Colloids and Surfaces B: Biointerfaces 141 (2016) 576–583

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Development and characterization of an innovative heparin coating to stabilize and protect liposomes against adverse immune reactions

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ARTICLE INFO

Article history:
Received 30 December 2015
Accepted 5 February 2016
Available online 8 February 2016

Keywords:
Drug delivery system
Cationic liposomes
Surface coating
Novel heparin complex
Complement system

ABSTRACT

Liposomes have been recognized as excellent drug delivery systems, but when they come in direct contact with different blood components they may trigger an immediate activation of the innate immune system. The aim of the present study was to produce long-circulating, blood-compatible liposomes by developing a construct of liposomes covered by a novel unique heparin complex (CHC; 70 heparin molecules per complex) to avoid recognition by the innate immune system. Unilamellar, cationic liposomes were produced by hand extrusion through a 100-nm polycarbonate membrane. Coating of liposomes with the macromolecular CHC was accomplished by electrostatic interactions. Dynamic light scattering as well as QCM-D measurements were used to verify the electrostatic deposition of the negatively charged CHC to cationic liposomes. The CHC-coated liposomes did not aggregate when in contact with lepirudin anticoagulated plasma. Unlike previous attempts to coat liposomes with heparin, this technique produced freely moveable heparin strands sticking out from the liposome surface, which exposed AT binding sites reflecting the anticoagulant potentials of the liposomes. In experiments using lepirudin-anticoagulated plasma, CHC-coated liposomes, in contrast to non-coated control liposomes, did not activate the complement system, as evidenced by low C3a and sC5b-9 generation and reduced leakage from the liposomes. In conclusion, we show that liposomes can be successfully coated with the biopolymer CHC, resulting in biocompatible and stable liposomes that have significant application potential.
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1. Introduction

Liposomes that basically mimic biological membranes have been recognized as excellent drug delivery systems, since they can entrap hydrophilic drugs in their aqueous core as well as insert hydrophobic drugs in their lipid bilayer. These phospholipid bilayer vesicles, can range in size from about 20 nm to several tens of micrometers [1]. One of their crucial advantages beside biocompatibility is their capacity to be adaptable to individual needs. Liposomal formulations can differ in many factors, including amongst others, composition, bilayer fluidity, diameter, surface charge or lamellarity.

Even though liposomes have promising drug carrying potential, several obstacles, such as low encapsulation efficiency, short circulation half-life, instability and limited shelf life, aggregation, poor reproducibility as well as limited sterilization possibilities may accompany their production and application. In addition, liposomes entering the circulation may trigger an immediate activation of the innate immune system on direct contact with different blood components depending on liposomal size, shape, charge and composition [2].

The complement system as well as immunoglobulins play a significant role in an enhanced liposomal blood clearance, which has important consequences for the application potential of liposomes as drug delivery systems. Receptor-mediated recognition of opsonized liposomes paves the way for the phagocytic uptake by macrophages, monocytes or neutrophils and results in rapid clearance of liposomes from the circulation [3–6]. Especially the liposomal surface net charge dictates which route of the

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http://dx.doi.org/10.1016/j.colsurfb.2016.02.014
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complement system is activated. Negatively charged liposomes activate the complement cascade *via* the classical pathway, positively charged liposomes elicit complement *via* the alternative pathway [7,8]. Neutral liposomes also activate a recently described non-proteolytic activation process [9]. Furthermore, recognition of non-coated liposomes by the immune system leads to adverse reactions. The response is a hypersensitivity, or infusion reaction, recently described as complement activation-related pseudoallergy (CARPA) [10].

A common strategy to prevent complement-mediated clearance by the mononuclear phagocyte system is to prevent the interaction with humoral blood components by shielding the liposomal surface. So far, poly-ethylene glycol (PEG), which is a non-toxic, linear polyether diol, has been widely used as a liposomal stabilizer against unwanted activation of the immune system [11]. However, it is well known that sterically stabilized PEG liposomes trigger the phenomenon of accelerated blood clearance (ABC) upon repeated injection within certain intervals [12]. Ishida and colleagues proposed that anti-PEG IgM, in response to a first injection, selectively binds to the PEG chains upon a second injection administered several days later leading to complement activation and an enhancement of the uptake of the second dose by Kupffer cells [13,14].

An alternative is to use biological polymers for shielding the liposomal surface. One such biopolymer is heparin, a highly sulfated glycosaminoglycan, which is present naturally in mast cells. Even though it is mainly known for its anticoagulant characteristics, heparin also has anti-inflammatory and immunomodulatory properties [15]. Sahli et al. showed that liposomal conjugation with heparin H108 was correlated with increased liposome stability, reduced leakage and improved haemocompatibility [16–18]. Recently, Chen et al. published promising results for cancer metastasis treatment by applying low molecular weight heparin-coated doxorubicin-loaded liposomes in an experimental melanoma lung metastasis model [19].

In the present study we wanted to reach an improved “stealth effect” and produce optimally long-circulating, non-activating liposomes as drug delivery system *via* liposome-surface modification by using a novel heparin complex (CHC). This novel heparin complex comprises about 70 molecules of heparin, with each having a molecular weight of ca. 13,000 g/mol, that are covalently bound to a polyamine carrier backbone chain [20]. The advantage with this complex is that the multiple arm structure allows many of the antithrombin (AT) binding sequences on heparin not to be involved in the interaction and anchoring of the complex to the surface creating a surface with freely movable heparin strands compared to surfaces coated with single chain heparin (Fig. 1). This improves the blood compatibility of a material surface with regard to coagulation, complement, and platelet activation within the heparin surface concentration interval corresponding to 6–12 pmol antithrombin/cm^2 [21]. By employing an electrostatic technique we coated liposomes with CHC and created highly blood compatible liposomes.

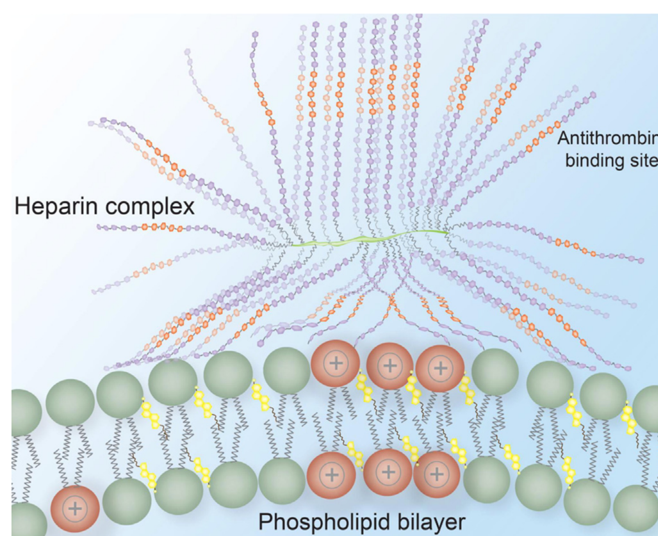

Fig. 1. Scheme of the electrostatic deposition of the negatively charged novel heparin complex on a cationic phospholipid bilayer.

2. Materials and methods

2.1. Material

Dimethyldioctadecylammonium bromide ($C_{38}H_{80}NBr$; DDAB) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine ($C_{40}H_{80}NO_8P$; DPPC) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol, 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) and *p*-xylene-bis(*N*-pyridinium bromide) (DPX) were purchased from Sigma–Aldrich (Munich, Germany). All lipids were used without extra purification. Phosphate buffered saline (PBS) tablets were obtained from Medicago AB (Sweden). The novel heparin complex CHC (Corline Heparin complex) was provided by Corline Biomedical AB (Uppsala, Sweden). Centrisart columns with a molecular weight cut off of 300 kDa were purchased from Sartorius (Göttingen, Germany). Dialysis cassettes (Slide-A-Lyzer) with a molecular weight cut off of 20 kDa were obtained from Thermo Fisher Scientific (Rockford, IL, USA) and Float-A-Lyzer G2 Dialysis devices with a molecular weight cut off of 1000 kDa were obtained from Spectrum Laboratories, Inc. (Rancho, CA, USA).

2.2. Liposome preparation

Unilamellar, cationic liposomes were prepared by mechanical extrusion. For conjugation with the novel heparin complex the following types of liposomes were prepared, namely DPPC:Cholesterol:DDAB 55:40:05, 50:40:10 or 40:40:20, each in a total concentration of 20 mM (approx. 12 mg total mass). Briefly, weighted amounts of DPPC, cholesterol as well as DDAB were dissolved in ethanol after which the organic solvent was evaporated under a gentle nitrogen stream. For each experiment liposomes were freshly prepared by hydration of the lipid film in phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4) followed by hand extrusion 25 times through a 100 nm diameter polycarbonate filter (Nuclear track-etch membrane, Whatman Ltd., UK) using the Avanti Mini Extruder (Avanti Polar Lipids, Inc., USA) at a temperature higher than the phase transition temperature of DDAB (T_m 61 °C). After preparation an overnight resting phase was assured.

2.3. Size, polydispersity and zeta potential measurements

Photon correlation spectroscopy, based on dynamic light scattering, was used to determine the average liposomal size distribution as well as liposomal polydispersity, whereas zeta potential measurements were used to investigate the particle surface charge. For size measurements all samples were diluted 10 times and for zeta potential measurements samples were diluted 50 times in PNS, pH 7.4. Each sample measurement was run in triplicate at 25 °C at an angle of 173° with the Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Data acquisition and analysis was performed with the software Malvern v7.03.

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