Enhancing Conjugation Yield of Brush Polymer-Protein Conjugates by Increasing Linker Length at the Polymer End-Group

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Polymers with oligoethylene glycol side chains are promising in therapeutic protein-polymer conjugates as replacements for linear polyethylene glycol (PEG). Branched PEG polymers can confer additional stability and advantageous properties compared to linear PEGs. However, branched PEG polymers suffer from low conjugation yields to proteins, likely due to steric interactions between bulky side chains of the polymer and the protein. In an effort to increase yields, the linker length between the protein-reactive functional end-group of the polymer chain and branched PEG side chain was systematically increased. This was accomplished by synthesizing four well-defined poly(poly(ethylene glycol methyl ether) acrylates) (pPEGA) with pyridyl disulfide end-groups by reversible addition-fragmentation chain transfer (RAFT) polymerization mediated by chain transfer agents (CTAs) with different linker lengths. These, along with linear PEG and poly(N-isopropylacrylamide) (pNIPAAm), were conjugated to two model proteins, bovine serum albumin (BSA) and beta-lactoglobulin (βLG). The conjugation yields were determined by gel electrophoresis. The length of the linker affected conjugation yield for both proteins. For BSA, the conjugation yield step increased from 10% to 24% when the linker was altered from 1 ethylene glycol (EG) unit to 3, with no additional increase for 4 and 6 EG units. In the case of βLG, the yield gradually increased from 9% to the 33% when the linker length was increased from 1 to 6. PEG and pNIPAAm reacted with yields as high as 75% further emphasizing the effect of steric hindrance in lowering conjugation yields.

Introduction

Recombinant DNA technology has greatly increased opportunities and interest in tailoring proteins for therapeutics use.[1] However, protein therapeutics suffer from several limitations, mainly short half-lives *in vivo* and immunogenicity.[2] In 1977, Abuchowski demonstrated the potential of covalently attaching straight chain, linear poly(ethylene glycol) (PEG) to proteins as a method to improve the pharmacokinetic properties of protein drugs.[3] Consequently, there are currently 11 FDA-approved linear PEG-drugs, which includes 10 PEG-protein conjugates, comprising a large market.[4-6] For example, Neulesta®, a granulocyte colony-stimulating factor (G-CSF) conjugated with the linear PEG, generated over four billion dollars of sales in 2014,[7] and is an important part of the chemotherapy treatment regime.[8]

The development of new polymerization techniques has greatly expanded the chemical makeup of the polymer component of bioconjugates. For instance, controlled radical polymerization (CRP) methods, such as atom transfer radical polymerization (ATRP) or reversible addition-fragmentation chain transfer (RAFT) polymerization, allow for the development of novel polymers with various monomers and functional end-groups, as well as differing architectures.[9-17] CRP techniques also yield polymers with narrow molecular weight distributions, which is an important factor for therapeutic use. CRP techniques readily polymerize methacrylate or acrylate based PEG monomers. These and other branched polymers, when conjugated to proteins, offer improved *in vivo* half-life and stability properties over linear PEG-protein conjugates.[5, 18-24] Brush polymers can also display reduced viscosity, which is beneficial in therapeutic delivery,[25] since the majority of protein therapeutics are delivered intravenously. Moreover, utilizing bioorthogonal end-groups on the polymers, which are easily introduced by CRP, offers the possibility of conjugating to specific sites on proteins so as to retain bioactivity.[26-29] As a result, brush PEG polymers have been increasingly synthesized and explored.[5]

However, one difficulty associated with brush PEG polymers is poor conjugation yields compared to linear polymers. Previous work from Kasko and coworkers utilizing linear PEG indicated that the protein size and accessibility were the largest factors in reaction rate and overall conjugation yield, and that the molecular weight of the polymer itself was a minor factor.[30] Yet brush polymers have additional steric hindrance from the side chains of the branched repeating units. Low conjugation yield for branched polymers can be circumvented by various approaches such as modifying the protein with an initiator and polymerizing directly from the protein forming the conjugate *in situ*, the *grafting from* approach.[31-40] Or for *grafting to* methods, this can be accomplished by modifying the biomolecule to extend the desired functional group away from the protein surface. For example, Hoogenboom, De Geest, and coworkers found that introducing additional thiols onto bovine serum albumin or ovalbumin (OVA) using N-succinimidyl-S-acetylthiopropionate (SATP) strongly correlated with higher

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conjugation yields.[41] New orthogonal polymer end-groups that can be conjugated to proteins in high efficiency are also under study to increase conjugation yields.[42-46] Another possible solution to improving yield in *grafting to* approaches is to increase the size of linker at the chain end of the branched polymer. Herein we detail a systematic study of the effect of linker length and polymer bulk on the disulphide exchange yields for the preparation of protein-polymer conjugates.

Experimental

Materials

All the chemicals and proteins were purchased from Sigma-Aldrich and Fisher Scientific and used without further purification unless mentioned otherwise. Monofunctionalized 20 kDa pyridyl disulfide PEG (PDS-PEG) was purchased from Laysan Bio Inc. (Arab, Alabama) and used without further modification. N-Isopropylacrylamide (NIPAAm) was obtained from Sigma-Aldrich, recrystallized from hexanes and dried under vacuum before use. Azobisisobutyronitrile (AIBN) was recrystallized from acetone prior to use. 2-(Ethylsulfanylthiocarbonyl sulfanyl)-propionic acid was prepared according to literature procedure.[47] **CTA1-CTA4** were synthesized directly or with slight modifications of previous literature reports (see SI for details).[48]

Analytical Techniques

NMR spectra were obtained on a Bruker AV 500 MHz and DRX 500 MHz spectrometers. For ^{1}H NMR spectra, a relaxation time of 2 seconds was used for small molecules and 30 seconds for polymers. Gel permeation chromatography was conducted on a Shimadzu high performance liquid chromatography (HPLC) system with a refractive index detector RID-10A, one Polymer Laboratories PLgel guard column, and two Polymer Laboratories PLgel 5µm mixed D columns. Eluent was DMF with LiBr (0.1 M) at 40 °C (flow rate: 0.6 mL/min) Calibration was performed using near-monodisperse poly(methyl methacrylate) standards (Polymer Laboratories). UV measurements were performed on a BioMate 5 (Thermo Spectronic) instrument and a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific). HPLC purification of chain transfer agents was conducted on a Shimadzu HPLC system with a refractive index detector RID-10A and one Luna µm C18(2) column with methanol and water as eluents. The UCLA Pasarow Mass Spectrometry Facility performed mass spectrometry analysis. SDS-PAGE analysis was performed using 10% poly(acylamide) gels (Bio-Rad).

Typical PEGA RAFT polymerization.

CTA4 (25 mg, 0.042 mmol), PEGA (1.16 g, 416.76 mmol) and AIBN (0.68 mg, 0.007 mmol) were dissolved in dimethylformamide (DMF, 1.353 mL). Three cycles of freeze-pump-thaw were performed and the polymerization was initiated by heating the solution to 65 °C. The polymerization was monitored by ^{1}H-NMR and stopped at 65% conversion (6 h) by rapidly cooling the solution in liquid nitrogen. The product (**pPEGA4**) was then purified by dialysis (MWCO 3,500) against H_2O for 3 days followed by MeOH for 1 day and dried under vacuum to produce a viscous yellow oil.

Characterization of pPEGA1

^{1}H-NMR (500 MHz, $CDCl_3$) δ: 8.42 (1H), 7.63 (2H), 7.07 (1H), 4.78 (1H), 3.73-3.40 (m, 104H), 3.33, 3.00, 2.36, 2.32, 2.25, 1.85, 1.57, 1.42, 1.30 (t, J = 7.23 Hz), 1.10 (q, *J* = 4.96 Hz). M_n (NMR) 24.4 kDa. M_n (GPC) 14.9 kDa. Đ(GPC) 1.32.

Characterization of pPEGA2

^{1}H-NMR (500 MHz, $CDCl_3$) δ: 8.33 (1H), 7.67 (1H), 7.56 (1H), 7.00 (1H), 4.70 (1H), 4.58-3.82 (m, 99H), 3.66-3.38 (m), 3.25, 3.11, 2.87, 2.56, 2.18, 1.77, 1.50, 1.35, 1.22 (t, *J* = 7.34 Hz), 0.99 (q, *J* = 3.69 Hz). M_n (NMR) 18.8 kDa. M_n (GPC) 13.8 kDa. Đ(GPC) 1.26.

Characterization of pPEGA3

^{1}H-NMR (500 MHz, $CDCl_3$) δ: 8.40 (1H), 7.73 (1H), 7.63 (1H), 7.05 (1H), 4.78 (1H), 3.74-3.46 (m, 84H), 3.74-3.46 (m), 3.33, 3.19, 2.95, 2.63, 1.4220, 1.30 (t, *J* = 7.33 Hz), 1.09 (q, *J* = 4.96 Hz). M_n (NMR) 20.7. M_n (GPC) 14.2 kDa. Đ(GPC) 1.29.

Characterization of pPEGA4

^{1}H-NMR (500 MHz, $CDCl_3$) δ: 8.41 (1H), 7.76 (1H), 7.66 (1H), 7.07 (1H), 4.07 (1H), 4.16-3.89 (m, 99H), 3.37-3.45 (m), 3.33, 3.18, 2.95, 2.63, 2.25, 1.84, 1.57, 1.14, 1.29 (t, J = 10.8 Hz), 1.07-1.03 (m). M_n (NMR) 21.3 kDa. M_n (GPC) 15.3 kDa. Đ(GPC) 1.27.

pNIPAAm RAFT polymerization mediated by CTA2

CTA2 (61 mg, 0.13 mmol), N-isopropylacrylamide (2.83 g, 25.01 mmol) and AIBN (2.17 mg, 0.013 mmol) were dissolved in DMF (2.61 mL). Three cycles of freeze-pump-thaw were performed and the polymerization initiated by heating the solution to 70 °C. The polymerization was monitored by ^{1}H-NMR and stopped at 65% conversion (2 h) by rapidly cooling solution in liquid nitrogen. The product was purified by precipitation in cold hexanes three times followed by freeze-drying with benzene on a vacuum line. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 8.36 (1H), 7.75 (1H), 7.65 (1H), 7.08 (1H), 7.00-5.57 (broad s), 4.19-3.78 (s, 164H), 3.75-3.28 (m), 2.96 (t, J = 12.48), 2.93 (s), 2.85 (s), 2.49-0.92 (m). M_n (NMR) 15.0 kDa. M_n (GPC) 20.0 kDa. Đ(GPC) 1.15.

BSA-Polymer Conjugations

BSA was reduced prior to conjugation using a literature procedure.[32] A 1 mg/mL stock solution was prepared in Delbarton phosphate buffered saline (D-PBS). BSA solution was separately mixed with **pPEGA1-pPEGA4, pNIPAAm** and **PDS-PEG** (100 eq polymer to BSA). The reaction was incubated at 4 °C for 24 h. Conjugation yield was quantified by SDS PAGE analysis according to literature procedure as described below.[49]

βLG-Polymer Conjugations

βLG exists naturally as a dimer and was reduced prior to use following a literature procedure.[30] A βLG solution (1 mg/mL in pH 8, 100 mM PBS) was reduced with freshly made 1 mg/mL dithiothreitol (0.95 eq) solution and allowed to incubate for 30 min at 4 °C. **pPEGA1-4, pNIPAAm** and **PDS-PEG** were dissolved into βLG solution separately (100 eq polymer to the protein). The reaction was cooled to 4 °C and incubated for 3 h.

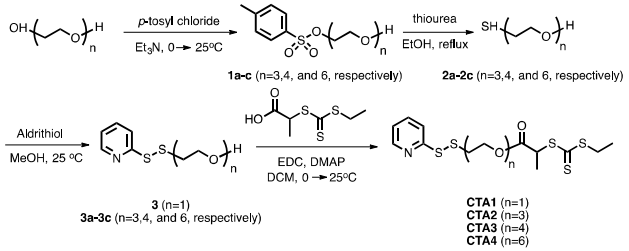
Analysis of Conjugation Yield

SDS-PAGE gels were scanned using an EPSON Perfection 2480 scanner and analysed using ImageJ software. Each lane was selected by the rectangular selection tool, and plotted with the gel analysis function. The percentage peak area of the conjugate divided by the sum of the conjugate and unmodified protein is the conjugation yield. Yields were calculated independently by three people and averaged. The error bars in Table 2 reflect the error in the calculation technique. The differences in the error of the

measurement were determined using one-way Anova with Tukey correction.

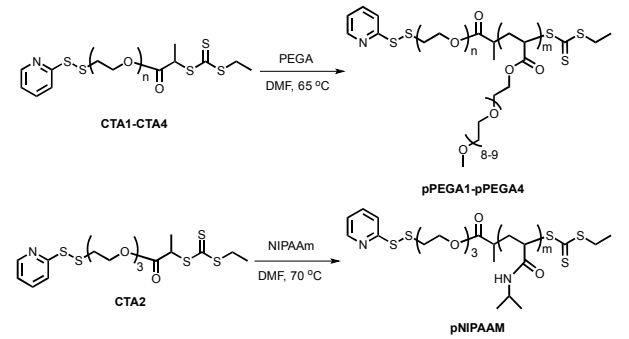
Results

In order to explore the effect of the extension of the chain end functionality from the branched polymer chain, we synthesized four CTAs (**CTA1-4**) with different linker lengths (Scheme 1). Each CTA was functionalized with a cysteine reactive, pyridyl disulfide end-group. The pyridyl disulfide functionality has been extensively demonstrated in literature as an effective means to achieve protein conjugation via a reversible disulfide bond.[50] Conjugation via free cysteine residues allows for site-specific protein modification, making comparisons between different polymers straightforward and allowing for yield quantification via SDS-PAGE.



Scheme 1 Synthesis of CTA1 through CTA4.

Polymers (**pPEGA1-4, pNIPAAm**) with varying linkers lengths, from 1 to 6 ethylene glycol units between the protein-reactive pyridyl disulphide end-group and branched PEG side chains were prepared by RAFT polymerization using AIBN as an initiator (Scheme 2). Polymerizations of PEGA were initiated at a temperature of 65 °C and **pNIPAAm** at 70 °C. A slightly lower temperature was utilized in the polymerization of PEGA because we have found that lower temperatures for this monomer provide better control.[51] Monomer conversions were monitored by ^{1}H-NMR spectra. In order to ensure that differences in observed conjugation yields were a function of linker length rather than molecular weights, a M_n value of 15 kDa was targeted for all polymers. Indeed, all of the resulting **pPEGA1-4** had similar molecular weights with M_n ranging from 13.8 to 15.3 kDa and molecular weight dispersity (Đ) values between 1.26 and 1.32. The pNIPAAm was slightly larger at 20 kDa. pNIPAAm is well known to conjugate to proteins in high yield, and was utilized as a positive control. Linear polymer **PDS-PEG** (20 kDa) was employed as another positive control. Molecular weights and dispersity (Đ) indices for **pPEGA1-4, pNIPAAm** are provided in Table 1 (Figure S1-S5 for ^{1}H NMR spectra and Figure S6 for GPC traces).



Scheme 2 Preparation of **pPEGA**s and **pNIPAAM** by RAFT polymerization.

Table 1 Summary of prepared polymers

Polymer	CTA	#of EG units in spacer	M_n by GPC (kDa)	Đ by GPC
pPEGA1	**CTA1**	1	14.6	1.32
pPEGA2	**CTA2**	3	13.8	1.26
pPEGA3	**CTA3**	4	14.2	1.29
pPEGA4	**CTA4**	6	15.3	1.27
pNIPAAm	**CTA2**	3	20.0	1.15
PDS-PEG	-	-	20.0	-

BSA and βLG were used as model proteins. Both proteins contain a free cysteine that can be exploited for conjugation and are considerably different in size (66.5 kDa for BSA and 18.4 kDa for βLG). Prior to conjugation, BSA and βLG were subject to reducing conditions, in order to increase available free thiols, and maximize conjugation efficiency. We have found that reduction of BSA with TCEP powder, as described in literature, increases the availability of free thiols from 0.5 thiols/protein to approximately 3 thiols/protein.[32] βLG exists naturally as an 18.4 kDa disulfide-linked dimer and was reduced with a 0.95 eq of 1 mg/mL dithiothreitol solution for 30 min prior to conjugation. DTT was used as the limiting reagent as described in literature so that unwanted reduction upon subsequent conjugation of the polymer would not occur.[30] Based on our observations, this resulted in cysteine activated βLG with approximately 0.8 cysteine residues/protein by Ellman's assay.

SDS-PAGE was utilized to allow direct and facile quantification of conjugation yield. (Figure 1a and Figure 1b). For BSA, a step increase in conjugation yield was observed between **pPEGA1** and **pPEGA2-4**. Specifically, a low conjugation was observed for the polymer with the shortest linker (**pPEGA1**, 10%). Yields improved with **pPEGA2** through **pPEGA4** to 23-24% (Table 2, Figure S7). Longer ethylene glycol linkers also improved conjugation yield to βLG; however the yield progressively increased with linker length (Table 2, Figure S7), although the conjugation of pPEGA1 and pPEGA2 are within the error of the measurement.

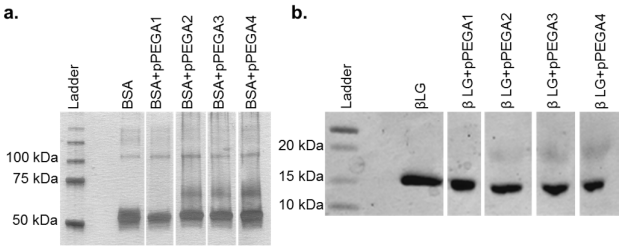


Figure 1a BSA-pPEGA conjugation visualized by SDS-PAGE. **Figure 1b**. βLG-pPEGA conjugation visualized by SDS-PAGE. All reactions were undertaken with 100 equivalent of polymer to protein.

Table 2 Polymer-Protein Conjugation Yield*

Polymer	Conjugation Yield (BSA)	Conjugation Yield (βLG)
pPEGA1	10% (±5)	9% (±3)
pPEGA2	24% (±7)	15% (±2)
pPEGA3	23% (±7)	21% (±1)

pPEGA4	23% (±4)	33% (±4)
pNIPAAm	33% (±4)	77% (±4)
PDS-PEG	56% (±3)	58% (±3)

* The numbers in parenthesis are the errors from the experimental measurement.

Despite the improvement in yield with increasing linker lengths, the pPEGA with the longest linker (**pPEGA4**, with 6 ethylene glycol repeats) had lower conjugation yields compared to the positive controls, **PDS-PEG** and **pNIPAAm** (SDS Pages, Figure 2a and Figure 2b). For BSA and βLG, the yields with linear PEG were 56% and 58%, respectively, while, pNIPAAm conjugated in 33% yield to BSA and 77% yield to βLG.

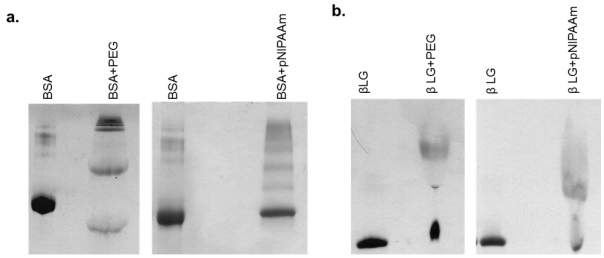


Figure 2. a. BSA-PDS-PEG and BSA-pNIPAAm conjugation visualized by SDS-PAGE. b. βLG-PDS-PEG and βLG-pNIPAAm conjugation visualized by SDS-PAGE. All reactions were undertaken with 100 equivalent of polymer to protein.

Discussion

The results demonstrate that linker length plays a role in influencing conjugation yield of polymer with bulky side chains. For BSA **pPEGA1-pPEGA4** conjugations, an enhancement in conjugation yield was observed between the shortest ethylene glycol linker length of pPEGA (**pPEGA1**) and the other polymers with longer ethylene glycol units. It is known that BSA's free cysteine (Cys-23) lies within a hydrophobic pocket approximately 6 Å deep within the protein tertiary structure.[52] The environment of Cys-23 impedes conjugation. Increasing the linker from one ethylene glycol unit (**pPEGA1**) to three units (**pPEGA2**) allowed the bulky polymer to conjugate by enabling the end group to reach the buried thiol. Interestingly, conjugation yield did not continue to increase even as the linker was lengthened to four and six ethylene glycol units (**pPEGA3** and **pPEGA4**) as shown in Figure 3. Unlike BSA, the conjugation yield between βLG and the different linker length polymers showed steady increase after **pPEGA1 and pPEGA2**, which are within error of the measurement to each other. This may be due to the environment of the free cysteine in βLG (Cys-122). Cys-122 is located at the surface of the protein and is more accessible to the polymer end-group compared with BSA. Conjugating **pPEGAs** after incubating BSA with guanidinium-HCl was attempted to test the effect of altering BSA tertiary structure, however, the yield was lower (data not shown).

While increasing the ethylene glycol linker length correlated with improvements in yield, linear **PDS-PEG** had higher conjugation yields than any of the tested **pPEGAs**. This suggests that steric bulk of the polymer chain is a critical factor and that increasing the spacer length of **pPEGA** further may be necessary to obtain higher conjugation yields. In this study, the yield of PEG-βLG and PEG-BSA were similar. A previous study by Kerr et al. indicated that conjugation efficiency was higher to βLG than BSA.[30] In their case the BSA was not reduced prior to reaction. When the maximum yield possible was taken into account the yields between the two proteins were similar and higher (~70%) than in this study. This difference is likely a result of the significantly larger molecular weight of the PEG used in this study.

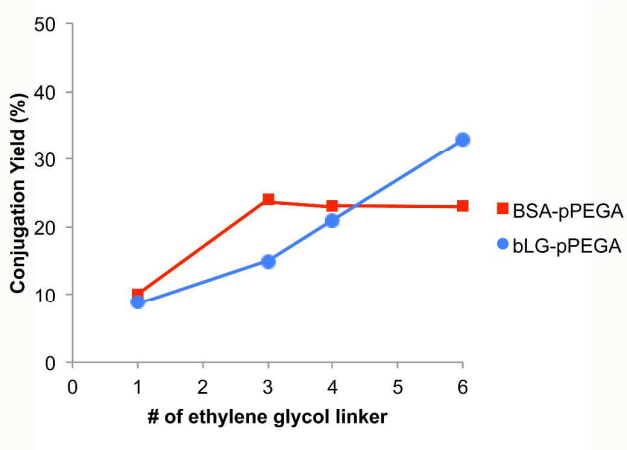


Figure 3. Conjugation yield of BSA and βLG with **pPEGA1-4**.

Beyond linear **PDS-PEG**, we also wanted to further explore the effect of polymer structure on conjugation yield. We selected **pNIPAAm** as a candidate since it has a smaller side chain compared to **pPEGA** and is known to conjugate in moderate to high yield to proteins.[53] For BSA-**pNIPAAm** with two ethylene glycol repeat units in the linker the conjugation yield was 33%, which was lower than linear PEG. However, for βLG, the opposite effect was observed. **pNIPAAm** conjugated in 77% yield, considerably higher than linear **PDS-PEG** and all of the **pPEGAs** studied. The distinction between **pNIPAAm** and **pPEGAs** likely results from the difference in the side chain sizes. In addition, the results indicate that the yield is also protein and polymer dependent. The results together suggest that linker length does have an effect on protein conjugation yields, but that the steric bulk of the side chain is the dominating factor.

Conclusions

These results provide the role of oligoethylene glycol linkers in improving conjugation efficiency between brush PEG side chain polymers and proteins. In both model proteins, BSA and βLG, pPEGA with longer spacer lengths between the bulky side chains and protein-reactive groups conjugated in higher yield than those with shorter linker lengths, offering a facile way to improve yield. However, BSA displays a step increase in conjugation yield between the two shortest linker length examples of pPEGA (**pPEGA1 and pPEGA2**) suggesting that factors such as thiol environment and location play important roles in influencing conjugation efficiency. Increasing linker length did not compensate for steric hindrance of bulky side chains as evidenced by the significantly higher yields for linear PEG and pNIPAAm to the model proteins. These results reinforce the need to consider protein architecture, polymer composition and thiol environment during branched polymer conjugation.

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Enhancing Conjugation Yield of Brush Polymer-Protein Conjugates by Increasing Linker Length at the Polymer End-Group

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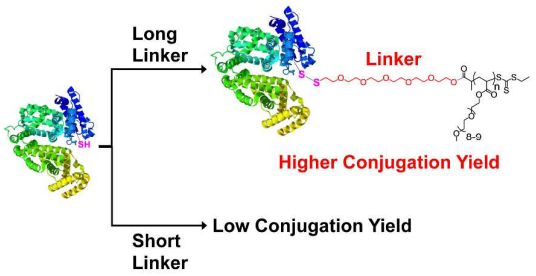

Increasing the linker length between oligoPEG side chain polymer and end group can enhance the yield of the protein conjugate.