tronic applications. Indeed, the bioconjugates of gold nanoparticles with the proteins in solution might show novel nonlinear optical properties. We believe the biggest advantage of this method based on fungal enzymes is the possibility of developing a rational approach for the synthesis of nanoparticles over a range of compositions such as oxides or nitrides, and we are currently working towards this end.

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[8] *Fusarium oxysporum* biomass (10 g) was taken in a conical flask containing distilled water (100 mL). A carefully weighed quantity of $HAuCl_4$ was added to the conical flask to yield an overall $AuCl_4^-$ ion concentration of 10^{-3} M in the aqueous solution; the reaction was carried out in the dark. UV/Vis spectroscopy measurements in this study were performed on a Shimadzu dual-beam spectrophotometer (model UV-1601PC) operating at a resolution of 1 nm while FTIR studies were carried out on a Shimadzu FTIR-8201PC instrument in the diffuse reflectance mode at a resolution of 4 cm^{-1}. TEM measurements were made on a JEOL 1200EX instrument operating at a voltage of 80 kV.
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OmpA Membrane Domain as a Tight-Binding Anchor for Lipid Bilayers

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biosensors · liposomes · membrane proteins · proteoliposomes · streptavidin

Liposomes have been used extensively to analyze membrane protein functions because they are realistic membrane substitutes.[1] In many applications liposomes are immobilized on solid supports,[2] which often causes protein malfunctions.[3] This problem was alleviated by suspending the lipid bilayers by long tethers that extend from the solid support to a lipid molecule immersed in the bilayer.[4] Such a lipid, however, cannot grip the bilayer tightly enough to yield high stability. Here we report the large-scale production of an engineered version of the small membrane protein OmpA, which fastens the outer membrane of *Escherichia coli* to the cell wall.[5] The presented OmpA derivative attaches liposomes to streptavidin molecules wherever these are located.

OmpA consists of an integral membrane domain (residues 1 – 171) and a periplasmic peptidoglycan-binding domain (residues 172 – 325). A fourfold mutant (F23L/Q34K/K107Y with an additional methionine residue at the N terminus) of the membrane domain[6] forms an eight-stranded β barrel with an aliphatic waist bordered by two girdles of aromatic side chains (Figure 1). This β barrel is tightly anchored in the membrane. We added the mutation N26C, which introduces a cysteine residue at the tip of one of the four long and mobile extracellular loops. The gene for this cysteine-containing fivefold mutant "OmpAfc" was verified by DNA sequencing. OmpAfc can be mass-produced in *E. coli* inclusion bodies and (re)natured therefrom.[7] Labeling Cys 26 with a tethered biotin[8] permits a tight association of membranes to streptavidin and their release by disulfide reduction.

The accessibility of Cys 26 was checked photometrically by Ellman's reaction,[9] which resulted in 0.7 thiols per OmpAfc. The disulfide exchange reaction with tethered biotin (Scheme 1) yielded 80 % "OmpAfc – biotin" as determined by photometric detection of the leaving pyridine-2-thione. Given the length of the spacer arm and the exposed position of Cys 26 (Figure 1), the maximum distance between the biotin and the membrane center amounts to about 70 Å.

Solutions of OmpAfc – biotin in buffer A (20 mM tris(hydroxymethyl)aminomethane (Tris) – HCl (pH 8.5), 140 mM NaCl, 0.6 % (w/w) octyltetraoxyethylene (C_8E_4)) were tested for streptavidin binding by titration of a fixed amount of OmpAfc – biotin with streptavidin. Streptavidin and OmpAfc – biotin keep their overall

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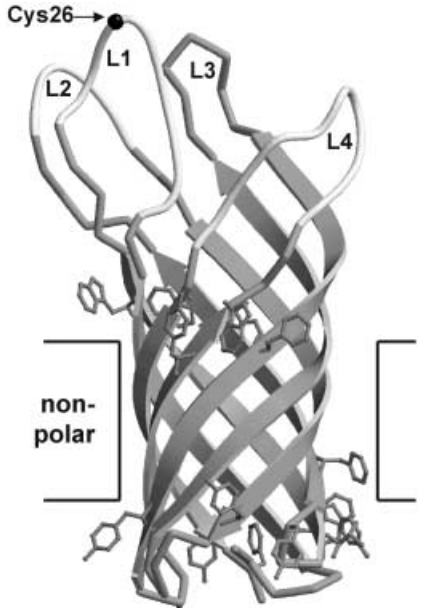
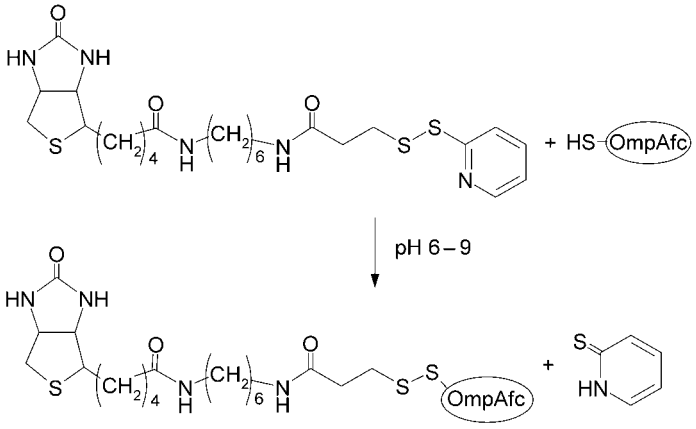


Figure 1. *Model of OmpAfc with Cys 26 in one of the four mobile extracellular loops, L1. The nonpolar moiety of the lipid bilayer faces the ribbon of aliphatic side chains running around the waist between the two girdles of aromatic residues.*[6]



Scheme 1. *Reaction scheme for the production of OmpAfc – biotin from OmpAfc and biotin – HPDP.*

structure as well as their binding function in the presence of 1 % sodium dodecylsulfate (SDS) at 50 °C and migrate as single bands when polyacrylamide gel electrophoresis (PAGE) is carried out (Figure 2). The OmpAfc – biotin band dropped as streptavidin concentration was increased and five new bands appeared. At low concentrations, streptavidin is fully decorated with OmpAfc – biotin, which gives rise to the slow-moving complex $\alpha_4\beta_4$, while higher concentrations of streptavidin resulted in several complexes which are putatively assigned in Figure 2 according to their apparent relative molecular weight ($^{app}M_r$; complex $\alpha_4\beta_2$ can form two distinct binding geometries). Complex $\alpha_4\beta_1$ migrates faster than streptavidin itself, which is atypical for fully denatured proteins but may happen with partially denaturated ones. This experiment demonstrates that

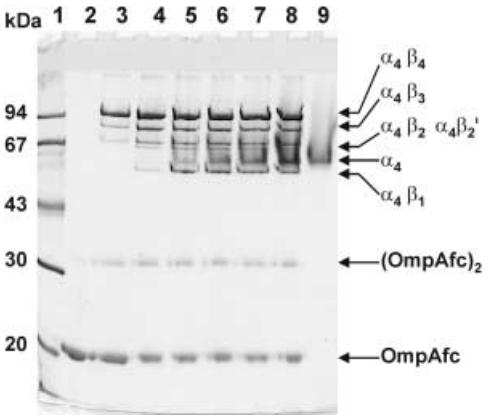


Figure 2. *SDS-PAGE of streptavidin binding to OmpAfc – biotin micelles. Aliquots of OmpAfc – biotin (150 pmol) were mixed with streptavidin (with 0, 70, 140, 210, 280, 350, and 560 pmol of biotin sites), incubated at room temperature for 10 min, and applied to lanes 2 – 8, respectively. Lane 9 contained streptavidin with 280 pmol of biotin sites without OmpAfc – biotin. The five resolved bands are putatively assigned to the expected stoichiometries between the tetrameric streptavidin (α_4) and OmpAfc – biotin (β). Monomeric OmpAfc (possibly biotin-labeled) and disulfide-bridged dimeric OmpAfc bands are also present.*

streptavidin can bind up to four OmpAfc – biotin molecules in their micelles.

In a second experiment we incorporated OmpAfc – biotin into liposomes by using the detergent dialysis method.[10] Briefly, OmpAfc – biotin solubilized in C_8E_4 and lipid solubilized in octyl-β-D-glucopyranoside (β-OG) were mixed and then dialyzed against detergent-free buffer A. Both detergents are easily removed because they have a high critical micelle concentration (cmc). The OmpAfc – biotin concentration in the liposomes can be adjusted by changing the lipid-to-protein ratio.

The interaction of these proteoliposomes with streptavidin was quantified by native PAGE at pH 8.8.[11] Streptavidin (isoelectric point, pI = 6) migrates as a single band while proteoliposomes are too large to enter the gel. When streptavidin with 130 pmol biotin sites was titrated with proteoliposomes, the streptavidin band vanished at 520 pmol OmpAfc – biotin (Figure 3). This experiment demonstrated that streptavidin was not able to pull OmpAfc – biotin out of the lipid bilayers. Half of the observed fourfold excess is due to random OmpAfc – biotin orientation in the membrane, while the other half is probably caused by proteoliposome surface occlusion on aggregation. If a proteoliposome – streptavidin mixture was treated with β-mercaptoethanol (β-ME), the biotin labels were removed and the streptavidin was recovered (lane 6).

Electron microscopy investigation of negatively stained proteoliposomes showed vesicles up to 1 μm in size, the shape and sizes of which were similar to those produced with other membrane proteins.[12] In contrast to streptavidin, membrane-incorporated OmpAfc – biotin molecules are too small to be visualized with a negative stain (Figure 4a). Therefore, fresh proteoliposomes flattened on the support were exposed to a streptavidin solution and then negatively stained, which resulted in particles with the typical size and shape of streptavidin (Figure 4b). This experiment confirmed that the biotin labels of the proteoliposomes are available for binding. Moreover, the

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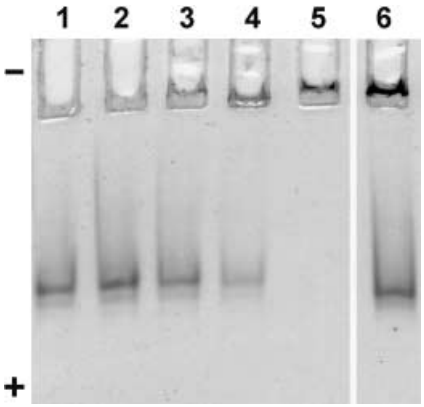

Figure 3. *Native PAGE at pH 8.8 demonstrating streptavidin binding to OmpAfc – biotin immersed in proteoliposomes. Streptavidin (with 130 pmol of biotin sites) was titrated with proteoliposomes that contained 0, 65, 130, 260, and 520 pmol of OpmAfc – biotin (lipid-to-protein ratio 5:1 (w/w)), incubated for 10 min at room temperature and applied to lanes 1 – 5, respectively. The excess streptavidin forms the band moving toward the anode. In lane 6, the biotin labels were shaved off by addition of 1% β-ME to the proteoliposomes that contained 260 pmol of OmpAfc – biotin. The arrested proteoliposomes in the gel pockets are only partly stained because material is lost in the Coomassie staining baths.*

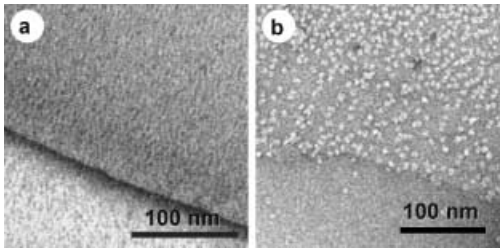

Figure 4. *Electron micrographs of negatively stained proteoliposomes produced from OmpAfc – biotin and DOPC at a lipid-to-protein ratio of about 1:1. a) A proteoliposome (top) on carbon support (bottom). b) A proteoliposome (top) that was washed with a streptavidin solution after deposition on the carbon support (bottom) shows streptavidin decoration. Note that the OmpAfc – biotin molecules are evenly dispersed in the lipid bilayer. The separation of these molecules at the center is somewhat larger than expected from the applied lipid-to-protein ratio. DOPC = 1,2-dioleoyl-sn-glycero-3-phosphocholine.*

results demonstrate that the OmpAfc – biotin molecules are dispersed in the membrane and do not aggregate.

Next, we produced a fresh proteoliposome solution, (slightly turbid, Figure 5 a) and added a small amount of streptavidin.

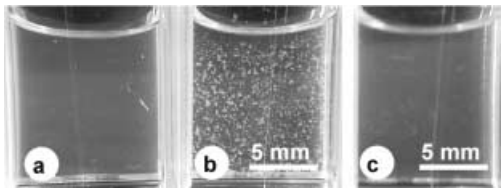

Figure 5. *Streptavidin-mediated reversible aggregation of proteoliposomes. a) Slightly turbid proteoliposome solution produced from OmpAfc – biotin and DOPC at a lipid-to-protein ratio of 5:1 (w/w). b) The same solution as in (a) flocculates several minutes after mixing with streptavidin (0.05 mg mL^{-1}). c) Treatment with 1% β-ME removes the biotin labels and the flocculate dissolves again after several minutes.*

Within a few minutes, this caused a visible flocculation (Figure 5 b) because streptavidin had cross-linked the proteoliposomes through the OmpAfc – biotin anchors to yield "snap-together vesicles".[13] The subsequent addition of β-ME to the flocculate cleaved the biotin labels off and dissolved the aggregates again (Figure 5 c). A control that used proteins other than streptavidin did not lead to flocculation.

In conclusion, proteoliposomes containing functionalized membrane protein anchors have been produced and used for reversible manipulations. They can be bound to solid surfaces that present streptavidin and can then be released by β-ME. The integral membrane protein OmpAfc attaches to the lipid bilayer much more tightly than immersed lipids. Since the distance between biotin and the bilayer center extends to 70 Å, a soft streptavidin cushion would suffice to provide a nearly native environment for additional membrane proteins, which would permit various membrane protein analyses such as atomic force microscopy[14] or biosensor measurements.[15]

Experimental Section

OmpAfc production and labeling: Streptavidin and DOPC were from Sigma (Steinheim) and *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (biotin-HPDP) was from Pierce (Rockford/IL/USA). All other chemicals were the best available commercial grade. The expression vector pET3b – OmpA derived from pET3b (Pharmacia, Freiburg) was mutated with the oligonucleotide primer 5′-CCATGATACTGGTTTAATCAAC*TGC*AATGGCCCGACCCATGAAAACC-3′ and its complement (MWG, München) by using the QuikChange kit (Stratagene, La Jolla). DNA was sequenced by SEQLAB (Göttingen). OmpAfc was overexpressed in inclusion bodies in *E. coli* BL21Star (DE3) Invitrogen, Groningen), (re)natured, and purified[7] to yield about 30 mg of protein per liter of culture. Contrary to the protocol[7] β-ME (10 mM) was added to the (re)naturation buffer. Excess β-ME was removed by repeated concentration to 1 – 2 mg mL^{-1} (10 000 Da cut-off; Vivascience, Göttingen) and dilution to 0.05 mg mL^{-1} in buffer A. OmpAfc concentrations were established photometrically ($\varepsilon_{280nm} = 46\,400\,M^{-1}cm^{-1}$). The free thiol was determined by using 200 μM Ellman's reagent and monitoring 2-nitro-5-thio-benzoate ($\varepsilon_{412nm} = 13\,600\,M^{-1}cm^{-1}$) production. Labeled OmpAfc – biotin was produced from desalted 1 – 2 mg mL^{-1} OmpAfc in buffer A, which was incubated at room temperature for 10 min with 200 μM biotin – HPDP and pyridine-2-thione ($\varepsilon_{343nm} = 8080\,M^{-1}cm^{-1}$) release was measured. The resulting OmpAfc – biotin was dialyzed against 200 volumes of buffer A.

Proteoliposomes: OmpAfc – biotin (0.5 – 2.0 mg mL^{-1}) in buffer A was mixed with DOPC in 2% β-OG at lipid-to-protein ratios of 0.5:1 – 5:1 (w/w). The mixture (50 – 800 μL) was dialyzed twice against detergent-free buffer A (100 volumes) for a total of 24 h.

Transmission electron microscopy: Electron microscopy was carried out with a LEO CEM 912 transmission electron microscope operating at 120 keV and equipped with a slow-scan CCD camera. Proteoliposome solutions (0.1 – 0.5 mg mL^{-1}) were deposited onto hydrophilized (by glow discharge in air) carbon-coated parlodion film supported by a copper grid. The loaded film was washed three times in water and negatively stained with 1% uranyl acetate in water.

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An Efficient Combinatorial Method for the Discovery of Glycosidase Inhibitors**

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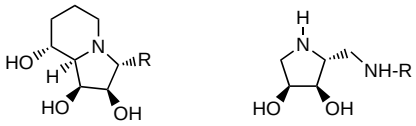
KEYWORDS:

carbohydrate mimics · combinatorial chemistry · imines · inhibitors · mannosidases

The specific inhibition of *N*-linked glycoprotein-processing α-mannosidases may provide a useful anticancer strategy.[1] Clinical trials have shown that swainsonine (**1**; Scheme 1), a natural α-mannosidase inhibitor that contains a 4-amino-4-deoxy-manno-



1 R = H
2 R = CH_2CH_2Ar

3 R = Bn
4 R = H

Scheme 1. *Structure of swainsonine and α-mannosidase inhibitors. Bn = benzyl.*

furanoside moiety,[2, 3] reduces solid tumor and hematological malignancies.[4] In order to avoid problems that arise from co-inhibition of lysosomal mannosidases, analogues of swainsonine such as **2** have been prepared and shown to have interesting properties.[5] Simpler synthetic analogues are also potent α-mannosidase inhibitors.[3, 6] We have reported that (2*R*,3*R*,4*S*)-3,4-dihydroxypyrrolidin-2-yl derivatives such as **3** are selective α-mannosidase inhibitors.[7, 8] The search for better inhibitors implies the multistep synthesis of a large number of analogues

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[**] *The concept and part of the text of this work were presented at the XIth Eurocarb Conference in Lisbon on September 7, 2001, under the title "An Efficient Combinatorial Method for the Discovery of Glycosidase Inhibitors. Imines Equilibrating with (2R,3R,4S)-2-Aminomethyl pyrrolidine-3,4-diol and Aldehydes are Inhibitors of α-Mannosidases and Models for the Inhibitory Activity of Corresponding Amines".*

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