Nanoreactor

DOI: 10.1002/anie.201306798

A Chaperonin as Protein Nanoreactor for Atom-Transfer Radical Polymerization**

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Abstract: *The group II chaperonin thermosome (THS) from the archaea Thermoplasma acidophilum is reported as nanoreactor for atom-transfer radical polymerization (ATRP). A copper catalyst was entrapped into the THS to confine the polymerization into this protein cage. THS possesses pores that are wide enough to release polymers into solution. The nanoreactor favorably influenced the polymerization of N-isopropyl acrylamide and poly(ethylene glycol)methylether acrylate. Narrowly dispersed polymers with polydispersity indices (PDIs) down to 1.06 were obtained in the protein nanoreactor, while control reactions with a globular protein–catalyst conjugate only yielded polymers with PDIs above 1.84.*

A reactor confines a chemical reaction into a defined volume. On the macroscopic scale, this is important to control and modulate the reaction through parameters such as temperature, stirring, and feeding rates of substrates. If, however, a reaction is confined in a vessel with dimensions on the nanoscale, that is, into a yocto liter ($=10^{-24}$ L) volume in a nanoreactor, the course of a chemical reaction can be influenced in unique ways.[1] Reaction rates can be enhanced by bringing substrate and catalyst in close proximity,[2] side reactions can be suppressed by limiting the number of reactive species that encounter each other,[3] the reactor can act as a template that defines the size of the formed products,[4] and the nanoreactor can provide a cavity to influence single-chain folding of synthetic polymers.[5] Furthermore, reactions can be carried out in environments where the reaction would normally not occur, such as biocatalysis in organic solvents[6] or in living cells.[7] Nanoreactors also offer the possibility to investigate reactions on the single-molecule level in order to gain insight into reaction mechanisms and catalysis.[8]

Several nanovessels have been investigated as potential nanoreactors, including swollen domains in polymer networks,[6,9] lipid and polymer vesicles,[10] and hollow protein complexes, so-called protein cages.[1a–e,11] They have in common that they enclose catalysts, most often enzymes, into a cavity surrounded by a shell or interface. This boundary layer is either permeable for substrates and products, or it encompasses pores that allow for the exchange of matter between the inside and the outside of the nanoreactor. Thus, the activity and the substrate selectivity of the enclosed catalyst do not only depend on the intrinsic properties of the catalyst, but also on the tailored properties of the nanoreactor.[1e] Nature provides intriguing nanovessels with defined pores in the form of protein cages, the best known being ferritin and viral capsids.[1a–e] Although some protein cages act as gated nanoreactors in living cells, for example, the microbial microcompartments,[12] implementations in non-natural settings with nonnative catalytic species are still a challenge. Successful examples are a peroxidase enclosed in the cowpea chlorotic mottle virus[8a] or alcohol dehydrogenase encapsulated into the capsid of bacteriophage P22.[13]

Here, we introduce the thermosome (THS), a group II chaperonin from the archaea *Thermoplasma acidophilum*,[17] as a nanoreactor for polymerization reactions. THS is a hexadecameric protein complex that consists of eight α and eight β subunits. In its closed conformation it is a spherical protein cage about 16 nm in diameter that encloses two cavities of approximately 130 nm^3 each (Scheme 1 a).[14] It provides a folding chamber to refold denatured proteins and can cycle between open and closed states by the consumption of ATP.[17] An advantage of THS compared to other nanoreactors is that the chaperonin, when in its open conformation, possesses pores that are large enough to allow macromolecules to enter and leave the cavities (Scheme 1 b).[14,17,18] Thus, it should be an ideal nanoreactor to synthesize polymers in its interior with the possibility to release the formed macromolecules into solution.

In the field of polymer synthesis, atom-transfer radical polymerization (ATRP) has proven to be one of the most versatile and successful controlled/living radical polymerization techniques, because it tolerates the presence of numerous functional groups, biomacromolecules, and reaction media.[19] Polymers synthesized by ATRP have been used, for example, as building blocks for nanostructures,[20] to form protein–polymer conjugates,[21] and in drug-delivery systems.[22] Although ATRP in aqueous media has been demonstrated,[19,21c,23] working in pure water still presents a challenge since the polymerization can be too fast and side

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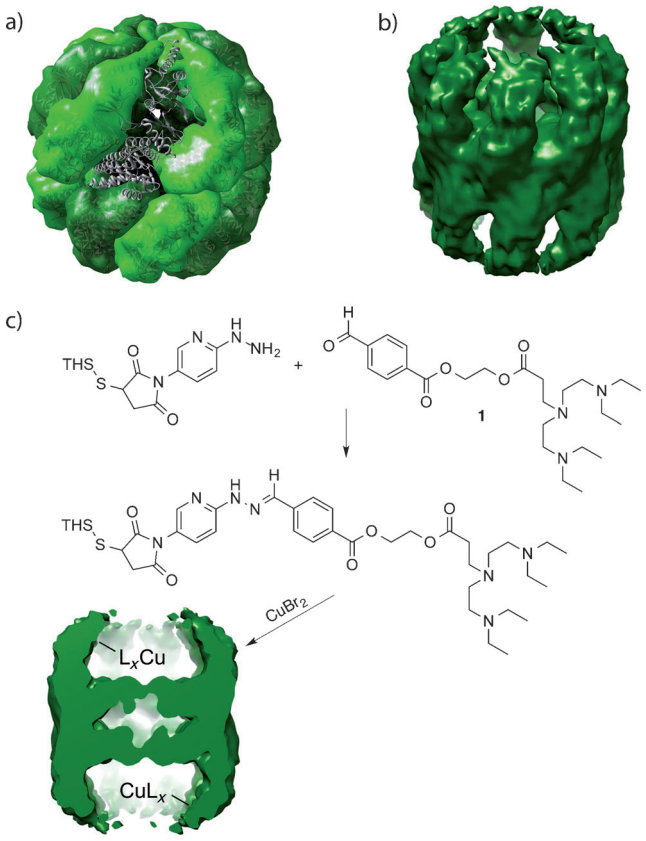
[**] This research was supported by a Marie Curie Intra European Fellowship and a Marie Curie European Reintegration Grant, by the Holcim Stiftung Wissen, by the Swiss National Science Foundation, and by the NCCR Nanoscale Science. We thank W. Meier (Department of Chemistry) for his support, M. Zehringer (Kantonales Laboratorium Basel-Stadt) and J. Jourdan (Department of Physics) for the NAA, V. Olivieri (Zentrum für Mikroskopie) for the TEM images, and T. Eaton for proofreading the manuscript.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201306798.



Scheme 1. Structure of THS and strategy to entrap catalysts into the protein cage. a) Representation of THS in its closed conformation.[14] b) Cryo-electron microscopy (cryo-EM) density map of a thermosome in its open conformation (Data from *Methanococcus maripaludis*,[15] since no structural data of the THS in this conformation is available in the databases. This structure is in good agreement with the cyro-EM data of the THS.[16]). c) Coupling strategy to covalently bind a TEDETA-derived ligand (**1**) into the cavities of THS. This ligand was used to complex Cu^{II}, thereby resulting in a protein–catalyst conjugate, that is, a nanoreactor for ATRP.

reactions tend to play a more important role than in other reaction media.[19b,24] Confining the reaction space within the small volume of a nanoreactor should increase the degree of control over the polymerization and increase the degree of functionalization at the chain ends of the products.[25]

A strategy to sequester ATRP into a protein nanoreactor is to conjugate initiators to protein cages.[26] However, this grafting-from approach does not allow for a continuous production of polymers since only one polymer chain per protein-bound initiator is formed and the chain is covalently attached to the protein. In order to use THS as a nanoreactor for ATRP, we therefore chose to entrap an ATRP catalyst into the THS and start chain growth from soluble initiators that diffuse into the THS. Thus, polymer chains that form in the protein cage are able to leave the cavities and make room for further chains to grow.

An organometallic catalyst was conjugated into the cavities of the THS by using bisaryl hydrazone linker chemistry (Scheme 1c), similar to our reported method to entrap fluorescent proteins into THS.[18] A THS mutant that displays one free cysteine residue on the inside surface of each β subunit was completely modified with a 25-fold excess (with respect to thiol groups) of 3-maleimido-6-hydraziniumpyridine hydrochloride (MHPH) as determined by UV/Vis spectroscopy (see the Supporting Information). After purification, the modified THS was reacted with **1**, a derivative of the ligand *N*,*N*,*N*′,*N*′-tetraethyldiethylene triamine (TEDETA) featuring an aromatic aldehyde moiety (Scheme 1c). The reaction yields a stable bisaryl hydrazone bond between the protein and the ligand.[27] The formation of this linker can be monitored by UV/Vis spectroscopy. The spectrum of the conjugate in Figure 1a shows the typical absorption band of proteins at 280 nm as well as the band of the bisaryl hydrazone link with a maximum at 354 nm. The deconvolution of this spectrum allows calculating the average number of bisaryl hydrazone bonds and gave a ratio of ligands per THS of 3.9 ± 0.1. The attached ligand was used to complex Cu^{II} ions, followed by purification of the modified THS (THS-L_xCu) by size-exclusion spin centrifugation. The linker can only have formed inside the THS because of the location of the engineered cysteine residues. Therefore, the TEDETA–Cu complex must be entrapped in the cavity of the chaperonin. This was confirmed by small-angle X-ray scattering (SAXS) measurements. THS-L_xCu showed an increased

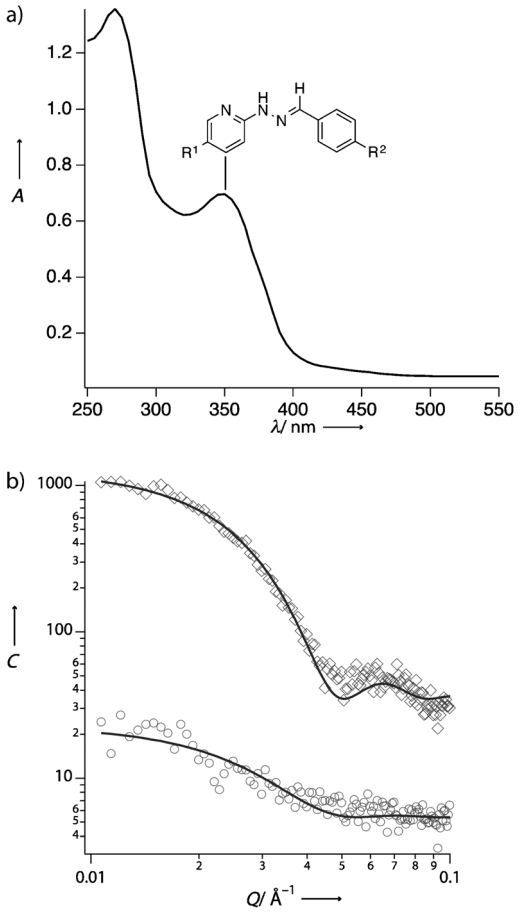


Figure 1. Characterization of THS-L_xCu. a) The UV/Vis spectrum shows absorption bands of the protein and of the bisaryl hydrazone that links the catalyst to the protein. b) SAXS data of THS-L_xCu (◇) in comparison to nonmodified THS (○) shows a higher contrast induced by the electron-dense copper that is bound to the ligand in the THS.

contrast compared to the nonmodified THS (Figure 1b), thus indicating that the former is a nanoobject with higher electron density than the THS alone, that is, the engineered THS contained copper ions. The best fit of the scattering data gave a diameter of 17.2 nm for THS-L_xCu, which is in agreement with the diameter of the protein in the apo state.[16] Therefore, it can be concluded that the electron-rich copper ions reside in the inner cavities of the THS. To elucidate whether the THS alone, that is, without a TEDETA ligand, also binds copper, nonmodified THS was incubated in a $CuBr_2$ solution and purified as described above. The comparison of SAXS data of this sample with data of THS that had not been exposed to Cu^{II} shows no difference in shape (Figure S1 in the Supporting Information). This indicates that copper is not complexed by the THS itself.

The protein cage was analyzed for its structural integrity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and by native PAGE (Figure S2a and b). Both gels show the same bands for THS, independent of their modification with the ATRP catalyst. Moreover, transmission electron microscopy (TEM) images of the protein–catalyst conjugate show spherical objects with a diameter of approximately 16 nm, which are typical for fully assembled THS (Figure S2c).[16] Thus, the overall structure of THS was unaffected by the encapsulated ATRP catalyst.

Polymerizations were conducted by adding THS-L_xCu to an aqueous buffered solution (100 mM sodium acetate, 150 mM NaCl, 80 mM $MgCl_2$, pH 5.2) of the water-soluble initiator 2-hydroxyethyl-2-bromobutyrate (HEBIB) and the monomer *N*-isopropylacrylamide (NiPAAm) in a molar ratio of [monomer]/[initiator]/[THS-L_xCu] $67:1:1.5\times10^{-5}$ under argon atmosphere (reaction scheme see Figure S3). An excess of ascorbic acid (ratio [THS-L_xCu]/[ascorbic acid] $1:2.7\times10^{5}$) was added to reduce Cu^{II} in situ to its catalytically active form THS-L_xCu^{I} and to regenerate Cu^{II} that could accumulate during the polymerization, according to the activators regenerated by electron-transfer (ARGET) ATRP method.[19a] The polymerization was stopped after 20 h by exposure to air and addition of non-deoxygenated buffer solution. Polymerizations were performed using the THS nanoreactor in its apo state, that is, in the absence of ATP. In this state both lids of the protein cage are open.[15,17] Thus, any macromolecules that are synthesized within the THS can leave the cavities after polymerization. Poly(*N*-isopropyl acrylamide) (pNiPAAm) was separated from the protein cage by size-exclusion spin centrifugation, leaving the THS in the supernatant while the polymer could be collected from the flow-through. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) of the latter showed a series of signals spaced $\Delta(m/z)$ 113.2, with a number average molecular weight (M_n) of 1500 g mol^{-1} and a polydispersity index (PDI) of 1.11 (Figure 2a). Since the molecular weight of the monomer is 113.16 g mol^{-1}, these data show that the reaction yielded pNiPAAm. The low PDI indicates that the polymerization proceeded with a good degree of control. A detailed analysis of the peaks is shown in Figure S4.

To set our findings into perspective, polymerizations were conducted under the same conditions, but with the ATRP

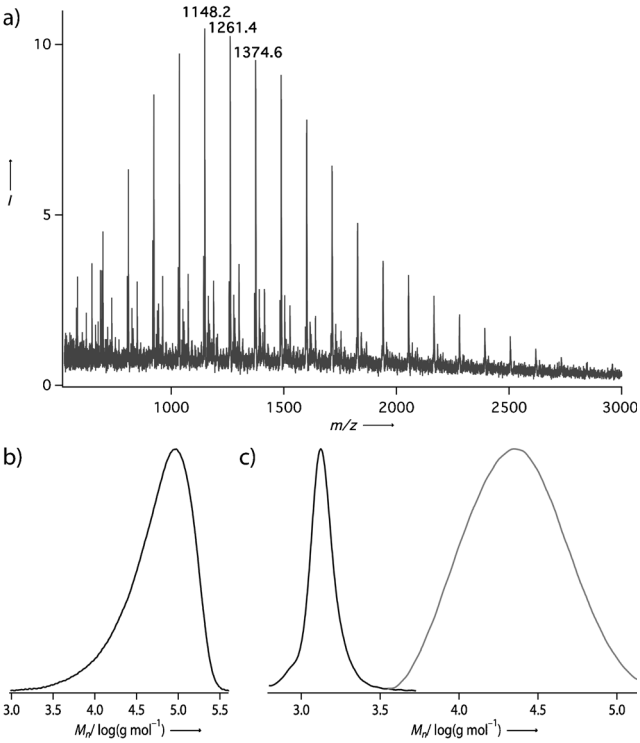


Figure 2. Characterization of pNiPAAm and pPEGA synthesized with THS-L_xCu and BSA-L_xCu. a) MALDI-ToF MS data of pNiPAAm synthesized with THS-L_xCu in aqueous buffer. b) GPC trace of pNiPAAm synthesized by BSA-L_xCu in aqueous buffer. c) Comparison of GPC traces from pPEGA synthesized with THS-L_xCu (black) or with BSA-L_xCu (gray) in a water/THF (7:3 *v*/*v*) mixture.

catalyst conjugated to the outside of the globular protein bovine serum albumin (BSA-L_xCu; characterization see Figure S5). ARGET ATRP with BSA-L_xCu yielded pNiPAAm with an apparent molecular weight M_n of 42 600 g mol^{-1} (calibrated against poly(methyl methacrylate)) and a PDI of 1.94 as determined by gel permeation chromatography (GPC; Figure 2b). By comparing the two different catalytic systems (THS as protein nanoreactor versus the globular protein BSA) we draw the conclusion that the synthesized polymers are substantially shorter and have a lower PDI if produced in the nanoreactor. A reason for the broad molecular-weight distribution of polymers synthesized with BSA-L_xCu may be the very low concentration of catalyst in the reaction. This can be neglected in the THS since the growing polymer chain stays in close proximity to the catalyst inside the nanoreactor. Therefore, the synthesis of pNiPAAm, which is intrinsically difficult to control in aqueous solution,[24] benefits from the confined reaction space. The lower M_n of polymers synthesized in the nanoreactor could be due to diffusion limitations of the monomer into the protein cage, due to space constrictions of the growing chain within the cavities, or due to a less probable reactivation of a dormant chain once it has diffused out of the nanoreactor.

The scope of THS as nanoreactor for polymerizations was further explored by conducting the experiments with a different monomer. Poly(ethylene glycol) methyl ether acrylate (PEGA) with a number average molecular weight of

480 g mol^{-1} was polymerized with THS-L$_x$Cu under the same ARGET ATRP conditions used for pNiPAAm, yielding pPEGA with a poly(ethylene oxide)-apparent M_n of 14700 g mol^{-1} and a PDI of 1.95, as analyzed by GPC (reaction scheme in Figure S3). In comparison, the BSA-conjugated catalyst produced pPEGA with apparent M_n of 119400 g mol^{-1} and a PDI of 3.12. In addition, the polymerization of PEGA with THS-L$_x$Cu was run in the presence of ATP, which can cause the main pores of THS to cycle between open and closed states.[17] The reaction resulted in pPEGA with an apparent M_n of 17500 g mol^{-1} and a PDI of 2.50. The addition of ATP had no beneficial effect on the polymerization, possibly because hydrolysis of ATP by THS is too slow at room temperature, that is, at the reaction temperature of the polymerizations.[28]

Addition of organic cosolvents to aqueous ATRP can result in better performance of a given catalyst, since side reactions, for example, disproportionation of the ATRP activator, loss of the halide ligand by the ATRP deactivator as well as fast chain propagation are suppressed, thus resulting in better-controlled polymerizations.[19b] Therefore, PEGA was polymerized in a 7:3 (*v*/*v*) mixture of water and THF, by using THS-L$_x$Cu as catalyst (see Figure S3). The product of this experiment resulted in an apparent M_n of 1400 g mol^{-1} and a PDI of 1.06 (Figure 2c). BSA-L$_x$Cu yielded pPEGA with an apparent M_n of 14900 g mol^{-1} and a PDI of 1.84. The PDI and the average molecular weight of pPEGA synthesized in the presence of organic cosolvent are lower than the ones obtained without cosolvents. In addition, these experiments confirm that reactions carried out in nanoreactors yield polymers with a narrower distribution of molecular weight.

For the ATRP to be confined into the THS, the protein cage has to be stable during the polymerization. The most harsh reaction condition was the one with THF as cosolvent. It was therefore assessed whether or not the THS retained its structure in a typical PEGA polymerization in the water/THF mixture. After the reaction, SDS and native PAGE (Figure 3a, b) show the distinct bands of the two subunits and of the fully assembled THS. Moreover, TEM (Figure 3c) indicates that the protein cage remained intact. Thus, the protein cage was stable in this reaction.

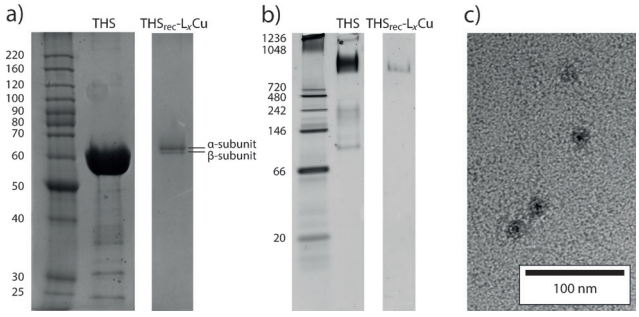


Figure 3. Characterization of THS-L$_x$Cu after the polymerization in a water/THF (7:3 *v*/*v*) mixture. a) SDS gel electrophoresis, b) native gel electrophoresis, and c) TEM images of THS-L$_x$Cu recovered after polymerization (THS$_{rec}$-L$_x$Cu). These data allow the conclusion that THS remained stable during polymerization and retained its structure.

A set of control reactions was carried out to ensure that only the Cu–TEDETA complex in the THS bears copper that is catalytically active in ATRP. Nonmodified THS was incubated with $CuBr_2$ solution. Then, size-exclusion spin centrifugation was used to remove the unbound copper ions. The resulting THS was used to polymerize NiPAAm and PEGA under the conditions described above. The reactions did not yield polymer products as checked by ^{1}H NMR and GPC. These experiments, in combination with the SAXS data discussed above (Figure S1), indicate that copper ions are not complexed by the protein. Further, we assessed whether the bisaryl hydrazone linker could complex copper ions in an ATRP-active form. For this purpose an analogue of the BSA-L$_x$ was produced that did bear the linker, but no TEDETA ligand (Figure S6). The modified protein BSA-4FB was mixed with $CuBr_2$ solution. After size-exclusion spin centrifugation it was used as catalyst for ARGET ATRP of NiPAAm with the same reaction conditions as described above. The polymerization with BSA-4FB did not yield polymer as checked by ^{1}H NMR and GPC. This control reaction with BSA-4FB indicates that, even though copper ions might be complexed by the linker, only TEDETA in combination with copper ions results in complexes that are able to catalyze ATRP. This underscores the necessity for conjugating a suitable Cu-binding ligand into the THS to obtain an ATRP catalyst inside of the protein cage.

In conclusion, we introduced the chaperonin THS as a nanoreactor and conducted ATRP in the protein cage by binding an ATRP catalyst to the inner cavities of the protein. To our knowledge, this is the first reported protein–catalyst conjugate for ATRP. THS is particularly appealing for this application because it has pores that are large enough to allow macromolecules to enter and leave the cavities. The synthesized polymers are therefore released into the surrounding solution without having to disassemble the protein cage into its subunits. We showed that the confined space within the nanoreactor results in polymers with narrow molecular weight distributions. Such a positive effect on the polymerization is most probably due to the proximity of the ATRP reagents and is consistent with simulations of other nanoreactor systems.[25b] Protein nanoreactors could influence polymerizations in some unique ways, for example, by imparting selectivity for certain monomers, or by modulating the rate of the polymerization through triggers that close or open pores in the protein wall. As chaperonins are the folding chambers of nature, they could beneficially affect the folding of (block)copolymers when sequestered single polymer chains are synthesized within their cavities. In addition, the use of protein nanoreactors could enable mechanistic studies of ATRP on a single-molecule level.

Received: August 3, 2013
Revised: September 18, 2013
Published online: December 20, 2013

Keywords: chaperone proteins · controlled/living radical polymerization · nanoreactor · polymerization · thermosome

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