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Visible light induced fast synthesis of protein–polymer conjugates: controllable polymerization and protein activity†

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Herein visible light is used to induce RAFT polymerization from protein for preparing protein–polymer conjugates at ambient temperature. Polymerization is fast and can be conveniently controlled with irradiation time. By site-specific polymerization of NIPAm to protein, the protein activity is maintained and in certain cases it presents an efficient on–off-switchable property.

Proteins and peptides play an essential role in both highly functional and structural biological applications. An ever increasing number of therapeutic drugs in development and site specific delivery agents are protein/peptide based. As bio-macromolecules with a very precise structure and conformation, the control of activity and stability of proteins is the key for their applications. Often therapeutic peptides and proteins are administered by intravenous methods directly into the blood so as to avoid complications from digestion *via* action of proteases. Even when delivered in this direct fashion their lifetime in the body is often low due to renal filtration and degradation *via* interactions with the immune system. Conjugation of synthetic polymers with proteins/peptides is a commonly employed method to improve protein efficacy by increasing lifetimes *in vivo* and decreasing immunogenicity. However, this often results in reduced bioactivity and there is an ever increasing need for conjugation to be site specific where interference with the active site is at a minimum. In addition, there is increasing interest in the conjugation with stimuli responsive polymers where the activity of protein can be turned "on" and "off" under appropriate conditions.[1] There are two approaches for protein conjugation – "grafting to" and "grafting from". For example, Hoffman and coworkers successfully prepared a variety of responsive protein–polymer conjugates *via* "grafting to" methods which involve the reaction of a polymer that contains a suitable and complementary functional group for reaction at an amino acid residue such as lysine, tyrosine or cysteine.[2] However, these reactions are often inefficient leading to the requirement of the use of an excess of functional polymers in the reaction. This produces a mixture of conjugates and excess polymers not linked with the protein which can lead to difficult, time consuming and often expensive separation techniques being required in this "grafting to" approach. The "grafting from" strategy is promising as it can simplify the purification as a low molecular weight unreacted monomer is often more easily removed from the bioconjugate and also the steric limitations associated with coupling the two large macromolecules, the polymer and protein, are reduced.[3] The preparation of conjugates *via* "grafting from" has been significantly aided by the progress of living radical polymerization techniques. Among them, atom transfer radical polymerization (ATRP) has been successfully employed for grafting polymers from proteins with[4] and without sacrificing initiators.[5] However, the introduction of copper ions can introduce concerns regarding cytotoxicity, as reported when comparing polymers prepared by ATRP and reversible addition-fragmentation chain transfer polymerization (RAFT).[6] RAFT, without using metal catalysts, became more popular for making protein–polymer conjugates *via* a "grafting from" approach, where proteins are modified with the Z group of chain transfer agents (CTA)[7] or the R group.[3b,8] Although traditional RAFT was carried out under mild conditions for preparing protein conjugates, polymerization from proteins often requires unsuitable long reaction times, which is not always ideal for maintaining the bioactivity. Visible light induced RAFT came to our mind, as it can lead to well controlled polymers at acceptably low reaction temperatures with very acceptable fast polymerization rates.[9] Thus, the ability to prepare protein conjugates *via* visible light induced RAFT in aqueous media at temperatures below 30 °C with an understanding of how this influences protein activity is important with potential in many therapeutic areas.

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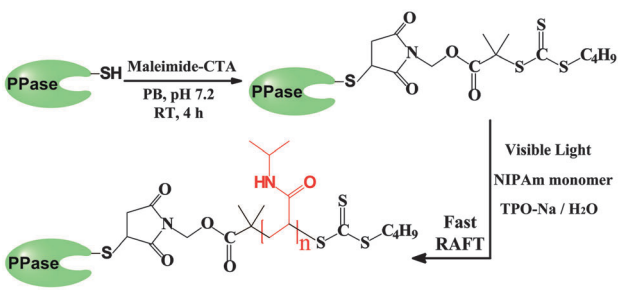
Site-specific attachment of stimuli responsive polymers near the active site can provide a sensitive and quite precise environmental control of the protein activity.[2c] Escherichia coli inorganic pyrophosphatase (PPase) is an important protein that provides energy and phosphate substrates for certain biosynthesis reactions by catalyzing the hydrolysis of pyrophosphate.[10] There are only two Cys residues in the *E. coli* PPase, and both are buried within the three-dimensional protein structure and show no significant response to changes in the redox state of the environment. Therefore, here we used site-directed mutagenesis to introduce a Cys residue into the specific Lys148 site, which is within a conserved sequence near the active site and is exposed to the surface of the PPase. Site-specific modified PPase was functionalized with a RAFT agent and subsequently used as a macro-CTA. Visible light induced RAFT polymerization of *N*-isopropylacrylamide (NIPAm) was performed and the activity of the protein–polymer conjugate was evaluated at different temperatures (Scheme 1).

The maleimide functional chain transfer agent was immobilized to PPase *via* its R-group to afford PPase-macroCTA, and the R-group approach is preferred compared to the Z-group approach and the trithiocarbonate moiety is readily accessible for RAFT chain transfer with propagating chains in solution.[8a] Polymerization was carried out in H_2O at 20 °C, using (2,4,6-trimethylbenzoyl)phenyl phosphonic acid sodium (TPO-Na) as a photoinitiator under visible light radiation at $\lambda = 420$ nm with a mild intensity of 0.2 mW cm^{-2}. The reaction was monitored using aqueous GPC (Fig. 1(a)). The GPC traces clearly show that after 10 min, new conjugates are formed. The molecular weights of conjugates increased with radiation time (Fig. 1(b)) according to both GPC and NMR. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis confirmed the results obtained *via* GPC and NMR as shown in Fig. 2(a). The PPase-macroCTA mediated aqueous RAFT polymerization is rather fast and after 30 min radiation, the molecular weight of PNIPAm on the conjugates reaches about 150 kDa according to GPC. It should be noted that molecular weight obtained by GPC may not be precise due to the difference in samples and standards. By NMR, the molecular weight of PNIPAm after 30 minutes is ~40 kDa. Polymerization mediated by maleimide-CTA without protein has been carried out and it also proceeds very fast with a steady increase of molecular weight (Fig. S6, ESI†), indicating that light instead of protein is the key element for the fast polymerization. Control polymerization reactions by replacing PPase-macroCTA with PPase turned out to be uncontrollable and no polymers were conjugated to PPase (Fig. S10, ESI†).



Scheme 1 Visible light assisted fast synthesis of protein–polymer conjugates.

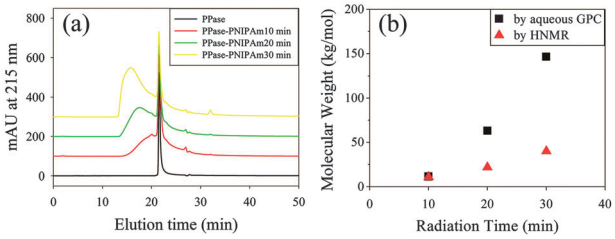


Fig. 1 Results of conjugates from visible light induced RAFT polymerization: (a) aqueous GPC traces for PPase and PPase–PNIPAm conjugates with different radiation time; (b) polymer molecular weight of PPase–PNIPAm conjugates calculated from standard samples by aqueous GPC (black) and ^{1}H NMR (red).

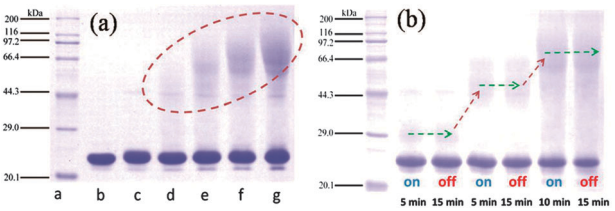


Fig. 2 (a) SDS-PAGE results of conjugates with different radiation time (lane a: molecular weight marker, lane b: PPase, lane c: PPase-macroCTA, lane d–g: 5 min/10 min/20 min/30 min radiation conjugates); (b) SDS-PAGE results with light-on–off periodically.

To investigate the effect of visible light on the polymerization, a periodic light-on–off switch process was employed (Fig. 2(b)). The light was periodically turned off for 15 minutes between each 5 or 10 minutes radiation. PAGE results of the obtained conjugates show that the polymerization did not occur during the light-off periods as the molecular weights of conjugates remained unchanged during the dark periods. After turning on the visible light, the molecular weight increased significantly indicating that the polymerization is turned on again with light. The periodic polymerization on–off process demonstrates that the visible light exerts good control over the PPase-macroCTA mediated aqueous RAFT polymerization. It indicates that the fast polymerization reactions occur not only through photo-initiation, but also by significantly activating the fragmentation of intermediate radicals. The on–off feature may also be desirable for making protein conjugates with polymers of multi-blocks or more complicated structures.

PPase-macroCTA mediated RAFT under visible light radiation can be carried out under mild conditions to afford protein–polymer conjugates in a fast and facile fashion. And the effect of the approach on the activity of protein is essential for its application. In addition, the ligand–protein recognition process and biological activity of proteins can be controlled by conjugating polymers near the active site.[11] PNIPAm is a widely used thermo-sensitive polymer with a lower critical solution temperature (LCST) = 32 °C, while due to the combination of PNIPAm, the obtained conjugates have an LCST = 35 °C (Fig. S7, ESI†), which is a little higher due to the hydrophilicity of protein. Here the effect and controllability on the activity of PPase–PNIPAm conjugates were investigated. PPase catalyzes the hydrolysis of inorganic

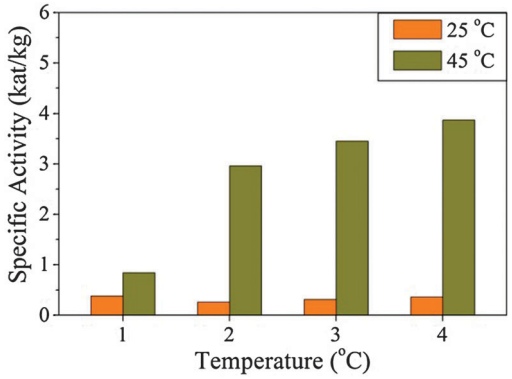


Fig. 3 Specific activity of (1) PPase-macroCTA, (2–4) PPase–PNIPAm conjugates with polymerization time 10 min/20 min/30 min after incubation at 25 or 45 °C for 10 min and measured at the same temperature.

pyrophosphate (PPi) into inorganic phosphate (Pi). Its catalytic mechanism primarily relies on the lysine, arginine, tyrosine, aspartic acid and glutamic acid residues in the active center. The PPase catalytic activity assay for the hydrolysis of sodium pyrophosphate were used to test the specific activity of conjugates. We first mixed PPase with free PNIPAm and found that the free polymer has no impact on PPase activity, no matter below or above LCST (Fig. S9, ESI†). By chemically attaching the CTA molecule or the polymer near the active site, the activity of macro-CTA and conjugate decreased to an extremely low level (approximate 0.3 kat kg^{-1}) at 25 °C (Fig. 3), as compared to that of original PPase (3.35 kat kg^{-1}, Table S1, ESI†) due to the change in the local environment (Fig. S11, ESI†). At 45 °C, a temperature above the LCST of the PPase–PNIPAm conjugate, the activity increased, but in different amounts. For PPase-macroCTA without a defined LCST, activity of the proteins increased only about twofold as the catalytic rate accelerated by temperature rise, which was consistent with the PPase sample. However, under the same conditions, the activity of conjugates increased about eleven fold. This indicates that in a collapsed state of PNIPAm at 45 °C, the polymer acts as a switch and the activity of PPase was turned on due to the change in the local environment. Meanwhile, it is interesting to find that above LCST the activity of conjugates with higher molecular weight PNIPAm presents higher specific activity, confirming the conclusion that the polymer molecular weight has an important influence on the conjugate activity.[2a,11a] Although the detailed mechanism of the activity control by attaching PNIPAm near the active site still needs further investigation, the effect of the visible light induced polymerization itself on the protein activity has been found to be negligible, and the protein activity can be switched by changing the temperature and can also be tuned by changing the molecular weight of conjugates, which can be easily obtained with varying polymerization time.

In conclusion, visible light assisted synthesis of protein–polymer conjugates *in situ* was carried out in a fast and controllable fashion at ambient temperature for the first time. The molecular weights of the conjugates increased with polymerization time, and in addition temporal control was achieved with the molecular weight increase turned on and off by controlling light radiation, which is desirable for making conjugates of polymers bearing multi-blocks and more complicated structures. By attaching PNIPAm site-specifically to the position near the active site of protein PPase the activity can be tuned by changing temperature and the molecular weight of conjugates. In certain cases, the activity is like being turned on-and-off between two temperatures.

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