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Research paper

PDMS-*b*-PMOXA polymersomes for hepatocyte targeting and assessment of toxicity

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ABSTRACT

Nanoparticles, such as polymersomes, can be directed to the hepatic asialoglycoprotein receptor to achieve targeted drug delivery. In this study, we prepared asialofetuin conjugated polymersomes based on the amphiphilic di-block copolymer poly(dimethylsiloxane)-*b*-poly(2-methyloxazoline) (PDMS-*b*-PMOXA). They had an average diameter of 150 nm and formed monodisperse vesicles. Drug encapsulation and sustained release was monitored using the hydrophilic model compound carboxyfluorescein. Asialoglycoprotein receptor specific uptake by HepG2 cells *in vitro* was energy dependent and could be competitively inhibited by the free targeting ligand. Mechanistic uptake studies revealed intracellular trafficking of asialofetuin conjugated polymersomes from early endosomes and to the lysosomal compartment. Polymersomes showed no toxicity in the MTT assay up to concentrations of 500 μg/mL. In addition, acute toxicity and tolerability of our PDMS-*b*-PMOXA polymersome formulations was assessed *in vivo* using zebrafish embryos as a vertebrate screening model. In conclusion, a hepatocyte specific drug delivery system was designed, which is safe and biocompatible and which can be used to implement liver-specific targeting strategies.

1. Introduction

Liver diseases such as hepatocellular carcinoma, viral hepatitis, or genetic and metabolic disorders belong to the leading causes of death, and the incidence rates are still increasing [1]. Though the liver is well known for its high drug uptake, available pharmacotherapies are often not adequate. Rapid drug elimination including P-glycoprotein mediated efflux and non-specific drug uptake by Kupffer cells makes the treatment of liver diseases difficult [2]. Therapeutic approaches using conventional small molecular weight drugs or novel therapeutic compounds such as nucleic acids, proteins, or peptides suffer from drawbacks including dose-limiting side effects, low bioavailability and rapid clearance from the blood stream. To overcome these challenges, there is an urgent need to develop alternative strategies for efficient drug delivery to the liver. Hepatocytes represent more than 80% of the resident hepatic cells and for several diseases they act as the most relevant target. Therefore, a specific delivery of pharmaceutical compounds to this cell type would be highly desirable [2,3].

In the search for alternative drug delivery systems, nanomedicines (including different classes of formulations such as drug-protein conjugates, drug-polymer conjugates, liposomes, micelles and polymersomes) have a great potential [4,5]. Among the particulate drug nanocarriers, liposomes are the most extensively studied, and there is now a range of clinically approved liposomes-based products that improve therapeutic outcome in patients. In 2016, only a few polymer-based nanocarriers were on the market, for example paclitaxel-loaded micelles. (For an extensive review on nanoparticle-based medicines see Refs. [4,6,7]).

Targeted nanomedicines [8] offer the possibility to increase the efficiency of drug treatment and to minimize toxic side effects since local drug accumulation and specific uptake reduce off-target exposure and allow for reduction of the administered dose. In terms of liver diseases, the asialoglycoprotein receptor (ASGPR) provides an opportunity to target hepatocytes [9]. The ASGPR is abundantly expressed on the surface of parenchymal liver cells

Abbreviations: ASGPR, asialoglycoprotein receptor; AF, asialofetuin; BSA, bovine serum albumin; CF, carboxyfluorescein; Cryo-TEM, cryogenic transmission electron microscopy; DLS, dynamic light scattering; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; DPBS, Dulbecco's phosphate buffered saline; EDAC, N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride; F, fetuin; FACS, fluorescence-activated cell sorting; FCS, fluorescence correlation spectroscopy; hpf, hours post fertilization; MFI, mean fluorescence intensity; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MWCO, molecular weight cut off; PCC, Pearson's correlation coefficient; PDI, polydispersity index; PDMS-*b*-PMOXA, poly(dimethylsiloxane)-*b*-poly(2-methyloxazoline.

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but minimally on extra-hepatic cells [10]. Up to now, different nanomaterials have been modified with asialofetuin such as PLGA/DOPE hybrid nanoparticles [11] and liposomes [12] in order to target the ASGPR.

Recently, polymersomes have been studied as nanocarriers for drug delivery [13]. Polymersomes are vesicles formed by self-assembly of amphiphilic block copolymers. Similar to liposomes, polymersomes offer the ability to encapsulate hydrophilic compounds in the aqueous core and to integrate hydrophobic or amphiphilic molecules into the hydrophobic part of their surrounding membrane. Compared to phospholipid based liposomes, polymersomes are characterized by a thicker membrane (8–21 nm) [14], which leads to enhanced membrane stability concurrent with lower permeability and improved storage properties. The precise biological and physicochemical characteristics of polymersomes are highly tunable (for review see [15–17]). By appropriate selection of the chemical composition of each polymer block, a broad range of block copolymers can be synthesized that confer polymersomes with a variety of specific properties such as improved drug encapsulation, biocompatibility, long circulation in blood stream, or stimuli-responsiveness. Chemical groups on the surface of polymersomes that allow conjugation of targeting moieties provide additional chemical versatility. No polymersome formulations have achieved FDA approval to date, and there are only few clinical trials in progress [4,6,7].

Recently, several reports on polymersomes formed by block copolymers consisting of poly(dimethylsiloxan) (PDMS) and poly (2-methyloxazoline) (PMOXA) have been published. The individual polymer blocks have been reported to be biocompatible [18] and polymersomes based on PDMS and PMOXA copolymers exhibited low cytotoxicity in various *in vitro* models [18–21]. PMOXA-decorated liposomes displayed increased circulation times in blood [22]. Konradi et al. [23] suggested that PMOXA could serve as a potential PEG substitute for rendering surfaces resistant to protein adsorption, i.e., adding stealth properties to the surfaces. In accordance to these findings, polymeric nanoreactors made of tri-block PMOXA-*b*-PDMS-*b*-PMOXA did not induce a macrophage-mediated inflammatory response *in vitro* and *in vivo* [24]. Antibody or peptide functionalized PDMS-*b*-PMOXA polymersomes have recently been used for cellular targeting. The ligand-targeted PDMS-*b*-PMOXA polymersomes were shown to bind specifically to their target cells, followed by rapid cellular uptake [19,21,25]. This suggests that polymersomes made of PDMS-*b*-PMOXA present a promising biocompatible and versatile platform to design novel specific drug targeting systems.

In this study, we present various formulations of polymersomes based on PDMS-*b*-PMOXA. We incorporated carboxyfluorescein as a model compound and demonstrated a slow drug release from these vesicles *in vitro*. In addition, we focused on the formulation of PDMS-*b*-PMOXA polymersomes for targeted drug delivery to hepatocytes and assessed potential toxicity of the resulting polymersomes. As a hepatocyte specific target, we chose the ASGPR. Its naturally occurring ligand asialofetuin, was selected as targeting moiety [9,12] and was covalently attached to PDMS-*b*-PMOXA polymersomes. We investigated the specific cellular uptake of AF modified polymersomes and we confirmed their biocompatibility *in vitro* using the human hepatocarcinoma derived HepG2 cell line. In addition, we used zebrafish embryos as a vertebrate model to assess *in vivo* toxicity [26,27].

2. Materials and methods

2.1. Materials

Poly(dimethylsiloxane)$_{67}$-block-poly(2-methyloxazoline)$_{15}$ and amino end functionalized poly(dimethylsiloxane)$_{67}$-block-poly (2-methyloxazoline)$_{15}$-NH_2 diblock copolymers (PDMS-*b*-PMOXA and PDMS-*b*-PMOXZ-NH_2, respectively) were obtained from Polymer Source Inc. (Montreal, Canada). Asialofetuin type II, fetuin, N -ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC), 2-iminothiolane, 5(6)-carboxyfluorescein, Hoechst 33342, and other reagents were of analytical grade and were obtained by Sigma-Aldrich (Buchs, Switzerland). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethylsulfoxide (DMSO) were of analytical grade and purchased from Carl Roth GmbH (Karlsruhe, Germany). HiLyte Fluor 488 NHS ester was obtained from Anaspec Inc. (Fremont, CA, USA). p-maleimidophenyl isocyanate was obtained from Thermo Fisher (Zug, Switzerland).

Amicon Ultra-4 centrifugal filters (MWCO of 30 kDa and100 kDa) were from Sigma Aldrich (Buchs, Switzerland). Whatman Nucleopore Polycarbonate filters were from VWR International GmbH (Dietikon, Switzerland). Spectra/Pore dialysis membranes (MWCO 3.5 kDa and 14 kDa) were from Carl Roth GmbH (Karlsruhe, Germany). Superose 6 prep grade was from GE Healthcare (Otelfingen, Switzerland).

Rabbit anti-EEA1 (Ab2900) and rabbit anti-LAMP1 (Ab24170) polyclonal antibodies were obtained from abcam (Cambridge, UK), goat anti-rabbit Dylight 633 conjugate and CellMask deep red plasma membrane stain were from ThermoFisher Scientific (Waltham, MA, USA).

Dulbecco's modified Eagle medium (DMEM, high glucose), Dulbecco's phosphate buffered saline (DPBS without calcium and magnesium, pH 7.2), 0.25% Trypsin/EDTA, 100× Penicillin/Streptomycin solution, and poly-D-lysine hydrobromide (MW 70,000–150,000) were obtained from Sigma Aldrich (Buchs, Switzerland). Fetal calf serum was purchased from Amimed (Bioconcept, Allschwil, Switzerland).

2.2. Formulation of polymersomes

Polymer vesicles were prepared using the thin film rehydration method as described by Egli et al. [19]. For rehydration of the polymer film DPBS was used and the resulting opalescent suspension was consecutively extruded through a series of polycarbonate filters with an average pore diameter of 0.4, 0.2 and 0.1 μm (each 5 extrusions; LipEx 10 mL extruder, Transferra Nanosciences Inc., BC, Canada).

2.3. Coupling of asialofetuin or fetuin to polymersomes

1.5 mg asialofetuin (AF) or fetuin (F) were dissolved in 500 μL DPBS/1 mM EDTA, pH 8.0. To thiolate the proteins, a 430-fold molar excess of 2-iminothiolane (Traut's reagent) was added and the reaction was carried out under stirring (350 rpm) at RT for 1 h. The resulting thiolated proteins were purified using Amicon Ultra-4 centrifugal filter units (MWCO 30 kDa) and concentrated to approximately 120 μL in DPBS/1 mM EDTA.

In parallel, PDMS-*b*-PMOXA polymersomes (1 mL, 5 mg/mL) were activated by adding 10 μL of p-maleimidophenyl isocyanate solution (7.84 mM). The reaction was carried out overnight at RT with stirring (350 rpm). Unreacted p-maleimidophenyl isocyanate was removed using Amicon Ultra-4 centrifugal filter units (MWCO 100 kDa) and the final maleimide activated polymersomes were re-suspended in 1 mL DPBS/1 mM EDTA.

Proteins were then covalently linked to the polymersomes via Michael addition. 1 mL of activated polymersome suspension was mixed with the thiolated proteins (120 μL) and stirred (350 rpm) at RT overnight. The resulting protein modified polymersomes were purified by gel filtration chromatography (Superose 6 Prep grade, elution buffer DPBS). Polymersome containing fractions were pooled and re-concentrated to a final volume of 1 mL in DPBS