Functional biohybrid amphiphiles

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INTRODUCTION

In order to stabilize the protein structure and to make enzymes more accessible, *e.g.* as catalysts for (organic) synthesis, these biomacromolecules have been coupled to synthetic polymers. Enzyme-polymer conjugates are generally constructed by non-selective coupling of the polymer to peripheral enzymatic amino acid residues. Recently, the synthesis of well-defined polymer-enzyme hybrids has been reported.[1-3] These compounds can act as giant amphiphiles when the synthetic polymer is hydrophobic.[2,3] They display interesting aggregation behavior and the catalytic activity of the enzyme is often retained. Therefore, these biohybrids have great potential for the construction of catalytically active nano-sized assemblies. Procedures to synthesize giant amphiphiles, among others, comprise a thiol-maleimide coupling between lipase (Cal B) and maleimide terminated polystyrene (PS)[2] and cofactor reconstitution of horseradish peroxidase by heme functionalized PS.[3] Both procedures are selective and site-specific, yet their modularity and general applicability is limited. Therefore, new synthetic strategies are desirable for the preparation of a range of functional giant amphiphiles. Towards this goal we investigated the application of the Cu(I) catalyzed azide-alkyne [3+2] cycloaddition reaction.[4] This so-called click reaction is in particular efficient in water[5] and can also be applied to polymer end groups.[6] Moreover, terminal alkyne and azide functionalized polymers can readily be prepared[6] and both moieties can be successfully incorporated into proteins through expression of non-natural amino acids.[7]

EXPERIMENTAL

Materials. Unless otherwise stated, chemicals were obtained from commercial sources and used without further purification. Styrene was stirred over CaH_2 and distilled under vacuum prior to use. THF was distilled under nitrogen from sodium/benzophenone. BSA was obtained from *Sigma*. Deionized water was used for the biological procedures.

Analytical techniques. Monomer conversions in ATRP were determined by gas chromatography. The molecular weight of PS was analyzed with size exclusion chromatography (SEC) calibrated on PS standards using $CHCl_3$ as the eluent. Purity and functionalization of the polymers was confirmed by ^{1}H-NMR and FT-IR. Protein-polymer conjugates were analyzed with size exclusion FPLC using phosphate buffer (20 mM, pH 7.2) as an eluent. Aggregation behavior of the amphiphilic biohybrids was studied with TEM. Samples for TEM were prepared using carbon coated copper grids and structures were visualized by Pt shadowing.

ATRP of styrene. In a typical procedure a Schlenk vessel loaded with CuBr was evacuated and back-filled with argon three times. Argon purged styrene, anisole (10% v/v) and PMDETA were added via a syringe. The reaction vessel was placed in a thermostatically controlled oil bath at 90 °C. After 5 min the initiator was added such that the ratio between copper, ligand and initiator was 1:1:1. Monomer conversion was monitored by GC, using anisole as the internal standard. When the desired conversion was reached the mixture was cooled to room temperature and diluted with CH_2Cl_2. After washing with aqueous EDTA (0.065 M) the polymer was precipitated in methanol, yielding ω-bromo-polystyrene as a white solid.

ω-Azido PS (3). To a solution of ω-bromo-polystyrene in THF (0.03 M) was added azidomethylsilane (10 eq.) and TBAF (1 M solution in THF, 10 eq.). The reaction mixture was stirred overnight at room temperature under an argon atmosphere and the polymer was precipitated in methanol, yielding a white solid (94 %).

α-carboxy ω-azido PS (6). α-*tert*-Butoxycarbonyl ω-azido PS (tBuO-CO-PS-N_3) was synthesized *via* ATRP starting from *tert*-butyl-2-bromoisobutyrate as the initiator and subsequent end group modification as described above. To a solution of tBuO-CO-PS-N_3 in CH_2Cl_2 (0.01 M) TFA (50 eq.) was added and the mixture was stirred overnight. The mixture was diluted with CH_2Cl_2 and washed twice with sat. aqueous $NaHCO_3$. After concentration under reduced pressure, HOOC-PS-N_3 (**6**) was precipitated in methanol, yielding 86 % of a white solid.

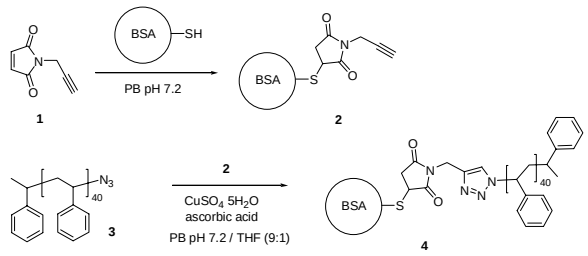
Alkyne functionalized BSA (2). To a 0.6 μM solution of BSA in phosphate buffer (PB) (20 mM, pH 7.2) was added *N*-alkyne functionalized maleimide **1** (60 eq.).[8] The mixture was shaken overnight excluding light and subsequently dialyzed against PB using a molecular weight cut-off (MWCO) of 12 – 14 kDa.

Click reaction between 2 and PS-N_3 (3). To a solution of **2** in PB (20 mM, pH 7.2) was added PS-N_3 in THF (50 eq.), $CuSO_4$ (6 eq.) and ascorbic acid (30 eq.), affording a final volume of 2.5 mL (16% THF in PB (20 mM, pH 7.2)) with a BSA concentration of 0.24 μM. The mixture was shaken for 24 hours. Subsequently the mixture was diluted with THF and dialyzed against PB using a MWCO of 66 kDA, resulting in a white precipitate and a slightly turbid phase. SEC confirmed that the precipitate was PS-N_3. The slightly turbid phase was used for further characterization.

Peptide coupling between Lipase and HOOC-PS-N_3 (6). HOOC-PS-N_3 in THF (2 eq.) and EDC in acetate buffer (50 eq.) (20 mM, pH 5.5) were added to a solution of lipase in acetate buffer, resulting in a final volume of 1.6 mL (60% THF and 40% acetate buffer (20 mM, pH 5.5)) with a lipase concentration of 0.012 μM. The mixture was shaken for 24 hours and analyzed without further purification.

RESULTS AND DISCUSSION

Recently, we demonstrated that azide functionalized PS can be coupled to alkyne functionalized bovine serum albumin (BSA) **2** by 'click' chemistry using copper sulfate and ascorbic acid as the catalyst system.[4] BSA was used as a model protein because it can easily be functionalized *via* the exposed free thiol of the CYS34 residue (scheme 1).



Scheme 1. Preparation of BSA-PS conjugates by the Cu(I) catalyzed 'click' reaction.

The conjugation reaction was monitored using size exclusion fast performance liquid chromatography (FPLC), which clearly revealed a peak corresponding to a product with higher molecular weight than **2** (figure 1). In addition, the ratio of UV absorption between λ = 254 nm and λ = 280 nm is higher for the product than for BSA, indicating the presence of PS for which the absorption maximum is at λ = 254 nm while for BSA this is at λ = 280 nm.

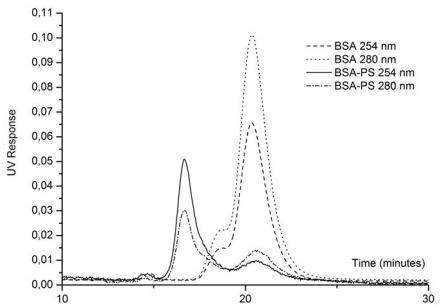
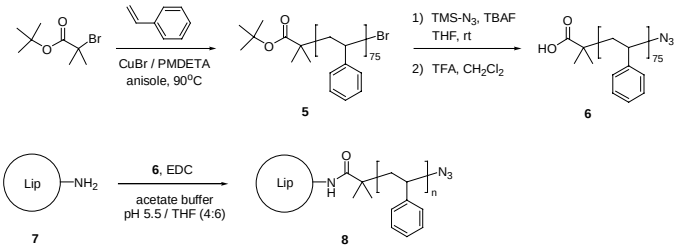


Figure 1. Size exclusion FPLC analysis of **4**.

After purification by dialysis, the aggregation behavior of the BSA-PS amphiphiles was studied with the help of transmission electron microscopy (TEM) and spherical aggregates in the range of 30-70 nm were observed (not shown).

The procedure described above holds great promise for the preparation of a host of functional giant amphiphiles, but should also allow the synthesis of more complex architectures such as giant bola-amphiphiles; *e.g.* a synthetic polymer that has proteins attached to both its ends. Towards this latter class of amphiphiles, hetero telechelic PS (**6**) with both a terminal azide and a terminal carboxylic acid moiety was prepared in three steps by atom transfer radical polymerization (ATRP) and subsequent end group modifications (Scheme 2).



Scheme 2. Synthetic route for the preparation of lipase-PS-N_3 conjugates.

A mutated lipase bearing a single primary amine[9] was conjugated to the carboxylic acid end of the polymer by employing a standard peptide coupling reaction in a THF/buffer mixture. Formation of the product was confirmed by FPLC analysis which clearly showed a peak at higher molecular weight than the lipase (Figure 2). A fractionated sample of the higher molecular weight peak was studied using TEM and spherical aggregates in the range of 100-130 nm were observed. Furthermore, initial activity studies showed that the coupled lipase retained 86% of its original activity.

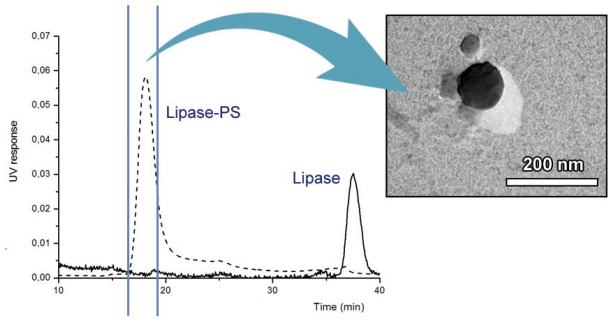


Figure 2. Size exclusion FPLC analysis and TEM analysis of **8**.

Currently the possibilities to use the other end of the polymer for cross-linking or further functionalization with a second protein are under investigation. In the latter case, combined protein assemblies can be obtained which are potentially capable of performing cascade-type reactions.

CONCLUSIONS

Biohybrid amphiphiles composed of a PS and protein block can be constructed in a straightforward way from monofunctionalized proteins and polymers using 'click' chemistry or conventional peptide coupling methods. Starting from a mutated lipase and hetero telechelic PS, catalytically active giant amphiphile assemblies were obtained which have an extra handle for further functionalization. Current research is aimed at studying this additional modification as well as evaluating the physical and catalytic properties of the PS-lipase amphiphiles.

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