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COMMUNICATION

Lighting up the PEGylation Agents via the Hantzsch Reaction

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

DOI: 10.1039/x0xx00000x

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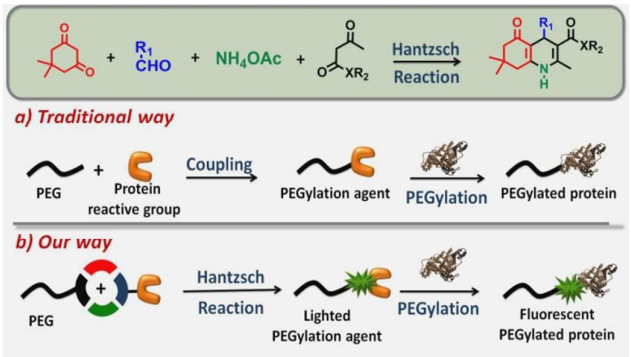
Fluorescent PEGylation agents have been facilely synthesized through the Hantzsch reaction. Different from other methods, the protein reactive group has been linked at the PEG chain ends through a Hantzsch ester which is inherent fluorescent to *in situ* generate fluorescent protein-reactive PEGs. Therefore, after conjugation with protein, the multifunctional polymers can not only act as the protective umbrella to the protein like normal PEGylation agents, but also introduce new function to the protein conjugates to achieve multifunctional protein conjugates. The multifunctional protein conjugates are directly visible under UV and remain nearly intact bioactivity, preliminary suggesting the new application of the 'old' Hantzsch reaction in other areas (polymer chemistry, chemical biology) outside the organic chemistry.

PEGylation is the process of linking polyethylene glycol (PEG) on therapeutic protein/peptides to protect them from the proteolytic, thus prolongs the metabolic half-life and reduces the administration frequency of the protein pharmaceuticals[1-3]. As one of the most successful pharmaceutical technologies, PEGylation has been rapidly developed since the initial research in the 1970s to achieve significant therapeutic benefits and great market success[4-8]. By now, the clinical value of PEGylation has been widely recognized, and the development of new PEGylation agents is an important subject for both fundamental research and practical application[9-23].

Traditional PEGylation agents are prepared through coupling reactions to modify the PEG chain ends with protein reactive groups (Scheme 1a). The first-generation PEGylation agents[4,24,25] (linear PEG derivatives) and the second-generation PEGylation agents[26, 27] (mid-functional branched PEG derivatives) mainly focus on the protective role of the PEGs to the target protein. With the rapid development of modern clinical medicine, the concept of third-generation PEGylation agents (multifunctional PEG derivatives) has been proposed[28,29]. In the new generation PEGylation technology, the polymer and protein should collaborate mutually as a team, the PEG is not only a protective umbrella of the therapeutic protein, but also involved in the diagnosis or therapy process. For example, incorporation of imaging agents into the PEGylation agents might make it possible to directly track the circulation and working site of the PEGylated therapeutic proteins through modern diagnosis methods, leading to more rapid and efficient evaluation of the protein drugs during the clinical trial.

However, through traditional multi-step synthetic strategy, the preparation of multifunctional PEG derivatives is normally laborious and time-consuming due to the unavoidable onerous purification processes, greatly hampering the development and application of the third-generation PEGylation agents and subsequent PEGylated proteins. How to efficiently synthesize multifunctional PEG derivatives containing both protein reactive group and other functions is still a great challenge to polymer chemists[28]. Several strategies have been developed to realize the third-generation protein conjugation technology. For example, Haddleton and O'Reilly et al. used dibromomaleimides to yield fluorescent conjugates upon conjugation to disulfide bridges to achieve simultaneous PEGylation and fluorescent labeling of disulfide-containing proteins[16]. Our group utilized the multicomponent reactions (MCRs), such as the tetra-component Ugi reaction[28] and the thiolactone chemistry[29], to prepare multifunctional PEG agents for fluorescent PEGylation.



Scheme 1. a) Traditional and b) Multifunctional PEGylation agents for protein conjugation.

In recent years, MCRs have been introduced into polymer chemistry to synthesize new functional polymers[30-32]. MCRs can be recognized as efficient coupling reactions to introduce several functional groups to achieve multifunctional polymers in one shot, thus avoid the laborious multi-step operations and save solvents,

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Electronic Supplementary Information (ESI) available: Detailed experimental procedures, GPC and ^{1}H NMR of the polymers, HPLC results, emission and excitation spectra of polymer. See DOI: 10.1039/x0xx00000x

chemicals and time[28,29]. Furthermore, MCRs can generate some specific groups to additionally enrich the property of thereof obtained polymers[33-37]. For example, Hantzsch reaction, a green and efficient four-component reaction which can form 1,4-dihydropyridines (1,4-DHPs) with excellent yields only using common starting materials (aldehyde, β-ketoester, ammonia)[38-40] has been introduced into polymer chemistry by our group to synthesize well-defined poly(1,4-dihydropyridine)s[34]. During the study of the Hantzsch reaction, we are deeply impressed by the fluorescence of 1,4-DHPs although it is normally neglected in the previous research about this old reaction (> 130 years). Herein, we reported our attempt to employ this phenomenon in polymer chemistry to *in situ* generate fluorescent PEGylation agents by linking protein reactive group to the PEG chain ends through the Hantzsch reaction (Scheme 1b). Different from other strategies, the current strategy avoids the synthesis of the fluorescent reactant, and the PEG chain ends have been modified with the protein reactive group through a 1,4-DHP linkage to directly achieve the fluorescent PEGylation agents. As the result, after conjugation, the protein-polymer conjugates showed obvious fluorescence while remained almost intact bioactivity, indicating the potential application of the Hantzsch reaction in the preparation of new generation PEGylation agents.

Under the similar condition as our previous report, the fluorescent PEGs containing the dithiopyridine (PDS) which is an efficient protein reactive group[41] have been synthesized via the Hantzsch reaction. Briefly, a difunctional compound containing benzaldehyde and dithiodipyridine (CHO-PDS), dimedone and ammonium acetate were mixed with the 1,3-dione terminated methoxypoly(ethylene glycol) (mPEG-dione, M_n ~ 5000) which was synthesized as our previous report[42] (Figure S1b, Figure S2). The reactant feeding ratio was set as: [CHO-PDS]/[dimedone]/[ammoniumacetate]/[mPEG-dione] = 1/1/1.5/1 in acetonitrile, excess amount of ammonium acetate (1.5 equiv to mPEG-dione) was added to guarantee the smooth Hantzsch reaction and glycine (10% to the mPEG-dione) was used as the catalyst. The mixture was heated at 70 °C for 4 h, and the fluorescent protein-reactive PEG derivative mPEG-1,4-DHP-PDS could be easily obtained through simple passing a short neutral alumina column and precipitation in diethyl ether (Figure 1a).

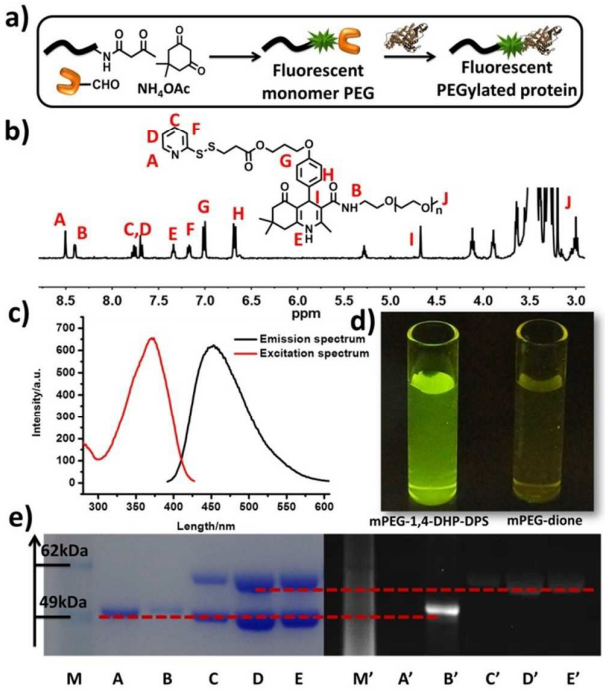

Figure 1. Synthesis of mPEG-1,4-DHP-PDS via the Hantzsch reaction and the subsequent protein conjugation. (a) Reaction conditions: [mPEG-dione] = 0.4 mM/mL, [mPEG-dione]/[Dimedone]/[CHO-PDS]/[Ammoniumacetate]/[Glycine] = 1/1/1/1.5/0.1, CH_3CN as solvent, 70 °C, 4 h. (b) ^{1}H NMR spectrum (DMSO-d_6, 400 MHz, portion) of the fluorescent mPEG-1,4-DHP-PDS. (c) Excitation and Emission spectra of mPEG-1,4-DHP-PDS. (d) The imaging of mPEG-1,4-DHP-PDS and mPEG-dione aqueous solutions (2 mg/mL) under UV ~ 312 nm. (e) SDS−PAGE stained with coomassie bright blue and under the UV ~ 365 nm (lanes M, M': marker; lanes A, A': native BSA; lanes B, B': BSA−FITC; lanes C, C', D, D', E, E': 1/1, 1/2, 1/4 of [thiol]/[polymer]).

From the ^{1}H NMR spectrum of the purified polymer (Figure 1b), the characteristic peaks of the 1,4-DHP group including the protons of CH (4.70 ppm) and NH (7.30 ppm), and the aromatic ring (6.70, 7.00 ppm) could be clearly identified, and the PDS moiety (8.50, 7.76, 7.69, 7.17 ppm) can also be clearly observed in the purified polymer, suggesting the successful Hantzsch reaction.

From the MALDI-TOF-MS analyses (Figure S1), the molecular weight difference (~ 480) between mPEG-dione (~ 5090, Figure S1b) and mPEG-1,4-DHP-PDS (~ 5573, Figure S1c) can be clearly detected, indicating the smooth Hantzsch reaction. The spectrum of the mPEG-1,4-DHP-PDS (Figure S1c) showed main series of peaks were separated by 44 mass units, consistent with the molar mass of the PEG repeat unit. The enlarged section (Figure S1c) only showed two types of ion peaks which represent the $[M+H]^+$ and $[M+Na]^+$, respectively, confirming the smooth conversion of the polymer chain-end through the Hantzsch reaction.

The protein reactive PEG derivative (mPEG-1,4-DHP-PDS) has excitation wavelength (370 nm) and emission wavelength (450 nm) (Figure 1c, 0.2 mg/mL in H_2O, 25 °C) and shows obvious fluorescence under UV ~ 312 nm (Figure 1d), suggesting the in situ generated fluorescent PEGylation agent via incorporation of the 1,4-DHP ring in the polymer. The absolute quantum yield of fluorescence (Φ_{FL}) value of mPEG-1,4-DHP-PDS was evaluated as 10.30% (H_2O, 25 °C) by the integrating sphere method[43,44], which is

quite high comparing with general fluorophores. Meanwhile, from the gel permeation chromatography (GPC) analyses, the mPEG-1,4-DHP-PDS could be tested using both RID and UV detectors (400 nm) while the mPEG-dione only showed a signal with the RID detector (Figure S3), further confirming that the 1,4-DHPs ring has been successfully included in the polymer structure. The GPC trace of the mPEG-1,4-DHP-PDS showed some interesting polymer coupling (Figure S3b), which might be attributed to the decreased reaction rate during polymer chain-end modification due to the diluted reactant concentration and the giant polymer chain, thus led to more or less coupling between two mPEG-dione chains.

The albumin from bovine serum (BSA) has been chosen as the model protein to react with the mPEG-1,4-DHP-PDS, and the active thiol group (cysteine residue) on BSA surface has been determined by the Ellman's assay as 57.8% (unpresented data) prior to the experiment. To optimize the reaction condition, a series of conjugation reactions with different thiol/polymer ratios were performed. Different volumes of mPEG-1,4-DHP-PDS solution (11.45 mg mL^{-1} in PBS buffer, pH 7.0) were added to three plastic vials containing freshly prepared BSA solution (0.5 mL, 2.0 mg mL^{-1} in PBS buffer, pH 7.0, 8.6×10^{-6} mmol active thiol group), and the final ratios of the [thiol]/[polymer] in the three vials were 1/1, 1/2, and 1/4, respectively. The vials were incubated at 37 °C with gentle shaking for 4 h. After removing salts through centrifugal filtration (MWCO: 30 k), the concentrated solutions were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses through different imaging methods. When the gel was exposed under 365 nm UV light (Figure 1e), the fluorescent bands (lanes C', D', E') corresponding to the protein conjugates could be clearly observed at higher molecular weight positions than the fluorescein BSA marker (lane B', isothiocyanate modified BSA, BSA-FITC), suggesting the generation of fluorescent protein-polymer conjugates.

After stained with coomassie brilliant blue (Figure 1e), the PEGylated proteins showed increased molecular weights (~ 59 kDa, lanes C, D, E) compared to the native BSA (~ 52 kDa, lane A), confirming the successful and efficient linkage of polymer on protein surface for all three reactant ratios. There are no obvious detectable differences by the naked eyes between the three protein conjugates, indicating the high efficiency of the coupling reaction between the cysteine residue on the protein surface and the PDS moiety at the polymer chain end, and protein conjugates could be efficiently obtained even with an equal [thiol]/[polymer] ratio.

After the primary study, the conjugation reaction was optimized as: [thiol]/[polymer] = 1/2, 37 °C, 4 h, excess of polymer was used to guarantee the complete conjugation. After removing salts through centrifugal filtration (MWCO: 30 k), the concentrated solution was used for SDS−PAGE and MALDI−TOF MS analyses. When the gel was exposed under UV light (365 nm), the fluorescent band corresponding to the BSA−mPEG conjugates (lane C') appeared at a higher molecular weight position than the BSA−FITC marker (lane B') (Figure 2a), suggesting the linkage of polymer on protein surface. After reduction by DL-Dithiothreitol (DTT), the disulfide linkage between polymer and protein was cleaved and the fluorescent signal disappeared (lane F'), confirming the fluorescence of the protein conjugates comes from the cleaved mPEG-1,4-DHP-PDS. Similarly, after stained with coomassie brilliant blue, the BSA−mPEG conjugates can be clearly observed at the higher molecular weight position (~ 59 kDa, lane C) than the native BSA (~ 52 kDa, lane A) (Figure 2a). After cleaving the disulfide linkage between polymer and protein by DTT, the reduced conjugates (lane F) stayed at the same position as the reduced BSA (~ 60 kDa, lane D). Moreover, from the MALDI−TOF MS spectrum (Figure 2b), the unreacted BSA and the conjugated protein are both visible, and the molecular weight difference (~ 5.5 kDa) between BSA (~ 66.4 kDa) and BSA−mPEG conjugates (~ 71.9 kDa) can be clearly detected, suggesting the successful protein conjugation.

In addition, high performance liquid chromatography (HPLC) has also been employed to analyze the protein conjugates (Figure 2c). After conjugation, the experiment mixture showed two peaks under 280 nm corresponding to unreacted BSA (23.20 min) and PEGylated BSA (23.55 min). However, only the protein conjugates peak (23.55 min) could be observed under 360 nm due to the 1,4-DHP group in the protein–polymer conjugate, further confirming the formation of the Hantzsch ester contained PEGylated protein.

As the continue research, the PEGylated protein dimer has also been studied. The dimerization and oligomerization of many proteins are important for their physiological functions as the enzyme, receptor, ion channel and transcription factor etc.[45,46]. Therefore, the multiple-conjugation of protein is of great value for the simulation of the protein multivalent interactions in biological processes[47-50].

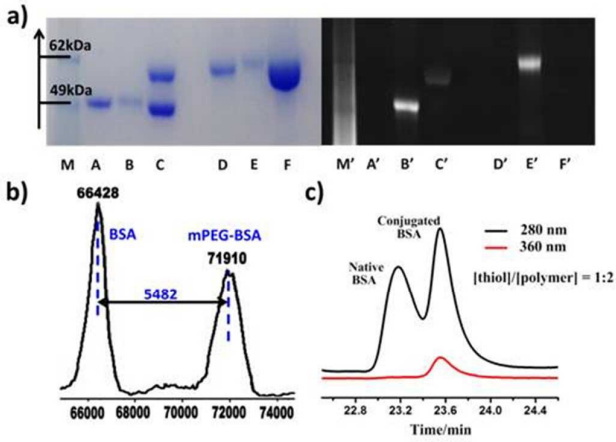

Figure 2. (a) SDS−PAGE analysis of the BSA−mPEG conjugates ([thiol]/[polymer] = 1:2) with coomassie bright blue staining and under UV ~ 365 nm (M, M': marker; A, A': native BSA; B, B': BSA−FITC; C, C': BSA−mPEG conjugates; D, D': reduced BSA; E, E': reduced BSA−FITC; F, F': reduced BSA−mPEG conjugates). (b) MALDI−TOF MS analysis of the BSA−mPEG conjugates. (c) HPLC results of the BSA−mPEG conjugates.

The telechelic dione-PEG-dione (M_n ~ 5000, Figure S4b, Figure S5) has been synthesized and used to react with dimedone, ammonium acetate and CHO-PDS in acetonitrile for 4 h (glycine as catalyst, 70 °C), and the fluorescent protein-reactive PEG dimer could be easily obtained by the simple fractional precipitation in diethyl ether/THF mixture (Figure 3a). In the ^{1}H NMR spectrum (Figure 3b), the characteristic peaks of the 1,4-DHPs structure, PDS moiety and PEG could be clearly observed. The MALDI-TOF MS spectra (Figure S4c) showed that the chain-end functionalization is almost complete. The polymer is fluorescent (Figure S6) and can also be observed by the GPC with a UV detector (400 nm) (Figure S7), confirming the successful integration of 1,4-DHPs at the both

ends of the polymer. The Φ_{FL} value of PEG-1,4-DHP-PDS was evalusted as 11.13% (H_2O, 25 °C).

The preliminary study has also been carried out to optimize the conjugation condition. Different ratios of [thiol]/[polymer] (2/1, 4/1, 8/1) have been investigated, and protein conjugates have been efficiently achieved (Figure 3c). However, the mono-PEGylated protein seemed the major with the 2/1 ratio of [thiol]/[polymer] (lanes C, C', Figure 3c), and too much unreacted BSA ([thiol]/[polymer] = 8/1) counteracted the protein dimer conjugates for clearly visible (lanes E, E', Figure 3c). The HPLC analyses are also in accordance with the SDS−PAGE results (Figure S8). Therefore, the 4/1 ratio of [thiol]/[polymer] has been chosen to achieve the PEGylated protein dimer, and the optimized conjugation condition was set as: pH 7.0, [thiol]/[polymer] = 4:1, 37 °C, 4 h. After conjugation, the salts were removed through centrifugal filtration (MWCO: 30 k) and the concentrated solution has been used for SDS−PAGE and MALDI−TOF MS analyses.

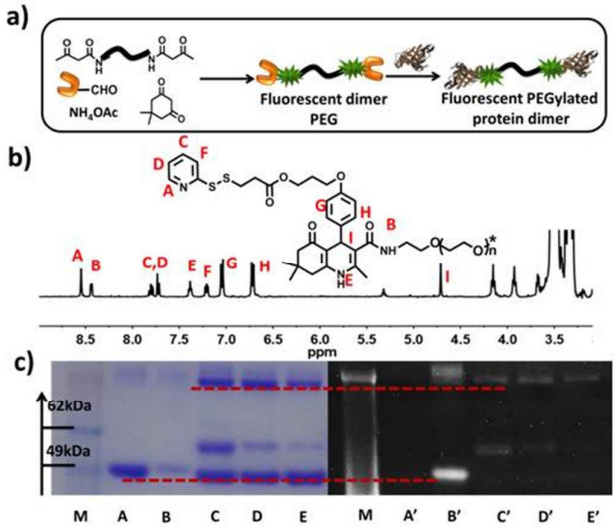

Figure 3. Synthesis of telechelic PEG-1,4-DHP-PDS via the Hantzsch reaction and the subsequent protein conjugation. (a) Reaction conditions: [dione-PEG-dione] = 0.2 mM/mL, [dione-PEG-dione]/[Dimedone]/[CHO-PDS]/[Ammoniumacetate]/[Glycine] = 0.5/1/1/1.5/0.1, CH_3CN as solvent, 70 °C, 4 h. (b) ^{1}H NMR spectrum (DMSO-d_6, 400 MHz, portion) of the telechelic PEG dimer. (c) SDS−PAGE of the BSA−PEG conjugates with coomassie bright blue staining and under UV ~ 365 nm, (lanes M, M': marker; A, A': native BSA; B, B': BSA−FITC; lanes C, C', D, D', E, E': [thiol]/[polymer]: 2/1, 4/1, 8/1).

When the gel was exposed under the UV light (365 nm), the spots (Figure 4a) corresponding to the monomeric and dimeric protein-polymer conjugates could be observed (lane C), and disappeared after reduction by DTT (lane F), suggesting the successful linkage of fluorescent polymer on protein surface via the disulfide bond. After stained with coomassie brilliant blue (Figure 4a), the gel showed two new higher molecular weight spots (~ 59 kDa, ~ 100 kDa, lane C), respectively, which are attributed to the monomeric and dimeric protein-polymer conjugates. After reduction, those two spots were all disappeared (lane F) as expected due to the removal of the conjugated polymer. From the MALDI−TOF MS spectrum (Figure 4b), the dimeric protein conjugates (BSA−PEG−BSA, ~ 143.0 kDa) can be clearly observed. By all those fully analyses, the conclusion could be drawn that the desired fluorescent dimeric protein-polymer conjugates have been successfully generated.

BSA can work like an esterase to catalyze the hydrolysis of 4-nitrophenylacetate and the esterase-like catalytic activity has been reported depending on the structural integrity of the protein. Therefore, the bioactivity of the obtained fluorescent PEGylated proteins has been tested as previous literatures[15,18,28,29,48]. All PEGylation reaction mixtures were tested using the native BSA as the control. And the mixture (BSA-polymer conjugates and unreacted BSA) showed almost the same bioactivity (97~106%) as the native BSA (Figure 5), semi-qualitatively suggesting the integrity of the protein structure during the conjugation, and the excess polymer has negligible effect on the protein bioactivity.

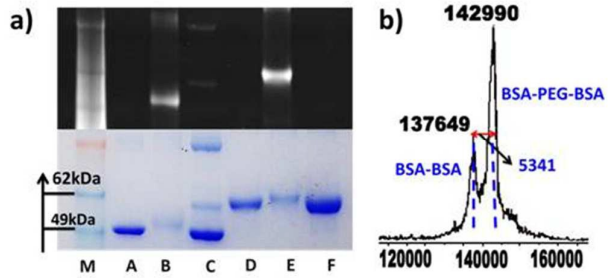

Figure 4. (a) SDS−PAGE analyses of the BSA−PEG conjugates ([thiol]/[polymer] = 4:1) with coomassie bright blue staining and under UV ~ 365 nm, (M: marker; A: native BSA; B: BSA−FITC; C: BSA−PEG conjugates; D: reduced BSA; E: reduced BSA−FITC; F: reduced BSA−PEG conjugates). (b) MALDI−TOF MS analysis of the BSA−PEG conjugates.

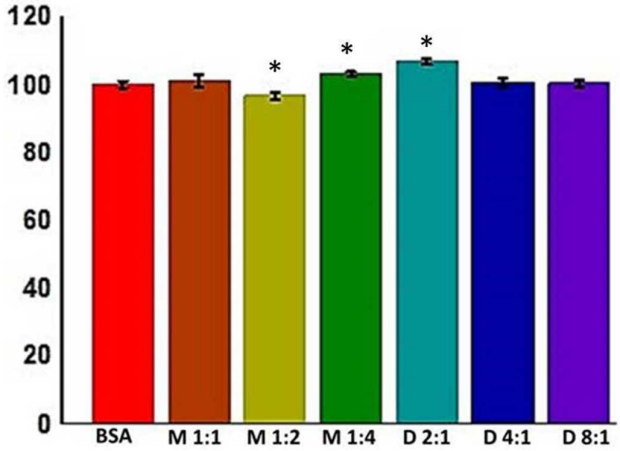

Figure 5. Structural integrity test of BSA conjugates with different multifunctional PEG agents. Data represent mean±SD (n = 5, * indicates $p < 0.05$) (fluorescent PEGylated protein monomer: M 1/1, M 1/2, M 1/4; fluorescent PEGylated protein dimer: D 2/1, D 4/1, D 8/1, [thiol]/[polymer]).

Conclusions

In summary, the Hantzsch reaction has been successfully applied to prepare multifunctional PEGylation agents containing both fluorescent and protein-reactive groups at the chain end. Without additional introducing fluorophore, the fluorescent PEGs could be *in situ* generated, then efficiently conjugated on protein surface to form corresponding multifunctional protein-polymer conjugates which remain almost intact bioactivity like the native protein. Compared to

traditional PEGylation technology, this method is straightforward to prepare detectable PEGylation agents for protein conjugation. Given the simple operation and spontaneously generated fluorophore, this method might have potential in the new generation PEGylation technology and other biomaterial areas.

ACKNOWLEDGMENT

This research was supported by the National Science Foundation of China (21574073, 21534006).

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Lighting up the PEGylation Agents via the Hantzsch Reaction

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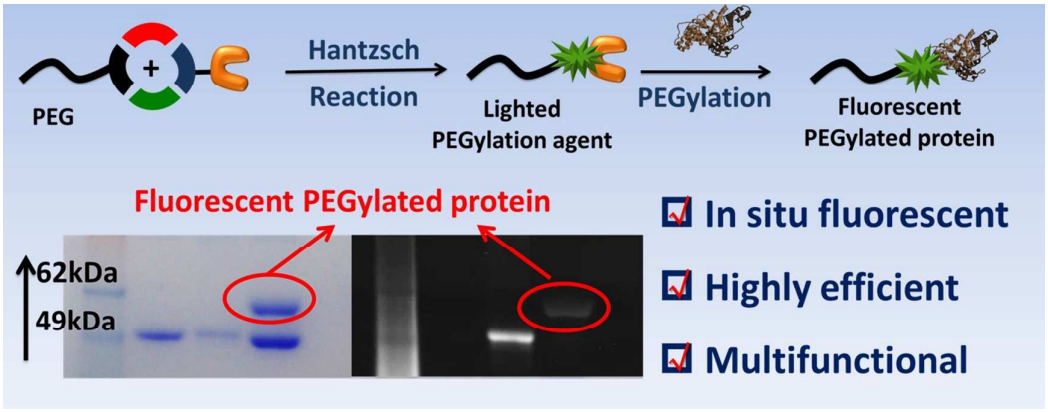

PEG chain ends have been modified with the protein reactive-group through the Hantzsch reaction to *in situ* achieve fluorescent PEGylation agents for protein conjugation.