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Research Paper

Biodistribution of size-selected lyophilisomes in mice

Etienne van Bracht [a,1], René Raavé [a,1], Igor Y. Perevyazko [f], Elly M. Versteeg [a], Theo G. Hafmans [a], Ulrich S. Schubert [b,c,d], Egbert Oosterwijk [e], Toin H. van Kuppevelt [a], Willeke F. Daamen [a,*]

[a] Radboud University Medical Center, Radboud Institute for Molecular Life Sciences, Department of Biochemistry, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands
[b] Friedrich Schiller University Jena, Laboratory of Organic and Macromolecular Chemistry (IOMC), Humboldtstr. 10, 07743 Jena, Germany
[c] Friedrich Schiller University Jena, Jena Center for Soft Matter (JCSM), Philosophenweg 7, 07743 Jena, Germany
[d] Dutch Polymer Institute (DPI), P.O. Box 902, 5600 AX Eindhoven, The Netherlands
[e] Radboud University Medical Center, Radboud Institute for Molecular Life Sciences, Department of Urology, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands
[f] Department of Molecular Biophysics and Physics of Polymers, St. Petersburg State University, Ulianovskaya Str. 1, 198504 St. Petersburg, Russia

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ABSTRACT

Lyophilisomes are a novel class of proteinaceous biodegradable nano/microparticle capsules developed for tumor drug delivery. The *in vivo* characteristics of lyophilisomes are unknown and, therefore, the time course of biodistribution of sized albumin-based lyophilisomes in CD1 mice after intravenous administration was studied. Lyophilisomes, prepared from Dylight680-labeled albumin, were sized using a sucrose gradient centrifugation methodology and four fractions with a mean size of approximately 200 nm, 400 nm, 550 nm, and 650 nm were pooled for *in/ex vivo* localization, (immuno)histochemistry and biochemical analysis. Lyophilisomes were rapidly taken out of the circulation by the liver and spleen. Immunohistochemistry revealed that lyophilisomes were taken up in the liver by F4/80 positive macrophages, and in the spleen by Sign-R1 positive macrophages specifically located in the marginal zones. Lyophilisomes were most likely degraded by the liver and spleen and subsequently excreted via the urine, as high levels of degraded Dylight680-labeled albumin were detected in the urine. This was corroborated by electron microscopy of the spleen, which showed intact lyophilisomes in the marginal zone 5 and 30 min after injection, but not after 2 h. In conclusion, IV injected lyophilisomes are rapidly entrapped by liver and splenic macrophages, biodegraded, and excreted in the urine.

1. Introduction

Nanoparticles and their potential application as drug delivery systems for pharmaceuticals have been extensively studied [1,2]. Research has focused on drug delivery systems assembled from a range of materials including natural or synthetic lipid liposomes, metal particles, polymeric particles, amino acid-, sugar- and nucleotide-based micelles, and protein-based particles [3]. Sizes range from a few nanometers for dendrimers and micelles [4], up to hundreds of nanometers to micrometers for liposomes, polymersomes, and (protein-based) nanospheres [5–7]. Despite the abundant research on these drug delivery systems, only few are currently clinically applied.

For cancer therapy, an effective strategy to deliver anti-tumor drugs may be intravenous injection of drug loaded nanoparticles. This methodology exploits the enhanced permeability and retention (EPR) effect that is associated with hyperpermeability of tumor vasculature and lack of lymphatic drainage [8–10]. Through normal endothelium, nanoparticles cannot extravasate as it is prevented by tight endothelial junctions (5–10 nm) between endothelial cells [11]. However, the cut-off size for permeability in individual tumors is increased to 200–800 nm [12]. Due to this EPR effect, drug delivery particles (<800 nm) can extravasate from blood vessels to tumor tissue and accumulate, creating high local drug concentrations [13], with the potential to eliminate tumor cells more efficiently while reducing drug exposure to healthy cells.

In a quest for stable and efficient carrier systems, we have developed a drug delivery system named 'lyophilisomes' [14]. Lyophilisomes are prepared by a freezing, annealing, and lyophilization process that results in stable nano to micrometer sized capsules. As base material, a wide range of water-soluble

* Corresponding author at: Department of Biochemistry (280), Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. Tel.: +31 24 3610557; fax: +31 24 3616413.
E-mail addresses: Etienne.vanBracht@Radboudumc.nl (E. van Bracht), Rene.Raave@Radboudumc.nl (R. Raavé), Igor.Perevyazko@uni-jena.de (I.Y. Perevyazko), Elly.Versteeg@Radboudumc.nl (E.M. Versteeg), Theo.Hafmans@Radboudumc.nl (T.G. Hafmans), Ulrich.Schubert@uni-jena.de (U.S. Schubert), Egbert.Oosterwijk@Radboudumc.nl (E. Oosterwijk), Toin.vanKuppevelt@Radboudumc.nl (T.H. van Kuppevelt), Willeke.Daamen@Radboudumc.nl (W.F. Daamen).
[1] These authors contributed equally to this work.

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macromolecules can be used (*e.g.* albumin, elastin, and heparin), making it an adaptable carrier system toward multiple drug delivery applications. The system is flexible, allowing the incorporation of virtually any biomolecule in the wall and/or lumen. For instance, enzymes incorporated either in the capsule wall or the lumen remain biological active, partly due to mild preparation conditions [14].

Recently, we investigated lyophilisomes prepared from bovine serum albumin. Albumin as a core material for lyophilisomes harbors a number of beneficial characteristics for drug delivery systems, as it is biocompatible, biodegradable to yield only innocuous degradation products, non-toxic and non-immunogenic. Furthermore, albumin is easy to purify and widely available, making it a suitable candidate for nanoparticle preparation [15–19]. We have shown that albumin-based lyophilisomes can be efficiently loaded with doxorubicin or curcumin and are able to efficiently eliminate tumor cells *in vitro* [20]. In order to obtain a selective drug delivery system, albumin-based lyophilisomes have also been modified with antibodies resulting in specific targeting of the cell of interest and facilitating the uptake of lyophilisomes *in vitro* [21]. Moreover, lyophilisomes conjugated with cell penetrating peptides increased cellular association and internalization in tumor cells [22]. These data indicate that albumin-based lyophilisomes may be used as a targeting system for the delivery of therapeutic agents. Although many parameters of lyophilisomes have been investigated *in vitro*, little is known about their characteristics *in vivo*.

In this study, the biodistribution of lyophilisomes was investigated. Property sized lyophilisomes were administered intravenously and the organ distribution and degradation characteristics were studied using *in vivo* fluorescence, light/electron microscopy and biochemical assays.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA) was purchased from PAA Laboratories (Linz, Austria). Dylight680 (amine reactive) was purchased from Pierce (Rockford, IL, USE). Glutaraldehyde, formaldehyde and 4',6-diamidino-2-phenylindole (DAPI) were obtained from Merck (Darmstadt, Germany). Rat anti-mouse F4/80 monoclonal antibody (MCA497RT, clone Cl:A3-1, lot 0212) and rat-anti mouse Sign-R1 (MCA2394Tl, clone Cl:ER-TR9, lot 0413) were obtained from AbD Serotec (Bio-Rad Laboratories, Hercules, CA, USA). Goat anti-rat IgG (H + L) Alexa fluor 488 conjugated antibody (A-11006, lot 52955A) and goat anti-rat IgM (μ chain) Alexa fluor 488 conjugated antibody (A-21212, lot 1252825) were purchased from Molecular Probes (Life Technologies, Grand Island, NY, USA).

2.2. Methods

2.2.1. Preparation of lyophilisomes

To image the lyophilisomes *in vivo*, Dylight680 (amine reactive) was conjugated to the free amine groups in albumin, applying the manufacturer's protocol, and after dialysis in 0.01 M acetic acid, labeled and non-labeled albumin were mixed in a 1:4 ratio. Albumin lyophilisomes were prepared as described [20]. Briefly, droplets of a solution of 0.25% (w/v) (labeled) BSA in 0.01 M acetic acid were frozen in liquid nitrogen (−196 °C) and then incubated at −10 to −20 °C for 3 h (annealing step), followed by lyophilization. This procedure results in hollow nano/micro spheres (''lyophilisomes''). Generally, we used 40 mL of a 0.25% BSA solution, which corresponds to 100 mg albumin (2.5 mg/mL). One batch of 40 mL was sufficient to perform the animal experiment. To stabilize lyophilisomes, they were vapor crosslinked with glutaraldehyde and formaldehyde. Subsequently, lyophilisomes were incubated in 1% (w/v) glycine (Scharlau, Barcelona, Spain) in phosphate buffered saline (PBS; pH 7.4) for 1 h under rotation to quench free aldehydes. Next, lyophilisomes were washed three times (17,000*g*, 5 min, 4 °C) with 0.1% (v/v) Tween-20 (Sigma Aldrich, Steinheim, Germany) in phosphate buffered saline (PBS-T, pH 7.4). The final lyophilisome preparation was centrifuged three or four times at 60*g* for 3 min to remove large lyophilisomes and sheet-like structures, until no pellet was observed. After this procedure, about 30% of the original weight of lyophilisomes remained. Lyophilisomes (1 mg/mL) were stored in PBS-T (pH 7.4).

2.2.2. Selecting appropriate sized lyophilisomes by density gradient centrifugation

In order to sort nanoparticles out of the lyophilisome population prepared as described above, centrifugation was used with a linear sucrose density gradient [23]. The linear sucrose density gradients were prepared in 14 mm diameter and 13.2 mL capacity ultracentrifuge tubes (*ultra clear* tubes, Beckman, Indianapolis, IN USA) by a gradient device that is composed of two chambers connected via a channel with a stopcock. To create a border for aggregated particles, 0.5 ml of 60% (w/w) sucrose solution was placed on the bottom of the tube. Subsequently, the gradient was prepared by mixing 5.6 mL of 10% (w/w) and 5.6 mL of 40% (w/w) sucrose solution in a mixing chamber of which the 10% sucrose solution is loaded first. The final gradient volume was 11.7 mL. The prepared gradient was stored for one hour at ambient temperature and loaded with the lyophilisome suspension (0.5 mL, 1.0 mg/ml). The tubes were placed in a swinging bucket rotor (SW 41 Ti rotor, Beckman) and centrifuged at 1543*g* in a Beckman Optima L-XP ultracentrifuge (Beckman) for 10 min. After centrifugation, fractions were collected using a peristaltic pump and a narrow tube, inserted from top to bottom in the centrifuge tube. For each fraction, 0.5–1.0 mL of the solution was collected. Dynamic light scattering (DLS), qNano and analytical ultracentrifugation (AUC) were used to analyze the mean size of the fractioned lyophilisomes. The lyophilisome concentration of each fraction was determined by optical density (λ = 280 nm) to investigate recovery.

2.2.3. Particle characterization

2.2.3.1. Dynamic light scattering (DLS). DLS was performed on a DynaPro Plate Reader Plus (Wyatt Technology Corporation, Santa Barbara, CA) equipped with a 60 mV linearly polarized gallium arsenide (GaAs) laser (λ = 832.5 nm), operating at an angle of 156°. DLS data were collected from lyophilisomes dispersed in PBS-T (pH 7.4). Data were analyzed using Dynamics software ver.6.10 by the method of cumulants [24].

2.2.3.2. qNano. The qNano (Izon, Science Ltd., Burnside, New Zealand) was used to measure particle size of lyophilisomes [20,25]. To ensure a continuous flow of particles, two different pore sizes were used. For unsorted lyophilisomes, a pore size of approximately 600–2000 nm was used, while for sorted lyophilisomes a pore size of approximately 100–800 nm was used. Data were analyzed with Izon Control Suite 2.1 software.

2.2.3.3. Analytical ultracentrifugation. Sedimentation velocity experiments were performed with a ProteomeLab XLI analytical ultracentrifuge (Beckman Coulter, Brea, CA), using conventional double-sector Epon centerpieces of 12 mm optical path length and a four-hole rotor (AN-60Ti). Rotor speed was set at 3000–6000 rpm, depending on the sample. The rotor was equilibrated in advance for approximately 1 h at 20 °C in the centrifuge. Cells were filled with 420 μL sample solution and 440 μL solvent (0.5% BSA/0.02% sodium azide in PBS). Sedimentation (s) profiles were