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DISSERTATION

Fabrication and Characterization of Hemoglobin Based Oxygen Carriers

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Abstract (English)

Currently, extensive research has been performed to design a suitable blood substitute. Most of these approaches have focused on the development of Hb-based oxygen carriers (HBOCs). HBOCs represent a propitious type of blood substitute to transport oxygen throughout the body while also acting as a carrier in biomedical applications. Although HBOCs have been developed and clinically investigated, it remains some safety concerns such as short circulatory half-life, oxidative stress, hypertension etc. Due to HBOCs in blood are recognized and are rapidly scavenged by the innate immune systems, HBOCs require a surface modification that provides protection against detection and elimination in order to prolong circulation time. In this study, we fabricated hemoglobin submicroparticles (HbMPs) using the CCD technique as HBOCs and investigated different surface modification of HbMPs. Double/triple precipitation as well as adsorption of human serum albumin, hyaluronic acid and pluronic were applied. The influence of these surface modifications was investigated determining the functional Hb and the binding of anti-Hb antibodies, immunoglobulin and haptoglobin to HbMPs. Additionally, we fabricated and characterized HbMPs by incorporation of polydopamine in order to protect Hb against oxidation. Finally, we established a novel fabrication process of HbMPs using oxidized-dextran as cross-linker and characterized their properties. The particle size, zeta-potential and morphology of all synthesized HbMPs were analyzed using zeta sizer, CLSM, SEM, and TEM. The results revealed that all fabricated HbMPs have a submicron size with a negative charge in PBS. For the functional Hb, there are no significant differences between surface-modified HbMPs and Odex-HbMPs. The functional Hb of PD-HbMPs was shown twice higher than unmodified HbMPs due to the protective antioxidant effect of PD. The specific binding of anti-Hb antibodies, immunoglobulin and haptoglobin to the surface-modified HbMPs was reduced. This indicates that the particles are protected again detection and elimination processes by the immune system and therefore it can be assumed that the circulation half-life will increased. The PD-HbMPs illustrated a high scavenging activity of free radicals including H_2O_2, suggesting promising HBOCs with antioxidation activity. The new fabrication technique of Odex-HbMPs minimizes the fabrication time and is a very efficient fabrication method. Odex-HbMPs maintained their ability to be oxygenated/deoxygenated and they are stable at least for 90 days. The results presented in this work suggest a great potential of HbMPs to become useful HBOCs.

Abstract (Deutsch)

Gegenwärtig wurden umfangreiche Untersuchungen durchgeführt, um eine geeignete und alternative Quelle für Blutblutersatz zu finden. Die meisten dieser Ansätze haben sich auf die Entwicklung von Hb-basierten Sauerstoffträgern (HBOCs) konzentriert. HBOCs stellen eine günstige Art von Blutersatz dar, um Sauerstoff durch den Körper zu transportieren und gleichzeitig als Träger in biomedizinischen Anwendungen zu fungieren. Obwohl HBOCs entwickelt und klinisch untersucht wurden, müssen nach der Infusion weitere Sicherheitsaspekte beachtet werden, wie z. B. kurze Kreislaufhalbwertszeit, oxidativer Stress, Bluthochdruck usw. HBOCs im Blut werden erkannt und werden vom angeborenen Immunsystem schnell abgefangen. Oberflächenmodifikationen, die vor dem Erkennen und der Elemination der HBOCs schützen, sollen die Zirkulationszeit verlängern. Wir untersuchten verschiedene Oberflächenmodifikationen von Hämoglobin-Mikropartikeln (HbMPs) durch Doppel- / Dreifachpräzipitation sowie durch Adsorption von Humanserumalbumin, Hyaluronsäure und Pluronsäure unter Verwendung der CCD-Technik, um herauszufinden, wie die verschiedenen Oberflächenmodifikationen die Funktion von Hb und die Bindung von Anti-Hb-Antikörpern, Immunglobulin und Haptoglobin an HbMPs beeinflussen. Zusätzlich stellten wir HbMPs her und charakterisierten sie, indem wir Dopamin in den HbMP polymerisierten, um Hb vor Oxidation zu schützen. Schließlich wurde ein neues Herstellungsverfahren für HbMPs unter Verwendung von oxidiertem Dextran als Vernetzer etabliert und die Eigenschaften der Odex-HbMP charakterisiert. Die Partikelgröße, das Zeta potential und die Morphologie aller hergestellten HbMPs wurden unter Verwendung des Zetasizers, CLSM, SEM und TEM analysiert. Die Ergebnisse zeigten, dass alle hergestellten HbMPs eine Größe im Submikronbereich mit einer negativen Ladung in PBS aufweisen. Für das funktionelle Hb wurden keinen signifikanten Unterschied zwischen oberflächenmodifizierten HbMPs und Odex-HbMPs gefunden. Das funktionelle Hb von PD-HbMPs war aufgrund der schützenden antioxidativen Wirkung von PD doppelt so hoch wie das in unmodifizierten HbMP. Die spezifische Bindung von Anti-Hb-Antikörpern, Immunglobulin und Haptoglobin war bei den oberflächenmodifizierten HbMPs reduziert. Dies deutet auf ein Beschichtungsdesign hin, das in der Lage ist, die Partikel vor dem Nachweis und den Eliminierungsprozessen durch das Immunsystem zu schützen, und daher kann davon ausgegangen werden, dass es zu einer Verlängerung der Halbwertszeit im Blutkreislauf kommen wird. Die PD-HbMPs besitzen eine hohe Aktivität freie Radikale, einschließlich H_2O_2 zu eliminieren, womit HBOCs mit Antioxidationsaktivität zur Verfügung stehen. Die neue Herstellungstechnik von Odex-HbMPs minimiert die Herstellungszeit und bietet eine sehr effiziente Herstellungsmethode. Odex-HbMPs konservieren ihre Fähigkeit zur Sauerstoffbindung/Sauerstoffabgabe und sind mindestens 90 Tage lang stabil. Insgesamt können die in dieser Arbeit vorgestellten Ergebnisse als Beleg für ein großes Potenzial der HbMPs als künstliche Sauerstofftransporter betrachtet werden.

1. Introduction

Blood transfusion is a potentially life-saving procedure and used in many therapeutic applications. Several risks, such as the transmission of infectious disease, immunomodulation, etc., accelerated the development of blood substitutes as alternatives to human blood [1,2]. The development of blood substitutes has mainly focused on the idea to carry oxygen, no toxicity and side effects and a sufficient circulation time [3–5]. During the last decades, the use of blood substitutes has been extensively investigated especially with the attention to hemoglobin-based oxygen carriers (HBOCs).

HBOCs were fabricated by using hemoglobin (Hb) extracted from human or mammalian red blood cells for delivering oxygen to human tissues. The development and modification of HBOCs for clinical trials are still on the way [4,6,7]. Currently, the different approaches to fabricate HBOCs have been investigated for a primary consideration in stabilizing the Hb molecule and improving the oxygen-carrying capabilities by cross-linked hemoglobin, polymerized hemoglobin, hemoglobin conjugated to macromolecules and encapsulated hemoglobin [8,9]. HBOCs are expected to increase the total oxygen-carrying capacity and oxygen delivery of blood because of an increase in total Hb concentration. Therefore, the oxygen-carrying behavior of HBOCs should be considered and investigated so far. During clinical trials, the investigated HBOCs showed side effects after infusions such as hypertension, elevation in pancreatic and liver enzymes, renal and neural toxicity, and oxidative stress [8,10]. Hypertension was attributed to nitric oxide (NO) extravasation and premature release of O_2 in the arterioles, both of which cause vasoconstriction [4,10]. Oxidation of Hb to Met-Hb, which releases reactive ferryl ions and radicals, was a common cause for oxidative stress and tissue toxicity [11]. Due to these safety concerns, new HBOCs are under development to avoid the above-mentioned negative side effects.

To date, our group published data of HbMPs fabricated by a Co-precipitation-Crosslinking-Dissolution technique (CCD-technique) with high oxygen affinity and low immunogenicity [12–14] for use as HBOCs. In particular, when isolated mice glomeruli were perfused with our HbMPs in *vitro*, no significant vasoconstriction occurred [14]. However, in animal experiments, one main problem seems to be the short half-life of HBOCs after their transfusion [15]. Surprisingly, there are no literature data available, which show and explain the reason for the elimination of the HBOCs. It is known that free Hb is detected by haptoglobin (HP) and the Hb-HP complex is recognized by the Kupffer cells very fast [16,17]. HP is a glycoprotein that is mostly synthesized and secreted by liver cells and plays a vital rule with a high affinity to Hb or HBOCs in the circulation [18]. Therefore, HBOCs should preclude Hb-HP complex formation to avoid liver uptake and depletion of HP in the plasma. To overcome this obstacle, the modification of the surface properties of carriers, also known as ‘stealth coatings’, have been developed and carried out to prevent particles from protein absorption and immune recognition [19]. The properties of the coating polymer should be flexible and hydrophilic. Many materials and coating approaches have been utilized for modifying the surface of particles. For instance, human serum albumin (HSA) [20–22], natural polysaccharides (e.g. hyaluronic acid) [23,24] or synthetic polymers (e.g., PEG-based copolymers such as pluronic [25]) have wildly used as coating materials. Therefore, it was investigated, which modifications of the surface of HbMPs influence the oxygen binding and the adsorption of antibodies, especially the anti-Hb antibodies, Immunoglobulin (IgG) and the glycoprotein HP. The surface modifications were performed by double as well as triple precipitation as well as by adsorption of human serum albumin (HSA), hyaluronic acid (HA), and pluronic (Plu). The use

of HSA, HA and Plu as surface coating materials is a promising approach for drug delivery carrier. We hypothesized that these polymers can absorbed in a layer of HbMP to avoid IgG antibodies and HP binding.

Despite all the efforts undertaken in the development of viable HBOCs, there are still concerns over their potential to promote oxidative tissue injury [26]. The issues of oxidative toxicity of Hb, its compatibility with biological systems and the preservation of Hb ability to bind and deliver oxygen have not been completely resolved. It is therefore essential to focus on modification of Hb with antioxidant materials to attenuate the oxidative toxicity of Hb and scavenging of NO. Our design of HBOCs also incorporated antioxidant molecules to protect Hb from oxidation and to increase its oxygenation levels. As an antioxidant, we used polydopamine (PD) due to its reported biocompatibility, biodegradability, free radical scavenging properties and ability to attach to almost any type of surface due to abundance of various functional groups [27].

In order to progress in synthesis and formulation of HBOCs, our group develops a novel, simple, efficient and cost-effective method of HBOCs fabrication based on the previously introduced CCD technique. The CCD technique provides pure protein particles with uniform morphology and narrow size distribution. Glutaraldehyde (GA) is widely used to cross-link proteins and other biopolymers for medical applications, but there are many reports concerning the undesired properties of GA-cross-linked biomaterials such as auto-fluorescence as well as its toxicity [28–30]. For that reason, “oxidize dextran (Odex)” cross-linkers are now being investigated as alternatives for GA to reduce the risks of side effects [31–33]. Therefore, we developed a novel Hb particle fabrication method based on the CCD technique by applying Odex as a cross-linker for Hb. Additionally, the functionality of the Hb entrapped in the Odex-HbMP as well as the storage stability of Odex-HbMP in a salt solution was determined.

The aim of the investigations was the improvement of HBOCs in respect of their oxygen carrying capacity and preventing their recognition by antibodies by means of surface modification and incorporation of antioxidants and the modification of the fabrication procedure.

2. Materials and Methods

Table 1: List of all substances

Substances	Abbreviation	Company
Human serum albumin	HSA	Baxalta Deutschland GmbH, Unterschleissheim, Germany
Fresh bovine whole blood	-	Biophyll GmbH, Berlin, Germany
Manganese chloride tetrahydrate	$MnCl_2.4H_2O$	Sigma-Aldrich, Munich, Germany
Sodium carbonate	Na_2CO_3	Sigma-Aldrich, Munich, Germany
Ethylenediaminetetraacetic acid	EDTA	Sigma-Aldrich, Munich, Germany
Glutaraldehyde	GA	Sigma-Aldrich, Munich, Germany
Sodium borohydride	$NaBH_4$	Sigma-Aldrich, Munich, Germany
Hyaluronic acid sodium salt	HA	Sigma-Aldrich, Munich, Germany
Kolliphor®P407	Plu	Sigma-Aldrich, Munich, Germany
Pronase from Streptomyces griseus	-	Roche Diagnostics GmbH, Mannheim, Germany
Potassium hexacyanoferrate (III)	$K_3(Fe(CN)_6)$	Sigma-Aldrich, Darmstadt, Germany
Sodium chloride	NaCl	Merck KGaA, Darmstadt, Germany
Ringer's acetate solution	-	Serumwerk Bernburg AG, Bernburg, Germany
Ampuwa®	-	Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany
Dextran (M.W. 40, 70 kDa)	Dex	AppliChem GmbH, Darmstadt ,Germany
Sodium hydroxide	NaOH	Carl Roth GmbH, Germany
Sodium (meta) periodate	$NaIO_4$	Sigma-Aldrich, Darmstadt, Germany
Glycine	-	Sigma-Aldrich Darmstadt, Germany
Dopamine hydrochloride	$C_8H_{11}NO_2.HCl$	Sigma-Aldrich, Darmstadt, USA
2,2-diphenyl-1-picrylhydrazyl	DPPH	Sigma-Aldrich, USA
2,2-azio-bis(3-ethylbenzthiazoline-6-sulfonic acid) antioxidant assay kit	ABTS	Sigma-Aldrich, USA
Phosphate buffered saline (10X)	PBS	Fisher Scientific , USA
Tris-HCl buffer (10 mM, pH 8.5)	Tris -HCl	Beijing Leagene Biotech , China
Triton™ X-100 solution	-	Sigma-Aldrich,USA
Rabbit Anti-Bovine Hemoglobin anti-body	-	LS Bio, Washington, USA
APC-conjugated rabbit polyclonal anti-body to human Haptoglobin	-	LS Bio, Washington, USA
Goat anti-rabbit IgG conjugate to APC	-	Columbia biosciences, Frederick, MD, USA
Haptoglobin from human blood pool	-	Sigma–Aldrich, Saint Louis, MO, USA
Human IgG isotype control (Biotin)	-	Novus Biologicals, Bio-Techne Brand, Centennial, CO, USA.
APC- streptavidin High concentration	-	BioLegend Inc. San Diego, CA, USA.

Table 2: List of equipment/facility

Equipment/facility	Company
Flow cytometry	FACS-Canto II, Becton and Dickinson, Franklin Lakes City, USA.
Magnetic Stirrer	Bibby Scientific CB161, Bibby Sterilin Ltd., Stone, UK.
UV-vis-spectrophotometer (U2800)	HitachHitachi High-Technologies Corporation, Tokyo, Japan
Zetasizer nano instrument	Malvern Instruments Ltd., Malvern, UK
Hematocrit centrifuge	Mikro 22R, Hettich GmbH & CoKG, Germany
Centrifuge	Thermo Electron Biofuge Primo R, Germany
Microplate reader	PowerWave 340 ,BioTek Instruments, USA.
Oxygen Microsensor (PM-PSt7)	PreSens- Precision Sensing GmbH, Regensburg, Germany
Portable oxygen meter (Microx 4)	PreSens -Precision Sensing GmbH, Regensburg, Germany
Heating and Drying Oven	Thermo Scientific (OVEN UT20 FUNCTION LINE 210L),UK
Confocal Laser Scanning Microscope (CLSM)	CLSM Zeiss LSM 510meta, Zeiss Micro Imaging GmbH, Jena, Germany
Scanning Electron Microscope (SEM)	Gemini Leo 1550 detector, Oberkochen, Germany
Transmission Electron Microscope (TEM)	Zeiss EM Omega 912 instrument, Carl Zeiss AG, Oberkochen, Germany
Transmission Electron Microscope (TEM)	Zeiss EM Omega 906 microscope, Carl Zeiss AG, Oberkochen, Germany

2.1 Particle Preparation

2.1.1 Preparation of Surface-modified HbMPs

2.1.1.1 Preparation of Hemoglobin Submicron Particles (HbMPs)

Hemoglobin (Hb) was extracted from bovine red blood cells by hypotonic hemolysis [34]. Fresh bovine whole blood, anticoagulated with EDTA was centrifuged (2,500 g, 10 min, 4 °C), were washed three times with PBS and then 5 volumes of ice-cold Ampuwa were added to 1 volume of washed red blood cells. The solution was stirred at 4°C overnight and then centrifuged (10,000 g, 4 °C) for 1 hour. The supernatant was filtered through a 0.1 µm polyethersulfone filter (Sartorius AG, Göttingen, Germany) and stored at -80 °C as a stock solution for further use.

HbMPs were fabricated using a protocol based on the CCD-technique as previously described [13,14,35]. Briefly, 5 mg/mL Hb and 0.125 M $MnCl_2$ solution were mixed rapidly with 0.125 M Na_2CO_3 and keep stirring for 30 seconds to obtain Hb-$MnCO_3$ particles (first precipitation). These particles were washed twice with distilled water by centrifugation (3,000 g, 3 min) and re-dispersed in 0.9% NaCl solution. The Hb-$MnCO_3$ particles were incubated with 0.02 % GA solution for 1 hour at room temperature. After that, the obtained particles were washed and re-suspended in water. These particles were used for a second as well as third precipitation. A short description of each particle type and the corresponding abbreviations are listed in Table 3.

Table 3. Specification of the different surface modifications of HbMPs.

Particle type	Abbreviation
Hemoglobin Submicron particles	HbMPs
Double precipitated HbMP in presense of HSA: Hb precipitation first, HSA absorption before second precipitation step.	HSA-d-HbMPs
Double precipitated HbMP in presense of HA: Hb precipitation first, HA & HSA absorption before second precipitation step.	HA-d-HbMPs
Double precipitated HbMP in presense of Pluronic: Hb precipitation first, Plu & HSA absorption before second precipitation step.	Plu-d-HbMPs
Triple precipitated HbMP in presense of HSA: Hb precipitation first, HSA absorption before second and third precipitation step.	HSA-t-HbMPs
Triple precipitated HbMP in presense of HA: Hb precipitation first, HA & HSA absorption before second and third precipitation step.	HA-t-HbMPs
Triple precipitated HbMP in presense of Pluronic: Hb precipitation first, Plu & HSA absorption before second and third precipitation step.	Plu-t-HbMPs

2.1.1.2 Preparation of HSA-d-HbMPs and HSA-t-HbMPs

Double precipitated HbMPs (HSA-d-HbMPs): For the second co-precipitation step, the washed Hb-$MnCO_3$ particles were suspended in 0.04 M $MnCl_2$ and 0.4% HSA was added to adsorb on the surface of the particles for 5 min. After adding 0.04 M Na_2CO_3, the obtained particles were washed with distilled water by centrifugation (3,000 g, 3 min) and were re-suspended in 0.9% NaCl solution. The double co-precipitated HSA-d-Hb-$MnCO_3$ particles were cross-linked again with 0.02% GA at room temperature under moderate stirring. 0.25 M EDTA was used to dissolute the $MnCO_3$ templates. 0.2 mg/mL $NaBH_4$ in 0.1 M NaOH solution were added and incubated for 30 min. The resulting HSA-d-HbMPs were washed three times with 0.9% NaCl solution supplemented with 0.2% HSA (centrifugation 10,000 g, 10 min) and were re-suspended in ringer's acetate solution and stored at 4 °C for further use. Triple precipitated HbMPs (HSA-t-HbMPs): The additional co-precipitation step of $MnCO_3$ with HSA for the fabrication of HSA-t-HbMPs was performed after the second crosslinking step followed by cross-linking with GA in the same manner and concentrations before performing the dissolution step

2.1.1.3 Preparation of HA-d-HbMPs and HA-t-HbMPs

The HA-d-HbMPs were prepared in the same manner as HSA-d-HbMPs using 0.04% HA in addition to the 0.4% of HSA. The fabrication of HA-t-HbMPs was performed after the second crosslinking step by co-precipitation of $MnCO_3$ with 0.04% HA and 0.4% HSA followed by cross-linking with GA in the same manner and concentrations before performing the dissolution step.

2.1.1.4 Preparation of Plu-d-HbMPs and Plu-t-HbMPs

The Plu-d-HbMPs were also prepared in the same manner as HSA-d-HbMPs using 0.4 % Pluronic in addition to the 0.4% of HSA. The fabrication of Plu-t-HbMPs was performed after the second crosslinking step by co-precipitation of $MnCO_3$ with 0.4% Pluronic and 0.4% HSA followed by cross-linking with GA in the same manner and concentrations before performing the dissolution step.

2.1.2 Preparation of HbMPs with incorporated Polydopamine (PD-HbMPs)

Firstly, HbMPs were synthesized by co-precipitation of Hb with $MnCO_3$ template. Briefly, 0.125 M Na_2CO_3 and 0.125 M $MnCl_2$ solution including 50 mg/mL Hb solution were rapidly stirred for 30 s. To prevent their agglomeration, 5 mg/mL HSA solution was introduced during 5 min stirring. Afterward, the particles were dissolved into 20 mL of 1 mg/mL GA solution after centrifugation (3,000 g, 3 min) and washing 2 times with 0.9% NaCl solution and then incubated for 1 hour at room temperature for the cross-linking. The $MnCO_3$ template was removed by the resuspension of Hb-$MnCO_3$-MPs with 0.25 M EDTA solution. The obtained HbMPs were dispersed in 0.9% NaCl containing 20 mg/mL HSA solution, followed by centrifugation and washing three times. The resulting HbMPs were re-suspended in ringer acetate solution for the next step.

To fabricate the PD-HbMPs, HbMPs were prepared as mentioned above. Then, the particles were added into dopamine hydrochloride solution with concentrations 0.75, 1, and 2.5 mg/mL in 0.1 mM Tris-HCl buffer, pH 8.5. After 3 hours oxidation and self-polymerization under magnetic stirring, the excess of dopamine was removed by centrifugation (10,000 g, 5 min) and the PD-HbMPs were washed three times and re-suspended in PBS.

2.1.3 Preparation of Odex-HbMPs

At first, the Odex solution was prepared and characterized. Briefly, sodium periodate (6.6 g, 30.9 mmoL) was added into 50 ml of 10% of dextran (30.9 mmoL glucose subunits) in water, shielded from light, with constant stirring at room temperature. The reaction was allowed to proceed for 1 hour. Then, the reaction mixture containing the oxidized dextran (Odex) was transferred to a cellulose dialysis tube and dialyzed against water. Finally, the Odex solution was lyophilized by the freeze-drying method for further usage. The aldehyde group content of Odex was determined using the hydroxylamine hydrochloride titration method, and nonoxidized dextran samples were used as a control.

Next step, the Odex-HbMPs were fabricated by a modified protocol based on the CCD technique. Briefly, equal volumes of solution 1 consisting of 0.25M of $MnCl_2$ and 50 mg/mL of Hb were rapidly mixed with solution 2 containing 0.25M of Na_2CO_3 and 40 mg/mL of Odex in a beaker under vigorous stirring using a mechanical stirrer at room temperature. In this step, the Hb and the cross-linker Odex were precipitated together into the inorganic $MnCO_3$ allowing the cross-linking reaction to perform in the formulated submicron particles. After incubation for 30 seconds, 5 mg/mL of HSA was added to the suspension and incubated for 5 min under stirring as a result of the adsorption of HSA on the surface of particles. After HSA incubation, the resulting suspension particles were proceeded to the dissolution of the $MnCO_3$ template by 0.25M EDTA /0.05 M glycine for 30 min and the reduction by 0.4 mg/mL of $NaBH_4$. Finally, the obtained Odex-HbMPs were centrifuged, washed three times (6,000 g for 5 min) and suspended in sterile 0.9% NaCl solution.

2.2 Particle Characterization

2.2.1 Particle size, Zeta potential and Morphology

2.2.1.1 The hydrodynamic diameter and Zeta-potential

The hydrodynamic diameter and the zeta-potential of all HbMPs were measured by dynamic light scattering using a Zetasizer Nano ZS instrument. Each sample was diluted 1:200 using PBS. All measurements were carried out in triplicate. In addition, the zeta potential of PD-HbMPs was also determined in 0.9% NaCl solution and water.

2.2.1.2 Transmission Electron Microscopy (TEM)

TEM imaging of surface-modified HbMPs was performed using a Zeiss EM 906 Omega microscope at an acceleration voltage of 80 kV. For sample preparation, the water of particle suspensions was exchanged against ethanol. After the last centrifugation (1800 g, 6 min), the sample was infiltrated with LR-White Resin (ratio of Ethanol: LR-WR = 1:1) and incubated over night at room temperature. After that, the sample was exchanged with a fresh LR-WR and was transferred into gelatine capsules. Polymerization of the resin was performed with UV-light for 48 hours at room temperature. At the end of the process, ultrathin sectioning (60-70 nm on mesh-grids) were prepared. Sections were stained with 1% phosphotungstic acid for 15 min and 4% aqueous uranyl acetate for 15 min. Moreover, TEM investigations of PD-HbMPs were performed using a Zeiss EM Omega 912 instrument after drying the HbMPs suspension on carbon-coated copper grids. No additional staining was performed.

2.2.1.3 Scanning Electron Microscopy (SEM)

The morphology of Odex-HbMPs and PD-HbMPs was also observed by SEM. 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.

2.2.1.4 Confocal Laser Scanning Microscope (CLSM)

CLSM images of all HbMPs were acquired with a confocal microscope LSM 510 Meta applying excitation wavelength 488 nm for confirmation the suspension stability of the samples, e.g. absence of aggregation.

2.2.2 Determination of Hb Content

For Hb determination, the modified AHD-575 method was employed [36,37]. 2% (*v/v*) HbMP suspension was mixed with pronase to achieve a final concentration of 0.5 mg/ml pronase. The pronase–HbMP suspension was then incubated for 30 min at 45 ℃. Afterward, AHD reagent (25 mg/mL Triton X-100 in 0.1 M NaOH) was added at the volume ratio of 1:1 to the pronase–HbMP mixture for hemin conversion. Absorption spectra of the particles were recorded using a UV-VIS spectrophotometer at 574 nm.

2.2.3 Determination of Functional Hb (Oxygenated Hemoglobin: Oxy-Hb)

The ferricyanide method was applied for the measurement of the oxygen content of HbMPs [38,39]. Briefly, 2% (*v/v*) particle suspensions were filled into a glass vial containing a magnetic stirrer. A miniaturized optical needle type oxygen sensor (oxygen micro-sensor NTH-PSt7) connected to a portable oxygen meter with data logging (Microx 4, PreSens) was inserted into the stirred sample and the concentration of dissolved oxygen was recorded. 3 seconds after starting the measurement, 10% potassium ferricyanide was added and the change of the concentration of dissolved oxygen was measured until a stable value was reached. The difference between the final pO_2 value and the initial pO_2 corresponds to the oxygen bound to hemoglobin in the particles. The change in pO_2 was converted into the concentration of released oxygen (cO_2) with the following formula known as Henry's Law:

$$cO_2 = aO_2 * pO_2 \quad (1)$$

where aO_2 is the solubility coefficient of oxygen in blood: 0.0031 ml O_2/ mmHg O_2 / dL blood. Having derived the mass of released oxygen, the concentration of the Oxy-Hb was calculated as follows:

$$c(OxyHb) = \frac{mO_2}{\rho(O_2) * Hb_S} \quad (2)$$

where $\rho(O_2)$=1.43 g/L is the density of O_2 and Hb_s is the saturated Oxy-Hb content which equals 1.34 mL of O_2 per gram of Hb.

2.2.4 In vitro antibody binding assays of Surface-modified HbMPs

The successful surface modification of HbMP and the detection of bound anti-bovine Hb antibodies, Immunoglobulins (IgG) and haptoglobin (HP) were performed bymeans of flow cytometry. Six different types of surface modified HbMP and the unmodified HbMP as a control were investigated. Staining the particles with only secondary antibodies provided the non-specific binding, staining with both primary and secondary antibodies provided the total binding. Specific binding was calculated by subtracting the non-specific binding from the total binding.

2.2.4.1 Binding assay of Anti-Hb Antibodies

1 mL of 0.2 % (v/v) particle suspensions were incubated for 2 hours with the primary rabbit anti-bovine hemoglobin antibody at a final concentration of 2.5 µg/mL. The particle suspensions were then incubated with the secondary APC-conjugated goat anti-rabbit IgG (final concentration 1 µg/mL). Incubations were performed at room temperature. Between each incubation step, the particle suspensions were washed 2-3 times with PBS solution. Subsequently, the particle suspensions were incubated again in darkness for 2 hours, washed and re-suspended in 300 µL PBS solution for the investigation by flow cytometry.

2.2.4.2 Binding assay of Immunoglobulin (IgG)

The binding of IgG to particles was also performed and comprised the same procedure used for the binding assay of anti-Hb antibodies described above. The primary biotin conjugated human IgG isotype control was added into 1 mL of particle suspensions at a final concentration of 2.5 µg/mL and was incubated

for 2 hours at room temperature followed by PBS washings. After that, the particle suspensions were incubated for 2 hours in the dark with APC-streptavidin at final concentration of 1 µg/mL.

2.2.4.3 Binding assay of Haptoglobin (HP)

The accessibility of Hb of each surface-modified HbMP for the HP was investigated in the same manner as described above. Firstly, HP from pooled human plasma was added to 1 mL of particle suspensions at a final concentration of 2.5 µg/mL HP. Then, APC-conjugated rabbit polyclonal antibody to human haptoglobin (HP antibody) was added at a final concentration at 1 µg/mL (secondary antibody).

2.2.5 Anti-oxidative properties of PD-HbMPs

2.2.5.1 Oxidative protection of Hb in PD-HbMP from H_2O_2

The PD-HbMPs were incubated with 5mM H_2O_2 and the absorption spectra were recorded with an UV- Vis spectrophotometer Hitachi U2800.

2.2.5.2 Oxidative protection of Hb in PD-HbMPs from Met-Hb formation

Firstly, the total Hb content of PD-HbMPs was performed as mentioned in section 2.2.2. Next, the ferricyanide method was performed to determine the amount of Oxy-Hb in PD-HbMPs as mentioned in section 2.2.3. Knowing the total Hb content and the Oxy-Hb of PD-HbMPs, the percentage of Hb-bound O_2 amount were calculated.

2.2.5.3 ABTS antioxidant assay

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) substrate working solution was prepared as follows: 2 mL of 2 mM ABTS solution in phosphate buffer, 0.5 mL of 12.5M peroxidase, 0.5 mL of 3% H_2O_2 were mixed and diluted in PBS. Dopamine hydrochloride solution in 0.1mM Tris-HCl buffer was added to HbMP suspensions and stirred for 3 hours at room temperature. The final concentrations of Hb and PD in the PD-HbMPs were 2 mg/mL each. The solution of Trolox and PD were also adjusted to the concentration of 2 mg/mL. Then 10 mL of Trolox, PD, HbMPs and PD-HbMPs (0.03–1 mg/mL) were incubated with 200 µL ABTS working solution for 5 min. The absorption peak measured at 405 nm with a microplate reader indicated the presence of ABTS in the solution. The radical scavenging activity of the samples is indirect proportional to the absorbance. The antioxidant activity was calculated as follows:

$$\text{Antioxidant activity (\%)} = \frac{\mathrm{A} - (\mathrm{A_s} - \mathrm{A_r})}{\mathrm{A}} \times 100\ \% \qquad (3)$$

; As being the absorbance of the samples incubated with the ABTS solution, Ar being the absorbance of the samples without the ABTS solution and A being the absorbance of the pure ABTS solution.

2.2.5.4 DPPH antioxidant assay

2, 2-diphenyl-1-picrylhydrazyl (DPPH) working solution in 95% ethanol was prepared with absorbance of 0.7– 0.8 at 520 nm and 200 µL were pipetted into each well of a 96-well plate. Then, 10 µL of Trolox, HbMPs and PD-HbMPs with concentrations from 0.03 to 1 mg/mL were added and the endpoint

absorbance at 520 nm was measured with a microplate reader. The antioxidant activity was calculated in the same manner as ABTS antioxidant assay (A is the absorbance of the pure DPPH working solution. A_s is the absorbance of the samples incubated with DPPH working solution. A_n is the absorbance of the samples incubated with 95% ethanol.

2.2.6 Storage stability of Odex-HbMPs

The storage stability of Odex-HbMPs in 0.9% NaCl solution was characterized by the size distribution, the Hb content and the amount of functional Hb (Oxy-Hb) at day 0, 30 and 90 after fabrication. The individual aliquots were kept at 4℃ during storage.

2.3 Statistical analysis

All results were presented as means ± SD. The data were compared using ANOVA-like test. GraphPad Prism 6 software (GraphPad, La Jolla, CA) was employed for graphs and statistical analyses. P-Values < 0.05 were considered statistically significant.

3. Results

We have successfully fabricated Hb-based oxygen carrying particles by surface modification using a modified CCD-technique. Surface modifications of HBOCs play a vital role for facilitating efficacy and minimize toxicity. The results of our HbMPs showed *(i)* a low binding of anti-Hb antibodies, IgG and HP to surface-modified HbMPs, indicating a possible half–life extension in circulation, (ii) a protection of Hb against oxidation from PD- HbMPs, and (iii) a novel and simple fabrication process of HbMPs with a long-term storage stability using the macromolecule Odex instead of the small molecule GA.

Six types of surface-modified HbMPs were prepared by means of double and triple precipitation in presence of HSA, HA and Plu (HSA-d-HbMPs, HSA-t-HbMPs, HA-d-HbMPs, HA-t-HbMPs, Plu-d-HbMPs and Plu-t-HbMPs), respectively. PD-HbMPs prepared with the three different initial concentrations of monomeric dopamine (concentrations 0.75, 1, and 2.5 mg/mL) were specified as PD-HbMP-1, PD-HbMP-2 and PD-HbMP-3, respectively. The Odex-HbMPs fabricated with oxidized dextran MW of 40,000 (40T) and dextran MW 70,000 (70T) are named 40T-HbMP and 70T-HbMP, respectively.

3.1 The hydrodynamic particle size and Zeta potential

The particle size of surfaced-modified HbMPs, Odex-HbMPs and PD-HbMPs measured by dynamic light scattering were submicron size (around 800 to 1000 nm) which corresponds to the observations by TEM and SEM (Figure 1). No significant differences in the hydrodynamic size of surface-modified HbMPs, Odex-HbMPs and PD-HbMPs could be found when compared with unmodified HbMPs.

The zeta potential of surface-modified HbMPs, Odex-HbMPs and PD-HbMPs suspended in PBS (conductivity 18–20 mS/cm) showed a negative potential between -9 and -12 mV. Moreover, the effect of the dopamine polymerization on the zeta potential of PD-HbMPs was investigated in different dispersion solution. The zeta-potential of HbMPs (control) dispersed in water was -32.1 ± 0.92 mV. In contrast, the zeta potential of PD-HbMPs in water was positive (approx. 7-10 mV). As expected, with increasing ionic

strength the zeta potential of the HbMPs was reduced to -11.4 ± 0.81mV in PBS and to -8.46 ± 0.93mV in 0.9% NaCl solution. More interestingly, the zeta-potential of the PD-HbMPs in 0.9% NaCl turned to strongly negative values of approx. -20 mV. In contrast, the Zeta-potential values of the PD modified particles in PBS were similar to the values obtained for the unmodified HbMPs.

3.2 Morphology

The morphology of surface-modified HbMPs in presence of HSA was investigated by TEM. The HSA-d-HbMPs and HSA-t-HbMPs almost show a peanut- like shape (Figure 1B & 1C). The unmodified HbMP clearly show the porous structure compared to the surface-modified HbMPs with HSA. The HSA-d-HbMPs and HSA-t-HbMPs appeared denser core particle surrounded by a thick dark rim compared to un-modified HbMPs. This result corresponds to the HSA coating on a surface of HbMPs. The inserts provides CLSM images of HbMPs with different HSA surface modifications and demonstrate the high homogeneity of particles. For PD-HbMPs, SEM images showed an ellipsoidal with the average longest diameter and shortest diameter of 750 ± 55 nm and 550 ± 76 nm, respectively. Observed by TEM, the resulting on PD-HbMPs shows a color change after polymerization of dopamine (Figure 1F) when compared to unmodified HbMPs (Figure E). For Odex-HbMPs, SEM images show also clearly a peanut-like shape with shortest and longest diameter of about 700 and 1,000 nm (Figure 1G & 1H).

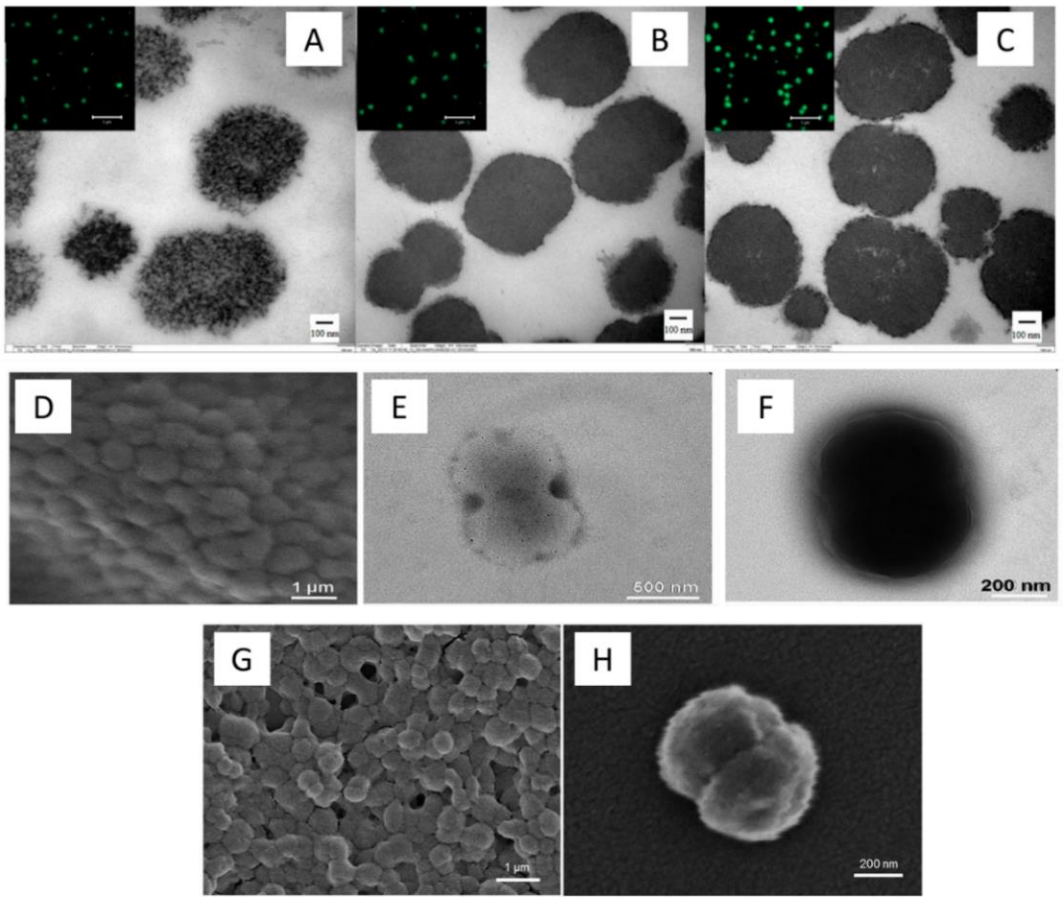

Figure 1. *The TEM images (scale bar: 100 nm) together with CLSM imaging (inserts, scale bar: 5µm) of unmodified HbMP (A) and surface-modified HbMPs with HSA adsorption by double precipitation technique (B) and triple precipitation technique (D). Example of SEM image (D) and TEM image (F) of PD-HbMPs compared with TEM image of HbMPs (E). Example of SEM images of 40T Odex- HbMPs (F,G).*

3.3 Hb Content and Functional Hb (Oxy-Hb)

In Figure 2A, the total Hb concentration of all surface-modified HbMP revealed no statistically significant difference compared to HbMPs (approx. 35 - 40 mg/mL as calculated into 20% Hct). The determination of Oxy-Hb by performing the ferricyanide method, each surface-modified HbMPs resulted in a range between 9 to 12 mg/mL calculated for a suspension with a Hct of 20%. The double precipitated HbMPs (HSA-d-HbMPs, HA-d-HbMPs and Plu-d-HbMPs) showed no statistically significant changes compared to HbMPs. In contrast to double precipitated HbMPs, the Oxy-Hb of triple precipitated HbMPs showed a significant decrease by 33 % , 32 % and 35% for HSA-t-HbMPs, HA-t-HbMPs and Plu-t-HbMPs, respectively, when compared to HbMPs (Figure 2B).

For Odex-HbMPs, the concentration of total Hb entrapped per particle was 28 - 30 mg/mL as calculated for a suspension with a Hct of 20% (Figure 2C). The values for 40T-HbMP and 70T-HbMP did not significantly differ from each other (28.8 ± 2.2 and 30.4 ± 2.1, respectively). The amount of functional Hb for 40T-HbMP and 70T-HbMP was 11.59 ± 0.6 and 12.53 ± 0.9 mg/mL, respectively (Figure 2D). For PD-HbMPs, the percentage of functional Hb to total Hb content was calculated and described in section 3.5.2.

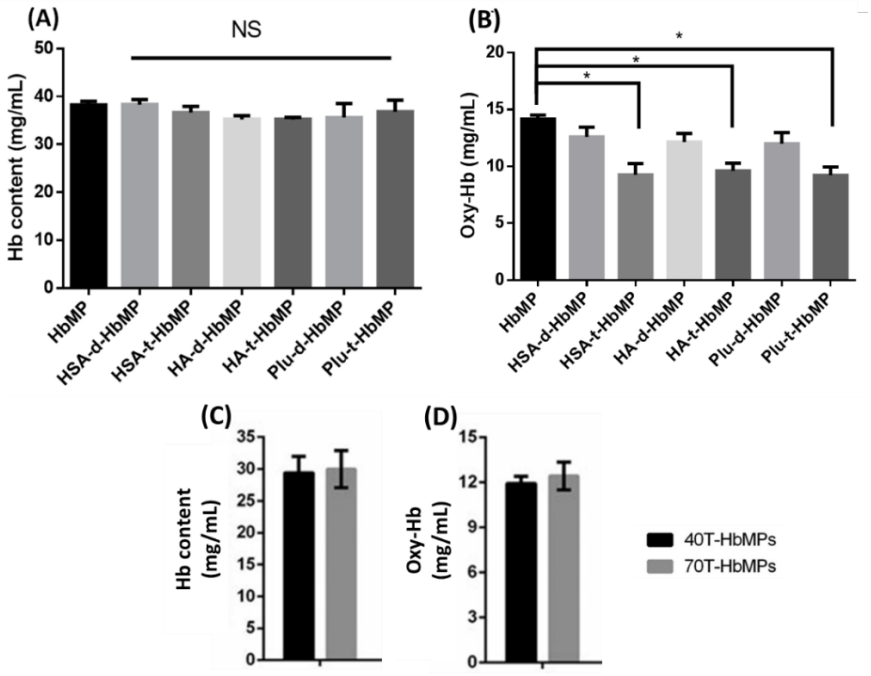

Figure 2. *The Hb content (A) and Oxy-Hb (B) of surface-modified HbMPs Data are represented as mean ± SD (n = 3). "NS" indicates not significant, whereas the asterisk indicates significant difference when compared to HbMPs (*P < 0.05). Moreover, The Hb content (C) and Oxy-Hb (D) of 40T and 70T Odex-HbMPs as calculated for a suspension with the Hct of 20%*

3.4 Binding assay of Anti-Hb antibodies, IgG and Haptoglobin to Surfaced-modified HbMPs

3.4.1 Binding of Anti-Hb Antibodies to Surface-Modified HbMP

The quality of the surface modification of the HbMPs was tested using indirect immunofluorescence staining with anti-Hb antibodies (Figure 3A). Unmodified HbMPs present Hb molecules on their surface that

will be recognized by the rabbit anti-bovine Hb antibodies (primary antibodies). The fluorescently labelled secondary goat anti-rabbit antibodies recognize subsequently the primary antibodies on the surface of the HbMPs and the fluorescence signal can be detected and analysed by flow cytometry. Figure 3B shows the percentage of non-specifically (only with secondary antibodies) and specifically stained particles with anti-Hb antibodies in each group (% events). The positive % events for non-specific binding were low (5 - 10 %), with no statistically significant differences between the different samples. The positive % events for specific anti-Hb antibody binding were very high for the non-modified HbMPs (approx.80%) and for HSA-d-HbMP (approx.70%), with no statistically significant differences. In the samples (HA-d-HbMPs and Plu-d-HbMPs) where double precipitation was performed the % positive events of specific anti-Hb antibody binding were statistically significantly decreased when compared to HbMPs (by 36% and 33%, respectively). Most importantly, the specific anti-Hb antibody binding in the samples modified by triple precipitation, HSA-t-HbMPs, HA-t-HbMPs and Plu-t-HbMPs, was decreased by 54%, 56% and 67%, respectively when compared to the unmodified HbMPs. Therefore, the surface-modifications by additional precipitations, especially by the triple precipitations, shield the Hb molecules and inhibits their recognition by the anti-Hb antibodies.

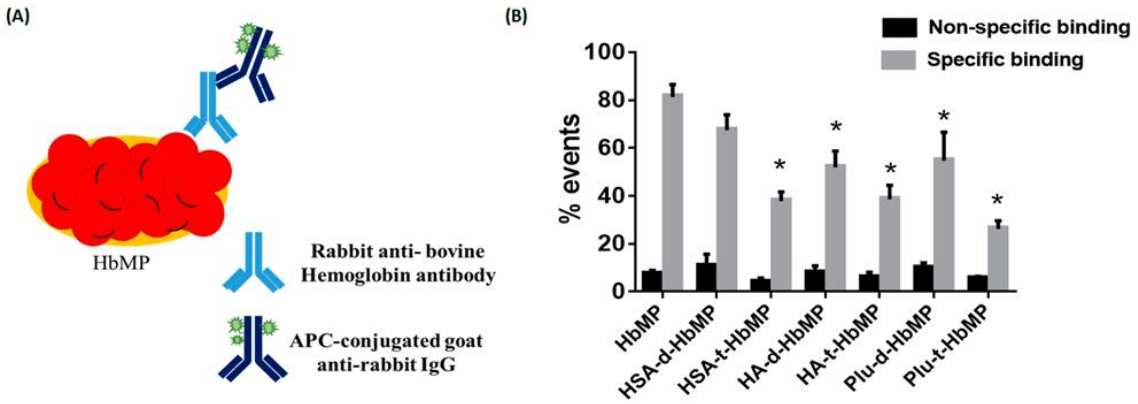

Figure 3. *Immunofluorescence assay with rabbit anti-bovine-Hb antibodies. (A) Schematic drawing representing a model of indirect immunofluorescence staining with anti-Hb antibodies. (B) The % positive events of non-specific and specific binding of anti-Hb antibodies on each surface-modified HbMPs. Specific binding was calculated by subtracting the non-specific binding from the total binding. Data represent the mean ± SD and asterisks indicate the significance of differences when compared to HbMPs (*P < 0.05).*

3.4.2 Binding of IgG to Surface-Modified HbMPs

The binding of IgG on the surface of particles leads to opsonization and recognition of the particles by the immunocompetent cells in the blood and tissues. Therefore, a high-quality surface modification should prevent the particles from IgG adsorption. Here we incubated our unmodified and modified HbMPs with biotinylated human IgG which was subsequently detected by APC-labelled streptavidin (Figure 4A) and analyzed by flow cytometry. The results presented in Figure 4B show that IgG binds to the unmodified HbMPs (roughly 50% positive events of specific binding). In contrast, all surface modified samples show statistically significant decreased events of specific binding by 80-95%. Therefore, the surface-modifications by additional precipitations successfully inhibit IgG binding to the particle surface.

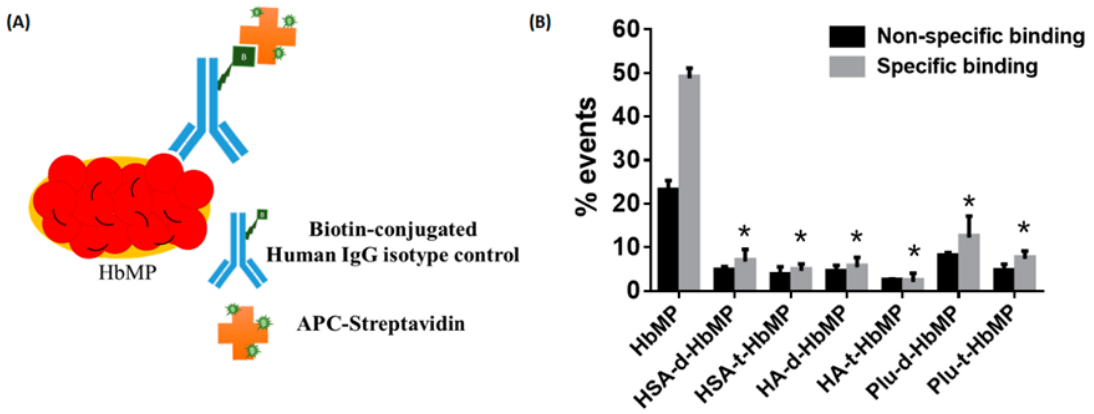

Figure 4. *Immunofluorescence assay of the binding of IgG (biotinylated) to surface-modified HbMPs. (A) Schematic drawing representing the staining procedure with APC-Streptavidin. (B) The % positive events of non-specific and specific binding of APC-Streptavidin on each surface-modified HbMPs. Specific binding was calculated by subtracting the non-specific binding from the total binding. Data represent the mean ± SD and asterisks indicate the significance of differences when compared to HbMPs (*P < 0.05).*

3.4.3 Binding of Haptoglobin to Surface-Modified HbMPs

HP is a glycoprotein that is mostly synthesized and secreted by liver cells and plays a vital role with a high affinity to free Hb in the circulation. It is responsible for the rapid clearance of acellular Hb from the blood circulation forming a complex with the β-chains of Hb which is then recognized by the Kupffer cells. Presumably, it can bind also to the Hb molecules on the surface of the HbMPs, which has to be avoided in order to achieve long circulation times of the HBOCs. We incubated our unmodified and modified HbMPs with human HP, which was subsequently detected by APC-labelled rabbit anti-human-HP antibodies (Figure 5A) and analyzed by flow cytometry. The results presented in Figure 5B show that HP binds to the unmodified HbMPs (roughly 25-30% positive events of specific binding). In contrast, all surface modified samples show statistically significant decreased events of specific binding by 80-95%. Therefore, the surface-modifications by additional precipitations successfully inhibit HP binding to the particle surface.

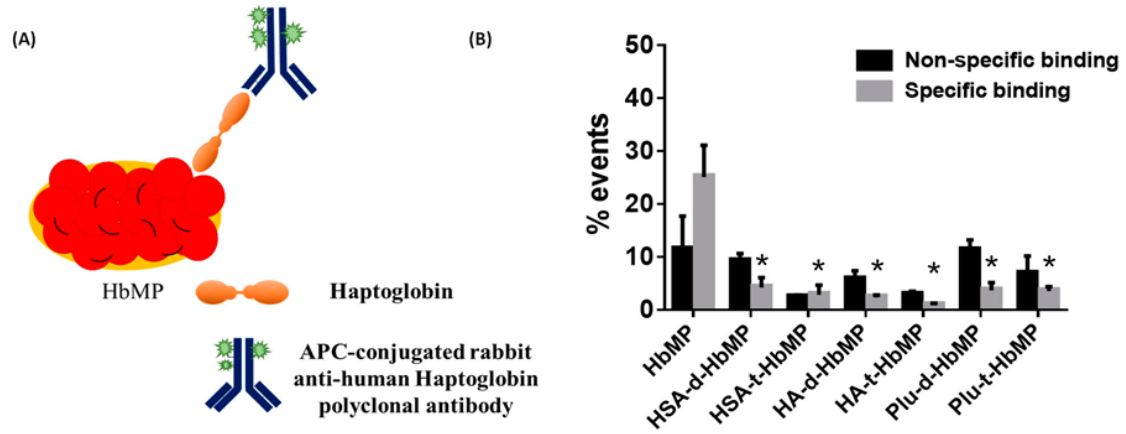

Figure 5. *Immunofluorescence assay of haptoglobin (HP) binding to surface-modified HbMPs. (A) Schematic drawing representing the staining procedure. (B) The % positive events of non-specific and specific binding of anti-HP antibodies on each surface-modified HbMPs. Specific binding was calculated by subtracting the non-specific binding from the total binding. Data represent the mean ± SD and asterisks indicate the significance of differences when compared to HbMPs (*P < 0.05).*

3.5 Anti-oxidative properties of PD-HbMPs

3.5.1 Degree of the polymerization of the PD-HbMPs

PD as the major pigment of eumelanin, typically shows broad-band absorption ranging from the ultraviolet to the visible region [40]. Hong et al. [41] reported the characteristic absorption peaks at 283 nm corresponding to the catechol moiety in the dopamine monomer. The peaks at 330 nm were observed as the reaction processed because of the oxidation of the catechol groups. Since our results suggested a linear relationship between the concentration of PD and absorption values at 320 nm, the concentration of PD attached to the HbMPs particles was determined by the absorption values at 320 nm after washing steps to remove the excess dopamine or PD. The results presented showed increasing the initial concentration of dopamine does apparently help with the amount of PD on the substrates. The amount of PD in one particle demonstrated higher polymerization from PD-HbMPs 1 to PD-HbMPs 3. It should be noted that the degree of polymerization with same reaction time is 75%, 58% and 26% for PD-HbMPs 1, PD-HbMPs 2 and PD-HbMPs 3, respectively.

3.*5.2 Oxidative protection of Hb in PD-HbMPs from Met-Hb formation*

The percentage of Hb-bound O_2 amount in the HbMPs was 27.6 ± 7 % with the 2 - 4% MetHb in the initial Hb solution. After the modification with PD the amount of Hb-bound O2 was 50.6 ± 7 % and 45.02 ± 12 % for PD-HbMPs-1 and for PD-HbMPs-2, respectively. However, the percentage of Hb-bound O_2 for PD-HbMPs-3, which have been treated with the highest concentration of monomeric dopamine, was 22.01± 5% and showed a decreased level when compare to unmodified HbMPs.

3.5.3 Oxidative protection of Hb in PD-HbMPs from H_2O_2

The capacity of oxidative protection of Hb against H_2O_2 was detected by the UV-Vis spectrum before and after H_2O_2 treatment. From the result (Figure 6B,6C), the absorbance of HbMPs was strongly reduced after incubation with H_2O_2 leading to the oxidation of Hb. In contrast, the absorbance reduction was far less in the presence of PD.

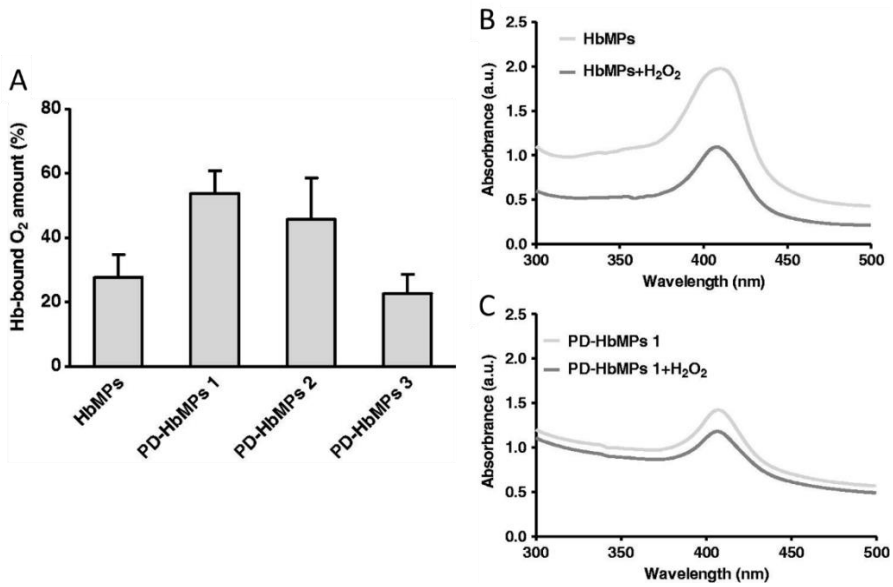

Figure 6. *The amount of Hb-bound O_2 from HbMPs and PD-HbMPs (A). Examples of UV-Vis spectra of (B) HbMPs and (C) PD-HbMPs 1 before and after the treatment with 5mM H_2O_2.*

3.5.4 Antioxidant activity of PD-HbMPs

The ability of the PD-HbMPs to scavenge free radicals was investigated with the DPPH and ABTS assay. As shown in Figure 7A for the DPPH assay, the PD-HbMPs exhibited a similar antioxidant activity like Trolox reaching 41% at the concentration of 1 mg/mL as compared to 55% for Trolox at the same concentration. It should also be pointed out that besides DPPH radicals, Trolox and PD-HbMPs showed also a similar antioxidant activity to scavenge ABTS radicals with up to 100% above the concentration of 0.5 mg/mL (Figure 7B). In contrast, Hb and HbMPs showed only a low antioxidant activity for both the DPPH and ABTS radicals.

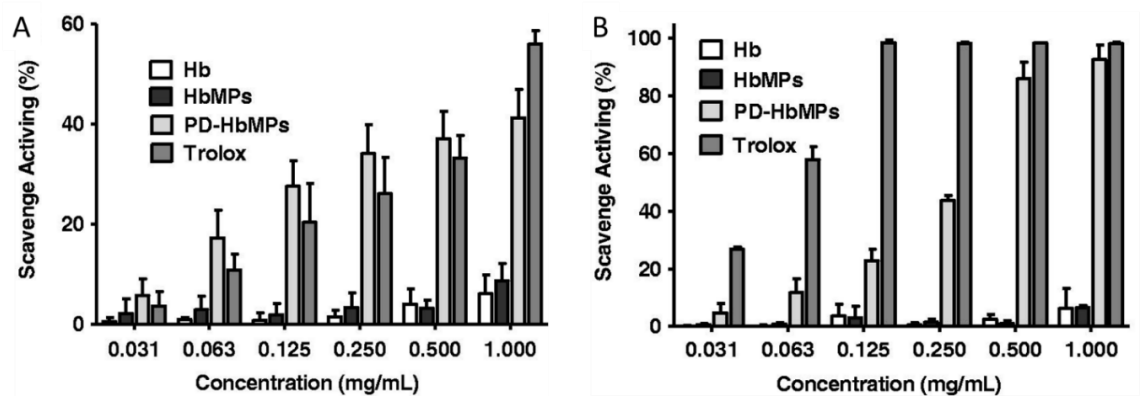

Figure 7. *Antioxidant activity of the Hb, HbMPs, PD-HbMPs and Trolox measured with (A) the DPPH assay (n=3) and (B) the ABTS assay.*

3.6 Stability of Odex-HbMPs

The stability of Odex-HbMPs was characterized by their Hb content, the fraction of functional Hb and the hydrodynamic size. The result showed that there was no change in size and the amount of functional Hb in saline solution at 4˚C during the period of 90 days (Figure 8).

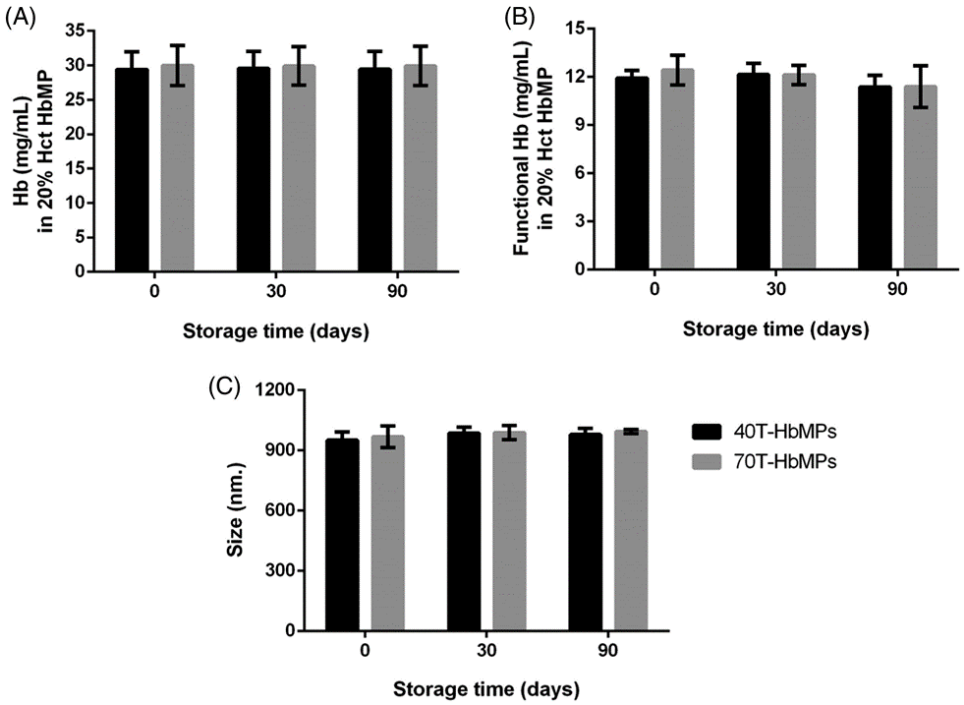

Figure 8. *Stability of Odex-HbMPs over 90 days. (A) Hb content of Odex-HbMPs, (B) concentration of functional Hb and (C) size stability by DLS analysis. Data are presented as mean ± SD (n=3).*

4. Discussion

The current development of blood substitutes, especially HBOCs, concentrates on investigations of their oxygen transport, immunogenicity, extended circulation half-life, lack of toxicity etc. We fabricated HbMPs and modified their surface applying the CCD-technique and characterized their physiochemical properties. Surface modifications of HbMPs were performed by double and triple precipitation with polymer as well as HSA adsorption and, moreover, by incorporation with PD for protection Hb against oxidation. Additionally, we established a novel and simple method for HbMP fabrication (one pot formulation technique) which optimizes the CCD technique using Odex as a cross-linker instead of the small molecule GA.

In order to fabricate the surface-modified HbMP, three biopolymers (HSA, HA and Plu) were used to cover the surface of HbMPs to shield the Hb within the HbMP against detection by antibodies or HP. Albumin and HA form complexes [42] and the amino groups of albumin are cross-linked by GA at the surface of HbMPs. That means HA will be immobilized at the surface, which was demonstrated by the zeta potential. In presence of HA the zeta potential become more negative compared with that of only albumin. Pluronic also interacts with albumin [43] and the same mechanism should work as for HA although only an indirect proof of the immobilization of Plu could be performed. The surface-modified HbMPs appeared as a peanut-shape with a particle size of around 1 micron. An aggregation of particles was not visible in each surface-modified HbMPs suspension, as shown in the CLSM images and a low polydispersity index by the Zetasizer measurement. Moreover, TEM images shown that the morphology of surface-modified HbMPs in presence of HSA produced is obviously different from the unmodified HbMPs. These results were also demonstrated in reasonable agreement with previously published work of our group [13,14,44]. From the ability of HSA, considering to be non-antigenic and biodegradable, and readily available, it has been more attractive and widely used as a biomaterial for particle preparation. In our fabrication process, HSA was adsorbed into HbMPs after the second and third precipitation and we assumed that HSA can cover the surface of HbMPs and help to avoid particle aggregation. The negative charges of HSA are also detected contributing to negative zeta-potential. From the result of the zeta potential of surface-modified HbMPs of about -11 mV the HSA-d-HbMPs and HSA-t-HbMPs were most likely covered with HSA because no significant difference could be found compared to HbMPs. In particular, the morphology of surface-modified HbMP in presence of HSA was visualized by TEM to confirm that the surface-modified HbMPs in presence of HSA were covered with HSA, exhibiting a thick dark rim on the surface of particles (Figure 1B & 1C). The HA surface modification had a significant influence on the zeta potential, resulting in a higher negative charge. No statistically significant effects of HA-HbMPs particle size compared to HbMP were found. Similar results are reported in our previous studies about the influence of HA on the $MnCO_3$ templated Hb/BSA-HA particles [45].

Generally, the oxygen binding capacity of HBOCs is commonly determined by the oxygen dissociation curves (ODC), which demonstrate the relationship between oxygen partial pressure and the oxygen fractional saturation [8]. However, this method requires a specific and expensive equipment, gas flow apparatus and gas tanks. Therefore some research groups attempted to overcome the high-cost instrument problem and proposed simpler method for the analysis of the oxygen binding capacity in the laboratory [46]. In this study, we applied the ferricyanide method to determine the concentration of Oxy-Hb as simple and inexpensive approach. The ferricyanide causes a conversion from oxyhemoglobin into Met-Hb with the

release of oxygen. The pO_2 in the surrounding medium corresponds to the oxygen amount bound to hemoglobin in the particles. Taking into consideration the reduction of the Oxy-Hb in triple precipitated HbMPs, the fabrication process could be the reason for it. The additional time for the third precipitation could lead that a part of the Hb converts into Met-Hb where the iron in the heme group goes over into the ferric state. Furthermore, the triple precipitated HbMPs had a higher GA concentration than HbMPs, so it can have an impact on the Oxy-Hb level due to increasing GA concentration during the cross-linking step can increase the level of Met-Hb in HBOCs [14,47].

Based on our previously reports of preclinical studies, HbMPs fabricated by CCD technique plays a more promising HBOCs for using as a blood substitutes by demonstrating their higher affinity to oxygen, avoiding vasoconstriction and non-mutagenicity [12,13]. Although the results of HBOCs in non-clinical studies have shown a good biocompability and safety, the circulatory clearance after administration is still a limitation and should be investigated for further clinical trial. In this study, we hypothesized that surface modification of HbMPs should be a promising approach and avoiding a rapid clearance. Therefore, it is critical to assess and investigate whether Hb of HbMPs after surface modification is completely shielded or not. We tested this hypothesis by using the immunofluorescence staining with anti-Hb antibodies, IgG and HP. The immunofluorescence staining is a common and efficient technique, which is based on the use of specific antibodies which have been conjugated to fluorescent dyes. Our data demonstrated that the double/triple precipitated HbMP in presence of HSA, HA and Plu can be successfully employed to shield the Hb resulting in a significant decrease of the specific binding compared to unmodified HbMPs. In particular, the triple precipitated HbMPs with surface coating by polymer showed the best promising approach and to reduce the binding of anti-Hb antibodies, IgG and HP. It can be noted that our surface modification can protect the HbMPs from the endogeneous Hb removal mechanisms and avoiding rapid clearance in the circulation.

The fabrication of PD-HbMPs is based on the mild self-polymerization reaction of PD under alkaline conditions following co-precipitation of Hb with $MnCO_3$. The color of the suspension changed from red to brown and continuously became black because of the polymerization of dopamine [48]. In our protocol, the dependence of the polymerization process on the starting dopamine concentration were taken into account. The efficiency of polymerization decreased with increasing starting concentration of dopamine at the same reaction time. These particles of submicron size (about 800 nm) cannot diffuse through the endothelium barrier and scavenge endothelial NO. Moreover, the size which is less than 1 µm can avoid excessive phagocytosis for a long circulation [49].

The excess of negative charges on the HbMPs surface could be attributed to the reaction of the amino groups of Hb with GA as well as the further attachment of new carboxyl groups to the particle surface after quenching with glycine [14], while the positive charges of PD-HbMPs could refer to protonated amino groups of quinone and catecholic groups from PD involved in the non-covalent binding to Hb [27]. Therefore, the zeta-potential of PD-HbMPs in PBS, NaCl and water was investigated due to the influence of the ionic strength of the suspending medium on the zeta potential. The zeta-potential is highly negative in water and becomes less negative in NaCl solution or in PBS. PD-HbMPs exhibit a positive zeta potential in water, because PD is highly positive charged and can compensate the negative charges of proteins. Important is that this change in the sign becomes visible only in water, which means PD is in the inner part of HbMPs

and not at the surface. This is confirmed by the determined zeta potential in NaCl solution, which is comparable with the zeta potential of HbMPs, because the Cl ions can completely shield the positive charges of PD.

In addition, the protection of Hb against autoxidation is a significant factor to maintain the function of Hb during and after the preparation of HBOCs. PD has been well-known as an efficient antioxidant appropriate for biological systems due to the presence of the distinct hydroquinone moiety [50]. PD-HbMPs could be proved to prevent the incorporated Hb from oxidation to Met-Hb. Moreover, PD-HbMPs exhibited the antioxidant capacity scavenging free radicals including H_2O_2. In conclusion, the synthesis of submicron Hb particles modified with PD provided a promising novel type of HBOCs with anti-oxidation activity.

For Odex-HbMPs fabrication, a novel one-pot fabrication technique was introduced and performed for protein microparticles based on the CCD procedure. The protein are precipitated and cross-linked in "one step" because the macromolecular cross-linker (Odex) precipitates and is entrapped in the particles together with the Hb. From this approach, one preparation step and several respective washing steps are excluded, which significantly reduces the preparation time. In comparison Hb content with HbMPs prepared by CCD technique using GA as a cross-linker [13,14,44], Odex-HbMP contain a comparable amount of Hb. The entrapped Hb amount could not be enhanced by increasing the initial concentration of the protein. In addition, the entrapped protein by $MnCO_3$ using a one-pot procedure had no significant difference in the entrapment efficiency of the CCD procedure (data not shown). In comparison with other fabrication methods, the new one-pot procedure provides easily and simple prepared HbMP.

Odex is promising as a highly effective macromolecular cross-linker because the carbonyl groups of the sugar open chains are capable of reacting with the free-NH_2 groups on the protein [51, 52]. The Odex-protein cross-linking process occurs simultaneously during the precipitation. This is obtained 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 [53]. The Odex concentration during co-precipitation influences the stability of Hb. At the Odex concentration of 2%, HbMPs exhibits 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. [54] reported earlier that dextran was able to decrease the autoxidation rate of Hb and could structurally stabilize Hb to avoid the oxidation process of the heme-containing protein and its denaturation. Odex-HbMPs fabricated by "one-pot" procedure fit to the criteria for the biomedical usages [55]. This procedure provided the uniform morphology, monodisperse and sub-micron size with negative zeta potential. The stability of Odex-HbMPs showed that there was no significant change of the particle size, size distribution and the total Hb content and the fraction of functional Hb of the Odex-HbMPs during at least 3 months storage. These results indicate a long shelf- life of Odex-HbMPs.

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Eidesstattliche Versicherung (Affidavit)

„Ich, Ausanai Prapan, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: Fabrication and Characterization of Hemoglobin Based Oxygen Carriers selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen werden von mir verantwortet.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

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Datum Unterschrift

Anteilserklärung an den erfolgten Publikationen

Ausanai Prapan hatte folgenden Anteil an den aufgeführten Publikationen:

Publication 1: **Prapan, A**.; Suwannasom, N.; Kloypan, C.; Chaiwaree, S.; Steffen, A.; Xiong, Y.;Kao,I.; Pruß, A.; Georgieva, R.; Bäumler, H. Surface Modification of Hemoglobin Based Oxygen Carriers Reduces the Recognition by Haptoglobin, Immunoglobulin and Hemoglobin Antibodies. Coatings 2019, 9(7), 454
IF: 2.330 (2018)

Contribution in detail: 70%. Performed experiments including particle fabrication, particle characterization, determination of functional Hb and Hb content, binding assay of antibodies and haptoglobin, analyzed and interpreted all the data together with the co-authors, wrote the draft of the manuscript. Results presented in figures 1, 3 to 7.

Publication 2: Kloypan, C.; **Prapan, A**.; Suwannasom, N.; Chaiwaree, S.; Kaewprayoon, W.; Steffen, A.; Xiong, Y.; Georgieva, R.; Bäumler, H. Improved Oxygen Storage Capacity of Haemoglobin Submicron Particles by One-Pot Formulation. *Artif. Cells, Nanomedicine,* Biotechnol. 2018, 46(sup3):1-9
IF: 3.026 (2017)
Contribution in detail: 25%. Partly performed experiments with Chiraphat Kloypan including particle fabrication and particle characterization as well as collecting these data and analyzing the results. Result presented in figure 4, 6, 7 (partly).

Publication 3: Baidukova, O.; Wang, Q.; Chaiwaree, S.; Freyer, D.; **Prapan, A.**; Georgieva, R.; Zhao, L.; Bäumler, H. Antioxidative Protection of Haemoglobin Microparticles (HbMPs) by Polydopamine, *Artif. Cells, Nanomedicine Biotechnol.* 2018, 46:sup3, 1–9.
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Contribution in detail: 5%. Partly performed experiments with Olga Baidukova and Quan Wang including particle fabrication and particle characterization as well as collecting these data and analyzing the results. Results (partly) presented in figures 2, 3, 5.

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Unterschrift, Datum und Stempel des betreuenden Hochschullehrers/der betreuenden Hochschullehrerin

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Printed copies of the selected publications

1. ***Publication 1***

Prapan, A.; Suwannasom, N.; Kloypan, C.; Chaiwaree, S.; Steffen, A.; Xiong, Y.;Kao,I.; Pruß, A.; Georgieva, R.; Bäumler, H. Surface Modification of Hemoglobin Based Oxygen Carriers Reduces the Recognition by Haptoglobin, Immunoglobulin and Hemoglobin Antibodies. Coatings 2019, 9(7), 454. https://doi.org/10.3390/coatings9070454

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2. ***Publication 2***

Kloypan, C.; **Prapan, A**; Suwannasom, N.; Chaiwaree, S.; Kaewprayoon, W.; Steffen, A.; Xiong, Y.; Georgieva, R.; Bäumler, H. Improved Oxygen Storage Capacity of Haemoglobin Submicron Particles by One-Pot Formulation. Artif. Cells, Nanomedicine, Biotechnol. 2018, 46:sup3, 1–9. https://doi.org/10.1080/21691401.2018.1521819

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3. ***Publication 3***

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Curriculum vitae

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

Complete list of Publications

1. **Prapan, A.;** Suwannasom, N.; Kloypan, C.; Chaiwaree, S.; Steffen, A.; Xiong, Y.;Kao,I.; Pruß, A.; Georgieva, R.; Bäumler, H. Surface Modification of Hemoglobin Based Oxygen Carriers Reduces the Recognition by Haptoglobin, Immunoglobulin and Hemoglobin Antibodies. Coatings 2019, 9(7), 454. **IF: 2.330 (2018)**

2. Kloypan, C.; **Prapan, A.**; Suwannasom, N.; Chaiwaree, S.; Kaewprayoon, W.; Steffen, A.; Xiong, Y.; Georgieva, R.; Bäumler, H. Improved Oxygen Storage Capacity of Haemoglobin Submicron Particles by One-Pot Formulation. Artif. Cells, Nanomedicine, Biotechnol. 2018, 46(sup3):1-9. **IF = 3.026 (2017)**

3. Baidukova, O.; Wang, Q.; Chaiwaree, S.; Freyer, D.; **Prapan, A.**; Georgieva, R.; Zhao, L.; Bäumler, H. Antioxidative Protection of Haemoglobin Microparticles (HbMPs) by Polydopamine. Artif. Cells, Nanomedicine Biotechnol. 2018, 46:sup3, 1–9. **IF = 3.026 (2017)**

4. 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. 2018; 47, 41-9. **IF= 3.026 (2017)**

5. Suwannasom, N.; Smuda, K.; Kloypan, C.; Kaewprayoon, W.; Baisaeng, N; **Prapan, A.**; Chaiwaree, S; Georgieva, R.; Bäumler, H. Albumin submicron particles with entrapped riboflavin – fabrication and characterization. Nanomaterials.2019; 9(3):482. **IF= 4.034 (2018)**

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