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Fluoresent PEGylation Agent by Thiolactone-based One-pot Reaction: A New Strategy for Theranostic Combines

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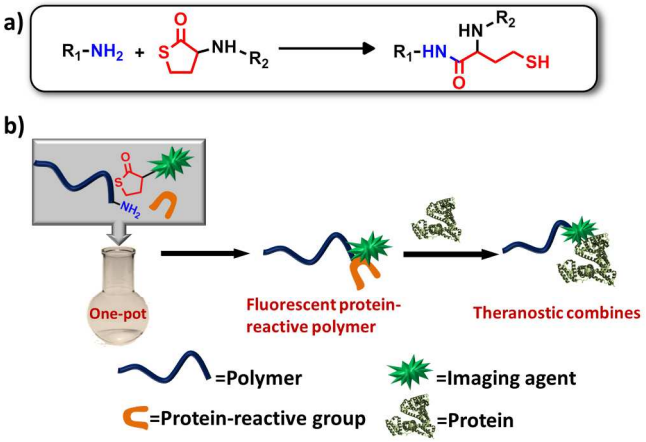
Yuan Zhao, Bin Yang, Yaling Zhang, Shiqi Wang, Changkui Fu, Yen Wei, Lei Tao*

Theranostic combines usually contain the imaging, the therapeutic and the cloaking components as a whole to simultaneously fulfil the diagnostic and therapy functions. As the upgraded PEGylation technology, a straightforward one-pot strategy based on the thiolactones ring-openning has been developed to facilely synthesize a mutifunctional PEGylation agent: fluorescent protein-reactive poly(ethylene glycol) (PEG), which can subsequently react with a model therapeutic protein to form a fluorescent PEGylated protein as a model of sophisticated theranostic combines.

Introduction

Proteins/peptides have become a major new class of phamaceutics since the early 1980s[1,2]. However, many proteins/peptide can be easily degraded by proteolytic digestion and rapidly cleaned through renal excretion, limiting their direct clinical application. PEGylation, a process to covalently attach poly(ethylene glycol) (PEG) on therapeutic proteins, can improve the safety and efficiency of those protein therapeutics. Through reducing immunogenicity, minimizing proteolytic cleavage, and increasing serum halflife, PEGylation can significantly increase drug efficacy and reduce injection frequency[3,4]. Therefore, PEGylation has greatly prompted the progress of protein drugs and gained remarkable achievement in both basic research[5-10] and markets[11,12]. For example, well-defined synthetic polymers as PEG analogues through atom transfer radical polymerization (ATRP)[13-15] and reversible addition fragmentation chain transfer (RAFT) polymerization[16-19] for protein conjugation are becoming the hot research topic in both polymer science and protein therapeutics areas[20,21].

With the rapid development of clinical medicine and the emerging concept of theranostic, PEGylation is facing new opportunities and challenges. The complexity of diseases makes it difficult to defeat a disease efficiently and selectively using diagnostic or therapy alone. Theranostic hence emerged as a new concept to simultaneously combine diagnostic imaging agents and therapeutic drugs within a single dose[22,23]. By this strategy, theranostic not only simplify the diagnosis and treatment processes, but also has the potential to overcome undesired incompatibility and unselectivity that currently exist between diagnostic imaging and therapeutic agents. Usually, typical theranostic combines should contain the imaging, the therapeutic and the cloaking components[24,25], following this principle, the PEG derivatives containing both imaging element and protein-reactive group for protein conjugation will offer not only protection, but also new function to the protein. Thus, the protein-polymer conjugates will be updated from single therapeutics to sophisticated theranostic combines[26-28], which will motivate the current PEGylation technology to a higher level.



Scheme 1. a) The thiolactone-based reaction to form an amide and a free thiol, b) the fluorescent protein-reactive polymer through one-pot thiolactone-based reaction and its conjugation to proteins.

By now, multifunctional PEG derivatives are normally prepared through laborious and time-consuming multi-step synthesis strategy, limiting the research and application of those multifunctional PEG agents and subsequent PEGylated proteins. How to efficiently and simply prepare multifunctional PEG containing both imaging group and therapeutic reactive group is still a practical challenge to polymer chemists. Recently, muticomponent reactions (MCRs)[29-34] and one-pot reactions[35] have been introduced into polymer science to prepare new multifunctional (co)polymers[36-38]. Compared with traditional step-by-step approaches, MCRs and one-pot reactions can easily incorporate new functions to synthesized polymers due to their multicomponent nature. For example, in the thiolactone–based one-pot reaction, the ring of thiolactone can be simply opened by an attacking amino to in situ generate an amide, and a free thiol for next reaction[35] **(Scheme 1a)**. This elegant one-pot reaction has been introduced by Filip Du Prez and coworkers into polymer science for polymer post-modification, sequence-defined oligomers synthesis,

polycondensation et al., demonstrating its potential for new multifunctional polymer preparation[40-45].

Inspired by abovementioned pioneer research, we attempt to employ this thiolactone-based reaction to facilely synthesize multifunctional polymer precursor which simultaneously contains imaging element, drug-reactive group and cloaking polymer for theranostic combines preparation. Briefly, in the presence of 2,2'-dithiodipyridine[46,47], a thiolactone derivative with a fluorescent moiety (a model fluorochrome) reacted with the amino terminated methoxy-poly(ethylene glycol) (mPEG-NH_2) to synchronously generate the fluorescent mPEG and a free thiol group, the new generated thiol group immediately reacted with 2,2'-dithiodipyridine in same reactor to finally form the desired multifunctional polymer: a PEG derivative containing both fluorescent and protein reactive groups at the chain end (**Scheme 1b**). The multifunctional PEG derivative through this convenient one pot method was subsequently used for protein conjugation using bovine serum albumin (BSA) as a model therapeutic protein. The obtained protein-polymer conjugate with imaging element (fluorescent group), cloaking polymer (PEG) and therapeutics (protein) can be recognized as a model of theranostic combines. Considered the simple operation and easily available starting materials, this thiolactone-based one pot strategy should be a general method to prepare sophisticated multifunctional polymers which might have potential applications in material science and biology fields.

Results and Discussion

One-pot synthesis of the fluorescent protein-reactive PEG derivative

5-(Dimethylamino)-N-(2-oxotetrahydrothiophen-3-yl) naphthalene-1-sulfonamide (dansyl thiolactone) was simply synthesized by the coupling reaction between dansyl choloride and DL-homocysteinethiolactone hydrochloride in the presence of triethylene amine (Experiment section, **ESI†**). Then, a one pot reaction system was constructed by mixing the dansyl thiolactone, the mPEG-NH_2 (M_{nNMR} ~ 5000 g/mol) in the presence of 2,2'-dithiodipyridine ([mPEG-NH_2]/[dansyl thiolactone]/[2,2'-dithiodipyridine] = 1/10/30, CH_2Cl_2 as solvent, 40 °C) (**Fig. 1a**).

Excess dansyl thiolactone and 2,2'-dithiodipyridine were added to reduce the possible coupling reaction between thiol terminated polymer intermediate, and ensure the complete reaction to generate target polymer. After 18 hours' reaction and simple precipitation in diethyl ether, the pure fluorescent protein-reactive PEG derivative could be easily obtained.

The multifunctional PEG derivative (mPEG-dansyl-PD) has an excitation wavelength at 331 nm and an emission wavelength at 544 nm (**Fig. 1b**), suggesting the incorporation of the fluorescent group in the polymer. Meanwhile, the gel permeation chromatography (GPC) analyses also reveal the polymer structure information. With a RID detector, both mPEG-NH_2 and mPEG-dansyl-PD showed curves at almost same position (**Fig. S1, ESI†**), but with an UV detector, the original mPEG-NH_2 has no absorption at 360 nm while mPEG-dansyl-PD still showed a peak (**Fig. 1c**), further confirming the fluorescent element has been successfully included in the polymer structure. From ^{1}H NMR spectrum (**Fig. 1d**), the signals of the fluorescent moiety (~7.23–8.43 ppm, the same position as the dansyl thiolactone, **Fig. S2, ESI†**) and the characteristic pyridine peak (~6.96 ppm) could be clearly observed. The integral ratio between the pyridine peak and the methyl group at the PEG chain end ($I_{6.96}/I_{3.38}$) was 0.31 and the integral ratio between the fluorescent peak (g) and the methyl group at the PEG chain end ($I_{8.32}/I_{3.38}$) was 0.32 (theoretical value: 0.33), indicating 94-97% modification of the polymer chain end. All these analytical data support the conclusion that the sophisticated multifunctional mPEG-dansyl-PD has been successfully prepared through this simple thiolactone-based one pot strategy.

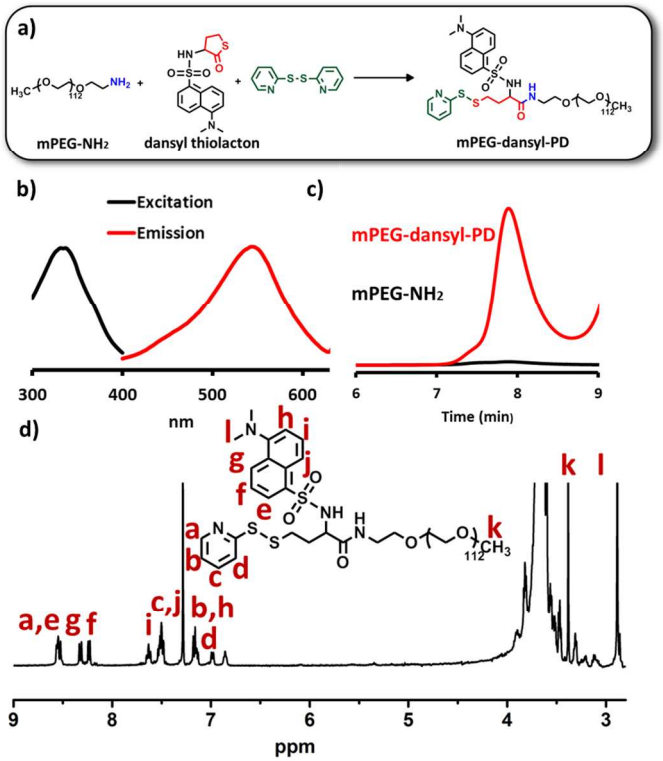


Fig. 1. One-pot synthesis of the fluorescent protein-reactive PEG derivative (mPEG-dansyl-PD). a) Reaction conditions: [mPEG-NH_2]/[dansyl thiolactone]/[2,2'-dithiodipyridine] = 1/10/30, CH_2Cl_2 as solvent, 40 °C, 18 h. b) Fluorescence spectrum of mPEG-dansyl-PD. c) GPC of the polymers before and after the one-pot reaction *via* an UV-detector (360 nm). d) ^{1}H NMR spectrum ($CDCl_3$, 400 MHz, portion) of the mPEG-dansyl-PD.

Primary study of the reactivity between the polymer and BSA

It is well-known that the 35 cysteines in BSA sequence constitute 17 disulfide bridges and leave a free cysteine residue (thiol group, Cys-34) which is our desired reactive group with the new PEG derivative. Before the protein conjugation, Ellman's assay[48,49] was carried out to calculate the reactivity of the free thiol group on BSA surface as 57.8% (**Fig. S3, ESI†**).

As the primary experiment to understanding the reaction efficiency between protein and polymer, a series of conjugation reactions with different protein/polymer ratios were performed, i.e. four plastic vials containing freshly prepared BSA solution (1.5 mL, 2.6×10^{-5} mmol active thiol group, 2.0 mg/mL in PBS buffer, pH 7.0) were respectively added different volume mPEG-dansyl-PD solution (15 mg/mL in PBS buffer, pH 7.0), the final ratios of active thiol/mPEG-dansyl-PD in four vials are 1/1, 1/2, 1/5, 1/10, respectively (Experiment section, **ESI†**). The vials were incubated at 37 °C with gentle shaking for 4 h. After removing salts through centrifugal filtration (MWCO: 30 kD), the concentrated solutions were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis through different imaging

methods (**Fig. 2**). When the gel was exposed under 312 nm UV light (**Fig. 2a**), the fluorescent bands (lane C, D, E, F) corresponding to the protein conjugates appeared at higher molecular weight position than the fluorescent BSA-FITC marker (lane B), and there is no any fluorescent signal of mPEG-dansyl-PD (lane G) because there is no charge on the polymer chain, suggesting the bright signal comes from the fluorescent moiety on the PEGylated protein. After stained with coomassie brilliant blue (**Fig. 2b**), the PEGylated protein showed increased molecular weight (~ 60 kD, lane C, D, E, F) compared to the native BSA (~52 kD, lane A), confirming the successful and efficient linkage of polymer on protein surface in all four different ratio reactions. Meanwhile, there are no obvious differences between four protein conjugates by native eyes, indicating the highly efficient coupling reaction between the cysteine residue on protein surface and the PD moiety at the polymer chain end. Thus, protein conjugate could be efficiently obtained even with equal [thiol]/[polymer] ratio.

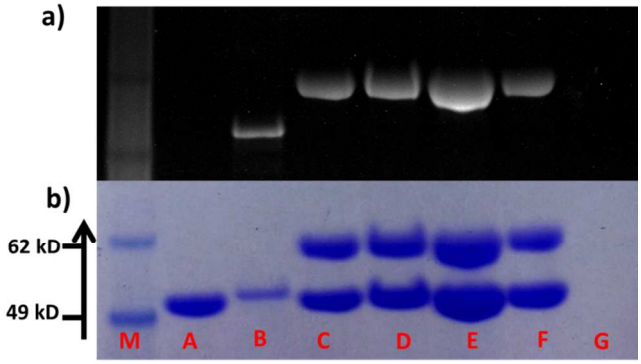


Fig. 2. SDS-PAGE analysis of the reactions between BSA and different amount mPEG-dansyl-PD. a) SDS-PAGE under the gel imaging system with 312 nm UV. b) SDS-PAGE stained with coomassie brilliant blue. (lane M: marker; lane A: BSA; lane B: BSA-FITC; lane C-F: BSA + mPEG-dansyl-PD (1/1, 1/2, 1/5, 1/10); lane G: mPEG-dansyl-PD).

Full analyses of the protein-conjugate

After the primary study, the PEGylated BSA under the 1/2 [thiol]/[polymer] ratio was chosen for further analyses, excess polymer was used to guarantee the complete protein conjugation.

From SDS-PAGE, under the 312 nm UV light, the fluorescent band of BSA conjugate (**Fig. 3a**, lane C) is higher than the band of BSA-FITC (**Fig. 3a**, lane B) as previous described, however, after incubation with dithiothreitol (DTT), the fluorescent band of protein conjugate completely disappeared (**Fig. 3a**, lane C') due to the cleavage of the disulfide linkage between the protein and polymer while the reduced BSA-FITC still showed bright signal (**Fig. 3a**, lane B'), suggesting the polymer provided fluorescence to the PEGylated protein. After stained with coomassie brilliant blue, the bands of native BSA, BSA-FITC and unreacted BSA appeared at the same lower molecular weight position (**Fig. 3b**, lanes A, B, C), and the PEGylated protein appeared at higher molecular weight position (**Fig. 3b**, lane C). After reducing, native BSA, BSA-FITC moved to higher position (**Fig. 3b**, lanes A', B') due to the loose structure after cleavage of disulfide bridges in protein structure, and the PEGylated BSA band disappeared while only a band corresponding to the regenerated BSA appeared at the same position as the markers (**Fig. 3b**, lanes C'), confirming the BSA and the fluorescent PEG were linked together through the reversible disulfide bond.

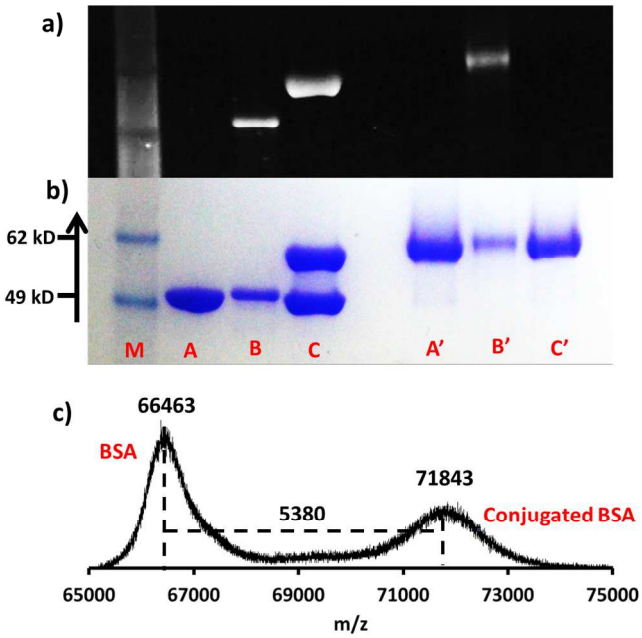


Fig. 3. a) SDS-PAGE under the gel imaging system with 312 nm UV. b) SDS-PAGE stained with coomassie brilliant blue. (lane M: marker; lane A: native BSA; lane B: native BSA-FITC; lane C: BSA + mPEG-dansyl-PD; lane A': reduced BSA; lane B': reduced BSA-FITC; lane C': reduced BSA + mPEG-dansyl-PD) c) MALDI-TOF result of the BSA conjugate.

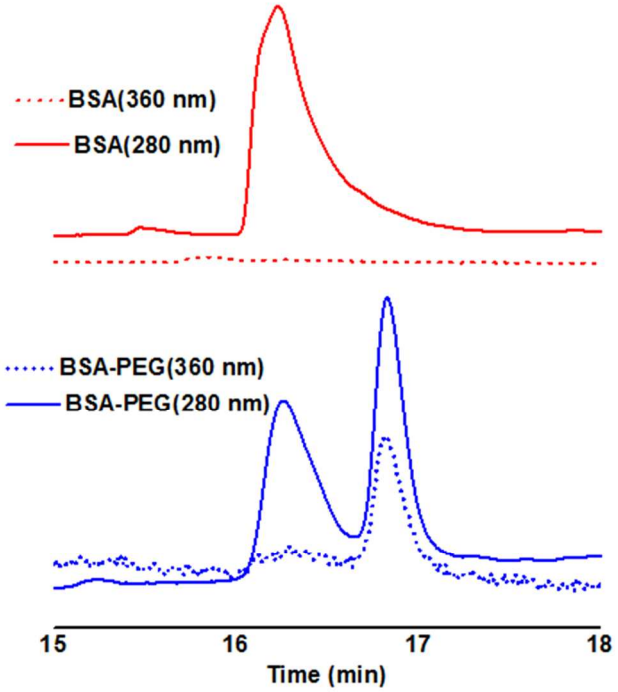


Fig. 4. HPLC results of BSA and the BSA conjugate. Red lines: BSA, blue lines: BSA conjugate; the solid lines: 280 nm, the dotted lines: 360 nm.

Other analytical methods were also employed to characterize the PEGylated protein. From the MALDI-TOF MS spectrum (**Fig. 3c**), the original BSA (m/z = 66463) and the conjugated protein (m/z = 71843) are clearly visible. The molecular weight difference between the conjugated and original proteins is 5380, consistent with the

molecular weight of the mPEG-dansyl-PD, verifying the successful protein conjugation.

Moreover, the reverse phase high performance liquid chromatography (RP-HPLC) was also utilized to analyze the protein conjugate (**Fig. 4**). From the RP-HPLC results, with different UV wavelength, the native BSA demonstrates a peak at 16.25 min under 280 nm and nothing under 360 nm (**Fig. 4**, red curves). The fluorescent PEGylated BSA showed two peaks corresponding to unreacted BSA (16.25 min) and BSA conjugate (16.83 min), respectively at 280 nm, however, with 360 nm, only the peak of protein conjugate could be observed due to the fluorescent group contained in the protein-polymer complex, indicating the formation of fluorescent PEGylated protein.

Bioactivity evaluation of the fluorescent PEGylated protein

PEGylation is a double-edged sword to protect protein but more or less reduce the bioactivity of protein since the polymer chains might prevent the protein from the approaching substrate. It is important for PEGylated proteins to at least partly remain bioactivity for their future pharmaceutical application. Therefore, the protein conjugates from different protein/polymer ratios were tested to evaluate the influence of excess PEG derivative to the remained bioactivity of protein conjugate. BSA can work like an esterase to catalyze the hydrolysis of 4-nitrophenylacetate, this esterase-like activity has been known dependent on the structural integrity of the protein[9]. All PEGylation reaction mixtures were tested using native BSA as the control. As shown in Fig. 5, the bioactivities of all conjugates are all near 100%. Although the BSA conjugates are not separated from the unreacted protein, there is no obvious bioactivity decrease observed, qualitatively suggesting the integrity of protein structure during the conjugation even with excess polymer.

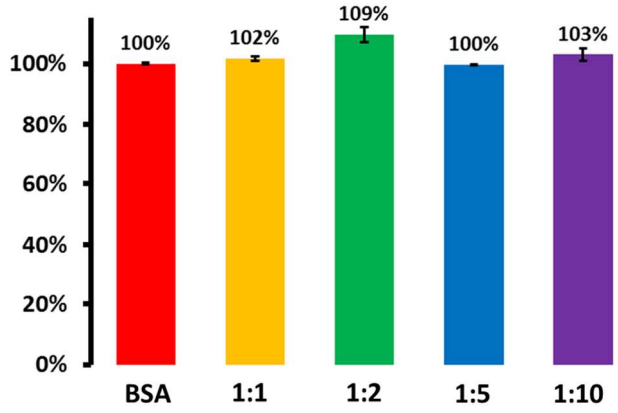


Fig. 5. The structural integrity test of BSA conjugates with different quantity of mPEG-dansyl-PD (1/1, 1/2, 1/5, 1/10), mean ± SD.

Conclusions

In summary, through the one-pot reaction based on the thiolactones ring-opening reaction, we successfully prepared a multifunctional PEG derivative containning both fluorescent and protein-reactive groups at the chain end. This polymer could be efficiently conjugated onto the protein surface to form a fluorescent protein conjugate which could be recognized as a model theranostic combines. Meanwhile, the structural integrity of the protein was almost completely reserved, implying the excellent balance between protein protection and bioactivity reservation through this method to prepare the theranostic combines. In our future work, the near infred dyes and mutant therapeutic protein with cysteine residue at suitable position will be included to prepare theranostic combines for *in vivo* experiment.

Experimental

One-pot synthesis of the fluorescent protein-reactive PEG derivative

The mPEG-NH_2 (M_{nNMR} ~ 5000, 100 mg, 0.02 mmol), dansyl thiolactone (70 mg, 0.2 mmol), and 2,2'-dithiodipyridine (132 mg, 0.6 mmol) were charged into a dry EP tube along with CH_2Cl_2 (1.0 mL). The EP tube was kept in a thermo-shaker (40 °C) for 18 hours. Then, the mixture was precipitated from CH_2Cl_2 to cold diethyl ether for 3 times, and dried under vacuum to obtain the pure polymer (64 mg, 59.2%) for further characterizations and protein conjugation.

^{1}H NMR (400 MHz, $CDCl_3$, δ/ppm): 8.74-8.42 (m, 2H, CHCS, NCH), 8.32 (d, 1H, J = 8.4 Hz, SCCHCHCHC), 8.23 (d, 1H, J = 8.4 Hz, SCCHCHCHC),7.68-7.57 (m, 1H, NCCHCHCH), 7.57-7.43 (m, 2H, NCCHCHCH, NCHCHCH), 7.23-7.09 (m, 2H, NCCHCHCH, NCHCHCH), 6.99 (d, 1H, J = 6.8 Hz, NCHCHCHCH), 3.39 (s, 3H, OCH_3), 2,89 (s, 6H, NCH_3).

PL: λ_{max}^{ex} = 331 nm, λ_{max}^{em} = 544 nm.

Conjugation between the polymer and BSA

Freshly prepared BSA solution (1.5 mL, 2.6 × 10^{-5} mmol active thiol group, 2.0 mg/mL in PBS buffer, pH 7.0) was added to four small plastic vials, followed by the addition of different volume mPEG-dansyl-PD solution (15 mg/mL in PBS buffer, pH 7.0): 8.7 μL, 17.5 μL, 44 μL and 87 μL, respectively. The ratios of [SH]/[mPEG-dansyl-PD] were 1/1, 1/2, 1/5, 1/10. The vials were incubated at 37 °C with gentle shaking for 4 h. Then, 250 μL of BSA/mPEG-dansyl-PD mixture in each vial was transferred into other vials for bioactivity analysis. The salts in remained mixture in each vials were removed through centrifugal filtration (MWCO: 30 kD, 7 times, 8000 rpm, 10 min per time), and the concentrated solutions were used directly for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Bioactivity evaluation of the fluorescent PEGylated protein

All samples were tested through same method, typically: 250 μL of BSA/mPEG-dansyl-PD mixture was put in a vial, then, freshly prepared 4-nitrophenylacetate solution in acetonitrile (1 M, 5 μL) was added. The mixture was diluted to 1.0 mL with water, then incubate at 25 °C for 2 min prior to the analysis by UV (405 nm) for five times. The data was presented as mean ± SD. Native BSA was test by the same way and the value was defined as 100%.

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Notes and references

The Key Laboratory of Bioorganic Phosphorus Chemistry & Chemical Biology (Ministry of Education), Department of Chemistry, Tsinghua University, Beijing 100084, P. R. China.
E-mail: leitao@mail.tsinghua.edu.cn

† Electronic Supplementary Information (ESI) available: Detailed experimental procedures, GPC of the polymer, measure of the quantity of thiol group, et al. See DOI: 10.1039/b000000x/

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