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Research paper

Albumin-derived perfluorocarbon-based artificial oxygen carriers: A physico-chemical characterization and first *in vivo* evaluation of biocompatibility

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ABSTRACT

Until today, artificial oxygen carriers have not been reached satisfactory quality for routine clinical treatments. To bridge this gap, we designed albumin-derived perfluorocarbon-based nanoparticles as novel artificial oxygen carriers and evaluated their physico-chemical and pharmacological performance.

Our albumin-derived perfluorocarbon-based nanoparticles (capsules), composed of an albumin shell and a perfluorodecalin core, were synthesized using ultrasonics. Their subsequent analysis by physico-chemical methods such as scanning electron-, laser scanning- and dark field microscopy as well as dynamic light scattering revealed spherically-shaped, nano-sized particles, that were colloidally stable when dispersed in 5% human serum albumin solution. Furthermore, they provided a remarkable maximum oxygen capacity, determined with a respirometer, reflecting a higher oxygen transport capacity than the competitor Perftoran®. Intravenous administration to healthy rats was well tolerated. Undesirable effects on either mean arterial blood pressure, hepatic microcirculation (determined by *in vivo* microscopy) or any deposit of capsules in organs, except the spleen, were not observed. Some minor, dose-dependent effects on tissue damage (release of cellular enzymes, alterations of spleen's micro-architecture) were detected.

As our promising albumin-derived perfluorocarbon-based nanoparticles fulfilled decisive physico-chemical demands of an artificial oxygen carrier while lacking severe side-effects after *in vivo* administration they should be advanced to functionally focused *in vivo* testing conditions.

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1. Introduction

Allogenic erythrocyte transfusions represent a life-securing intervention in modern medicine. However, the availability of erythrocyte concentrates (ECs) is limited and they are discussed critically because of potential infection risks and immunomodulation phenomena [1–3]. The implementation of restrictive transfusion triggers [1] as well as of more global measures called "patient blood management" [2] are still not sufficient to resolve the shortage of ECs, caused by the demographic change and the co-occurring declining willingness for blood donation of the population [4–6]. However, efficient supply of molecular oxygen (O_2) to the tissue with a simultaneous removal of carbon dioxide from the organism, is impaired not only in anemic or hemorrhagic

Abbreviations: ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; CK, creatine kinase; DAPI, 4′,6-diamidin-2-phenylindole; FITC, fluorescein isothiocyanate; ^{19}F-NMR, ^{19}F-nuclear magnetic resonance spectroscopy, HSA, human serum albumin; HSA, , human serum albumin; IVM, *intravital* microscopy; LDH, lactate dehydrogenase; LSM, laser scanning microscopy; MAP, mean arterial blood pressure; PFC, perfluorocarbon; PFD, perfluorodecalin; PI, polydispersity index; SEM, scanning electron microscopy; EC, erythrocyte concentrate; pO_2, pCO_2 oxygen and carbon dioxide partial pressure; RI, refractive index; Vis, viscosity; ANOVA, analysis of variance.

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patients [1,7,8] but also at blood shortage scenarios [9]. In such situations, artificial oxygen carriers dispersed in plasma-like media would be an urgently awaited alternative to ECs. Most importantly, ECs change their physico-chemical qualities during storage while until today the consequences of those changes on patients as well as the optimal parameters of storage of ECs are still not defined unanimously [5,10]. Although artificial oxygen carriers are available in a couple of countries such as Russia, Ukraine, Mexico, Kyrgyzstan and Kazakhstan (Perftoran®, perfluorocarbon-based) [11,12] as well as South Africa and Russia (Hemopure®, hemoglobin-based) [13], authorities in Europe, Japan or USA still reject those drugs because of unbalanced risk-benefit analysis [13]. However, the use of perfluorocarbon-based artificial oxygen carriers is particularly attractive as perfluorocarbons (elsewise than hemoglobin-based drugs) may be used not only to bridge blood loss but also for therapy of decompression sickness [14,15] and smoke poisonings [16,17] as they remain functional even in the presence of flue gases, e.g. carbon monoxide. For intravenous use, perfluorocarbons must be processed to become compatible with the aqueous medium blood [18]. This is achieved either by emulsification or encapsulation [11,19–23]. However, both alternatives go along with various undesirable side-effects that have been attributed to biological incompatibility of the emulsifiers or to immune reactions against the synthetic polymers that have been used for encapsulation [19,22,24–27]. In contrast, the biopolymer albumin qualifies in particular for medical research purposes due to the absence of toxicity and antigenicity [28]. Because of the amphiphilic character of albumin, there is no need to introduce any additional emulsifier [29]. Albumin also appears in the context of artificial oxygen carriers; in fact in both classes: hemoglobin-based [30–32] and perfluorocarbon-based artificial oxygen carriers [33]. However, the perfluorocarbons used in the past led to severe side-effects [34] and exhibited excessively long organ retention (not tolerated by regulatory authorities) [11]. Meanwhile it is known, that other perfluorocarbons, e.g. perfluorodecalin (PFD) or perfluorooctyl bromide, show acceptable organ retention times and less severe side-effects [35,36]. This is why we combined for the first time the medical appropriate perfluorocarbon PFD with the most promising synthesis procedure (ultrasonics in the presence of albumin) resulting in albumin-derived perfluorocarbon-based artificial oxygen carriers (capsules). The present study was to conduct an *in vitro* characterization and first *in vivo* evaluation of the novel capsules to prove their feasibility for intravenous administration. Therefore, their physico-chemical properties including the absolute oxygen capacity as well as effects on blood viscosity were studied *in vitro*. Furthermore, effects of the capsules on acid base status, tissue damage, mean arterial blood pressure, hepatic microcirculation as well as their organ distribution were studied *in vivo*.

2. Materials and methods

2.1. Materials

5% human serum albumin solution (5% HSA, containing 5% human serum albumin, 0.75% NaCl, 0.11% sodium-N-acetyltryptophanoate, 0.07% sodiumcaprylate) was purchased from Baxter (Unterschleissheim, Germany). Perfluorodecalin (PFD) was from Fluorochem Chemicals (Derbyshire, UK), CASO Bouillon from Carl Roth (Karlsruhe, Germany), 1.5% agar from Merck (Darmstadt, Germany) and limulus amebocyte lysate reagent water from Lonza (Walkersville, MD, USA). NaCl (0.9%) was obtained from B. Braun (Melsungen, Germany). All other chemicals were purchased from Sigma Aldrich (Steinheim, Germany).

2.2. Synthesis of capsules

The synthesis of capsules was based on a method of Sloviter et al. [33]. In detail, 5 ml of 5% HSA and 1 ml PFD were combined in a reaction tube with a total capacity of 15 ml. The reaction tube was cooled in an ice bath and the mixture was sonicated for 90 s using a sonotrode with a tip diameter of 3 mm associated with a UP 400S ultrasonic processor (Hielscher, Teltow, Germany). For sonication the tip of the sonotrode was placed at the PFD–water interface. At a power of 400 W, ultrasonic amplitudes with 210 μm and a frequency of 24 kHz were generated. After synthesis, capsules were adjusted to 32 vol% or 64 vol% using micro-hematocrit glass capillary tubes (d = 1.15 mm, Brand, Wertheim, Germany) and a centrifuge (Universal 320R, Hettich, Tuttlingen, Germany) with a hematocrit rotor.

2.3. Scanning electron microscopy (SEM)

For SEM measurements, lager capsules (0.8 μm diameter) were synthesized, washed with and stored in purified H_2O (purified with a Milli-Q® Integral System from Merck Millipore).

For routine SEM, capsules, suspended in an aqueous solution, were fixed with 2.5% glutaraldehyde in purified H_2O for 30 min. After centrifugation, the specimens were dropped on poly-lysine covered glass slips, routinely dehydrated in a graded series of ethanol followed by critical point drying (CPD 7501, Polaron) and sputtered with platinum/palladium (208HR, Cressington). Capsules were analyzed in a Hitachi S-4000 SEM and images were obtained with a DISS5 (Point Electronics) analysis system.

2.4. Laser scanning microscopy (LSM)

For LSM procedure, capsules were synthesized using a combination of 90% HSA and 10% fluorescein isothiocyanate-labeled (FITC) HSA. Therefore, human albumin derived from rice was labeled with FITC and purified according to the method of Brookes and Kaufman [37]. To obtain larger capsules for better visualization the power of the sonotrode and the sonification time were reduced to 160 W and 30 s, respectively. A laser-scanning microscope (LSM 510, Zeiss, Oberkochen, Germany) equipped with an argon laser was used to study the capsules. The objective lens was a 100× NA 1.30 oil Fluar. Image processing and evaluation were performed using the software of the LSM 510 imaging system.

2.5. Flow cytometric analysis

The total number of capsules/μl equivalent to a 32 vol% capsule-dispersion was determined with flow cytometric analysis using FITC-labeled capsules. To that purpose, human albumin derived from rice was labeled with FITC and purified according to the method of Brookes and Kaufman [37]. FITC-HSA-spectrum was checked afterwards on a fluorometer (RF-1501, Shimadzu, Duisburg, Germany) before a mixture of HSA and FITC-HSA (9:1) was used for standard capsule synthesis (see above). Six different batches of FITC-labeled capsules (32 vol%) were produced, transferred into purified H_2O and measured in repeated determination. Flow cytometric data was acquired on a Navios cytometer (Beckman Coulter, Krefeld, Germany). Labeled capsules were detected in FL1 channel, each FITC-labeled event was considered as capsule. Proper controls were used for standardization. For quantitation and standardization purposes, Accu check counting beads were used (Invitrogen, Life Technologies, Darmstadt, Germany). Fifty μl of capsules (diluted 1:10 with purified H_2O) were mixed with 400 μl purified H_2O and 100 μl Accu check counting beads. Data was analyzed by using Kaluza Version 1.2 (Beckman Coulter).

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