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Investigation on PEGylation strategy of recombinant human interleukin-1 receptor antagonist

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Abstract—Although PEGylation is a potential approach to prolong the half-lives and reduce the dosing frequency of therapeutic proteins, conjugation behaviors of polymer have pivotal effects on the remaining bioactivities of the derivatives. In this study, the PEGylation strategy of recombinant human interleukin-1 receptor antagonist was investigated. The random conjugation of polyethylene glycol to amino groups on the protein resulted in a severe loss of activity and only retained 9.8% of the activity. In contrast, the PEGylation at the thiol groups had moderate effects on the bioactivity of protein and 40% of activity was conserved. The results suggested that the thiol-target PEGylation was more beneficial for IL-1ra.
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1. Introduction

Interleukin-1 (IL-1) is one of main cytokines in the course of inflammation, infection, and tissue damage. It induces signal transduction and cell activation by binding to the cellular receptors and executes pro-inflammatory actions. Conversely, interleukin-1 receptor antagonist (IL-1ra) competes against IL-1 to bind the receptors and is devoid of any signal transduction, serving as a very important anti-inflammatory cytokine.[1] Recombinant human IL-1ra (rhIL-1ra) has been approved by FDA for the treatment of rheumatoid arthritis (RA) in adults who had failed to respond adequately to any of the disease-modifying anti-rheumatic drugs (DMARD).[2] It is potent to reduce signs and symptoms and to slow the progression of structural damage in active RA. However, rhIL-1ra shows a short plasma circulating half-life (4–6 h) in vivo and necessitates administration daily to patients for optimal effectiveness.[3] The long-term administration was well known to reduce patient compliance and to increase the probability of infection. Therefore, the development of long-acting candidates of rhIL-1ra would be of significant benefit to patients.

It was well known that attachment of polyethylene glycol (PEG) to therapeutic proteins (referred to as PEGylation) would effectively prolong their half-lives, reduce antigenicity and immunogenicity, and improve their pharmacokinetic and pharmacodynamic properties.[4,5] The different PEGylation strategies have been successfully applied to such proteins as interferon-α2a (PEGasys from Hoffman),[6] interferon-α2b (PEG Intron from Schering-Plough),[7] granulocyte colony stimulating factor (Neulasta from Amgen),[8] and asparaginase (Oncospar from Enzone),[9] and rendered them the significantly prolonged circulating half-lives.

In PEGylation of pharmaceuticals, the design of appropriate conjugation strategy was essential to confer the desired properties to parent protein. In some proteins, when lysines were far from the active sites of proteins, the modification strategy was usually non-specific and mainly targeted at the prevalent lysine residues using PEG reagents such as succinimidyl carbonate PEG (SC-PEG), succinimidyl ester of the carboxymethylated

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PEG (SCM-PEG), and succinimidyl succinate PEG (SS-PEG).[10] However, when some lysine residues were within or near the active sites of proteins such as lysozyme and interleukin-15, the random conjugation of PEG would result in the significant decrease even loss of bioactivities.[11,12] Furthermore, the random conjugation of polymer with lysine residues of parent protein often produced a complicated mixture of positional isomers,[13,14] which showed inconsistently therapeutic effects and was undesired in PEGylated pharmaceuticals.[14] In these cases, site-selective PEGylation of proteins was preferred and many of the important benefits could be achieved by conjugating PEG at the tailored sites of proteins. The designed polymer location chemistry, combined with efficiently chromatographic techniques, could yield the highly homogeneous, characteristic, and reproducible conjugate.[10]

To our knowledge, untill now, there is no systematic report on the development of PEGylated IL-1ra. To develop a favorable conjugate, therefore, the investigation on PEGylation of rhIL-1ra was conducted. In this work, we chose two PEG reagents to modify the rhIL-1ra: one was lysine-target succinimidyl ester of carboxymethylated PEG (SCM-PEG), and the other was thiol-target PEG derivatives with maleimide group (MBS-PEG). We purified the two monomeric conjugate, characterized their conjugation sites, investigated on their binding activities to the cellular receptors, and established the PEGylation strategy of rhIL-1ra.

2. Chemistry

The SCM-PEG was facilely achieved by activating CM-PEG into the corresponding succinimidyl ester (SCM-PEG, yield: 98%), which was well known to react preferentially with the free amino group. As PEG reagent, it was extensively used to modify protein in a random manner, especially targeted at the available lysine residues under basic conditions.

The synthesis routes of thiol-selective MBS-PEG were shown in Figure 1 (Graph a), which was achieved by further derivatization of the SCM-PEG. In that case, SCM-PEG was reacted with 1,6-hexanediamine to add a free amino group at the terminus of the polymer, which facilitated the subsequent introduction of MBS. In this step, however, the cross-linking reaction between SCM-PEG and unblocked 1,6-hexanediamine would occur as a major side reaction and produce white precipitation when they were simultaneously mixed. Therefore, an effective approach was to add dropwise SCM-PEG (dissolved in dried methylene chloride) into the free 1,6-hexanediamine under stirring. In this case, 1,6- hexanediamine always kept predominant excesses over SCM-PEG to minimize the cross-linking and to maximize the yield of mPEG-NH_2 (72.2%). Monitoring the reaction mixture by RP-HPLC, none cross-linking product was observed (Fig. 2, line b). Alternatively, the mPEG-NH_2 can be prepared by activating mPEG-OH by *p*-nitrophenyl chloroformate to then react with diamine.[15,16] However, the intermediate achieved by such means possessed a susceptible ester linkage, which would be unfavorable in the further derivatization reaction. The problem was resolved in this product, where stable amide linkage was constructed between CM-PEG and 1,6-hexanediamine.

The SCM-PEG was extremely reactive, and the traced amount of water in solvent would result in the rapid hydrolysis of a portion of active polymer to release the CM-PEG and hydroxysuccinimide. In the products, the hydrolyzed CM-PEG was detected in the resultant mPEG-NH_2 mixture (Fig. 2, line b). The macromolecular impurity had unfavorable interference in the conjugation of MBS-PEG to protein. Therefore, the purification procedure was necessary. The reaction mixture was first dialyzed against pure water to remove small molecules. Then the mixture was purified by cationic exchange resin. The charged mPEG-NH_2 was adsorbed on the resins, and non-charged CM-PEG flowed through the column. CM Sepharose Fast Flow resins performed well to achieve mPEG-NH_2 with a purity of >99% as shown by RP-HPLC (Fig. 2, line c).

MBS was a heterobifunctional reagent, the two-headed functionalities of which directed toward amino (hydroxysuccinimidyl ester, NHS) and thiol groups (maleimide, Mal) (Fig. 1, Graph a), respectively. The reaction between purified mPEG-NH_2 and MBS was readily conducted under neutral aqueous solution. The resulting MBS-PEG was achieved by removing free MBS using Sephadex G25 resin and lyophilization (yield: 92%). The method described was simple and effective to convert hydroxyl group of mPEG-OH to amino groups and to prepare the thiol-selective PEG reagent.

3. Results and discussion

3.1. Preparation and purification of SCM-PEG conjugate

SCM-PEG is notable as one of non-selective reagents to randomly react with lysine residues on the surface of protein under basic conditions and form stable amides (Fig. 1, Graph b) in PEGylation chemistry,[10] and used to prepare PEGylated rhIL-1ra derivative at amino-group. The conjugation reaction was conducted for 2 h at 4 °C and the reaction mixture was purified using SP Sepharose Fast Flow resin. The buffer of 40 mM NaAC-HAC (pH 5.0) was preferred as the equilibration solution in purification procedure. In those cases, both of unreacted protein and conjugates were adsorbed on the resin and the residual PEG reagent flowed through the column due to its weak interaction with the resins. Subsequently, the linear salt gradient of 0.4 M NaCl in NaAC-HAC (pH 5.0) over eight column volumes was developed to elute the bound proteins, and three fractions were achieved as *F*1, *F*2, and *F*3 (Fig. 3, Graph a). Due to non-baseline separation between *F*1 and *F*2, therefore, we only collected their peak summits to perform the following analysis.

3.2. Preparation and purification of MBS-PEG conjugate

The MBS-PEG possesses a maleimide group at its terminus, which specifically reacts with the thiol group of

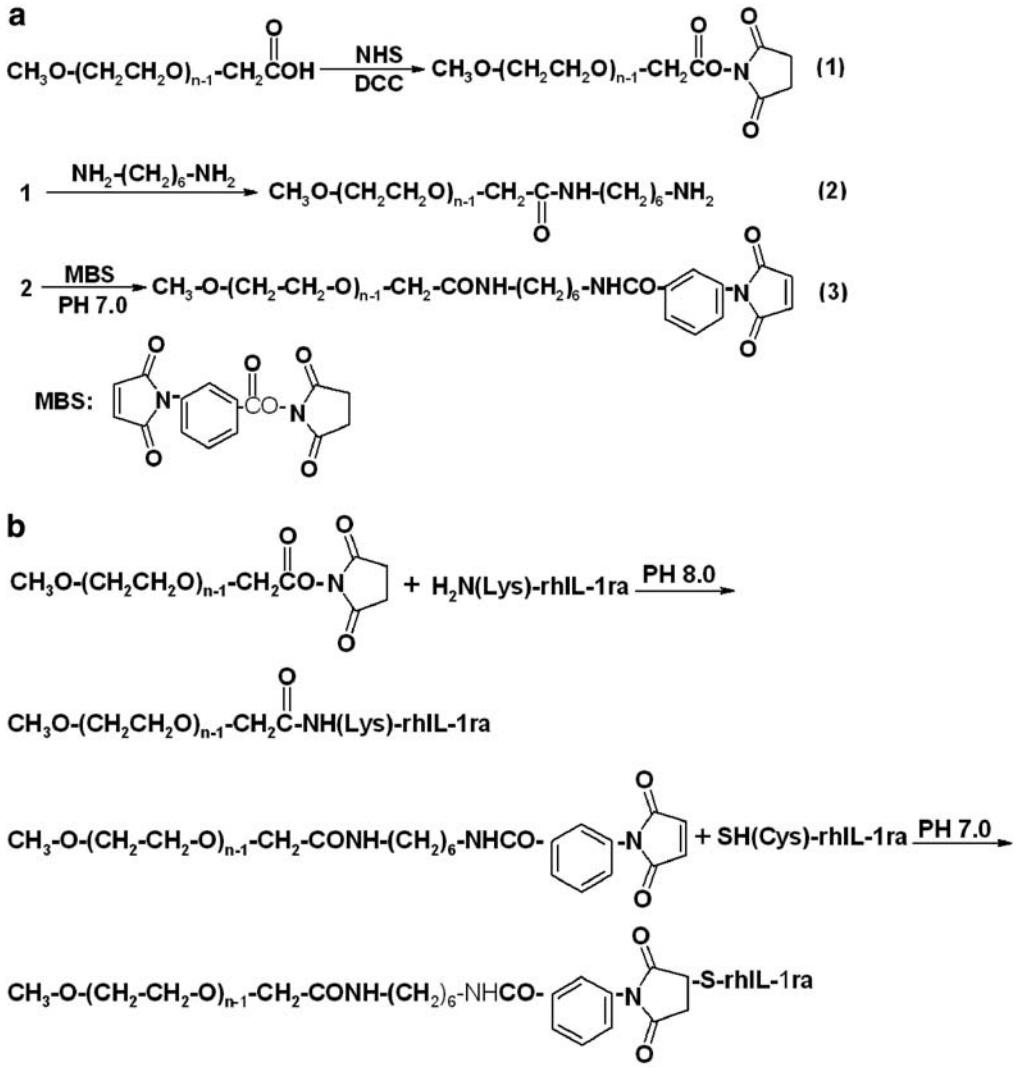

Figure 1. Synthesis of SCM-PEG and MBS-PEG and their conjugation to rhIL-1ra. Graph a: (1) Activation of CM-PEG into its hydroxysuccinimidyl ester (SCM-PEG); (2) reaction of SCM-PEG with diamine to add a free amino group at its terminal (mPEG-NH_2); (3) conjugation of mPEG-NH_2 to MBS to produce the thiol-selective reagent, MBS-PEG. MBS, 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester. Graph b: Scheme of random and thiol-selective conjugation of PEG reagents to rhIL-1ra. The thioether linkage was formed in thiol-selective conjugate and amide structure was produced in random species.

proteins especially at neutral pH and forms the stable thioether linkage (Fig. 1, Graph b) and is used widely to derivatize varying forms of PEGs for cysteine modification of proteins in PEGylation chemistry.[17] Here, the MBS-PEG was used to prepare the thiol-selectively PEGylated rhIL-1ra derivative. The conjugation reaction was performed for 4 h at 4 °C and the reaction mixture was purified using SP Sepharose Fast Flow resins as mentioned above. Two fractions were achieved as *P*1 and *P*2 in a baseline resolution when the linear salt gradient of 0.4 M NaCl in NaAC-HAC (pH 5.0) over six column volumes was developed (Fig. 3, Graph b).

3.3. Characterization of two PEGylated rhIL-1ra derivatives

The fractions *F*1 and *F*2 showed the tense and single band on the SDS–PAGE at the position of approximate 43 kDa and 32 kDa, which, respectively, corresponded to the di- and mono-PEGylated conjugate (Fig. 4, Graph a, lanes 1 and 2). The *F*3 showed a single and tense band on the SDS–PAGE at the position of approximate 22 kDa (Fig. 4, Graph a, lane 3), which had similar migration rate to that of wild rhIL-1ra (Fig. 4, Graph a, lane 5) and was determined to be unreacted protein.

Similarly, the fraction *P*1 was assigned to be mono-PEGylated derivatives (Fig. 4, Graph b, lane 3), and *P*2 was to unreacted protein (Fig. 4, Graph b, lane 4) according to their relative positions in the gel. In order to perform side-by-side comparison, only the mono-PEGylated derivatives were used as the source materials for the following studies.

The monomeric conjugates were further characterized by size exclusion chromatography (SEC). Either random (a) or thiol-selective (b) derivative showed the single and

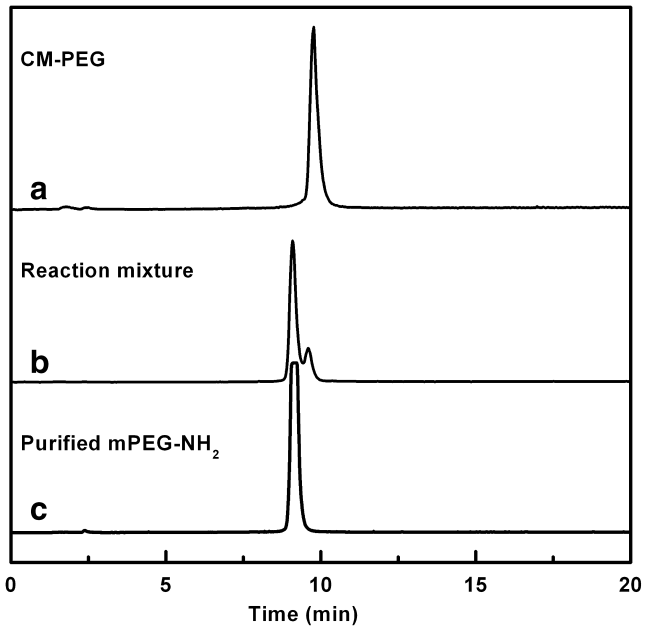

Figure 2. RP-HPLC profiles of PEG derivatives (symmetry shield RP18 column, 3.9 × 150 mm id). A 5 μl volume of each sample (w/v, 5 mg/ml) was injected and the time program was designed such as 20–35% B in 5 min, 35–50% B in 15 min, 50–100% B over 5 min. Solvent A consisted of 0.1% TFA in ultra-pure water and solvent B was 0.1% TFA in acetonitrile. Detector: ELSD. (a) CM-PEG; (b) reaction mixture; (c) purified mPEG-NH_2.

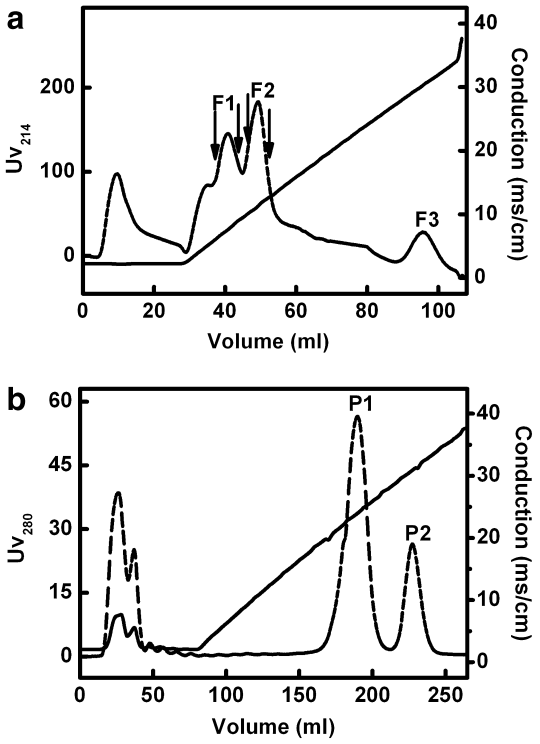

Figure 3. Purification profiles of the PEGylated rhIL-1ra derivatives. Conjugation reaction mixture was adjusted to pH 5.0 and applied to the column packed with SP Sepharose Fast Flow resin, which was pre-equilibrated with 0.04 M sodium acetate (buffer A, pH 5.0). After washed with buffer A until the conduction returned to baseline, the column was developed with a linear gradient to 40% of the same buffer containing 1.0 M sodium chloride (buffer B, pH 5.0) over eight column volumes to achieve *F*1, *F*2, and *F*3 and over six column volumes to obtain *P*1 and *P*2. Graph a, flow rate is 1.5 ml/min, absorbance was recorded at 214 nm, UV_{214} (---) and conduction (—), fractions were collected between arrows for *F*1 and *F*2; Graph b, the flow rate is 1.0 ml/min, absorbance was recorded at 280 nm, UV_{280} (---) and conduction (—).

symmetrical peak on SEC chromatogram (Fig. 5). Both techniques suggested that the conjugates had the purity of >95%, which provided good conditions for the side-by-side PK studies on bioactivities of derivatives.

3.4. Analysis of location site in SCM-PEG conjugate

Due to the random attachment of polymer and highly similar properties of positional isomers in non-selective conjugate, the precise determination of conjugation sites was difficult to conduct.[10,13,14] However, it could be readily determined whether polymer was only conjugated at lysine residues in the species. The fluorescamine assay was usually used to determine the modification degree of proteins' primary amines when multiple PEG strands were attached in non-selective manner such as ADAGEN and Pegvisomant.[18,19] In this case, the method was exquisitely employed to corroborate the modification of polymer at lysine residues in the conjugate. The results suggested that the attachment of PEG resulted in substitution of 10.4% of primary amino groups in the monomeric conjugate, which corresponded to an average attachment of 1.04 PEG strand per derivative (rhIL-1ra contains nine lysine residues and one N-terminus amino group). Namely, each rhIL-1ra was attached with 1.04 polymer strand in the monomeric conjugate, which suggested that the polymer was only located at the lysine residues of rhIL-1ra.

3.5. Analysis of location site in MBS-PEG conjugate

RhIL-1ra contains four free cysteines, all of which have the possibility to be modified by the polymer strand in the conjugation reaction. In order to determine the location site of polymer in the thiol-target conjugate, the species was digested by trypsin and the PEGylated peptide fragments were analyzed employing MALDI-TOF techniques, with the digestion of unreacted protein as reference. The rhIL-1ra was digested by trypsin into ten peptide fragments with various molecular weights, the mass of which was analyzed by LC–MS, and labeled from T1 to T10, respectively (see Table 1). The longest peptide fragment (T9) contributed to the mass of 4510.06 Da and the smallest peptide fragment (T8) to the mass of 652.79 Da. Therefore, the mass of the PEGylated peptide was always larger than 5000 Da (calculated mass of PEG-MBS: 5309 Da).

The scan was performed in the mass range of 5000–15,000 Da, and only a broad and bell-shaped peak with the mass of 10,465 Da was presented on the MALDI-TOF chromatogram in the digested products of thiol-selective conjugate (Fig. 6). The peak was easily judged to be the PEGylated peptide due to the well-known polydispersal feature of PEG. The peak showed the molecular weight of 10,465 Da, which was similar to

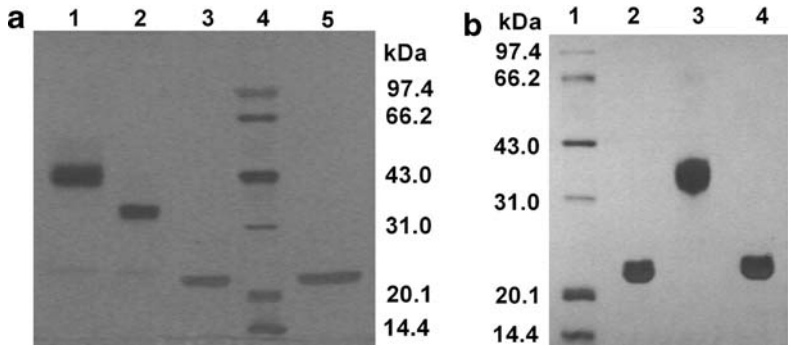

Figure 4. Non-reducing SDS–PAGE analysis of purified conjugates. Samples (about 10 μg) were electrophoresed on a separation gel of 12% concentration and stained with silver. Graph a: lane 1, *F*1 (dimeric non-selective conjugate); lane 2, *F*2 (monomeric non-selective conjugate); lane 3, *F*3 (unreacted rhIL-1ra); lane 4, molecular weight markers; lane 5, wild rhIL-1ra; Graph b: lane 1, molecular weight markers; lane 2, wild rhIL-1ra; lane 3, *P*1 (monomeric thiol-selective conjugate); lane 4, *P*2 (unmodified rhIL-1ra).

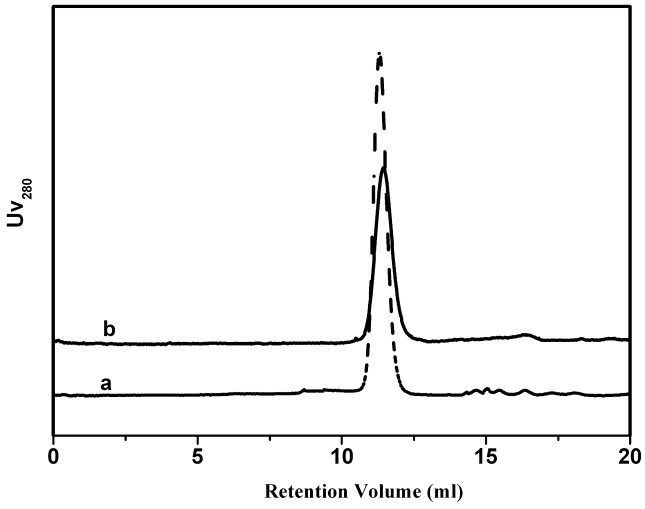

Figure 5. Size exclusion HPLC of conjugate (Superdex 75, 1.0 × 30 cm id, 50 mM sodium phosphate containing 0.15 M sodium chloride, pH 7.0, 0.5 ml/min). Absorbance was recorded at 280 nm. (a) Non-selective conjugate; (b), thiol-selective conjugate.

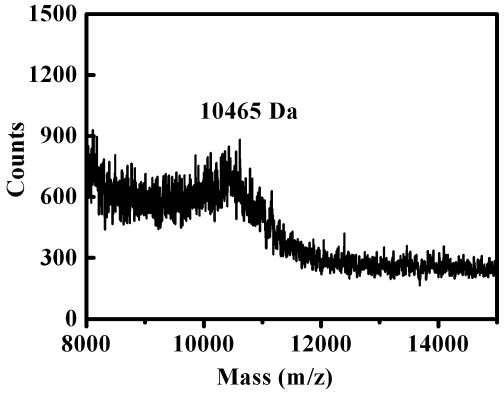

Figure 6. MALDI-TOF mass spectrum of the conjugate peptide fragment. The mass spectrum was obtained on a Voyager DE Pro mass spectrometer (Applied Biosystem, Foster City, USA) equipped with a pulsed nitrogen laser (337 nm, 3-ns pulse) in the positive linear mode at 25 kV. The matrix α-cyano-4-hydroxycinnamic acid was used in a saturated solution of 50% acetonitrile and 0.1% trifluoroacetic acid.

that of T8 + T9 + MBS-PEG (10,472 Da). Therefore, the polymer strand was determined to be attached at the cysteine in T9 fragment. Further digestion was not carried out to determine the position of either 116 or 122 in T9 by other enzymes due to hindrance of polymer. T8 was close to T9 in the position in the denatured protein, and it failed to be digested by trypsin from T9 fragments. This suggested that polymer had significant steric hindrance to neighbor groups, and it was difficult for trypsin to access the Arg site between T8 and T9. Similarly, the results also indicated that the steric hindrance of polymer might greatly interfere with the acces-

Table 1. Tryptic fragments of unmodified and thiol-specific PEGylatd rhIL-1ra

Amino acid no.	Tryptic peptide	Calculated mass (Da)	Observed mass (Da)	Sequence
rhIL-1ra				
16–22	T1	902.02	902.54	IWDVNQK
23–27	T2	698.82	699.53	TFYLR
28–46	T3	2100.32	2102.70	NNQLVAGYLQGPNVNLEEK
47–65	T4	2012.38	2011.20	IDVVPIEPHALFLGIHGGK
66–72	T5	783.05	783.50	MCLSCVK
73–78	T6	663.64	661.68	SGDETR
79–93	T7	1714.89	1717.18	LQLEAVNITDLSENR
99–103	T8	652.79	653.47	FAFIR
104–146	T9	4510.06	4513.77	SDSGPTTSFESAACPGWFLCTAMEADQPVS LTNMPDEGVMVTK
147–153	T10	977.00	977.58	FYFQEDE
Thiol-target conjugate		10,472	10,465	T8 + T9 + MBS-PEG

sion of conjugate to the receptors and lead to the significant decrease or loss of binding to the receptors, when the polymer was located at or near active sites.

3.6. The binding activity of SCM-PEG conjugate to the receptors

The binding activity of SCM-PEG conjugate to the receptors was determined to be 9.8% (0.95×10^4 UI/mg) of wild rhIL-1ra (9.72×10^4 UI/mg), employing EL-4/CTLL-2 cells. The decrease of bioactivity was significantly marked, and the random attachment of PEG resulted in the 10.2-fold decrease of the binding activity of protein. The great loss of activity often resulted from the occupation of important lysine residues such as those of lysozyme and interleukin-15 in the random manner.[11,12] For rhIL-1ra, there were nine lysine residues in the 153-amino acid protein, and they were, respectively, located at the position of 6, 9, 21, 45, 64, 71, 93, 96, and 145 of rhIL-1ra (the methionine added at the N-terminus of protein was denoted as the position of 0 in the recombinant protein). Among them, the lysine residue at the position of 145 (K145) was involved in the VTKFYF motif of rhIL-1ra, which competed with IL-1 for the cellular receptors.[20] In addition, the mutation of K145 in IL-1ra into Asp residue produced an analog (IL-1raK145R) with partial agonist activity.[21] The lysine at the position of 21 (K21) was also close to the interaction domain encircled by five residues W16, Q20, Y34, Q36, and Y147 (Fig. 7), which was involved in the interaction with the receptors and their mutagenesis would result in reduced binding activities to the receptors.[20,22,23] When the polymer was attached at K145 or K21, the resultant conjugates might lose the capability of accessing to and binding with the receptors. For some lysines far from the binding domain of protein, it was possible that the unfavorable conformations were formed and the polymer adversely masked the hydrophobic patches of the conjugate.[20,22,24] The non-specific shield hindered the interaction of protein with receptors and caused the significant decrease of activities of the isomers. And the binding activity of the SCM-PEG conjugate was mainly contributed by the rest isomers, in which the polymer was free from the shield of hydrophobic patches and from the occupation of sites near active sites of rhIL-1ra. Unfortunately, an endeavor to separate the positional isomers failed using high-performance Mono S column (HR 5/5, 5 mm × 5 cm id, GE Healthcare, USA) under a very slow gradient, which was attributed to the high similarity of properties of isomers (data not shown). The low remaining bioactivity suggested that random conjugation of PEG to rhIL-1ra was undesirable.

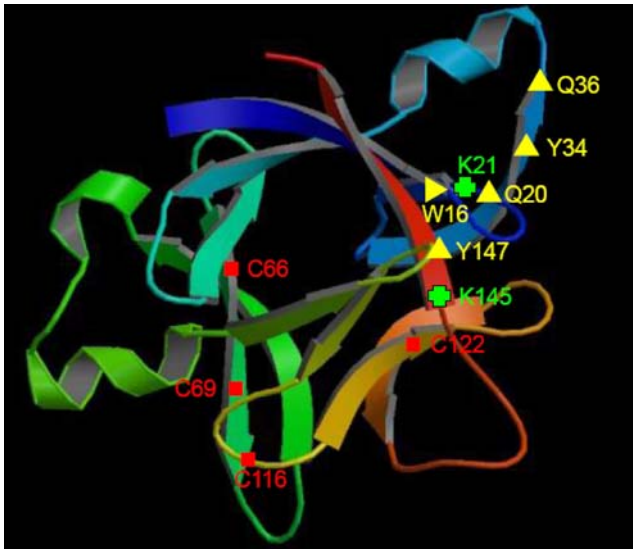

Figure 7. Crystal structure model of interleukin-1 receptor antagonist (IL-1ra) (required from PDB, ID:1ILT). Five active sites of IL-1ra were labeled with the signal (▲), four cysteines with (■), K21 and K145 with (■) along with their approximate position at the primary sequence of IL-1ra.

3.7. The binding activity of MBS-PEG conjugate to the receptors

The binding activity of MBS-PEG conjugate to the receptors was determined to be 40% (3.89×10^4 UI/mg) of native rhIL-1ra (9.72×10^4 UI/mg). The results suggested that the attachment of polymer at the cysteine of rhIL-1ra only had moderate effects on the binding to the receptors and only 2.5-fold decrease of activity was shown. The decrease of bioactivities was very common in PEGylated proteins, which generally resulted from the steric hindrance effect of the polymer. The bioactivity might be attributed by one isomer, in which polymer was only attached to any of C116 and C122, and the other cysteine was inaccessible due to the spatial conformation of protein. If it was the case, thiol-target derivative was highly homogeneous. On the other hand, it was also possible that the derivative was composed of two isomers, in which polymer was, respectively, attached to C116 and C122. In two derivatives, one retained very low activity and the other had relatively high activity, and the 40% bioactivity was the mean value of activities of two isomers. No matter in what way the polymer was attached, the conjugation showed moderate effects on the bioactivities of conjugate and rendered the derivative more appealing activity, compared with random conjugation.

The decreased in vitro bioactivity could be greatly comprised by the increased stability and prolonged residence time in vivo of pharmaceuticals. For instance, the PEGasys only retained 7% in vitro bioactivity of unmodified protein, however, the serum half-life was significantly prolonged (from 72–96 to 6–9 h).[25] Therefore, although the thiol-target conjugation of polymer caused moderate decrease of activity on rhIL-1ra, it might render protein a long-acting half-life in vivo.

3.8. Establishment of PEGylated strategy for rhIL-1ra

The thiol-target rhIL-1ra derivative could be achieved as relatively homogeneous (only two/one conjugation sites), characteristic, and reproducible conjugate and retained more appealing binding activity to the receptors. In contrast, the non-selective counterpart might be composed of complicated ingredients and showed lower

Table 2. Comparison of physicochemical properties of lysine-target and thiol-target PEGylated rhIL-1ra

	Lysine-target conjugate	Thiol-target conjugate
Reaction buffer	Sodium borate (50 mM, pH 8.0)	Sodium phosphate (50 mM, pH 7.0)
PEG reagents	SCM-PEG	MBS-PEG
Mole ratio of PEG/protein	7.9	2.45
Linkage	Stable amide	Stable thioether
Product ingredient	Di- and mono-PEGylated derivatives	Mono-PEGylated derivative
Target product	Monomeric conjugate	Monomeric conjugate
Product purity	>95%	>95%
Conjugation site	Lysine residues	Cysteine residues
Possible isomers	K6, K9, K21, K45, K64, K71, K93, K96, and K145	C116 and C122
Binding activity	9.8%	40%

binding activity (see Table 2). Therefore, the thiol-selective conjugate was more promising to serve as an appealing candidate of parent protein, compared to the random species. The pharmacokinetic and pharmacodynamic assay of the conjugate is in progress.

4. Conclusion

In summary, the tailored conjugation of polymer rendered the protein more appealing bioactivity, whereas random conjugation gave relative low bioactivity. The tailored sites, far from active domain of rhIL-1ra, may be responsible for the moderate decrease of bioactivity of thiol-target derivative. In contrast, the attachment of polymer to the lysine residues within or near active domain of protein or the formation of unfavorable conformation may be correlated to the great loss of bioactivity of counterpart derivative. The results indicated that thiol-target conjugate was much potent to be explored into a long-acting candidate of rhIL-1ra.

5. Experimental

5.1. Materials

NMR spectra were recorded on a Bruker AVANCE 400 (400 MHz) with chemical shifts δ in ppm. All reagent-grade chemicals were purchased from Beijing Chemical Reagents Co., (Beijing, China) except for those specified. The mPEG of molecular weight 5000, 1,6-hexanediamine, *N*-hydroxysuccimide (NHS), *N*-dicyclohexylcarbodiimide (DCC), and fluorescamine were acquired from Fluka Chemicals (Ronkonkoma, NY). The 3-maleimidobenzoic acid *N*-hydroxy-succinimide ester (MBS) was obtained from pierce Biotechnology (Rockford, IL). RPMI 1640, calcium ionophore A23187, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and α-cyano-4-hydroxycinnamic acid (α-CHCA) were purchased from Sigma Chemicals (St. Louis, MO). Carboxymethylated monomethoxypolyethylene glycol (CM-PEG) was prepared as previously described.[26] EL-4 cells and CTLL-2 cells were from Cell Bank of Chinese Academy of Sciences (Shanghai, China). The rhIL-1ra was prepared as previously described with a purity of >95%.[27] All aqueous solutions were prepared with ultra-pure water (Millipore, Bedford, MA).

5.2. Activation of CM-PEG into SCM-PEG

CM-PEG (5 g, 1 mmol) was dissolved in 30 ml of dried methylene chloride. To this solution was added *N*-hydroxysuccinimide (138 mg, 1.2 mmol) and stirred in the ice-water bath for 1 h. *N*-Dicyclohexylcarbodiimide (216 mg, 1.05 mmol) was subsequently added to this solution. The reaction mixture was allowed to stand overnight at room temperature. The dicyclohexyl urea was removed by filtration and the succinimidyl ester of CM-PEG (SCM-PEG) was precipitated with cold ether and purified by recrystallization from isopropyl alcohol and dried in vacuum (yield: 4.9 g, 98%). The SCM-PEG was used as non-selective reagent of rhIL-1ra and the intermediate to synthesize mPEG-NH_2 as follows. ^{1}H NMR (chloroform-d, 400 MHz), δ (ppm): 3.40 (s, 3H, PEG CH_3O—CH_2—CH_2—O—); 3.61 (s, multi H, PEG backbone —OCH_2CH_2—); 2.84 (s, 4H, —CO—CH_2—CH_2—CO— of NHS); 4.15 (s, 2H, PEG-O—CH_2COO-NHS).

5.3. Synthesis and purification of mPEG-NH_2

The 1,6-hexanediamine (2.3 g, 20 mmol) was dissolved in 10 ml of dried methylene chloride. To this solution was added dropwise the SCM-PEG (4.5 g, 0.9 mmol, dissolved in 10 ml of dried methylene chloride). The reaction mixture was allowed to stand for 30 min at room temperature. The solution was filtered, concentrated, and the mPEG-NH_2 was precipitated with cold ether (yield: 3.25 g, 72.2%). The resulting polymer was redissolved in purified water (w/v, 4%), then filtered and exhaustively dialyzed (cutoff molecular weight of bag filter: 3400 Da) against pure water.

The mPEG-NH_2 solution was further purified on the CM Sepharose Fast Flow resin (26 × 500 mm id, GE Healthcare, USA). The column was fully equilibrated with 8 mM borate buffer (pH 8.5) and 3.2 g of reaction polymer mixtures was loaded. The acidic CM-PEG flowed through the column and basic PEG-NH_2 was absorbed on the resin under those conditions, which was revealed by iodine assay.[28] The PEG-NH_2 was eluted with 20 mM NaCl and extracted with methylene chloride. The desired polymer was precipitated with cold ether with a yield of 61% (1.95 g). The product was subjected to analysis by reversed-phase high-performance liquid chromatography (RP-HPLC). ^{1}H NMR (chloroform-d, 400 MHz), δ (ppm): 3.40 (s, 3H, PEG

$CH_3O—CH_2—CH_2—O—$); 3.59 (s, multi H, PEG backbone $—OCH_2CH_2—$); 4.21 (s, 2H, PEG-$O—CH_2CO—NH$); 8.01 (t, 1H, $—CO—NH—$); 2.95 (m, 2H, $—CONH—CH_2—$); 2.17 (s, 2H, $—CH_2—NH_2$); 1.25 (m, 2H, 1,6-hexanediamine γ CH_2); 1.58 (m, 2H, 1,6-hexanediamine β CH_2).

5.4. Synthesis of MBS-PEG

Purified mPEG-NH_2 (100 mg, 20 μmol) was dissolved in 2.0 ml of phosphate buffer salt (50 mM, pH 7.0). To this solution was added 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester solution (MBS, 7.0 mg, 24 μmol) dissolved in *N*,*N*-dimethylformamide (DMF, 30 μl). The reaction was allowed to stand for 30 min at 30 °C. The resultant MBS-PEG was purified from free MBS by size exclusion chromatography using a Sephadex G-25 column (16 × 120 mm id, GE Healthcare, USA), and ultra-pure water was eluant. The MBS-PEG eluted at the void volume of the column was collected and immediately lyophilized to yield a yellowish powder (yield: 92 mg, 92%). 3.41 (s, 3H, PEG $CH_3O—CH_2—CH_2—O—$); 3.59 (s, multi H, PEG backbone $—OCH_2CH_2—$); 4.18 (s, 2H, PEG-$O—CH_2CO—NH$); 7.92 (m, 2H, $—CO—NH—$); 3.15 (m, 4H, $—CONH—CH_2—$); 1.29 (m, 2H, 1,6-hexanediamine γ CH_2); 1.56 (m, 2H, 1,6-hexanediamine β CH_2); 7.85 (m, 4H, $—C_6H_4—$); 6.75 (s, 2H, $—N—CH=CH—N—$ of maleimide group).

5.5. Reversed-phase high-performance liquid chromatography (RP-HPLC)

RP-HPLC of various forms of polymers was conducted on an Agilent 1100 HPLC System (Agilent Technologies, Palo Alto, CA) equipped with evaporation and light scattering detection (ELSD). Nitrogen was used as carrier gas, and the pressure at the nebulizer was set to 2.0 bar. The temperature of the evaporator oven was 40 °C. The Symmetry Shield RP18 column (3.9 × 150 mm id, Waters, USA) was equilibrated with 80% solvent A (0.1% trifluoroacetic acid in ultra-pure water) and 20% of solvent B (0.1% trifluoroacetic acid in acetonitrile) at the flow rate of 0.5 ml/min. Five microliters of each sample (w/v, 5 mg/ml) was injected and the elution program was set up as 20–35% B in 5 min, 35–50% B in 15 min, 50–100% B over 5 min.

5.6. Preparation of non-selective rhIL-1ra conjugate

RhIL-1ra (6.3 mg, 0.364 μmol) at 2.0 mg/ml in 0.05 M sodium borate (pH 8.0) was modified with 2.88 μmol mPEG-NHS. The reaction was conducted at 4 °C for 2 h and then the solution was brought to pH 5.0 with 0.5 M acetic acid. The conjugate was purified by cationic exchange chromatography on SP Sepharose Fast Flow resin, using an AKTA Purifier 10 Protein Purification Systems (GE Healthcare, USA). The XK 16/30 column (16 × 120 mm id, GE Healthcare, USA) was equilibrated with buffer A (0.04 M sodium acetate, pH 5.0) for three column volumes at a flow rate of 1.0 ml/min and the conjugation mixture was loaded. After the column was washed with buffer A until the conduction returned to baseline, the column was developed with a linear gradient to 40% buffer B (buffer A containing 1.0 M sodium chloride, pH 5.0) over eight column volumes. The peak fractions were collected, concentrated with Amicon ultrafiltration cell (YM 10 membrane, Beverly, MA), and determined by non-reducing SDS–PAGE.

5.7. Preparation of thiol-selective rhIL-1ra conjugate

RhIL-1ra (20 mg, 1.14 μmol) at 2.0 mg/ml in 0.05 M sodium phosphate (pH 7.0) was modified with 2.8 μmol MBS-PEG. The reaction was conducted at 4 °C for 4 h and then the solution was brought to pH 5.0 with 0.5 M acetic acid. The conjugate was purified by cationic exchange chromatography as mentioned above. The XK 26/30 column (26 × 10 mm id, GE Healthcare, USA) was equilibrated with buffer A for three column volumes and the conjugation mixture was loaded, at a flow rate of 1.5 ml/min. After the column was washed with buffer A until the conduction returned to baseline, the elution was applied with a linear gradient to 40% buffer B over six column volumes. The peak fractions were collected, concentrated with Amicon ultrafiltration cell (YM 10 membrane, Beverly, MA), and determined by non-reducing SDS–PAGE.

5.8. Non-reducing SDS–PAGE

Non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli.[29] The total polyacrylamide concentration was 5% in the stacking gel and 12% in the separating gel with gel thickness of 0.75 mm. Samples (20 μl, about 10 μg) were mixed with 5-fold oxide buffer (5 μl), boiled for 5 min, and loaded into each well using Mini-Protein III (Bio-Rad, USA). Silver staining was used to visualize the proteins.

5.9. Size exclusion chromatography

High-performance size exclusion chromatography (HP-SEC) was performed on an AKTA purifier 10 Protein Purification Systems (GE Healthcare, USA). Samples were analyzed with an isocratic mobile phase of 0.05 M phosphate buffer containing 0.15 M sodium chloride, pH 7.0, on a Superdex 75 column (10 mm × 300 mm id, GE Healthcare, USA). The flow rate was 0.5 ml/min and the absorbance at 280 nm was monitored.

5.10. Fluorescamine assay

The assay was performed as described by Stocks et al.[30] Samples (1.0 mg/ml, 10 μl), 50 mM phosphate buffer (pH 7.4, 200 μl), double distilled water (90 μl), and fluorescamine solution (1 mM fluorescamine in acetonitrile, 100 μl) were mixed and incubated for 10 min in the dark. Fluorescene was measured at excitation/emission wavelengths of 390/490 nm on Hitach F-4500 fluorescence spectrometer (Hitachi, Japan). The unmodified rhIL-1ra containing nine lysine residues was used as reference, the number of free amino groups of the monomeric random conjugate was determined.

5.11. Tryptic peptide mapping of conjugate

Site-selectively PEGylated and native rhIL-1ra solutions (1.0 mg/ml, 0.5 ml) were dialyzed against 0.1 M Tris–HCl containing 2.0 M urea at pH 8.0. The digestion of samples was initiated by the addition of trypsin (5 μl of 1.0 mg/ml solution in purified water) and the mixture was incubated at 37 °C for 4 h. A second equal aliquot of trypsin was then added and the incubation was continued at 37 °C overnight. The analysis of the rhIL-1ra digest was subsequently conducted on an Agilent 1100 system (Agilent Technologies, Palo Alto, CA) equipped with a diode array detector (DAD). A Vydac C4 reversed-phase column (4.6 × 250 mm id, Hisperia, USA) was equilibrated with 90% solvent A (0.1% TFA in ultra-pure water) and 10% solvent B (0.1% TFA in acetonitrile) at a flow rate of 0.75 ml/min. The eluted gradient was set up as 10–80% B over 80 min. The outlet of the column was connected with the ion source of mass spectrometer (Finnigan LCQ ion trap mass spectrometer, Thermo electron, San Jose, CA) equipped with an electrospray ionization source. The spray voltage was 3.5 kV, the heat capillary was kept at 275 °C, and the scan range was set from *m*/*z* 300 to 2000. The zoom scan and tandem MS (MS/MS) functions were performed in data dependent mode. The collision energy value was 35–40%.

5.12. MALDI-TOF

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) was performed to analyze the PEGylated peptides of thiol-selective conjugate. It was conducted on a Voyager DE Pro mass spectrometer (Applied Biosystem, Foster City, USA) equipped with a pulsed nitrogen laser (337 nm, 3-ns pulse), operating in the positive linear mode. The accelerating voltage in the ion source was 25 kV. Time-to-mass conversion was achieved by the calibration using Mass Standard Kits. The α-cyano-4-hydroxycinnamic acid (α-CHCA) was used as matrix in a saturated solution of 50% acetonitrile and 0.1% trifluoroacetic acid. Digested thiol-selective conjugate was dropped onto the target plate (5 μl) and subsequently the equal aliquot of matrix solution was added to produce protein/matrix crystal.

5.13. Receptor-binding assay

The competitive receptor-binding assay was performed in two stages as described before.[31,32] Briefly, EL-4 cells were washed three times with RPMI 1640 and adjusted to 2×10^6 cells/ml with RPMI 1640 containing 5% fetal calf serum. Each well of 96-well plates was distributed 100 μl of cells and serial 2-fold dilutions of samples in RPMI 1640 containing 5% foetal calf serum were, respectively, added in triplicate to the wells of a 96-well plate. The volume was made up to 200 μl. Calcium ionophore A23187 was dissolved in dimethylsulfoxide with a concentration of 1×10^{-3} M and diluted into a concentration of 1×10^{-6} M with RPMI 1640, and added to each well (50 μl). The cells were harvested after 12-h incubation at 37 °C and 5% CO_2 and centrifuged at 2000 rpm for 10 min. 100 μl of culture supernatants from each well was transferred to a second 96-well plate.

The CTLL-2 cells were washed to remove any IL-2 and resuspended in RPMI 1640 containing 5% fetal calf serum at 1×10^5 cells/ml. 100 μl of CTLL-2 cell suspension was added to each well of the second 96-well plate. After cultured for 20 h, to each well was added 5 μl of MTT (10 mg/ml, dissolved into phosphate buffer salt) and the plate was incubated for a further 4 h. The proliferation was assessed using the colorimetric methods as described by Mosmann.[33] The concentrations of various forms of rhIL-1ra were determined by Bradford assay with a Hitachi U-3200 spectrophotometer at 595 nm,[34] using bovine serum albumin as reference.

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