Formation of giant amphiphiles by post-functionalization of hydrophilic protein–polymer conjugates†‡§

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A novel, generic method for the synthesis of families of tri-block protein–polymer giant amphiphiles was designed and developed. We have synthesized a hydrophilic α-maleimido poly-1-alkyne with M_n = 9.5 kDa (^{1}H-NMR) and narrow PDi (1.15 as measured by SEC) *via* ATRP (Atom Transfer Radical Polymerization). This polymer was succesfully coupled to BSA to afford a hydrophilic multifunctional bioconjugate which was isolated using protein purification techniques and fully characterized. Following the post-functionalization approach, we introduced hydrophobicity to the resulting hydrophilic biohybrid by a straightforward, high yield ''click''-chemistry cycloaddition step. The resulting tri-block protein–polymer amphiphiles were isolated and showed interesting aggregation patterns (TEM, confocal microscopy).

1 Introduction

Following the first conjugation of a protein to poly(ethylene glycol) (PEG) by Abuchowski *et al.*[1] (PEGylation), the area of polymer bioconjugation has flourished.[2–7] Numerous approaches have been developed for both specific and non-specific protein functionalization, whilst the range of proteins and polymers that can be employed is now diverse.[8–12] Several protein–polymer bioconjugates have already found biomedical and biotechnological applications, or are in the process of approval *e.g.* PEGylated interferon alfa-2a is now commonly used as an injectable treatment for hepatitis C infection.

One, recently developed, subclass of bioconjugates is the so-called giant amphiphiles.[13–16] These biohybrids differ from the other protein/enzyme–polymer conjugates in the sense that the conjugation position is precisely known and the hydrophobicity of the polymer moiety conveys an overall amphiphilic character responsible for bioconjugate self-assembly. Additionally, giant amphiphiles have been shown to retain part of the catalytic activity of the enzyme component while exhibiting aggregation properties similar to those of their lower molecular weight counterparts.

Whilst several methods have already been developed for synthesis of giant amphiphiles, practical limitations can hamper efficient synthesis in high yields.[13–15] The main limitation may be attributed to the incompatibility (mainly in terms of solubility) of the two components (*i.e.* the hydrophobic polymer and the hydrophilic protein) while several constraints are also being posed to ensure that the protein structures will remain intact.

In this work, we report on a novel approach for the synthesis of tri-block giant amphiphiles in which hydrophobicity is introduced by post-functionalization of an appropriately designed, polymer pre-functionalized, biohybrid. More specifically, for the development of our method a hydrophilic α-maleimido terminated polymer bearing multiple 1-alkyne terminated side chains on its backbone (**I**, Scheme 1) was designed and synthesized.[17] This multifunctional polymer was specifically coupled to the protein to initially form a hydrophilic multifunctional protein–polymer bioconjugate. The hydrophobicity was introduced in a second step, by performing multiple ''clicking'' of low molecular weight alkyl azides onto the 1-alkyne functionalities of the bioconjugate using the copper-catalysed Huisgen's 1,3 dipolar cycloaddition (often referred to as ''click''-chemistry).[18–21] It is demonstrated that this final synthetic step offers the advantage of introducing hydrophobicity under the mild conditions required for click-chemistry in aqueous solutions and avoids problems such as the denaturation of proteins, which is often noticeable in organic solvents. As a result, a significant increase in the total reaction yields and applicability of the method is introduced. To the best of our knowledge, this is the first generic method reported in the literature which leads to a new generation of tri-block protein–polymer giant amphiphiles with high efficiency and yields.

2 Experimental

2.1 Materials and measurements

All chemicals were purchased from Fluka or Sigma-Aldrich (unless otherwise specified) and used without further purification. Cu(I)Br was purified as reported by Keller and Wycoff.[22] *N*-(*n*-Propyl)-2-pyridylmethanimine[23] and 2-methyl-acrylic acid 2,2-dimethyl-[1,3]dioxolan-4-ylmethyl ester[24] were prepared as described earlier and stored at 0 °C. Protected

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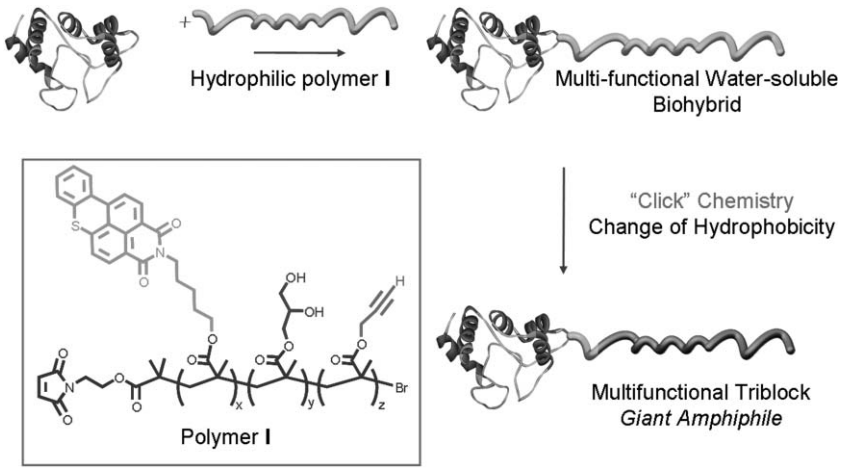

Scheme 1 Schematic representation of the post-functionalization synthetic approach for the creation of giant amphiphiles.

maleimido initiator,[11] protected alkyne monomer,[17] fluorescent hostasol co-monomer[25] and 1-azidodecane[26] and benzyl-azide were synthesized according to the literature (see ESI§). Triethylamine was dried over KOH pellets. Deuterated solvents were obtained from Cambridge Isotope Laboratories. Bovine Serum Albumin (BSA) was purchased from Sigma Aldrich. Polymerizations were carried out using standard Schlenk techniques under an inert atmosphere of oxygen-free nitrogen, unless otherwise stated. Yields of the reactions were not optimized.

SEC (Size Exclusion Chromatography) were measured on a Shimadzu VP HPLC system equipped with a Thermo Biobasic SEC-300 column eluting with a solvent mixture of 70% phosphate buffer 5 mM pH 7.4, 30% acetonitrile (unless otherwise noticed). NMR spectra were recorded on a Bruker 300 MHz and a Bruker 400 MHz spectrometer system. All chemical shifts are reported in ppm (δ) relative to tetramethylsilane, referenced to the chemical shifts of residual solvent resonances (^{1}H and ^{13}C). The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, m = multiplet. The number average molecular weights, M_n, are calculated by comparing the integrals of the chain-end signals and appropriate peaks related to the polymer backbone. Infrared absorption spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer (using a Golden Gate diamond or a NaCl cuvette). UV-Vis spectra were recorded on a CARY 1 BIO UV-visible spectrophotometer. Solutions were sonicated in a Bandelin Sonorex RK 100 apparatus. MALDI-TOF MS measurements were performed in the SVS-MS Mass Spectrometry Core Facility using an Axima CFR+ MALDI-TOF (Shimadzu Biotech, Manchester, UK) in positive ionization mode and HABA as the matrix.

2.1 General polymerization procedure: synthesis of polymer VI

In a Schlenk tube, the initiator **II** (609 mg, 1.70 mmol), solketal monomer **III** (9.99 g, 49.9 mmol), fluorescent hostasol co-monomer **V** (236 mg, 0.499 mmol) and the *N*-(*n*-propyl)-2-pyridylmethanimine (ligand) (488 mg, 3.29 mmol) were dissolved in anisole (20 mL). The mixture was subjected to five freeze–pump–thaw cycles and then cannulated into a second Schlenk tube containing a magnetic stirrer and Cu(I)Br (236 mg, 1.65 mmol), previously evacuated and filled with nitrogen (t = 0). The resulting mixture was stirred at ambient temperature ($\sim$20 °C) and aliquots were removed at regular intervals of time in order to monitor the evolution of both the conversion (^{1}H-NMR) and the molecular weight (SEC). At 54% conversion, the protected trimethylsillyl alkyne **IV** was added as the second monomer (3.0 g, 15 mmol). The reaction was stopped at 80% overall conversion. The reaction mixture was bubbled with air for 24 h, passed through a neutral alumina pad and precipitated by dropwise addition to a large amount of petroleum ether ($\sim$20 : 1 v : v ratio with respect to anisole). The pale orange powder was dissolved in the minimum amount of dichloromethane, passed through a short neutral alumina pad (which was subsequently washed with additional dichloromethane) and precipitated again in petroleum ether to give, after filtration, the pure product as a pale orange powder (for ^{1}H-NMR and FT-IR spectra see ESI§).

2.2 Retro Diels–Alder reaction: synthesis of polymer VII

The polymer **VI** (1.06 g, 0.0922 mmol) was dissolved in toluene (10 ml) and the solution refluxed overnight. The resulting polymer was precipitated in a large amount of petroleum ether (20 : 1 v : v with respect to the toluene) to afford a thin pale yellow powder; volatiles were removed under reduced pressure to give the resulting maleimido-terminated polymer **VII** in close to 100% yield (1.05 g) (for ^{1}H-NMR and FT-IR spectra see ESI§).

2.3 Deprotection of 1-alkyne units: synthesis of polymer VIII

An aqueous 1.0 M acetic acid solution (1.5 equiv. mol/mol with respect to the alkyne-trimethylsilyl groups, 1.11 mL, 1.11 mmol) was added to a solution of the maleimido deprotected polymer **VII** (1.05 g, 0.0922 mmol) in THF (25 mL) in a round bottom flask. Nitrogen was bubbled through the solution (*ca*. 10 min) and the yellowish solution was cooled to −20 °C. A 1.0 M solution of TBAF·3H_2O in THF (1.5 equiv. mol/mol with respect to the alkyne-trimethylsilyl groups, 1.11 mL, 1.11.mmol) was added dropwise over a period of *ca*. 2–3 min. The mixture was stirred at this temperature for 30 min and then at ambient temperature overnight. The resulting solution was passed through a silica

pad (to remove the excess TBAF) and the pad washed with THF. Removal of the volatiles gave the product as a brown oil. Finally, the polymer was precipitated in petroleum ether to give the pure polymer **VIII** as a fine yellow powder in close to 100% yield (1.0 g) (for ^{1}H-NMR and FT-IR spectra see ESI§).

2.4 Hydrolysis of ketal functions: synthesis of polymer I

Polymer **VIII** (1.00 g, 0.0922 mmol) was dissolved in 1,4-dioxane (80 mL) in a round bottom flask and the resulting clear solution cooled to 0 °C. The mixture became turbid upon addition of 33 mL of a 1 M HCl aqueous solution, and was subsequently allowed to slowly warm up to ambient temperature overnight and stirred for a further 24 h.

The reaction medium was neutralised by using a 1 M phosphate buffer pH 7.4 solution, at 0 °C. Freeze-drying of the resulting mixture gave an orange powder which was dissolved in the minimum amount of dry methanol. Final filtration through a pad of neutral alumina, washing with dry methanol and removal of the solvent under vacuum afforded the final, totally deprotected polymer **I** as an orange powder (800 mg, 0.0842 mmol, yield = 91%).

2.5 Coupling of BSA to the maleimide appended polymer I: synthesis of the BSA–PA(I) biohybrid IX

A total of 1.9 ml of a 0.3 mM solution of Bovine Serum Albumin (BSA) in 20 mM phosphate buffer (PB) pH 7.4 was added to a solution of 360 µl of 20 mM PB pH 7.4 and 112 µl of a 50 mM solution of the polymer **I** in the same buffer. The reaction mixture was stirred gently at 7 °C for 1 d. Removal of the unreacted polymer was achieved by filtration of the reaction mixture using Microcon® Centrifugal Filter Units (Millipore NMWCO 30000). Isolation of the biohybrid from the unreacted BSA was carried out on a Superdex 150 column eluting with 20 mM phosphate buffer pH 7.4. The enriched fractions were freeze-dried and analyzed by SEC (mobile phase: 70% 10 mM phosphate buffer pH 7.4, 30% CH_3CN), electrophoresis under native and denaturating conditions and MALDI-TOF MS (Fig. 1, ESI§). Samples of the pure biohybrid were used for the coupling experiments and imaging with TEM and confocal microscopy.

2.6 Huisgen [3 + 2] cycloaddition ("click"-chemistry reaction) of 1-azidodecane on the BSA–polyalkyne adducts: formation of the giant amphiphiles BSA–PA@$C_{10}H_{21}N_3$ (X)

1-Azidodecane (48 µL) was added to 166 µL of 20 mM phosphate buffer pH 7.4 and the resulting biphasic mixture was sonicated for *ca.* 10 min. Subsequently 250 µL of a 0.24 mM solution of BSA–polyalkyne **IX** in 20 mM phosphate buffer pH 7.4 were added. Finally 12 µL of a 100 mM sodium ascorbate solution in 20 mM PB pH 7.4 and 24 µL of a 100 mM solution of $CuSO_4$ in PB 20 mM pH 7.4 were added. The reaction mixture was stirred gently for 2 d at 7 °C, in the dark. The course of the reaction was monitored by SEC-HPLC. The final giant amphiphiles were characterized by electrophoresis and SEC and their aggregation patterns imaged with TEM and confocal microscopy.

2.7 Huisgen [3 + 2] cycloaddition ("click"-chemistry reaction) of azidomethyl-benzene on the BSA–polyalkyne adducts: formation of the giant amphiphiles BSA–PA@$C_7H_7N_3$ (XI)

Azidomethyl-benzene (35 µL) was added to 153 µL of 20 mM phosphate buffer pH 7.4, the resulting biphasic mixture was sonicated and was "clicked" to BSA–polyalkyne **IX** using the same conditions as in 2.6.

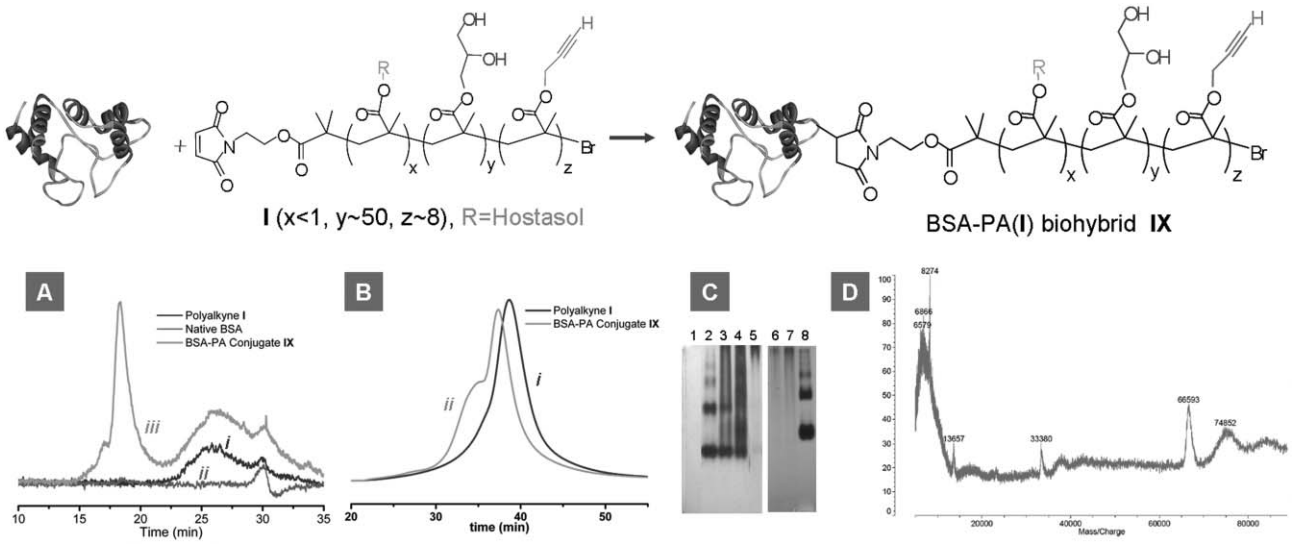

Fig. 1 Synthesis and characterization of the BSA–PA biohybrid **IX**. (A) SEC traces (466 nm) of polyalkyne **I** (i), BSA (ii) and BSA–PA(**I**) **IX** reaction mixture (iii) (Thermo Biobasic SEC-300 column eluting at 0.50 mL min^{-1} with a solvent mixture 70% phosphate buffer 5 mM pH 7.4, 30% acetonitrile). (B) SEC traces (fluorescence detection) of the polyalkyne **I** (i) and BSA–PA(**I**) **IX** (ii) (two Phenomenex BioSep-SEC-S3000 columns connected in series, using H_2O : MeCN : TFA 65 : 35 : 0.1 (vol : vol : vol) at 0.50 mL min^{-1} as the mobile phase. (C) Native gel electrophoresis (Silver Staining), lane 1: polyalkyne **I**, lane 2: native BSA, lane 3: BSA + **I** (blank), lane 4: BSA–**I** bioconjugate, lane 5: BSA–**I**@$C_{10}H_{21}N_3$ giant amphiphiles, lane 6: BSA–**I**@$C_7H_7N_3$ giant amphiphiles, lane 7: BSA–**I**@$C_{10}H_{21}N_3$ giant amphiphiles, lane 8: native BSA. (D) MALDI-TOF MS of the BSA–PA reaction mixture.

3 Results and discussion

3.1 Synthesis of the multifunctional polymer I

The synthesis of the hydrophilic polymer (**I**, Fig. 2) was achieved using Atom Transfer Radical Polymerization (ATRP).[27,28] This technique allows the synthesis of polymers with high levels of control over their molecular weight and polydispersity index (PDi) and is very tolerant towards a variety of functional groups. Intending to specifically couple this polymer to proteins *via* cysteine (Cys) residues, we incorporated in the retrosynthesis a terminal-protected maleimide functionality to the ATRP initiator (**II**). This protection was necessary since it had already been demonstrated that α-maleimido-terminated polymers and initiators have the tendency to copolymerize under ATRP conditions by addition to the maleimide moiety.[11] The hydrophilic character of the polymer was designed to originate from a solketal methacrylate monomer (SMA, **III**) while a gradient of protected propargyl methacrylate (PMA, **IV**) led to the multiple 1-alkyne functions. Gradient polymerization was utilized to assure the formation of a water soluble polymer rather than an amphiphilic block copolymer. Finally, to facilitate the characterization procedure a visibly fluorescent tag (Hostasol yellow, **V**) was incorporated as a co-monomer in the polymerization feed. As previously reported, a small percentage of this fluorescent co-monomer ($\leqslant 1\%$ mol/mol) is sufficient to confer to the

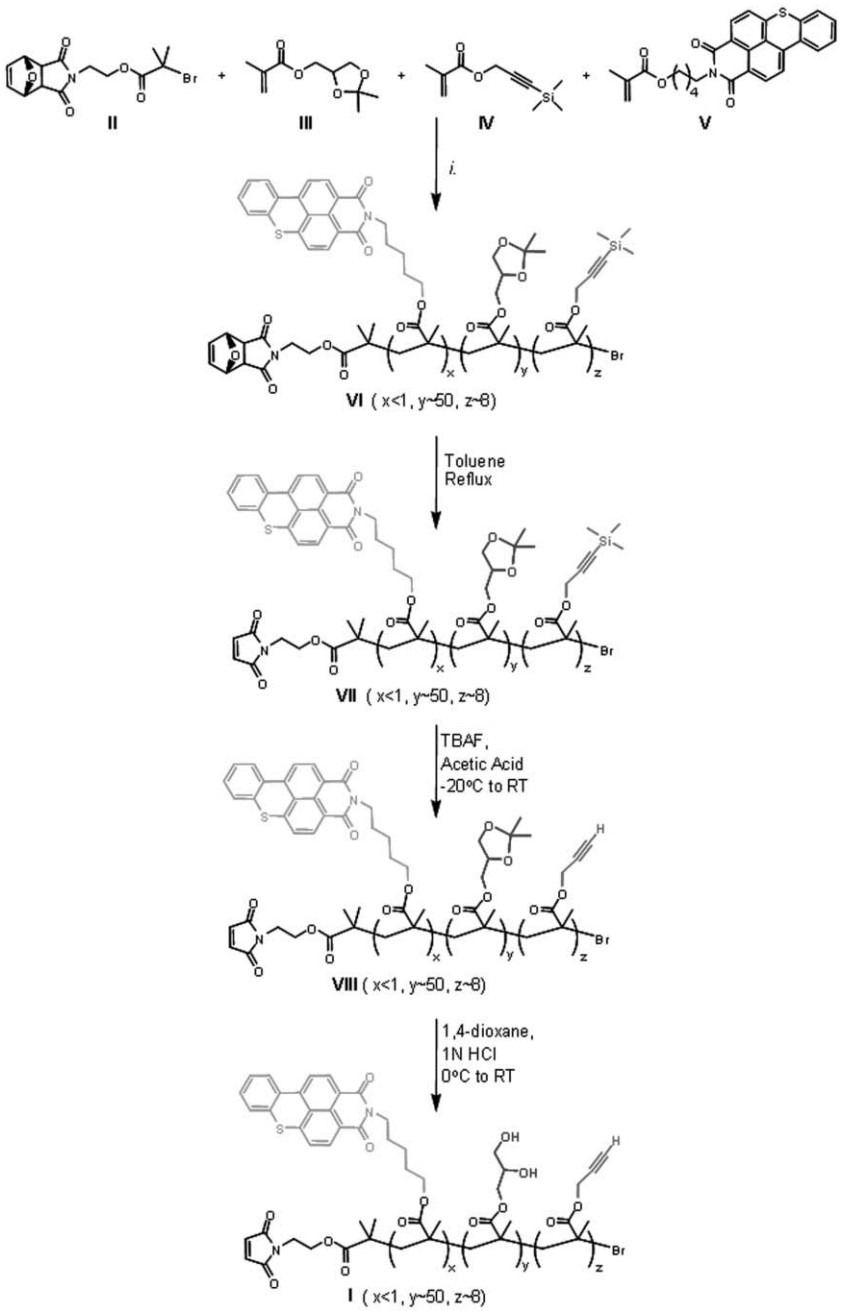

Fig. 2 Synthesis of the α-maleimido-poly-1-alkyne polymer **I**. (i) Polymerization procedure: Cu(I)Br, propyl-pyridin-2-ylmethylene-amine, anisole, ambient temperature.

polymer an intense yellowish visibly fluorescent colour.[29] The synthesis and characterization of the initiator and the various monomers is reported in full detail in the ESI§.

Polymerization of the SMA (**III**) and a small percentage of the hostasol yellow co-monomer was initiated using the Diels–Alder protected maleimido-initiator (**II**). Monomer conversion was followed by ^{1}H-NMR and the PMA co-monomer (**IV**) was added upon reaching approximately 50% conversion. The polymerization reaction allowed control over the molecular weight which is dependant upon the reaction time. The fully protected polymer (**VI**) product had a M_n = 11.5 kDa (^{1}H-NMR) and narrow polydispersity (PDi = 1.15 as measured by SEC) (full characterization data by ^{1}H-NMR, SEC and FT-IR spectroscopy in ESI§).

Three different deprotection steps were necessary to obtain the desired hydrophilic polymer (**I**) containing the 1-alkyne side chains. In the first step, the retro Diels–Alder removal of furan from the oxanorbornene group was achieved by refluxing polymer **VI** overnight in toluene. The trimethylsilyl protecting groups were subsequently removed using TBAF in THF in the presence of acetic acid, acting as a buffer to maintain an acidic pH. Finally, the deprotection of the ketal functions of solketal groups was performed by stirring the polymer in 1,4-dioxane in the presence of 1 M aqueous HCl. The final product **I** was characterized with ^{1}H-NMR, SEC and FT-IR (see ESI§).

3.2 "Click"-chemistry derivatization of 1-alkyne-deprotected polymer I

In order to determine the conditions and to investigate the efficiency of the click-chemistry reaction with **I**, we first studied the reaction of low molecular weight azides with a precursor of **I** which was deprotected only on the 1-alkyne functions (see ESI§). This polymer was selected in order to eliminate the possibility of cycloaddition of the azido-compounds on the maleimide terminal unit.[30] The assay was performed with the low molecular weight alkyl, 1-azidodecane ($C_{10}H_{21}N_3$) and aryl, benzyl-azide ($C_7H_7N_3$), azides using the Cu(II)SO_4/sodium ascorbate Cu(I) generating system and conditions compatible with the envisioned protein coupling.[11] We followed the course of the reaction using FT-IR spectroscopy until the C≡C–H stretching band disappeared. ^{1}H-NMR of the product verified the formation of the clicked copolymer through the disappearance of the 1-alkyne characteristic peak and the shifting of the protons in the position α- to the triple bond of the polymer and α- to the azide group towards higher fields. Furthermore, SEC analyses (in THF : TEA 95 : 5 eluent with DRI (Differential Refractive Index) detection, see ESI§) clearly showed the formation of a new polymer with higher molecular weight and a virtually unchanged PDi (for spectra, SEC traces and conditions see ESI§).

3.3 Synthesis of BSA–PA(I) hydrophilic bioconjugates IX

Having proved the efficiency of clicking azides to the poly-1-alkyne precursor of **I**, we studied the formation of protein–**I** biohybrids utilizing the 66 kDa globular carrier protein BSA (BSA has one free cysteine residue at position 34) as a model protein. The fully-deprotected hydrophilic polymer **I** was designed to specifically react with the free thiol function of cysteine residues in proteins and form hydrophilic polymer–protein conjugates. The coupling reaction was performed by incubation of a 10 molar excess solution of the polymer **I** with BSA in 20 mM PB pH 7.4 at ambient temperature for 24 h.

The conjugation reaction was followed by SEC [Fig. 1(A), ESI§]. Measurements at λ = 466 nm (maximum absorption of the fluorescent tag[31]) indicated the formation of the biohybrid **IX**. Both native gel electrophoresis [Fig. 1(C)] and SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis, see ESI§) also verified the formation of the BSA–PA(**I**) biohybrid (**IX**) through the appearance of a new broad band migrating at higher molecular weights than that of the native BSA. Furthermore, examination of the gels under UV-light at λ = 366 nm confirmed the presence of the bioconjugates through the observation of fluorescence in this broad single band attributed to the Hostasol tag incorporated in the polymeric backbone. To exclude the possibility of non-specific interactions between the polymer and the protein, a control sample, prepared by mixing a solution of native BSA and a solution of polyalkyne **I**, was also included in the electrophoresis. As expected, the protein band displayed no shift and remained non-fluorescent after irradiation by UV-light at 366 nm excluding the possibility of the formation of non covalent complexes between BSA and **I**.

The BSA–**I** bioconjugate was purified with extensive dialysis [30 kDa nominal molecular weight cutoff (NMWCO)] followed by gel filtration (Sephadex G150, see ESI§) and the biohybrid (**IX**) was characterized with SEC [Fig. 1(B)] and MALDI-TOF MS [Fig. 1(D)]. From these measurements, we were able to verify the formation of the biohybrid **IX** as the main product of the coupling reaction (MW 74.8 kDa, MALDI-TOF MS). A small percentage of a by-product that was detected is attributed to non-specific coupling of the polymer with the protein (most probably with one of the exposed lysines on the surface of BSA) or to the presence of an impurity at the native BSA samples (see MALDI-TOF MS, ESI§). The BSA–PA(**I**) was subjected to a second chromatographic purification (Superdex 75), the pure BSA–PA fractions were freeze-dried and subsequently further functionalized by clicking low molecular weight azides ($C_{10}H_{21}N_3$ and $C_7H_7N_3$) to the terminal 1-alkyne moieties through a copper-catalysed [3 + 2] Huisgen's cycloaddition.

3.4 "Click"-chemistry reaction: synthesis of the giant amphiphiles X and XI

Click-chemistry reactions were carried out in 20 mM phosphate buffer using a 40 molar excess of the alkyl ($C_{10}H_{21}N_3$) or benzyl azide ($C_7H_7N_3$) over the BSA–PA **IX** in the dark for ~2 days at 7 °C (see experimental sections 2.6 and 2.7). During the course of the reaction, the mixtures became opaque indicating the formation of aggregates. Native gel electrophoresis confirmed the quantitative formation of giant amphiphiles [see Fig. 1(C), ESI§] which due to aggregation did not migrate on the gel and retained the fluorescence of the Hostasol tag. It is worth noting that the only purification step required at this point was a single dialysis (30 kDa NMWCO) to remove the unreacted azide and the catalyst. This facilitated the quantitative recovery of pure fractions.

3.4 Aggregation studies

The giant amphiphiles **X** and **XI** were investigated with TEM which revealed the formation of well-defined spherical aggregates [Fig. 3(A–D)].¶ The formation of such micellar superstructures is in good aggrement with previous reports on BSA–polystyrene giant amphiphiles.[16] It is noted that the BSA–**I**@$C_{10}H_{21}N_3$ giant amphiphiles **X** were rather uniform in diameter [mean diameter *ca.* 150 nm, Fig. 3(A), (B)], while the diameters of the BSA–**I**@$C_7H_7N_3$ giant amphiphiles **XI** varied between 20 and 200 nm [Fig. 3(C), (D)]. This dispersity difference of the resulting superstructures in water is attributed to the more dynamic behaviour of the alkyl chains. This is the first report of a difference in aggregation behaviour in giant amphiphiles arising from the different hydrophobic tail attached.

Laser confocal microscopy allowed the observation of the superstructures together with the fluorescence arising from the Hostasol tag which is incorporated on the polymer moiety.¶

Furthermore, when the conjugate **X** was suspended in a 1 : 1 (v : v) mixtute of water and ethanol, upon addition of a small quantity of decane and vigorous shaking, a milky emulsion was formed. The analysis of this emulsion by confocal microscopy clearly shows the biohybrid orienting itself at the decane–water interface, a fact that is attributed to its amphiphilic nature.

4 Conclusions

In summary, we have described the design and synthesis of a maleimide-appended hydrophilic polymer (**I**) bearing multiple 1-alkyne units under standard ATRP conditions and with narrow PDi. The specific conjugation of **I** with protein cysteine residues led to the formation of multifunctional biohybrids which could be isolated using standard protein purification protocols. We have demonstrated that these bioconjugates can be in turn post-functionalized by a straightforward, high yield click-chemistry cycloaddition step to afford tri-block protein–polymer amphiphiles. Furthermore, we were able to prove with TEM and confocal microscopy that these new tri-block giant amphiphiles exhibit aggregation properties similar to those reported for the direct coupling of a protein to a hydrophobic polymer.[13–16]

This represents a new and generic method for the synthesis of giant amphiphiles. The creation of a series of multi-1-alkyne polymers bearing alternate initiator groups specifically aiming at different functionalities of protein structures, as well as the cycloaddition of a large variety of azido-compouds to introduce both hydrophobicity and secondary functions can be easily envisioned and is currently in progress.

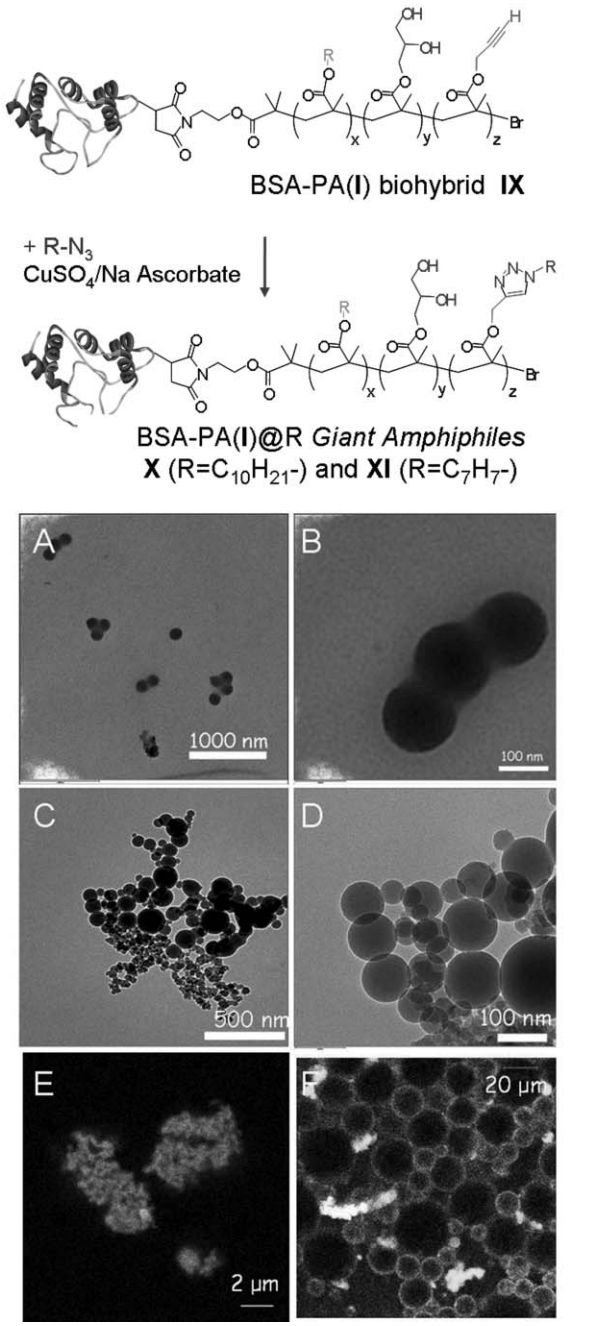

Fig. 3 Schematic representation of the click-chemistry reaction leading to the giant amphiphiles **X** (R = $C_{10}H_{21}$–) and **XI** (R = C_7H_7–). (A), (B) TEM micrographs of the BSA–**I**@$C_{10}H_{21}N_3$ giant amphiphiles. (C), (D) TEM micrographs of the BSA–**I**@$C_7H_7N_3$ giant amphiphiles. (E) Confocal microscopy image of the BSA–**I**@$C_{10}H_{21}N_3$ (excitation with 514 nm Argon-Krypton laser line). (F) Confocal microscopy image of the BSA–**I**@$C_{10}H_{21}N_3$ emulsion in a water–ethanol–decane mixture.

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¶ Blank experiments performed under the same conditions with BSA, the polymer **I**, the BSA–PA(I) bioconjugate **IX** or either of the azides, did not reveal any aggregation pattern (see ESI§).

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