Preparation of biohybrid amphiphiles *via* the copper catalysed Huisgen [3 + 2] dipolar cycloaddition reaction†

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Biohybrid amphiphiles have been prepared from terminal azide functionalised polystyrene and an alkyne functionalised peptide or protein *via* a Cu(I) catalysed Huisgen [3 + 2] dipolar cycloaddition reaction.

Amphiphiles composed of a polar headgroup and an apolar tail (*e.g.* soaps, surfactants) are technologically important compounds, which have been the subject of intensive studies for many years. Traditionally, much attention has been given to the classical low molecular weight amphiphiles, but more recently these studies have been extended to macromolecular surfactants as well.[1] This latter class of amphiphiles comprises amphiphilic block copolymers, referred to as 'super amphiphiles', and 'giant amphiphiles' which consist of a hydrophobic polymer as a tail and a protein or enzyme as a head group. Many examples of super amphiphiles constructed from purely synthetic blocks have been described in the literature.[2] Reports on biohybrid amphiphiles, on the other hand, are still limited.[3,4] Since these compounds may have great potential for the construction of biologically active nano-assemblies, versatile methods for their preparation are desired.

Generally, two synthetic strategies have been employed to build up well-defined polymer-bioconjugates from peptides and proteins. The first, which has recently drawn much attention, involves the growing of a polymer tail from a peptide or protein initiator.[5] The second strategy relies on the efficient coupling of a synthetic polymer to a bio(macro)molecule.[4a,6] One of the first reported routes in the latter category is the covalent attachment of a maleimide functionalised polystyrene (PS) to a reduced S–S bridge on the surface of a lipase (Cal B).[4a] Other routes involve cofactor reconstitution and coupling with the help of streptavidin and biotin.[4b,c] The modularity of all these approaches is limited and new procedures to prepare amphiphilic biohybrid macromolecules are therefore desirable. Towards this goal we decided to investigate the application of the Cu(I) catalysed azide–alkyne [3 + 2] cycloaddition reaction. This so-called "click" reaction has recently received a lot of attention and is particularly efficient in water.[7,8]

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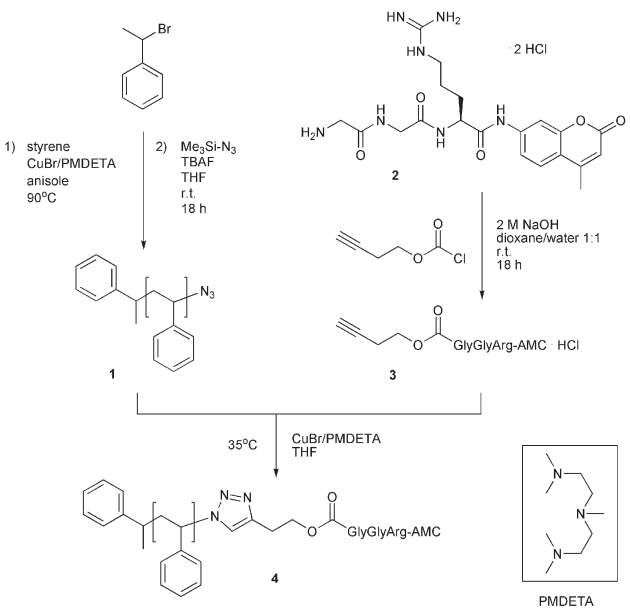
Following up on our previous research on PS–peptide amphiphiles[3a] we first chose to focus on H-GlyGlyArg-(7-amino-4-methylcoumarin) (H-GlyGlyArg-AMC, **2**) as the peptide part, which is easily accessible and moreover, the AMC fluorophore is a powerful tool in the characterisation of the biohybrid.

The alkyne functionalised peptide block was synthesised from the tripeptide (**2**)[9] by linking the *N*-terminus with 3-butynyl chloroformate (Scheme 1). Terminal azide functionalised PS of well-defined length (Mn = 4150 Da, PDI = 1.15) was readily synthesised using atom transfer radical polymerisation (ATRP) and subsequent end group modification with azidotrimethylsilane and tetrabutylammonium fluoride (TBAF).[10] The peptide and polymer blocks were subsequently linked together with the help of the Cu(I) mediated click reaction in THF (Scheme 1).

Initial attempts using $CuSO_4 \cdot 5H_2O$/ascorbic acid as the catalyst system in THF/water mixtures were unsuccessful. We reasoned that this might be caused by the coordinating nature of the arginine residue in the peptide and therefore we examined the strong CuBr/PMDETA complex as the catalyst in pure THF at 35 °C. Although the peptide is only poorly soluble in THF the reaction was complete in 36 hours, as was judged from thin layer



Scheme 1 Preparation of polymer–peptide conjugate PS-GlyGlyArg-AMC **4**, from alkyne and azide functionalised building blocks.

chromatography (TLC). Furthermore, size exclusion chromatography (SEC) in combination with UV detection (AMC group, λ_{abs} = 330 nm) clearly showed the formation of the coupled product **4**.[11] Formation of the super amphiphile was further confirmed by MALDI-TOF mass spectrometry, which revealed a clear shift in the molecular weight distribution with respect to the starting PS-N_3.[11] In addition, end group analysis of the molecular mass distribution was in line with the presence of the tripeptide.

The PS-GlyGlyArg-AMC biohybrid amphiphiles were observed to form vesicles in water upon injection from a THF solution as seen by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (Fig. 1). The sizes of the aggregates were found to increase upon standing from 150–500 nm (Fig. 1A, B and D) to 2 μm (Fig. 1C).

In order to further study the scope of the Cu(I) mediated click strategy the construction of giant amphiphiles from alkyne functionalised bovine serum albumin (BSA) and PS-N_3 was investigated (Scheme 2). In naturally occurring BSA the thiol of the Cys-34 residue is exposed and can be used to introduce functionalities. BSA was reacted with an alkyne bearing maleimide (**5**)[12] affording the singly alkynated protein **6**. As a test reaction first the cycloaddition of **6** with an azide functionalised coumarin dye (**7**) to give **8** was examined using $CuSO_4 \cdot 5H_2O$/ascorbic acid as the catalyst system in a THF/phosphate buffer solution (PB, pH 7.2). After extensive dialysis and size exclusion column chromatography UV-Vis spectroscopy clearly showed the presence of both the dye and BSA.[13]

Subsequently, the reaction between PS-N_3 (**1**) and the alkyne functionalised BSA (**6**) was carried out using the conditions applied for the conjugation of **6** with the coumarin dye. Although the polymer was barely soluble in the aqueous mixture, size exclusion fast performance liquid chromatography (FPLC) analysis clearly revealed the presence of a peak corresponding to a product with a higher molecular weight than **6** (Fig. 2).[14] Furthermore, the UV intensity of this new peak was higher at

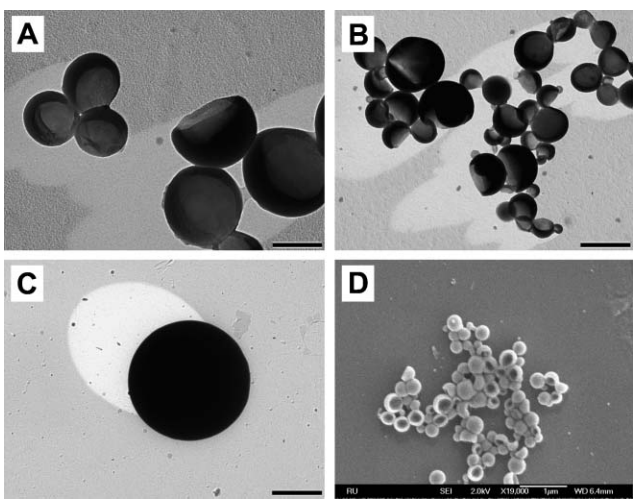
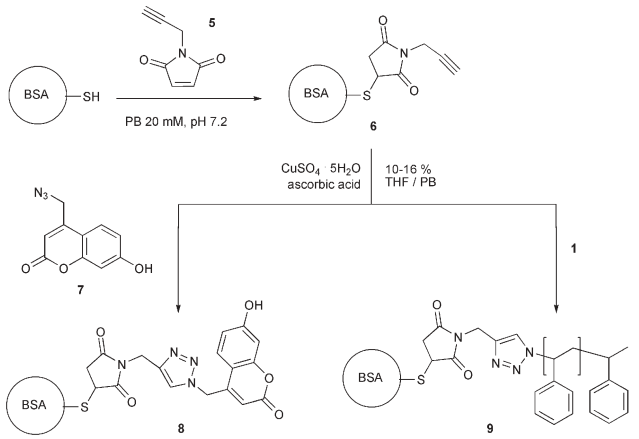


Fig. 1 Electron microscope images of the aggregates formed after injecting a solution of **4** in THF into water; A, B) TEM image taken directly after injection (Pt shadowing, scalebars represent 200 and 500 nm for A and B, respectively); C) TEM image taken 18 hours after injection (Pt shadowing, scalebar represents 1 μm); D) SEM image directly taken after injection.



Scheme 2 Preparation of alkyne functionalised BSA **6**, BSA-coumarin **8** and BSA-PS **9**.

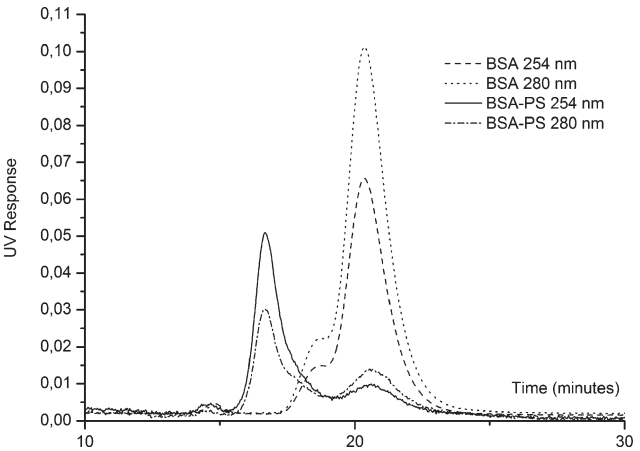


Fig. 2 FPLC of BSA-PS (**9**) (reaction mixture) and BSA (**6**) using PB 20 mM pH 7.2 as an eluent.

λ = 254 nm than at λ = 280 nm, while for BSA derivative **6** the opposite was true. This can be attributed to the presence of the PS block, which has a higher absorption at λ = 254 nm than at λ = 280 nm, whereas for BSA the absorption maximum is at λ = 280 nm. The expected residual peak of uncoupled BSA (20.4 min) results from protein material that is not available for functionalisation, because not all Cys-34 groups in commercial batches of BSA are known to be accessible for reaction.[15]

The aggregation behaviour of the giant amphiphiles (**9**) was studied in aqueous solution with the help of TEM. The reaction mixture was purified by dialysis against PB (20 mM, pH 7.2) and a sample was prepared without further treatment.[11] Spherical aggregates in the range of 30–70 nm (Fig. 3) were observed, suggesting the formation of protein–PS micelles. The size of the protein spheres fits the micellar model as the estimated length of an amphiphile with a fully stretched PS block is *circa* 18 nm.[16]

In conclusion, we have shown that biohybrid amphiphiles composed of a polystyrene block and a peptide or protein block can be straightforwardly constructed *via* "click" chemistry. In the case of the GlyGlyArg-AMC derived amphiphiles potentially interesting biologically active assemblies were obtained, which are currently under investigation. Furthermore, the synthetic strategy

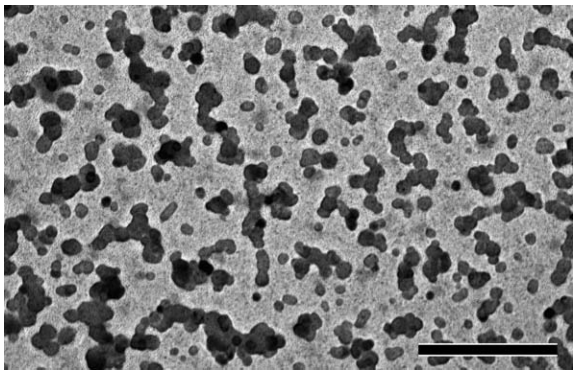


Fig. 3 TEM image (without shadowing or staining) of BSA-PS giant amphiphiles in an aqueous phosphate buffer (20 mM, pH 7.2), scalebar represents 500 nm.

employed here holds great promise for the construction of a host of giant amphiphiles.

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