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Probing Polymersome-Protein and -Cell Interactions: Influence of Different End-Groups and Environments

Regina Bleul,[1] *Diana Bachran,*[2] *Raphael Thiermann,*[1] *Annabelle Bertin,*[1] *Hendrik Fuchs,*[2] *Michael Maskos**[1]

Summary: Developing polymersomes for drug delivery purposes requires a deeper understanding of their behavior in physiological environment. We performed the self-assembly and in-situ loading of polybutadiene-*block*-polyethyleneoxide (PB-b-PEO) polymersomes in a continuous process using micromixers. Varying the length and end-groups of the starting block copolymer allows us to control the polymer membrane thickness and surface functionalities (hydroxyl or carboxylic acid), required to realize a further coupling with specific cell targeting ligands. To get a deeper understanding of these polymersomes in physiological environment, we studied the cellular response (HeLa cells) in presence of various polymersomes, and showed by cytotoxicity tests the relative biocompatibility of the systems. Flow cytometry experiments at 4 °C in PBS buffer showed a different behavior of hydroxyl-functionalized vesicles compared to carboxylic acid-functionalized vesicles. On the contrary cell binding in DMEM medium supplemented with 10% FCS was almost completely blocked with both kinds of polymersomes. Protein adsorption measurements by dynamic light scattering confirmed that protein binding occurs in all cases, which apparently influences the particle-cell interaction. This study contributes towards a deeper understanding of polymersomes in biological environment and further investigations will help us to design highly effective polymersomes for *in vitro* as well as *in vivo* applications.

Keywords: drug delivery; end-group functionality; particle-cell interaction; physiologic environment; polymersomes; protein adsorption

Introduction

Interest in nanomedicine has dramatically increased in recent years, but still little is known about the behavior of nanoparticles after introduction into physiological environment. Particularly, the dynamic process of protein adsorption plays a key role in the biological response[1,2] by influencing the particle biodistribution and biocompatibility.[3]

Polymersomes (vesicles self-assembled from block copolymers) are a relatively new kind of soft nanoparticles, which have already shown great promises as drug carriers.[4,5,6] Compared to liposomes, consisting of natural phospholipids, polymersomes provide much more variability, stability and functionality due to the good synthetic control of polymer chemistry.[7,8,9] Furthermore, polymersomes possess the ability to load hydrophilic molecules, as well as hydrophobic ones, which offer advantages over spherical polymer micelles.[10,11] Nevertheless the requirements for polymersomes as drug delivery systems are very challenging and include (i) protection and transport of the therapeutic agent, (ii) stability in blood stream, (iii) specific binding to diseased cells, (iv) internalization, endosomal escape

[1] BAM Federal Institute for Materials Research and Testing, 12205 Berlin, Germany
E-mail: Michael.Maskos@bam.de
[2] Institut für Laboratoriumsmedizin, Klinische Chemie und Pathobiochemie – Universitätsmedizin Charité, 12200 Berlin, Germany

and drug release. The defined goal is to minimize undesired side effects, reduce the toxicity and get a better control of dosage and pharmacokinetics.

To design effective drug carriers it is very important to get a deeper understanding of nanocarriers in biological environment. Nanoparticles in biological fluids almost invariably become coated with proteins.[2] This process called opsonization strongly influences the biological response including toxicity, biodistribution and cell entering. Indeed, protein adsorption affects the clearance of the particles by the reticuloendothelial system (RES, also called MPS - mononuclear phagocyte system) and therefore the biodistribution of the carrier.[1,3]

The polymer polyethylene oxide (PEO) is generally accepted to be biocompatible and relatively resistant to protein adsorption. PEO is commonly used to extend the blood circulation time of nanocarriers by protecting those from RES clearance.[12,13,14] The circulation time of polymer vesicles containing PEO as hydrophilic block has already been shown to be dependent on the PEO molecular weight and the PEO density,[14,15] additionally further characteristics of the entire PEO protection layer such as thickness, charge and conformation can affect the interaction with opsonins.[13]

In this study, we investigated the influence of different PEO end-groups (hydroxyl or carboxylic acid) on the interaction of polymersomes with proteins as well as the cell binding behavior depending on the surface functionality and the environment. We performed experiments in the standard buffer PBS (Phosphate buffered saline) as well as in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FCS (Fetal calf serum) as it is often used in *in vitro* studies and cell experiments.

Experimental Part

Materials

Bovine serum albumin (BSA), phosphate buffer solution (PBS), Phloxin b, tetrahydrofuran (THF) and fluorescein diacetate were purchased from Sigma Aldrich (Germany). DMEM media was purchased from PAA Laboratories GmbH, Germany. All chemicals were used without further purification. The water used in all experiments was prepared in an arium® Ultrapure Water System (Sartorius Stedim Biotech). The synthesis of poly(butadiene)-b-poly(ethylene oxide) was performed as described previously.[16]

Preparation and Characterization of Loaded Polymersomes

The controlled self-assembly of the vesicles has been carried out with a microfluidic mixing device employing syringe pumps (Postnova, Germany) and a stainless steel IMM (Institute of Microtechnology Mainz) Caterpillar (R300) micromixer (Figure 1).[17]

Polymer solutions were prepared with $PB_{130}PEO_{66}$-H (sample code 1a) or $PB_{130}PEO_{66}$-(CO)$-CH_2-CH_2-$COOH (sample code 1b) in 50% (v/v) of THF in water. Self-assembly was performed at room temperature with 2 g/L starting polymer concentration, 0.9 ml/min flow rate and with a mixing ratio 1:2 (polymer solution/water). The final polymer concentration after evaporation of the non-selective solvent was 1.3 g/L. In-situ loading of the polymersomes was performed by adding the fluorescence dye Phloxin b (0.08 g/L) to the starting polymer solution. The exterior excess of Phloxin b was removed by gel filtration with a DextraSEC PRO10 column (Applichem, Germany).

Vesicles composed of the $PB_{32}PEO_{29}$-H (sample code 2a) or $PB_{32}PEO_{29}$-(CO)$-CH_2-CH_2-$COOH (sample code 2b) were prepared in pure water (or with 0.08 g/L Phloxin b) by sonication and extrusion through a polycarbonate membrane with 100 nm pore size (Avestin Europe GmbH, Germany). Purification was carried out by gel filtration.

The polymer solutions were filtered through 0.45 μm filters (LCR or PVDF, Millipore, Germany) and characterized by dynamic light scattering (DLS) with an ALV-CGS-3 MD goniometer system,

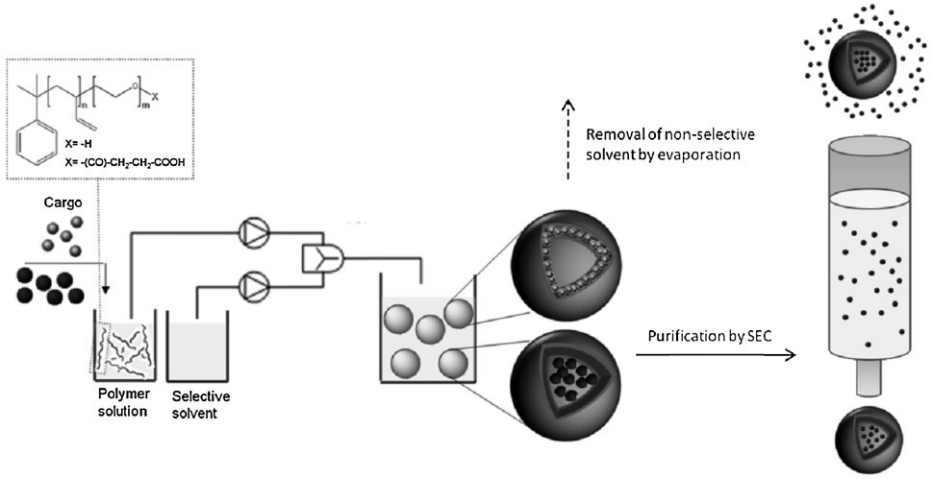

Figure 1.
Schematic illustration of the continuous synthesis of loaded polymersomes. The starting polymer solution (PB-b-PEO) is diluted with a selective solvent in a micro structured mixing device. Resulting morphologies of the polymeric particles can be controlled by varying different parameters: polymer composition, end-group functionality, solvent ratio, flow rate. Carboxyl end-groups enable further surface functionalization e.g. to incorporate specific targeting moieties. Moreover an in situ loading can be performed by adding the cargo (fluorescent or drug molecules) to the starting polymer solution. Depending on the hydrophilicity of these molecules, they will be incorporated in the hydrophobic part of the membrane or encapsulated in the hydrophilic inner lumen of the vesicle.

632 nm HeNe laser (22 mW), APD, dual ALV-7004 Multiple Tau Digital Real Time Correlator (ALV, Germany).

For TEM imaging samples were γ–irradiated to fix the structures and morphologies by cross-linking of the PB part as described before.[16] The measurements were performed using a Philips Electron Microscope EM420 on carbon coated copper grids at 120 kV acceleration voltage and the images were taken with a CCD camera.

Cell Culture

Cell culture experiments were performed with the human cervical carcinoma cell line HeLa (obtained from ATCC, The American Type Culture Collection, Manassas).

Cytotoxicity Assay

Cells were washed twice with PBS, trypsinized and after resuspension in PBS seeded in 96-well plates at concentrations of 10,000 cells per well in 100 μL of culture medium 24 h before treatment. The conditioned medium was replaced by 180 μl fresh medium and 20 μL polymersomes (1g/L final polymer concentration). Cells were incubated for 48 h at 37 °C (5% CO_2) and relative survival was measured by a cytotoxicity assay[18] based on the cleavage of fluorescein diacetate by living cells. Cells were washed twice with PBS and incubated for 1 h with 200 μl fluorescein diacetate (10 μg/ml; Sigma, Germany) in PBS. Developing fluorescence was measured in a microplate reader (Spectra Max Gemini, Molecular Devices, Ex 485 nm, Em 538 nm). Relative survival was calculated as the percentage of living cells in treated wells in relation to untreated cells.

Flow Cytometry

Adherent cells were harvested via gentle shaking in 5 mM EDTA (diluted in PBS) for 10 minutes at 37 °C. Cells were counted and 1×10^6 cells/mL were placed in eppendorf caps, centrifuged at 0.2 rcf for 5 minutes, washed twice with PBS, and re-suspended in PBS or DMEM +10%

FCS. Phloxin B loaded polymersomes (0.5 g/L) were incubated with 1×10^6 cells/mL for 1 h at 4 °C. Cells were washed three times with PBS, and re-suspended in 1 mL PBS. Cell samples were analyzed with a Beckman Coulter Epics XL flow cytometer. Data were acquired with the fsc channel in linear mode and all other channels in log mode. A gate was placed on the forward scatter-side scatter plot that excluded cellular debris. Fluorescence intensity was measured using the 488-nm laser source, and emission intensity was collected in the FL2 channel (575 nm/bandpass).

Protein Adsorption Experiments

Polymersomes (0.1 g/L) were incubated with BSA in PBS buffer (3 g/L) or with 10% FCS in DMEM with gentle shaking at room temperature for 1 h. Hydrodynamic radii of polymersomes treated with proteins and untreated were determined by dynamic light scattering.

Results and Discussion

Polymersome Formation and Loading

The preparation of polymersomes based on polybutadiene-*block*-poly(ethylene oxide) (PB-b-PEO) was performed as illustrated in Figure 1. The obtained vesicles are well defined in terms of size and morphology. Using $PB_{130}PEO_{66}$-(CO)$-CH_2-CH_2-$COOH as starting polymer under optimized conditions we obtain relatively monodisperse vesicles with hydrodynamic radii of about 43 nm (Table 1). The TEM image confirms the DLS measurement and shows the hydrophobic part of the membrane with about 20 nm (Figure 2). Surface functionality (carboxylic acid or hydroxyl) and membrane thickness can be controlled by the composition of the starting polymer (Table 1).

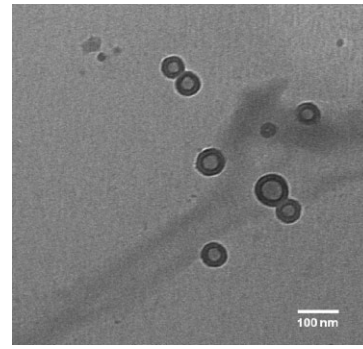

Figure 2.
TEM images of crosslinked nanoparticles from PB-b-PEO polymer. Polymer vesicles (sample 1b) were obtained using $PB_{130}PEO_{66}$-(CO)$-CH_2-CH_2-$COOH (2 g/L) dissolved in 50% (w/w) THF in water as starting solution, diluting the polymer with water in a 1:1 ratio in the micro mixer with a total flow rate of 1 mL/min.

The loading of the polymersomes with fluorescence dye Phloxin b was successful and confirmed by fluorescence spectroscopy.[10]

Interaction of the Polymersomes with Cells and Serum Proteins

HeLa cells exposed to polymersomes (1 g/L) composed of polymers of different length and end-group functionality showed no significant ill-effects after 48 h incubation (Figure 3). Cell viability was tested

Table 1.
Polymer composition and features of the tested polymer samples. Sample code refers to different numbers of repeating units and different endgroups. Average hydrodynamic radius of the polymersomes was measured by angle-dependent dynamic light scattering. The second cumulant μ_2 can be attributed to the polydispersity of the sample (cumulant method,[19,20] normalized $\mu_2 = 0.05$ at 90° scattering angle, corresponding to a polydispersity in radius of approximately 25%.). The thickness of the hydrophobic part of the membrane was determined by TEM imaging.

Sample	Polymer	R_H/nm	μ_2 (90°)	Membrane thickness/nm
1a	$PB_{130}PEO_{66}$-H	44.2	0.08	20
1b	$PB_{130}PEO_{66}$-(CO)$-CH_2-CH_2-$COOH	43.2	0.06	20
2a	$PB_{32}PEO_{29}$-H	62.6	0.14	13
2b	$PB_{32}PEO_{29}$-(CO)$-CH_2-CH_2-$COOH	69.3	0.11	13

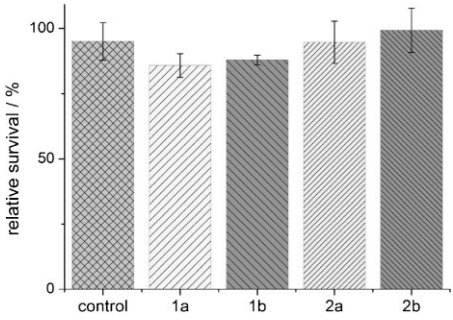

Figure 3.
Cytotoxicity in presence of different PB-b-PEO copolymer nanoparticles. Cytoxicity assay is based on cleavage of fluoerescein diacetate by living cells and was performed as described by Nygren et al.[18]. HeLa cells were incubated with nanoparticles (1g/L polymer, specification of particles as described in Table 1) for 48 h. Relative survival was calculated as the percentage of living cells in treated wells in relation to untreated cells. Error bars indicate S.E.M of 3 replicates.

with a fluorescein diacetate assay,[18] the relative survival was above 85% for all cells, treated with polymersomes and control cells. The non-toxicity of PB-b-PEO polymersomes to several cell lines of PB-b-PEO polymersomes was already confirmed by other groups.[21,22]

Binding behavior to HeLa cells depending on surface functionality in different environment was investigated by flow cytometry. We performed the cell binding experiments in PBS and DMEM (supplemented with 10% FCS) as they are widely used in numerous *in vitro* experiments. Both physiological environments have comparable pH value and ionic strength, while DMEM cell culture media contains additionally sugars and amino acids. For cell culture experiments DMEM is usually supplemented with 10% FCS to supply the cells with proteins and growth factors. We chose these different physiological environments because they are examples of standard media for *in vitro* methods.

Incubation in PBS buffer showed significant differences between vesicles with hydroxyl or carboxylic acid end-groups with comparable polymer length. While in PBS buffer carboxyl polymersomes (sample 2b) tend to bind stronger to HeLa cells than hydroxyl polymersomes (sample 2a), in DMEM media in presence of 10%

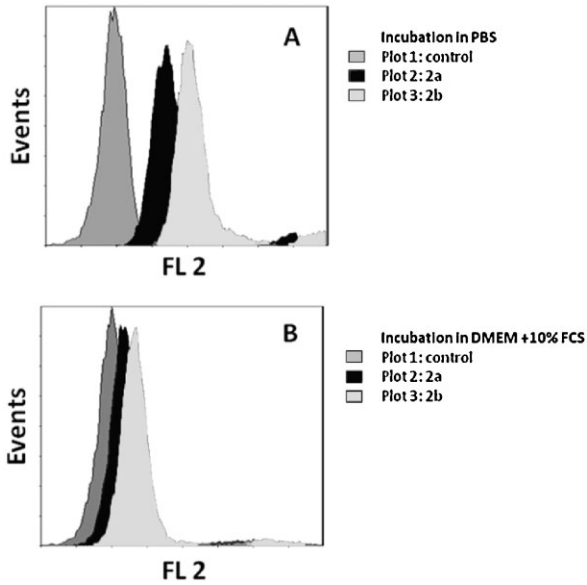

Figure 4.
Flow cytometry analysis. Histogramms of untreated HeLa cells, HeLa cells incubated with Phloxin b loaded polymersomes (0.5 g/L) with hydroxl (2a) or carboxyl (2b) end-groups. Binding experiments were performed with 10^6 HeLa cells in PBS (A) or DMEM medium with 10% FCS (B) 60 min at 4 °C.

Table 2.
Hydrodynamic radii of polymersomes (0.1 g/L) incubated with BSA (3g/L in PBS buffer) or with FCS (10% in DMEM media). Samples (sample code Table 1.) were incubated 1h with gentle shaking at room temperature.

Sample	1a	1a+BSA	1a+FCS	1b	1b+BSA	1b+FCS	2a	2a+BSA	2b	2b+BSA	BSA
R_H/nm	44.2	47.0	46.3	43.2	45.6	46.7	62.6	65.5	69.3	75.3	3.8
μ_2 (90°)	0.08	0.09	0.20	0.06	0.08	0.10	0.14	0.16	0.11	0.19	0.06

FCS almost no binding of both kinds of polymersomes was detected. PEO is known to be relatively resistant to protein binding,[12,23] but as it was already shown by Photos et al.[15] the in vivo circulation time depends on the chain length of the polymer. As we observed in our study the cell binding behavior in PBS depends also on surface functionalization of the polymersomes (Figure 4). This fact can be very important if *in vitro* tests are performed in PBS in absence of proteins. Binding behavior of polymersomes to cells is apparently strongly dependent on the environment.

The surface charge effect during incubation in DMEM media with 10% FCS is attenuated compared to the incubation in PBS. Only slight differences between the hydroxyl- and the carboxyl-polymersomes were observed. Cell binding was almost blocked completely. This fact hints at a protein adsorption process, which results in similar protein corona for hydroxyl- and carboxyl-polymersomes. Thus, the cells apparently do not recognize differences between both types of polymersomes.

Further investigations concerning the protein adsorption process to the polymersomes were made by DLS measurements. Polymersomes were incubated with BSA (in PBS buffer), as a major component of FCS, or with 10% FCS (in DMEM media) for 1h. Changes in hydrodynamic radii of the polymersomes were determined by dynamic light scattering. Slight increases of size were observed with all kinds of polymersomes incubated with BSA or with FCS (Table 2).

Conclusion

We synthesized nanoparticles based on polybutadiene-*block*-poly(ethylene oxide) (PB-b-PEO) for drug delivery purposes. The micromixing technology allows us to control the size and features of the carrier system, which is very promising to open up new possibilities for personalized medicine in future. Furthermore, it enables the adjustment of dose and release profiles individually and a point-of-care preparation could be feasible.

Nevertheless, designing these functional polymersomes for biomedical application requires a deeper understanding of their behavior in physiological environment. Thus, first we confirmed the non-toxicity of the carrier by a cell viability assay. Investigations on cell binding behavior showed that the polymersome-cell interaction depends on surface functionality and incubation media, which is apparently induced by protein adsorption processes.

Further investigations on polymersome-protein and -cell interaction will help to design highly effective and functional nanoparticles for biomedical applications with improved properties in terms of bioavailability, biodistribution and biocompatibility.

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