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Biodegradable polymersomes as a basis for artificial cells: encapsulation, release and targeting

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Abstract

The encapsulation of biofunctional compounds, release properties and targetability of polymersomes of amphiphilic block-copolymers based on poly(ethylene glycol) (PEG) and biodegradable polyesters or polycarbonate are described. Carboxyfluorescein (CF), as a model for hydrophilic biofunctional compounds, could be readily incorporated in the polymersomes by adding the compound to the aqueous phase during polymersome preparation. The release of encapsulated material from the polymersomes can be adjusted by changing the copolymer composition, especially the molecular weight and type of hydrophobic block of the copolymer. The presence of plasma proteins other than albumin suppressed the release of CF. CF release in PBS both at room temperature and at 60 °C followed first order kinetics, confirming that the CF containing polymersome system is a membrane controlled reservoir system. These biodegradable polymersomes have the potential to be targeted to specific sites in the body as shown by the specific interaction of anti-human serum albumin immobilized polymersomes with a human serum albumin coated sensor surface.

Keywords: Biodegradable polymersomes; Block copolymers; Artificial cells; Carboxyfluorescein

1. Introduction

Artificial cells refer to water-insoluble man-made particles that can perform a specific biological function in the body without being recognized by the defense system. Polymersomes are of particular interest as a basis for artificial cells because of the large compartment for encapsulation of biofunctional compounds, tunable membrane properties, and stability. The development of biodegradable polymersomes makes them even more attractive [1,2].

For an artificial cell, biofunctionality means that the biofunctional compound included in the cell can locally alter physiological events or a physical parameter, such as preventing the growth of tumor cells by delivering anticancer drugs, treating enzyme defects in inborn problems of metabolism by deliver-

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ing encapsulated enzymes, transporting O_2/CO_2 or locally changing the electron density allowing visualization of certain cells or organs by imaging techniques. An artificial cell body can be provided with biofunctionality by encapsulation or immobilization of biofunctional compounds such as drugs, enzymes, peptides, antibodies, DNA. Encapsulation of biofunctional compounds [3–10] in the artificial cell body can be performed during its formation. Immobilization of biofunctional compounds onto the artificial cell surface [11,12] can be done via covalent binding [13] or adsorption. Compared to immobilization, encapsulation has the advantage that large amounts of biofunctional compounds can be entrapped.

For the potential application of site-specific release of compounds, it is important that the release from the artificial cell can be controlled. A wide range of drug delivery systems has been developed over the years, including membrane-controlled reservoir systems, diffusion-controlled monolithic systems, biodegradable systems, systems of which the release is controlled by osmosis, swelling, erosion or external control (e.g., temperature, magnetic, ultrasonic or electrical stimulation), and (bio)chemically controlled systems. The release of the incorporated biofunctional compound from the artificial cells can be controlled by modifying the properties of the membrane of the cells.

Homing devices on the cell body surface can guide the cell to the specific site where it performs its function. By applying proteins including antibodies, antibody fragments and lipoproteins, lectins, hormones, mono-, oligo- and polysaccharides as targeting moieties, microcapsules, microparticles, liposomes, and micelles could be successfully used for targeted drug delivery [14–18]. Providing a particle surface with monoclonal antibodies is one of the most effective ways to target antigen expressing cells [15,19] and depending on the dimensions, specific uptake of particles by target cells has been observed [20]. This approach has been applied to improve the therapeutic efficacy of anticancer drugs [15,21] and to design advanced diagnostic systems [22]. Of particular interest is the attachment of antibodies through the distal end of poly(ethylene glycol) (PEG) chains [15,23]. By using PEG as a spacer the binding of the antibody located on the artificial cell to the target cell is not sterically hindered, and non-specific binding is reduced [24]. Recombinant protein A and G can be used in order to provide site-specific immobilization of an antibody due to the specific interaction with the Fc portion of the antibody without affecting the antigen–antibody interaction.

We have demonstrated that biodegradable polymersomes can be formed from amphiphilic PEG-polyester and PEG-polycarbonate diblock copolymers in either chloroform/water systems [1,2] or water miscible solvent/aqueous phase systems [1,2]. To complete these polymersomes as artificial cells, they should also be provided with a biofunctional compound and a homing device. In this study, carboxyfluorescein (CF) which self quench [25–27] was applied as a model hydrophilic substance for encapsulation and release studies using polymersomes that were prepared from THF/ethyl acetate system [1,2]. Anti-human IgG (a-HIgG) or anti-human serum albumin (a-HSA) was covalently immobilized via carboxyl groups present at the polymersome surface using the carbodiimide method. a-HIgG and a-HSA were also coupled to the polymersome surface via protein G, which was first covalently immobilized onto the carboxyl group containing polymersome surface. The targetability of these polymersomes was evaluated by following the interaction of these polymersomes with their corresponding immobilized antigens, i.e., human IgG and HSA by imaging surface plasmon resonance (iSPR).

2. Materials and methods

2.1. Materials

5(6)-Carboxyfluorescein (CF, 97%) was purchased from Molecular Probes (Eugene, OR, USA). Sodium deoxycholate (DOC) was purchased from Aldrich. Freshly frozen, citrated, human plasma was obtained from the Blood Transfusion Service Twente-Achterhoek (Enschede, The Netherlands). 1-Dimethylaminopropyl-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxy-succinimide (NHS), 2-(*N*-morpholino)ethanesulfonic acid (MES), human albumin, 2-amino-2-(hydroxymethyl)-1,3-propanediol and tris(hydroxymethyl)aminomethane hydrochloride were obtained from Sigma (St. Louis, MO, USA).

Human albumin antibody (a-HSA) in PBS (pH 7.2, 0.5 mg/mL), developed in goat, was bought from Bethyl Laboratories (Montgomery, TX, USA). Goat anti-human IgG (γ chain specific) (a-HIgG), protein G (recombinant), human IgG (HIgG, ≥95%), human serum albumin (HSA, 99%), bovine serum albumin (BSA) were bought from Sigma. Rabbit antibody (polyclonal Rabbit anti(human PSA), affinity-purified) in phosphate buffer (1.18 mg/mL, pH 7.4) was purchased from Cortex Biochem (San Leandro, CA). Mouse IgG (29 mg/mL in PBS) was bought from Future Diagnostics (Wijchen, The Netherlands).

The PEG-polyester copolymers used in this study (Table 1) were prepared using the ring-opening polymerization of D,L-lactide, ε-caprolactone and trimethylene carbonate using zinc bis[bis(trimethylsilyl)amide] and monomethoxy PEG as an initiator [1,2]. α-Carboxyl poly(ethylene glycol)-*b*-poly(D,L-lactide) copolymer with PEG molecular weight 3400 and PDLLA molecular weight of 29,600 was denoted as C-PEG-PDLLA3.4-30 and the synthesis was described elsewhere [1]. Deionized water (DI water) was obtained from a Milli-Q water purification system (Millipore, Molsheim, France). All other chemicals were from Merck (Darmstadt, Germany) and used as received. MES buffer (0.05M, pH 5.4, containing 0.02 wt.% NaN_3), and phosphate buffer (PB, 0.01M, pH 7.4, containing 0.02 wt.% NaN_3) were used in the immobilization or targeting experiments.

Table 1
Characteristics of polymersomes of different copolymers made in the presence of CF[a]

Copolymers	Count rate[b]	Size (nm)	Encapsulated CF per preparation (nmol/%)[c]
PEG-PDLLA5.8-15	157±4	255±2	4.9±0.7/2.5
PEG-PDLLA5.8-24	236±1	258±4	5.3±0.3/2.7
PEG-PDLLA5.8-48	457±20	250±3	9.0±1.0/4.5
PEG-PDLLA5.8-48[d]	1921±10	248±2	30.1±1.3/15.1
PEG-PCL5.8-24	251±1	261±6	5.0±0.5/2.5

[a] Data presented are the average value of duplicate experiments followed by the variation, except the third entry of this table, of which the data is the mean value followed by the standard deviation.

[b] Count rate of the original CF-PS dispersions based on the DLS measurement of its dilutions.

[c] Calculation based on the fluorescence measured after DOC treatment.

[d] For the preparation of these polymersomes, 0.25 mL instead of 0.05 mL of PEG-PDLLA5.8-48 solution was used.

2.2. Preparation of CF encapsulated polymersomes (CF-PS)

CF encapsulated polymersomes (CF-PS) were prepared by injecting a solution of a block-polymer (see Table 1) in THF (0.05 mL, 10 mg/mL) into an aqueous solution with the tip of the pipette or syringe immersed close to the bottom of the bottle. The aqueous solution consisted of 1.225 mL of 165 mM CF in Tris (20 mM, pH 7.4) and 1.225 mL of an aqueous solution of ethyl acetate (8 vol.%) in a glass bottle. The osmolarity of the final solution was approximately 290 mosM. After 15–30 min, the bottle was inverted twice to obtain a homogenous opaque dispersion. Subsequently, the CF-PS was separated from free CF by gel filtration using a PD-10 column (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) at room temperature applying TRIS (10 mM, pH 7.4) as eluent.

2.3. Preparation of carboxylic acid group containing polymersomes (C-PS)

Carboxylic acid group containing polymersomes (C-PS) were made by injecting 0.5 mL of a solution of C-PEG-PDLLA3.4-30 in THF (10 mg/mL) into 5.0 mL of MES buffer (0.05M, pH 5.4, with 0.02 wt.% NaN_3) with the tip of the pipette or syringe immersed close to the bottom of the bottle. After 15 min without agitation, the bottle was gently inverted a few times and a homogenous bluish polymersome dispersion was obtained. The dispersion was then subjected to overnight dialysis against MES buffer using VISKING® dialysis tubing (MWCO 12,000–14,000, ϕ 16 mm) (SERVA Electrophoresis, Heidelberg, Germany).

2.4. Immobilization of antibodies directly onto C-PS (Ab-PS)

Solid NHS (5.75 mg) and EDC (38.3 mg) were subsequently added to 2.0 mL of a C-PS dispersion in a reaction tube. Invertion of the tube ensured complete dissolution of NHS and EDC. The reaction was allowed to proceed for 10 min before ultracentrifugation (60,000×*g*, 18 min, Centrikon T-2180, Kontron Instruments, Watford, UK) at 10 °C. The supernatant was removed using a pipette. a-HIgG or a-HSA

solution (0.8 mL, 0.1 mg/mL) in MES buffer was added to the tube. The polymersomes were re-dispersed by aspirating/dispensing. The coupling reaction was allowed to proceed for 2.5 h at room temperature under mild shaking.

Free antibody was separated from the Ab-PS by gel filtration of the dispersion over a Sepharose® 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) column (volume 12 mL), which had been equilibrated with 25 mL of PB buffer before use. The Ab-PS dispersion (0.8 mL) was loaded on the column which was then eluted with 20 mL of PB buffer. Fractions (n=20, V=1.0 mL) were collected, and the count rate in DLS measurements and protein content of each fraction were determined. The fractions that only contained Ab-PS were pooled. The obtained polymersomes are denoted as a-HIgG-PS and a-HSA-PS, respectively.

2.5. Immobilization of antibodies via protein G onto C-PS (Ab-G-PS)

Protein G immobilized polymersomes (G-PS) were prepared and purified in the same way as mentioned for Ab-PS only the antibody solution was replaced by a protein G solution (0.1 mg/mL) in MES. The purified G-polymersomes in PB buffer were stored at 4 °C for 16 h, and then centrifuged (60,000×g, 18 min) at 10 °C to remove the supernatant. The pellet was re-suspended in a-HIgG or a-HSA solution (0.1 mg/mL, 1.0 mL) in PB buffer and the polymersomes were incubated for 2 h under mild shaking. The obtained Ab-G-PS, denoted respectively as a-HIgG-G-PS and a-HAS-G-PS, were purified from free antibody by gel filtration as described for the preparation of the Ab-PS.

2.6. Characterization of polymersomes

The size and zeta potential of C-PS, Ab-PS and Ab-G-PS in PB buffer were measured at 25 °C using a Malvern Zetasizer 4000 and 2000 (Malvern, Malvern, UK), respectively. Determination of the polymersome size was performed at a wavelength of 633 nm and a scattering angle of 90°. The CONTIN method was applied for data processing. The size, count rate and polydispersity index (PDI) were determined. The zeta potential of polymersomes in PB buffer was measured using Laser Doppler Velocimetry (LDV), in which the velocity of particles moving in a fluid that is exposed to an electric field is measured. By applying the Smoluchowski equation [28] the ξ-potential can be determined. Measurements were carried out at 25 °C using a 1000 Hz modulator frequency, and a 120 V cell drive voltage. Results are the mean of data obtained with three samples, which were each measured four times.

2.7. CF release from CF-PS

The fluorescence emission of CF is quenched to a marked extent [25–27] when the molecules are confined at high concentration. Thus a marked increase in fluorescence intensity results when CF is released from the CF-PS to the surrounding aqueous medium.

The release of CF from polymersomes in different media at different temperatures was monitored as a function of time using a Perkin-Elmer LS-3 fluorescence spectrometer (Perkin-Elmer, Wellesley, MA, USA). Gel filtrated CF-polymersome dispersions (0.2 mL) were mixed with different media (1.1 mL) at room temperature (PBS, 4 wt.% human albumin in PBS, 15 and 85 vol.% human plasma in PBS) or at 60 °C (PBS) under stirring. The time between the gel filtration of the polymersomes and the start of the release experiment was 20–30 min. This time was not included in the release time.

At periodic intervals, 0.1 mL of the mixture was withdrawn and diluted with 1.0 mL of tris buffer (10 mM, pH 7.5), and then the fluorescence (F) was measured. The total fluorescence of CF encapsulated (F_T) was determined by adding DOC (0.1 mL, 3 wt.%) to 0.05 mL of a freshly gel filtrated dispersion, boiling briefly (ca. 2 min) and diluting with 0.95 mL of tris buffer before the fluorescence was measured. The total amount of encapsulated CF can be calculated from F_T, a calibration curve of CF fluorescence as a function of CF concentration, and the dilution factor. The quenching of the fluorescence inside the polymersomes initially was more than 95% [27], and even at 90% release, the fluorescence from the CF in the polymersomes is only 3–5% of the total fluorescence, therefore its contribution to the total fluorescence was neglected. The CF release (%) over time is presented by $100 \times F/F_T$.

2.8. Immobilization of the C-PS on an aminated sensor surface

The reaction of C-PS with an aminated surface was studied by SPR. An aminated SPR sensor disk (P-NH_2, IBIS Technologies, Hengelo, The Netherlands) was equilibrated in MES buffer (150 μL, 0.05 M, pH 5.4) in the cuvette sensor compartment of an IBIS II SPR instrument (IBIS Technologies). After the removal of MES buffer, C-PS dispersion (150 μL) in MES buffer was added to both channels and the interaction of the polymersomes with the sensor surface was studied under continuous mixing. After 20-min interaction, the C-PS dispersions were drained from both channels followed by four times rinsing with MES buffer. Thereafter C-PS (150 μL) in MES buffer was added to the reference channel, and the activated C-PS (150 μL) were added to the reaction channel. C-PS (150 μL in MES) were previously activated for 5 min by the addition of NHS and EDC subsequently with a final molar EDC to NHS ratio of 4 and [EDC] of 0.4 M. After incubation 1 h under continuous mixing, both channels were rinsed with MES buffer for five times. During the whole experiment the SPR angle in milli-degree (m°) was monitored.

2.9. Preparation of antigen-spotted surfaces

Human serum albumin (HSA), bovine serum albumin (BSA), rabbit IgG (RIgG), mouse IgG (MIgG), and human IgG (HIgG) in MES buffer (50 mM, pH 5.4) were spotted on a pre-activated SPR sensor (iSPR-P-AE-sp, IBIS Technologies) in an array of 6×4 protein spots (1 nL) using a TopSpot™ contactless spotter (HSG IMIT, Villingen-Schwenningen, Germany). From left to right, the columns corresponded to HSA (0.5 mg/mL), BSA (0.5 mg/mL), RIgG (0.5 mg/mL), MIgG (0.5 mg/mL), HIgG (0.5 mg/mL) and HIgG (0.2 mg/mL). Directly after spotting, the sensor was transferred to a humidity chamber and incubated at 4 °C overnight. Subsequently, un-reacted active esters were quenched with ethanol amine (0.1 M, pH 8.5) for 10 min at room temperature and the sensor surface was rinsed with PBS and stored in PBS at 4 °C until use.

2.10. Interaction of Ab-PS or Ab-G-PS with protein immobilized surface

Targetability of the polymersomes was investigated by studying the interaction of the Ab-PS and Ab-G-PS with the protein spotted sensor surfaces by imaging SPR (IBIS iSPR, IBIS Technologies). In a typical experiment, the sensor surface was rinsed with PB buffer and incubated with 150 μL of PB buffer for 10 min before regeneration with HCl (100 μL, 4 mM) followed by PB (150 μL) rinsing five times. PB buffer (100 μL) was then removed from the cuvette and polymersome dispersion (100 μL) was added and incubated for 1 h. Subsequently, the polymersomes were removed and the surface was rinsed with PB (150 μL) for five times before regeneration with 4 mM HCl followed by five times rinsing with PB (150 μL). Thereafter, the sensor surface was reused to study the interaction with other polymersome dispersion following the same procedure. During the whole procedure the SPR shift was followed as a function of time.

3. Results and discussion

3.1. Encapsulation of CF

The fluorescence emission of CF is quenched to a marked extent [25–27] when the molecules are confined at high concentration, for example inside polymersomes. Thus, a marked increase in fluorescence intensity is observed when CF is released from the polymersomes to the surrounding aqueous medium. The total fluorescence is determined after disruption of the polymersomes with the detergent DOC [25]. CF has an excitation maximum at ~491 nm at pH 7.4 and an emission maximum at ~520 nm, which is independent of the presence of DOC.

The injection of a solution of block copolymers in THF (Table 1) into an aqueous phase, which contained CF and ethyl acetate, yielded polymersomes with incorporated CF. Un-trapped CF was removed by gel filtration over a PD-10 column. Gel filtration of the polymersomes did not cause loss of particles as evidenced by the DLS measurements. The polymersomes were disrupted by adding DOC in combination with brief boiling and the encapsulated

CF was liberated. Table 1 lists the count rate of the polymersome dispersions, the average size of the polymersomes and the total amounts of CF that were incorporated in the polymersomes. For all copolymers a similar polymersome size was obtained. With an increase of molecular weight of the hydrophobic block (HB) of the copolymer, CF encapsulation increased due to the formation of more polymersomes. No effect of the type of HB (PDLLA or PCL) on the amount of encapsulated CF was observed. The percentage of CF encapsulation, however, is generally very low.

3.1.1. The release of CF

The release of CF from the polymersomes into different media at different temperatures was studied by measuring at different time intervals the fluorescence of the media in which the polymersomes were incubated. The presence of plasma proteins suppresses the fluorescence [27] and the extent of the suppression depends on the protein concentration. This dependency was studied and all data were corrected for this fluorescence suppression. Even at 90% release, the fluorescence from the CF inside the polymersomes is still quenched 40% and only accounts for 3–5% of the total fluorescence, as calculated based on the quenching factor at different CF concentrations [27]. Also, the initial CF concentration used is very high giving more than 95% quenching, therefore, the fluorescence contribution of the CF inside the polymersomes is neglected.

The release of CF from all polymersomes that were studied had the same trend and typical release profiles of CF at room temperature in different media are shown in Fig. 1. All the profiles show an initial rapid release followed by a gradual decrease of the release rate, which practically decreased to zero after 5–6 days (Fig. 1A). CF release profiles of the first 4 h are "magnified" and shown in Fig. 1B. At the first time point measured (2 min) the release was already 30%. It should be noted however, that the time between the gel filtration of the polymersomes and the start of the release experiment (20–30 min) was not taken into account.

The CF containing polymersomes can be considered as a membrane-controlled reservoir device system. If the device contains an unsaturated solution, the release rate, dM_t/dt, which is proportional to the mass of the encapsulated agent and declines exponentially with time [29], can be described by

$$\frac{dM_t}{dt} = k(M_0 - M_t) = kM_0\exp(-kt)$$

where M_0 and M_t is the mass of agent in the device at time t=0 and the mass released at time t, respectively, and k is a constant.

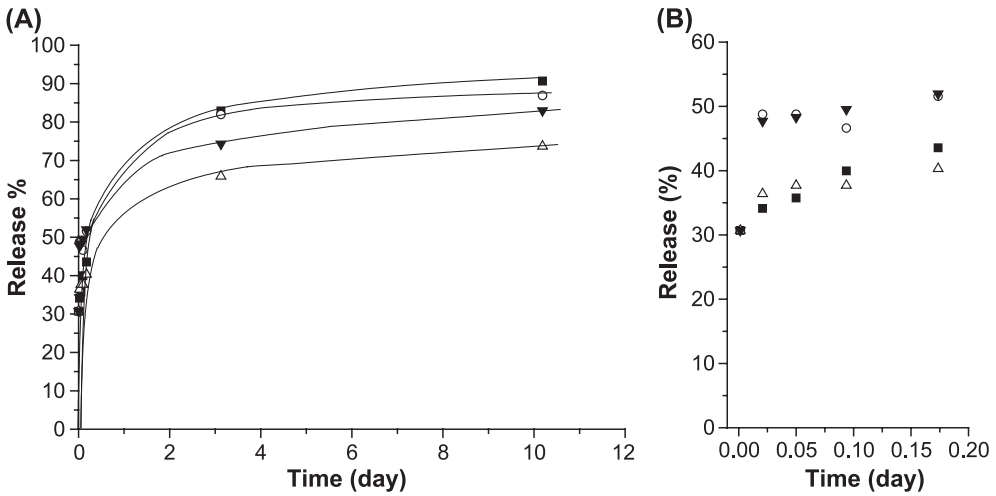

Fig. 1. CF release profiles of polymersome of PEG-PDLLA5.8-48 in different media at room temperature (A): ■ PBS, ○ human serum albumin in PBS (4 wt.%), ▼ human plasma in PBS (85 vol.%) and △ human plasma in PBS (15 vol.%). N=3, standard deviation is <12%. (B) is the magnified part of the release of the first 4 h. The lag time (20–30 min) between the gel filtration of the polymersomes and the start of the release experiment was not taken into account.

To validate this, the data for the CF release in PBS at 20 °C was re-plotted as $\ln(F_T/(F_T-F))$ as a function of the release time. This resulted in a linear relation (R=0.996, see Fig. 2, dashed line). This confirms that the CF containing polymersome system is a membrane-controlled reservoir system, with CF in an unsaturated solution inside the polymersomes. As CF is released its concentration decreases, resulting in a release rate that declines exponentially, giving a first-order release profile [29].

The release of CF in PBS at 20 °C lasted about 2 weeks. The release of CF in a physiological albumin solution (4 wt.% in PBS) was almost identical to the release of CF in PBS. This indicates that albumin adsorption and/or albumin insertion in the polymersome membrane does not take place or does not affect CF release. However, CF release into plasma dilutions (15 and 85 vol.% in PBS) was slower than in PBS and surprisingly the release in 85 vol.% plasma was faster than in 15 vol.% plasma. This can be ascribed to the complex process of protein adsorption from plasma dilutions onto the polymersomes. Some proteins other than albumin may penetrate the PEG layer and stick to the hydrophobic part resulting in a thicker membrane, thus slowing down the release of CF. The reason that 15 vol.% plasma has a larger influence on the CF release than 85 vol.% plasma, is probably due to the adhesion and insertion of certain proteins that is inhibited at high plasma concentration. For example, Fn adsorption onto poly(ethylene teraphthalate) and Teflon films reached a maximum value at a serum concentration of ~0.1% [30], and was significantly decreased or completely blocked by other proteins when the serum concentration was higher than 1%. A faster CF release in PBS than in plasma dilutions was also observed for multi-lamellar liposomes [31], where the rate of CF release was 3–8 fold less in the presence of up to 25 vol.% human serum as compared to the release in PBS. This was ascribed to the association of serum proteins with the outermost lipid bilayer, which led to stabilization of the liposomes (less bilayer defects), resulting in restricted permeability of the lipid membrane [31].

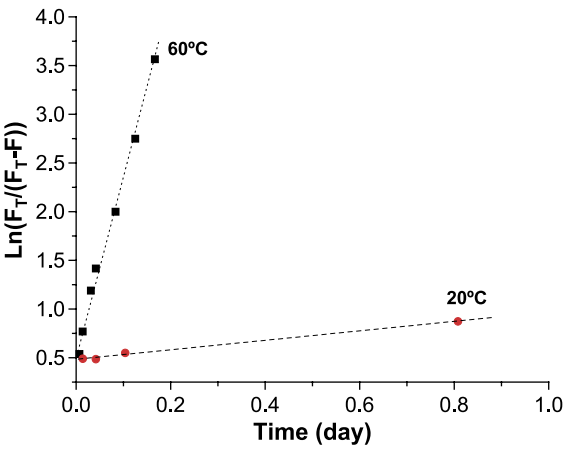

Fig. 2. The kinetics of CF release from polymersomes of PEG-PDLLA5.8-48 in PBS at 20 °C (●, the dashed line is the linear fit for Y=0.482+0.486X, R=0.997) and 60 °C (■, the dotted line is the linear fit for Y=0.492+18.470X, R=0.996).

This also indicates that the pegylated surface of the polymersomes does not fully prevent non-specific protein adsorption. Besides influencing the release properties of the polymersomes, non-specific protein adsorption may also lead to clearance of the particles from the body by the mononuclear phagocytic system. It is anticipated that it is not easy to tune the PEG surface concentration of the polymersomes by varying the polymersome formation conditions. Therefore, the use of a PEG block with a higher molecular weight may be a better alternative to further suppress non-specific protein interactions with the polymersomes.

When the temperature was increased from 20 to 60 °C, the release of CF in PBS was enhanced drastically (Figs. 2 and 3). At 20 °C polymersomes of PEG-PDLLA5.8-48 completely released the encapsulated CF within ca. 12 days, whereas at 60 °C release was complete within 4 h. The kinetics of CF release in PBS at 60 °C also showed a linear relationship (R=0.997) between $\ln(F_T/(F_T-F))$ and the release time (Fig. 2, dotted line), suggesting a first order release profile of CF. In addition, the CF containing polymersomes retained their integrity also at 60 °C.

Assuming that the T_g of the PEG-PDLLA copolymer in the dry state (35–37 °C) is the same as of the polymer in an aqueous environment, the difference in release at these two temperatures may be (partially) explained by the T_g.

Although the CF release profile was the same for polymersomes of all block-copolymers that are listed in Table 1, the composition of the block-copolymer had an influence on the release rate of CF. The lower

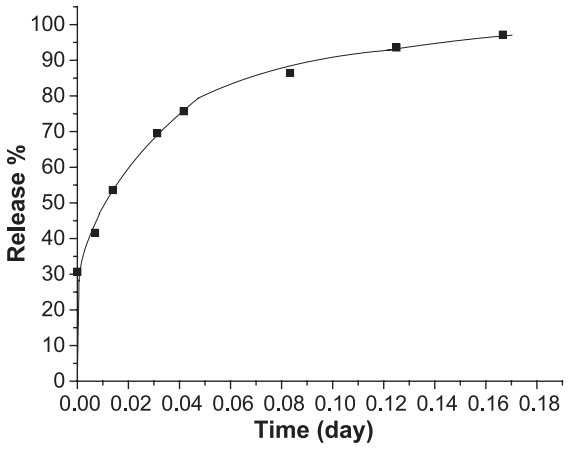

Fig. 3. The release profile of CF from polymersomes of PEG-PDLLA5.8-48 in PBS at 60 °C.

the Mn of the whole copolymer (with similar HF/HB ratio), the higher the release rate (data not shown). This is probably due to the relatively thinner membrane of the polymersomes of copolymers with a low Mn of HB [1]. The CF release rate decreased in the order: PEG-PCL5.8-24>PEG-PDLLA5.8-24> PEG-PDLLA5.8-48. This sequence can be expected considering the softness and/or thickness of polymersome membranes, i.e., PCL has a low glass transition temperature as compared to PDLLA and polymersomes of PEG-PDLLA5.8-24 may have thinner membranes than those of PEG-PDLLA5.8-48.

In conclusion, hydrophilic substances can be readily encapsulated inside these biodegradable polymersomes. At room temperature (20 °C) and 60 °C the CF release of the polymersomes followed first order kinetics, suggesting that the CF containing polymersome system is a membrane-controlled reservoir system. The release can be adjusted by the copolymer composition (Mn and/or type of HB).

3.1.2. Targetability of the polymersomes

A targeting moiety present on the polymersome surface can render it site specific and thus reduce unwanted side effects. In this section, the accessibility of the carboxylic acid groups of the polymersomes for the immobilization of antibodies as targeting moieties is evaluated. Furthermore, the preparation and purification of Ab-PS and Ab-G-PS, as well as their interaction with immobilized antigens will be addressed.

3.2. Availability of carboxyl groups of C-PS

To prepare polymersomes with covalently immobilized antibodies, polymersomes with –COOH groups on the surface were prepared from copolymer C-PEG-PDLLA3.4-30. The availability of the –COOH groups of the C-PS was evaluated by zeta potential and SPR measurements. The zeta potential of C-PEG-PDLLA3.4-30 polymersomes in MES buffer and in PBS were -39.5 ± 0.4 and -37.2 ± 0.5 mV, respectively, which are ~20–25 mV less than those of PEG-PDLLA3-24 polymersomes, suggesting that –COOH end groups of the PEG block of the copolymer are present at the surface of the polymersomes.

This was further confirmed by an SPR measurement (Fig. 4). Incubation of the aminated surface with C-PS in MES for 20 min did not induce any adhesion of polymersomes to the surface. Incubation of the surface with EDC/NHS activated C-PS generated a large shift of the SPR angle of which most was due to the refractive index change by the dispersion of EDC/NHS activated polymersome. Washing five times with MES buffer eliminated the bulk effect, and an SPR shift of ca. 475 m° was left which can be attributed to the covalent immobilization of the C-PS to the aminated sensor surface. In the reference channel, where exactly the same procedure was performed except that un-activated C-PS were applied, no significant SPR shift was detected. The covalent attachment of C-PS to the sensor surface in MES buffer was also confirmed by tapping mode AFM measurements (results not shown).

These results confirm the proposed structure of the polymersome model, in which the wall of the polymersomes consists of a layer of stacked coiled polymer chains held together by non-covalent forces with the PEG blocks protruding into the aqueous phases and the entangled hydrophobic block forming the core of the polymersome wall [1,2]. Most interestingly, the availability of the –COOH groups on the polymersome surface allows polymersome surface modification for instance for targeting purposes.

3.3. Interaction of Ab-PS, Ab-G-PS and G-PS with protein patterned surface

After the purification of the Ab-PS, G-PS and Ab-G-PS dispersions by gel filtration, the obtained

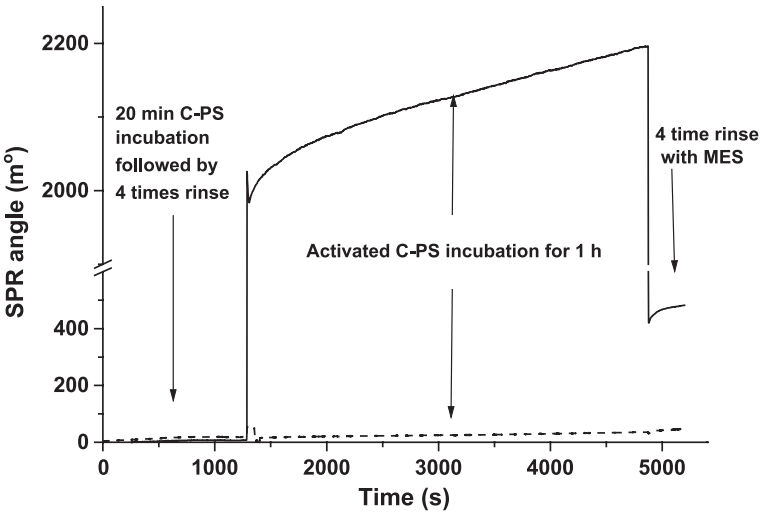

Fig. 4. SPR profile of the interaction between C-PS and an aminated sensor surface. The solid line is the SPR angle of the reaction channel where the interaction of EDC/NHS activated C-PS with the surface takes place, and the dashed line represents the SPR angle of the reference channel where the aminated surface was incubated with non-activated C-PS.

polymersomes were free of free antibodies/protein G as shown by determination of the protein concentration in the collected fractions. Table 2 shows the characteristics of these purified polymersomes. Immobilization of protein G (42 kDa) did not cause much increase in the polymersome size, but an increase in zeta potential (9 mV) was observed, indicating the substitution of the –COOH groups on the polymersome surface. a-HSA-PS had a larger size (~20 nm) and a higher zeta potential (~14 mV) than the starting C-PS. With a similar increase in zeta potential (8 mV), Ab-G-PS were larger (100 nm) and had a much higher polydispersity (PDI) than the C-PS. The large increase in size of polymersomes can be ascribed to the immobilization of antibodies on the surface and to partial polymersome aggregation. Upon the immobilization of the antibodies to the G-PS surface, which involved an additional ultracentrifugation step, it was difficult to redisperse the polymersomes.

The targetability of the polymersomes was investigated by following the interaction of purified Ab-PS and Ab-G-PS with surface immobilized antigens in time by iSPR. SPR shifts due to the interaction of protein surfaces with polymersomes are shown in Fig. 5. All the spots showed some non-specific interaction when the surface was incubated with C-PS, probably due to electrostatic interactions. The interaction of a-HSA-PS with HSA spots only gave a slightly higher SPR angle shift than for other protein spots. This is probably due to the low amount of a-HSA that was immobilized onto the polymersomes and/or to the random immobilization of the a-HSA on the polymersome surface.

Recombinant protein G can be used to provide site specific immobilization of an antibody due to its specific interaction with the Fc portion of the antibody without affecting the antigen–antibody interaction. Therefore, coupling of an antibody via

Table 2
Size and zeta potential of different polymersomes in PB buffer

	C-PS	a-HSA-PS	G-PS	a-HSA-G-PS	a-HIgG-G-PS
Zeta potential (mV)	−26.1±1.5	−12.0±0.4	−17.0±0.2	−17.7±0.1	−17.9±0.1
Size (nm)	288±2	310±1	286±0	382±4	392±5
PDI	0.12±0.02	0.16±0.05	0.13±0.03	0.45±0.08	0.31±0.05

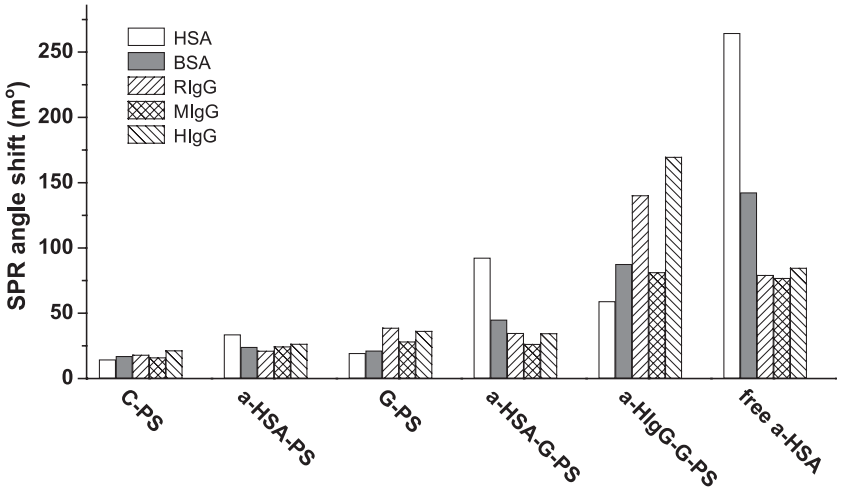

Fig. 5. SPR angle shift as a result of the interaction of different polymersome preparations (C-PS, a-HSA-PS, G-PS, a-HSA-G-PS, a-HIgG-G-PS) and free a-HSA with different immobilized protein spots (HSA, BSA, rabbit IgG, mouse IgG and Human IgG).

protein G can improve the orientation of the antibody on the polymersome surface and thus enhance the binding efficiency. The results show that antibody immobilization via protein G is indeed more efficient than direct immobilization of antibody, whereas the G-PS only gave a small SPR shift, especially the a-HSA-G-PS showed specific interaction with immobilized HSA. The interaction of these polymersomes with BSA may be non-specific or due to some cross-reactivity of the antibody. The pattern of the interaction between a-HSA-G-PS and surface immobilized IgG's was the same as for the G-PS, showing that a-HSA-G-PS had no affinity for the immobilized IgG's.

The same trend as was observed for a-HSA-G-PS was observed for the interaction of free a-HSA with the immobilized protein spots. However, the SPR angle shifts caused by the free a-HSA were much larger. This is probably due to a smaller diffusion constant of the polymersome immobilized a-HSA. For the interaction of free a-HSA with immobilized HSA, an SPR angle shift of 265 m° was obtained which corresponds to ~0.22 μg/cm^2, indicating that less than a monolayer of a-HSA was present on the surface.

The a-HIgG-G-PS showed a strong interaction with the immobilized HIgG spots. However, also the interaction with the albumin spots and RIgG and MIgG spots was rather large. This is probably due to the fact that the a-HIgG that was used for the immobilization was not cross-adsorbed.

Although these targeting experiments are preliminary, it can be concluded that the targeting of polymersomes by immobilization of antibodies is possible and that site-specific immobilization through protein G is favoured over direct immobilization of the antibodies.

4. Conclusions

As shown by the encapsulation of carboxyfluorescein (CF), hydrophilic biofunctional materials can be readily incorporated in polymersomes of amphiphilic biodegradable block copolymers simply by adding biofunctional materials to the aqueous phase during the polymersome preparation. The CF release in PBS at room temperature was complete within ca. 2 weeks and the presence of plasma proteins other than albumin decreased the release rate. The release of encapsulated material from the polymersomes can be tailored by changing the copolymer composition, especially the MW and type of hydrophobic block of the copolymer. CF release in PBS both at room temperature and at 60 °C followed first order kinetics, confirming that the CF containing polymersome system is a membrane controlled reservoir release system. These biode-

gradable polymersomes have potential for targeting to specific sites in vivo as shown by the specific interaction between a-HSA-G-PS and immobilized HSA in imaging SPR experiments.

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