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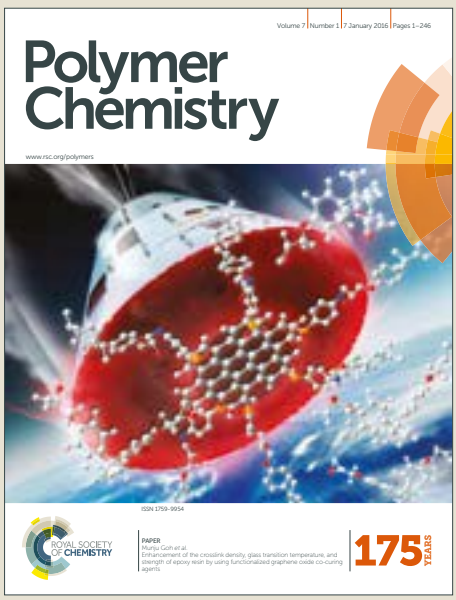

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Biocatalytic Atom Transfer Radical Polymerization in a Protein Cage Nanoreactor†

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Incorporation of the ATRP-catalyzing enzyme horseradish peroxidase (HRP) into the cavities of the group II chaperonin thermosome is demonstrated. The resulting nanoreactor was used to polymerize an acrylate under ARGET ATRP conditions. The confined space within the protein cage results in poly(ethylene glycol) methyl ether acrylate (PEGA) with lower molecular weights (poly(styrene)-apparent M_n = 4400 g mol^{-1}) as well as narrower molecular weight distributions ($Đ$ = 1.08) compared to polymerizations with the free ATRPase (M_n = 43700 g mol^{-1} and $Đ$ of 1.23).

Nanoreactors, such as protein cages,[1-10] lipid and polymer vesicles,[4,11-17] nanostructured polymer networks,[18] and hollow inorganic structures[19] present a unique opportunity to confine chemical reactions into compartments with yocto liter volumes (10^{-24} L). There they can be studied and influenced in ways that are not possible in free solution.[1-4,20] In particular, nanoreactors allow for a close proximity of catalyst and substrates and therefore enhanced reaction rates,[21] as well as diminished side reactions.[22] The shell of the reactor can impart size- or chemo-selectivity to a catalytic reaction.[23-26] Moreover, the reaction chamber itself can define the shape and the size of the synthesized molecules[27,28] and can enable reactions in untypical reaction environments, e.g. biotransformations in non-aqueous solvents[18] or in living cells.[29] Furthermore, nanoreactors have opened-up ways to study reaction mechanisms and catalysis on a molecular level.[30,31]

The protein cage thermosome (THS), a group II chaperonin from the archaea *Thermoplasma acidophilum*, is a hollow nanostructure of approx. 16 nm diameter in its fully closed conformation. Eight α and eight β subunits form two stacked rings that enclose two cavities. They are large enough to accommodate proteins of up to 50 kDa.[32] The THS can take up macromolecules into its cavities through large gated pores at the apex of each hemisphere. The pores are approx. 5.4 nm in diameter when they are open.[32] In its open conformation the THS diameter increases to up to 18 nm.[33] The native function of THS is to provide a folding chamber for partially unfolded proteins as part of a heat shock response. Because of the ability of THS to take up and release macromolecules, it is an intriguing structure in nanotechnology.[34-36] Recently, we used THS as nanoreactor for Cu(I)-catalyzed atom transfer radical polymerization (ATRP). To this end, a cupper complex was conjugated into the THS and *N*-isopropyl-acrylamide (NiPAAm) and poly(ethylene glycol) methyl ether acrylate (PEGA) were polymerized within the cage.[35] The effect of the confined reaction space manifested itself in smaller but more narrowly dispersed polymer products compared to polymers synthesized under comparable conditions with a catalyst that was conjugated to a globular protein.

We[37-40] and others[41-45] demonstrated that metalloproteins such as horseradish peroxidase (HRP),[37,39,42,44] hemoglobin,[38,45] catalase[42-44] and laccase[40-42,44] can catalyze ATRP of vinyl-type monomers under activators regenerated by electron transfer (ARGET) ATRP conditions. Thus, conventional catalysts for ATRP[46-49] can be replaced by non-toxic biological catalysts. Moreover, such ATRPases are able to polymerize monomers that can otherwise not be polymerized in a controlled way by ATRP, as demonstrated for *N*-vinylimidazole.[40] After the reaction, the biomolecules can be quantitatively removed from the polymers by easy means, allowing to produce polymers that do not contain residual traces of metal ions.[40]

Here, we aim to create an all-protein nanoreactor system for ATRP. To this end, we encapsulated HRP into the thermosome by covalent conjugation of the enzyme to the inner wall of the

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† Electronic Supplementary Information (ESI) available: Additional experimental details, including polymerization protocols, THS-HRP conjugation, UV/Vis data, and SDS-PAGE of THS-HRP. See DOI: 10.1039/x0xx00000x

THS. By combining the advantages of the THS nanoreactor with the functionalities of ATRPases, we aim to investigate the effect of confined space on ATRP-catalyzing enzymes.

A mutant of THS was used (K316C of the β-subunit and surface-exposed cysteines of all subunit replaced by alanines), because it features four cysteine residues as attachment points in the cavity of each hemisphere.[34,35] The THS was expressed in *E. coli* as described earlier.[34] Figure 1a shows the strategy to covalently bind the guest HRP into the cavities of the THS. In a first step, the cysteines on the β-subunits of the THS were modified with the heterobifunctional linker maleimido trioxa-6-formyl benzamide (MTFB) to introduce an aromatic aldehyde. In parallel, the HRP was modified via its lysines with succinimidyl 6-hydrazino-nicotinamide (HyNic), a heterobifunctional linker that introduced hydrazine residues to the surface of the enzyme. The modified THS and the modified HRP were coupled in a simple incubation step by formation of a pH- and temperature stable bisaryl hydrazone bond. To this end, a 25-fold excess of HRP was mixed with THS and incubated for 14 h at room temperature. The host-guest protein conjugate was purified by size-exclusion chromatography that separated the unreacted HRP from the THS-HRP.

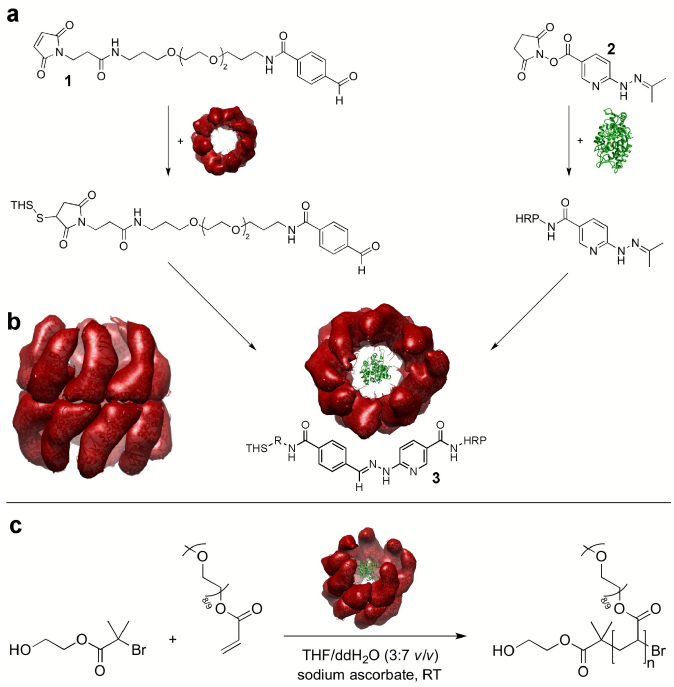


Fig. 1 Conjugation strategy to covalently bind HRP into the cavities of THS and reaction scheme for an THS-HRP-catalysed ATRP. a) Free cysteines in THS (red) were modified with MTFB (**1**). Lysines of HRP (green) were modified with HyNic (**2**). The modified protein cage and the modified enzyme were reacted to form THS-HRP conjugates via bisaryl hydrazone bonds (**3**). b) Side view of a chaperonin in its open confirmation. c) Reaction scheme of the polymerization of PEGA using THS-HRP as the catalyst under ARGET ATRP conditions.

The successful conjugation was confirmed by UV/Vis spectroscopy (Figure 2a). The spectrum shows three distinct bands corresponding to THS and HRP (280 nm), the bisaryl hydrazone linker (354 nm) and the Soret band of HRP (402 nm). These bands allow to calculate that 2.0 ± 0.2 HyNic-MTFB linkers formed per THS and that an average of 0.4 ± 0.1 HRP bound in each THS. Thus, not every THS cavity is filled with HRP. This is not surprising, because the THS adopts a mixture of fully closed, bullet-shaped (i.e. one hemisphere closed, the other one open) and fully open conformations when obtained from fermentations.[33,50,51] Only open hemispheres can take up proteins from solution. For comparison, the UV/Vis spectra of THS alone and of a conjugate of THS with a non-heme enzyme are shown in Figure 2a and Supporting Figure S1. The bis-aryl hydrazone bond at 354 nm is only present if an enzyme is encapsulated into the THS. The Soret band of HRP is only visible when HRP is encapsulated.

In addition, small angle X-ray scattering (SAXS) confirms that the HRP is located inside the THS (Figure 2b). The broad peak at 0.06 $Å^{-1}$ is a signature of the hollow spherical protein and the thickness of its protein shell. The empty THS has a pronounced peak, due to the good electron contrast between the shell and the water-filled interior, indicating a hollow spherical structure. The peak of THS-HRP is less pronounced, which indicates a reduced electron contrast. This is caused by the HRP which has a similar electron density than the THS and partially fills the hollow protein. The SAXS data resulted in diameters of 17.8 nm (THS) and 17.2 nm (THS-HRP) that are in good agreement with the reported size for the open conformation of THS[33] and indicate that HRP is bound within and not to the outside of the cage.

Transmission electron microscopy (TEM) images of THS-HRP display the typical hexadecameric structure of THS[33] showing that the protein cage remained stable after formation of THS-HRP conjugate (Figure 2c). A sodium dodecyl sulfate polyacrylamide electrophoresis gel of the purified THS-HRP (see Figure S2) does not show a band of free HRP, which indicates that the excess of HRP was quantitatively removed during purification of the conjugate.

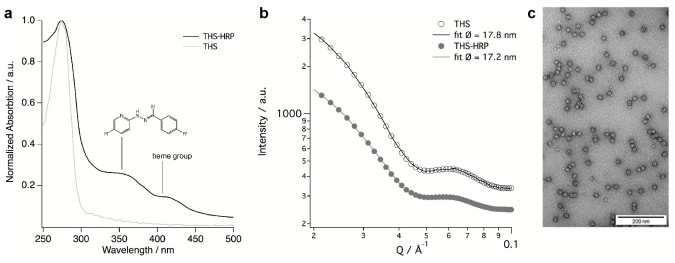


Fig. 2 Characterization of THS-HRP. a) UV/Vis spectra of THS-HRP and of unfunctionalized THS. b) SAXS measurements of empty THS and THS-HRP. For a better comparison curves were shifted in intensity. c) TEM image of THS-HRP.

The THS-HRP nanoreactors were used to polymerize the monomer PEGA (reaction scheme see Figure 1c). A 7:3 *v/v* mixture of water and THF was selected as reaction media for the polymerization because aqueous ATRP reactions tend to proceed with a better degree of control when conducted in mixtures of water and organic co-solvents.[35,48,52] 2-Hydroxyethyl-2-bromoisobutyrate (HEBIB) was used as the initiator and sodium ascorbate as the reducing agent. The molar ratio of the reagents and the biocatalyst was [Monomer]:[Initiator]:[NaAsc]:[THS-HRP] 67:1:0.12:3.4*10^{-4} (only the concentration of HRP is taken into account for the

ratio) in a total reaction volume of 1.9 ml. Polymerizations were carried out under an argon atmosphere. They were stopped after 20 h (conversion of 6% as determined by ^{1}H-NMR) by addition of 550 µl non-deoxygenated water and exposure to ambient air. Gel permeation chromatography (GPC) revealed poly[poly(ethylene glycol) methyl ether acrylate] (pPEGA) with a poly(styrene)-apparent number-average molecular weight (M_n) of 4400 g mol^{-1} and a dispersity ($Đ$) of 1.08 (Figure 3).

To assess the effect of the confined space in the THS nanoreactor, ARGET ATRP polymerizations were also performed with free HRP. ^{1}H-NMR showed a conversion of 18%, and GPC revealed pPEGA with a poly(styrene)-apparent molecular weight M_n of 43700 g mol^{-1} and Đ = 1.23 (Figure 3). The comparison of the THS-HRP nanoreactor versus the free ATRPase implies that the resulting polymers are significantly shorter and are more narrowly dispersed if synthesized in the nanoreactor. This result may be caused by the close proximity of the enzyme and the growing polymer chain inside the nanoreactor. Furthermore, diffusion limitations of monomers and dormant polymer chains into the nanoreactor may explain the smaller polymer chains and the lower conversion that resulted from the polymerization in the nanoreactors.

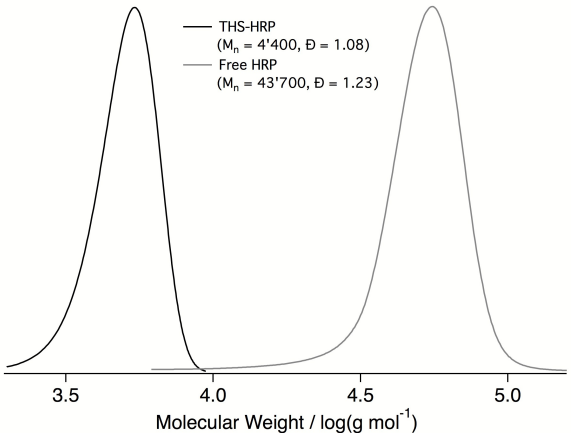


Fig. 3 GPC traces of pPEGA synthesized by biocatalytic ARGET ATRP with THS-HRP (black) and with free HRP (gray) in a water/THF (7:3 *v/v*) mixture.

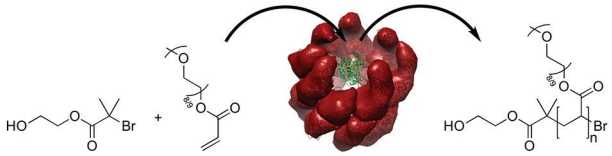
Concluding, we demonstrated the incorporation of the ATRPase horseradish peroxidase into the cavities of the chaperonin thermosome. The resulting all-protein nanoreactor was used to polymerize PEGA under ARGET ATRP conditions in a mixture of 7:3 *v/v* water and THF. The enzyme produced shorter and more narrowly dispersed polymers in the nanoreactor than in solution. These results are consistent with our previous reports on ATRP within THS catalyst by copper complexes,[35] and with simulations of other nanoreactor systems.[53] Replacing conventional Cu-catalysts with enzymes allows for heavy metal free products[40] and therefore renders the resulting polymers more compatible with biomedical, food grade and electronic device requirements. Moreover, this study paves the way to use THS and other protein cages to modify enzymatic polymerizations, e.g. by providing size or substrate selectivity through selective or gated pores in the protein shell.

This research was supported by the Swiss National Science Foundation (200021_140693, P2BSP2_151900, PP00P2_144697), by a Marie Curie Intra European Fellowship and a Marie Curie European Reintegration Grant, by the Holcim Stiftung Wissen and by the Swiss Nanoscience Institute. We thank W. Meier (Department of Chemistry, University of Basel) for his support, Vesna Oliveri (ZMB, University of Basel) for TEM expertise and E. Konishcheva for her help with GPC.

References

1 M. Uchida, M. T. Klem, M. Allen, P. Suci, M. Flenniken, E. Gillitzer, Z. Varpness, L. O. Liepold, M. Young and T. Douglas, *Adv. Mater.*, 2007, **19**, 1025–1042.
2 A. de la Escosura, R. J. M. Nolte and J. J. L. M. Cornelissen, *J. Mater. Chem.*, 2009, **19**, 2274–2278.
3 K. T. Kim, S. A. Meeuwissen, R. J. M. Nolte and J. C. M. van Hest, *Nanoscale*, 2010, **2**, 844–858.
4 K. Renggli, P. Baumann, K. Langowska, O. Onaca, N. Bruns and W. Meier, *Adv. Funct. Mater.*, 2011, **21**, 1241–1259.
5 M. Rother, M. G. Nussbaumer, K. Renggli and N. Bruns, *Chem. Soc. Rev.*, 2016, **45**, 6213–6249.
6 S. Abe, K. Hirata, T. Ueno, K. Morino, N. Shimizu, M. Yamamoto, M. Takata, E. Yashima and Y. Watanabe, *J. Am. Chem. Soc.*, 2009, **131**, 6958–6960.
7 D. P. Patterson, B. Schwarz, K. El-Boubbou, J. van der Oost, P. E. Prevelige and T. Douglas, *Soft Matter*, 2012, **8**, 10158–10166.
8 J. Lucon, S. Qazi, M. Uchida, G. J. Bedwell, Ben LaFrance, P. E. Prevelige Jr and T. Douglas, *Nat. Chem.*, 2012, **4**, 781–788.
9 M. L. Hovlid, J. L. Lau, K. Breitenkamp, C. J. Higginson, B. Laufer, M. Manchester and M. G. Finn, *ACS Nano*, 2014, **8**, 8003–8014.
10 R. Frey, T. Hayashi and D. Hilvert, *Chem. Commun.*, 2016, **52**, 10423–10426.
11 A. Jesorka and O. Orwar, *Annu. Rev. Anal. Chem.*, 2008, **1**, 801–832.
12 R. J. R. W. Peters, I. Louzao and J. C. M. van Hest, *Chem. Sci.*, 2012, **3**, 335–342.
13 A. Lu and R. K. O'Reilly, *Curr. Opin. Biotechnol.*, 2012, **24**, 1–7.
14 J. Gaitzsch, D. Appelhans, L. Wang, G. Battaglia and B. Voit, *Angew. Chem. Int. Ed.*, 2012, **51**, 4448–4451.
15 M. Spulber, A. Najer, K. Winkelbach, O. Glaied, M. Waser, U. Pieles, W. Meier and N. Bruns, *J. Am. Chem. Soc.*, 2013, **135**, 9204–9212.
16 J. Gaitzsch, X. Huang and B. Voit, *Chem. Rev.*, 2016, **116**, 1053–1093.
17 K. T. Kim, J. J. L. M. Cornelissen, R. J. M. Nolte and J. C. M. van Hest, *Adv. Mater.*, 2009, **21**, 2787–2791.
18 N. Bruns and J. C. Tiller, *Nano Lett.*, 2005, **5**, 45–48.
19 G. Prieto, H. Tüysüz, N. Duyckaerts, J. Knossalla, G.-H. Wang and F. Schüth, *Chem. Rev.*, 2016, **116**, 14056–14119.
20 M. Marguet, C. Bonduelle and S. Lecommandoux, *Chem. Soc. Rev.*, 2013, **42**, 512–529.
21 P. Cotanda, N. Petzetakis and R. K. O'Reilly, *MRS Commun.*, 2012, **2**, 119–126.
22 B. Helms, S. J. Guillaudeu, Y. Xie, M. McMurdo, C. J. Hawker and J. M. J. Fréchet, *Angew. Chem.*, 2005, **117**, 6542–6545.
23 T. Ueno, M. Suzuki, T. Goto, T. Matsumoto, K. Nagayama and Y. Watanabe, *Angew. Chem.*, 2004, **116**, 2581–2584.
24 Z.-A. Qiao, P. Zhang, S.-H. Chai, M. Chi, G. M. Veith, N. C.

Gallego, M. Kidder and S. Dai, *J. Am. Chem. Soc.*, 2014, **136**, 11260–11263.
25 A. Liu, C. H. H. Traulsen and J. J. L. M. Cornelissen, *ACS Catal.*, 2016, **6**, 3084–3091.
26 I. Gitsov, L. Wang, N. Vladimirov, A. Simonyan, D. J. Kiemle and A. Schutz, *Biomacromolecules*, 2014, **15**, 4082–4095.
27 M. Okuda, Y. Suzumoto, K. Iwahori, S. Kang, M. Uchida, T. Douglas and I. Yamashita, *Chem. Commun.*, 2010, **46**, 8797–8799.
28 R. McHale, J. P. Patterson, P. B. Zetterlund and R. K. O'Reilly, *Nat. Chem.*, 2012, **4**, 491–497.
29 P. Tanner, V. Balasubramanian and C. G. Palivan, *Nano Lett.*, 2013, **13**, 2875–2883.
30 S.-H. Shin and H. Bayley, *J. Am. Chem. Soc.*, 2005, **127**, 10462–10463.
31 M. Comellas Aragonès, H. Engelkamp, V. I. Claessen, N. A. J. M. Sommerdijk, A. E. Rowan, P. C. M. Christianen, J. C. Maan, B. J. M. Verduin, J. J. L. M. Cornelissen and R. J. M. Nolte, *Nat. Nanotechnol.*, 2007, **2**, 635–639.
32 L. Ditzel, J. Lowe, D. Stock, K. O. Stetter, H. Huber, R. Huber and S. Steinbacher, *Cell*, 1998, **93**, 125–138.
33 M. Nitsch, J. Walz, D. Typke, M. Klumpp, L. O. Essen and W. Baumeister, *Nat. Struct. Mol. Biol.*, 1998, **5**, 855–857.
34 N. Bruns, K. Pustelny, L. M. Bergeron, T. A. Whitehead and D. S. Clark, *Angew. Chem. Int. Ed.*, 2009, **48**, 5666–5669.
35 K. Renggli, M. G. Nussbaumer, R. Urbani, T. Pfohl and N. Bruns, *Angew. Chem. Int. Ed.*, 2014, **53**, 1443–1447.
36 M. G. Nussbaumer, J. T. Duskey, M. Rother, K. Renggli, M. Chami and N. Bruns, *Adv. Sci.*, 2016, **3**, 1600046.
37 S. J. Sigg, F. Seidi, K. Renggli, T. B. Silva, G. Kali and N. Bruns, *Macromol. Rapid Commun.*, 2011, **32**, 1710–1715.
38 T. B. Silva, M. Spulber, M. K. Kocik, F. Seidi, H. Charan, M. Rother, S. J. Sigg, K. Renggli, G. Kali and N. Bruns, *Biomacromolecules*, 2013, **14**, 2703–2712.
39 M. V. Dinu, M. Spulber, K. Renggli, D. Wu, C. A. Monnier, A. Petri-Fink and N. Bruns, *Macromol. Rapid Commun.*, 2015, **36**, 507–514.
40 C. Fodor, B. Gajewska, O. Rifaie-Graham, E. A. Apebende, J. Pollard and N. Bruns, *Polym. Chem.*, 2016, **7**, 6617–6625.
41 Y.-H. Ng, F. di Lena and C. L. L. Chai, *Polym. Chem.*, 2011, **2**, 589–594.
42 Y.-H. Ng, F. di Lena and C. L. L. Chai, *Chem. Commun.*, 2011, **47**, 6464–6466.
43 A. Simakova, M. Mackenzie, S. E. Averick, S. Park and K. Matyjaszewski, *Angew. Chem. Int. Ed. Engl.*, 2013, **52**, 12148–12151.
44 G. Gao, M. A. Karaaslan, J. F. Kadla and F. Ko, *Green. Chem.*, 2014, **16**, 3890–3898.
45 Y. Sun, H. Du, Y. Lan, W. Wang, Y. Liang, C. Feng and M. Yang, *Biosens. Bioelectron.*, 2016, **77**, 894–900.
46 W. A. Braunecker and K. Matyjaszewski, *Prog. Polym. Sci.*, 2007, **32**, 93–146.
47 M. Ouchi, T. Terashima and M. Sawamoto, *Chem. Rev.*, 2009, **109**, 4963–5050.
48 K. Matyjaszewski, *Macromolecules*, 2012, **45**, 4015–4039.
49 A. Simakova, S. E. Averick, D. Konkolewicz and K. Matyjaszewski, *Macromolecules*, 2012, **45**, 6371–6379.
50 T. Waldmann, E. Nimmesgern, M. Nitsch, J. Peters, G. Pfeifer, S. Müller, J. Kellermann, A. Engel, F. U. Hartl and W. Baumeister, *Eur. J. Biochem.*, 1995, **227**, 848–856.
51 I. Gutsche, O. Mihalache, R. Hegerl, D. Typke and W. Baumeister, *FEBS Lett.*, 2000, **477**, 278–282.
52 M. Fantin, A. A. Isse, A. Gennaro and K. Matyjaszewski, *Macromolecules*, 2015, **48**, 6862–6875.
53 P. B. Zetterlund, *Polym. Chem.*, 2011, **2**, 534–549.



ATRP-catalyzing enzyme horseradish peroxidase was encapsulated into the protein cage thermosome resulting in an all-protein nanoreactor system for controlled radical polymerizations