**In-vitro** haemocompatibility of dextran-protein submicron particles

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**ABSTRACT**

Blood compatibility is a key requirement to fulfil for intravenous administration of drug and oxygen carrier system. Recently, we published the fabrication of oxidised-dextran (Odex)-crosslinked protein particles by one-pot formulation. In the current study we investigate the haemocompatibility of these Oxex - particles including albumin particles (Odex-APs) and haemoglobin particles (Odex-HbMPs). Oxex-APs and Oxex-HbMPs have a submicron size ranged 800–1000 nm with peanut-like shape and a negative surface charge. In vitro haemocompatibility assays included haemolysis test, indirect phagocytosis test and platelet activation test in human blood. Oxex-APs and Oxex-HbMPs did not provoke any undesirable effects on the blood cells. Firstly, the ratio of haemolysis after contacted with Oxex-crosslinked protein particles were less than 5% and therefore the particles may be considered non-haemolytic. Secondly, the incubation of leukocyte with Oxex-APs/HbMPs did not influence the phagocytosis of leukocyte. We conclude that our particles are not recognized by monocytes or granulocytes. Finally, exposure of Oxex-APs/HbMPs to platelets did not cause an activation of platelets. Additionally, Oxex-HbMP/AP did not enhance or attenuate agonist-induced platelet activation. We conclude that Oxex-crosslinked protein particles exhibit a very good haemocompatibility and represent highly promising carriers for drugs or oxygen.

**Introduction**

A major part of the micro- and nanometer size particles in clinical and preclinical investigations are biomolecule-based particles fabricated from polysaccharides, polypeptides, proteins or lipids. Among them, protein-based micro- and nanoparticles have been extensively investigated [1] as a site-specific drug carrier for cancer [2–7] as well as haemoglobin-based oxygen carriers (HBOCs) [8–16].

However, questions concerning 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. Materials are considered to be haemocompatible, if they 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 [17]. For instance, the rupture of red blood cells (RBCs) and subsequent haemoglobin releasing can cause the symptoms haemoglobinuria or anuria followed by renal failure [18]. 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. 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 [19]. 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 haemocompatibility of particles is mainly affected by their physicochemical characteristics such as chemical composition, size, shape, surface charge, hydrophobicity or hydrophilicity [20–22]. The fabrication techniques and selection of biomaterials are the fundamental steps, which can drastically impact the haemocompatibility of the particles as well as the efficacy of the proteins in the particles. Recently, we described a new promising formulation of protein submicron
of solution 1 consisting of 0.25 M of MnCl$_2$ and 50 mg/mL of HSA or Hb were rapidly mixed with solution 2 containing 0.25 M of Na$_2$CO$_3$ and 40 mg/mL of Odex (40 T or 70 T, individually) in the beaker under vigorous stirring at room temperature. After 30 s, 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 PBS (6000 g for 5 min) and finally suspended in sterile PBS (final particle concentration $2 \times 10^{11}$/mL). APs and HbMPs crosslinked with 40T-Odex and 70T-Odex are named 40T-APs, 70T-APs, 40T-HbMPs and 70T-HbMPs, correspondingly.

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).

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.

**Blood collection**

Venous blood anticoagulated by lithium heparin or sodium citrate was collected from healthy volunteers. Informed consent was obtained from all donors in written form. The blood samples were withdrawn in accordance with the transfusion law of Germany. The use of donor blood samples for scientific purposes was approved by the ethics committee of the Charité – Universitätsmedizin Berlin (# EA1/137/14). Immediately after blood collection, tubes were slowly agitated to ensure an appropriate mixing of anticoagulant and blood and discarded if there was any evidence of clotting. The assays were performed within 2 h after blood collection.

**Haemolysis test**

The haemolytic test is based on the release of haemoglobin from damaged erythrocytes *in vitro*. Human heparinized erythrocytes were washed (3000 g, 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. This ratio of particles to RBC corresponds to an exchange of 50% blood with particle 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:

$$\text{% Haemolytic ratio} = \frac{(\text{OD}_{\text{test}} - \text{OD}_{\text{NC}})}{(\text{OD}_{\text{PC}} - \text{OD}_{\text{NC}})} \times 100 \quad (1)$$

All results were estimated from the data of three individual experiments, and all data were expressed as the mean ± SD [26]. The value of the positive control (water) was set to 100% haemolysis.

**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. 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 heparinised-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 10^8 bacteria per mL from PHAGOBURST kit) 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 10^8 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 leucocyte 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.

**Platelet activation test**

The influence of Odex-AP and Odex-HbMP on human platelets was investigated in platelet-rich plasma (PRP) samples. Citated human whole blood was centrifuged at 150 g for 15 min at 20–25°C, 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): mölLab 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 (GPIibα) 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 [27,28].

**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_ Values <.05 was considered statistically significant.

**Results**

**Particle characterisation**

Odex-APs and Odex-HbMPs were successfully fabricated by the One-pot procedure. Like the CCD-technique using MnCO₃ as inorganic template, the morphology and the size of the obtained haemoglobin and albumin particles by the One-pot procedure were not significantly different as observed by SEM (Figure 1). The dynamic light scattering analysis of the particles is represented in Figure 2. 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 results of flow cytometry analysis in Figure 3 also demonstrated that Odex-particles had no auto-fluorescence in contrast to the protein particles prepared using the CCD technique and crosslinked by glutaraldehyde \[8–10,29,30\].

**Figure 2.** 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 3.** 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).

**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 3h 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 did not deteriorate significantly the RBCs.

**Phagocytosis test**

The commercial phagotest kit allows determinations 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 part Materials and Methods. Non-fluorescent *E. coli*, Odex-APs and Odex-HbMPs were added to the whole blood sample and incubated at 37 °C for 10–120 min in order to reach a saturated phagocytosis by the granulocytes and monocytes. Then, the standard test with FITC-labeled *E. coli* at 37 °C was performed with all samples. Results of 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 4 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 behavior with an increased number of non-fluorescent cells.

The summarized results in dependency on the pre-incubation time are shown in Figure 5. 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 decrease in the percentage of the cells engulfing FITC-*E. coli* in a time-dependent manner, due to the phagocytosis of non-fluorescing *E. coli*.

**Platelet activation test**

To evaluate whether our Odex-APs and Odex-HbMPs alter the haemostasis system, their influence on the activation of

| 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).

![Figure 4](image-url)
platelets was investigated. Figure 6 shows results of the flow cytometry analysis of platelets using the platelet-specific membrane receptor CD42b (GPIba), 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.

Figure 7 summarizes the results of the flow cytometry analysis. Obviously, 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.
Discussion

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. The incorporation of toxic compounds or active ligands for recognition and triggering of biological receptors has been well described. Small particles or positive charge caused thrombocyte and granulocyte activation, and haemolysis. In contrast, negatively charged particles larger than 60 nm hydrodynamic diameter appear to be considerably less haemotoxic than smaller ones. Our new particles fit these criteria. Additionally, a successful perfusion of isolated mouse glomeruli with concentrated HBMP suspensions of the same size in vitro without vasoconstriction of the afferent arterioles could be shown. 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. Therefore, it is very important to utilize procedures that allow preparation of particles with high degrees of uniformity, and with control over their physical and chemical characterisations.

Odex-APs and Odex-HbMPs fabricated by One-pot formulation have a size in the submicron range, uniform morphology and negative surface charge. The coating of the particles with human serum albumin improves significantly their blood compatibility by reducing the adsorption of other proteins as well as interaction with platelets and leukocytes. 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 blood stream 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.

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. According to the ISO 10993-4:2017, the haemolysis assays of Odex-AP and Odex-HbMP 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).

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 the 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. Our 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 circulatory system.

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 dextran used as plasma expander influences the function of platelets due to its adsorption on platelets surface. 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-HbMP is covalently bound to haemoglobin and not free available for adsorption. But the moieties of dextran partially presented on the particle surface contribute to a repulsive force against other macromolecules.

Therefore, the interaction between platelets and particle 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 hemolysis [18]. We assume that the negative surface charge as well as the albumin coating of Odex-APs and Odex-HbMPs prevent interaction between the particles and the platelets and therefore we do not expect negative effects on haemostasis.

Conclusions

In the current study, we demonstrated the in vitro evaluation of the compatibility of Odex-APs and Odex-HbMPs fabricated by "One-pot formulation" with human blood. The results of the haemolysis test, the direct phagocytosis test and of the platelet activation tests reflected good haemocompatibility. No biologically relevant alterations during blood contact were observed and therefore Odex-APs and Odex-HbMPs fabricated by "One-pot formulation" fulfill the requirements according to ISO 10993-4 for blood contacting materials. Therefore, their application potential is worthwhile to be further developed and explored.

Disclosure statement

No potential conflict of interest was reported by the authors.

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