Vesicles

DOI: 10.1002/ange.201004003

Triggered Templated Assembly of Protein Polymersomes**

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There is a strong demand for robust targetable nanosized containers in biomedical applications. The development of a general strategy to assemble functional biomolecules into stable nanostructures with desired size and shape is challenging. Liposomes for instance are extremely fragile and have low stimuli responsiveness and chemical diversity.[1] Most polymersomes, on the other hand, lack biofunctionality, which restricts their ability to interact with cells or tissues. Herein we present a versatile method to make stable biocompatible protein polymersomes by a triggered templated self-assembly route. Pluronic vesicles (see Figure 1), routinely fabricated with a narrow size distribution that ranges from 50–2000 nm in diameter, serve as a matrix that can take up large quantities of biosynthetic triblock copolymers CS^XS^XC in their unilamellar shell. The middle block S^XS^X of the protein has silk-like repeats, where X stands for the chargeable amino acids glutamic acid (E) or histidine (H). In response to a change of the pH value (down or up, respectively), the S block of the protein polymers becomes hydrophobic and inserts into the template, thus leading to the formation of protein polymersomes. Alternatively, by adding negatively charged CS^ES^EC or siRNA to the positively charged CS^HS^HC, co-assembly and co-insertion occurs at neutral pH values. The C block forms the stabilizing corona, is collagen-like, and has been shown to be hypoallergenic. Hence, biocompatible[2] multifunctional capsules for drug- and gene-delivery applications are obtained.

Our method may be characterized as "triggered templated assembly" (TTA). In a first step, we fabricate medically compatible Pluronic vesicles[3] with tunable diameters in the range of 0.1–2 μm in the presence of fully water-soluble protein CS^XS^XC polymers (Figure 1).[4] In a second step, we trigger the assembly by neutralizing the charge X on the S block. The protein adopts a β-sheet secondary structures and inserts itself into the vesicle walls. We refer to these capsules as protein polymersomes. Polymersomes based on polypeptides have received a great deal of interest in recent years.[5–7] Typical examples of protein polymersomes are vesicles made of chemical synthetic polypeptide block copolymers[2,5] or virus capsides.[7] These protein polymersomes generally have excellent biocompatibility, but one drawback is that it is rather tedious and time-consuming to make the functionalized starting material. Our TTA system, however, overcomes this problem.

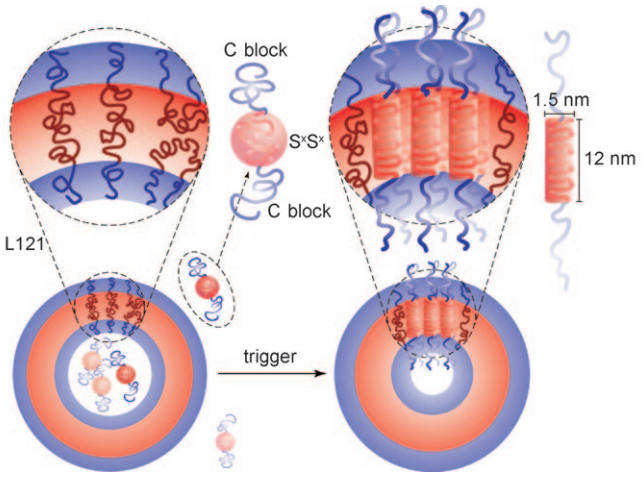

Figure 1. Triggered templated assembly of protein polymersomes. Pluronic L121 vesicles (light red core with thin blue corona) surrounded by triblock peptide copolymer CS^XS^XC (S block: red, C block: blue). After a trigger, the X groups (spherical dots) become uncharged. The S blocks adopt a β-sheet secondary structure, the hydrophobicity of which drives the insertion of the protein polymers into the capsule.

All the molecules for the TTA system are available in large amounts. The primary vesicles that form the template material are made of Pluronic L121 (PEO_5–PPO_{68}–PEO_5), which is a thermosensitive amphiphilic block copolymer with a large, marginally hydrophobic poly(propylene oxide) middle block and two very short oligo(ethylene oxide) outer blocks.[4] This polymer spontaneously forms rather unstable unilamellar vesicles in a small temperature window: below 15 °C the polymer is molecularly dissolved and above 25 °C the bare vesicles quickly aggregate. Around room temperature the fragile membrane is composed of loosely packed polymers and acts as the template ready to host the polypeptide block copolymers. These peptides give stability to the otherwise fragile polymersomes.

Herein we employ designed protein block copolymers, in which chosen amino acid sequences are expressed in an appropriate host organism. With this method, absolute control over the polymer length and sequence provided by a biosynthetic approach is retained, and biocompatible products with large quantities and unprecedented specifications are produced.[8] Two such protein polymers, which may be

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[**] This research is supported by NanoNed, a national nanotechnology program coordinated by the Dutch Ministry of Economic Affairs.
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201004003.

recognized as twins because they have an almost identical primary sequence of amino acids, are applied in our study. Both molecules were separately expressed in yeast, and the twins have a modular CS^XS^XC structure. The C block carries few charges and is water-soluble at all pH values used. The central motive S^X has a number of silk-like repeats separated by a chargeable amino acid X, which differ for the twin compounds (histidine: CS^HS^HC, glutamic acid: CS^ES^EC). If the groups are charged (neutral pH) the S block has no secondary structure and is water-soluble (Figure 1). However, in the absence of the charge, folding takes place and β rolls or in some cases β sheets form through intramolecular hydrogen-bonding.[9] These secondary structure elements have hydrophobic faces and they may further assemble into ribbonlike aggregates in a rather slow "nucleation and growth" process that can easily take several hours (depending on the actual protein concentration). Extensive investigations of these objects were recently reported.[8,10] A key observation is that one dimension of the ribbon, namely the distance that separates the two collagen-like blocks, is approximately 12 nm. This dimension is compatible with the PPO block of the Pluronic vesicle (Figure 1). Therefore it is reasonable to assume that hydrophobic interactions drive these protein polymers to enter the Pluronic vesicle membrane once the charges in the S block are neutralized.

Indeed, under such conditions, the protein polymers readily associate with the vesicle template and then form protein polymersomes. For facile imaging of the protein, we fluorescently labeled the C blocks of the protein polymers. Fluorescein isothiocyanate (FITC) dyes were exclusively attached to the lysine amino group by isothiocyanate coupling, and the labels do not interfere with the driving force for the self-assembly of the protein polymers. Results for giant polymersomes stabilized by labeled protein polymers CS^ES^EC are shown in Figure 2. Upon pH triggering, remarkably monodisperse vesicles are clearly visible from the confocal microscopy image. In this particular example, we also added the hydrophobic fluorescent label Nile red, which highlights the hydrophobic regions (Figure 2a). The location of FITC-labeled proteins is shown in Figure 2b. The overlay of the images (Figure 2c) proves that, at the resolution of the optical microscope, most of the protein polymers are at the same location as the Pluronic vesicles.

We realized that the fragile Pluronic vesicles can be extruded through a polycarbonate membrane and then adopt a smaller size. The triggered assembly of protein polymers in extruded polymersomes gives relatively monodisperse protein polymersomes with a controlled diameter of, for example, up to 100 nm (see Figure S1 in the Supporting Information). Dynamic light scattering (DLS) shows that the size is kept constant for at least two weeks (Figure 3). A very small

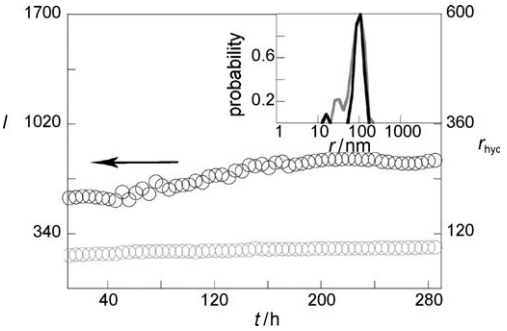

Figure 3. DLS stability study of 100 nm extruded vesicles. Top curve (relating to right ordinate): hydrodynamic radius of vesicles by cumulant fitting; bottom curve (relating to left ordinate): scattered intensity of the vesicles; the inset shows Contin software analysis results, 24 h after preparation (gray curve) and 280 h after preparation (black curve).

trend in the data indicates that the average size of the vesicles increases and also the intensity of the scattered light grows slowly. This observation is consistent with a slow addition of protein polymers to the vesicle membrane over time. Interestingly, the Contin software analysis shows that the vesicles become progressively more monodisperse.

It is important to note that the protein polymers are most likely not simply molecularly dissolved in the hydrophobic vesicle membrane phase. All protein polymers are extremely identical, chirally pure, and monodisperse. These unique properties give the protein polymers the extraordinary ability to assemble into intermolecular aggregates. Such protein ordering is expected to continue until very large protein to Pluronic ratios are reached. Small angle neutron scattering (SANS) measurements confirm that the thickness of the bilayer core significantly increases from 6 nm to 16 nm with the addition of 2 mg mL^{-1} CS^ES^EC; the thickness of the bilayer core even reaches 20 nm after addition of another 0.25 mg mL^{-1} CS^HS^HC (Figure 4). The change in bilayer core thickness indicates that the silk-like domain is inserted into the Pluronic vesicle membrane, while keeping the collagen-like domains in the aqueous phases. Indeed, the build-up of these layers is assumed to be responsible for the improved stability of the capsules.

The time needed for the protein polymers to be inserted into the vesicle membrane is apparently shorter than that needed for protein self-assembly into long rigid ribbons. The instant stabilization of Pluronic vesicles also indicates that the insertion process is fast. As proven by CD spectroscopy (see Figure S3 in the Supporting Information), the protein polymers adapt themselves to the polymersome structure by intramolecular hydrogen-bonding, β sheet, or β rolls secondary structures. By using fluorescence correlation spectroscopy (FCS), we could quantitatively determine the speed and

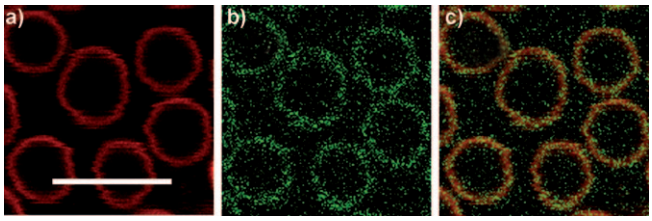

Figure 2. Confocal microscopic images of: a) vesicle membrane labeled with Nile red; b) CS^ES^EC labeled with FITC; c) overlapping of the first two images. Scale bar: 5 μm.

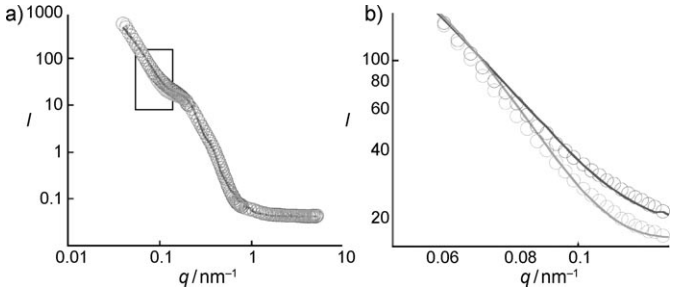

Figure 4. SANS measurements of 100 nm extruded vesicles with different proteins.[12] a) From top to bottom: 8 mg mL^{-1} L121 with 2 mg mL^{-1} CS^{E}S^{E}C (dark gray), 2 mg mL^{-1} CS^{E}S^{E}C and 0.25 mg mL^{-1} CS^{H}S^{H}C (light gray). b) Expansion of the marked area in (a).

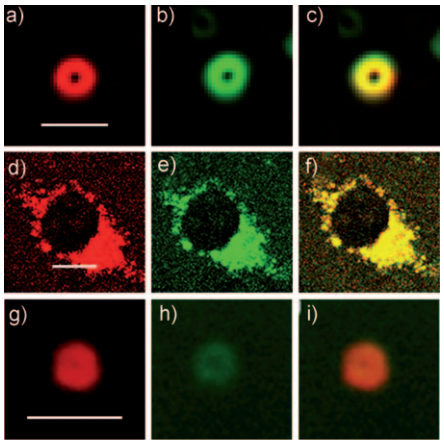

Figure 6. Coassembly of protein polymersome and their delivery into living cells. Capsules with both negatively and positively charged protein polymers: a) CS^{H}S^{H}C labeled with Fluor^{TM}647; scale bar: 5 μm. b) CS^{E}S^{E}C labeled with FITC. c) Overlay of the first two images. Uptake of capsules with $r = 100$ nm stabilized by CS^{H}S^{H}C and CS^{E}S^{E}C in caco-2 cells. The protein polymersomes enter the cytoplasm not the nucleus. d) Color of Fluor^{TM}647 labeled CS^{H}S^{H}C, scale bar: 10 μm. e) Color of FITC labeled CS^{E}S^{E}C. f) Overlay of the images (d) and (e). Coassembly of Pluronic vesicles with CS^{H}S^{H}C and siRNA. g) CS^{H}S^{H}C labeled with Fluor^{TM}647; scale bar: 2 μm. h) FITC-labeled siRNA. i) Overlay of the images (g) and (h). See Figure S4 in the Supporting Information for images with more cells.

the amount of protein polymers that participate in the build-up of the vesicle membrane (Figure 5). Pluronic vesicles are initially permeable for the protein polymer; no protein is

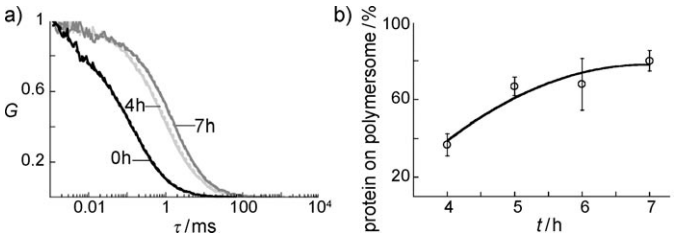

Figure 5. FCS measurements of 100 nm extruded vesicles with FITC-labeled CS^{E}S^{E}C. a) Autocorrelation curve measured at different times after sample preparation. b) Amount of protein on polymersome bilayer as a function of time. Numbers were subtracted from two-component model fitting. The solid exponential curve was plotted to guide the eye.

detected on the vesicle membrane directly after sample preparation. However, roughly 40% of the overall amount of protein participates in the formation of the vesicle membrane within four hours, and the incorporation of proteins levels off at approximately 70% seven hours after sample preparation.

This new method to fabricate stable capsules is versatile in the sense that we can incorporate various biologically active materials into the protein polymersome by selection of the triggering method. For instance, the positively charged protein polymer CS^{H}S^{H}C (Figure 6a–c) can be triggered by addition of negatively charged polyelectrolytes, such as siRNA (Figure 6g–h), which then makes a stable biocompatible gene container. In this case, the biologically active species is also responsible for the stability of the capsules (and gives the capsules a cooperative release mechanism). This approach presents many possibilities to assemble multifunctional biomolecules into stable nanostructures with desired size and shape. Moreover, the protein polymersome that contains both CS^{E}S^{E}C and CS^{H}S^{H}C polymer is delivered into human cells, because both protein species are taken up by the cytoplasm of the cell (Figure 6d–f). No significant cell death was observed under the utilized experimental conditions. This result suggests that biomolecules incorporated into these vesicles can easily be transported in vivo. Note that effective in vivo application of siRNA leads to various novel therapeutic approaches.[11]

Since the protein polymer CS^{X}S^{X}C is expressed in yeast we easily can produce large quantities (grams) of proteins. This possibility is a significant improvement over synthetic polypeptides. In addition, other functional groups can be designed and incorporated into the protein polymer, if they are either produced in yeast or as a post-chemical modification. For example, an appropriate targeting sequence can be inserted in the C block of the protein polymers, thus allowing the capsules to deliver their contents in a very controlled way. Our TTA method leads to new multifunctional capsules for drug delivery and gene-delivery applications.

Received: July 1, 2010
Published online: November 23, 2010

Keywords: block copolymers · drug delivery · gene delivery · protein polymersome · vesicles

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