ChemComm

Cite this: *Chem. Commun.*, 2012, **48**, 1586–1588

www.rsc.org/chemcomm

COMMUNICATION

# Multifunctional *Giant Amphiphiles via* simultaneous copper(I)-catalyzed azide–alkyne cycloaddition and living radical polymerization†‡

**Eleftheria Daskalaki,[a] Benjamin Le Droumaguet,[b] David Gérard[b] and Kelly Velonia*[a]**

***Received 16th August 2011, Accepted 19th September 2011***
**DOI: 10.1039/c1cc15075h**

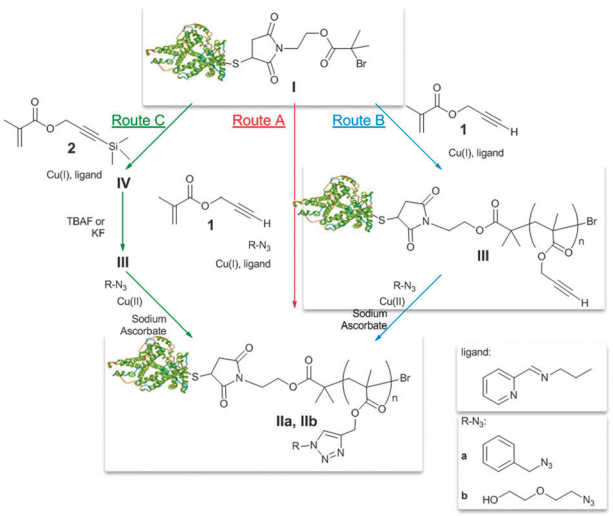


**A novel class of chemically addressable, multifunctional *Giant Amphiphiles* was synthesized in excellent yields and polydispersity following simultaneous or sequential living radical polymerization and the *click*, copper(I)-catalysed azide–alkyne cycloaddition (CuAAC). This new approach allows chemical tailoring of the biomacromolecules and *in situ* formation of nanocontainers.**

The combination of the unique functional and structural properties of biomolecules with those of synthetic molecules and macromolecules, and the application of such chimeric bioconjugates in medicine, bio- and nanotechnology have been an area of intense research during the recent decades.[1] One of the most straightforward approaches toward this direction involves covalent coupling of single or multiple polymeric chains to biomolecules with the most pronounced example being protein PEGylation which has led to bioconjugates with enhanced physical and pharmacological properties and wide applications in medicine.[1,2]

Protein–polymer hybrids are conventionally prepared either through the direct conjugation of appropriately functionalized macromolecules to amino acids[3–8] or cofactors,[9] or through bioaffinity couplings.[10] These methods have nevertheless proven to be less efficient in the case of *Giant Amphiphiles*,[3–5,8–10] *i.e.* the subclass of protein–polymer bioconjugates in which a hydrophobic polymer moiety conveys an overall amphiphilic character to the biohybrids responsible for their interesting aggregation architectures. Practical limitations posed to the synthetic protocols primarily by this amphiphilic character or by restrictions applied to ensure protein integrity, hamper efficient classical synthesis and therefore limit further investigations to unravel *Giant Amphiphiles* full application potential. The recent application of living radical polymerization techniques for the *in situ* preparation of polymer–protein conjugates has revolutionized the area. Atom-Transfer Radical polymerization (ATRP)[11,12] and Reversible Addition–Fragmentation chain Transfer polymerization (RAFT)[13,14] of appropriately modified biomacroinitiators were shown to bypass the multiple synthetic and purification steps used in classical bioconjugations and facilitate purification. We recently demonstrated that ATRP is the method of choice also for the family of *Giant Amphiphiles* as it overcomes the intrinsic synthetic limitations of the system and displays excellent control over the polydispersity indices of the resulting bioconjugates.[8] More importantly, it allows the hierarchical *in situ* encapsulation of guest molecules (*e.g.* other proteins) for the construction of nanoreactors.

One underlying goal of protein–polymer self-assembly is the construction of multifunctional nanoarchitectures following efficient protocols and a key approach to this direction involves bioconjugation of multifunctional polymer moieties. Herein we demonstrate how the facile and high-yielding ATRP mediated *grafting* of the appropriate monomer *from* a protein–macroinitiator **I** can be directly combined with the "*click*" copper(I)-catalysed azide–alkyne cycloaddition (CuAAC)[15] for the *in situ* formation of such novel multifunctional bioconjugates (Scheme 1, Route A). We also demonstrate how ATRP and CuAAC can be combined in sequential steps (Route B) and in a chemical sequence comprising other simple chemical methodologies (Route C). The efficiency of this




**Scheme 1** Schematic representation of one-pot or sequential ATRP/CuAAC synthesis of *Giant Amphiphiles*.

[a] *Department of Materials Science and Technology, University of Crete, University Campus Voutes, 71003 Heraklion, Crete, Greece. E-mail: velonia@materials.uoc.gr; Fax: +30 2810 39 4273; Tel: +30 2810 39 4036*
[b] *Department of Organic Chemistry, University of Geneva, Quai Ernest Ansermet 30, Sciences II, CH 1211 Geneva 4, Switzerland*

† This article is part of the *ChemComm* 'Emerging Investigators 2012' themed issue.

‡ Electronic supplementary information (ESI) available: Full experimental conditions and characterization data. See DOI: 10.1039/c1cc15075h

approach not only proves the pertinence of the *grafting from* living polymerization techniques for the creation of protein–polymer bioconjugates, it more importantly gives rise to a new family of chemically addressable amphiphilic bioconjugates (**II**, **III** and **IV**) and allows the *in situ* formation of biohybrid nanocontainers.

In previous studies[5] we synthesized a hydrophilic gradient copolymer bearing pendant alkyne-1 groups to introduce multifuntionality to proteins and, in a later step, linked the polymer to a protein. In the current study we reasoned that the combination of ATRP with CuAAC should provide the means to avoid independent polymer synthesis, improve reaction yields, control bioconjugate polydispersity and simplify isolation of the products.[8,16] The choice of the methacrylate monomer **1** for the polymerization reaction was dictated by the expected simplicity and orthogonality of the CuAAC aimed at their tailoring. BSA was utilized as a model protein for the purposes of our experiments since it contains a unique, accessible cysteine group (Cys 34) permitting specific couplings.[17] The synthesis of the biomacroinitiator **I**, the propargyl methacrylate monomer **1**, the trimethylsilyl protected propargyl methacrylate **2**, the azides and the copper ligand, *N*-(*n*-propyl)-2-pyridylmethanimine was achieved in high yields following established protocols (ESI‡).[5,6]

The ATRP *grafting from* polymerization reactions were performed in buffered aqueous solutions, under oxygen free conditions and ambient temperature, using the copper(I) bromide/*N*-(*n*-propyl)-2-pyridylmethanimine catalyst system and without the presence of any "sacrificial" initiator.[5,6,8] More specifically, in the novel one-pot approach (Route A) we utilized prop-2-ynyl methacrylate **1** as a monomer in the presence of an azide (benzyl azide or azido-triethylene glycol) to achieve simultaneous CuAAC and living radical polymerization (ESI‡).[16] In order to fully exploit this new approach, we also performed the sequential ATRP and CuAAC described in Route B as well as the synthetic approach described in Route C which incorporates an intermediate deprotection step and trimethylsilyl protected propargyl methacrylate **2** as a monomer. The CuAAC in both latter cases was investigated using standard conditions,[5,16] *i.e.* a large excess of the azide over the biohybrid and the $CuSO_4$/sodium ascorbate copper(I) generating system. The deprotection step of Route C was systematically pursued using TBAF, KF or $K_2CO_3$ which were selected on the basis of common reaction conditions found in the literature with a significant factor directing the choice of the reagent, the retention of protein integrity. Additional polymerization reactions were performed under the same conditions and in the presence of the non-polymerizable, fluorescent dye carboxyfluorescein (CF) aiming to prove the ability of the resulting *Giant Amphiphiles* to concurrently form hierarchically assembled nanocontainers through the statistical encapsulation of CF within the superstructures.

For the one-pot Route A, the SEC chromatographic behaviour of the dialyzed reaction mixtures revealed quantitative formation of the biomacromolecules **IIa** and **IIb** through the formation of new peaks possessing shorter retention times, larger hydrodynamic volume, than BSA (Fig. 1A) and similar molecular weight distribution as judged by their broadness. In all SEC measurements the Refractive Index (RI) and UV traces were in excellent agreement. The efficiency of Route A becomes more pronounced when comparing the SEC chromatographs with those obtained for Routes B and C (Fig. 1B–D). In Route B an, at least, bimodal distribution is observed for the case of the azido-triethylene glycol clicking, while wide polymodal peaks point to multiple final products in the chromatographs of Route C. The formation of the *Giant Amphiphiles* **IIa**, **IIb**, **III** and **IV** was also supported by gel electrophoresis (Fig. 1E). The polyacrylamide gels revealed for all reaction products typical *Giant Amphiphile* behaviour, *i.e.*, electrophoretic mobility hampered by the amphiphilicity of the biomacromolecules. Silver staining of these gels revealed minute quantities of the BSA–macroinitiator **I** and the non-reacting BSA dimer only in the case of Route C. When electrophoresis was performed on agarose gels, a clear difference in the migration between **I** and **IIa**,**b** was observed.

Infrared spectroscopy (IR) validated the structures and provided a direct proof for the efficiency of our synthetic approach through the absence or presence of the characteristic stretching vibration of the triple bond emerging at 2130 $cm^{-1}$.

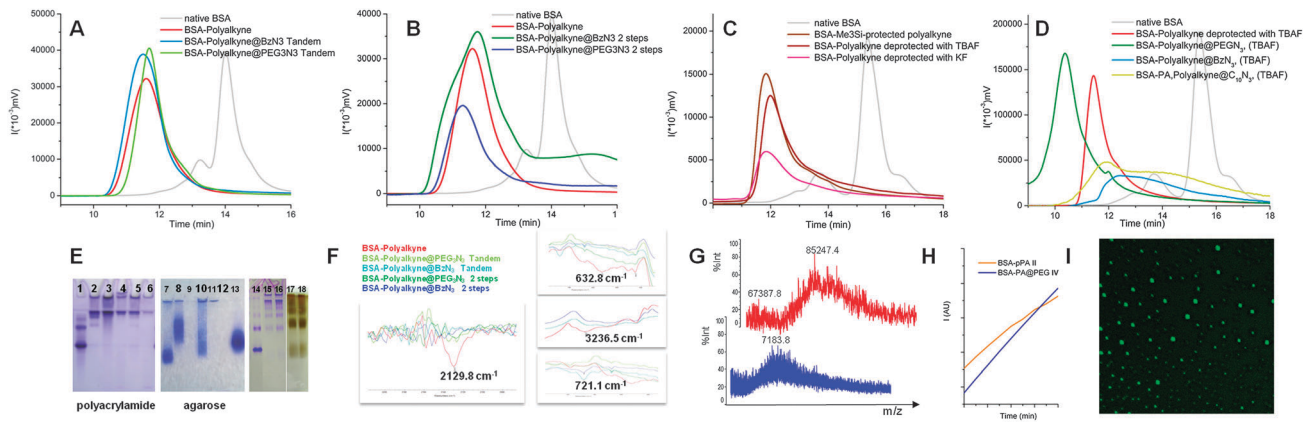



**Fig. 1** Characterization of *Giant Amphiphiles*. Representative: SEC traces (A): for Route A, (B): Route B, (C) and (D): Route C, (E): Polyacrylamide Gel Electrophoresis, lanes 1 and 14: native BSA, lane 2: **III**, lane 3 and 5: **IIa**, lane 4 and 6: **IIb**, lane 15: **IV**, lane 16: **III**. Agarose: lane 7: **III**, lanes 8 and 9:**IIb**, lanes 10 and 11: **IIa**, lane 13: native BSA (F): FT-IR spectra, (G): MALDI-TOF spectra of the *Giant Amphiphiles* **III** (red) and the isolated polyalkyne (blue), (H): BSA enzyme-like activity for **III** and **IIb**, (I): CFM images of **III** formed in the presence of CF.

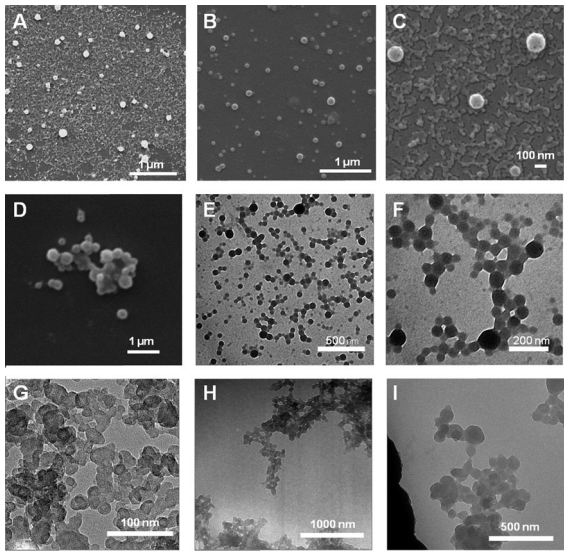



**Fig. 2** FE-SEM micrographs (A): **III**, (B) and (C): **IIb**, (D): **IIa**. TEM micrographs (E) and (F): **IV** (G): **III** upon TBAF deprotection of **IV** (H) and (I): **IIa**.

This peak was present only in the BSA–polyalkyne **III** and was accompanied by, less clear due to the nature of the products, peaks at 3300 $cm^{-1}$ and 632 $cm^{-1}$ (Fig. 1F). All peaks disappeared upon multiple CuAAC. Furthermore, the peak attributed to the aromatic C–H bend at 721 $cm^{-1}$ emerged only in the reaction products obtained by clicking of the benzyl azide. MALDI-TOF analysis, often problematic in the case of *Giant Amphiphiles*, afforded limited data with the most reliable showing a $m/z$ signal of around 85 kDa for the protein–polymer hybrid **IV** and a signal of approximately 7 kDa $m/z$ for the polymer moiety itself when recovered through HCl mediated protein degradation of **II** (Fig. 1G, ESI‡). Aggregation studies using Transmission Electron Microscopy (TEM) and Field Emission Scanning Electron Microscopy (FE-SEM) established the amphiphilic nature of the products by revealing the formation of well-defined spherical aggregates with diameters varying from 30 to 150 nm in all products (Fig. 2). No difference was observed for the superstructures formed in the presence of CF (Fig. 2F). In the latter, CFM imaging demonstrated the statistical encapsulation of CF within the superstructures (Fig. 1I), proving the ability of the produced *Giant Amphiphiles* to host non-polymerizable guests. Given that both CuAAC and ATRP could be detrimental for protein structure integrity due to copper poisoning, we performed enzyme-like activity tests in products and starting materials of all routes which established retention of activity indicating structure integrity. For the specific step of the alkyne deprotection in Route C, the TBAF and the KF deprotected samples retained enzyme-like activity, showed slightly disturbed spherical superstructures in TEM, while CFM verified the existence of discrete fluorescent aggregates.

In conclusion, this is to the best of our knowledge, the first synthesis of protein–polymer conjugates using simultaneous or sequential living radical polymerization and copper catalyzed [3 + 2] Huisgen cycloaddition. Equally important, we presented the synthesis of a series of novel, chemically addressable, multifunctional protein–polymer biohybrids in excellent yields and polydispersity. This approach allows chemical tailoring of the protein–polymer hybrids and *in situ* formation of nanocontainers and could be implemented in several applications. Our current efforts are focused on the full exploitation of such biohybrids by the introduction of secondary (catalytic) functions for the creation of multifunctional nanoreactors.

We thank the Swiss National Science Foundation (200020-113804/1) for partial financial support, D.-H. Tran and the SVSMS/University of Geneva for MALDI-TOF, Mrs K. Tsagaraki, IESL/FORTH for FE-SEM, Mr A. Askounis and D. Kritsiotakis for assistance with experimental measurements.

## Notes and references

1 F. M. Veronese, *Biomaterials*, 2001, **22**, 405; D. W. Pack, A. S. Hoffman, S. Pun and P. S. Stayton, *Nat. Rev. Drug Discovery*, 2005, **4**, 581.
2 F. M. Veronese and J. M. Harris, *Adv. Drug Delivery Rev.*, 2002, **54**, 453, and references cited therein.
3 K. Velonia, A. E. Rowan and R. J. M. Nolte, *J. Am. Chem. Soc.*, 2002, **124**, 4224.
4 A. J. Dirks, S. S. van Berkel, N. S. Hatzakis, J. A. Opsteen, F. L. van Delft, J. J. L. M. Cornelissen, A. E. Rowan, J. C. M. van Hest, F. P. J. T. Rutjes and R. J. M. Nolte, *Chem. Commun.*, 2005, 4172.
5 B. Le Droumaguet, G. Mantovani, D. M. Haddleton and K. Velonia, *J. Mater. Chem.*, 2007, **17**, 1916.
6 J. Nicolas, G. Mantovani and D. M. Haddleton, *Macromol. Rapid Commun.*, 2007, **28**, 1083; G. Mantovani, F. Lecolley, L. Tao, D. M. Haddleton, J. Clerx, J. J. L. M. Cornelissen and K. Velonia, *J. Am. Chem. Soc.*, 2005, **127**, 2966.
7 S. Balan, J. Choi, A. Godwin, I. Teo, C. M. Laborde, S. Heidelberger, M. Zloh, S. Shaunak and S. Brocchini, *Bioconjugate Chem.*, 2007, **18**, 61.
8 B. Le Droumaguet and K. Velonia, *Angew. Chem., Int. Ed.*, 2008, **47**, 6263.
9 M. J. Boerakker, J. M. Hannink, P. H. H. Bomans, P. M. Frederik, R. J. M. Nolte, E. M. Meijer and N. A. J. M. Sommerdijk, *Angew. Chem., Int. Ed.*, 2002, **41**, 4239.
10 J. M. Hannink, J. J. L. M. Cornelissen, J. A. Farrera, P. Foubert, F. C. De Schryver, N. A. J. M. Sommerdijk and R. J. M. Nolte, *Angew. Chem., Int. Ed.*, 2001, **40**, 4732.
11 K. L. Heredia and H. D. Maynard, *Org. Biomol. Chem.*, 2007, **5**, 45; D. Bontempo and H. D. Maynard, *J. Am. Chem. Soc.*, 2005, **127**, 6508; K. L. Heredia, D. Bontempo, T. Ly, J. T. Byers, S. Halstenberg and H. D. Maynard, *J. Am. Chem. Soc.*, 2005, **127**, 16955.
12 J. Nicolas, V. San Miguel, G. Mantovani and D. M. Haddleton, *Chem. Commun.*, 2006, 4697; B. S. Lele, H. Murata, K. Matyjaszewski and A. J. Russell, *Biomacromolecules*, 2005, **6**, 3380.
13 C. Boyer, V. Bulmus, J. Liu, T. P. Davis, M. Stenzel and C. Barner-Kowollik, *J. Am. Chem. Soc.*, 2007, **129**, 7145.
14 P. De, M. Li, S. R. Gondi and B. S. Sumerlin, *J. Am. Chem. Soc.*, 2008, **130**, 11288.
15 H. C. Kolb, M. G. Finn and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2001, **40**, 2004; C. W. Tornoe, C. Christensen and M. Meldal, *J. Org. Chem.*, 2002, **67**, 3057.
16 J. Geng, J. Lindqvist, G. Mantovani and D. M. Haddleton, *Angew. Chem., Int. Ed.*, 2008, **47**, 4180.
17 J. Janatova, J. K. Fuller and M. J. Hunter, *J. Biol. Chem.*, 1968, **243**, 3612.