Aus dem Institut für Transfusionsmedizin
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

One-pot Formulation of Protein Submicron Particles and Their Haemocompatibility

zur Erlangung des akademischen Grades
Doctor rerum medicinalium (Dr. rer. medic.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

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Datum der Promotion: 13.12.2019

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Abstrakt (Deutsch)

Das Verfahren zur Herstellung von Proteinsubmikropartikel mittels der Coprecipitation-Crosslinking-Dissolution-Technik (CCD-Technik) besteht im wesentlichen aus drei voneinander unabhängigen Herstellungsschritten. Mit der Einführung eines makromolekularen Vernetzers, periodatoxidiertem Dextran (Odex, MG von 40 und 70 kDa), konnte die Copräzipitation und Vernetzung des Proteins in einem einzigen Schritt erfolgen. Für den Proof of Principle wurden Humanserumalbumin (HSA) und Rinderhämoglobin (Hb) verwendet. Die resultierenden Albumin-Partikel (Odex-APs) und Hämoglobin-Partikel (Odex-HbMPs) sind verformbar und weisen eine enge Größenverteilung zwischen 800-1000 nm mit einer erdnussähnlichen Form und einer negativen Oberflächenladung. Interessanterweise konnten mehr als 40% des Hb in den Odex-HbMPs über eine Lagerzeit von 90 Tagen Sauerstoff transportieren. Außerdem wurden *In-vitro*-Hämokompatibilitätsassays mittels Hämolysetest, indirekten Phagozytosetest und Blutplättchenaktivierungstests in menschlichem Blut, durchgeführt. Odex-APs und Odex-HbMPs verursachten keine unerwünschten Wirkungen auf die Blutzellen. Die Hämolyserate der Erythrozyten nach Kontakt mit Odex-vernetzten Proteinpartikeln war Kleiner als 5%. Deshalb können die Partikel als nicht hämolytisch betrachtet werden. Die Inkubation von Leukozyten mit Odex-APs/HbMPs beeinflusst die Phagozytose der Leukozyten nicht. Die Ergebnisse deuteten darauf hin, dass unsere Partikel von Monozyten oder Granulozyten nicht erkannt werden. Die Exposition von Odex-APs/HbMPs verursacht bei Blutplättchen keine Aktivierung. Die Odex-APs/HbMPs verminderten weder die durch Agonisten-induzierte Blutplättchenaktivierung noch verstärkten sie dioese. Zusammenfassend zeigten Odex-vernetzte Proteinpartikel, die durch Eintopfformulierung hergestellt wurden, eine sehr gute Hämokompatibilität und stellten vielversprechende Träger für Medikamente oder Sauerstoff dar.

Abstract (English)

The coprecipitation-crosslinking-dissolution (CCD) technique for protein submicron particle fabrication consists of three independent fabrication steps. Introducing a macromolecular crosslinker, periodate-oxidised-dextran (Odex, M.W. of 40 and 70 kDa), coprecipitation and crosslinking could be performed within one step. For the proof of principle, human serum albumin (HSA) and bovine haemoglobin (Hb) were used. The resulting albumin particles (Odex-APs) and haemoglobin particles (Odex-HbMPs) are deformable and have a peanut-like shape, a narrow size distribution ranging between 800-1000 nm and a negative surface charge. Interestingly, more than 40% of Hb in the Odex-HbMPs was able to carry oxygen over a storage period of 90 days. The *in vitro* haemocompatibility assays included haemolysis test, indirect phagocytosis test and platelet activation tests in human blood. Odex-APs and Odex-HbMPs did not provoke any undesirable effects on the blood cells. After incubation of erythrocytes with Odex-crosslinked protein particles, the ratio of haemolysis was less than 5%. Therefore, the particles may be considered as non-haemolytic. The incubation of leukocyte with Odex-APs/HbMPs did not influence the phagocytosis of leukocyte. The results suggested that our particles were not recognized by monocytes or granulocytes. Finally, exposure of Odex-APs/HbMPs to platelets did not cause the activation of platelets. Additionally, Odex-APs/HbMPs did neither enhance nor attenuate agonist-induced platelet activation. In conclusion, Odex-crosslinked protein particles fabricated by One-pot formulation exhibited a very good haemocompatibility and represented highly promising carriers for drugs or oxygen.

1. Introduction

In the last decades, protein particles have been proposed for a number of biomedical applications such as drug delivery systems and haemoglobin-based oxygen carriers [1]. Many techniques are available for the fabrication of protein nano- and micro-particles including spray drying, emulsification, layer-by-layer assembly, solvent displacement and polymersome formation [2]. However, each of these techniques has serious drawbacks which impair its function and/or biocompatibility. For example, spray drying or lyophilisation techniques deliver protein particles in dry powder formulation but their polydispersity is usually high [3,4] and entrapment efficiency is typically poor in protein particles prepared by solvent displacement [5]. Apart from that, tertiary and quaternary structures of proteins need to be preserved to maintain protein function that is hard to fulfil by many of these conventional methods due to exposure to high temperature, high ionic strength, non-physiological pH, organic solvents or hydrophobic interfaces.

Previously, fabrication method for a micro- and submicro- particle, which consists of three main steps called co-precipitation-crosslinking-dissolution (CCD) were described. Firstly, protein and inorganic template are precipitated together followed by a crosslinking step where glutaraldehyde (GA) was used to crosslink the proteins. Finally, EDTA was used to remove the inorganic template to obtain protein micro-particles [6–8]. This technique is favourable to fabricate in mild condition and provides pure protein particles with uniform morphology and narrow size distribution. However, the cross-linking of protein with glutaraldehyde needs to be taken into consideration.

GA can react with several functional groups of proteins, such as amine, thiol, phenol, and imidazole and is very effective as a proteins cross-linker because the most reactive amino acid side-chains are nucleophiles [9]. It, therefore, has been widely used to cross-link proteins and other biopolymers for medical applications. However, there are many reports concerning the undesired properties of glutaraldehyde cross-linked biomaterials such as auto-fluorescence as well as its toxicity [10,11]. For that reason, “biocompatible” cross-linkers are now being investigated as alternatives for glutaraldehyde to reduce the risks of side effects.

Polysaccharides are natural biopolymers which have a great number of reactive

groups on their molecular chains, such as hydroxyl, carboxyl, and amino groups which can make them be easily modified chemically and biochemically. They are highly safe, non-toxic, stable, biodegradable, and biocompatible. Besides, polysaccharides have abundant resources in nature and low cost in their processing. There are various sources from animal (chitosan, chondroitin), plant (e.g. pectin, cellulose), alga (e.g. alginate), and microorganisms (e.g. dextran). The polysaccharide is desirably oxidised to extent sufficient to provide the aldehyde groups capable of promoting rapid cross-linking of the polymers. For that reason, there are several usages of polysaccharide in the biomedical application as a biopolymer cross-linker, for instance, oxidised-hydroxyethylstrach [12], oxidised-raffinose (*O*-raffinose) [13–15] and oxidised-dextran (Odex) [16,17] were used as a cross-linker for haemoglobin conjugation.

Dextran is a glucose homopolymer, produced by the bacteria called *Leuconostoc mesenteroides*. It is consisting essentially of α-1,6 linked D-glucopyranose residues with a few per cent of α-1,2, α-1,3, or α-1,4-linked side chains. Dextran is selected in many biomedical applications because it is slowly degraded by human enzymes compared to other polysaccharides (e.g. glycogen with α-1,4 linkages) and cleaved by microbial dextranases in the gastrointestinal tract. It has been primarily used as a plasma expander, a macromolecular carrier for the delivery of drugs and proteins, to increase the durability of therapeutic agents in the blood circulation and to decrease the *in vivo* immunogenicity of proteins and enzymes [18,19]. It is also an excellent candidate as a bio-cross-linker because of its chemical properties such as high water solubility, high stability, high content of hydroxyl groups, which are suitable for derivatization and can be subsequently used for chemical cross-linking of proteins [20].

Oxidation of dextran by periodate ions is a common dextran functionalizing modification reaction which yields a purified product with simple dialysis method. Glucose residue of dextran has vicinal diol which houses two oxidisable bonds. The oxidation of those bonds forms an aldehyde in C_3, which is susceptible to periodate oxidation on account of the presence of the hydroxyl groups in C_2 and C_4 and subsequently leads to a double oxidation of the same residue. This leads to the opening of the glucose ring and formation of double aldehyde groups, which may react with amino groups creating Schiff's base (Maillard reaction). The dextran oxidation reaction may perform under mild conditions with high efficiency but no catalyst is needed [21]. For this reason, oxidised-dextran (Odex) has recently been investigated

as an “alternative biocompatible hydrophilic cross-linker” for biopolymers i.e. hydrogel formation, tissue engineering and drug delivering particles [22–24]. Nevertheless, to the best of our knowledge, Odex was not used for the fabrication of micro- or nano-protein particles.

However, questions concern the safety of prolonged use of nano- and micro-particles have been raised since most biomedical nanoparticles, for therapeutic and/or diagnostic purposes, are typically intravenously administrated and directly interact with the blood. Hence, the haemocompatibility of the nano- and micro- particles becomes important and critical. Haemocompatible are materials, which are able to remain effectual after being exposed to blood but do not bring about any form of toxicity to the blood cells and do not cause any changes in composition and viscosity of the blood plasma [25]. For instance, the rupture of red blood cells (RBCs) and subsequent haemoglobin releasing can cause the symptoms haemoglobinuria or anuria followed by renal failure [26]. Besides, the phagocytic cells (e.g. granulocytes and monocytes) can proficiently engulf particles in the blood which results in serious limitation of their blood circulation time and extravasation into target tissues [27]. Additionally, platelets are critical to haemostasis by virtue of their ability to adhere, aggregate and release the contents of their granules as well as their capacity to alter their surface characteristics to support blood coagulation. Therefore, thrombotic and thromboembolic complications, as well as bleeding risks associated with the disseminated intravascular coagulopathy (DIC) remain of serious concern. Therefore, to avoid these events, the use of biocompatible and biodegradable materials are crucial factors for the fabrication of particles suitable for clinical applications.

The hypothesis is that dextran may able to improve not only the protein function and stability but also the haemocompatibility of the particles because of its protein-rejecting and cell repelling abilities. In addition, the multivalent nature of dextran is advantageous for surface immobilization of biologically active molecules. Therefore, the aim of this study was to develop a novel biopolymer micro-particle fabrication method based on the CCD technique by applying Odex as a cross-linker for proteins including human serum albumin (HSA) or bovine haemoglobin (Hb). Here, the co-precipitation and the crosslinking steps are combined in one single step since the cross-linker, Odex, is co-precipitated together with the protein. The physiochemical properties of the resulting Odex-crosslinked albumin particles (Odex-APs) and

haemoglobin particles (Odex-HbMPs) were investigated by several techniques including scanning electron microscopy (SEM), atomic force microscopy (AFM) and dynamic light scattering (DLS), zeta-potential measurements, spectrophotometry. The functionality of the haemoglobin entrapped in the Odex-HbMPs, as well as the stability of Odex-HbMPs in a salt solution and their deformability were determined. Additionally, *in vitro* assays with human erythrocyte, leucocytes and thrombocyte were performed to test the haemocompatibility of Odex-APs and Odex-HbMPs using several methods including RBCs haemolysis, phagocytic activity of leukocytes and activation/aggregation of thrombocytes, *in vitro*.

2. Methods

2.1 Synthesis of Odex-crosslinked Protein Particles

50 mL of 10% dextran solutions (30.9 mmoL; MW 40 and 70 kDa named as 40T and 70T, respectively) were oxidised by sodium periodate (6.6 g, 30.9 mmoL) for 1 hour at room temperature [28]. Then, the resulting oxidised dextran (Odex) was transferred to a cellulose dialysis tube (MWCO of 12,000: Carl Roth GmbH, Germany) and dialysed against water. The amount of aldehyde in Odex was quantified by hydroxylamine hydrochloride titration method with unmodified dextran as a reference [23].

Albumin particles (APs) and haemoglobin (HbMPs) were prepared by the one-pot procedure using 40T- and 70T-Odex as crosslinking [29]. Briefly, equal volumes of solution 1 consisting of 0.25 M of $MnCl_2$ and 50 mg/mL of human serum albumin (HSA, Baxalta Deutschland GmbH, Germany) or bovine haemoglobin (Hb, Biophyll GmbH, Germany) were rapidly mixed with solution 2 containing 0.25 M of Na_2CO_3 and 40 mg/mL of Odex (40T or 70T, individually) in the beaker under vigorous stirring at room temperature. After 30 seconds, 5 mg/mL of HSA was added to the suspension and incubated for 5 min under stirring to allow the HSA to absorb into particles surface. The resulting particle suspensions were then proceeded to the dissolution of the $MnCO_3$ template by 0.25 M EDTA/0.05 M Glycine for 30 min and the reduction by 0.4 mg/mL of $NaBH_4$. Finally, the obtained Odex cross-linked albumin particles (Odex-APs) or haemoglobin particles (Odex-HbMPs) were washed three times with phosphate buffered saline (PBS, 6000g for 5 minutes) and finally suspended in sterile PBS. APs and HbMPs crosslinked with 40T-Odex and 70T-Odex are named 40T-APs, 70T-APs, 40T-HbMPs and 70T-HbMPs, correspondingly.

2.2 Particles Characterization

2.2.1 Scanning Electron Microscopy

For SEM imaging, samples were prepared by applying a drop of particles suspension onto glass slide followed by drying overnight. After sputtering with gold, measurements were conducted at an operation voltage of 3 keV using Gemini Leo 1550 instrument (Oberkochen, Germany) and ImageJ 1.44p software (Wayne Rasband, National Institute of Mental Health, Bethesda, MD, USA).

2.2.2 Particle Size and Zeta-potential

The hydrodynamic diameter and the zeta-potential of the Odex-APs and Odex-HbMPs were measured by dynamic light scattering using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Malvern, UK) in PBS solution. Additionally, Odex-crosslinked particles were dispersed in PBS and analysed for autofluorescence using flow cytometry (BD FACSCanto II), FITC and PE channel were gated. BD FACSDIVA software (BD Biosciences, USA) was employed for data analysis.

2.2.3 Haemoglobin Content in Odex-HbMPs

For Hb determination, modified Alkaline Hemin Detergent-575 (AHD-575) method was employed. 2% (v/v) Odex-HbMPs suspension was mixed with pronase to achieve a final concentration of and 0.5 mg/ml pronase. The particles were digested by pronase with the purpose to terminate the binding of Odex and haemoglobin in order to reduce the noise scattering from of particles. The pronase-HbMPs suspension was then incubated for 30 min at 45ºC. Afterwards, AHD reagent was added at the volume ratio of 1:1 to the pronase-HbMPs mixture for hemin conversion. Absorption spectra of the particles were recorded using a UV−VIS spectrophotometer at 575 nm. (Hitachi U2800, Hitachi High-Technologies Corporation).

2.2.4 Determination of Oxygen Carrying Capacity of Odex-HbMPs

The haemoglobin solution was diluted into different concentrations (0.75-6 mg/mL). 1 mL of each concentration was filled into a 2-mL glass vial containing a magnetic stirrer. After an equilibration time of 5 min, a miniaturized optical needle type oxygen sensor (oxygen microsensor NTH-PSt7, PreSens–Precision Sensing GmbH, Germany), which was connected to a portable oxygen meter with data logging (Microx 4, PreSens–Precision Sensing GmbH, Germany), was inserted into the stirred sample. The concentration of dissolved oxygen was recorded at the starting point. Then, 50 µL

of 10% potassium ferricyanide ($K_3[Fe(CN)_6$) dissolved in water were added, and the change in concentration of dissolved oxygen was measured and recorded every second. After reaching a stable value, the measurement was stopped. An increase of dissolved oxygen concentration after adding ferricyanide is dependent on the haemoglobin concentration-manner. The change in pO_2 is determined by subtracting the pO_2 from the beginning and the end of the measurement. The released oxygen was previously bound to haemoglobin and defined as a functional haemoglobin. A calibration curve demonstrated the ratio of haemoglobin concentration vs pO_2. For Odex-HbMPs, the particle volume concentration was measured by the haematocrit **(**Hettich Mikro 22R; at 20,000g for 10 min) and then adjusted to 2%. The measurement of 2% of Odex-HbMP was performed by using the same procedure as described for standard haemoglobin. The functional Hb in the particles compared with a determined calibration curve [30,31]. Finally, the percentage of functional haemoglobin to total haemoglobin was calculated using the identical sample.

2.2.5 Particles Deformability

Particle compression and dilation behaviour were measured by using an automatic measuring system based on an analytic centrifuge with an integrated optoelectronic sensor (LUMiFuge, LUM GmbH, Berlin, Germany). The integrated transmitted light intensity during the centrifugation at different accelerations allows quantification of the suspension stability by means of an instability index:

$$\text{Instability index (\%)} = \frac{\text{clarification as a function of time}}{\text{maximal possible clarification}} \quad (1)$$

where clarification is the integral of transmission along the cuvette during the centrifugation. The maximal possible clarification is obtained at the highest possible centrifugation force until no changes in the profile will be detected.

In total, 10% haematocrit of particles was placed into the cuvette. The centrifugal force was applied to particles as a cycle to observe the compression and relaxation of particles [32]. Particles were first equilibrated at the beginning with a centrifugal force of 5g for 10 min. Then, the centrifugal force up to 2300g was applied to compress particles for 4 hours. Finally, the centrifugal force was reduced to 5g again for 4 hours to observe the relaxation of particles. The profiles are simultaneously registered along the cuvette by the STEP-Technology as a function of time and of the applied centrifugal force and analysed using the software SEPVIEW 6.

2.2.6 Atomic Force Microscopy (AFM)

Atomic force microscopy (AFM) images were obtained using a Nanoscope III Multimode AFM (Digital Instrument Inc., Santa Barbara, CA, USA) in taping mode as described earlier [33]. Briefly, Odex-HbMP were applied into polyethylene imine (25 kDa, 1mM) pre-treated mica substrate. After allowing the particles to attach, the substrate was rinsed with deionized water and dried under a gentle stream of nitrogen. The particles were first scanned in the dry state, then a drop of deionized water was added, and the particles were scanned again in the wet state. The height of each particle in the dry and wet state was measured applying the Nanoscope software as the height difference between the support and the highest point of the particle surface profile.

2.2.7 Storage Stability of Odex-HbMPs

The storage stability of Odex-HbMPs in 0.9% NaCl was determined at day 0, 30 and 90 after fabrication. The individual aliquots were kept at 4°C. Then, the size distribution, the Hb content and the amount of functional Hb of the stored Odex-HbMPs were evaluated.

2.3 Haemocompatibility Tests

2.3.1 Haemolysis Test

The haemolytic test is based on the release of haemoglobin from damaged erythrocytes *in vitro*. Human heparinized erythrocytes were washed (3000g, 5 min) in PBS until the supernatant was clear and colourless. RBCs were then to obtain a cell suspension with a volume concentration of 2% in PBS. 0.5 mL of 2% Odex-crosslinked particles suspension was mixed with 0.5 mL of the 2% washed human erythrocyte suspension. 0.5 mL of double distilled water and PBS were employed as the positive (PC) and negative (NC) control, respectively. After incubation at 37°C for 3 h, the erythrocyte suspensions were centrifuged at 3000*g* for 5 min, the supernatants were collected and pipetted into a 96-well plate. The haemolytic ratio was determined by measuring the absorbance of the supernatants at 545 nm using a microplate reader (Cytation™ 3 Cell Imaging Multi-Mode Reader, BioTek) and calculated according to the following equation:

$$\%\text{Haemolytic ratio} = \frac{(OD_{test}-OD_{NC})}{(OD_{PC}-OD_{NC})} \times 100 \quad (2)$$

All results were estimated from the data of three individual experiments, and all data were expressed as the mean±SD [34]

2.3.2 Phagocytosis Test

The activation of phagocytosis performance of granulocytes and monocytes in whole blood was investigated using an indirect method based on a modified PHAGOTEST (Glycotope Biotechnology GmbH, Germany). The method is suitable for non-fluorescing particles. The different particles (10 µL of 2 x 10^{11} per mL) were added to 50 µL of heparinized-whole blood and incubated at 37°C for 0, 10, 30, 60, 120 min to allow uptake by the leucocytes. Samples with non-fluorescent *Escherichia coli* (2 x 10^9 bacteria per ml from PHAGOBURST kit, Glycotope Biotechnology GmbH, Germany). and PBS were used as a positive and negative control, respectively. After reaching the corresponding incubation time, the standard test for phagocytosis activity (PHAGOTEST) was performed with all samples. Briefly, 10 µL of fluorescein isothiocyanate labelled *E. coli* (FITC-*E. coli*, 2 x 10^9 bacteria per ml from Phagotest kit) were added to each sample and incubated at 37°C for further 10 min. Afterwards, the fluorescence of non-phagocytosed *E. coli* was quenched, the RBCs were lysed, and the leucocytes were fixed and DNA-stained by propidium iodide. Washing steps with PBS were performed between each preparation step. Finally, the leukocyte populations were analysed by flow cytometry (BD FACSCanto II) to obtain the percentage of granulocytes and monocytes which have phagocytosed FITC-*E. coli*. Leukocytes previously saturated with non-fluorescent bacteria or particles are not able to uptake the FITC-*E. coli*, and therefore decreased phagocytic activity to FITC-*E. coli* indicates phagocytic activity to the non-fluorescent bacteria or particles.

2.3.3 Platelet Activation Test

The influence of Odex-APs and Odex-HbMPs on human platelets was investigated in platelet-rich plasma (PRP) samples. Citrated human whole blood was centrifuged at 150g for 15 minutes at 20-25 ºC, and the PRP fraction was collected. The platelet amount was detected before the test using a haematology analyser ABX Micros 60 (HORIBA Europe GmbH, Germany). 45 µL of PRP was gently mixed with 5 µL of particle suspensions (to a ratio of 10 = particles per 1 platelet). Platelets incubated with PBS were used as a control. The samples were incubated at 37°C for 30 min with gently shaking. Then, the pre-incubated human platelets were activated with platelet

agonists (0.5 mg/mL arachidonic acid (AA), 0.2 mg/mL collagen and 0.01 mM epinephrine (Epi): möLab GmbH, Germany) to induce platelet activation and aggregation. The mixtures were incubated at 37°C for further 30 min with shaking and the samples were then fixed by 0.5% formaldehyde in PBS. Finally, the platelets were stained by APC anti-human CD42b (GPIbα) antibody and Alexa Fluor® 488 anti-human CD62P (P-Selectin) antibody (BioLegend, San Diego, USA) and analysed by flow cytometry (BD FACSCanto II). Double-stained events were counted as activated platelets [35,36].

2.4 Statistical Analysis

Data were presented as means ± standard deviation (SD), and statistical differences between groups were compared using ANOVA-like test. GraphPad Prism 6 software (GraphPad, La Jolla, CA, USA) was employed for graphs and statistical analyses. *P-value <0.05* was considered statistically significant.

3. Results

3.1 Fabrication and Characterisation of Odex-crosslinked Protein Particles

Odex-APs and Odex-HbMPs were successfully fabricated by the One-pot procedure (Figure 1). The morphology and the size of the obtained albumin and haemoglobin particles by the One-pot procedure were not significantly different as observed by SEM. Figure 2 shows clearly a peanut-like shape with a shortest and longest diameter about of 700 and 1000 nm. The dynamic light scattering analysis of the particles is represented in Figure 3. The results showed the size of Odex-APs and Odex-HbMPs was about 800-1000 nm and the zeta-potential was approximately -13 mV and -9 mV as measured in PBS (conductivity 18-20 mS/cm) for Odex-APs and Odex-HbMPs, respectively. No significant differences in size and zeta-potential could be detected for the particles prepared with 40T- and 70T-Odex.

The flow cytometry analysis in Figure 4 also yet demonstrated that Odex-particles had no auto-fluorescence in contrast to the protein particles prepared using the CCD technique and crosslinked by glutaraldehyde (GA) [6–8,37,38].

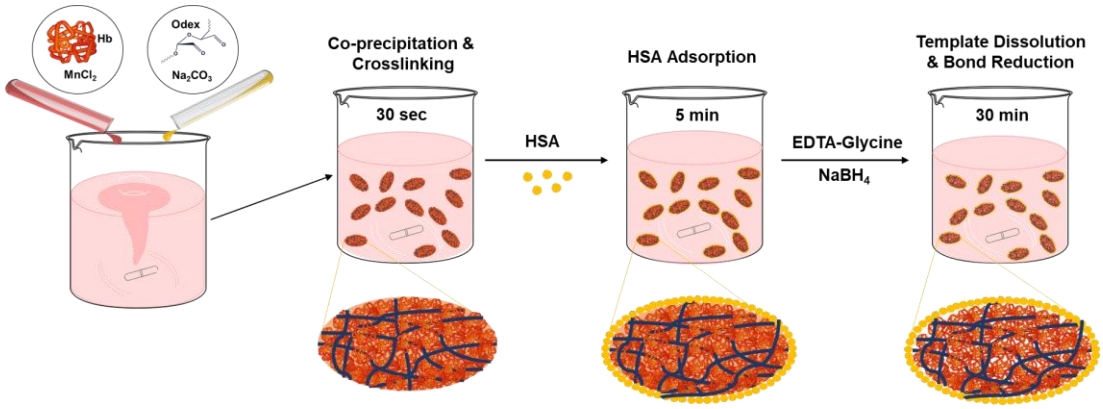

Figure 1. Scheme of particle preparation through the one-pot fabrication technique. Two mixtures of inorganic solutions, $MnCl_2$ containing haemoglobin (or albumin) and Na_2CO_3 with Odex, are mixed together. Here, $MnCO_3$ particles are formed with entrapped biopolymers, meanwhile, the Hb are crosslinked by Odex in the particle. HSA was absorbed to into particle surface. The $MnCO_3$ template is then dissolved and the pure biopolymer particles remain in the solution.

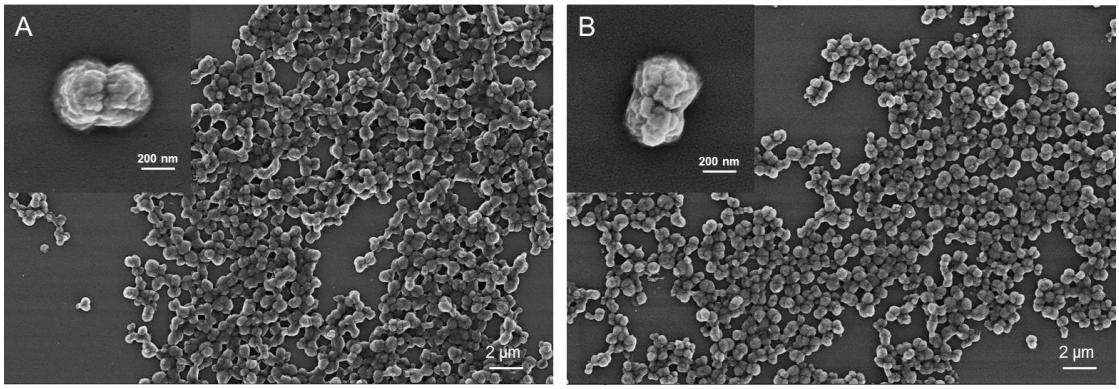

Figure 2. SEM images of Odex-APs (A) and Odex-HbMPs (B) fabricated by One-pot formulation. The inlets show peanut-like single particles. There is no significant difference in morphology compared with Odex M.W. of 40T and 70T.

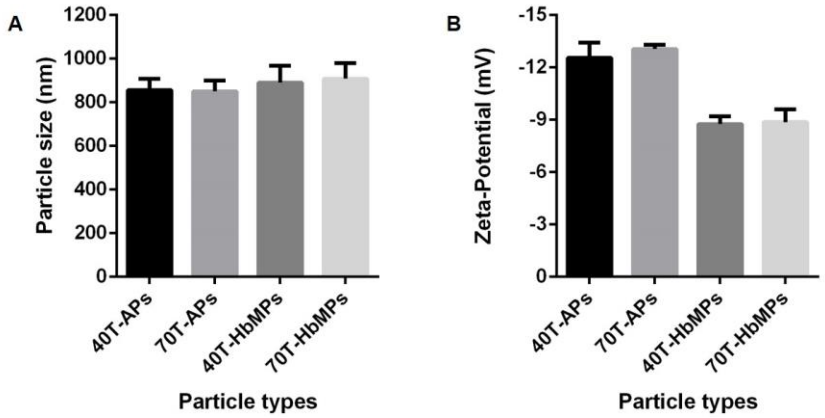

Figure 3. Characteristics of Odex-crosslinked protein particles. (A) Size and (B) Zeta-potential measured in 10 mM PBS (conductivity 18-20 mS/cm) at room temperature by Dynamic Light Scattering analysis. Data are presented as mean ± SD (n = 5)

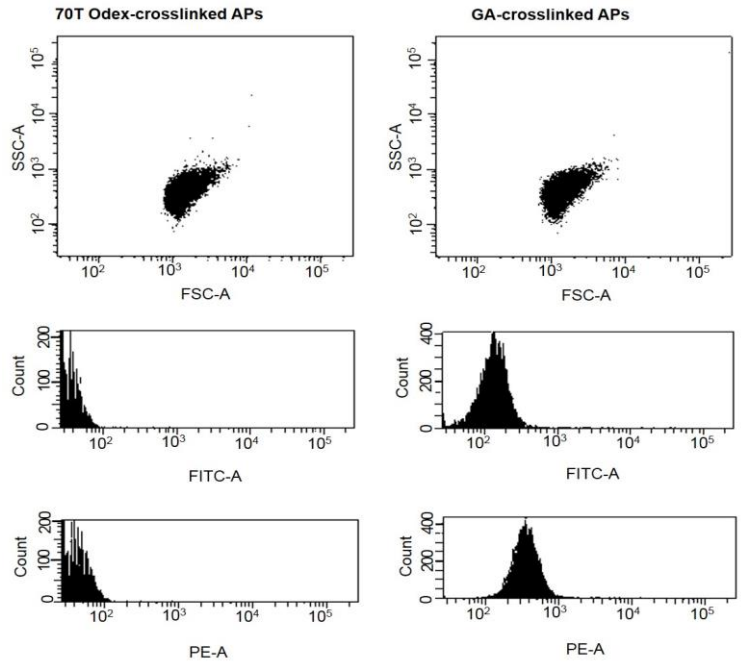

Figure 4. The side vs forward scatter dot plot of 70T-APs and GA-crosslinked APs (GA-APs). The stronger fluorescence intensity of GA-APs was observed compared to Odex-crosslinked particles. (APs and HbMPs crosslinked with both 40T and 70T Odex have the same phenomenon, figures were not shown)

The deformability of the Odex-HbMPs cross-linked with both 40T- and 70T-Odex was studied using an analytical centrifuge with the integrated optoelectric sensor system. If the particles are exposed to the high centrifugal force at 2300 g, rapidly packing is obtained which results in a constant thickness of the sediment layer. This becomes visible in the high instability index (Figure 5(A)). After exposure to a low centrifugal force at 5 g, the particles are decompressed, which increases the thickness of the sediment layer and results in a decreased instability index. This phenomenon demonstrates the deformability of these particles.

To confirm the deformability of the Odex-HbMPs, AFM scans in the dry and wet state were performed. Example of these scans is shown in Figure 5(B). Figure 5(C) represents the measured size of the particles at the long axis and Figure 5(D) present their thickness measured as the vertical distance between the mica support and the highest point of the height profiles. The mean thickness determined from scans of particles in the dry state of 40T- and 70T-HbMPs was 214 ± 40 and 213 ± 12 nm and in the wet state 308 ± 15 and 321 ± 18 nm, respectively. It can be clearly seen that the particles are swollen in the wet state.

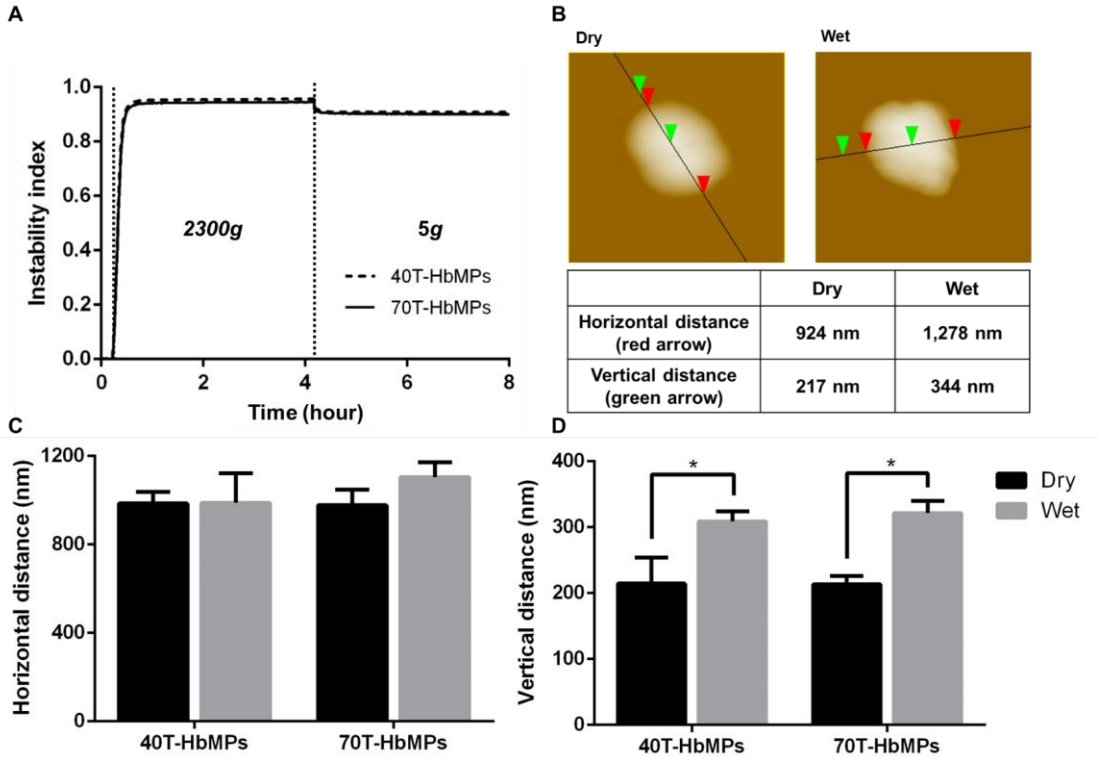

Figure 5. Deformability of Odex-crosslinked particles. (A) Compression and dilation behaviour of particles, the profile from the analytical centrifuge. (B) AFM images of 40T-HbMPs in dry as well as wet state. (C) The particles width is defined as the horizontal distance and (D) the thickness of the particles is given as the vertical distance between the mica support and the top of the particle. Dried HbMPs swelled after water was added. Data are presented as mean ± SD (n = 5), *p<0.05.*

The concentration of total Hb entrapped in Odex-HbMPs was 28–30 mg/mL as calculated for a suspension with a Hct of 20%. The values for 40T-HbMPs and 70T-HbMPs did not significantly differ from each other (28.8 ± 2.2 and 30.4 ± 2.1, respectively). The amount of functional Hb was 11.59 ± 0.6 and 12.53 ± 0.9 mg/mL, respectively. The ability of oxygenation and deoxygenation of the Odex-HbMPs was confirmed using UV-VIS spectrophotometric analysis as shown in Figure 6. Odex-HbMPs have similar spectra and spectral changes compared to free Hb in the oxygenated and deoxygenated state.

The stability of Odex-HbMPs was characterized by their size, the Hb content and the fraction of functional Hb as shown in Figure 7. The results demonstrated that there was no obvious change in size and in the amount of functional Hb in saline solution at 4ºC during the period of 90 days.

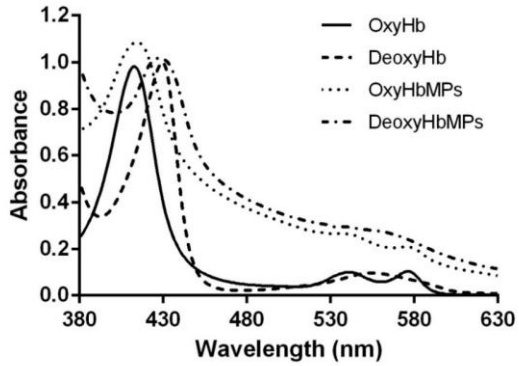

Figure 6. Absorption spectra of oxygenated Hb (OxyHb), deoxygenated Hb (DeoxyHb), oxygenated HbMPs (OxyHbMPs) and deoxygenated Hb. (DeoxyHbMPs). A shift toward higher absorption of Odex-HbMPs is a result of particles' light scattering.

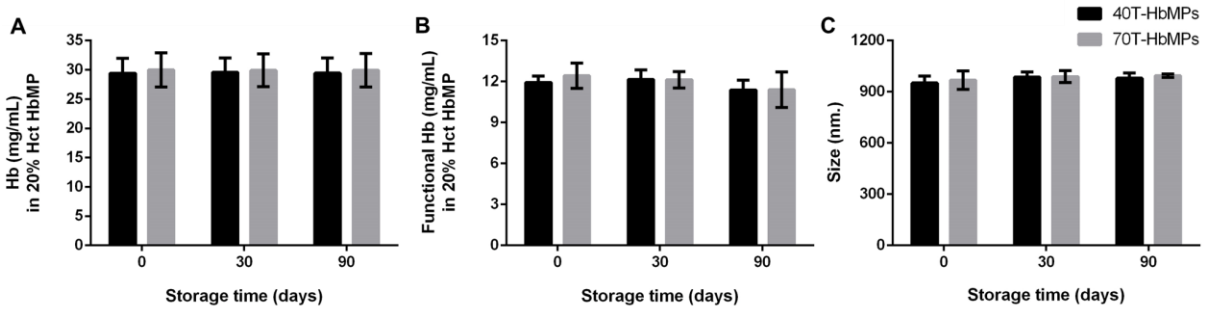

Figure 7. Stability of HbMPs over 90 days. (A) Hb content of Odex-HbMPs (B) Concentration of functional Hb and (C) Size stability by DLS analysis. Data are presented as mean±SD (n = 3)

3.2 Haemocompatibility Tests

3.2.1 Haemolysis Test

Haemolysis refers to the release of haemoglobin from RBCs due to damage of RBCs membrane, which is extensively applied to evaluate the biosafety of particles. A haemolytic ratio higher than 5% is considered as a significant damage of RBCs. The haemolytic ratio of the RBCs incubated with different Odex-crosslinked particles for 3 hours at 37°C is demonstrated in Table 1. It can be seen clearly that the haemolysis ratio for the samples with Odex-APs and Odex-HbMPs was between 1% and 2% and so far, they obviously do not damage significantly the RBCs.

Table 1. The haemolytic ratio of the Odex-crosslinked particles

Test sample	Haemolysis ratio (%)
40T-APs	1.14±0.36
70T-APs	1.03±0.16
40T-HbMPs	1.67±0.58
70T-HbMPs	1.77±0.52
PBS	0±0.00
Water	100±0.00

Data are presented as mean ± SD (n = 3).

3.2.2 Phagocytosis Test

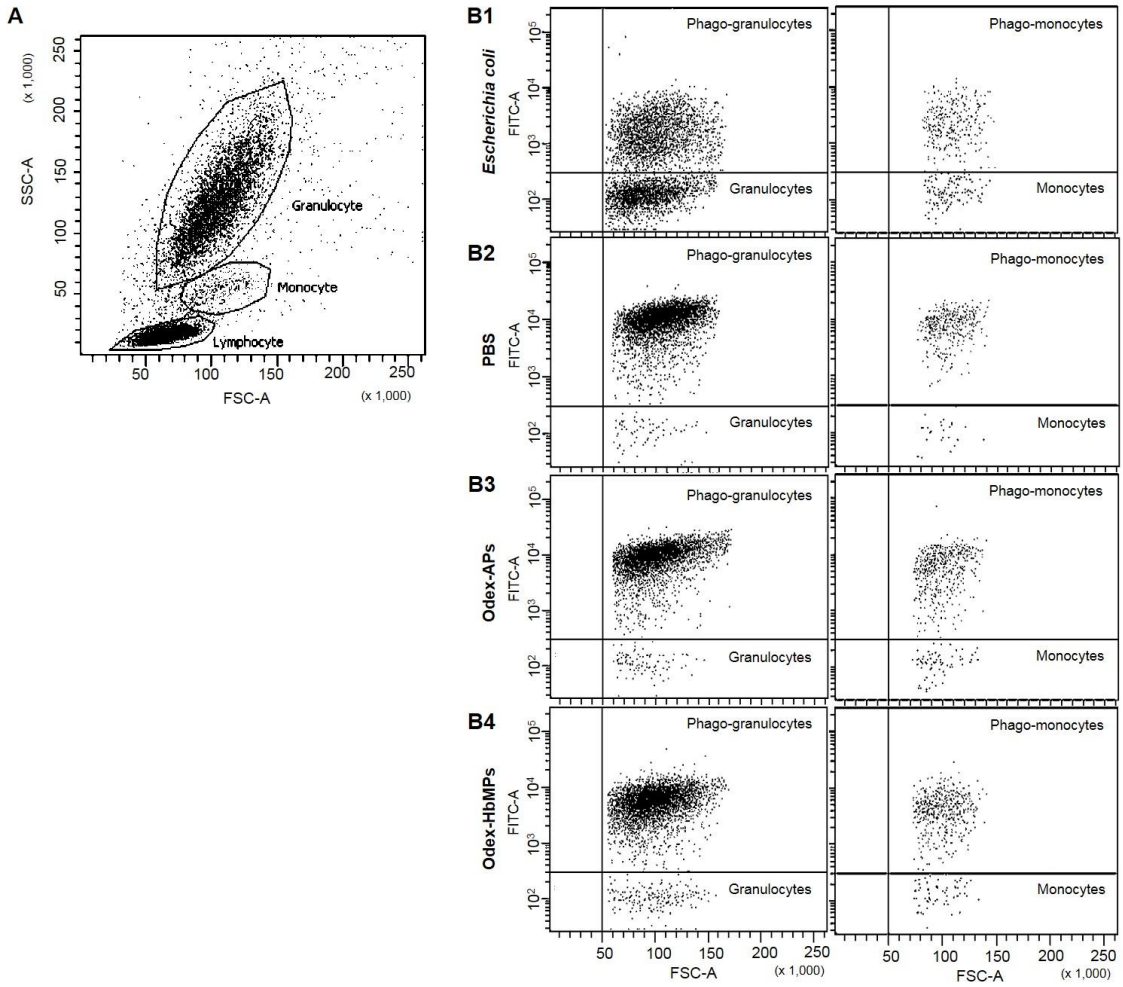

Figure 8. Flow cytometric dot plot of human peripheral blood leukocytes. (A) Sub-populations of leukocytes can be distinguished based on forward scatter (relative size) and side scatter (relative complexity). Granulocyte are large and complex; monocytes are slightly larger than lymphocytes and less complex than granulocyte, while lymphocytes are smaller and less complex. (B) Dot plots FITC/FSC demonstrate the phagocytic activity of granulocytes (left panel) and monocytes (right panel) detected with FITC-labelled *E. coli*. Preincubation with different particles was performed for 120 min. (B1) Preincubation of leukocytes with non-stained *E. coli* (positive control), (B2) Preincubation of leukocytes with PBS (negative control), (B3) Preincubation of leukocytes with Odex-APs, (B4) Preincubation of leukocytes with Odex-HbMPs.

The indirect phagocytic assay was carried out in vitro. The commercial phagotest kit allows determination of the percentage of phagocytes (in whole blood samples) which ingest FITC-labelled opsonized *E. coli* bacteria and is used as a diagnostic tool for functional testing of leukocytes. The test can be directly used to determine the phagocytosis of fluorescent particles by the phagocytes in the blood (mainly granulocytes and monocytes). However, in the case of the non-fluorescent particles, an additional fluorescent staining is needed which may alter their surface properties

and influence the phagocytosis. Therefore, we employed an indirect method as described in the Materials and Methods. Non-fluorescent *E. coli*, Odex-APs and Odex-HbMPs were added to the whole blood and incubated at 37°C for 10 to 120 min in order to reach a saturation of the granulocytes and monocytes. Then, the standard test with FITC– labelled *E. coli* for 10 mins at 37°C was performed with all samples. The flow cytometry analysis of samples pre-incubated with non-stained *E. coli*, PBS, Odex-APs and Odex-HbMPs for 120 min is presented in Figure 8 as dot plots of granulocyte and monocyte populations detectable in the FITC channel. It can be seen that the distribution of the FITC-labelled cells is similar for the samples pre-incubated with PBS, 40T-APs and 40T-HbMPs. Only the positive control (pre-incubated with non-fluorescent *E. coli*) shows different behaviour with an increased number of non-fluorescent cells.

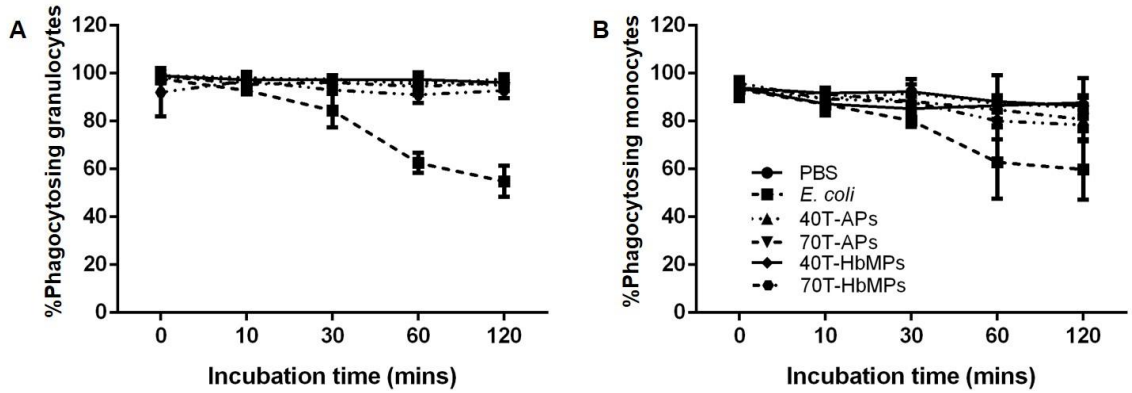

Figure 9. Percentage of phagocytic activity of (A) granulocyte and (B) monocyte on Odex-APs and Odex-HbMPs compared with PBS (negative control) and *E. coli* (positive control). On one hand, the fluorescence signal of detective FITC-*E. coli* decreased grammatically across incubation periods in positive control. On the other hand, leukocyte preincubated with PBS as well as Odex-APs and Odex-HbMPs showed a strong detective FITC-*E. coli* and there was no change over the incubation times. Data are presented as mean ± SD (n = 3)

The summarized results in dependency on the pre-incubation time are shown in Figure 9. The number of granulocytes and monocytes phagocytosing FITC-*E. coli* was not significantly different for Odex-crosslinked particles and PBS (negative control) over all pre-incubation times. In contrast, the positive control, *E. coli*, demonstrated a gradually decrease in the percentage of the cells engulfing FITC-*E. coli* in a time-dependent manner, due to the previously performed phagocytosis of non-fluorescing *E. coli.*

3.2.3 Platelet activation Test

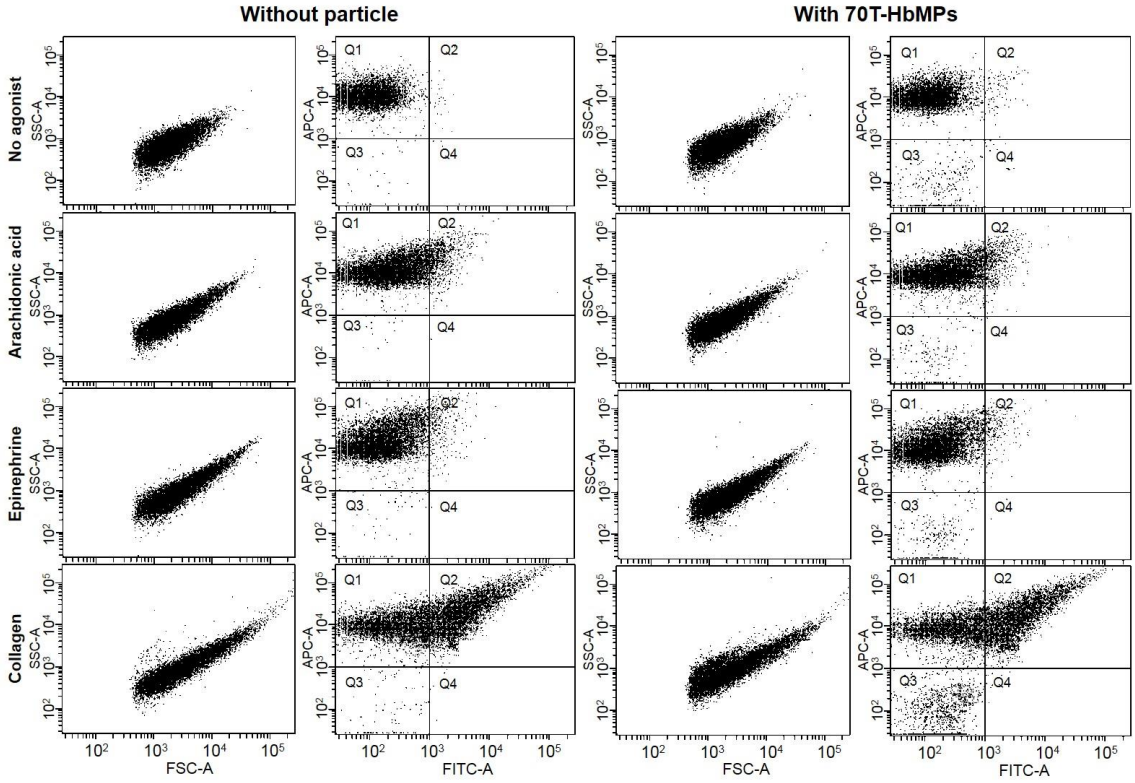

Figure 10. Flow cytometric analysis of the activation of platelets. Example of 70T-HbMPs compared to negative control. The events of the forward and side scatter as well as APC and FITC fluorescence channel are shown as dot plot. Platelets stained with APC-Antihuman CD42b (GPIbα) are enclosed in quadrant Q1. Double-stained with APC Anti-CD42ba and AlexaFlour 488 Antihuman-CD62P (P-selectin) events in quadrant Q2 represent activated-platelets. Non-stained particles were presented in Q3.

To evaluate whether Odex-APs and Odex-HbMPs alter the haemostasis system, their influence on the activation of platelets was investigated. Figure 10 shows flow cytometry analysis of platelets using the platelet-specific membrane receptor CD42b (GPIbα), and then distinguished those platelets that were activated using the activation marker CD62P. The results showed that the platelet activation assay was able to distinguish between platelets that are resting with high fluorescence level for CD42b and lower fluorescence levels for activation markers, CD62P, and those that have been activated by agonists with higher fluorescence levels for the two activation markers, CD42b and CD62P. Simultaneously, upon the stimulation of platelets by agonists, the aggregation of platelets was also observed as the shift of forward scatter/side scatter positioning compared with the control.

The Odex-APs or Odex-HbMPs did not cause activation of the platelets as compared to the control sample which was incubated with PBS. Additionally, platelet activation induced by agonists including arachidonic acid, collagen and epinephrine of pre-incubated PRP with particles was comparable to the control samples. It can be concluded that Odex-APs or Odex-HbMPs do not influence the function of platelets and therefore no negative side effects on the haemostasis are expected.

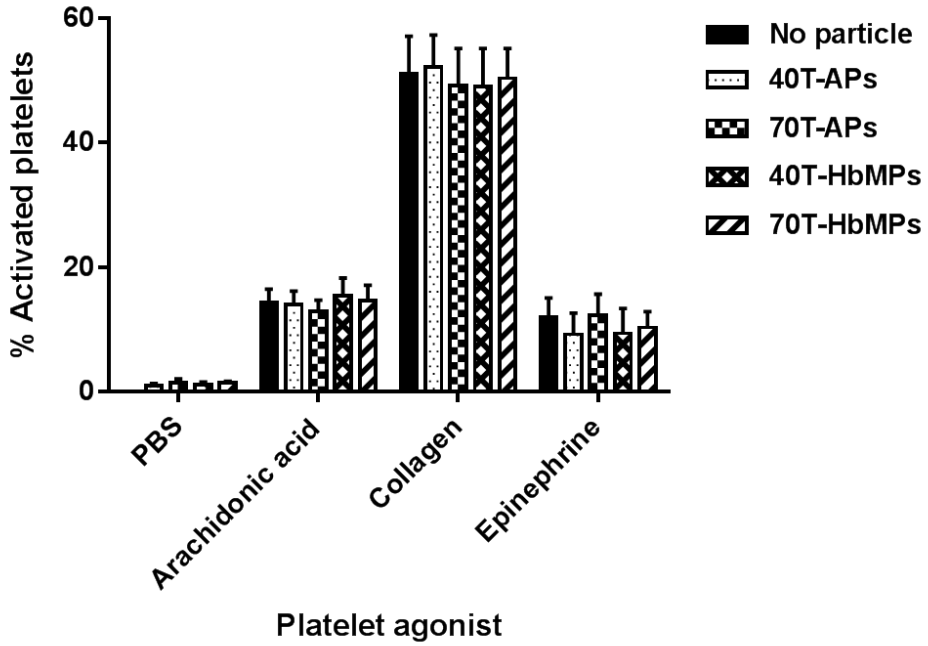

Figure 11. Platelet activation (as determined by %CD62P expression) is not influenced by Odex-APs and Odex-HbMPs. Fresh citrated PRP was treated with the different particles or PBS as a control. The presence of particles did not influence on arachidonic acid-, collagen- and epinephrine-induced platelet activation. Non-stimulated labelled platelets are shown as a baseline for spontaneous activation. Data are presented as mean ± SD (n = 3)

4. Discussion

The new one-pot fabrication technique for protein microparticles is based on the coprecipitation-crosslinking-dissolution (CCD) procedure, which exploits the ability of insoluble carbonates template to capture proteins and other macromolecules that are present in the aqueous solution during precipitation [38].

The proteins are precipitated and crosslinked in “one step” because the macromolecular crosslinker (Odex) precipitates and is entrapped in the particles together with the proteins (Figure 1). Odex is a promising as a highly effective macromolecular crosslinker because the carbonyl groups of the sugar open chains are capable of reacting with the free –NH2 groups on the protein; the relatively fast formation of Schiff base results in creating a crosslinked network. [24,28,39,40].

In such a way, one preparation step and several respective washing steps are omitted, which significantly reduces the preparation time. $MnCO_3$ and bovine haemoglobin were selected as a model to demonstrate this novel fabrication procedure. Haemoglobin content in Odex-HbMPs was estimated through photometric and quantitative measurement by a photometric method using the modified pronase-AHD method. The method is the combination of pronase enzyme and alkaline haematin D-575 (AHD) together. In comparison with HbMP-700 [6,37] prepared by CCD technique using GA as a crosslinker, Odex-HbMPs contain a comparable amount of Hb. The entrapped haemoglobin amount could not be enhanced by increasing the initial concentration of the protein. Additionally, the entrapped protein by $MnCO_3$ using a one-pot procedure had no significant difference in the entrapment efficiency of the CCD procedure as earlier reported by Xiong et al [7,37]. In comparison with other fabrication methods, the new one-pot procedure provides easily prepared particles with high protein content under ambient condition and no organic solvents or catalysts are needed. The reagents are neither toxic and nor expensive. Henceforward, this new technique is fast, simple, efficient and cost-effective.

The Odex-protein crosslinking process occurs simultaneously during the precipitation. This is achieved by the large molecular size of Odex and its substantial excess in the reaction. Odex can rapidly cover the protein surface avoiding protein-protein interaction (Figure 12) [41]. The Odex concentration during coprecipitation influences the stability of haemoglobin. At the Odex concentration of 2%, Odex-HbMPs exhibit a

higher amount of functional Hb. The reason could be that the dextran moiety hinders the steric expansion of Hb that would inhibit the denaturation rate of Hb. Additionally, Zhang et al. reported earlier that dextran was able to decrease the autooxidation rate of Hb and could structurally stabilize Hb to avoid the oxidation process of the heme-containing protein and its denaturation [42,43]. Cross-linking of Hb by GA has many drawbacks such as a reduction of redox potential as well as an increase of its autoxidation rate [44]. This could be the reason for the different amount of functional Hb in GA-HbMPs and Odex-HbMPs (40% in Odex-HbMPs and 30% in GA-HbMPs). This value was stable during storage for Odex-HbMPs but not for GA-HbMPs where the functional Hb decreased rapidly with the storage time.

The results of analytical centrifugation clearly showed the deformability of Odex-crosslinked particles, they were compressed when applied high centrifugal force. However, they were dilated when the force was reduced. The deformability of particles was also confirmed by scanning several Odex-HbMPs by AFM in dry and wet states. An increase in thickness gives some evidence for a sponge-like structure of the particles with a high water content. Upon drying, the particles shrink but recover their thickness quickly when water is added. The deformability of particles is very important for the intravenous administration because they need to circulate through the bloodstream and pass through microvessels.

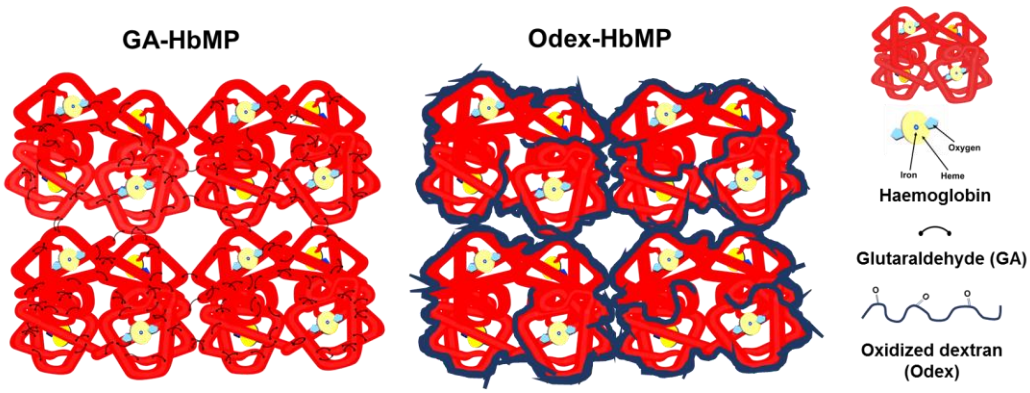

Figure 12. Schematic presentation of Hb crosslinking by Odex vs. crosslinking by GA.

In terms of protein functionality, the UV-VIS spectrophotometric analysis in Figure 6 exhibited the ability of Odex-HbMPs to bind and release oxygen in comparison to stroma-free haemoglobin. Oxygenated Odex-HbMPs demonstrated three absorption peaks at 414, 542, and 576 nm, which correspond to the oxygenated Hb (OxyHb). After deoxygenation of the Odex-HbMPs using 1 mg/mL sodium dithionite (SDT), a red

shift of the 414 nm peak (the soret peak) to 432 nm was observed. A peak at 556 nm was detected instead while the peaks at 542 and 576 nm disappeared. The absorption spectra of Odex-HbMPs showed the characteristic shape of oxygenated/deoxygenated Hb and confirmed the functionality of Odex-HbMPs. The shift to higher absorption values of Odex-HbMPs spectra compared to the Hb solution is due to the scattering of Odex-HbMPs.

The stability of particles is one of the most important factors influencing their medical and pharmaceutical applications. We could show that there was no significant change of the particle size, size distribution as well as of the total haemoglobin content and the fraction of functional haemoglobin of the Odex-HbMPs during at least 3 months storage. This information indicated the long shelf-life of new particles which is a huge benefit.

Prior to all intravenous administration applications, the influence of particles on the blood cells needs to be evaluated. The haemocompatibility of particles is affected by their physical attributes, including size, shape, and flexibility, as well as their chemical composition, for instance, the incorporation of toxic compounds or active ligands for recognition and triggering of biological receptors. It has been well described that small particle size and positive charge caused thrombocyte and granulocyte activation, and haemolysis. Negatively charged particles larger than 60 nm hydrodynamic diameter appear to be considerably less haemotoxic than smaller ones [45].

Particles fabricated by the new “one-pot” procedure fit to the criteria for the biomedical usages [45]. This procedure provided the uniform morphology, monodisperse, have a submicron size range between 800-1000 nm. The Odex-particles have negative zeta-potential, suggestive that the particles have a tendency to repel each other, rendering the colloid stable. Additionally, a successful perfusion of isolated mouse glomeruli with concentrated HbMPs suspensions of the same size *in vitro* without vasoconstriction of the afferent arterioles could be shown [37]. As the size of a particle decreases, its surface area per unit volume (or mass) increases and also allows a greater proportion of its atoms or molecules to be displayed on the surface rather than the interior of the material [46,47]. Therefore, it is very important to utilize procedures that allow the preparation of particles with high degrees of uniformity, and with control over their physical and chemical characterisations.

Additionally, the coating of the particles with human serum albumin improves significantly their blood compatibility by reducing the adsorption of other proteins as well as the interaction with platelets and leukocytes [48]. Due to the covalent binding of albumin to haemoglobin, the stability of the coating is sufficient to protect the particles during their circulation in the bloodstream against non-specific adsorption of other plasma proteins.

Currently described *in vitro* assays for haemocompatibility testing of particulate materials are not standardized. We evaluated the haemocompatibility of the Odex-crosslinked particles by testing haemolysis (destruction of RBCs), phagocytosis by leucocytes and platelet activation in order to explore possible adverse effects of Odex-crosslinked protein particles to blood cells. [49]

RBCs are the most abundant compartment in the bloodstream and are an important component determining the haemocompatibility. Once they are lysed, they release not only haemoglobin but also procoagulant factors which can cause serious adverse effects [50]. According to the ISO 10993-4:2017, the haemolysis assays of Odex-APs and Odex-HbMPs were considered to be non-haemolytic because these particles induced less than 5% haemolysis. Particle-induced haemolysis can be caused by the release of toxic substances from a biomaterial surface or from the interaction between particles and RBCs which result in the disruption and integrity of the RBC membrane and release of haemoglobin into the plasma. Additionally, it is generally agreed that surface properties (especially surface charge) are important, and there are several studies which have demonstrated this. For example, among a set of similar-sized fullerenes (C60-derivatives) bearing different numbers of anionic and cationic surface moieties, those with negative surface charge were not haemolytic, and haemolytic tendency increased in proportion to the number of attached cationic surface groups (positive surface charge) [26].

The charge of particles stemming from distinct surface chemistries influences opsonisation, circulation times and interaction with resident macrophages of organs comprising the phagocytic system. On one hand, positively charged particles more prone to sequestration by macrophages in the lungs, liver and spleen. On the other hand, neutral and slightly negatively charged nanoparticles have longer circulation lifetimes and less accumulation in the aforementioned organs [51]. The indirect

phagotest assay showed that Odex-APs and Odex-HbMPs particles were not recognized by phagocytic cells including granulocytes and monocytes. As the adsorption of plasma proteins on the nanoparticle surface can have an important influence on the interactions between cells and the particles. Therefore, diminishing the susceptibility of particles to recognition by the phagocytes through coverage of their surface with hydrophilic polymers such as polyethylene glycol, dextrans or mimic the surface using human serum albumin is another strategy to prolong the residence time of particles in the circular system. [52].

Activation of platelet plays a crucial role in the coagulation cascade. They are very sensitive to the presence of foreign materials that can enhance or attenuate the activity of platelets and further affect the blood coagulation.

It should also be considered that, on the one hand, dextran used as plasma expander influences the function of platelets due to its adsorption on platelets surface [53]. On the other hand, dextran forms not only a depletion layer around the blood cells and reduces the adsorption of proteins but can also be adsorbed to the cell surface. The dextran in the Odex-HbMPs is covalently bound to haemoglobin and not freely available for adsorption. But the moieties of dextran partially presented on the particle surface contribute to a repulsive force against other macromolecules [54]. Therefore, the interaction between platelets and particles is an important evaluation of biomaterials for blood compatibility. In this study, the presence of Odex-APs and Odex-HbMPs influenced neither the platelet activation/aggregation nor the agonist induced-platelet activation. The mechanisms through which particles induce platelet aggregation are largely unknown. Nevertheless, trends observed in studies of polymer-based nanoparticles are similar in their charge-dependence to those described above for haemolysis [18]. The negative surface charge, as well as the albumin coating of Odex-APs and Odex-HbMPs, might be able to prevent interaction between the particles and platelets and therefore prevent the negative effects on haemostasis.

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Affidavit

I, **CHIRAPHAT KLOYPAN**, certify under penalty of perjury by my own signature that I am writing the proposed dissertation on the topic: **One-pot Formulation of Protein Submicron Particles and Their Haemocompatibility.** I wrote this thesis independently and without undisclosed assistance from third parties, and no sources other than listed sources and resources.

All passages that are literally or literally based on publications or presentations by other authors are identified as such in the proper citation (see "Uniform Requirements for Manuscripts (URM)" of ICMJE -www.icmje.org). The sections on methodology (in particular practical work, laboratory conditions, statistical work-up) and results (in particular figures, graphics and tables) correspond to the URM (s.o) and are the responsibility of me.

My interest in the selected publications is the same as those stated in the joint statement with the supervisor below. All publications that have emerged from this dissertation and in which I am an author comply with the URM (s.o) and are responsible for me.

The importance of this affidavit and the criminal consequences of a false affidavit (§156.161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

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Date Signature

Detailed declaration of contribution

Chiraphat Kloypan hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: Kloypan C, Prapan A, Suwannasom N, Kaewprayoon W, Steffen A, Xiong Y, Baisaeng N, Georgieva R, Bäumler H. Improved Oxygen Storage Capacity of Haemoglobin Submicron Particles by One-Pot Formulation. Artif Cells, Blood Substitutes, Biotechnol. 2018; doi:10.1080/21691401.2018.1521819. **IF: 3.026**

Beitrag im Einzelnen:

Das Studiendesign wurde mit dem Supervisor nach einer ausführlichen Literaturrecherche diskutiert und festgelegt. Er stellte Hämoglobin-Submikropartikel nach einem in der AG konzipierten neuartigen Verfahren her, welches erlaubt, die Partikel im Gegensatz zum bisher genutzten 3 Schritt CCD-Verfahren in einem sogenannten Eintopf-Verfahren herzustellen (Fig 2, Fig 3). Der Schwerpunkt seiner Arbeiten war zunächst auf die Herstellung und Charakterisierung des makromolekularen Crosslinkers gerichtet, wobei insbesondere die je Makromolekül vorhandenen für die Quervernetzung der Proteine erforderlichen aktiven Gruppen bestimmt werden mussten. Das im nächsten Schritt zu lösende Problem war die Optimierung der bei der Herstellung der Mikropartikel zu verwendenden Protein und makromolekularen Crosslinker zu verwendenden Konzentrationen, um Partikel mit einer engen Größenverteilung zu erhalten. Bei der Herstellung musste eine Aggregation der Partikel verhindert werden (Fig. 5). Außerdem wurde der Nachweis erbracht, dass die Partikel ein Schwammartiges Verhalten aufweisen (Fig. 5) und deformierbar sind. Da die Partikel als Sauerstofftransporter konzipiert wurden, musste außerdem sichergestellt werden, dass das Hämoglobin während des Herstellungsprozesses nicht in Methämoglobin umgewandelt wird (Fig. 6). Konventionelle Blutgasanalysatoren konnten für die Bestimmung der verschiedenen Hämoglobinfraktionen in den Partikeln nicht verwendet werden, weshalb physiko-chemische Methoden zur Bestimmung der Hb-Fraktionen etabliert werden mussten (Fig. 7). Die Sauerstofftransportkapazität der Partikel wurde erfasst und quantifiziert. C.K. diskutierte mit allen genannten Autoren die experimentellen Befunde und schrieb das Manuskript. Eine Erklärung für die verbesserte Hämoglobinfunktion in den Partikeln wurde gegeben (Fig. 8).

Publikation 2: Kloypan C, Suwannasom N, Chaiwaree S, Prapan A, Smuda K, Baisaeng N, Pruß A, Georgieva R, Bäumler H. In-vitro haemocompatibility of dextran-protein submicron particles. Artif Cells, Nanomedicine, Biotechnol. 2019;47(1):241–9. **IF: 3.026**

Beitrag im Einzelnen:

Das Studiendesign des zweiten Teils der Untersuchungen der nach einem neuartigen Verfahren hergestellten Hämoglobin-Submikropartikel wurde mit dem Supervisor diskutiert und festgelegt. Die Untersuchungen zur Hämokompatibilität der Partikel untergliederte sich in die Untersuchungen der Wechselwirkung der Partikel mit Blutzellen. C.K. konzentrierte sich dabei insbesondere auf die Phagozytose der Partikel durch Monozyten und Granulozyten. Da die Partikel keine Autofluoreszenz (Fig. 3) aufweisen, kann die Phagozytose nicht direkt erfasst werden. Die Markierung der Partikel mit einem Fluoreszenzfarbstoff (z.B. FITC) führt zur Opsonierung der Partikel und damit zu falschen Ergebnissen. Deshalb etablierte C.K. einen indirekten Phagozytosetest, der die Phagozytose der Partikel durch Monozyten und Granulozyten mit Hilfe des Durchflußzytometers zu quantifizieren vermag (Fig. 5). Des Weiteren bestimmte er spektralphotometrisch die Hämolyserate nach der Inkubation von Blut/Erythrozytensuspensionen mit den Partikeln (Table 1). In der Publikation beschrieb er ausführlich die etablierten Methoden und unter Einbeziehung aller Autoren die Ergebnisse sowie die Diskussion der experimentellen Befunde.

Publikation 3: Suwannasom N, Smuda K, Kloypan C, Kaewprayoon W, Baisaeng N, Prapan A, et al. Albumin Submicron Particles with Entrapped Riboflavin—Fabrication and Characterization. Nanomaterials. 2019;9(3):482. **IF: 3.504**

Beitrag im Einzelnen:

C.K. führte in Abstimmung mit N.S. die Untersuchungen zur Hämolyse von Blut/Erythrozytensuspensionen vor und nach Inkubation derselben mit den Albuminpartikeln durch (Fig. 5). Außerdem untersuchte er den Einfluss der Partikel auf die Phagozytose von Monozyten und Granulozyten. Da Riboflavin fluoresziert konnte hierzu der direkte Phagozytosetest benutzt werden (Fig. 6).

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Signature, date and stamp of the supervising university lecturer

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Signature of the doctoral candidate

Printed copies of selected publications

Publication 1

Kloypan C, Prapan A, Suwannasom N, Kaewprayoon W, Steffen A, Xiong Y, Baisaeng N, Georgieva R, Bäumler H. Improved Oxygen Storage Capacity of Haemoglobin Submicron Particles by One-Pot Formulation. Artif Cells, Nanomedicine, Biotechnol. 2018; DOI:10.1080/21691401.2018.1521819
https://doi.org/10.1080/21691401.2018.1521819

Publication 2

Kloypan C, Suwannasom N, Chaiwaree S, Prapan A, Smuda K, Baisaeng N, Pruß A, Georgieva R, Bäumler H. *In-vitro* haemocompatibility of dextran-protein submicron particles. Artif Cells, Nanomedicine, Biotechnol. 2019;47(1):241–9. https://doi.org/10.1080/21691401.2018.1548476

Publication 3

Suwannasom N, Smuda K, Kloypan C, Kaewprayoon W, Baisaeng N, Prapan A, et al. Albumin Submicron Particles with Entrapped Riboflavin—Fabrication and Characterization. Nanomaterials. 2019;9(3):482. https://doi.org/10.3390/nano9030482

Curriculum vitae

My CV is not included in the electronic version of the dissertation for data protection reasons.

Complete list of publications

Research articles

Kloypan C, Prapan A, Suwannasom N, Kaewprayoon W, Steffen A, Xiong Y, Baisaeng N, Georgieva R, Bäumler H. Improved Oxygen Storage Capacity of Haemoglobin Submicron Particles by One-Pot Formulation. Artif Cells, Blood Substitutes, Biotechnol.2018; DOI:10.1080/21691401.2018.1521819. **IF: 3.026**

Kloypan C, Suwannasom N, Chaiwaree S, Prapan A, Smuda K, Baisaeng N, Pruß A, Georgieva R, Bäumler H. In-vitro haemocompatibility of dextran-protein submicron particles. Artif Cells, Nanomedicine, Biotechnol. 2019;47(1):241–9. **IF: 3.026**

Suwannasom N, Smuda K, Kloypan C, Kaewprayoon W, Baisaeng N, Prapan A, et al. Albumin Submicron Particles with Entrapped Riboflavin—Fabrication and Characterization. Nanomaterials. 2019;9(3):482. **IF: 3.504**

Patent

Bäumler H, Geogieva R, Kloypan C, Axel S, Xiong Y. One-pot formulation of biopolymer particles. European patent 2018:23384P-EP.

Oral Presentation

Kloypan C, “Properties of Protein Particles of Submicron Size” in The International Symposium on Nano, Transfusion and Translational Medicines at Charité – Universitätsmedizin Berlin on 6th-7th September 2018.

Kloypan C, “Determination of Hemoglobin Concentration in Hemoglobin-microparticles (HbMPs)” in DiNAMiT meeting (European project) at Department of Polymer Science & Engineering, Zhejiang University, Hangzhou, China, 23rd June 2016.

Acknowledgement

First, I would like to express my sincere gratitude to **PD Dr. rer. nat. Hans Bäumler**, my supervisor, for providing me with the golden opportunity to join and work with AG-Bäumler as a doctoral student at Institut für Transfusionsmedizin, Charité – Universitätsmedizin Berlin. I am thankful not only for his continued and tireless support for my study and related works but also his patience, motivation, encouragement, inspiration and immense knowledge.

My sincere thanks also goes to my co-supervisor, **Dr. rer. nat. Radostina Georgieva**, for her kind and valuable suggestions and inputs for our publications. I am gratefully indebted for her valuable and helpful comments for all of my papers.

I am also grateful for the continued interest and support of **Prof. Dr. Axel Pruß** in the progress of my work in the Institut für Transfusionsmedizin.

I particularly thank my labmates from Thailand namely **Nittiya Suwannasom, Aussanai Prapan, Saranya Chaiwaree and Waraporn Kaewprayoon** for cooperation, stimulating discussions, mental support and all the enjoyment we had shared in the last three years of friendship.

I also thank scientists and technicians of AG-Bäumler including **Kathrin Smuda, Wanit Chaisorn**, **Axel Steffen, Yu Xiong, Josephine Waade, Anya Schnabel and Anne Mohr** who gave access to the laboratory and research facilities. Thanks for helping and good working atmosphere. Without they precious support it would not be possible to conduct this research. In addition, I would like to express my special thanks to **Kathrin Smuda** (AG-Bäumler)**,** and **Pamela Glowacki** (the officers of Charité International Cooperation: ChIC) for solving the life problems and public affairs, making me live easily in Berlin.

A very special gratitude goes out to **University of Phayao**, Thailand for financial support for my doctoral study in Germany. I would like to also thank my lovely colleague from University of Phayao, **Dr. rer. nat. Nuttakorn Baiseang**, for introducing me to my supervisor, PD Dr. rer. nat. Hans Bäumler.

With a special mention to my former advisors, **Dr. Serm Surapinit** from University of Phayao and **Assistant Prof. Dr. Chanchai Boonla** from Chulalongkorn University for academic guidance and continued support throughout my research, it was such a scientific experience that had groomed me.

Finally, I would like to express my wholehearted love to my mother **“Jiraporn Kloypan”**, my father **“Samret Kloypan”**, my sister **“Nattida Kloypan”**, who had offered moral and emotional support throughout my study time in Germany. I am also thankful to my partner **“Chinnaphan Roekchamnong”** for unfailing support and continuous encouragement through thick and thin during my research and paper writing. This accomplishment would not have been possible without them. Thank you.