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Research Paper

Calcein Release from Polymeric Vesicles in Blood Plasma and PVA Hydrogel

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Purpose. The main goal of this study was to show the long-term stability of vesicles from poly(2-methyl oxazoline-block-polydimethylsiloxane-block poly(2-methyl oxazoline) (PMOXA-PDMS-PMOXA) in PBS, blood plasma and the feasibility to use these vesicles for drug release from PVA hydrogels.
Methods. The vesicle formation properties and loading efficiency was evaluated using fluorescent dyes. The stability of the vesicles was evaluated in buffer at pH 7 at room temperature and in 50% blood plasma at 37°C. The calcein loaded vesicles were dispersed in a UV crosslinked PVA hydrogel. The stability of the vesicles in the hydrogel was observed over one week, before the vesicles were ruptured with Triton X-100.
Results. The vesicles are very stable in buffer, blood plasma, and the PVA hydrogel. In plasma 50% of the calcein is released in 48 h in the presence of sodium azide. The vesicles can be evenly dispersed in PVA and are stable. The release can be triggered and the calcein diffuses afterwards quickly throughout the gel.
Conclusion. Polymeric vesicles can be used as diffusion barrier in hydrogels for the controlled release of water soluble drugs.

KEY WORDS: controlled release; drug delivery system; hydrogel; PVA; vesicles.

INTRODUCTION

Hydrogels consist of a water-swollen 3D-network of crossslinked polymer chains, like poly(vinyl alcohol) (PVA), poly(ethylene glycol), polyacrylates, polypeptides or polysaccharides. Due to their good biocompatibility, adjustable mechanical, temperature and pH stability, there is considerable interest for their use as scaffolds in tissue engineering, contact lenses or sustained-release delivery systems for a variety of therapeutic agents targeting different sites in the body. Since hydrogels consist of up to 99% water, the release of water-soluble drugs is mainly controlled by Fickian diffusion, therefore a particular challenge remains, to reduce and control their release rate.

Although for specific applications a delivery time of a few hours, *e.g.*, in order to avoid rapid clearance of proteins from systemic circulation is limiting enough (1,2) considerable efforts have been made to introduce additional functionalities for controlling the release rate beyond that. The introduction of hydrophilic polymer fillers (3), varying the crosslinking density of the gels or introducing gradients in the mesh-size of the hydrogel (3,4), making use of triggered swelling/deswelling transitions, exploiting electrostatic interactions between oppositely charged drugs and polymer backbones (5), introducing degradable bonds between the gel and the drug (6,7), use of degradable release depots (8) or environmentally sensitive gels (9–11) are strategies that have been tried to reduce and control the release rate of hydrophilic drugs from hydrogels. Most of these approaches are however limited to specific drugs or do not allow controlled release beyond 24 h.

An interesting alternative is to introduce a compartmentalized inner structure with hydrophobic diffusion barriers into the hydrogel by incorporation of drug loaded vesicles such as liposomes. Vesicles with dimensions in the range of few hundreds of nanometers can be physically trapped inside the mesh-structure of a hydrogel and stay in place. Here the release of hydrophilic substances is either controlled by their diffusion across the wall-forming bilayer membranes of the vesicles or the time needed to rupture the vesicles

Dispersing drug loaded vesicles in a hydrogel matrix offers therefore the following advantages:

- The release kinetics can be adjusted independently of other properties of the hydrogel.
- The diffusion of the active agent into the desired environment is not hindered.
- The hydrogel protects the vesicles from clearance without affecting the release.
- Several release mechanisms can be superimposed
- Several drugs can be combined without an interaction of the drugs in the hydrogel

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Feasibility of this concept with liposomes has successfully been demonstrated by various groups (12–16). However it is well-known that the stability of liposomes is rather limited and can easily be affected by the environment, the nature of the gel, temperature, pH, mechanical stress and components in body fluids.

As a more robust alternative vesicles based on self-assembly of amphiphilic block copolymers find an increasing interest. Here the wide diversity of block copolymer chemistry offers the possibility to adjust the properties of the resulting aggregates by varying the chemical constitution, the block length ratio or the molecular architecture of the polymeric building blocks (17,18). Nanometer sized polymer vesicles have proven to be promising candidates for *in vivo* therapeutic use due to their low immunogenicity, prolonged blood circulation times and low cytotoxicity (19–21).

Here we demonstrate for the first time that nanometer-sized unilamellar polymeric vesicles formed by poly(2-methyloxazoline)-block-poly(dimethylsiloxane-block poly(2-methyloxazoline) (PMOXA-PDMS-PMOXA) triblock copolymers can be immobilized in a PVA matrix. The PVA matrix is composed of UV crosslinkable PVA macromers with pendent acrylamide groups (3,7,22). The same type of macromer is used to produce contact lenses, microspheres for embolotherapy or a sprayable wound dressing.

UV–Vis spectroscopy, fluorescence spectroscopy and confocal microscopy have been used to demonstrate the successful immobilization of the vesicles as well as to quantify the encapsulation and release of calcein under various conditions.

MATERIALS AND METHODS

Materials

Calcein, SEC beads (Sepharose 4B), PBS, and polyethylene glycol tert-octylphenyl ether (Triton-X 100) were purchased from Sigma, BODIPY-630 was purchased from Molecular Probes. Irgacure 2959 was purchased from Ciba SC. Photocrosslinkable polyvinyl alcohol (16.8 wt.% in water, M_w 67,000, average seven acrylamide groups per PVA-molecule (3,7,22) and $PMOXA_5$–$PDMS_{22}$–$PMOXA_5$ were provided by BioCure Inc. (23).

Preparation of Polymeric Nanovesicles

The amphiphilic triblock copolymer poly(2-methyloxazoline)-block-poly(dimethylsiloxane-block poly(2-methyloxazoline) (PMOXA-PDMS-PMOXA) was synthesized and characterized using an established procedure (23).The molar mass distribution and the composition were determined by GPC and ^{1}H-NMR, respectively. The number average molar mass of the polymer was found to be M_n=2600 g/mol with two times five repeat units of the 2-methyl oxazoline and 22 repeat units of the hydrophobic dimethylsiloxane at average. The polydispersity index of the polymer was 1.48.

Two previously established methods were used for the preparation of the polymeric nanovesicles (NVs) (23). In the 'co-solvent method' the polymer (10 mg/50 μL) was dispersed in ethanol and then added with a syringe to a PBS buffer (pH 7.4)) containing 50 mM calcein, giving a final polymer concentration of 10 mg/mL. The polymeric suspension was stirred at least 10 h and then filtered ten times each with a 0.45 μm and a 0.20 μm filter respectively to narrow the size distribution of vesicles and to remove bigger aggregates. The extruded polymeric NVs were further purified using size exclusion chromatography (Sepharose 4B, PBS buffer) in order to remove the free dye and the micelle fraction.

In case of the 'film hydration method' the polymer (5 mg/mL in ethanol) was uniformly deposited on the flask walls by allowing ethanol evaporation on a rotavapor followed by drying *in vacuo*. Then 50 mM calcein in PBS was added to achieve the final polymer concentration of 2–3 mg/mL. The flask was allowed to shake overnight. This led to spontaneous budding of vesicles from the thin film into the aqueous solution. The remaining procedure was similar to the co-solvent method. For preparation of giant vesicles we applied the same procedure was applied without the extrusion steps.

The fluorescence maximum of encapsulated calcein was measured after incubation of an aliquot of the vesicle stock solution with 3% Triton-X solution followed by tenfold dilution.

Giant vesicles were obtained by film hydration in bidistilled water without further filtration.

Dynamic Light Scattering (DLS)

DLS studies were carried out using a commercial goniometer (ALV-Langen) equipped with He–Ne laser (λ= 632.8 nm) at scattering angles between 30 and 150°. An ALV-5000/E correlator calculates the photon intensity autocorrelation function $g^2(t)$. The experiments were performed at T= 293 K. The data for DLS were analyzed by second order cumulant fit.

Encapsulation Efficiency

Calcein concentration in the prepared NVs solution was determined from the absorption at 495 nm considering ε equal to 80,000 M^{-1} cm^{-1} (24).

In order to find out the encapsulated volume (V_{encaps}) of the 50 mM calcein hydration solution we used following equation:

$$V_{encaps} = c_f \times V_f/50$$

where c_f is a calcein concentration in the formulation and V_f is a formulation volume.

The maximum theoretical encapsulated volume (V_{theor}) was estimated from the hydrodynamic radius R_h of the vesicles obtained from DLS experiments and assuming that the membrane thickness is 10 nm and the polymer density 1 g/cm^3.

Encapsulation efficiency (EE) was estimated as $V_{encaps}/V_{theor}\times 100\%$.

Incorporation of Vesicles into the Hydrogel (HG) and Stability Study

Vesicles were incorporated into the hydrogel by photocrosslinking a polyvinyl alcohol macromer solution in the presence of vesicles. The macromer used was synthesized from Mowiol 3–83 (M_W=14,000 g/mol) modified with

0.45 meg/g of N-acryloylamino-acetataldehyde dimethylacetal (22). The PVA macromer was used as a 16.8 wt% solution. In a typical experiment 1 g of 16.8 wt.% photocrosslinkable polyvinyl alcohol (PVA) solution was mixed with 150 μL Irgacure 2959 (5 mg/mL in PBS), 850 μL PBS and 1 mL vesicle dispersion, followed by irradiation for 3 min with a 200 W Oriel UV lamp (150 mW/cm^2) at a distance of 5 cm.

For the stability study the prepared hydrogel sample was incubated with 5 mL of PBS. There was not visible swelling of the hydrogel observed after crosslinking and incubation. The calcein fluorescence in an aliquot of the supernatant PBS solution was subsequently measured over time.

Confocal Laser Scanning Microscopy (CLSM)

Studies were performed with a Zeiss 510 Confocal 2 setup equipped with an argon-ion laser (maximum power, 30 mW). The 488 nm line of the argon-ion laser was used for the excitation of calcein, while the 633 nm HeNe laser-line was applied for the excitation of BODIPY for the giant vesicles imaging.

Calcein Release from NVs and HG

Calcein fluorescence intensity I_t (λ_{em}=495 nm, λ_{ex}= 520 nm) was monitored on FluoroMax-2 as a function of time (t) starting immediately after NVs or HG preparation. Calcein release over time was normalized to the fluorescence level using the equation:

$$Y = (I_t - I_0) \times 100/(I_\infty - I_0)$$

in which I_0, is the fluorescence intensity right after vesicles preparation and I_∞ is the final fluorescence intensity after breaking the vesicles upon addition of Triton-X.

Blood Plasma Experiments

For the calcein release from NVs in blood plasma, pig blood plasma was sterile filtered with 0.20 μm filter then mixed with an aliquot of NVs in a 1:1 ratio. To preserve bacterial growth NaN_3 solution was added (0.1% final concentration). The samples were incubated at 37°C. To measure the fluorescence intensity due to release of calcein an aliquot was withdrawn after the corresponding time and diluted five times with PBS. As a reference in parallel the same experiments were done with PBS instead of blood plasma.

RESULTS

Giant-Vesicles Formation

To examine vesicle formation from this rather short triblock copolymer, a thin film of polymer was hydrated with bi-distilled water. The PDMS part of the polymer was stained with the hydrophobic dye BODIPY to visualize the resulting aggregates. For this, the aqueous sample was plated on a microscopy slide and the polymer morphology was examined. Fig. 1 illustrates the formation of multilamellar giant vesicles after 12 h of hydration with diameters of up to 10 μm.

Encapsulation Efficiency: Cosolvent Method *vs* Film Hydration

To measure encapsulation efficiency, calcein was used as a model dye. Calcein was chosen since at a pH greater than 7, its four carboxylic groups are deprotonated. The hydrophobic part of the polymer membrane has a very low permeability for the tetra-anion, which therefore remains entrapped inside the vesicles during these measurements. Secondly, when loaded at concentrations high enough to allow self-quenching, the contribution to the fluorescence intensity from the trapped dye is negligible, since also upon dilution of the vesicle dispersion it locally (*i.e.*, inside the vesicles) remains at high concentration. Two different methods were used to determine the encapsulation efficiency. The first method consisted in the dissolution of the polymer in ethanol and drop-wise addition of the latter into a calcein solution followed by repeated extrusion through filters of defined pore size (see 'MATERIALS AND METHODS'). This method yielded a mixture, which, after SEC separation of the free dye, yielded two fractions containing polymeric

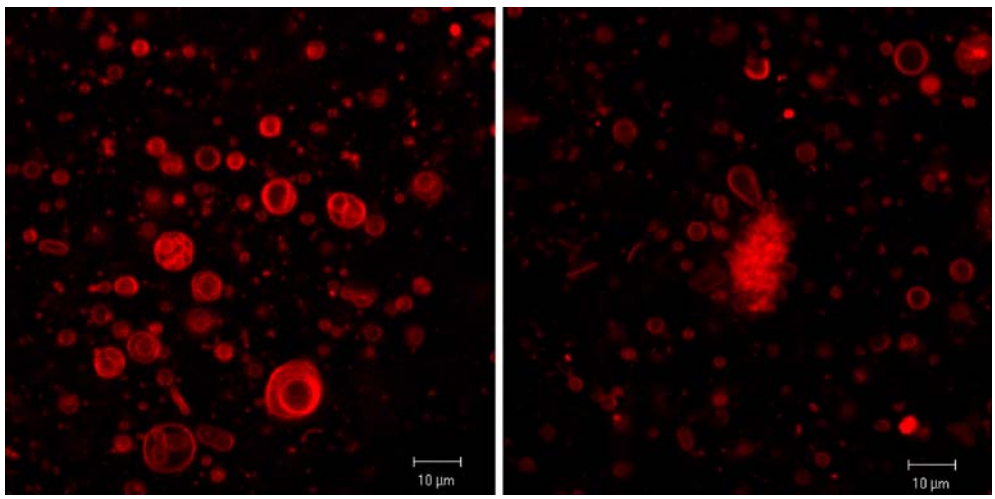

Fig. 1. CLSM image of polymersomes stained with BODIPY-630. The *scale bar* corresponds to 10 μm.

aggregates. DLS measurements gave a hydrodynamic radius of R_h=78 nm for the first polymer fraction and 18 nm for the second one. Upon disrupting the aggregates by addition of Triton-X we observed a tenfold increase of fluorescence intensity for the first fraction (red lines in Fig. 2A) and less than a factor of two for the second fraction (blue lines in Fig. 2B). Interestingly the fluorescence intensity of the first fraction after treatment with Triton X was 400 times greater than for the second fraction, *i.e.*, encapsulating a considerably higher concentration of encapsulated calcein. This and the measured dimensions of the aggregates suggests that the first fraction consisted of vesicles whereas the second fraction was composed of micelles.

Also the UV spectra for the two fractions (Fig. 2B) had different absorbance maxima. In case of the first fraction, the maximum absorbance of calcein was observed at 495 nm whereas for the second fraction, it was found to be at 505 nm. According to recent literature (24) an absorbance maximum of 495 nm was found for calcein encapsulated in liposomes in PBS buffer. This also supports that the first fraction consists of vesicular structures. The trapped volume of calcein buffer could be estimated from the calcein absorbance. This was used for the estimation of the encapsulation efficiency which corresponds to the ratio between the encapsulated volume, found from UV–Vis measurements, and the theoretical one assuming a quantitative yield of vesicles with 80 nm radius. The encapsulation efficiency for this vesicle preparation method was up to 10% (see supporting information in the Electronic Supplementary Material).

The encapsulation efficiency was also estimated for the film hydration method. A thin dry film of the triblock copolymer was formed on a vessel wall and afterwards hydrated with 50 mM calcein in PBS. To reduce the dimensions of the vesicles formed during this process the resulting suspension was repeatedly extruded through filters of defined pore size. It has to be emphasized that when vesicles were prepared with this method, only one fraction of polymer aggregate was obtained after SEC removal of the free dye. DLS measurements yielded a R_h of 85 nm and UV–Vis spectroscopy as well as the increase in the fluorescence level after incubation with Triton-X confirmed the presence of hollow cavities (*i.e.*, vesicular structures) in the formulation prepared by this method. Obviously the formation of micellar aggregates was induced by the presence of ethanol during the preparation by the 'cosolvent method'. The encapsulation efficiency of vesicles prepared by this method was found to be at least 15% (see supporting information in the Electronic Supplementary Material).

Stability Study

For stability studies, PMOXA-PDMS-PMOXA vesicles (NVs) were loaded with calcein at concentrations high enough to allow self-quenching using the co-solvent method. The untrapped dye and micellar fractions were removed by gel filtration chromatography equilibrated with PBS. The obtained vesicle dispersion was then kept at room temperature for four months and calcein release from vesicles was followed by the increasing fluorescence level over time. To find out the maximum fluorescence level, aliquots of the NVs solution were incubated with Triton-X to liberate all trapped calcein. The fluorescence was normalized considering the initial fluorescence level as a zero and 100% for the fluorescence level after NVs rupture, respectively. The release profile shows that only about 7% calcein release occurred during the first 2 months. Within experimental error no further release could be observed after this time (Fig. 3A).

DLS measurements showed that the morphology of vesicles was stable over time. The initial R_h was 78±9 nm and after 4 months the R_h was 81±10 nm. Since the change in R_h falls within the error, it can be assumed to be negligible.

It has to be emphasized that the rate of fluorescence increase during the first 2 months was very similar for the vesicles and micellar fractions (Fig. 3B). Also for micelles no further release could be detected after that time. This could be explained by the fact that a fraction of the dye entrapped in the aggregates during the preparation procedure or is non-specifically adsorbed to the hydrophilic/hydrophobic interface of the self-assembled superstructures formed by the polymers and slowly released during the incubation.

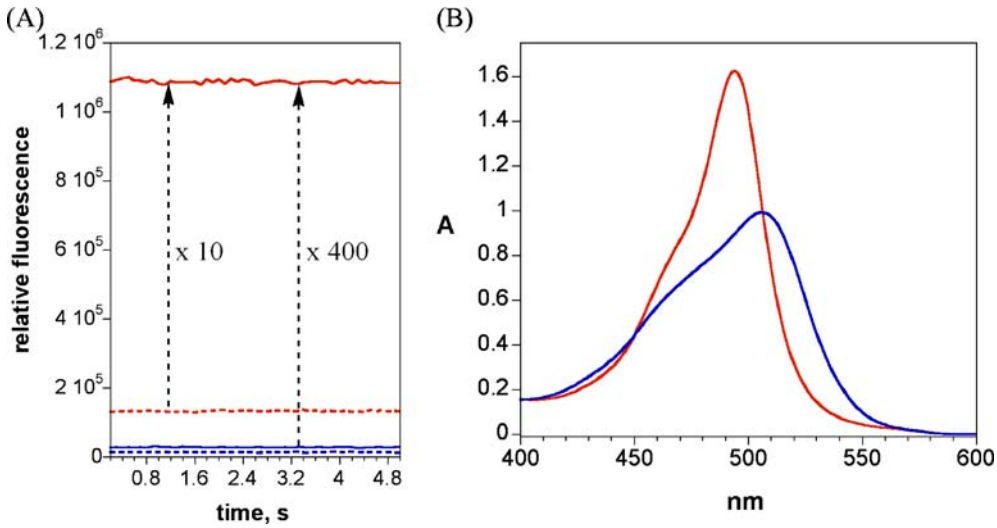

Fig. 2. **A** Fluorescence increase before (*dash line*) and after (*solid line*) incubation of an aliquot of vesicles (*red*) and micelles (*blue*) with Triton-X. **B** UV–Vis spectrum of vesicles (*red*) and micelles (*blue*).

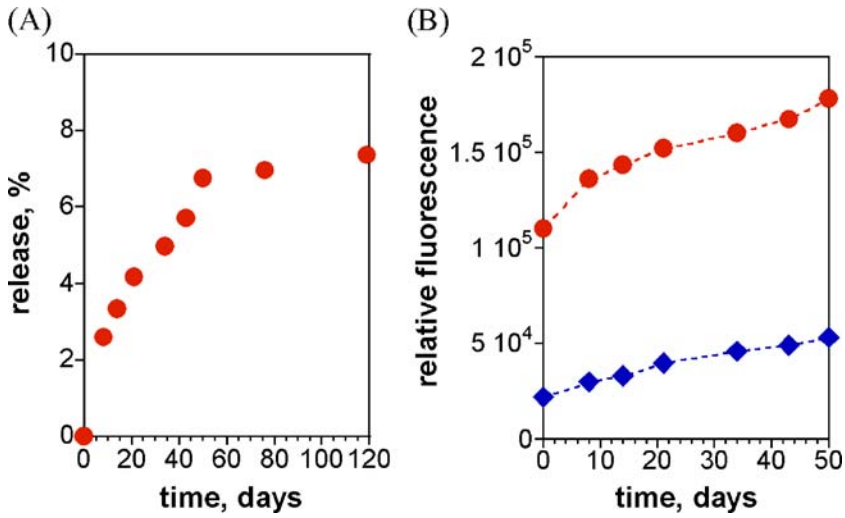

Fig. 3. **A** The release profile from polymer vesicles over 4 months. **B** Calcein release kinetics from vesicles (*red*) and micelles (*blue*).

Stability in Blood Plasma

The calcein release from NVs was also measured in a presence of 50% blood plasma at 37°C (Fig. 4A). An immediate release of about 20% of loaded calcein was observed within the first 90 min, followed by a phase of sustained release. Overall 50% of initial vesicle content was released over 48 h, suggesting that blood plasma destabilizes the polymer vesicles. However, they are considerably more stable than conventional unilamellar liposomes (25–27).

To examine the possible influence of the sterilization agent, NVs were incubated with blood plasma in the absence of NaN_3. It has to be emphasized that in this case no burst effect was observed (Fig. 4B). Unfortunately we could not follow the calcein release over longer periods of time due to the growth of bacteria in the non-sterile solutions. Nevertheless the experiments indicate a specific interaction or chemical reaction between the azide ion and our polymers that destabilizes the self-assembled superstructure. This will be systematically investigated in the future.

Distribution of Vesicles in Hydrogel and Release

A PVA hydrogel was loaded with vesicles containing calcein by inducing the crosslinking reaction of the PVA macromers in the presence of a vesicle dispersion. Confocal fluorescence microscopy (Fig. 5A) shows fluorescent spots that are evenly distributed in the hydrogel sample, which can be assigned to the trapped vesicles. Although the vesicles with their diameter of around 170 nm are below the resolution limit of the confocal microscope under epi-illumination, the calcein loaded vesicles still appear as fluorescent spots. After the hydrogel sample was incubated in 1% Triton-X solution, the LSM image showed after a short incubation time an even distribution of calcein due to the rupture of vesicles (Fig. 5B) thus indicating the possibility to

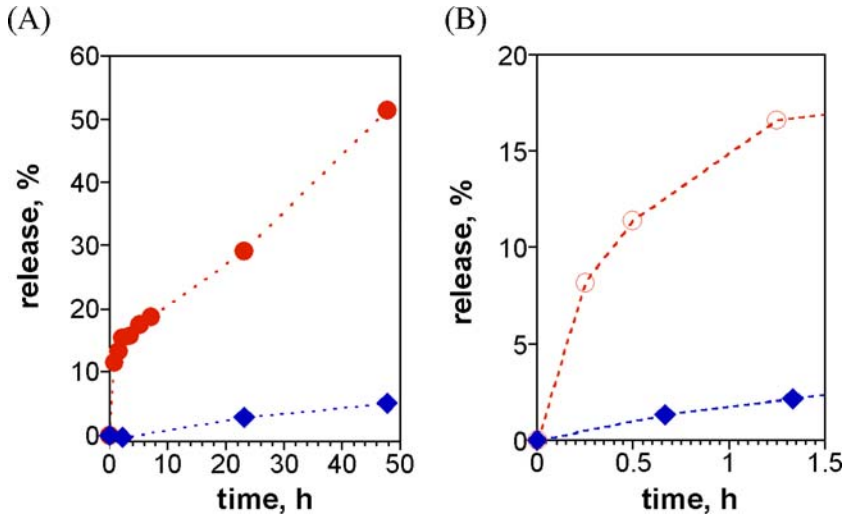

Fig. 4. **A** Calcein release from NVs after incubation with 50% blood plasma (*red*) and as a control (reference) with PBS (*blue*). **B** NVs incubation with 50% blood plasma in the presence (*red*) and absence (*blue*) of NaN_3.

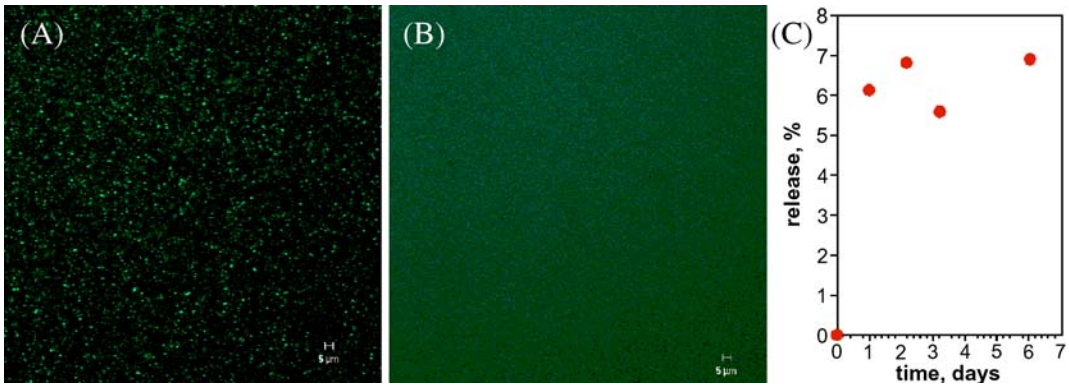

Fig. 5. **A** LSM image of a PVA hydrogel sample loaded with calcein polymersomes. **B** Image of the same sample after incubation with Triton-X. The *scale bar* corresponds to 5 μm. **C** Normalized calcein release from polymersomes loaded hydrogel incubated with PBS.

induce a triggered release from these systems. The stability of the vesicle containing hydrogel formulation was examined by incubating the hydrogel sample with PBS at room temperature. The fluorescence of the PBS supernatant was measured over 6 days. Afterwards the sample was treated with Triton-X to release all encapsulated dye. The normalized fluorescence profile is shown on Fig. 5C. We can see that 6% of calcein was released during the first 24 h. This is presumably due to mechanical stress acting on the vesicles during the cross-linking reaction, which partially may cause defects in their walls or disrupt them. It has to be noted that after the initial 'burst' no further release could be observed over at least one week.

DISCUSSION

The $PMOXA_5$–$PDMS_{22}$–$PMOXA_5$triblock copolymer investigated in this study forms vesicular structures in aqueous buffers that are tight and stable over 4 months at room temperature and pH 7.4 despite the rather low molar mass of the polymer used. It should be noted that the preparation procedure had considerable influence on the self-assembly behavior of the polymer. While film rehydration exclusively leads to the formation of vesicular structures the co-solvent method always results in a mixture of micelles and vesicles. This is presumably due to a certain interfacial activity of the ethanol present in these systems. Preliminary experiments (unpublished data) indicate that upon removing the ethanol from the solution, the micelles slowly reassemble over a period of several weeks finally also leading to exclusively vesicular structures.

The encapsulation efficiency for calcein was found to be in a range of 10–20% and needs to be further improved for potential applications. As already indicated by the slight differences for our experimental procedures development of an optimized preparation procedure for the vesicles will be crucial.

Interestingly the initial release of calcein from the aggregates was similar for vesicular and the micellar structures over 50 days. Since calcein is poorly soluble in ethanol, one can assume that in the co-solvent method some dye was trapped in the polymer due to the presence of ethanol, and slowly redissolves back into the aqueous medium over time or that a fraction of the dye was adsorbed to the surface of the aggregates and slowly dissociates off during the incubation time.

In blood plasma the polymer vesicles had a half life of at least 48 h, *i.e.*, were considerably more stable than conventional unilamellar liposomes (25–27). Control experiments revealed that the release from the vesicles during that period of time is mainly induced by the presence of NaN_3 since samples in NaN_3-free blood plasma were completely tight. Whether this is the result of specific interactions or even chemical reactions between our polymer and the azide ions has to be clarified in the future.

Confocal laser scanning microscopy clearly shows that intact and tight polymer vesicles can be entrapped in a PVA hydrogel and remain stable over at least 1 week at room temperature. The vesicles were evenly distributed throughout the gel and beside an initial burst, the calcein was trapped in the vesicles. This fast liberation of about 6% of the encapsulated calcein is presumably caused by density changes of the gel during the crosslinking reaction. This may cause mechanical stress on a part of the embedded vesicles, which induces defects or completely disrupts them, causing a release of their content. Upon adding Triton X-100 as a model for a specific trigger, the encapsulated calcein could be released in one shot and rapidly distributes within the hydrogel. Further work needs to go into the investigation of the cause for the initial release and also long-term stability studies should be conducted in order to investigate the applicability of such a drug release system.

CONCLUSIONS

The study clearly shows that polymeric vesicles are suited to be used for extended release of water soluble drugs in solution and from hydrogels. Due to the more robust nature of the polymeric vesicles compared to liposomes, their applicability should be more versatile with respect to the nature of the hydrogel and the possible release mechanism. The combination of the results from the stability study in blood plasma and the incorporation of these vesicles into the hydrogel suggests, that the vesicles would release their content slowly in an *in-vivo* application, such as a wound dressing. Although this study used the addition of a detergent to trigger the complete release of the encapsulated material,

many other possibilities exist. It is straightforward that polymer chemistry offers convenient ways to introduce, *e.g.* temperature-, pH-sensitive polymer blocks to induce a triggered release or simply by enhancing the permeability of the hydrophobic blocks of these polymers by switching from PDMS to more polar polymers like polyesters or polyamides.

In order to make this technology more widely applicable still many open questions (encapsulation efficiency, influence of NaN_3 on the vesicle stability, burst effect in the hydrogel, *etc.*) have to be addressed. In addition either more triggerable systems need to be developed, where the vesicles slowly degrade or rupture due to other external triggers such as pH, salt concentration, temperature or mechanical stress; or amphiphilic drugs can be encapsulated that diffuse slowly through the membrane of the vesicle into the hydrogel without the need for a trigger.

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