Vesicles

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Polymersomes in Polymersomes: Multiple Loading and Permeability Control**

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Polymer vesicles polymersomes are vesicles obtained from the self-assembly of amphiphilic block copolymers in aqueous solution as a result of free-energy minimization.[1] Their potential use as drug delivery systems,[2] sensors, and/or nanoreactors[3] has recently attracted a great deal of interest.[4] Polymersomes exhibit larger mechanical stability and lower permeability than liposomes, their structural analogues that often suffer from premature drug leakage.[5] To circumvent this limitation, Zasadzinski and co-workers developed liposomes in liposomes structures, also referred as "vesosomes".[6] With such a compartmentalized structure, a molecule encapsulated in the inner liposome, would have to permeate through two successive membranes, instead of a single one before leaking into the outside environment. This double-membrane effect was demonstrated by observing the serum half-life of ciprofloxacin drug increasing from 10 min in single liposomes to 6 h in vesosomes.[7] Other reports evidenced the biomedical impact of such vesosomes for transcutaneous,[8] and oral administration,[9] important areas in drug delivery and cancer therapy. More complex or compartmentalized structures in general, have started to appear because they enable an unprecedented level of control, in particular in the fields of drug delivery[10] and confined reactors.[11] However, it is still very challenging[12] to encapsulate multiple distinct components in a single compartment[13] and control their stability and release properties.

Such vesosome structures based on polymers that can be termed "polymersomes in polymersomes" have recently been reported. One of the most recent approaches consisted in forming the larger polymersomes by solvent-displacement method (or nanoprecipitation) with a suspension of smaller polymersomes previously formed by film rehydration as a water phase.[14] The drawback of this technique lies essentially in the poor encapsulation yield during nanoprecipitation. To overcome this limitation, other options, such as emulsions or double-emulsion techniques, have been investigated. The first team taking up this challenge used two successive emulsions.[15] Even if very original, such a process is not the most easy to use and may suffer from a lack of reproducibility and homogeneity. Weitz and co-workers formed another type of complex polymersomes, aggregates of polymersomes, or multicompartment vesicles using microfluidics.[13] Such an approach allows a high level of control and reproducibility that has recently been extended further to fully multicompartmentalized polymersomes.[16]

Herein, we demonstrate the generation of polymer vesosomes, that is, polymersomes in polymersomes, with an original, facile, versatile, reproducible, and low-time and low-product-consuming technique. Our method allows multiple compartment encapsulation and the formation of systems that have controlled permeability, as they present a significant decrease in the release rate of the anticancer drug doxorubicin (DOX) encapsulated in the inner polymersomes.

The inner polymersomes are formed by nanoprecipitation of poly(trimethylene carbonate)-*b*-poly(L-glutamic acid) (PTMC-*b*-PGA) synthesized following a reported method.[17] This suspension is then loaded in larger polymersomes of poly(butadiene)-*b*-poly(ethylene oxide) (PB-*b*-PEO) by emulsion–centrifugation[18] with a quantitative loading efficiency. The procedure for forming giant PB-*b*-PEO polymersomes was inspired from Li and co-workers.[19] Briefly (Scheme 1, Part 1), a small fraction of an inverted emulsion of aqueous solution (in this case a nanosize polymersome suspension of PTMC-*b*-PGA with 380 mOsm sucrose) in toluene is poured over an interface of toluene and an aqueous solution of 380 mOsm glucose. (Scheme 1, Part 2) The PB-*b*-PEO diblock copolymer is dissolved in toluene at 3 mg mL^{-1} and stabilizes the emulsion droplets (forming the inner leaflet of the final bilayer) of this interface. (Scheme 1, Part 3) In a final step, both centrifugal force and denser sucrose (as compared to glucose) inside the droplets, force these droplets to cross the interface and to be enveloped by a second leaflet of amphiphilic PB-*b*-PEO block copolymer, resulting in the final giant polymersomes or polymer vesosomes (if a nanosize polymersome suspension is used as aqueous inner solution as reported herein).

The control provided by this process enabled imaging the compartmentalized polymersome structure by spinning disk confocal microscopy. In Figure 1 the Alexa Fluor 568 (red) labeled inner polymersomes are visibly encapsulated in a green giant polymersome (with 10 wt % of an Alexa Fluor 488 conjugated polybutadiene). The red inner polymersomes in

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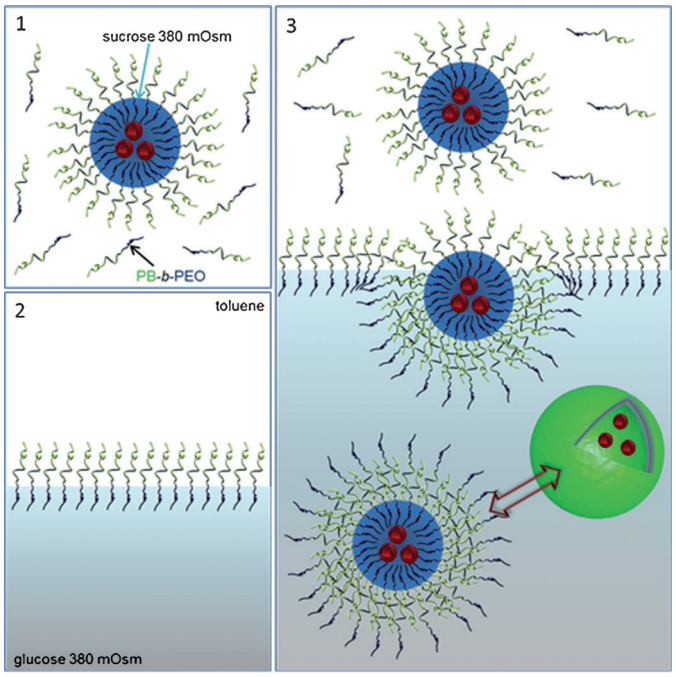
Supporting information for this article (detailed experimental section) is available on the WWW under http://dx.doi.org/10.1002/anie.201106410.



Scheme 1. Schematic representation of the emulsion–centrifugation process generating giant polymersomes or polymer vesosomes (the smaller red vesicles represent the inner nanosize polymersomes of PTMC-*b*-PGA in an aqueous solution at 380 mOsm sucrose). See text for details.

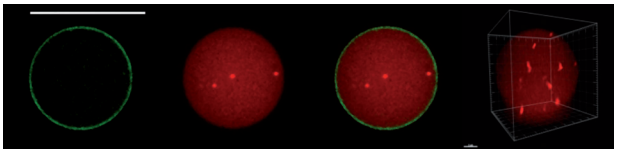


Figure 1. Spinning disk confocal microscopy images of "red" nanosize vesicles (Alexa fluor 568) in a "green" giant polymersome (Alexa Fluor 488). From left to right: green channel, red channel, overlay and 3D reconstruction (red channel). Scale bar: 20 μm.

Brownian motion can clearly be observed (see Movie 1 in Supporting Information). The giant vesicle has also been reconstructed in 3D for better visualization (see Movie 2 in Supporting Information). Such compartmentalized structures are of particular interest for the delivery of multiple active components.[20] To address this point, a 50/50 vol % solution of two different PTMC-*b*-PGA suspensions, labeled with red (Alexa Fluor 568) and green (fluorescein isothiocyanate (FITC)) fluorophores was encapsulated instead of a single nanosize vesicle suspension (Figure 2). The two nanosize polymersomes are clearly localized in the same giant polymersome; the Movie 3 (Supporting Information) shows the distinct motion of each kind of fluorescent nanosize polymersome. As long as the inner polymersome suspensions that are mixed are concentrated enough, this encapsulation can be extended to far more than two different populations. Finally, we were able to further increase the complexity of this system by co-encapsulating a large polymer, FITC-dextran (20000 g mol^{-1}), with a red nanosize vesicle suspension (Figure 3). As the FITC-dextran was already green fluorescent, the membrane was this time labeled in blue (with 10 wt % Alexa Fluor 405 conjugated polybutadiene), allowing separate imaging of the giant vesicles (blue), the inner nanosize vesicles (red) and the loaded dextran (green). Such a three-compartment encapsulation in vesicles is, to the best of our knowledge, the first reported to date.

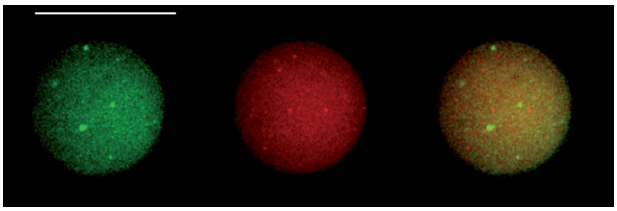


Figure 2. Spinning disk confocal microscopy images of red (Alexa fluor 568) and green (FITC) nanosize vesicles in a giant polymersome. From left to right: green channel, red channel, and overlay. Scale bar: 25 μm.

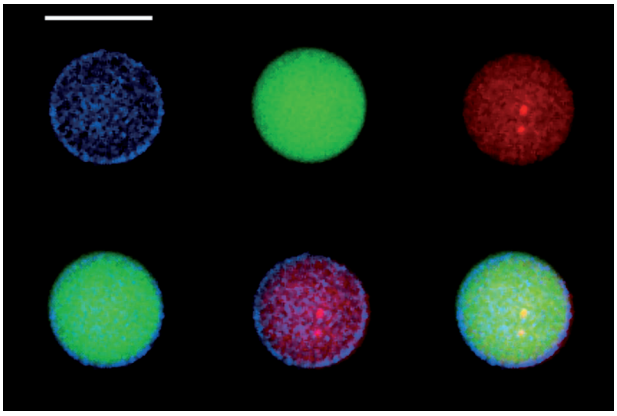


Figure 3. Spinning disk confocal microscopy acquisitions of red (Alexa fluor 568) nanosize vesicles and green FITC-dextran in a blue (Alexa Fluor 405) giant polymersome. From top left to bottom right: blue channel, green channel, red channel, overlay blue and green, blue and red, and finally blue, green, and red channels. Scale bar: 10 μm.

The double-membrane diffusion barrier effect of these polymer vesosomes was then studied by measuring the release profile of doxorubicin (DOX) as a model drug. DOX was encapsulated in the inner PTMC-*b*-PGA polymersomes.[21] The resulting suspension was concentrated as much as possible to have the highest possible drug concentration (loading content of 9 wt %). In vitro drug release experiments were then conducted on solutions of free DOX, of PTMC-*b*-PGA nanosize polymersomes loaded with doxorubicin (nano-DOX), and polymer vesosomes loaded with nano-DOX as inner polymersomes (veso-DOX). These solutions were placed in the donor chamber of a tailor-made drug release device inspired by Franz cells (Supporting Information, Figure S1). The release of DOX was followed by monitoring the absorption and fluorescence of the drug (Supporting Information, Figure S2).

A significant decrease of the DOX release rate from nano-DOX to veso-DOX can be observed at 37 °C (Figure 4), evidencing this double-membrane diffusion barrier effect and permeability tuning. Indeed, in veso-DOX, the drug has not

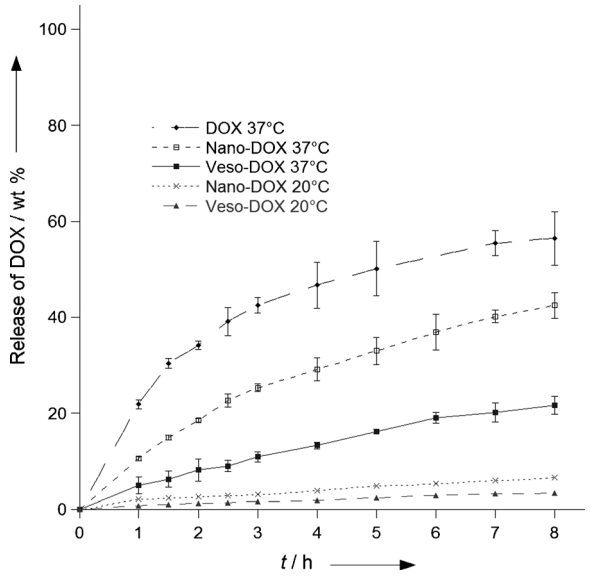


Figure 4. In vitro drug DOX release (wt%), from top to bottom, of: free DOX (◆, 10.34 μg), Nano-DOX (□, 9.25 μg), Veso-DOX (■, 9.95 μg) at 37°C, Nano-DOX (×, 9.33 μg) at 20°C and Veso-DOX (▲, 11.35 μg) at 20°C (experiments performed in duplicate, error bars represent standard deviation).

only a PTMC-*b*-PGA polymeric membrane but also a PB-*b*-PEO one to cross. At 20°C the release is negligible in the time frame of the measurement, this temperature being efficient for storage. The data can also be represented (Supporting Information, Figure S3) by an experimental law established by Peppas and Ritger,[22] where m_t and m_f represent the DOX release at time t and infinite time (initial amount in μg) respectively, k is a kinetic constant dependent of the matrix and the drug, and n is the release exponent [Eq. (1)].

$$\frac{m_t}{m_f} = kt^n \tag{1}$$

The release data so analyzed can be fitted by a linear regression, thus suggesting a fickian, purely diffusive release regime. Moreover, by extracting the kinetic constants k for each system, it can be determined, as expected, that the release rate of nano-DOX is about twice that of veso-DOX, for which there is an additional diffusion barrier.

In summary, polymersomes in polymersomes (or polymer vesosomes) were obtained by an original, versatile, and efficient method. Using such an emulsion centrifugation process, nanosize vesicles and any other material (nanoparticles, proteins, …) can be quantitatively loaded in larger polymersomes. Moreover, this highly controlled technique enabled encapsulating different nanosize polymersomes inside larger ones (constituting the first path towards the delivery of multiple and/or mutually incompatible actives), and also encapsulating molecules and (bio)macromolecules in at least three different compartments (in the membrane/lumen of the inner nanosize polymersomes, in the cavity, and in the membrane of the giant vesicles). Finally, the encapsulation of highly concentrated doxorubicin-loaded nanosize vesicles was performed. A double-membrane diffusion barrier effect has been demonstrated, allowing a specific stability and disruption in different environments. This strategy presents a straightforward approach and new opportunities for the use of these polymersomes in polymersomes in biomedical or cosmetic applications, where the encapsulation of multiple, distinct and fragile ingredients are required, together with specific release conditions.

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