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Construction of silica-enhanced S-layer protein cages

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ABSTRACT

The work presented here shows for the first time that it is possible to silicify S-layer coated liposomes and to obtain stable functionalized hollow nano-containers. For this purpose, the S-layer protein of *Geobacillus stearothermophilus* PV72/p2 was recombinantly expressed and used for coating positively charged liposomes composed of dipalmitoylphosphatidylcholine, cholesterol and hexadecylamine in a molar ratio of 10:5:4. Subsequently, plain (uncoated) liposomes and S-layer coated liposomes were silicified. Determination of the charge of the constructs during silicification allowed the deposition process to be followed. After the particles had been silicified, lipids were dissolved by treatment with Triton X-100 with the release of previously entrapped fluorescent dyes being determined by fluorimetry. Both, ζ-potential and release experiments showed differences between silicified plain liposomes and silicified S-layer coated liposomes. The results of the individual preparation steps were examined by embedding the respective assemblies in resin, ultrathin sectioning and inspection by bright-field transmission electron microscopy (TEM). Energy filtered TEM confirmed the successful construction of S-layer based silica cages. It is anticipated that this approach will provide a key to enabling technology for the fabrication of nanoporous protein cages for applications ranging from nano medicine to materials science.

1. Introduction

Nature has developed a broad range of nanometre-scale architectures based on the self-assembly of molecular building blocks [1,2]. While some of these nanometre scale three-dimensional (3-D) structures are artistically and ambitiously shaped morphologies, such as the well-known silica frustules of diatoms [3] and silica spicules of mineralized sponges [4], others impress by their simple geometrical shape. Prime candidates of the latter group are the protein shells of viruses (called capsids) exhibiting either helical or icosahedral structure [5,6]. Although bottom-up-based building principles are well understood and the building blocks are often fully characterized, only a few man-made biological architectures have been introduced into the scientific community. An example is the cavity of the cage-shaped protein apoferritin used for the synthesis of iron nanoparticles [7,8]. Another example that has been optimized by nature during billions of years of evolution is crystalline bacterial surface layers, termed S-layers [9–14]. S-layers are the most commonly observed cell surface structures in prokaryotic organisms and are found in a broad range of bacteria and archaea [11,12]. S-layers fulfil various functions for the bacterial or archaeal cell, such as providing molecular sieving characteristics in the ultrafiltration range [15–17] or acting as a rigid corset in the shape determination of various archaeal cells [18,19]. One of the most remarkable properties of S-layer proteins is their intrinsic tendency to form two-dimensional (2D) arrays in suspension [10,11], on solid supports [20,21], on the air–water interface [22,23], on planar lipid films [24,25], on liposomes [26–28] and on nanocapsules [29–31]. Although S-layer proteins reassemble only in plane (in monolayers or double layers), the coating of liposomes with S-layer proteins has demonstrated that closed 3-D protein containers may be fabricated in vitro, too [26–28]. Owing to the introduction of lattice faults such as disclinations, which are a necessity for 2D crystals to cover curved surfaces, the rigidity and isoporosity of the entire S-layer meshwork is maintained.

The work presented here is derived from the present authors' knowledge of making S-layer coated liposomes and shows for the first time that S-layer functionalized hollow nano-containers may be produced (Fig. 1a). The mechanical robustness of these spherically shaped containers was obtained by deposition of a thin layer of biogenic silica onto the S-layer [32]. It is anticipated that this approach will provide a key enabling technology for the fabrication of nanoporous protein cages, using self-assembling strategies common in nature. Applications will be found in the development of novel encapsulation techniques, controlled drug targeting and delivery, including hydrophobic substances, affinity matrices and the fabrication of nanoporous, spherical silica structures with controlled diameters, applicable in, for example, materials science.

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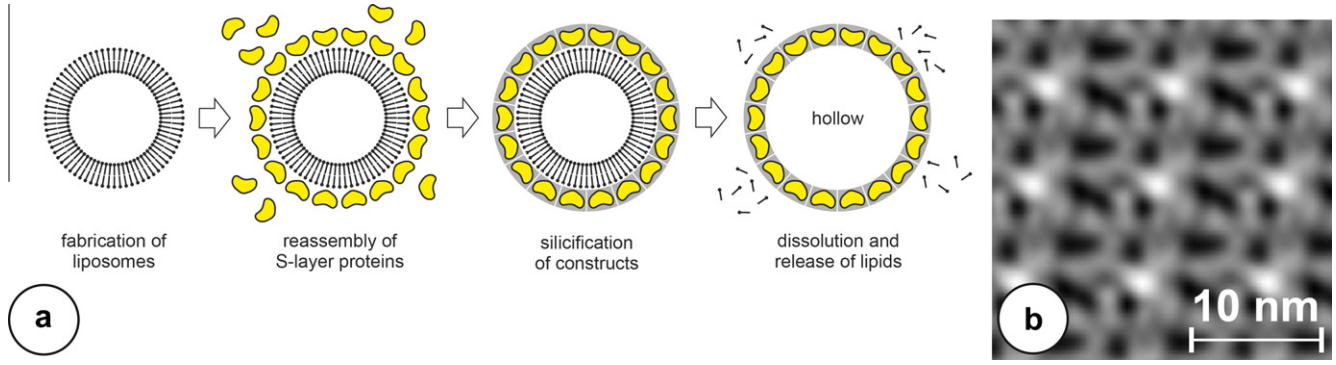

Fig. 1. (a) Schematic drawing of the consecutive preparation steps for making hollow, silica-enhanced S-layer cages. (b) TEM image of a negatively stained preparation of an S-layer protein monolayer of SbsB from *Geobacillus stearothermophilus* PV72/p2 (used in this work). Protein appears white to grey, pores are dark.

2. Experimental

2.1. Expression and purification of the recombinant S-layer protein

The S-layer protein SbsB of *Geobacillus stearothermophilus* PV72/p2 (Fig. 1b) was recombinantly expressed in *Escherichia coli*. Genetically modified bacteria were grown batchwise in M9ZB media using isopropyl-β-D-thiogalactopyranosid as an inducer for rSbsB expression [33]. The protein was purified under denaturing conditions, using guanidine hydrochloride solution (5 M in 50 mM Tris–HCl buffer pH 7.2), centrifuged at 40,000*g* for 20 min at 4 °C and refolded by dialysis against Milli-Q water. Analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis with Coomassie blue staining showed the full-length protein band migrating at ~95 kDa [34].

Protein concentration of the solution was determined in a spectrum photometer at 280 nm (NanoDrop ND-1000, Wilmington, DE 19810 USA) using the physicochemical parameters of rSbsB (extinction coefficient 47,790; molecular mass 94,876 Da) [35].

A detailed description of the expression and purification of the recombinant S-layer protein is provided as Supplementary data.

2.2. Fabrication of liposomes and 5-(6)-carboxyfluoresceine filled liposomes

A liposome preparation method slightly modified from that described by Kirby and Gregoriadis [36] was used to produce positively charged liposomes. The lipid mixture consisted of dipalmitoylphosphatidylcholine (DPPC; Avanti Polar Lipids, Alabaster, AL, USA), cholesterol (Sigma, St. Louis, MO, USA) and hexadecylamine (HDA; Fluka, Buchs, Switzerland) in a molar ratio of 10:5:4. Because of the HDA in the lipid composition, the liposomes have a positive surface charge that favours the reassembly of the S-layer protein, similar to bacterial cells. A lipid stock solution containing 30 mg ml^{-1} DPPC was prepared by dissolving all the lipids in 2 ml chloroform and was rotary evaporated at 60 °C under vacuum. The dry, lipid film was rehydrated in Milli-Q water. Liposomes were formed during rotation at room temperature followed by 30 min sonication in an ultrasonic bath (USC300T, VWR, Radnor, PA, USA). The liposome suspension was passed 11× through a symmetric polycarbonate membrane with pore size 200 nm (Nuclepore; Whatman plc; Maidstone; UK) using a LiposoFast pneumatic extruder (Avestin, Ottawa, Canada).

Carboxyfluoresceine liposomes (CFL) were prepared by lyophilization of the liposomes overnight and rehydrated with 50 mg ml^{-1} 5-(6)-carboxyfluoresceine (CF) (Molecular Probes, Eugene, OR, USA) in Milli-Q water adjusted to pH 7.5 with 1 M NaOH. After 30 min sonication in an ultrasonic bath (VWR USC300T), the CFL suspension was also downsized by extrusion (passing 11× through a symmetric polycarbonate membrane with pore size 200 nm, using a LiposoFast pneumatic extruder). To separate the CFL from free CF, gel permeation chromatography was performed with a G-25 PD-10 column (Pharmacia, Uppsala, Sweden). The column was equilibrated with 160 mM KCl and presaturated with empty liposomes. Only the most turbid fractions were collected and pooled.

2.3. Recrystallization of S-layer protein on liposomes

Liposomes and S-layer protein were mixed according to a DPPC-to-protein ratio of 1 mg:2 mg in Milli-Q water. Recrystallization of the S-layer protein on the liposomes was carried out for 2 h on a test tube rotator (Heidolph Reax 2, Germany) at 30 rpm at room temperature. Non-reassembled protein was removed with the supernatant after centrifugation at 20,000*g* for 10 min at room temperature [27].

2.4. Silicification of liposomes and S-layer coated liposomes

The process for silica deposition followed a two-step reaction. First, silicic acid monomers and silicate dimers form silicate trimers following apparent third-order reaction kinetics:

$$Si(OH)_4 + Si(OH)_3OSi(OH)_2O^- \rightarrow [Si(OH)_3OSi(OH)_2OSi(OH)_2O]^- + H_2O$$

After this stage, monomers reversibly condense into increasingly large oligomers, following apparent first-order reaction kinetics [37].

In order to obtain silicic acid oligomers, prehydrolysis of freshly prepared 1 M tetraethyl orthosilicate (TEOS; Sigma, St. Louis, MO, USA) in 50% ethanol with 10 mM HCl was performed. The solution was stirred for 15 min at room temperature and then immediately used. The condensing system consisted of the sample (liposomes or S-layer coated liposomes, all without previous crosslinking) and 30 mM prehydrolysed TEOS in 100 mM BIS–TRIS propane (Sigma, St. Louis, MO, USA)/citrate buffer pH 7.0. The condensation reactions were also performed in solution without the addition of any liposomes (background). Silicified liposomes and silicified S-layer coated liposomes were used for ultrathin sectioning, transmission electron microscopy (TEM) and titration experiments. The lipid concentration in plain (uncoated) and S-layer coated liposomes in the condensing solution (silicification mixture) was 3 mg ml^{-1} DPPC. The silicification reaction was stopped after defined periods of time (from 0 to 120 min) by adjusting the pH to 4 with 1 M HCl, followed by centrifugation at 20,000*g* and a washing step with Milli-Q water. CFL and S-layer coated CFL (S-CFL)