Bioconjugate Chemistry

Review

Cite This: *Bioconjugate Chem.* 2019, 30, 1604−1616

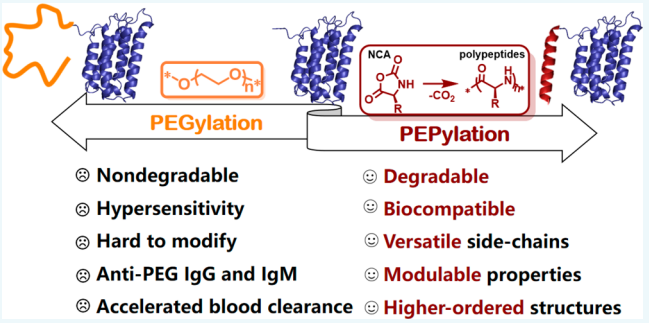
pubs.acs.org/bc

Protein PEPylation: A New Paradigm of Protein−Polymer Conjugation

Yingqin Hou[†] and Hua Lu*[,†]

[†]Beijing National Laboratory for Molecular Sciences, Center for Soft Matter Science and Engineering, Key Laboratory of Polymer Chemistry and Physics of Ministry of Education, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, People's Republic of China

ABSTRACT: Various polymers have been tested for protein conjugation with a goal of bridging the complementary advantages of both components. However, many of these polymers, including the most well-established PEG, are nondegradable, which raises potential concerns on their cumulative chronic toxicity. Moreover, the immunogenicity of PEG has recently evoked considerable controversy. Synthetic polypeptides, on the other hand, are biomimetic polymers with tunable degradability, versatile side chain functionalities, unique secondary structures, and fascinating self-assembly behaviors. These properties have made them promising materials in protein modification for various applications. In this Topical Review, we summarize recent advances and list a number of interesting future directions in protein−polypeptide conjugation, which we termed protein PEPylation.



INTRODUCTION

Proteins are biological macromolecules with their versatile functions mostly determined by their hierarchical structures. Because of their high potency and selectivity, proteins are extremely important in both industry and biomedicine.[1−6] Nevertheless, the biological activities of proteins are often vulnerable to environmental stresses including ionic strength, temperature fluctuation, pH variation, and organic solvents. This fragility necessitates special formulation and caution, and thus increased cost in the storage, transportation, and handling of proteins.[7] *In vivo*, many protein therapeutics suffer from rapid proteolytic degradation, renal filtration, and reticuloendothelial system (RES) clearance. Moreover, repeated administration of therapeutic proteins often elicits adaptive immune responses and the generation of antidrug antibodies (ADAs).[8−10] There has been strong clinical evidence correlating the lost efficacy and/or hypersensitivity of many monoclonal antibodies (mAb) with the presence of ADAs, even for those with humanized sequences. One vivid example is Infliximab (brand name Remicade), one of the top 10 drugs by global sales targeting the tumor necrosis factor-α (TNF-α) for various inflammatory diseases. It was reported that up to 51% patients receiving this chimeric antibody were found to develop ADAs, which were believed to associate with clinically observed hypersensitivity and loss of therapeutic response.[11] To this end, engineering approaches that can impart proteins enhanced stability and stealthy property *in vitro* and *in vivo* are highly desirable.

Polymer conjugation is one of the many approaches for protein modification. Synthetic polymers are usually inexpensive, highly stable, and more importantly, readily tunable, which are in sharp contrast to proteins.[12,13] Both the structures and properties of many synthetic polymers can be made responsive under variable external stimuli (e.g., pH, temperature, redox, electromagnetic field, and/or enzyme).[14−16] Protein−polymer conjugates (PPCs), developed with a goal of bridging the complementary advantages of both components, are thus widely exploited as hybrid materials and drugs for applications in biomedicine, biotechnology, and nanotechnology.[17−20] Particularly, protein PEGylation (i.e., the conjugation of polyethylene glycol (PEG) to proteins) has gained vast success in long-acting drugs.[21] Beyond that, the research field of protein−polymer conjugates has expanded enormously over the decades, ranging from the development of diversiform site-specific synthetic approaches, introduction of advanced characterization methods, to seeking alternatives to PEGylation for various industrial and biological applications. Many of these topics have been thoroughly discussed in recent excellent review articles.[22−30] Instead of duplicating with those previous works, this Topical Review paper will briefly revisit the achievements and limitations of traditional PEGylation, and mainly focus on recent advances in protein PEPylation, a term describing the modification of proteins with synthetic polypeptides (also known as poly(α-amino acid)s).

Achievements and Limitations of PEGylation. PEGylation has been the gold standard in protein−polymer conjugation. PEG is a unique synthetic polymer possessing the following properties: (1) cheap, narrowly dispersed, and

Received: March 31, 2019
Revised: May 1, 2019
Published: May 2, 2019

DOI: 10.1021/acs.bioconjchem.9b00236
Bioconjugate Chem. 2019, 30, 1604−1616

Table 1. FDA Approved PEGylated Protein Drugs

	PEG(M_w: Da)	protein	disease	conjugation site[a]	approved time
Adagen	5000	Adenosine deaminase	Severe combined immunodificiency (SCID)	amino group of lysine and/or the N-terminus	1990
Oncaspar	5000	Asparginase	Acute Lymphoblastic Leukemia (ALL)	amino group of lysine and/or the N-terminus	1994
PEG-lntron	12000	IFNα-2b	Hepatitis C	amino group of lysine or the N-terminus	2001
Pegasys	40000	IFNα-2a	Hepatitis C	amino group of lysine or the N-terminus	2002
Neulasta	20000	G-CSF	Neutropenia	N-terminal methionyl residue#	2002
Somavert	5000	Anolog of hGH	Acromegaly	amino group of lysine and/or the N-terminus	2003
Mircera	30000	EPO-β	Anemia Due To chronic kidney disease	amino group of lysine and/or the N-terminus	2007
Cimzia	40000	Anti-TNF Fab	Rheumatoid arthritis and Crohn's disease	Cysteine	2009
Krystexxa	10000	Recombinant uricase	Chronic gout	amino group of lysine and/or the N-terminus	2010
Sylatron	12000	IFNα-2b	Melanoma	amino group of lysine or the N-terminus	2011
Omontys	40000	Esatide	Anemia Due to Chronic Kidney Disease	ε-amino group of the C-terminal lysine#	2012
Plegridy	20000	IFNβ-1a	Multiple sclerosis	Amino group of the N-terminus#	2014
Adynovate	20000	Antihemophilic Factor VIII (Recombinant)	Hemophilia A	Amino group of lysine and/or the N-terminus	2015
Palynziq	20000	Recombinantphenylalanine ammonialyase	Phenylketonuria	Amino group of lysine and/or the N-terminus	2018
JIVI	60000	Antihemophilic Factor VIII (Recombinant)	Hemophilia A	K1804C#	2018

[a]NOTE: # indicates site-specific conjugation.

ease in large scale synthesis; (2) nonionic and neutral; (3) high hydration capacity: each repeating unit of PEG can coordinate ~6−7 water molecules, which imparts a hydrodynamic volume ~5−10-fold greater than those folded proteins of the same molar mass; (4) generally considered safe and low immunogenic.[31−33] All of these characters make PEG the most successful candidate for protein modification. Since the debut of the first protein−PEG conjugate in 1976, i.e., BSA-PEG,[34] numerous proteins have been PEGylated via various approaches.[21,35] The first PEGylated protein drug, pegademase bovine (Adagen), was approved by the Food and Drug Administration (FDA) in 1990.[36] Over the past three decades, more than a dozen PEGylated proteins have been available on the market treating diseases such as hepatitis, blood cancers, melanoma, autoimmune diseases, gout, and hemophilia.

Despite those successes, however, the limitations of PEGylation have recently drawn increasing concerns. Traditional PEGylation was usually realized through random bioconjugation reactions with the amino group of the lysine residue. The lack of site-specificity in PEGylation typically leads to drawbacks such as tedious purification, difficulties in characterization, dramatic loss in activity, and potential regulatory issues. For example, up to 15 isomers were found in PEG-INTRON after chromatography separation, with the relative bioactivity of these isomers ranging from 8% to 37%.[37] Similar results were also observed in PEGASYS[38] and PEGylated growth hormone.[39] From this point of view, the site-specific conjugations are increasingly pursued nowadays for merits such as well-defined structure, simpler characterization, and homogeneity. Indeed, 4 out of the 15 approved pegylated protein drugs listed in Table 1 are site-specific, and 3 out of the 4 site-specific PEGylated proteins are only introduced in the past decade: Neulasta (N-terminus, approved in 2002), Omontys (ε-amino group from a single lysine, approved in 2012), Plegridy (N-terminus, approved in 2014), and JIVI (K1804C at the cysteine amino acid position 1804, approved in 2018).

Although PEG is commonly considered safe, increasing evidence has shown that repeating injections of PEG-protein drugs induced vacuoles in organs such as liver, kidney, and spleen.[40] This has raised growing awareness of the chronic toxicity of PEG due to its nondegradable nature. In the pharmaceutical industry, many PEGylated proteins failed in preclinical and/or clinical trials due to the accumulative toxicity of PEG. Even more concerning, the repetitive administration of PEG has been shown to elicit immunological responses including hypersensitivity and the so-called accelerated blood clearance (ABC) effect.[41] The first indication of the PEG immunogenicity was reported using PEGylated liposomes.[42] Boerman and colleagues observed that the blood concentration of PEG-liposome at 4 h after the second infusion was significantly lower than that at the same time point after the first infusion. Similar effects were later confirmed in PEGylated proteins in both animal models and the clinic. Now, it is well-accepted that the generation of anti-PEG antibodies, particularly anti-PEG IgM, is the major reason for the ABC effect.[43−45] Furthermore, anti-PEG antibodies were shown to correlate with the loss of therapeutic efficacy in the clinic.[46] For example, in a clinic study using a commercial PEG-asparaginase conjugate (brand name Oncarspar), anti-PEG IgG or IgM antibodies were found in 13 (86.7%) out of the 15 patients who had undetectable asparaginase activity in plasma.[47] More worrisome, anti-PEG antibodies were detected in healthy people, and the positive population in pre-existing anti-PEG antibodies soared significantly form 0.2% in 1984 to 27% in 2003.[48−51] To address these issues, polymer

DOI: 10.1021/acs.bioconjchem.9b00236
Bioconjugate Chem. 2019, 30, 1604−1616

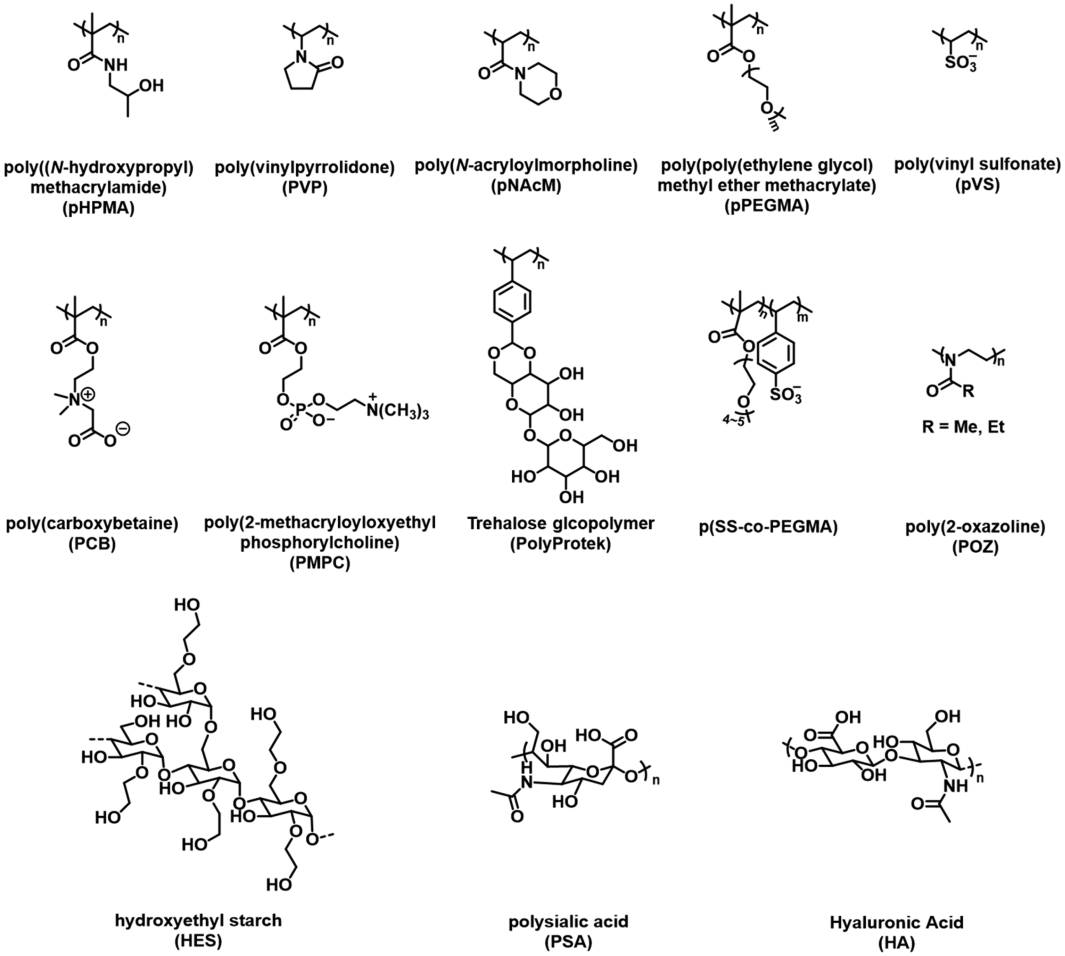


Figure 1. Examples of polymers that have been considered potential alternatives to PEG.

conjugation beyond PEGylation has drawn tremendous attention in the past decade.[24,52−54] Notable examples (Figure 1) include polyolefins carrying *N*-hydroxypropyl,[55−59] pyrrolidone,[60−62] oligo ethylene glycol,[63] sulfonate,[64] trehalose,[65,66] oxazoline,[67] or zwitterionic side chains,[68−70] poly(2-oxazoline),[67,71−73] hydroxyethyl starch (HES),[74−76] polysialic acid (PSA),[77] and hyaluronic acid (HA).[78] Those pioneering works have offered tremendous fundamental insight and redefined the frontiers of the protein−polymer hybrids.

Synthetic Polypeptides as Promising Biomaterials. Recombinant polypeptides such as XTEN, ELP, and PAS have been fused to proteins/peptides, which demonstrated different degree of success in long circulation and low immunogenicity.[79−85] These advances suggest a bright future of employing polypeptides for protein modification. Among many promising biopolymers, synthetic polypeptides have been attractive biomaterials owing to their unique structural characters.[86] First of all, polypeptides share identical peptidic backbone with proteins, making both chemical hydrolysis and proteolytic degradation possible. Second, the side chains of polypeptides can be chosen from both natural and unnatural sources, enabling the construction of large libraries. Indeed, the ring-opening polymerization (ROP) of amino acid *N*-carboxyanhydrides (NCAs) has been a powerful platform providing pools of new molecules. In the past decade, numerous functionalities with versatile physical properties have been efficiently installed to the side-chain of polypeptides via either direct monomer design and/or post-polymerization modifications.[87] Most importantly and different from many synthetic polymers, polypeptides can adopt higher ordered structures similar to proteins. For instance, polypeptides are known to have secondary structures such as α-helix and β-sheet via cooperative hydrogen bonding, from which highly compacted and ordered nanoscale architectures may be achievable via hierarchical supramolecular self-assembly.[88−90]

A variety of synthetic polypeptides have been investigated in biomedicine as drugs, additives, and delivery carriers. Glatiramer acetate, a random copolymer constituted of four canonical amino acids (tyrosine, lysine, alanine, and glutamate), is a FDA-approved drug for multiple sclerosis with the annual global sale reaching almost 4.0 billion USD in 2012.[91] Poly(γ-glutamic acid) (γPGA), a poly(amino acid) normally produced from bacteria or cnidarian, is a major constitute of the Japanese food nattō. First studied by Li and Wallace, γPGA was approved by the FDA for cosmetics.[92,93] The conjugate containing a 40 kDa poly(L-glutamate) (PGA) and paclitaxel advanced to phase III clinic trial (NCT00108745) in 2005.[92] Kataoka and co-workers have developed numerous self-assembled polymer micelles based on PGA and poly(L-aspartic acid) for delivering various bioactive drugs including doxorubicin (DOX) (NK911), paclitaxel (Ptx) (NK105), SN-38 (NK012), cisplatin (CDDP) (NC6004), and oxalipatin (NC-4016).[94] Moreover, polypeptides have been extensively tested as transfection materials,[95,96] hydrogels for controlled drug release and tissue engineering,[97−101] and antimicrobial materials.[102−107]

DOI: 10.1021/acs.bioconjchem.9b00236
Bioconjugate Chem. 2019, 30, 1604−1616

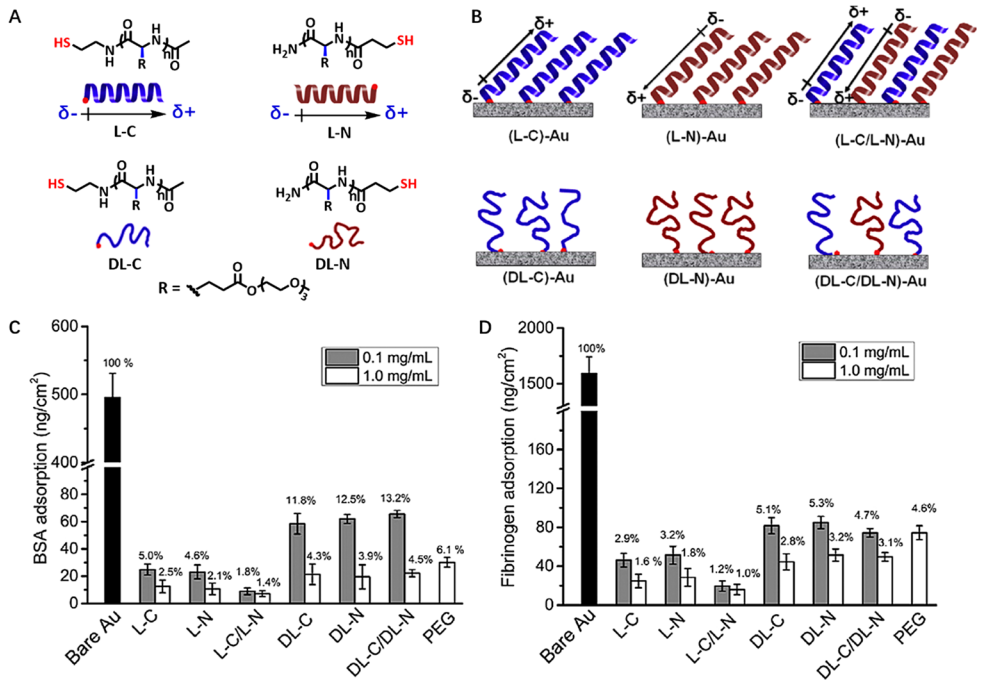


Figure 2. (A,B) Chemical structures (A) and cartoon illustration (B) of nonfouling polypeptides on gold surfaces. (C,D) Adsorption levels of BSA (C) or fibrinogen (D) on different polypeptide adlayers, measured by QCM-D.

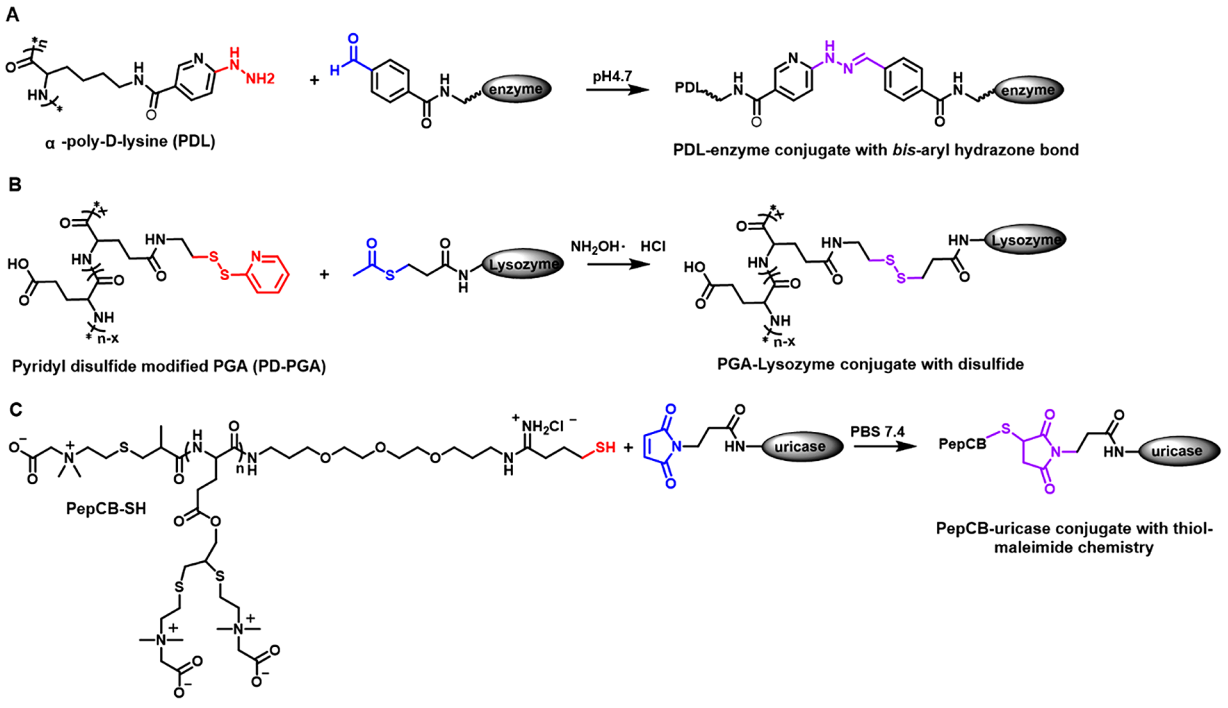


Figure 3. Synthetic methods for protein PEPylation using hydrazine bond formation (A), disulfide bond formation (B), and thiol-maleimide chemistry (C).

Ideal PEG alternatives are required to possess outstanding ability in preventing nonspecific biofouling, and many polypeptides have shown such promise. Klok et al. synthesized oligoethylene glycol modified (OEGylated) polylysine brushes through surface initiated ring opening polymerization, which showed effective prevention of nonspecific protein adsorption.[108] Recently, Lu and colleagues tested the antifouling property of PGA bearing OEGylated (PEG_3Glu) side chains and observed interesting effects on the helical conformation and anchoring orientation (Figure 2A,B).[109] Briefly, the polypeptides L-P(EG_3Glu) with a rigid α-helical conformation produced superior antifouling properties compared to DL-P(EG_3Glu) that bears the same side chain but with an unstructured conformation. The antifouling performance of L-P(EG_3Glu) can be further enhanced by anchoring antiparallel orientated helices on the surface (L-C/L-N in Figure 2C,D). By introducing a zwitterionic functional group such as carboxybetaine (CB) to the side chain of L-P(EG_3Glu),

DOI: 10.1021/acs.bioconjchem.9b00236
Bioconjugate Chem. 2019, 30, 1604−1616

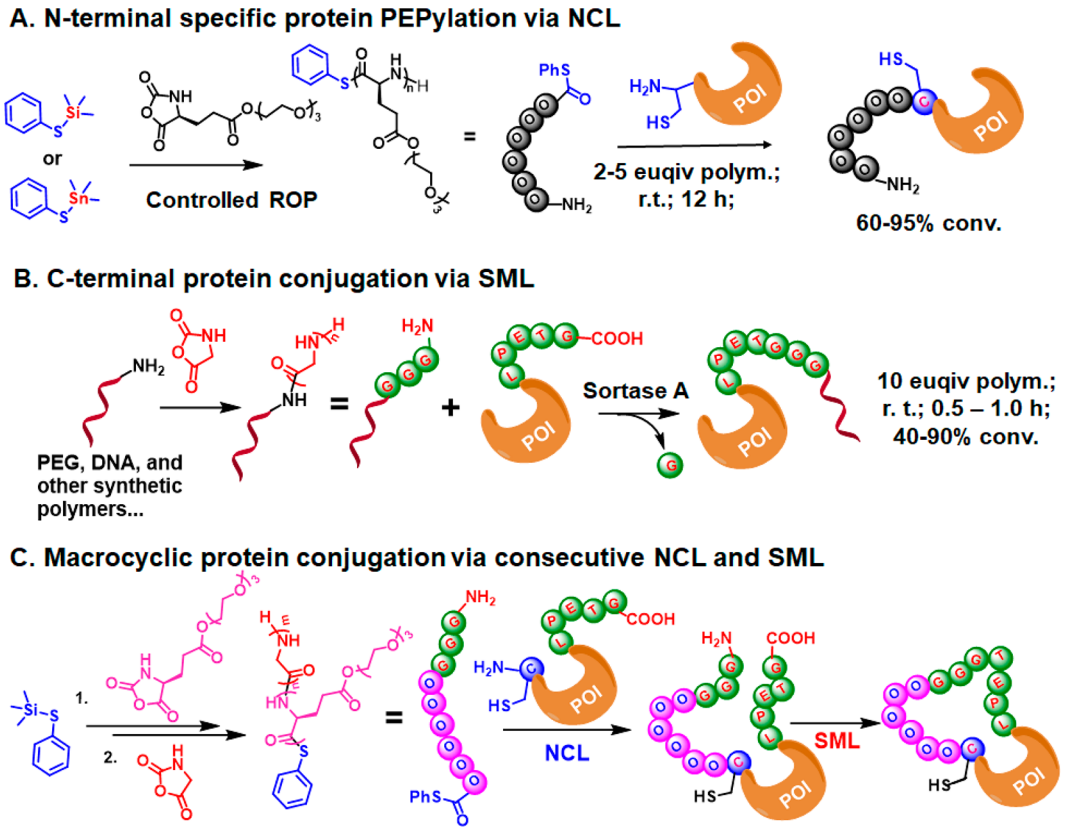


Figure 4. Site-specific protein PEPylation via native chemical ligation (A), sortase A mediated ligation (B), and the combination of the two for the synthesis of macrocyclic protein−polypeptide conjugates (C).

ultralow nonfouling surfaces were produced with further enhanced performances on antiprotein adsorption and anticell adhesion.[110] Together, those studies demonstrated that polypeptides, flexible or rigid, are potentially excellent partners for protein conjugation.

PROTEIN PEPYLATION

Synthetic Methods for Protein PEPylation. Protein PEPylation was infrequently explored previously, with only a few attempts made via random labeling (Figure 3). Walde and colleagues used the aldehyde-hydrazine chemistry to attach poly(D-lysine) (PDL) to enzymes such as α-chymotrypsin and horseradish peroxidase (Figure 3A).[111] The same approach was also pursued by Reilly and Winnik to produce antibody−polyglutamate conjugates.[112] Talelli and Vicent described a PGA-Lysozyme conjugate linked by disulfide bond (Figure 3B).[113] Recently, Jiang and colleagues used carboxybetaine polypeptide (PepCB) to modify uricase, a protein used for the treatment of gout, via the combination of thiol-maleimide and amine-NHS ester chemistries (Figure 3C).[114] Nevertheless, none of those pilot studies listed above employed a site-specific bioconjugation method, which may result in problems similar to those random PEGylations.

To enable site-specific protein PEPylation, one prerequisite is the introduction of biorthogonal functionalities to both macromolecular substrates. However, the conventional processes of introducing the highly reactive functionalities are typically time-consuming, labor-intensive, and multistep. Lu and colleagues have developed a concise approach for the site-specific protein PEPylation. To simplify the process and reduce steps in the synthetic route, a new concept involving the in situ generation of desired biorthogonal functionalities on the polypeptides is proposed, as shown in Figure 4.[115,116] In one example, a reactive phenyl thioester was installed to the polypeptides by using trimethylsilyl phenylsulfide (PhS-TMS) as the initiator for NCA polymerization. Later, the same group developed trimethylstannyl phenylsulfide (PhS-$SnMe_3$) that is more reactive and can produce ultrahigh-molecular-weight polypeptides, also bearing the in situ generated phenyl thioester.[117] Those polypeptides can be directly used for native chemical ligation (NCL, Figure 4A) with proteins engineered with a *N*-terminal cysteine, yielding *N*-terminal specific protein PEPylation in two steps. In another example, the ROP of glycine NCA can in situ create a short aminooligoglycine nucleophile, a substrate for the widely used transpeptidase enzyme sortase A, at the end of the polymer-of-interest. Subsequent sortase A-mediated ligation (SML, Figure 4B) of the polymer to proteins bearing a sorting motif LPXTG (X = any canonical amino acid) smoothly afforded polymer conjugates at the *C*-terminus of the protein. Remarkably, these two methods can be facilely combined for the synthesis of PEPylated proteins harboring complex topological structures. For example, a heterotelechelic polypeptide bearing a phenyl thioester on one end and an aminoglycine on the other end can be produced via PhSTMS-mediated tandem NCA polymerizations in a one-pot fashion (Figure 4C). The polypeptide can react with a protein flanked with a *N*-Cys and a *C*-terminal LPXTG to give otherwise inaccessible macrocyclic protein−polypeptide conjugates via successive NCL and SML. Other types of conjugates, i.e., one protein attached to two polymers or two proteins attached to

DOI: 10.1021/acs.bioconjchem.9b00236
Bioconjugate Chem. 2019, 30, 1604−1616

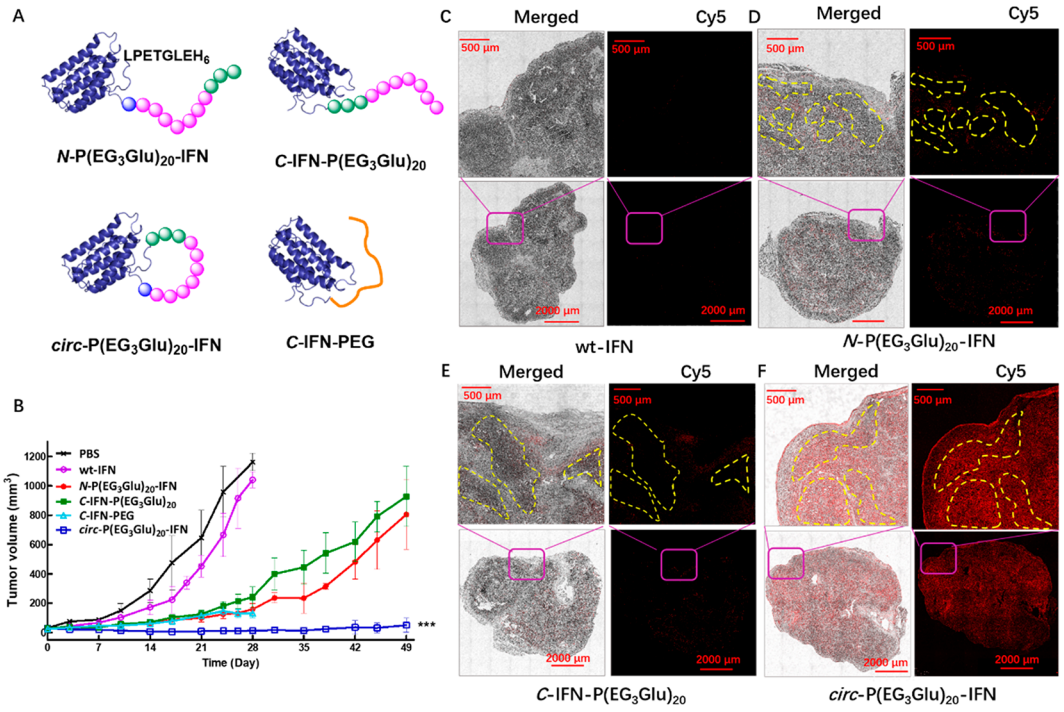


Figure 5. Cartoon illustration (A), antitumor efficacy (xenograft OVCAR3 mouse model) (B), and in vivo tumor penetration (C−F) of various topological PEPylated interferon conjugates. The red fluorescence represents IFN conjugates. The dotted yellow lines indicate the regions of tumor tissues with densely packed malignant cells. *N*-P(EG_3Glu)$_{20}$-IFN and *C*-IFN-P(EG_3Glu)$_{20}$ defines the linear IFN conjugates with the *N*- and *C*-terminus of IFN attaching the polypeptide L-P(EG_3-Glu)$_{20}$(M_n ~5 kDa), respectively; *circ*-P(EG_3Glu)$_{20}$-IFN denotes the macrocyclic IFN conjugates with both the *N*- and *C*-termini of IFN attached to L-P(EG_3-Glu)$_{20}$. *C*-IFN-PEGis the C-terminal specific IFN-PEG conjugate, included as a bench marker.

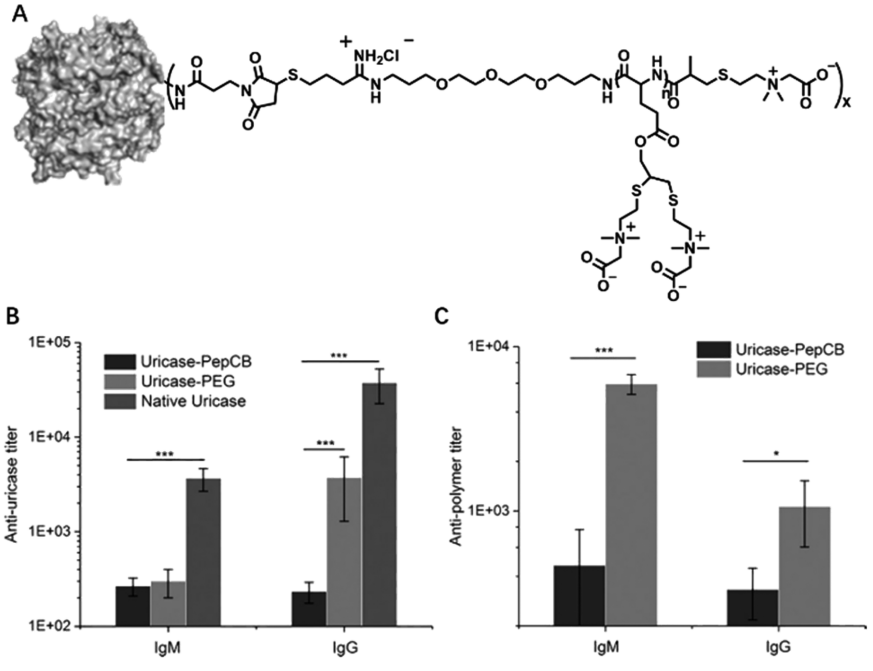


Figure 6. (A) Structure of the uricase-PepCB conjugate. (B,C) Antibody responses after the third immunization measured by enzyme-linked immunosorbent assay (ELISA): antiuricase antibodies (B); antipolymer antibodies (C).

one polymer, can also be facilely generated by using different combinations of the two methods.

PEPylated Proteins for Cancer Therapy. Polymer conjugation is commonly used to endow higher stability and longer half-life in vivo to the conjugated protein. However, this was usually achieved at the cost of reduced pharmacodynamics (PD) and diminished tissue permeability due to the steric hindrance created by the attached polymer. To address this dilemma, Lu et al. carefully compared several PEPylated interferon (IFN) with different topology in their proof-of-concept study (Figure 5A). They kept other structural parameters of the PEPylated IFN identical or similar in

DOI: 10.1021/acs.bioconjchem.9b00236
Bioconjugate Chem. 2019, 30, 1604−1616

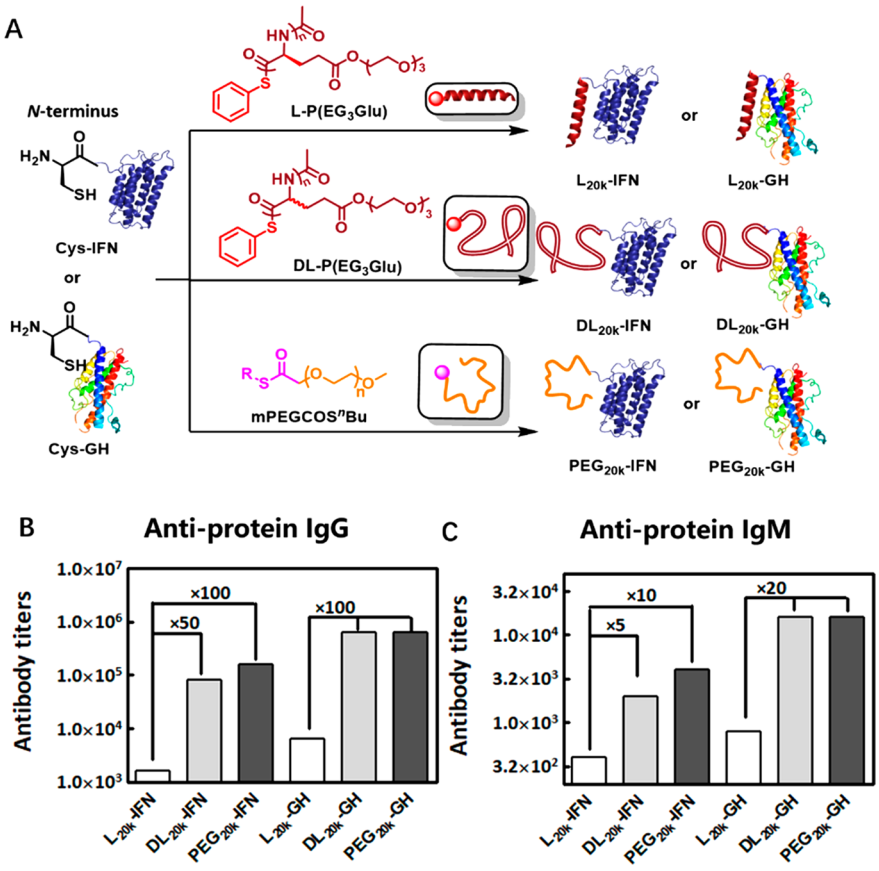


Figure 7. (A) *N*-Terminal specific PEPylation and PEGylation of engineered therapeutic proteins (IFN and GH) via native chemical ligation; the M_n of the two polypeptides, L-P(EG_3Glu) and DL-P(EG_3Glu), and the bench marker PEG are all ~20 kDa. (B,C) Antiprotein antibody titers (IgG in (B) and IgM in (C)) after the fourth immunization.

order to isolate the topology effect. The rationale was that by macrocyclizing the PEPylated proteins, the long acting conjugates would be more proteolytic stable and tissue permeable. This strategy has previously been vigorously exploited for peptides (mostly at cellular or ex vivo level), but never been tested for protein−polymer conjugates.[118−122] Systematic and pharmacological evaluation of those conjugates revealed that the macrocyclic IFN conjugate *circ*-P$(EG_3Glu)_{20}$-IFN did give significantly higher stability, stronger binding in vitro, and greater tumor inhibition ex vivo and in vivo (Figure 5B). Most interestingly, their results indicated a clear macrocyclization-promoted tumor penetration (Figure 5C−F) and profoundly enhanced antitumor efficacy in several tumor models.[123] This study thus provided strong experimental evidence favoring the topology engineering in protein−polymer conjugates.

Polypeptoids, analogues of polypeptides, have also been suggested to be excellent alternatives to PEG. For example, Polysarcosine (PSar), a highly hydrated, nontoxic and nonionic polypeptoid, has a neutral and random coil structure similar to PEG.[124] Previously, PSar has been extensively exploited as a stealthy polymer for nanoparticle coating[125−127] and small molecule conjugation.[128] However, it has not been frequently used for protein modification. In a preliminary study involving the head-to-head comparison of PSar-IFN and PEG-IFN conjugates, Lu et al. showed that the former outperformed the latter in a xenograft OVCAR3 ovarian tumor model in mice, underscoring the promise of PSar in protein therapeutics.[129]

Protein PEPylation for Reduced Immunogenicity. The cumulative toxicity and the anti-PEG antibody induced ABC effect have been the two major devastating reasons leading to clinical failure of PEGylated protein therapeutics. In the search of polymers "invisible" to the immune system and with better protection capability to the protein drugs, The Jiang group from University of Washington have pioneered many zwitterionic polymers as alternatives to PEG. Recently, they prepared a superhydrophilic polypeptide bearing high density of zwitterionic CB (PepCB) groups in the side chain (Figure 6A).[114] PepCB was shown to not induce detectable organ toxicity, while cytoplasmic vacuolation was clearly observed in kidney and spleen for the PEG group. PepCB and PEG were then separately conjugated to uricase, a highly immunogenic protein drug for gout treatment. They showed that the uricase-PepCB conjugate stimulated 16-fold lower blood antiuricase IgG titers than the uricase-PEG conjugate after three injections to immunocompetent animals (Figure 6B). Moreover, anti-PepCB antibody response was negligible in the former group whereas anti-PEG antibodies were abundant in the latter one (Figure 6C). The results provided strong evidence that polypeptides, despite of their seemingly antigen-like peptide backbone, are promising low immunogenic materials once the side chain structures are properly optimized.

The Lu group from Peking University examined the immunogenicity of polypeptides from the secondary structure point of view. Previously, flexible and unstructured polymers without a defined conformation are almost exclusively preferred for protein conjugation.[130] Typical examples

DOI: 10.1021/acs.bioconjchem.9b00236
Bioconjugate Chem. 2019, 30, 1604−1616

following this principle include PEG, XTEN, and ELP.[131−133] Kataoka reported that polypeptide micelles bearing helical bundles in the core afforded longer circulation and less accumulation in the liver as compared to similar micelles based on a racemic polypeptide.[134] Inspired by this interesting observation, Lu and colleagues hypothesized that synthetic polypeptides may be less immunogenic when the rigid helix backbone was shielded by long antifouling side chains. To this end, they systemically evaluated the effect of the helical conformation by setting up stringent control groups including the benchmark PEG (Figure 7A).[135] The work showed proteins such as IFN and human growth hormone (GH) conjugated to the helical L-P(EG_3Glu) gave better in vivo pharmacological results than those carrying flexible polymers such as DL-P(EG_3Glu) and PEG. Most interestingly, they found a clear helix-dependent effect in minimizing the generation of both antidrug and antipolymer antibodies (Figure 7B,C). Although this counterintuitive observation needs further validation in other protein drugs, antifouling polypeptides, and/or different animal models, the interesting helix effect may suggest a paradigm shift in the design principle of protein−polymer conjugates.

PERSPECTIVES

The current studies have highlighted the promise of protein PEPylation toward long-acting drugs. However, PEPylation is still in its infant stage, with numerous challenges and opportunities facing future researchers. From synthetic point of view, more robust methods are necessary to enable site-specific conjugation beyond the *N*- and *C*-termini.[136,137] Introduction of specific peptide tags for sequence-specific enzymatic reactions is a feasible route.[122,138−141] Genetic and/or metabolic incorporation of unnatural amino acids (UAA) with special biorthogonal reactivity are also powerful tools for such purposes.[142−144] More examples and new functionalities introduced by the in situ functionalization strategy will be useful to further facilitate PEPylation.[145] On the other hand, further development of recombinant polypeptides with well-defined sequence and self-assembly structures via either de novo design or bioinformatics will be extremely interesting approaches to construct genetically PEPylated proteins.[146−151] The versatile self-assembly behaviors of polypeptides can be another exciting strategy to advance and redefine the fronteirs of PEPylated proteins.[152,153] From the functional point of view, applications beyond long circulation such as imaging, sensing, diagnosis, intracellular delivery, catalysis, and self-assembly are highly desirable. To fulfill such diversified applications, one must significantly expand the arsenal of polypeptides suitable for protein modification. Thanks to recent advances in NCA chemistry, the past decade has witnessed the blossom of a wide variety of side-chain modified polypeptides.[86,154,155] Numerous stimuli-responsive, biomimetic, and membrane active polypeptides have been developed,[156−162] which lay the firm material foundation pursuing new applications of PEPylation. For example, on-demand switches of the catalytic activity of enzymes by polypeptides sensitive to external triggers will be the low hanging fruits. Increasing the stability, maintaining the activity, and recycling of enzymes under extreme stresses are important challenges for industrial applications.[163] Targeted cytosolic and/or nucelic delivery of functional proteins such as Cas9 represents a significant hurdle for many proteins acting on intracellular targets.[164−171] Protein conjugates modified with membrane-active polypeptides may offer a solution toward this challenge. Last but not the least, one long-standing challenge in the field is the lack of exhausted purification and comprehensive characterization for quality control and accurate structure−activity relationship analysis. New separation methods and characterization technologies are thus fundamentally important and can never be overlooked.

AUTHOR INFORMATION

Corresponding Author

*E-mail: chemhualu@pku.edu.cn.

ORCID

Hua Lu: 0000-0003-2180-3091

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China (21722401 and 21474004).

REFERENCES

(1) Leader, B., Baca, Q. J., and Golan, D. E. (2008) Protein therapeutics: a summary and pharmacological classification. *Nat. Rev. Drug Discovery 7* (1), 21−39.

(2) Muller, D. (2014) Antibody-cytokine fusion proteins for cancer immunotherapy: an update on recent developments. *BioDrugs 28* (2), 123−131.

(3) Sievers, E. L., and Senter, P. D. (2013) Antibody-drug conjugates in cancer therapy. *Annu. Rev. Med. 64*, 15−29.

(4) Liu, T., Du, J., Luo, X., Schultz, P. G., and Wang, F. (2015) Homogeneously modified immunoglobulin domains for therapeutic application. *Curr. Opin. Chem. Biol. 28*, 66−74.

(5) Ecker, D. M., Jones, S. D., and Levine, H. L. (2015) The therapeutic monoclonal antibody market. *MAbs. 7* (1), 9−14.

(6) Choi, J. M., Han, S. S., and Kim, H. S. (2015) Industrial applications of enzyme biocatalysis: Current status and future aspects. *Biotechnol. Adv. 33* (7), 1443−1454.

(7) Frokjaer, S., and Otzen, D. E. (2005) Protein drug stability: a formulation challenge. *Nat. Rev. Drug Discovery 4* (4), 298−306.

(8) Krishna, M., and Nadler, S. G. (2016) Immunogenicity to biotherapeutics - The role of anti-drug immune complexes. *Front. Immunol. 7*, 21.

(9) Rosenberg, A. S., and Sauna, Z. E. (2018) Immunogenicity assessment during the development of protein therapeutics. *J. Pharm. Pharmacol. 70* (5), 584−594.

(10) Yanover, C., Jain, N., Pierce, G., Howard, T. E., and Sauna, Z. E. (2011) Pharmacogenetics and the immunogenicity of protein therapeutics. *Nat. Biotechnol. 29* (10), 870−873.

(11) Van Stappen, T., Vande Casteele, N., Van Assche, G., Ferrante, M., Vermeire, S., and Gils, A. (2018) Clinical relevance of detecting anti-infliximab antibodies with a drug-tolerant assay: post hoc analysis of the TAXIT trial. *Gut 67* (5), 818−826.

(12) Hubbell, J. A. (2010) Drug development: Longer-lived proteins. *Nature 467* (7319), 1051−1052.

(13) Gauthier, M. A., Gibson, M. I., and Klok, H. A. (2009) Synthesis of functional polymers by post-polymerization modification. *Angew. Chem., Int. Ed. 48* (1), 48−58.

(14) Hoffman, A. S. (2013) Stimuli-responsive polymers: biomedical applications and challenges for clinical translation. *Adv. Drug Delivery Rev. 65* (1), 10−16.

(15) Cobo, I., Li, M., Sumerlin, B. S., and Perrier, S. (2015) Smart hybrid materials by conjugation of responsive polymers to biomacromolecules. *Nat. Mater. 14* (2), 143−159.

(16) Lutz, J. F., Lehn, J. M., Meijer, E. W., and Matyjaszewski, K. (2016) From precision polymers to complex materials and systems. *Nat. Rev. Mater. 1* (5), 1 DOI: 10.1038/natrevmats.2016.24.

DOI: 10.1021/acs.bioconjchem.9b00236
Bioconjugate Chem. 2019, 30, 1604−1616

(17) Zelikin, A. N., Ehrhardt, C., and Healy, A. M. (2016) Materials and methods for delivery of biological drugs. *Nat. Chem. 8* (11), 997−1007.
(18) Tucker, B. S., Stewart, J. D., Aguirre, J. I., Holliday, L. S., Figg, C. A., Messer, J. G., and Sumerlin, B. S. (2015) Role of polymer architecture on the activity of polymer-protein conjugates for the treatment of accelerated bone loss disorders. *Biomacromolecules 16* (8), 2374−2381.
(19) Fuhrmann, G., Grotzky, A., Lukic, R., Matoori, S., Luciani, P., Yu, H., Zhang, B. Z., Walde, P., Schluter, A. D., Gauthier, M. A., et al. (2013) Sustained gastrointestinal activity of dendronized polymer-enzyme conjugates. *Nat. Chem. 5* (7), 582−589.
(20) Schulz, J. D., Patt, M., Basler, S., Kries, H., Hilvert, D., Gauthier, M. A., and Leroux, J. C. (2016) Site-specific polymer conjugation stabilizes therapeutic enzymes in the gastrointestinal tract. *Adv. Mater. 28* (7), 1455−1460.
(21) Pfister, D., and Morbidelli, M. (2014) Process for protein PEGylation. *J. Controlled Release 180*, 134−149.
(22) Klok, H.-A. (2009) Peptide/Protein−synthetic polymer conjugates:Quo Vadis. *Macromolecules 42* (21), 7990−8000.
(23) Qi, Y., and Chilkoti, A. (2015) Protein-polymer conjugation-moving beyond PEGylation. *Curr. Opin. Chem. Biol. 28*, 181−193.
(24) Pelegri-O'Day, E. M., Lin, E. W., and Maynard, H. D. (2014) Therapeutic protein-polymer conjugates: advancing beyond PEGylation. *J. Am. Chem. Soc. 136* (41), 14323−14332.
(25) Russell, A. J., Baker, S. L., Colina, C. M., Figg, C. A., Kaar, J. L., Matyjaszewski, K., Simakova, A., and Sumerlin, B. S. (2018) Next generation protein-polymer conjugates. *AIChE J. 64* (9), 3230−3245.
(26) Welch, R. P., Lee, H., Luzuriaga, M. A., Brohlin, O. R., and Gassensmith, J. J. (2018) Protein-polymer delivery: Chemistry from the cold chain to the clinic. *Bioconjugate Chem. 29* (9), 2867−2883.
(27) Liu, X., Sun, J., and Gao, W. (2018) Site-selective protein modification with polymers for advanced biomedical applications. *Biomaterials 178*, 413−434.
(28) Gong, Y., Leroux, J. C., and Gauthier, M. A. (2015) Releasable conjugation of polymers to proteins. *Bioconjugate Chem. 26* (7), 1172−1181.
(29) Obermeyer, A. C., and Olsen, B. D. (2015) Synthesis and application of protein-containing block copolymers. *ACS Macro Lett. 4* (1), 101−110.
(30) Pelegri-O'Day, E. M., and Maynard, H. D. (2016) Controlled radical polymerization as an enabling approach for the next generation of protein-polymer conjugates. *Acc. Chem. Res. 49* (9), 1777−1785.
(31) Pasut, G., and Veronese, F. M. (2009) PEGylation for improving the effectiveness of therapeutic biomolecules. *Drugs Today 45* (9), 687−695.
(32) Pasut, G., and Veronese, F. M. (2012) State of the art in PEGylation: the great versatility achieved after forty years of research. *J. Controlled Release 161* (2), 461−472.
(33) Cui, J., De Rose, R., Alt, K., Alcantara, S., Paterson, B. M., Liang, K., Hu, M., Richardson, J. J., Yan, Y., Jeffery, C. M., et al. (2015) Engineering poly(ethylene glycol) particles for improved biodistribution. *ACS Nano 9* (2), 1571−1580.
(34) Abuchowski, A., Van Es, T., Palczuk, N. C., and Davis, F. F. (1977) Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. *J. Biol. Chem. 252* (11), 3578−3581.
(35) Nischan, N., and Hackenberger, C. P. (2014) Site-specific PEGylation of proteins: recent developments. *J. Org. Chem. 79* (22), 10727−10733.
(36) Booth, C., and Gaspar, H. B. (2009) Pegademase bovine (PEG-ADA) for the treatment of infants and children with severe combined immunodeficiency (SCID). *Biologics. 3*, 349−358.
(37) Wang, Y. S., Youngster, S., Grace, M., Bausch, J., Bordens, R., and Wyss, D. F. (2002) Structural and biological characterization of pegylated recombinant interferon alpha-2b and its therapeutic implications. *Adv. Drug Delivery Rev. 54* (4), 547−570.
(38) Foser, S., Schacher, A., Weyer, K. A., Brugger, D., Dietel, E., Marti, S., and Schreitmüller, T. (2003) Isolation, structural characterization, and antiviral activity of positional isomers of monopegylated interferon α-2a (PEGASYS). *Protein Expression Purif. 30* (1), 78−87.
(39) Finn, R. F. (2009) PEGylation of human growth hormone: strategies and properties, in *PEGylated Protein Drugs: Basic Science and Clinical Applications* (Veronese, F. M., Ed.) pp 187−203, Birkhäuser Basel, Basel.
(40) Xu, L., Yang, J. P., Xue, B., Zhang, C., Shi, L. L., Wu, C. W., Su, Y., Jin, X., Liu, Y. M., and Zhu, X. Y. (2017) Molecular insights for the biological interactions between polyethylene glycol and cells. *Biomaterials 147*, 1−13.
(41) Abu Lila, A. S., Kiwada, H., and Ishida, T. (2013) The accelerated blood clearance (ABC) phenomenon: clinical challenge and approaches to manage. *J. Controlled Release 172* (1), 38−47.
(42) Dams, E. T., Laverman, P., Oyen, W. J., Storm, G., Scherphof, G. L., Van Der Meer, J. W., Corstens, F. H., and Boerman, O. C. (2000) Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes. *J. Pharmacol. Exp. Ther. 292* (3), 1071−1079.
(43) Wang, X. Y., Ishida, T., and Kiwada, H. (2007) Anti-PEG IgM elicited by injection of liposomes is involved in the enhanced blood clearance of a subsequent dose of PEGylated liposomes. *J. Controlled Release 119* (2), 236−244.
(44) Ishida, T., Wang, X., Shimizu, T., Nawata, K., and Kiwada, H. (2007) PEGylated liposomes elicit an anti-PEG IgM response in a T cell-independent manner. *J. Controlled Release 122* (3), 349−355.
(45) Mima, Y., Hashimoto, Y., Shimizu, T., Kiwada, H., and Ishida, T. (2015) Anti-PEG IgM is a majorcontributor to the accelerated blood clearance of polyethylene glycol-conjugated protein. *Mol. Pharmaceutics 12* (7), 2429−2435.
(46) Zhang, P., Sun, F., Liu, S., and Jiang, S. (2016) Anti-PEG antibodies in the clinic: Current issues and beyond PEGylation. *J. Controlled Release 244*, 184−193.
(47) Muller, H. J., Loning, L., Horn, A., Schwabe, D., Gunkel, M., Schrappe, M., Von Schutz, V., Henze, G., Da Palma, J. C., Ritter, J., et al. (2000) Pegylated asparaginase (Oncaspar (TM)) in children with ALL: drug monitoring in reinduction according to the ALL/NHL-BFM 95 protocols. *Br. J. Haematol. 110* (2), 379−384.
(48) Richter, A. W., and Akerblom, E. (2004) Polyethylene glycol reactive antibodies in man: titer distribution in allergic patients treated with monomethoxy polyethylene glycol modified allergens or placebo, and in healthy blood donors. *Int. Arch. Allergy Immunol. 74* (1), 36−39.
(49) Armstrong, J. K., Leger, R., Wenby, R. B., Meiselman, H. J., Garratty, G., and Fisher, T. C. (2003) Occurrence of an antibody to poly(ethylene glycol) in normal donors. *Blood 102* (111), 556a−556a.
(50) Ganson, N. J., Povsic, T. J., Sullenger, B. A., Alexander, J. H., Zelenkofske, S. L., Sailstad, J. M., Rusconi, C. P., and Hershfield, M. S. (2016) Pre-existing anti-polyethylene glycol antibody linked to first-exposure allergic reactions to pegnivacogin, a PEGylated RNA aptamer. *J. Allergy Clin. Immunol. 137* (5), 1610−1613.
(51) Yang, Q., and Lai, S. K. (2015) Anti-PEG immunity: emergence, characteristics, and unaddressed questions. *Wiley Interdiscip Rev. Nanomed. Nanobiotechnol. 7* (5), 655−677.
(52) Knop, K., Hoogenboom, R., Fischer, D., and Schubert, U. S. (2010) Poly(ethylene glycol) in drug delivery: pros and cons as well as potential alternatives. *Angew. Chem., Int. Ed. 49* (36), 6288−6308.
(53) Pasut, G. (2014) Polymers for protein conjugation. *Polymers 6* (1), 160−178.
(54) Qi, Y. Z., and Chilkoti, A. (2015) Protein-polymer conjugation - moving beyond PEGylation. *Curr. Opin. Chem. Biol. 28*, 181−193.
(55) Duncan, R., and Vicent, M. J. (2010) Do HPMA copolymer conjugates have a future as clinically useful nanomedicines? A critical overview of current status and future opportunities. *Adv. Drug Delivery Rev. 62* (2), 272−282.
(56) Tao, L., Liu, J. Q., and Davis, T. P. (2009) Branched polymer-protein conjugates made from mid-chain-functional P(HPMA). *Biomacromolecules 10* (10), 2847−2851.

DOI: 10.1021/acs.bioconjchem.9b00236
Bioconjugate Chem. 2019, 30, 1604−1616

(57) Kopecek, J., and Kopeckova, P. (2010) HPMA copolymers: origins, early developments, present, and future. *Adv. Drug Delivery Rev. 62* (2), 122−149.
(58) Yang, J., and Kopecek, J. (2016) Design of smart HPMA copolymer-based nanomedicines. *J. Controlled Release 240*, 9−23.
(59) Liu, X. Y., Sun, M. M., Sun, J. W., Hu, J., Wang, Z. R., Guo, J. W., and Gao, W. P. (2018) Polymerization induced self-assembly of a site-specific interferon alpha-block copolymer conjugate into micelles with remarkably enhanced pharmacology. *J. Am. Chem. Soc. 140* (33), 10435−10438.
(60) Lee, W. Y., Sehon, A. H., and Von Specht, B. U. (1981) Suppression of IgE antibodies to the (4-hydroxy-3-iodo-5-nitrophenyl)acetyl (NIP) group and induction of NIP-specific suppressor cells with NIP-poly-N-vinylpyrrolidone conjugates. *Eur. J. Immunol. 11* (1), 13−17.
(61) Smorodinsky, N., Vonspecht, B. U., Cesla, R., and Shaltiel, S. (1981) A conjugate between a purified timothy allergen and poly(N-vinyl pyrrolidone) suppresses the specific IgEpesponse in ice. *Immunol. Lett. 2* (5−6), 305−309.
(62) Caliceti, P., Schiavon, O., and Veronese, F. M. (2001) Immunological properties of uricase conjugated to neutral soluble polymers. *Bioconjugate Chem. 12* (4), 515−522.
(63) Hu, J., Wang, G. L., Zhao, W. G., Liu, X. Y., Zhang, L. B., and Gao, W. P. (2016) Site-specific in situ growth of an interferon-polymer conjugate that outperforms PEGASYS in cancer therapy. *Biomaterials 96*, 84−92.
(64) Nguyen, T. H., Kim, S. H., Decker, C. G., Wong, D. Y., Loo, J. A., and Maynard, H. D. (2013) A heparin-mimicking polymer conjugate stabilizes basic fibroblast growth factor. *Nat. Chem. 5* (3), 221−227.
(65) Lee, J., Lin, E. W., Lau, U. Y., Hedrick, J. L., Bat, E., and Maynard, H. D. (2013) Trehalose glycopolymers as excipients for protein stabilization. *Biomacromolecules 14* (8), 2561−2569.
(66) Mancini, R. J., Lee, J., and Maynard, H. D. (2012) Trehalose glycopolymers for stabilization of protein conjugates to environmental stressors. *J. Am. Chem. Soc. 134* (20), 8474−8479.
(67) Viegas, T. X., Bentley, M. D., Harris, J. M., Fang, Z. F., Yoon, K., Dizman, B., Weimer, R., Mero, A., Pasut, G., and Veronese, F. M. (2011) Polyoxazoline: chemistry, properties, and applications in drug delivery. *Bioconjugate Chem. 22* (5), 976−986.
(68) Keefe, A. J., and Jiang, S. (2012) Poly(zwitterionic)protein conjugates offer increased stability without sacrificing binding affinity or bioactivity. *Nat. Chem. 4* (1), 59−63.
(69) Liu, S., and Jiang, S. (2016) Zwitterionic polymer-protein conjugates reduce polymer-specific antibody response. *Nano Today 11* (3), 285−291.
(70) Hu, J., Wang, G., Zhao, W., and Gao, W. (2016) In situ growth of a C-terminal interferon-alpha conjugate of a phospholipid polymer that outperforms PEGASYS in cancer therapy. *J. Controlled Release 237*, 71−77.
(71) Mero, A., Fang, Z., Pasut, G., Veronese, F. M., and Viegas, T. X. (2012) Selective conjugation of poly(2-ethyl 2-oxazoline) to granulocyte colony stimulating factor. *J. Controlled Release 159* (3), 353−361.
(72) Tong, J., Yi, X., Luxenhofer, R., Banks, W. A., Jordan, R., Zimmerman, M. C., and Kabanov, A. V. (2013) Conjugates of superoxide dismutase 1 with amphiphilic poly(2-oxazoline) block copolymers for enhanced brain delivery: synthesis, characterization and evaluation in vitro and in vivo. *Mol. Pharmaceutics 10* (1), 360−377.
(73) Li, J., Zhou, Y., Li, C., Wang, D., Gao, Y., Zhang, C., Zhao, L., Li, Y., Liu, Y., and Li, X. (2015) Poly(2-ethyl-2-oxazoline)-doxorubicin conjugate-based dual endosomal pH-sensitive micelles with enhanced antitumor efficacy. *Bioconjugate Chem. 26* (1), 110−119.
(74) Hey, T. K. H., and Vorstheim, P. (2012) Half-life extension through HESylation, in *Therapeutic Proteins*, pp 117, Wiley-VCH, Weinheim.
(75) Liebner, R., Mathaes, R., Meyer, M., Hey, T., Winter, G., and Besheer, A. (2014) Protein HESylation for half-life extension: Synthesis, characterization and pharmacokinetics of HESylated anakinra. *Eur. J. Pharm. Biopharm. 87* (2), 378−385.
(76) Lameire, N., and Hoste, E. (2014) What's new in the controversy on the renal/tissue toxicity of starch solutions? *Intensive Care Med. 40* (3), 427−430.
(77) Jain, S., Hreczuk-Hirst, D. H., McCormack, B., Mital, M., Epenetos, A., Laing, P., and Gregoriadis, G. (2003) Polysialylated insulin: synthesis, characterization and biological activity in vivo. *Biochim. Biophys. Acta, Gen. Subj. 1622* (1), 42−49.
(78) Yang, J. A., Park, K., Jung, H., Kim, H., Hong, S. W., Yoon, S. K., and Hahn, S. K. (2011) Target specific hyaluronic acid-interferon alpha conjugate for the treatment of hepatitis C virus infection. *Biomaterials 32* (33), 8722−8729.
(79) Ding, S., Song, M., Sim, B. C., Gu, C., Podust, V. N., Wang, C. W., McLaughlin, B., Shah, T. P., Lax, R., Gast, R., et al. (2014) Multivalent antiviral XTEN-peptide conjugates with long in vivo half-life and enhanced solubility. *Bioconjugate Chem. 25* (7), 1351−1359.
(80) Schellenberger, V., Wang, C. W., Geething, N. C., Spink, B. J., Campbell, A., To, W., Scholle, M. D., Yin, Y., Yao, Y., Bogin, O., et al. (2009) A recombinant polypeptide extends the in vivo half-life of peptides and proteins in a tunable manner. *Nat. Biotechnol. 27* (12), 1186−1190.
(81) Hu, J., Wang, G., Liu, X., and Gao, W. (2015) Enhancing pharmacokinetics, tumor accumulation, and antitumor efficacy by elastin-like polypeptide fusion of interferon alpha. *Adv. Mater. 27* (45), 7320−7324.
(82) Luginbuhl, K. M., Schaal, J. L., Umstead, B., Mastria, E. M., Li, X. H., Banskota, S., Arnold, S., Feinglos, M., D'Alessio, D., and Chilkoti, A. (2017) One-week glucose control via zero-order release kinetics from an injectable depot of glucagon-like peptide-1 fused to a thermosensitive biopolymer. *Nat. Biomed. Eng. 1* (6), 1 DOI: 10.1038/s41551-017-0078.
(83) Schlapschy, M., Binder, U., Borger, C., Theobald, I., Wachinger, K., Kisling, S., Haller, D., and Skerra, A. (2013) PASylation: a biological alternative to PEGylation for extending the plasma half-life of pharmaceutically active proteins. *Protein Eng., Des. Sel. 26* (8), 489−501.
(84) Petitdemange, R., Garanger, E., Bataille, L., Bathany, K., Garbay, B., Deming, T. J., and Lecommandoux, S. (2017) Tuning thermoresponsive properties of cationic elastin-like polypeptides by varying counterions and side-chains. *Bioconjugate Chem. 28* (5), 1403−1412.
(85) Petitdemange, R., Garanger, E., Bataille, L., Dieryck, W., Bathany, K., Garbay, B., Deming, T. J., and Lecommandoux, S. (2017) Selective tuning of elastin-like polypeptide properties via methionine oxidation. *Biomacromolecules 18* (2), 544−550.
(86) Song, Z., Han, Z., Lv, S., Chen, C., Chen, L., Yin, L., and Cheng, J. (2017) Synthetic polypeptides: from polymer design to supramolecular assembly and biomedical application. *Chem. Soc. Rev. 46* (21), 6570−6599.
(87) Deming, T. J. (2016) Synthesis of side-chain modified polypeptides. *Chem. Rev. 116* (3), 786−808.
(88) Machado, C. A., Smith, I. R., and Savin, D. A. (2019) Self-assembly of oligo- and polypeptide-based amphiphiles: Recent advances and future possibilities. *Macromolecules 52* (5), 1899−1911.
(89) Song, Z., Fu, H., Wang, R., Pacheco, L. A., Wang, X., Lin, Y., and Cheng, J. (2018) Secondary structures in synthetic polypeptides from N-carboxyanhydrides: design, modulation, association, and material applications. *Chem. Soc. Rev. 47*, 7401−7425.
(90) Bonduelle, C. (2018) Secondary structures of synthetic polypeptide polymers. *Polym. Chem. 9* (13), 1517−1529.
(91) Weinstock-Guttman, B., Nair, K. V., Glajch, J. L., Ganguly, T. C., and Kantor, D. (2017) Two decades of glatiramer acetate: From initial discovery to the current development of generics. *J. Neurol. Sci. 376*, 255−259.

DOI: 10.1021/acs.bioconjchem.9b00236
Bioconjugate Chem. 2019, 30, 1604−1616

(92) Li, C., and Wallace, S. (2008) Polymer-drug conjugates: recent development in clinical oncology. *Adv. Drug Delivery Rev. 60* (8), 886−898.
(93) Sung, M. H., Park, C., Kim, C. J., Poo, H., Soda, K., and Ashiuchi, M. (2005) Natural and edible biopolymer poly-gamma-glutamic acid: synthesis, production, and applications. *Chem. Rec. 5* (6), 352−366.
(94) Cabral, H., and Kataoka, K. (2014) Progress of drug-loaded polymeric micelles into clinical studies. *J. Controlled Release 190*, 465−476.
(95) Fang, H. P., Guo, Z. P., Lin, L., Chen, J., Sun, P. J., Wu, J. Y., Xu, C. N., Tian, H. Y., and Chen, X. S. (2018) Molecular strings significantly improved the gene transfection efficiency of polycations. *J. Am. Chem. Soc. 140* (38), 11992−12000.
(96) Gabrielson, N. P., Lu, H., Yin, L., Li, D., Wang, F., and Cheng, J. (2012) Reactive and bioactive cationic alpha-helical polypeptide template for nonviral gene delivery. *Angew. Chem., Int. Ed. 51* (5), 1143−1147.
(97) McHale, M. K., Setton, L. A., and Chilkoti, A. (2005) Synthesis and in vitro evaluation of enzymatically cross-linked elastin-like polypeptide gels for cartilaginous tissue repair. *Tissue Eng. 11* (11−12), 1768−1779.
(98) Li, C., Faulkner-Jones, A., Dun, A. R., Jin, J., Chen, P., Xing, Y., Yang, Z., Li, Z., Shu, W., Liu, D., et al. (2015) Rapid formation of a supramolecular polypeptide-DNA hydrogel for in situ three-dimensional multilayer bioprinting. *Angew. Chem., Int. Ed. 54* (13), 3957−3961.
(99) Anderson, M. A., O'Shea, T. M., Burda, J. E., Ao, Y., Barlatey, S. L., Bernstein, A. M., Kim, J. H., James, N. D., Rogers, A., Kato, B., et al. (2018) Required growth facilitators propel axon regeneration across complete spinal cord injury. *Nature 561* (7723), 396−400.
(100) Yu, S. J., Wang, C., Yu, J. C., Wang, J. Q., Lu, Y., Zhang, Y. Q., Zhang, X. D., Hu, Q. Y., Sun, W. J., and He, C. L. (2018) Injectable bioresponsive gel depot for enhanced immune checkpoint blockade. *Adv. Mater. 30* (28), 1801527.
(101) Xu, Q. H., He, C. L., Ren, K. X., Zhang, Z., and Chen, X. S. (2017) Injectable, biomolecule-responsive polypeptide hydrogels with triggered degradation capacity for cell encapsulation and facile cell recovery. *J. Controlled Release 259*, E112−E112.
(102) Zhou, C., Qi, X., Li, P., Chen, W. N., Mouad, L., Chang, M. W., Leong, S. S., and Chan-Park, M. B. (2010) High potency and broad-spectrum antimicrobial peptides synthesized via ring-opening polymerization of alpha-aminoacid-N-carboxyanhydrides. *Biomacromolecules 11* (1), 60−67.
(103) Engler, A. C., Shukla, A., Puranam, S., Buss, H. G., Jreige, N., and Hammond, P. T. (2011) Effects of side group functionality and molecular weight on the activity of synthetic antimicrobial polypeptides. *Biomacromolecules 12* (5), 1666−1674.
(104) Xiong, M., Lee, M. W., Mansbach, R. A., Song, Z., Bao, Y., Peek, R. M., Yao, C., Chen, L. F., Ferguson, A. L., Wong, G. C. L., et al. (2015) Helical antimicrobial polypeptides with radial amphiphilicity. *Proc. Natl. Acad. Sci. U. S. A. 112* (43), 13155−13160.
(105) Lam, S. J., Wong, E. H., O'Brien-Simpson, N. M., Pantarat, N., Blencowe, A., Reynolds, E. C., and Qiao, G. G. (2016) Bionano Interaction Study on Antimicrobial Star-Shaped Peptide Polymer Nanoparticles. *ACS Appl. Mater. Interfaces 8* (49), 33446−33456.
(106) Qian, Y. X., Qi, F., Chen, Q., Zhang, Q., Qiao, Z. Q., Zhang, S., Wei, T., Yu, Q., Yu, S., Adn Mao, Z. W., et al. (2018) Surface modified with a host defense peptide-mimicking beta-peptide polymer kills bacteria on contact with high efficacy. *ACS Appl. Mater. Interfaces 10* (18), 15395−15400.
(107) Bevilacqua, M. P., Huang, D. J., Wall, B. D., Lane, S. J., Edwards, C. K., Hanson, J. A., Benitez, D., Solomkin, J. S., and Deming, T. J. (2017) Amino acid block copolymers with broad antimicrobial activity and barrier properties. *Macromol. Biosci. 17* (10), 1600492.
(108) Wang, J., Gibson, M. I., Barbey, R., Xiao, S. J., and Klok, H. A. (2009) Nonfouling polypeptide brushes via surface-initiated polymerization of N(ε) -oligo(ethylene glycol)succinate-L-lysine N-carboxyanhydride. *Macromol. Rapid Commun. 30* (9−10), 845−850.
(109) Zhang, C., Yuan, J., Lu, J., Hou, Y., Xiong, W., and Lu, H. (2018) From neutral to zwitterionic poly(alpha-amino acid) nonfouling surfaces: Effects of helical conformation and anchoring orientation. *Biomaterials 178*, 728−737.
(110) Zhang, C., Lu, J., Hou, Y., Xiong, W., Sheng, K., and Lu, H. (2018) Investigation on the linker length of synthetic zwitterionic polypeptides for improved nonfouling surfaces. *ACS Appl. Mater. Interfaces 10* (20), 17463−17470.
(111) Grotzky, A., Manaka, Y., Kojima, T., and Walde, P. (2011) Preparation of catalytically active, covalent alpha-polylysine-enzyme conjugates via UV/vis-quantifiable bis-aryl hydrazone bond formation. *Biomacromolecules 12* (1), 134−144.
(112) Lu, Y. J., Mbong, G. N. N., Liu, P., Chan, C., Cai, Z. L., Weinrich, D., Boyle, A. J., Reilly, R. M., and Winnik, M. A. (2014) Synthesis of polyglutamide-based metal-chelating polymers and their site-specific conjugation to trastuzumab for auger electron radioimmunotherapy. *Biomacromolecules 15* (6), 2027−2037.
(113) Talelli, M., and Vicent, M. J. (2014) Reduction sensitive Poly(L-glutamic acid) (PGA)-protein conjugates designed for polymer masked-unmasked protein therapy. *Biomacromolecules 15* (11), 4168−4177.
(114) Zhang, P., Jain, P., Tsao, C., Yuan, Z. F., Li, W. C., Li, B. W., Wu, K., Hung, H. C., Lin, X. J., and Jiang, S. Y. (2018) Polypeptides with high zwitterion density for safe and effective therapeutics. *Angew. Chem., Int. Ed. 57* (26), 7743−7747.
(115) Yuan, J., Sun, Y., Wang, J., and Lu, H. (2016) Phenyl trimethylsilyl sulfide-mediated controlled ring-opening polymerization of alpha-amino acid N-carboxyanhydrides. *Biomacromolecules 17* (3), 891−896.
(116) Hou, Y., Yuan, J., Zhou, Y., Yu, J., and Lu, H. (2016) A concise approach to site-specific topological protein-poly(amino acid) conjugates enabled by in situ-generated functionalities. *J. Am. Chem. Soc. 138* (34), 10995−11000.
(117) Yuan, J., Zhang, Y., Li, Z., Wang, Y., and Lu, H. (2018) A S-Sn lewis pair-mediated ring-opening polymerization of α-Amino Acid N-Carboxyanhydrides: fast kinetics, high molecular weight, and facile bioconjugation. *ACS Macro Lett. 7*, 892−897.
(118) Wang, X. W., and Zhang, W. B. (2018) Chemical topology and complexity of protein architectures. *Trends Biochem. Sci. 43* (10), 806−817.
(119) Nguyen, G. K., Hemu, X., Quek, J. P., and Tam, J. P. (2016) Butelase-mediated macrocyclization of D-amino-acid-containing peptides. *Angew. Chem., Int. Ed. 55* (41), 12802−12806.
(120) Hemu, X., Qiu, Y., Nguyen, G. K., and Tam, J. P. (2016) Total synthesis of circular bacteriocins by butelase 1. *J. Am. Chem. Soc. 138* (22), 6968−6971.
(121) Nguyen, G. K., Kam, A., Loo, S., Jansson, A. E., Pan, L. X., and Tam, J. P. (2015) Butelase 1: A versatile ligase for peptide and protein macrocyclization. *J. Am. Chem. Soc. 137* (49), 15398−15401.
(122) Popp, M. W., Dougan, S. K., Chuang, T. Y., Spooner, E., and Ploegh, H. L. (2011) Sortase-catalyzed transformations that improve the properties of cytokines. *Proc. Natl. Acad. Sci. U. S. A. 108* (8), 3169−3174.
(123) Hou, Y., Zhou, Y., Wang, H., Wang, R., Yuan, J., Hu, Y., Sheng, K., Feng, J., Yang, S., and Lu, H. (2018) Macrocyclization of interferon-poly(alpha-amino acid) conjugates significantly improves the tumor retention, penetration, and antitumor efficacy. *J. Am. Chem. Soc. 140* (3), 1170−1178.
(124) Birke, A., Ling, J., and Barz, M. (2018) Polysarcosine-containing copolymers: Synthesis, characterization, self-assembly, and applications. *Prog. Polym. Sci. 81*, 163−208.
(125) Lau, K. H. A., Ren, C., Sileika, T. S., Park, S. H., Szleifer, I., and Messersmith, P. B. (2012) Surface-grafted polysarcosine as a peptoid antifouling polymer brush. *Langmuir 28* (46), 16099−16107.
(126) Fokina, A., Klinker, K., Braun, L., Jeong, B. G., Bae, W. K., Barz, M., and Zentel, R. (2016) Multidentate polysarcosine-based

DOI: 10.1021/acs.bioconjchem.9b00236
Bioconjugate Chem. 2019, 30, 1604−1616

ligands for water-soluble quantum dots. *Macromolecules 49* (10), 3663−3671.
(127) Chen, Y., Xu, Z., Zhu, D., Tao, X., Gao, Y., Zhu, H., Mao, Z., and Ling, J. (2016) Gold nanoparticles coated with polysarcosine brushes to enhance their colloidal stability and circulation time in vivo. *J. Colloid Interface Sci. 483*, 201−210.
(128) Sano, K., Ohashi, M., Kanazaki, K., Makino, A., Ding, N., Deguchi, J., Kanada, Y., Ono, M., and Saji, H. (2017) Indocyanine green -labeled polysarcosine for in vivo photoacoustic tumor imaging. *Bioconjugate Chem. 28* (4), 1024−1030.
(129) Hu, Y., Hou, Y., Wang, H., and Lu, H. (2018) Polysarcosine as an alternative to PEG for therapeutic protein conjugation. *Bioconjugate Chem. 29* (7), 2232−2238.
(130) Ekladious, I., Colson, Y. L., and Grinstaff, M. W. (2019) Polymer-drug conjugate therapeutics: advances, insights and prospects. *Nat. Rev. Drug Discovery 18*, 273−294.
(131) Zaman, R., Islam, R. A., Ibnat, N., Othman, I., Zaini, A., Lee, C. Y., and Chowdhury, E. H. (2019) Current strategies in extending half-lives of therapeutic proteins. *J. Controlled Release 301*, 176.
(132) Qin, G. K., Glassman, M. J., Lam, C. N., Chang, D., Schaible, E., Hexemer, A., and Olsen, B. D. (2015) Topological effects on globular protein-ELP fusion block copolymer self-assembly. *Adv. Funct. Mater. 25* (5), 729−738.
(133) Liu, M., Johansen, P., Zabel, F., Leroux, J. C., and Gauthier, M. A. (2014) Semi-permeable coatings fabricated from comb-polymers efficiently protect proteins in vivo. *Nat. Commun. 5*, 5526.
(134) Mochida, Y., Cabral, H., Miura, Y., Albertini, F., Fukushima, S., Osada, K., Nishiyama, N., and Kataoka, K. (2014) Bundled assembly of helical nanostructures in polymeric micelles loaded with platinum drugs enhancing therapeutic efficiency against pancreatic tumor. *ACS Nano 8* (7), 6724−6738.
(135) Hou, Y., Zhou, Y., Wang, H., Sun, J., Wang, R., Sheng, K., Yuan, J., Hu, Y., Chao, Y., Liu, Z., et al. (2019) Therapeutic protein PEPylation: The helix of nonfouling synthetic polypeptides minimizes antidrug antibody generation. *ACS Cent. Sci. 5* (2), 229−236.
(136) Hackenberger, C. P. R., and Schwarzer, D. (2008) Chemoselective ligation and modification strategies for peptides and proteins. *Angew. Chem., Int. Ed. 47* (52), 10030−10074.
(137) Schumacher, D., and Hackenberger, C. P. R. (2014) More than add-on: chemoselective reactions for the synthesis of functional peptides and proteins. *Curr. Opin. Chem. Biol. 22*, 62−69.
(138) Rashidian, M., Dozier, J. K., and Distefano, M. D. (2013) Enzymatic labeling of proteins: techniques and approaches. *Bioconjugate Chem. 24* (8), 1277−1294.
(139) Nguyen, G. K., Wang, S., Qiu, Y., Hemu, X., Lian, Y., and Tam, J. P. (2014) Butelase 1 is an Asx-specific ligase enabling peptide macrocyclization and synthesis. *Nat. Chem. Biol. 10* (9), 732−738.
(140) Antos, J. M., Truttmann, M. C., and Ploegh, H. L. (2016) Recent advances in sortase-catalyzed ligation methodology. *Curr. Opin. Struct. Biol. 38*, 111−118.
(141) Pang, Y., Liu, J. Y., Qi, Y. Z., Li, X. H., and Chilkoti, A. (2016) A modular method for the high-yield synthesis of site-specific protein-polymer therapeutics. *Angew. Chem., Int. Ed. 55* (35), 10296−10300.
(142) Liu, C. C., and Schultz, P. G. (2010) Adding new chemistries to the genetic code. *Annu. Rev. Biochem. 79*, 413−444.
(143) Li, J., and Chen, P. R. (2016) Development and application of bond cleavage reactions in bioorthogonal chemistry. *Nat. Chem. Biol. 12* (3), 129−137.
(144) Bi, X., Yin, J., Hemu, X., Rao, C., Tam, J. P., and Liu, C. F. (2018) Immobilization and intracellular delivery of circular proteins by modifying a genetically incorporated unnatural amino acid. *Bioconjugate Chem. 29* (7), 2170−2175.
(145) Huesmann, D., Klinker, K., and Barz, M. (2017) Orthogonally reactive amino acids and end groups in NCA polymerization. *Polym. Chem. 8* (6), 957−971.
(146) Luo, Q., Hou, C., Bai, Y., Wang, R., and Liu, J. (2016) Protein assembly: Versatile approaches to construct highly ordered nanostructures. *Chem. Rev. 116* (22), 13571−13632.
(147) Tang, N. C., and Chilkoti, A. (2016) Combinatorial codon scrambling enables scalable gene synthesis and amplification of repetitive proteins. *Nat. Mater. 15* (4), 419−424.
(148) Huang, P. S., Boyken, S. E., and Baker, D. (2016) The coming of age of de novo protein design. *Nature 537* (7620), 320−327.
(149) Silva, D. A., Yu, S., Ulge, U. Y., Spangler, J. B., Jude, K. M., Labao-Almeida, C., Ali, L. R., Quijano-Rubio, A., Ruterbusch, M., Leung, I., et al. (2019) De novo design of potent and selective mimics of IL-2 and IL-15. *Nature 565* (7738), 186.
(150) Ovchinnikov, S., Park, H., Varghese, N., Huang, P. S., Pavlopoulos, G. A., Kim, D. E., Kamisetty, H., Kyrpides, N. C., and Baker, D. (2017) Protein structure determination using metagenome sequence data. *Science 355* (6322), 294−297.
(151) Ljubetic, A., Lapenta, F., Gradisar, H., Drobnak, I., Aupic, J., Strmsek, Z., Lainscek, D., Hafner-Bratkovic, I., Majerle, A., Krivec, N., et al. (2017) Design of coiled-coil protein-origami cages that self-assemble in vitro and in vivo. *Nat. Biotechnol. 35* (11), 1094−1101.
(152) Barz, M., Luxenhofer, R., Zentel, R., and Vicent, M. J. (2011) Overcoming the PEG-addiction: well-defined alternatives to PEG, from structure-property relationships to better defined therapeutics. *Polym. Chem. 2* (9), 1900−1918.
(153) Bertin, A., Hermes, F., and Schlaad, H. (2009) Biohybrid and Peptide-Based Polymer Vesicles. *Adv. Polym. Sci. 224* (1), 167−195.
(154) Lu, H., Wang, J., Song, Z., Yin, L., Zhang, Y., Tang, H., Tu, C., Lin, Y., and Cheng, J. (2014) Recent advances in amino acid N-carboxyanhydrides and synthetic polypeptides: chemistry, self-assembly and biological applications. *Chem. Commun. (Cambridge, U. K.) 50* (2), 139−155.
(155) Hadjichristidis, N., Iatrou, H., Pitsikalis, M., and Sakellariou, G. (2009) Synthesis of well-defined polypeptide-based materials via the ring-opening polymerization of alpha-amino acid N-carboxyanhydrides. *Chem. Rev. 109* (11), 5528−5578.
(156) He, X., Fan, J., and Wooley, K. L. (2016) Stimuli-triggered sol-gel transitions of polypeptides derived from alpha-amino acid N-carboxyanhydride (NCA) polymerizations. *Chem. - Asian J. 11* (4), 437−447.
(157) He, C., Zhuang, X., Tang, Z., Tian, H., and Chen, X. (2012) Stimuli-sensitive synthetic polypeptide-based materials for drug and gene delivery. *Adv. Healthcare Mater. 1* (1), 48−78.
(158) Huang, J., and Heise, A. (2013) Stimuli responsive synthetic polypeptides derived from N-carboxyanhydride (NCA) polymerisation. *Chem. Soc. Rev. 42* (17), 7373−7390.
(159) Shen, Y., Fu, X., Fu, W., and Li, Z. (2015) Biodegradable stimuli-responsive polypeptide materials prepared by ring opening polymerization. *Chem. Soc. Rev. 44* (3), 612−622.
(160) Sun, Y. L., Hou, Y. Q., Zhou, X. H., Yuan, J. S., Wang, J. Y., and Lu, H. (2015) Controlled synthesis and enzyme-induced hydrogelation of poly(L-phosphotyrosine)s via ring-opening polymerization of alpha-amino acid N-carboxyanhydride. *ACS Macro Lett. 4* (9), 1000−1003.
(161) Xiong, W., Fu, X. H., Wan, Y. M., Sun, Y. L., Li, Z. B., and Lu, H. (2016) Synthesis and multimodal responsiveness of poly(alpha-amino acid)s bearing OEGylated azobenzene side-chains. *Polym. Chem. 7* (41), 6375−6382.
(162) Yuan, J. S., Zhang, Y., Sun, Y., Cai, Z. C., Yang, L. J., and Lu, H. (2018) Salt- and pH-triggered helix-coil transition of ionic polypeptides under physiology conditions. *Biomacromolecules 19* (6), 2089−2097.
(163) Panganiban, B., Qiao, B., Jiang, T., DelRe, C., Obadia, M. M., Nguyen, T. D., Smith, A. A. A., Hall, A., Sit, I., Crosby, M. G., et al. (2018) Random heteropolymers preserve protein function in foreign environments. *Science 359* (6381), 1239−1243.
(164) He, H., Chen, Y. B., Li, Y. J., Song, Z. Y., Zhong, Y. N., Zhu, R. Y., Cheng, J. J., and Yin, L. C. (2018) Effective and selective anticancer protein delivery via all-functions-in-One nanocarriers coupled with visible light-responsive, reversible protein engineering. *Adv. Funct. Mater. 28* (14), 1706710−1706720.

DOI: 10.1021/acs.bioconjchem.9b00236
Bioconjugate Chem. 2019, 30, 1604−1616

(165) Liu, X., Wu, F., Ji, Y., and Yin, L. C. (2019) Recent advances in anti-cancer protein/peptide delivery. *Bioconjugate Chem. 30* (2), 305−324.
(166) Cheng, L., Yang, L., Meng, F. H., and Zhong, Z. Y. (2018) Protein nanotherapeutics as an emerging modality for cancer therapy. *Adv. Healthcare Mater. 7* (20), 1800685−1800693.
(167) Ray, M., Lee, Y. W., Scaletti, F., Yu, R., and Rotello, V. M. (2017) Intracellular delivery of proteins by nanocarriers. *Nanomedicine (London, U. K.) 12* (8), 941−952.
(168) Mout, R., Ray, M., Yesilbag Tonga, G., Lee, Y. W., Tay, T., Sasaki, K., and Rotello, V. M. (2017) Direct cytosolic delivery of CRISPR/Cas9-ribonucleoprotein for efficient gene editing. *ACS Nano 11* (3), 2452−2458.
(169) Mout, R., Ray, M., Tay, T., Sasaki, K., Yesilbag Tonga, G., and Rotello, V. M. (2017) General strategy for direct cytosolic protein delivery via protein-nanoparticle co-engineering. *ACS Nano 11* (6), 6416−6421.
(170) Gao, J., Zhao, B., Wang, M., Serrano, M. A. C., Zhuang, J., Ray, M., Rotello, V. M., Vachet, R. W., and Thayumanavan, S. (2018) Supramolecular assemblies for transporting proteins across an immiscible solvent interface. *J. Am. Chem. Soc. 140* (7), 2421−2425.
(171) Su, S., Wang, Y. Y., Du, F. S., Lu, H., and Li, Z. C. (2018) Dynamic covalent bond-assisted programmed and traceless protein release: High loading nanogel for systemic and cytosolic delivery. *Adv. Funct. Mater. 28* (48), 1805287−1805295.

DOI: 10.1021/acs.bioconjchem.9b00236
Bioconjugate Chem. 2019, 30, 1604−1616