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Bionanoreactors

In Situ ATRP-Mediated Hierarchical Formation of Giant Amphiphile Bionanoreactors**

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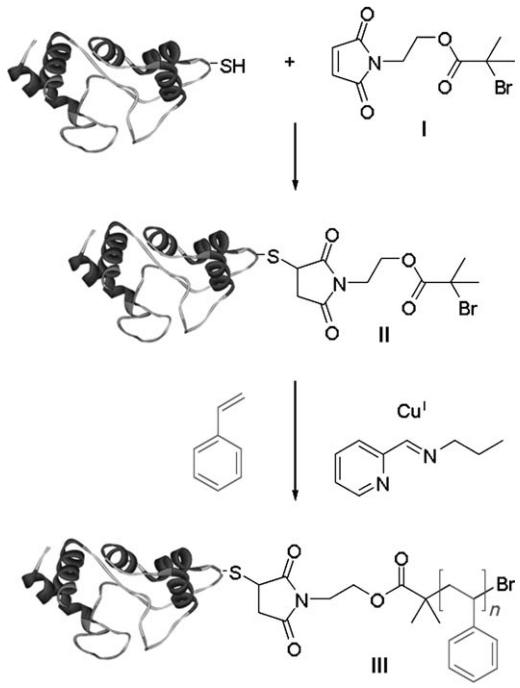
Hierarchical architectures with a broad range of lengths are ubiquitous in biological material systems. In nature, most of these multifunctional materials are produced in situ under ambient conditions and through ecologically balanced processes. Synthesized to mimic natural superstructures, giant amphiphiles,[1–3] a subclass of protein–polymer conjugates[4–10] in which the hydrophobicity of the polymer conveys overall amphiphilic character to the biohybrid, exhibit interesting aggregation properties and enormous potential in bio- and nanotechnology.

In the past, these bioconjugates have been prepared either through the direct conjugation of appropriately functionalized macromolecules to specific amino acids[1] or cofactors,[3] or through bioaffinity couplings.[2] The efficiency of their synthesis by these conventional pathways was hampered by practical limitations caused mainly by the solubility incompatibility between the protein and the hydrophobic polymer and by constraints posed to guarantee the stability of the protein itself. Moreover, concomitant to their synthesis, stable nondynamic superstructures formed, accounting for the tedious purification required and the low yields; this significantly limited any further exploration of the potential of giant amphiphiles in the creation of preprogrammed multifunctional systems.

To overcome the intrinsic limitations caused by either the synthetic approaches or the amphiphilic nature of such bioconjugates, we employed protein-initiated atom-transfer radical-mediated polymerization (ATRP), a method recently introduced for the preparation of hydrophilic[11,12] and smart[11] polymer–protein conjugates. We envisioned that a successful application of ATRP for the formation of giant amphiphiles would offer an efficient synthetic alternative, but more importantly it would allow for the first time their hierarchical programming into the construction of multienzyme superstructures.

We report here on the facile and high-yielding ATRP-mediated preparation of giant amphiphiles in situ (Scheme 1). We prove that by utilizing ATRP, quantitative amounts of



Scheme 1. General scheme for the in situ ATRP-mediated synthesis of giant amphiphiles.

bioconjugates possessing low polydispersities and the characteristic aggregation properties of amphiphilic biomacromolecules form. More importantly, we demonstrate for the first time the construction of hierarchically assembled bionanoreactors by the efficient one-pot incorporation of a guest protein within the formed superstructures.

The efficiency of in situ protein-initiated ATRP for the synthesis of giant amphiphiles was explored using the 66 kDa globular bovine serum albumin (BSA), which contains only one free cysteine residue at position 34.[13] Adapting the approach used by Maynard et al. for the formation of smart protein conjugates,[11] we synthesized the maleimido-capped ATRP initiator **I** (Scheme 1).[14] Its subsequent bioconjugation reaction was performed under mild conditions by the

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slow addition of a solution of **I** (40 mol equiv) in DMSO (4% final volume content) to BSA. The reaction mixture was overincubated (48 h at 7 °C) for maximum yields, and the bioconjugate **II** was easily purified by extensive dialysis.

The quantification of the reaction with Ellman's colorimetric assay[15] revealed practically no free conjugation sites (Cys) in the product solution; native gel electrophoresis revealed a new single band for **II**; and size-exclusion chromatographic (SEC) analyses showed a peak with same broadness as and a retention time only slightly different from that of native BSA. No trace for the free ATRP initiator **I** was detected upon extensive dialysis. The quantitative formation of **II** was verified by MALDI-TOF analyses, which revealed peaks at masses of 66313 and 66737 amu for native BSA and the BSA–macroinitiator **II**, respectively, a shift corresponding to the addition of one molecule of maleimido initiator **I** per protein (Figures S1–S3 in the Supporting Information).

The monomer employed for the subsequent polymerization was styrene, as it would lead to polystyrene giant amphiphiles known to exhibit a strong amphiphilic character.[1] ATRP on **II** was performed in aqueous solution, in the absence and presence of DMSO as cosolvent, under oxygen-free conditions, at ambient temperature, with the copper bromide/*N*-(*n*-propyl)-2-pyridylmethanimine catalyst system,[16] and without any "sacrificial" initiator.[11] Several sets of experiments were performed utilizing different ratios monomer to biomacroinitiator **II**, while a series of control experiments (in the absence of **II** or styrene or Cu^I, and in the presence of O_2) were conducted to ensure that the in situ polymerization proceeds on the designated BSA initiating position (see Table S1 in the Supporting Information). In all cases, the resulting bioconjugate solutions were subjected to extensive dialysis to ensure that all traces of reagents (styrene, copper, *N*-(*n*-propyl)-2-pyridylmethanimine) were removed.

When low monomer/**II** ratios were employed (50:1 and 500:1), SEC revealed mixtures of unreacted **II** and bioconjugates **III** with a hydrodynamic volume slightly higher than that of BSA (Figure 1). Native gel electrophoresis revealed a band of lower electrophoretic mobility than that of BSA (higher molecular weight). MALDI-TOF spectrometry verified these findings through *m*/*z* signals corresponding to unreacted **II**, along with signals ranging from 67 to 71 kDa for **III**. These reactions were accompanied by low yields (full data in the Supporting Information).

When high styrene/**II** ratios were utilized (1500:1 to 3000:1), SEC analysis revealed the quantitative formation of giant amphiphiles **III** having a larger hydrodynamic volume than BSA (Figure 1 A). In all cases, RI (RI = refractive index) and UV traces were in good agreement. The broadness of the peaks observed for BSA, the macroinitiator **II**, and the giant amphiphiles **III** was similar, indicating both the efficiency of the polymerization and the retention of dispersity. Native gel electrophoresis revealed behavior typical for giant amphiphiles, that is, migration hampered by the amphiphilic character of the bioconjugate and no trace of the starting materials. MALDI-TOF analyses showed *m*/*z* signals ranging from 71 to almost 80 kDa depending on the monomer/**II** ratio utilized (Figure 1 B) and no signal corresponding to the macroinitiator **II**. The TEM images verified the in situ formation of spherical aggregates with behavior similar to

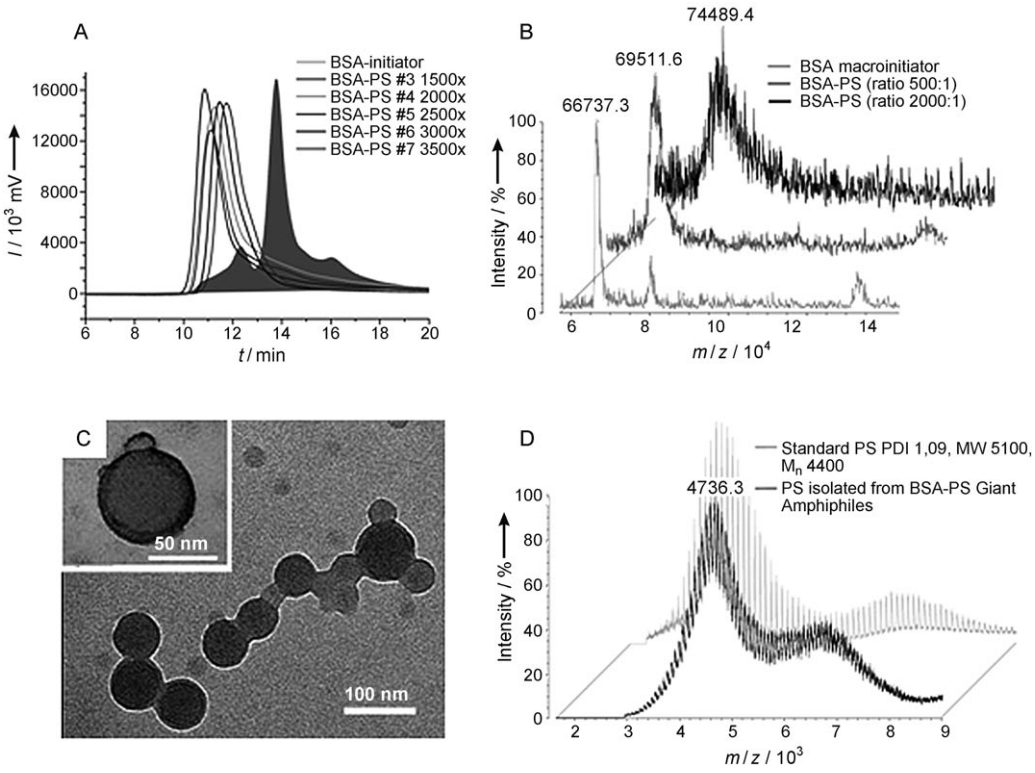

Figure 1. Characterization of the BSA–polystyrene biohybrids synthesized utilizing ATRP and high monomer/**II** ratios. Representative A) SEC traces, B) MALDI-TOF spectra, and C) TEM images. D) MALDI-TOF spectrum of isolated polystyrene.

that of conventionally synthesized giant amphiphiles[16b] and diameters ranging from 20 to 100 nm (Figure 1C; see the Supporting Information).

To further prove the formation of polystyrene, the biopolymers **III** were subjected to HCl-mediated protein degradation. The structure of the isolated polymer samples was verified by NMR spectroscopy and MALDI-TOF spectrometry (full data in the Supporting Information). It is worth noting that the MALDI-TOF spectrum revealed only two major distributions of the isolated polymer (Figure 1D), one of which was predominant and displayed a rather low polydispersity index in comparison to that of a standard polystyrene of known polydispersity. We attribute this low polydispersity to the selected synthetic approach.

Finally, this type of "grafting from" by means of the ATRP technique was successfully applied to two other proteins, namely, human serum albumin (HSA) and reduced human calcitonin, thus demonstrating the generality of this method for the formation of giant amphiphiles. (Full experimental details are included in the Supporting Information.)

We confirmed the nature of the in situ generated giant amphiphiles but more significantly their capacity to concurrently form hierarchically assembled nanocontainers by performing the polymerization reaction in the presence of a second nonpolymerizable protein. For this reason, fluorescently labeled papain or horseradish peroxidase (HRP) was integrated into the optimized reaction scheme (i.e. high styrene/**II** ratios) and a supplementary final dialysis step was added to remove the non-encapsulated proteins. In both cases, aliquots from the reaction mixtures and purified solutions were analyzed by SEC, which revealed the formation of the nanocontainers and the elimination of the non-encapsulated proteins by dialysis. The TEM micrographs (Figure 2B) showed aggregation patterns similar to those observed for the polymerizations of **II** in the absence of a second protein.

The presence of the fluorescently labeled papain within the spherical superstructures was demonstrated by confocal fluorescence microscopy (CFM). When these nanocontainers were subjected to a second, external labeling with Atto, CFM revealed the expected statistical presence of both fluorescent species in the superstructures (Figure 2A).

The catalytic efficiency and permeability of these hierarchically formed nanocontainers was tested using a solution of the purified biohybrid **III** loaded with HRP and by employing the standard TMB/H_2O_2 (TBM = 3,3′,5,5′-tetramethylbenzidine) chromogenic assay. Following a small lag time after the addition of the reagent solution, an intense blue color was observed (at 650 nm) along with a deep yellow color (at 450 nm) after the catalysis had been stopped with an acid solution; this provides direct proof of the catalytic capacity of the nanoreactors (Figure 2C).

In conclusion, the first efficient ATRP-mediated in situ preparation of BSA–polystyrene giant amphiphiles was accomplished; a family of bioconjugates was obtained in which the degree of polymerization is controlled by the monomer/biomacroinitiator ratio. Both the yields and the dispersities of the resulting giant amphiphiles were drastically improved and the need for an organic cosolvent was eliminated. We proved that by the creation of such chimeric systems in situ, the one-pot hierarchical incorporation of other species (such as enzymes) is possible without steps that would interfere with the protein integrity or the overall architecture of the aggregate. Furthermore, we demonstrated that these novel giant amphiphile nanocontainers are permeable and can be used as nanoreactors. Our current efforts are focused on the full exploitation of the ATRP technique for the preparation of functional polymer–biomolecule conjugates and multienzyme nanoreactors.

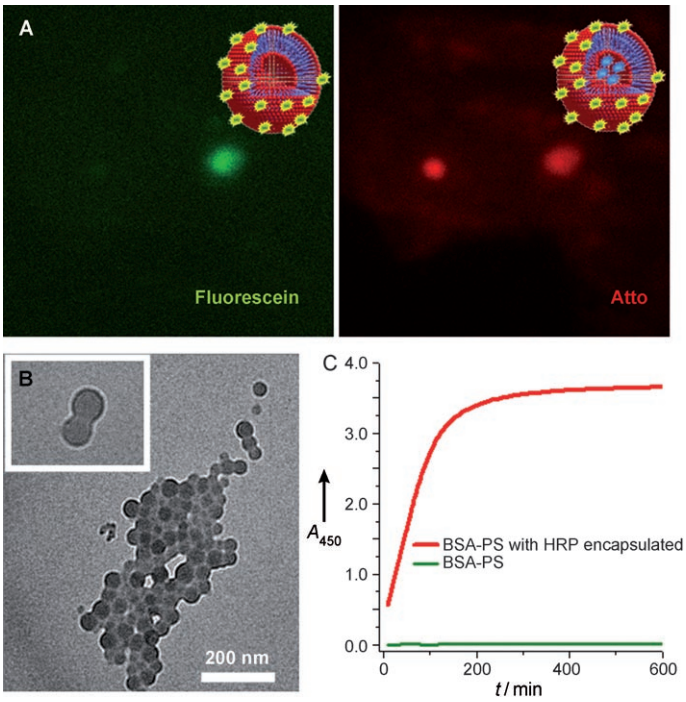

Figure 2. Hierarchically formed nanocontainers. A) CFM images and B) TEM images of papain-loaded nanocontainers labeled with fluorescein and Atto. C) Activity profile of HRP-loaded nanoreactors.

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[1] K. Velonia, A. E. Rowan, R. J. M. Nolte, *J. Am. Chem. Soc.* **2002**, *124*, 4224–4225.
[2] J. M. Hannink, J. J. L. M. Cornelissen, J. A. Farrera, P. Foubert, F. C. De Schryver, N. A. J. M. Sommerdijk, R. J. M. Nolte, *Angew. Chem.* **2001**, *113*, 4868–4870; *Angew. Chem. Int. Ed.* **2001**, *40*, 4732–4734.
[3] M. J. Boerakker, J. M. Hannink, P. H. H. Bomans, P. M. Frederik, R. J. M. Nolte, E. M. Meijer, N. A. J. M. Sommerdijk, *Angew. Chem.* **2002**, *114*, 4413–4415; *Angew. Chem. Int. Ed.* **2002**, *41*, 4239–4241.
[4] a) F. M. Veronese, *Biomaterials* **2001**, *22*, 405; b) D. W. Pack, A. S. Hoffman, S. Pun, P. S. Stayton, *Nat. Rev. Drug Discovery* **2005**, *4*, 581–593.
[5] V. Bulmus, Z. L. Ding, C. J. Long, P. S. Stayton, A. S. Hoffman, *Bioconjugate Chem.* **2000**, *11*, 78–83.

[6] P. Thordarson, B. Le Droumaguet, K. Velonia, *Appl. Microbiol. Biotechnol.* **2006**, *73*, 243 – 254.
[7] J.-F. Lutz, H. G. Börner, *Prog. Polym. Sci.* **2008**, *33*, 1 – 39.
[8] J. Nicolas, G. Mantovani, D. M. Haddleton, *Macromol. Rapid Commun.* **2007**, *28*, 1083 – 1111.
[9] H. G. Börner, H. Schlaad, *Soft Matter* **2007**, *3*, 394 – 408.
[10] H. G. Börner, *Macromol. Chem. Phys.* **2007**, *208*, 124 – 130.
[11] a) K. L. Heredia, H. D. Maynard, *Org. Biomol. Chem.* **2007**, *5*, 45 – 53; b) D. Bontempo, H. D. Maynard, *J. Am. Chem. Soc.* **2005**, *127*, 6508 – 6509; c) K. L. Heredia, D. Bontempo, T. Ly, J. T. Byers, S. Halstenberg, H. D. Maynard, *J. Am. Chem. Soc.* **2005**, *127*, 16955 – 16960; d) H. D. Maynard, K. L. Heredia, R. C. Li, D. P. Parra, V. Vazquez-Dorbatt, *J. Mater. Chem.* **2007**, *17*, 4015 – 4017; e) R. M. Broyer, G. M. Quaker, H. D. Maynard, *J. Am. Chem. Soc.* **2008**, *130*, 1041 – 1047.
[12] a) J. Nicolas, V. San Miguel, G. Mantovani, D. M. Haddleton, *Chem. Commun.* **2006**, 4697 – 4699; b) B. S. Lele, H. Murata, K. Matyjaszewski, A. J. Russell, *Biomacromolecules* **2005**, *6*, 3380 – 3387.
[13] J. Janatova, J. K. Fuller, M. J. Hunter, *J. Biol. Chem.* **1968**, *243*, 3612 – 3622.
[14] G. Mantovani, F. Lecolley, L. Tao, D. M. Haddleton, J. Clerx, J. J. L. M. Cornelissen, K. Velonia, *J. Am. Chem. Soc.* **2005**, *127*, 2966 – 2973.
[15] a) G. L. Ellman, *Arch. Biochem. Biophys.* **1959**, *82*, 70 – 77.
[16] a) D. M. Haddleton, M. C. Crossman, B. H. Dana, D. J. Duncalf, A. M. Heming, D. Kukulj, A. J. Shooter, *Macromolecules* **1999**, *32*, 2110 – 2119; b) B. Le Droumaguet, G. Mantovani, D. M. Haddleton, K. Velonia, *J. Mater. Chem.* **2007**, *17*, 1916 – 1922.

