	TMC Nano L (20%)	PEG-TMC Nano
Number of values	9	9	9	9
Minimum	53.15	87.29	48.48	38.85
25% Percentile	58.26	88.44	53.27	39.73
Median	65.17	90.59	60.74	61.49
75% Percentile	74.64	92.75	73.15	72.96
Maximum	77.49	94.7	78.68	79.72
Mean	66.1	90.68	62.04	59.08
Std. Deviation	8.634	2.484	10.71	15.92
Std. Error	2.878	0.8281	3.57	5.306
Lower 95% CI of mean	59.47	88.77	53.8	46.84
Upper 95% CI of mean	72.74	92.59	70.27	71.31
D'Agostino & Pearson omnibus normality test				
K2	0.8789	0.06577	1.617	1.988
P value	0.6444	0.9676	0.4455	0.3702
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes
P value summary	ns	ns	ns	ns
Skewness	-0.02033	0.05705	0.4084	-0.312
Kurtosis	-1.226	-0.529	-1.392	-1.559
Sum	594.9	816.1	558.3	531.7

Table D.60 – Repeated measures ANOVA of the percentage experimental particles bound to plasma proteins, with Bonferroni post-test.

P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	4				
F	30.14				
R squared	0.7902				
Was the pairing significantly effective?					
R squared	0.2278				
F	4.219				
P value	0.0028				
P value summary	**				
Is there significant matching? (P < 0.05)	Yes				
ANOVA Table	**SS**	**df**	**MS**		
Treatment (between columns)	5622	3	1874		
Individual (between rows)	2099	8	262.3		
Residual (random)	1492	24	62.18		
Total	9213	35			
Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
TMC Nano S (20%) vs TMC Nano S (60%)	-24.58	6.612	Yes	***	-35.27 to -13.89
TMC Nano S (20%) vs TMC Nano L (20%)	4.066	1.094	No	ns	-6.621 to 14.75
TMC Nano S (20%) vs PEG-TMC Nano	7.029	1.891	No	ns	-3.659 to 17.72
TMC Nano S (60%) vs TMC Nano L (20%)	28.65	7.706	Yes	***	17.96 to 39.33
TMC Nano S (60%) vs PEG-TMC Nano	31.61	8.503	Yes	***	20.92 to 42.30
TMC Nano L (20%) vs PEG-TMC Nano	2.962	0.7969	No	ns	-7.725 to 13.65