Zwitterlation improves efficacy and safety of therapeutic proteins

Yanjiao Han

A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2020

Reading Committee:

Shaoyi Jiang, Chair

Ying Zheng

Stéphanie Valleau

Program Authorized to Offer Degree:

Molecular Engineering & Science

University of Washington

Abstract

Zwitterlation improves efficacy and safety of therapeutic proteins

Yanjiao Han

Chair of the Supervisory Committee:

Professor Shaoyi Jiang

Department of Chemical Engineering

During the past decades, engineered proteins have been widely used as biopharmaceutical drugs (e.g., recombinant therapeutic proteins, enzymes, and monoclonal antibodies) and gene editing tools (e.g., zinc fingers, TALENs and CRISPR/Cas9). This has significantly transformed the pharmaceutical industry. Compared with conventional small molecule drugs, these biomacromolecules offer the advantages of higher specificity and potency. However, safety and efficacy concerns have been raised from different aspects. In this thesis, the author discusses the tactics to improve efficacy and safety of protein therapeutics. First, to protect the protein from body clearance and immune system, a poly(carboxybetaine) (pCB) polymer is conjugated to the protein surface. Compared with PEG conjugation, the zwitterionic coating more effectively shields protein epitopes, rendering the whole protein invisible to the body immune system. This strategy is proved to be effective in increasing protein stability, prolonging *in vivo* circulation half-life and

reducing immunogenicity, and is successfully applied to interferon-alpha (IFN-α). Compared with PEGylated IFN-α, Zwitterlated IFN-α largely retains its *in vitro* efficacy. In addition, the author further discusses the tactics to improve the safety and efficacy of nuclease for gene editing. To reduce the off-target effect of CRISPR/Cas9 system, the author discusses a novel method that conjugating CRISPR/Cas9 with zwitterionic pCB polymer to address the mismatch issue at the off-target sites. The author demonstrates the conjugation of CRISPR/Cas9 system with pCB polymer can both increase the efficacy of Cas9 nuclease and reduce non-specific interactions between gRNA and DNA (off-target DNA break). Finally, we converted the strategy to the mRNA version via a new strategy for reducing off-target effect of CRISPR/Cas9 systems that relies on fusing zwitterionic peptide poly(EK) extensions to the terminus of Cas9 via genetic engineering. The poly(EK) peptide is biodegradable and potentially safer for clinical applications. This technology was realized through mRNA delivery and will provide a robust and easily used strategy to reduce “off-target” mutations on a genome-wide scale and to improve the efficiency of a wide range of CRISPR/Cas9-based biological and clinical applications.

TABLE OF CONTENTS

Acknowledgements

For all the studies presented here, the author would like to thank her advisor, Professor Shaoyi Jiang for his support and inspiration. The author would also thank all members in Prof. Jiang's research group for their kind help and discussion and Profs. Ying Zheng, Stéphanie Valleau and Marjorie Olmstead for serving on the supervisory committee. The author would especially thank her family.

List of Tables

List of Figures

Chapter 1 Introduction

Bioengineered protein drugs have multiple advantages over conventional small-molecule drugs, such as target specificity, high potency, and low adverse effects.[1] The global market for bioengineered protein drugs is expected to reach $228.4 billion by 2021, rising at a compound annual growth rate (CAGR) of 5.8% from 2016 through 2021.[2] More than 210 biopharmaceutical products were marketed during the last few decades, with over 900 new products currently in the pipeline.[3] Despite the huge success achieved by biopharmaceutics, these structurally complex biomacromolecules usually face great challenges, including poor stability, inadequate circulation half-life, and strong immunogenicity.[4, 5] A low half-life limits therapeutic efficacy and requires frequent dosing regimen. Immune responses against many biological drugs not only result in accelerated blood clearance during chronic use but also threaten lives of patients with adverse effects, including anaphylaxis and infusion reactions.[6]

The covalent conjugation of poly(ethylene glycol) (PEG) to a protein (or PEGylation) is clinically proven to favorably alter the protein's pharmacokinetics by prolonging its circulation time and to decrease the protein's toxicity profile, especially with respect to the reduction of immunogenicity[7-11]. Additional advantages of PEGylation include the potential to increase water solubility and the storage stability of a protein. More than 10 PEGylated proteins have been brought to the market, and more than 15 are under clinical trials[12]. Although PEGylation has addressed several limitations and is clinically proven, there are a variety of obstacles and pitfalls to overcome in the continued

development of PEGylated protein drugs. In almost all cases, the *in vitro* biological activity of PEGylated proteins is lower, sometimes significantly lower, than that of the unmodified protein[8, 9]. This is particularly true for those proteins, which interact with large targets. For example, Pegasys, a PEGylated interferon alpha-2a (IFN-α2a) bearing a 40-kDa branched PEG, is a typical example of a popular protein drug that displays only 7% *in vitro* activity of the unmodified IFN-α2a[13]. Even for enzymes dealing with small substrates, a significant bioactivity loss can also be observed. For example, Adagen, a PEGylated adenosine deaminase, can only retain 18% bioactivity when compared to unmodified protein[14]. PEG is an amphiphilic polymer with both hydrophilic and hydrophobic characters. It was shown previously by our group from both experimental and simulation studies that protein bioactivity decreases after PEGylation due to the hydrophobic nature of PEG[15, 16]. PEG polymers are often randomly attached to proteins in a nonspecific way, some of which may be in or near protein active sites.

In contrast, zwitterionic materials, bearing simultaneously a pair of oppositely charged ions in the same moiety while maintaining overall neutral charge, have been identified recently as a class of extremely hydrophilic materials[17]. Taking poly(carboxybetaine) (pCB) as an example, its strong electrostatically induced hydration confers an ability with superior resistance to nonspecific protein adsorption[18]. Zwitterionic polymer coatings also endow stealth properties to nanoparticles, enabling significantly prolonged in vivo circulation half-lives[19]. In a previous study, pCB has been conjugated to the model enzyme chymotrypsin (CT) to test its protein stabilizing effect[15]. It has been shown that this pCB conjugation (or zwitterlation) exhibited a more robust ability than PEGylation to protect conjugated proteins against environmental

stressors. More importantly, conjugation of pCB not only preserves enzyme bioactivity but also increases its binding affinity slightly since pCB is composed of glycine betaine, which is a well-known protein stabilizer. However, it was shown that PEG conjugation decreased the binding affinity of the enzyme to a peptide substrate, particularly for larger substrates. A fundamental understanding of the difference between PEG and pCB conjugation was studied before[17]. For proteins, hydrophobic–hydrophobic interactions are significant for enzyme-substrate specific binding (or bioactivity). With PEG, its amphiphilicity reduces the hydrophobic–hydrophobic driving force of enzyme-substrate interactions while also imposing steric hindrance (Figure 1.1b). Molecular simulation results[17] also show the steric (or blocking) effects of PEG to the specific binding. In contrast, with the pCB conjugates, the binding affinity was either unaltered or even improved. Owing to the super-hydrophilicity of the polymer, water is drawn away from hydrophobic regions of the protein, shifting the equilibrium to allow the substrate and binding site to interact (Figure 1.1c). All of these results make pCB a candidate in substituting PEG for future design of polymer-protein conjugates.

Bioinformatics studies of over 1000 proteins have revealed the predominance of positively charged lysine (K) and negatively charged glutamic acid (E) residues on protein surfaces at balanced ratios[20]. It is believed that the distribution of equal amounts of oppositely charged E and K residues provides a zwitterionic layer to stabilize protein and resist non-specific adsorptions. Both random and alternating EK sequences have been shown to confer nonfouling zwitterionic characteristics to surfaces and nanoparticles[21-26]. A zwitterionic $(EK)_n$ peptides would provide protection to the protein in a similar way as a zwitterionic pCB polymer[21] and $(EK)_n$ peptides are also biodegradable. One major

advantage is that a simple alternating $(EK)_n$ peptides can be attached to a protein via genetic fusion in addition to chemical conjugation (or EKylation).

In this thesis, the author aims to improve the safety and efficacy of biological therapeutics for different applications. In Chapter 2, the author proposes to perform a systematic study of the role of zwitterionic polymers in the bioactivity of conjugated proteins and to demonstrate that Zwitterlation is better than PEGylation to retain protein bioactivity. IFN-α2a is a small (19.2 kDa) protein that is rapidly cleared from the body through renal filtration. PEGylation of IFN-α2a dramatically increases the circulation time. However, the bioactivity of PEGylated IFN-α2a is only 7% of the native form. In this work, IFN-α2a will be used as a model protein for various studies since IFN-α2a targets large cellular receptors on cell walls and its bioavailability is always an issue. In this work, linear pCB with different molecular weights will be chemically synthesized and linked to IFN-α2a at different locations of IFN-α2a. Detailed chemical and biological characterization is performed to reveal the mechanisms behind bioactivity retention. This work employs state-of-the-art biomaterials and protein conjugation methods to solve one long-standing issue, i.e., protein bioactivity is greatly reduced after PEGylation and PEGylated protein enzymes or ligands are not able to interact with large substrates or receptors.

In chapter 3, the author applies this new technology to gene editing. The clustered, regularly interspaced, short palindromic repeats (CRISPR)–associated protein 9 (Cas9) system is now widely used for genome editing in cultured cells and whole organisms[27, 28]. Unfortunately, it can also cause unwanted mutations at off-target sites that resemble the on-target sequence [29]. For example, a single guide RNA (sgRNA) which recognizes 20-bp target DNA sequences can tolerate mismatches at up to 5 nucleotide positions [29].

This suggests that there are up to thousands of off-target sites for a given nuclease in the human genome. Off-target DNA cleavages can lead to mutations at unintended genomic loci and to chromosomal rearrangements such as translocations[30, 31], deletions[32, 33] and inversions[34] which could induce cytotoxic effects on the host cell[35]. These mutations at unwanted sites might disable a tumor-suppressor gene or activate a cancer-causing one and the translocations is the cause of chronic myeloid leukemia[36]. Preventing, avoiding or at least minimizing these effects is crucial for the success of any genome editing application. Various strategies have been described to reduce genome-wide off-target mutations of the commonly used SpCas9 nuclease, including: truncated sgRNAs bearing shortened regions of target site complementarity[37, 38], SpCas9 mutants such as the recently described D1135E variant[39], paired SpCas9 nickases[40, 41], and dimeric fusions of catalytically inactive SpCas9 to a non-specific FokI nuclease[42-44]. However, these approaches are only partially effective, have as-yet unproven efficacies on a genome-wide scale, and/or possess the potential to create more off-target sites. Furthermore, some require expression of multiple sgRNAs and/or fusion of additional functional domains to Cas9, which can reduce targeting range and create challenges for delivery with viral vectors that have limits on nucleic acid payload size. Thus, a major challenge for the field remains the development of a robust and easily used strategy that eliminates off-target mutations on a genome-wide scale.

The “off-target” activity of the nucleases occurs fundamentally because the nucleases lack perfect specificity, i.e., they are able to bind to sequences similar to the intended DNA target, which is called mismatch. Therefore, it is hypothesized that off-target effects of SpCas9 might be minimized by decreasing non-specific interactions with its target DNA

sites. Zwitterionic polycarboxybetaine (pCB) polymers and alternating lysine (K) and glutamic acid (E) [poly(EK)] peptides are highly hydrated or super-hydrophilic and uniquely resistant to non-specific interactions[45]. Extending pCB to a more therapeutically relevant platform, we worked with a model polymer-protein conjugate. Results show that pCB conjugation not only preserves enzyme bioactivity, but also increases its binding affinity slightly since pCB is consisted of glycine betaine, which is a well-known protein stabilizer[46].

For proteins, hydrophobic–hydrophobic interactions are significant for enzyme-substrate specific binding (or bioactivity). Owing to the super-hydrophilicity of the polymer, water is drawn away from hydrophobic regions of the protein, shifting the equilibrium to allow the substrate and binding site to interact. Based on this finding, it is expected that a pCB conjugated object will reduce its (both specific and non-specific) interactions with its target. Since specific DNA-sgRNA matching is far stronger than non-specific interactions, if this pCB conjugation is able to reduce non-specific interactions mostly and still maintain strong specific interactions, then off-target will be eliminated or reduced.

Furthermore, besides chemical conjugation, bio-compatible and degradable zwitterionic poly(EK) peptide will be fused to Cas9 via genetic engineering and discussed in Chapter 4. In our previous studies, we demonstrated that the bio-inspired ‘EKylation’ method not only confers the stabilizing benefits of polyzwitterions, but also allows for rapid biosynthesis of target constructs through the genetic fusion of poly(EK). This 'one-step' strategy to produce conjugate or fused protein provides a broadly-applicable alternative to synthetic polymer conjugation. Poly(EK) is both bio-compatible and

degradable. Here the author develops a new strategy for reducing off-target effect of CRISPR/Cas9 systems that relies on fusing poly(EK) extensions to their termini via genetic engineering. The tails are treated and tested as a peptide analogue of the zwitterionic polymer pCB that exhibits similar resistance to non-specific mismatch between gRNA and DNA. The author appends poly(EK) tails of well-defined lengths to the C-terminus or both C- and N-terminus of the Cas9 protein and studies the on/off target effect of these constructs.

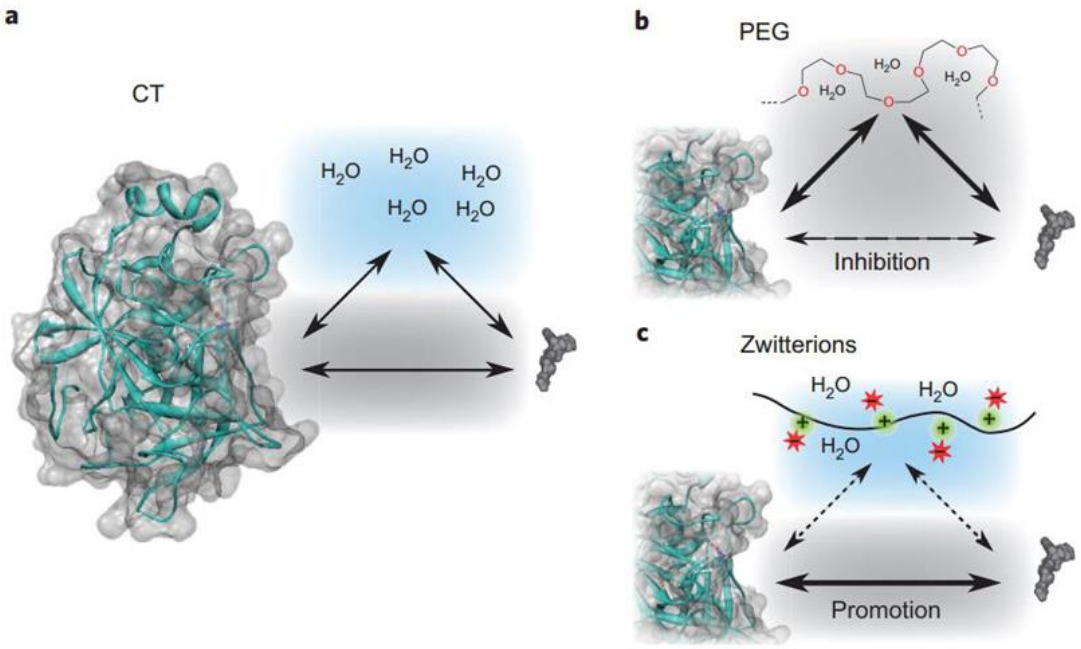


Figure 1.1. Mechanism of how PEG and pCB polymers influence binding affinity. a, Relationship between enzyme and substrate without polymer. b, PEG impedes affinity by reducing enzyme–substrate hydrophobic–hydrophobic interactions as a result of its amphiphilic characteristics. c, Super-hydrophilic pCB has a strong effect on the structure of water, creating a local environment that increases enzyme–substrate hydrophobic–hydrophobic interactions, thereby increasing the affinity of the substrate for the binding pocket.[47]

Chapter 2 Mitigates Protein Bioactivity Loss *in vitro* over PEGylation *via* Zwitterlation

Conjugation with poly(ethylene glycol) (PEG) or PEGylation is a widely used tool to overcome the shortcomings of native proteins, such as poor stability, inadequate pharmacokinetic (PK) profiles, and immunogenicity. However, PEGylation often accompanies an unwanted detrimental effect on bioactivity, particularly, resulting from the amphiphilic nature of PEG. This is especially true for PEGylated proteins with large binding targets. Pegasys, a PEGylated interferon alpha-2a (IFN-α2a) bearing a 40-kDa branched PEG, is a typical example that displays only 7% in vitro activity of the unmodified IFN-α2a. In this work, by employing IFN-α2a as a model protein, we demonstrated that a protein conjugated with zwitterionic polymers (or Zwitterlation) could significantly mitigate the antiproliferative bioactivity loss in vitro after polymer conjugation. The retained antiproliferative activity of Zwitterlated IFN-α2a is 4.4-fold higher than the PEGylated IFN-α2a with the same polymer molecular weight, or 3-fold higher than the PEGylated IFN-α2a with the similar hydrodynamic size. It is hypothesized that nonspecific interactions between zwitterionic polymers and IFN-α2a/ IFN-α2a receptor can be mitigated due to the super-hydrophilic nature of zwitterionic polymers. This, in turn, reduces the 'nonspecific blocking' between IFN-α2a and IFN-α2a receptor. In addition, we demonstrated that Zwitterlated IFN-α2a showed a prolonged circulation time and a mitigated accelerated blood clearance after repeated injections in rats.

2.1 Introduction

Advances in effective protein therapy are hindered by their poor stability, short circulating half-time, and immunogenicity.[1, 48] The covalent conjugation of poly(ethylene glycol) (PEG) to a protein or PEGylation is clinically proven to favorably alter protein pharmacokinetics by prolonging its circulation time and decreasing its toxicity profile, especially reduced immunogenicity.[5, 8-11] Additional advantages of PEGylation include the potential to increase protein solubility and its storage stability. At present, more than 10 PEGylated proteins have been brought to market, and more than 15 are under clinical trials.[12] Although PEGylation has addressed several limitations and is clinically proven, there are a variety of obstacles and pitfalls to overcome in the continued development of PEGylated protein drugs. In almost all cases, the in vitro biological activity of PEGylated proteins is lower, sometimes significantly lower, than that of the unmodified proteins.[8, 9] This is particularly true for those proteins, which interact with large binding targets whose size is bigger than 900Da[49]. For example, Pegasys, a PEGylated interferon alpha-2a (IFN-α2a) bearing a 40-kDa branched PEG, is a typical example of a popular protein drug that displays only 7% in vitro activity of the unmodified IFN-α2a.[13] Even for enzymes dealing with small substrates, a significant bioactivity loss can also be observed. For example, Adagen, for which PEGylation is used to decrease the immunogenicity of the non-human protein, can retain only 18% bioactivity when compared with the unmodified protein.[14] PEG is an amphiphilic polymer with both hydrophilic and hydrophobic characters. It was shown previously from both experimental and simulation studies that protein bioactivity decreases after PEGylation due to the hydrophobic nature of PEG.[16, 47] Although various efforts have been made to minimize this effect, such as

the site-specific PEGylation,[50, 51] the material issue remains unresolved over the last 40 years[52].

In contrast, zwitterionic polymers, bearing simultaneously a pair of oppositely charged ions in the same moiety while maintaining the overall neutral charge, have been identified recently as a class of extremely hydrophilic materials.[17] Taking poly(carboxybetaine) (pCB) as an example, its strong electrostatically induced hydration confers an ability to effectively resist nonspecific protein adsorption in complex media.[18] Due to their nonfouling characteristics, zwitterionic polymers have been used for inhibiting foreign body reactions[53], maintaining stem cell phenotypes[54], sustaining protein delivery[55] and stabilizing proteins[56, 57]. In addition, zwitterionic polymer layers significantly prolong the in vivo circulation half-life of nanoparticles including liposome, protein and gold particles.[19, 58-61] Among zwitterionic polymers, pCB is particularly unique because it is composed of glycine betaine, which is a well-known protein stabilizer. In a previous study, pCB has been conjugated to chymotrypsin (CT) to test its protein stabilizing effect.[47] It has been shown that pCB conjugation (or Zwitterlation) not only preserves enzyme bioactivity, but also slightly increase its binding affinity to peptide substrates. At the same time, it is found that PEGylation decreases the binding affinity of the enzyme. A fundamental understanding of the difference between PEGylation and Zwitterlation was studied before.[17] For proteins, hydrophobic-hydrophobic interactions are significantly important for enzyme-substrate specific binding (or bioactivity). With PEGylation, the amphiphilicity of PEG reduces the hydrophobic-hydrophobic driving force of enzyme-substrate interactions as confirmed from our molecular simulations.[17] In contrast, with Zwitterlation, its binding affinity is either unaltered or even improved

when the binding target of protein is a small molecule.[47] Owing to the super-hydrophilicity of pCB polymers, water is drawn away from hydrophobic regions of the protein, shifting the equilibrium to allow the substrate and binding site to interact. All these results make pCB a competitive candidate for protein conjugates. Previously, we have demonstrated that zwitterionic pCB conjugate is able to retain the bioactivity of enzyme with relatively small substrates. Here, we performed a systematic study of the effect of Zwitterlation on the bioactivity of protein whose binding target is substantially larger than Keefe studied previously.[47]

2.2 Experimental Section

2.2.1 Materials

All chemicals (unless otherwise specified) and DAUDI human cell line were purchased from Sigma—Aldrich (St. Louis, MO). Interferon-alpha 2a (IFN-α2a), Human was phurchased from Genescript (Piscataway, NJ). 10-kDa MW, 20-kDa MW and 40-kDa MW 2-arms branched MethoxyPEG COO NHS (NHS-mPEG) were purchased from NOF America Corporation (White Plains, NY). N-α-maleimidoacet-oxysuccinimide ester (AMAS) and Pierce Protein Concentrators were purchased from Thermo Fisher Scientific (Waltham, MA). Sprague Dawley rats were purchased from Charles River Laboratories (Burlington, MA). Daudi cells were purchased from American Type Culture Collection (ATCC). Human IFNα (multiple subtypes) bioluminescent ELISA kit was purchased from InvivoGen (San Diego, CA). Antibodies were purchased from Abcam (Cambridge, MA). 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was purchased from Bethyl Laboratories (Montgomery, TX).

2.2.2 Size-exclusion chromatography

The native IFN-α2a and IFN-α2a conjugates were analyzed through a 1260 Infinity binary high performance liquid chromatography (HPLC) system equipped with a UV detector (Agilent Technologies, Santa Clara, CA), a miniDAWN TREOS light scattering (LS) detector, and a Optilab T-rEX differential refractive index (dRI) detector (Wyatt Technology, Santa Barbara, CA). The flow rate was set at 1 mL/min with the mobile phase PBS (pH 7.4). A Waters Ultrahydrogel 1000 column (7.8 mm x 300 mm, 12 μm particle size) was used for polymer-protein conjugates. All samples were filtered through 0.2 μm PTFE filters prior to the experiment. Molecular weight was determined by generating a standard curve with six PEG samples of known MW and PDI.

2.2.3 Dynamic light scattering (DLS)

The hydrodynamic sizes of the native IFN-α2a and its conjugate samples were measured by a DLS method using a Malvern Nano Zetasizer in PBS buffer at RT. Samples were treated with a filter of 0.2 μm pore size before analysis.

2.2.4 Detection of anti-IFN and anti-polymer antibodies

Indirect enzyme-linked immunosorbent assay (ELISA) experimental conditions were performed to detect antibodies specific for IFN-α2a, PEG, or pCB. The antigens used here consisted of native IFN-α2a (for detection of anti-IFN antibody), PEG-BSA conjbugate (for detection of anti-PEG antibody), and pCB-BSA conjugate (for detection of anti-pCB antibody). PEG-BSA conjugate and pCB-BSA conjugate were made following the same procedure as PEG-IFN and pCB-IFN samples. An antigen solution (10 μg/mL) in sodium carbonate buffer (0.1 M, pH 9.5) is first adsorbed onto the plate at 100 μL antigen solution (10 μg/mL) per well for 12 h at 4 °C. After moving antigen solutions, the plate was washed

5 times using PBS (pH 7.4) and then blocked with blocking buffer (1% BSA solution in 0.1 M Tris buffer, pH 8.0) for 1 h at 4 ◦C. After another 5× wash, Serial dilutions of rat sera in PBS were added to the plate (100 µL/well) and incubated for 2 h at 37 °C. The plate was then washed 5 times with PBS. Secondary antibody (goat anti-rat IgM or IgG conjugated to HRP) diluted 1:20,000 was then added and incubated at RT for 1 h followed by 5× wash with PBS. The HRP substrate 3,3',5,5'-tetramethylbenzidine (TMB) solution was then added and the plate was shaken for 15 min and quenched with 100 µL stop solution (0.2 M H_2SO_4). Absorbance at 450 nm with 570 nm values as background subtraction was recorded by a microplate reader. Pre-experiment blood samples were used as negative control for all ELISA detections. Anti-PEG IgM serum concentrations were quantified using commercial mono-clonal rat anti-PEG IgM, which is also the positive control for the polymer-specific assays. Polymer-specific antibody detection assays were further validated via competition with free pCB or PEG polymer.

2.2.5 Synthesis of N-hydroxysuccinimide poly(carboxybetaine acrylamide) (NHS-pCBAA)

pCB-NHS was synthesized based on our previous procedure.[62] Briefly, we firstly synthesized 3-acrylamido-N-(2-(tert-butoxy)-2-oxoethyl)-N,N-dimethylpropane-1-aminium (CBAAM-1-tBu). Then, a typical reversible addition-fragmentation chain-transfer (RAFT) polymerization reaction was performed to yield the 10 kDa and 20kDa SH-pCB polymer. The final colorless NHS-activated polymer was formed by reaction with AMAS at 1:10 molar ratio in DI water (pH 6) for 30 min, followed by removal of unreacted AMAS via Amicon spin dialysis tubes and freeze-drying for 48 h.

2.2.6 Synthesis of protein conjugates

Conjugates of pCB-IFN and PEG-IFN were synthesized by reacting NHS ester groups of the polymer with available amine groups on the protein. In a typical conjugation reaction, IFN-α2a and NHS-pCB at 1:3 molar ratio were dissolved in 50 mM sodium borate buffer, pH 9.0. The final protein concentration was ~5 mg/mL. The reaction mixture was stirred for 2 hours at 4 °C and stopped by adjusting the pH of the mixture to 4.5 with glacial acetic acid. The polymer-protein conjugate was isolated via molecular weight cut-off (MWCO) spin dialysis membrane followed by ion exchange chromatography. The same conjugation and purification methods were used to generate PEGylated IFN-α2a. The protein concentrations of the prepared conjugates were determined with the Pierce™ 660nm protein assay reagent (ThermoFisher Scientific) and confirmed with Human IFNα (multiple subtypes) bioluminescent ELISA kit (InvivoGen).

2.2.7 *In vitro* antiproliferative activity

The procedures of in vitro proliferative activity measurement were employed with lymphoblast cells, Daudi. Daudi cells at passage 4 or 5 were cultured in RPMI 1640 medium containing 10% (vol/vol) fetal bovine serum (FBS) and antibiotics at 37℃ in a humidified, 5% CO_2 atmosphere. For bioassays, the cells were suspended in 2×10^5 cells/mL and seeded in a 96-well plate (Corning) (50 µL, 1×104 cells/well). Conjugated protein samples were diluted in culture medium at twice the desired concentrations (5, 10, 20, 50, 100, 500, 1000, 10000 pg/mL) and 50 µL added to each test cells. Wells without IFN-α2a were used as the negative control, defined as 100% cell viability. After incubation for 72 h, the viability of cells was determined by MTT assay.

2.2.8 Pharmacokinetics

The pharmacokinetics of native and conjugated IFN-α2a were studied using Sprague Dawley rats (female, body weight 101 – 125 g) as animal model. All animal experiments adhered to federal guidelines and were approved by the University of Washington Institutional Animal Care and Use Committee IACUC) under protocol #4203-01. The rats were randomly distributed to six groups (3 rats per group) and injected with IFN-α2a, pCB10K-IFN, pCB20K-IFN, PEG10K-IFN, PEG20K-IFN, PEG40K-IFN at a dosage of 100μg IFN-equivalent/kg body weight via tail vein. Blood samples were collected from the tail vein at 5 min, 4 h, 8 h, 24 h, 28 h and 72 h post injection to generate a circulation profile of the different formulations. A pre-dose sample (0 h) was drawn before the injection of the test proteins as a control sample. The concentration of IFN-α2a was determined by ELISA assay according to the instructions of human IFNα (multiple subtypes) bioluminescent ELISA kit (InvivoGen). Pharmacokinetic parameters were analyzed by PKSolver following the instruction. The IV injections and bleeding procedure were repeated 3 times, with one week between each injection. Five weeks after the first injection, 5 mL blood was drawn by using cardiac punch, and serum was prepared for antibody detections.

2.3 Results and Discussion

IFN-α2a is a small protein (19.2 kDa) which targets large cellular receptors on cell membrane. In clinical applications, unconjugated IFN-α2a can be rapidly cleared from the body through renal filtration due to their small size (19.2 kDa). PEGylation of IFN-α2a dramatically increases its circulation time. However, the bioactivity of PEGylated IFN-α2a

is only 7% of its native form. In this work, as illustrated in Figure 1a-b, zwitterionic polymers with different molecular weights were conjugated to IFN-α2a through a two-step method as we reported before,[62] and the schematic is illustrated in Figure 2.1a. To make a fair comparison between Zwitterlated and PEGylated conjugates, PEGylated IFN-α2a with both similar molecular weights and hydrodynamic sizes to Zwitterlated IFN-α2a were prepared and compared. The size differences with various polymers and IFN-α2a conjugates are presented in Figure 2.5 and Figure 2.1c. After purification, pCB-IFN and PEG-IFN with single-polymer conjugation were obtained. The successful synthesis of mono-PEGylated or mono-zwitterlated IFN was confirmed by SDS-PAGE analysis (Figure 2.6). The hydrodynamic size and polydispersity index of the polymer-IFN conjugates (Table 2.1) further confirmed the successful preparation and the monodispersity of the conjugates. The conversions and yields of the conjugate reactions were shown in Table 2.2.

IFNs are highly pleiotropic cytokines and exhibit potent antiproliferative properties.[63] To demonstrate whether Zwitterlation could reduce the bioactivity loss of IFN-α2a, we employed a widely accepted antiproliferation assay to study the in vitro bioactivity change of Zwitterlated and PEGylated IFN-α2a conjugates. In brief, the antiproliferation assay was performed by serial dilutions in 96-well microtiter plates seeded with Daudi cells, a human lymphoblastoid cell line. After 72 h incubation, cells were treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and analyzed microplate reader for viability. Antiproliferative activity, expressed as IC_{50}, was calculated by a nonlinear regression method (Figure 2.2a). As presented in Figure 2b, the antiproliferative activity of pCB-IFNs is much higher than PEGylated IFNs. Notably,

pCB_{20k}-IFN and PEG_{20k}-IFN have similar molecular weight, but the antiproliferative activity of pCB_{20k}-IFN (62.1%) is 4.4-fold higher than that of PEG_{20k}-IFN (14.2%). Considering the hydrodynamic volume difference, we also prepared PEG_{10k}-IFN, which has the similar hydrodynamic size to pCB_{20k}-IFN. The antiproliferative activity of PEG_{10k}-IFN is 20.9% and is about 1/3 of pCB_{20k}-IFN. These results indicate that pCB conjugation can mitigate bioactivity loss than PEGylation.

As presented in Figure 2.2c, we hypothesize that the bioactivity loss after PEGylation is mainly attributed to the steric effect and nonspecific interactions of amphiphilic PEG with both interferon binding domain and interferon receptor. This nonspecific blocking effect sometimes does not significantly affect the bioactivity when the protein size is large, and the substrate size is small (e.g., uricase). However, for conjugated proteins whose receptors have similar or larger size, this nonspecific blocking usually results in a significant bioactivity loss (e.g., interferon). In contrast to PEGylation, we hypothesize that zwitterionic conjugation could mitigate this bioactivity loss due to the reduced nonspecific interactions of pCB polymers with both IFN-α2a binding domain and IFN-α2a receptor (Figure 2.2c). Due to the tightly bound water layer around zwitterionic polymers, nonspecific interactions between either IFN-α2a or IFN-α2a receptor and zwitterionic polymers can be minimized. As a result, the bioactivity loss of pCB conjugated IFN-α2a can be reduced.

Monomeric PEGylated IFNs suffer from accelerated blood clearance upon repeated injections in monkey[64]. In this work, encouraged by the in vitro results, we further examined the in vivo performance of Zwitterlated, PEGylated and unconjugated IFN-α2a conjugates with same doses were injected every week for three weeks. The blood was

harvested at different time points to examine circulation time. As shown in Figure 2.3a, for the circulation profile of the first injection, both PEGylation and Zwitterlation significantly improve circulation time when compared with unconjugated IFN-α2a. In brief, we observed a 47.8-fold increase in $t_{1/2}$ when pCB_{20k} was conjugated. At the same time, a 26.6-fold increase was observed when PEG with similar molecular weight (PEG_{20k}) was conjugated and a 11.9-fold increase was observed the PEG with similar hydrodynamic size (PEG_{10k}) was conjugated. Notably, the circulation time of pCB_{20k}-IFN is comparable to that of PEG_{40k}-IFN (Pegasys) while its bioactivity is 9-fold higher although pCB_{20k} is half of PEG_{40K} in its molecular weight (Figure 2.2b). In this work, we found that the increased molecular weight of conjugated polymers could further improve the circulation time. As presented, the $t_{1/2}$ of pCB_{20k}-IFN shows a 2.3-fold increase over the $t_{1/2}$ of pCB_{10k}-IFN. Similar scenario was observed in PEG conjugation where a 2.2-fold increase was achieved when 10 kDa PEG was replaced with 20 kDa PEG.

Upon repeated injection, a significant difference was observed between pCB-IFNs and PEG-IFNs. As presented in Figure 2.3a and 2.3b, for unconjugated IFN-α2a, we noticed a 15.6% and a 36.2% decrease in $t_{1/2}$ after 2nd and 3rd injections, respectively when compared with the first injection. Despite PEG conjugation can significantly increase the circulation time of IFN-α2a after the 1st injection, the circulation profiles show a 20.0% and a 46.3% decrease in $t_{1/2}$ after 2^{nd} and 3^{rd} injections respectively for PEG_{10k}-IFN, and a 16.2% and a 34.9% decrease in $t_{1/2}$ after 2nd and 3rd injections respectively for PEG_{20k}-IFN. These results show the "accelerated blood clearance" effect[65] of PEG conjugation after repeated injection. For this reduced circulation time, PEG_{10k}-IFN is even more evident than unconjugated IFN-α2a. In sharp contrast to unconjugated IFN-α2a and PEGylated

IFN-α2a, Zwitterlated IFN-α2a do not show significant decline in $t_{1/2}$ after 2nd and 3rd injections.

It is known that the surface attachment of hydrophilic polymers like PEG to a protein can significantly prolong in vivo circulation time by increasing its hydrodynamic size to avoid rapid renal clearance (for small particles) and reducing interactions with both blood components (opsonization) and immune cells in early studies.[8, 12] However, several studies since then have found anti-PEG antibodies after treatment with PEGylated therapies, with titers strongly related to the modification density of PEG chains and the immunogenicity of the anchoring protein.[66, 67] The haptenic character of PEG is considered as the main culprit in the therapeutic efficacy loss of PEGylated products. Recent clinical studies of Pegloticase (PEGylated uricase) in refractory chronic gout patients unequivocally demonstrated that the production of anti-PEG antibodies are responsible for reduction in drug effectiveness—Pegloticase loses efficacy in more than 40% of patients, and the presence of anti-PEG antibodies doubles the risk of infusion reactions.[68, 69] Similar results have also been observed for other PEGylated proteins in clinical use such as PEG-asparaginase[70]. PEGylated nanoparticles such as liposomes also stimulate strong anti-PEG response, and these antibodies are found to cross-react with different PEGylated products.[71, 72] Furthermore, there is a concern that the prevalence of anti-PEG antibodies was found to be 72% (273/377) in healthy individuals, attributed to daily exposure to consumer products.[73] However, a cautious examination of whether these pre-existing antibodies have equivalent effect on different Pegylated proteins is needed. Furthermore, antibodies toward proteins (e.g., Peginterferon-alfa[74]) can also further reduce the therapeutic effect of Pegylated proteins. Altogether, these findings raise

concerns regarding the toxicity and efficacy of PEGylated drugs. As a result, alternatives to this PEGylation strategy are urgently needed.[75, 76] Systematic studies have shown that PEG immunogenicity strongly results from the hydrophobic characteristics of this amphiphilic polymer.[77-79] Due to the super-hydrophilic nature of zwitterionic pCB polymers, we hypothesized that Zwitterlation could mitigate the immunogenicity of IFN-α2a. At the same time, zwitterionic polymers do not elicit strong immune response in the body.

To confirm this hypothesis, we further proceeded to analyze the production of IgM and IgG antibodies after repeated injections. The results are summarized in Figure 2.4. As presented, both PEGylation and Zwitterlation can mitigate the generation of IFN-α2a-specific antibodies after repeated injections. For PEGylation, at the lowest dilution, the protein-specific IgM tier for PEG_{10k}-IFN and PEG_{20k}-IFN groups are 36.7% and 31.1% of that of the unconjugated IFN-α2a group. The protein-specific IgG tier for PEG10k-IFN and PEG20k-IFN groups are 57.4% and 51.1% of that of the unconjugated IFN-α2a group. At the same time, it is observed that Zwitterlation can further decrease the production of both protein-specific IgM and IgG. At the lowest dilution, the protein-specific IgM titer for pCB_{10k}-IFN and pCB_{20k}-IFN groups are 22.4% and 16.7% of that of the unconjugated IFN-α2a group. The protein-specific IgG tier for pCB_{10k}-IFN and pCB_{20k}-IFN groups are 26.9% and 23.5% of that of the unconjugated IFN-α2a group. These results also indicate that pCB polymers can better protect the conjugated IFN-α2a from immunosurveillance than PEG polymers. We further examined the production of specific IgM and IgG production against PEG and pCB polymers. As presented in Figure 2.4c-d, we observed a dramatic difference between PEG and pCB conjugates for their antibody production specifically against

polymers. At the lowest dilution, we found pCB-IFNs induce negligible anti-polymer production of either IgM or IgG compared with PEG-IFNs. These results indicate that the accelerated blood clearance of PEG-IFN is mainly attributed to the production of antibodies against both IFN-α2a and PEG polymer. For pCB-IFN, since pCB polymers can mitigate the antibody production against IFN-α2a and do not elicit specific antibody production against themselves in the body, the accelerated blood clearance can be significantly attenuated.

2.4 Conclusions

In summary, protein-polymer conjugation has been demonstrated to be an effective method to improve the therapeutic result of protein drugs in vivo. However, for proteins whose receptor/substrate have a large size, PEG polymer conjugation often results in a significant loss in protein bioactivity. In this work, we selected IFN-α2a as a model protein whose in vitro bioactivity can be reduced by more than 90% after PEGylation. We demonstrate that zwitterionic conjugation could significantly retain the in vitro antiproliferative bioactivity of conjugated IFN-α2a due to their super-hydrophilic nature which, in turn, the 'nonspecific blocking' issue in PEGylation can be mitigated. Furthermore, in vivo results show that zwitterionic conjugation prolongs IFN-α2a circulation time and attenuates its accelerated blood clearance significantly. Further analysis reveals that zwitterlation mitigates the production of antibodies toward both IFN-α2a and polymer. This work indicates that zwitterionic polymer conjugation may be particularly suitable for the conjugation of therapeutic proteins with large binding targets.

2.5 Tables

Table 2.1 Dynamic light scattering (DLS) analysis of various polymer-IFN conjugates.

	IFN	pCB_{10K}-IFN	pCB_{20K}-IFN	PEG_{10K}-IFN	PEG_{20K}-IFN	PEG_{40K}-IFN
Hydrodynamic size (nm)	2.34 ± 0.23	3.74 ± 0.41	5.16 ± 0.35	4.94 ± 0.46	7.28 ± 0.61	10.47 ± 0.52
PDI	0.31 ± 0.04	0.17 ± 0.05	0.2 ± 0.06	0.14 ± 0.03	0.17 ± 0.04	0.19 ± 0.05

Table 2.2 Conversion and yield of various polymer-IFN conjugates.

	IFN + pCB_{10k}	IFN + pCB_{20k}	IFN + PEG_{10k}	IFN + PEG_{20k}	IFN + PEG_{40k}
IFN conversion	61.4% ± 9.2%	55.3% ± 7.2%	52.7% ± 6.5%	51.1% ± 5.7%	46.6% ± 8.3%
Yield	54.5% ± 5.9%	47.6% ± 8.4%	45.4% ± 4.9%	47.5% ± 6.3%	40.2% ± 4.7%

2.6 Figures

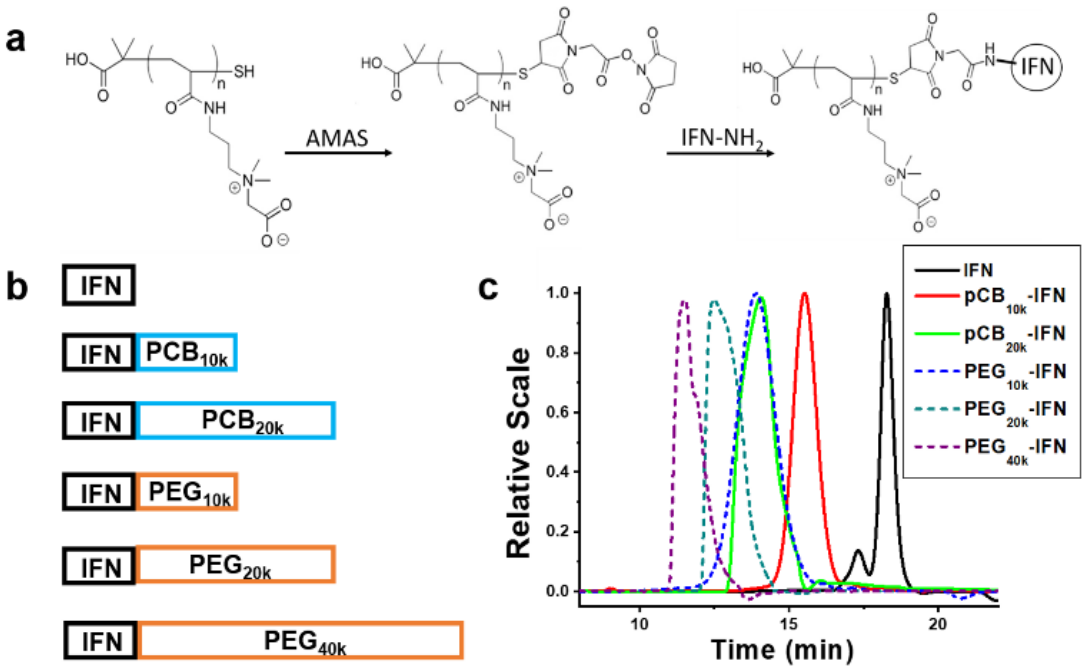
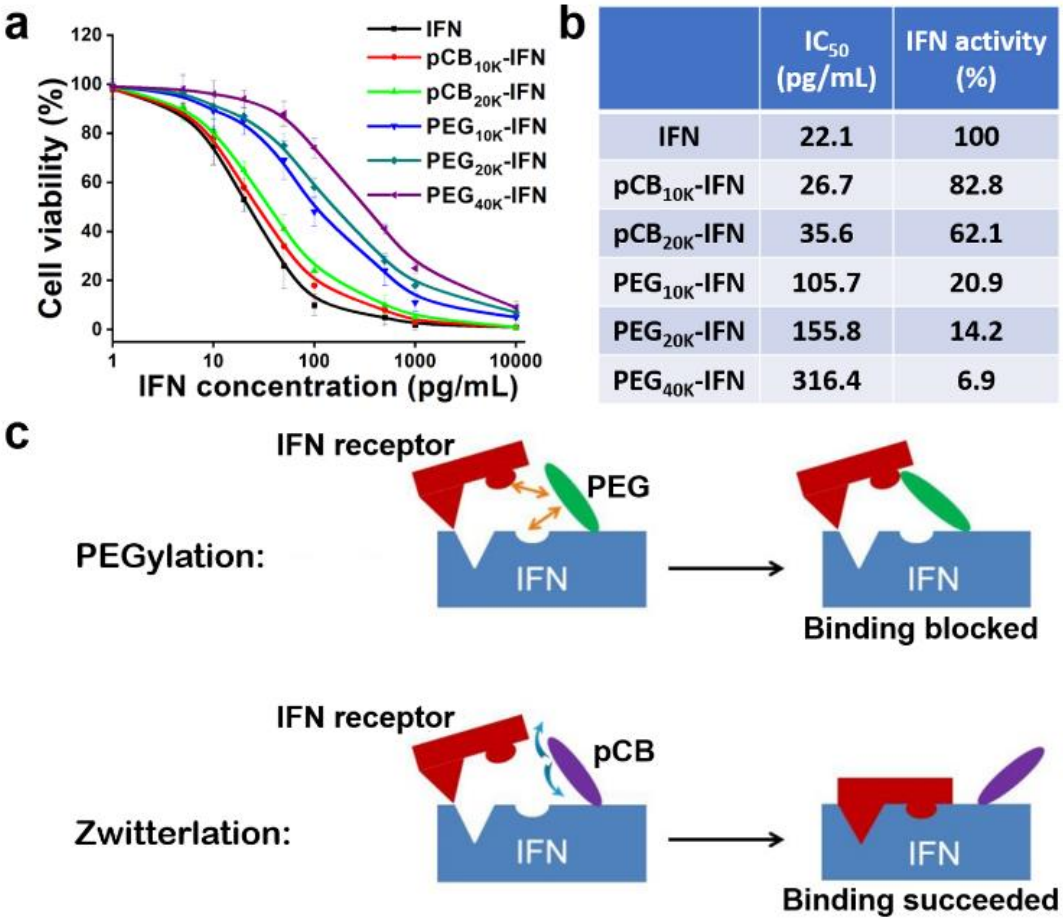


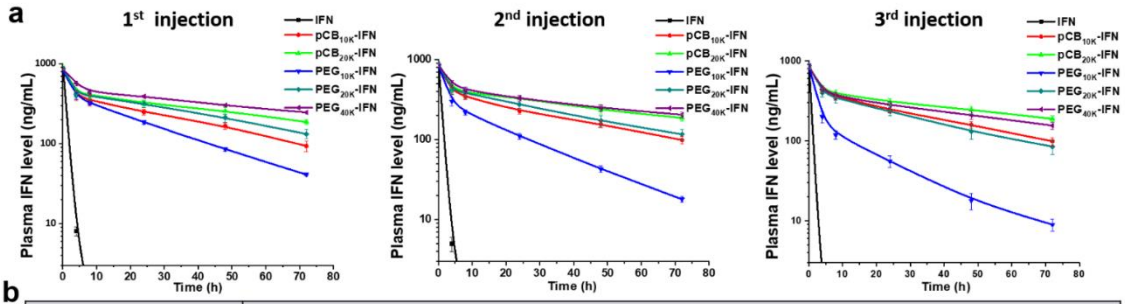
Figure 2.1 Synthesis and characterization of zwitterionic polymer-IFN conjugates. a) synthetic route of conjugates; b) Schematic illustration of conjugates of different molecular weights; c) Representative size-exclusion chromatogram for conjugates of different molecular weights.



	IC_{50} (pg/mL)	IFN activity (%)
IFN	22.1	100
pCB_{10K}-IFN	26.7	82.8
pCB_{20K}-IFN	35.6	62.1
PEG_{10K}-IFN	105.7	20.9
PEG_{20K}-IFN	155.8	14.2
PEG_{40K}-IFN	316.4	6.9

Figure 2.2 Zwitterlation retains the most bioactivity of conjugated IFN. a) Conjugates with serial dilutions were incubated with human Daudi cells for 4 days at 37 °C in 96-well tissue culture plates. MTT assay was used for cell viability assay; b) Antiproliferative activity; c) Schematic illustration of how a zwitterionic conjugate avoids bioactivity loss by effectively eliminating nonspecific interactions.

a



b

	$t_{1/2}$ (h)					
	IFN	pCB_{10K}-IFN	PCB_{20K}-IFN	PEG_{10K}-IFN	PEG_{20K}-IFN	PEG_{40K}-IFN
First injection	1.307	26.67	62.45	15.56	34.84	69.14
Second injection	1.104	27.56	60.57	12.45	29.23	61.35
Third injection	0.826	25.87	61.38	8.36	22.68	52.06

Figure 2.3 Zwitterlation mitigates the accelerated blood clearance. a) In vivo circulation profiles of native IFN-α2a and pCB or PEG conjugated IFN-α2a; b) Pharmacokinetic parameters after repeated injections.

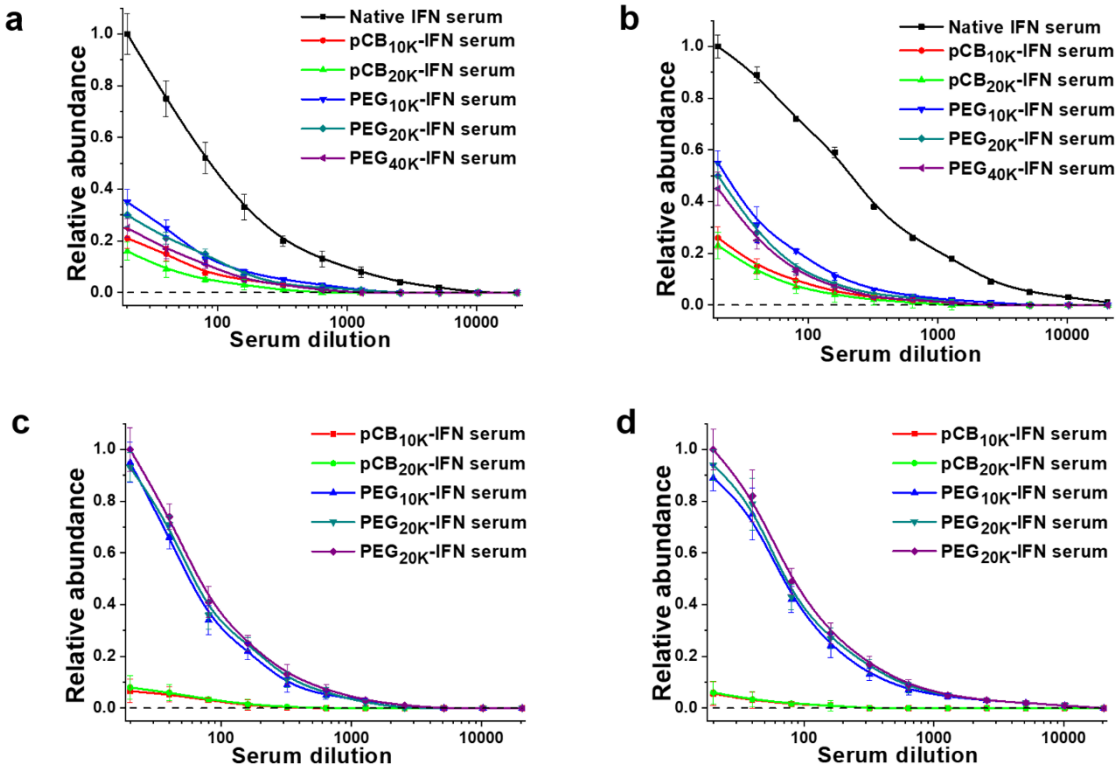


Figure 2.4 Zwitterlation mitigates the production of specific antibodies against polymer and IFN. After 3 injections, IFN-specific IgM (a) and IgG (b) antibodies and polymer-specific IgM (c), IgG (d) antibodies were analyzed.

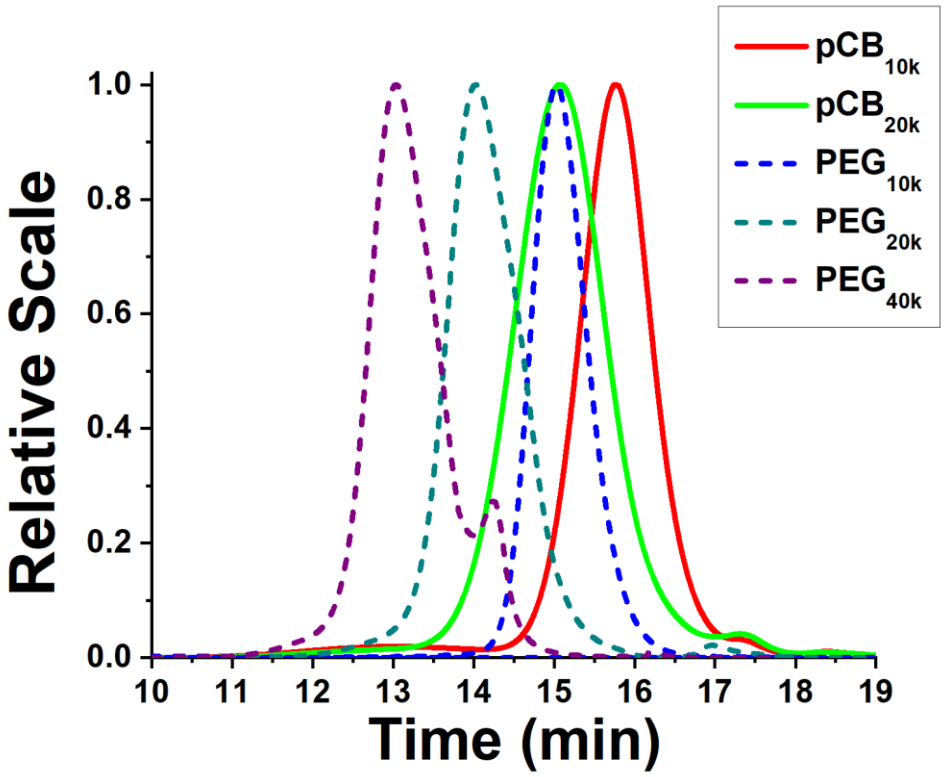


Figure 2.5 Representative size-exclusion chromatogram for different pCB and PEG polymers.

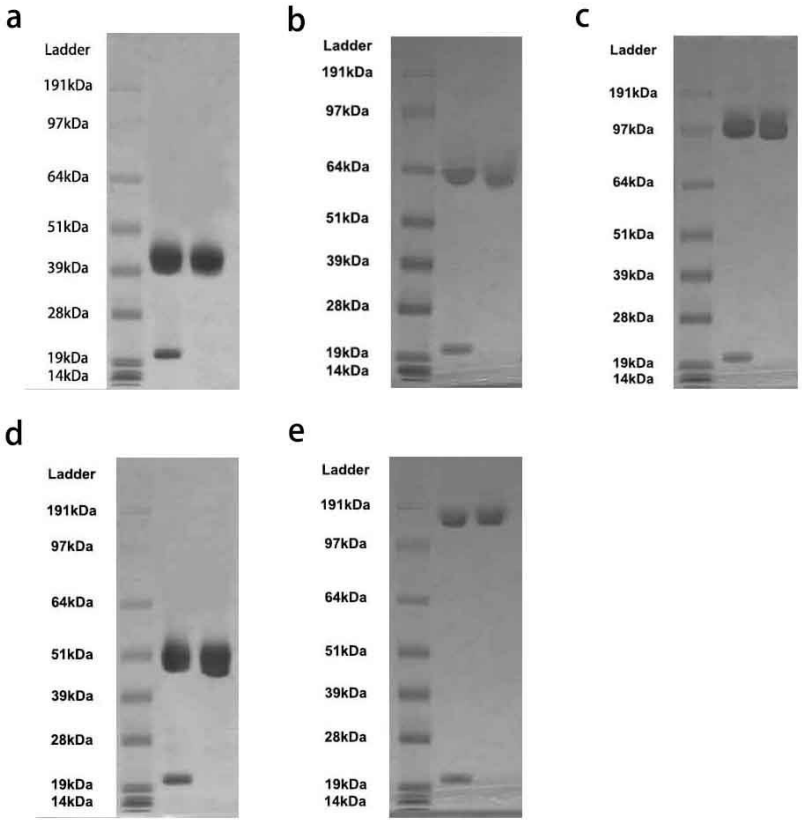


Figure 2.6 SDS-PAGE analysis of polymer conjugated IFN-α2a. a) PEG_{10k} conjugated IFN-α2a. Lane 1: Molecular weight marker proteins; Lane 2: Reaction mixture; Lane 3: purified PEG_{10k} -IFN. b) PEG_{20k} conjugated IFN-α2a. Lane 1: Molecular weight marker proteins; Lane 2: Reaction mixture; Lane 3: purified PEG_{20k} -IFN. C) PEG_{40k} conjugated IFN-α2a. Lane 1: Molecular weight marker proteins; Lane 2: Reaction mixture; Lane 3: purified PEG_{40k} -IFN. d) pCB_{10k} conjugated IFN-α2a. Lane 1: Molecular weight marker proteins; Lane 2: Reaction mixture; Lane 3: purified pCB_{10k} -IFN. e) pCB_{20k} conjugated IFN-α2a. Lane 1: Molecular weight marker proteins; Lane 2: Reaction mixture; Lane 3: purified pCB_{20k} -IFN.

Chapter 3 Minimizing Off-target Frequency of CRISPR/Cas9 System *via* Zwitterionic Polymer Conjugation

The clustered, regularly interspaced, short palindromic repeats (CRISPR)–associated protein 9 (Cas9) system is a powerful genome-editing tool that is widely used in many different applications. However, the high-frequency mutations induced by RNA-guided Cas9 at sites other than the intended on-target sites is a major concern that impedes therapeutic and clinical applications. A deeper analysis shows that most off-target events result from the non-specific mismatch between single guide RNA (sgRNA) and target DNA. Therefore, minimizing the non-specific RNA-DNA interaction can be an effective solution to this issue. Here we provide a novel method to minimize this mismatch issue by conjugating Cas9 with zwitterionic pCB polymers. The zwitterlated CRISPR/Cas9 ribonucleoproteins (RNPs) shows reduced off-target DNA editing but similar levels of on-target gene editing activity. To be more specific, the off-target efficiency of CRISPR/Cas9 is reduced on average by 70% and can be as high as 90% when compared with naive CRISPR/Cas9 editing. This approach is a simple and effective way to streamline the development of genome editing with the potential to accelerate a wide array of biotechnological and therapeutic applications of CRISPR/Cas9 technology.

3.1 Introduction

CRISPR/Cas9 system is now a widely used tool for genome editing in various organisms and cell types [27, 28]. Unfortunately, it can also cause unwanted mutations at off-target sites that resemble the on-target sequence [29]. The off-target mutations are

caused by the nonspecific recognition of DNA sequence by CRISPR/Cas9 RNPs [80]. It has been demonstrated that besides the optimal PAM sequence 5′-NGG-3′, Cas9 can also cleave sites with a 5′-NAG-3′ or 5′-NGA-3′ PAM although less efficiently [35]. In addition, a 20nt single guide RNA (sgRNA) can recognize DNA sequences that harbor as many as 3-5 base pair mismatches with the sgRNA, suggesting there are up to thousands of possible binding sites for a given nuclease in the human genome [29]. Furthermore, CRISPR/Cas9 can induce off-target cleavages with DNA sequences containing a few extra bases ('DNA bulge') or a few missing bases ('RNA bulge') compared to the RNA guide strand [81]. Off-target DNA cleavages can give rise to mutations at unintended genomic loci and to gross chromosomal rearrangements such as deletions [32, 33], inversions [34], and translocations [30, 31]. These mutations at unwanted sites might disable a tumor-suppressor gene or activate a cancer-causing gene. Translocations have been known to be a possible reason for chronic myeloid leukemia[36]. Preventing, avoiding, or at least reducing these off-target effects is crucial for the success of any downstream genome editing applications. Various strategies have been developed to reduce genome-wide off-target mutations of the commonly used Cas9 nuclease, including truncated sgRNAs bearing shortened regions of target complementarity [37, 82], Cas9 mutants [39], paired Cas9 nickases [40, 41], and dimeric fusions of catalytically inactive Cas9 to a non-specific FokI nuclease [42-44]. However, these approaches are only partially effective and/or possess the potential to create more off-target sites. Furthermore, they may also require the expression of multiple sgRNAs and/or fusion of additional functional domains to Cas9, which can reduce the targeting range and create challenges for delivery using viral vectors

which have a limited payload size of nucleic acids. Thus, a major challenge for the field remains the development of a simple robust strategy that can reduce the off-target effects of the CRISPR/Cas9 system.

The “off-target” activity of the nucleases occurs fundamentally because the Cas9/sgRNA complex possesses more energy than what is needed for the effective recognition of its intended target DNA site [82, 83]. As a result, the complex lacks high specificity and is able to bind sequences that are similar to the on-target DNA strand. Therefore, it is hypothesized that the off-target effects of CRISPR/Cas9 might be minimized by reducing the non-specific interactions with its target DNA sites. Zwitterionic poly(carboxybetaine) (pCB) polymers are highly hydrated and effectively resistant to non-specific interactions[45]. In our previous study, pCB polymers have been conjugated to chymotrypsin (CT)[46], uricase[84] and interferon-α2a[85] to preserve protein bioactivity. The super-hydrophilic nature of the polymer creates an environment to shift the equilibrium and favor the substrate and the binding site to interact. It has been demonstrated that a pCB conjugated protein exhibits reduced non-specific interactions with its surrounding environment. In our previous study, this reduction of nonspecific interactions was shown to significantly enhance protein circulation time and reduce protein-specific antibody production *in vivo*.

Since specific DNA-sgRNA matching is far stronger than non-specific interactions, we hypothesized that pCB conjugation was able to reduce non-specific interactions and still maintain specific interactions strong enough with on-target DNA strands. With this strategy, the off-target effects of CRISPR/Cas9 will be minimized. In this work, we conjugated Cas9 with pCB polymers and examined its on-target and off-target efficiency.

To assess the specificities of the pCB-conjugated CRISPR/Cas9 systems, we designed a series of mismatched sgRNAs containing single, double, or triple substitutions within multiple sgRNA-target DNA interfaces. Different endogenous human genes were tested. In this study, pCB conjugates showed decreased off-target activity compared with the unmodified Cas9, but similar levels of on-target gene editing efficiency. We believe that pCB polymer conjugate may provide a simple, safe, and robust strategy for CRISPR/Cas9 system-based gene editing.

3.2 Experimental Section

3.2.1 Synthesis of N-hydroxysuccinimidepoly(carboxybetaine acrylamide) (NHS-PCBAA)

pCB-NHS was synthesized based on our previous procedure. Briefly, we firstly synthesized 3-acrylamido-N-(2-(tert-butoxy)-2-oxoethyl)-N,N-dimethylpropan-1-aminium (CBAAM-tBu). Then, a typical reversible addition fragmentation chain transfer (RAFT) polymerization reaction was performed to yield the 10 kDa SH-pCB polymer. The final colorless NHS-activated polymer was formed by reaction with AMAS at 1:10 molar ratio in DI water (pH 6) for 30 min, followed by removal of unreacted AMAS via Amicon spin dialysis tubes and freeze-drying for 48 h.

3.2.2 Preparation and characterization of pCB-Cas9 conjugates

Conjugate of pCB-Cas9 was synthesized by reacting NHS ester groups of the polymer with available amine groups on the protein. In a typical conjugation reaction, Cas9 nuclease and NHS-pCB at 1:10, 1:20, or 1:50 molar ratio were dissolved in 50 mM sodium borate buffer, pH 9.0. The final protein concentration was ~5 mg/mL. The reaction mixture was

stirred for 2 hours at 4 °C and stopped by adjusting the pH of the mixture to 4.5 with glacialacetic acid. The polymer-protein conjugate was isolated via molecular weight cut-off (MWCO) spin dialysis membrane followed by ion-exchange chromatography. High performance liquid chromatography (HPLC) was used to measure the hydrodynamic size of the protein conjugates.

3.2.2 In vitro synthesis of sgRNA

The *in vitro* synthesis of sgRNA was carried out using EnGen® sgRNA Synthesis Kit using the manufacturer's recommended conditions. The gRNA product was purified using GeneJET RNA Cleanup and Concentration Micro Kit as described in the manual. The concentration of RNA was determined by measuring the absorbance at 260 nm on a microplate reader.

3.2.3 Mammalian cell culture

HEK 293-GFP cells were maintained in DMEM, medium supplemented with 10% FBS. U2OS cells were maintained in McCoy's 5A modified medium supplemented with 25 mM HEPES and 10% FBS. K562 cells were propagated in RPMI 1640 medium containing 10% FBS. After thawing, cells were passaged 4–5 times before using for transfection. When setting up the experiments for transfections, cultured cells were plated in 24-well format (500 µl volume) in complete growth medium at a cell density necessary to reach ~70% confluence the next day. Full serum media was replaced with the same media but containing no antibiotics at least 1 h before delivery. All cultures were maintained in 5% CO_2 at 37◦C in a humidified incubator.

3.2.4 In vitro co-delivery of Cas9 protein and sgRNA

For Cas9 protein transfection, 200 ng of purified Cas9 protein was added to 5 μl of Opti-MEM medium, followed by addition of 50 ng gRNA. The molar ratio of gRNA to Cas9 protein was kept at approximately 1 to 1.2: 1. The sample was mixed by gently tapping the tubes a few times and then incubated at room temperature for 10 min. In a separate test tube, 0.8 μl of Lipofectamine CRISPRMAX transfection reagent was diluted to 5 μl with Opti-MEM medium. The diluted transfection reagent was transferred to the tube containing Cas9 protein/gRNA complexes, followed by incubation at room temperature for 10 min and then the entire solution was added to the cells in a 24-well plate and mixed by gently swirling the plate. The plate was incubated at 37 ◦C for 48 h in a 5% CO_2 incubator.

3.2.5 Determination of on- and off-target mutation frequencies in human cells.

Genomic DNA was harvested 2 d after transfection from U2OS, HEK293 or K562 cells using the Quick-DNA Miniprep (Zymo Research), according to the manufacturer's instructions. 100 ng of isolated genomic DNA will be used as template to PCR amplify the targeted genomic sites with primer pairs. PCR products were purified with a PureLink™ PCR Purification Kit (Thermo Fisher) and quantified on a microplate reader. 250 ng of purified PCR DNA will be combined with 2 μl of NEBuffer 2 (NEB) in a total volume of 19 μl and denatured then re-annealed with thermocycling at 95 °C for 5 min, 95–85 °C at 2 °C/s; 85–20 °C at 0.2 °C/s. The re-annealed DNA will be incubated with 1 μl of T7 Endonuclease I (10 U/μl, NEB) at 37 °C for 30 min. Cas9-induced cleavage bands and the uncleaved band will be visualized under UV light and quantified using ImageJ software30. The peak intensities of the cleaved bands will be divided by the total intensity of all bands

(uncleaved + cleaved bands) to determine the fraction cleaved, which will be used to estimate gene modification levels. For each sample, transfections and subsequent modification measurements will be performed in triplicate on different days. Off-target analysis will be performed using a bioinformatics based search tool to select potential off-target sites, which will also be evaluated using the T7E1 mutation detection assay.

3.2.6 Sanger Sequencing

To better determine the mutation rate, the same purified PCR products used for T7EI assay were sequenced to observe the individual mutations and determine the mutational spectra. Sanger sequencing was used to confirm the gene modification frequencies for the modified and unmodified CRISPR/Cas9 systems. The results were analyzed by ICE Analysis (Synthego).

3.3 Results and Discussion

To examine the effect of the zwitterionic pCB polymer conjugation on the off-target efficiency of the CRISPR/Cas9 system, we prepared a series of pCB-Cas9 conjugates with different numbers of polymer chains per protein. Conjugates of pCB-Cas9 were synthesized by reacting N-Hydroxysuccinimide (NHS) ester groups of the polymer with available amine groups on the protein. The reaction scheme is illustrated in Figure 3.1. The polymer density was controlled by altering the molar ratio between Cas9 and NHS-pCB in the reaction. In this work, we synthesized pCB_{10}-Cas9, pCB_{20}-Cas9, and pCB_{50}-Cas9 at the molar ratio of 1:10, 1:20, and 1:50, respectively. A native (unconjugated) Cas9 protein was used for comparison throughout the studies. The difference in size between native Cas9 and pCB-Cas9 conjugates was shown in Figure 3.2, which confirms the successful

synthesis of the polymer-protein conjugates. In almost all cases, surface modification of a protein by covalent conjugation with polymers, such as PEGylation, lowers the in vitro biological activity of conjugated proteins.[26-27] Therefore, we first tested whether the presence of zwitterionic polymers would compromise the on-target activity after conjugation. For these experiments, we used a well-established Cas9-induced GFP disruption assay that enabled the rapid quantification of targeted nuclease activities.[3] In this assay, we targeted a genomic GFP reporter gene in human HEK293-GFP cells. The activities were quantified by measuring the loss of fluorescence signal in human HEK293-GFP cells, which is caused by the on-target CRISPR/Cas9 cleavage (Figure 3.3a). The cells were treated with 50ng sgRNA and 200ng native Cas9/Cas9-equivalent pCB-Cas9 conjugates with CRISPRMAX (Figure 3.4, Figure 3.5) in DMEM containing 10% FBS for 48 hours to induce the disruption of GFP reporter gene. As shown in Figure 3.3b, we found that pCB_{10}-Cas9 and pCB_{20}-Cas9 showed comparable editing efficiency to native Cas9, in which about 60% cells lost their GFP expression after the treatment. The encouraging results demonstrated that the presence of pCB polymer did not compromise the "on-target" editing efficiency of the CRISPR/Cas9 system. However, only 40% GFP negative cells were found when treated with pCB_{50}-Cas9 (Figure 3.3b). This is due to the presence of more pCB polymers, which can physically impede the binding between DNA and CRISPR/Cas9 RNP.

To explore the potential of pCB conjugates in reducing the off-target activity, we randomly generated variant sgRNAs for the target site with one, two, or three mismatched nucleotides and tested whether these mismatched sgRNAs could drive off-target GFP disruption in human cells (Figure 3.3c). If pCB conjugation could reduce off-targeting,

then pCB-Cas9 conjugates would be less tolerant of mismatches than native Cas9. As presented in Figure 3.3c, native Cas9 can still induce substantial GFP gene disruption in human cells when using mismatched sgRNA. In contrast, pCB-Cas9 conjugates showed a significant reduction of GFP disruption efficiency when mismatched sgRNAs were used (Figure 3.3c). pCB_{10}-Cas9 induced 35.6%, 21.9%, and 5.6% GFP disruption while pCB_{20}-Cas9 led to 14.2%, 8.9%, and 0, respectively when one, two, or three nucleotide mismatches were present in the sgRNA. pCB_{50}-Cas9 generated no detectable GFP disruption when 2 or 3 nucleotide mismatches were present. These data suggest that pCB conjugation can significantly reduce the off-target gene editing when mismatched sgRNAs are used. Taking both the on-target and off-target efficiency into consideration, we selected pCB_{20}-Cas9, which shows complete on-target efficiency and significantly reduce off-target efficiency. In the following study, pCB_{20}-Cas9 will be denoted as pCB-Cas9 thereafter.

To further evaluate the effects of mismatches on pCB-Cas9, we created more sgRNA bearing one, two, or three nucleotide mutations. As shown in Table 3.1, native Cas9 exhibited significant off-target editing in a single-mismatch scenario (57.4%, 60.6%, and 49.3%). These off-target editing efficiencies are very close to its on-target editing efficiency in the perfect-match scenario (62.6%) as we discussed above. In contrast, using the same mismatched sgRNAs, pCB-Cas9 conjugate showed a significantly reduced off-target editing efficiency (14.2%, 12.6%, and 6.8%). This editing efficiency is more than 80% lower than its editing efficiency in the perfect-match scenario (67.4%). This observation was confirmed in another two scenarios when the sgRNAs have two or three mismatches. When 3 mismatches are presented in a sgRNA, the native Cas9 still exhibited positive editing efficiencies (12.4%, 7.9%, 9.3%). In contrast, no “off-target” efficiency is

observed for pCB-Cas9 conjugate groups. This indicates that pCB-conjugated CRISPR/Cas9 has a high resolution in DNA editing and can distinguish the mismatch on a single-base level.

It is known that the "off-target" activity of the nucleases is fundamentally caused by the extra energy that the Cas9/sgRNA complex possesses, leading to the lack of perfect specificity.[14, 21] Such extra energy comes mainly from the nonspecific forces - hydrophobic and electrostatic in particular. Coating a protein with non-fouling polymeric materials can alter these nonspecific interactions and is the key to lower energy and promote specific binding.[28-29] Over the last several years, zwitterionic materials based on naturally occurring betaines such as pCB have particularly high hydration. As a result, ultra-low nonspecific adsorption in complex biological media has been observed in different scenarios.[22] Here we hypothesized that the conjugation of pCB to Cas9 could reduce the nonspecific binding force between the Cas9/sgRNA complex and the target DNA. As shown in Figure 3.6, for native Cas9, the energy that the Cas9/sgRNA complex possesses is much higher than the minimum energy required for on-target binding between the sgRNA and the DNA. As a result, the RNP complex still possesses enough energy even one or more mismatched nucleotides are present. With the pCB conjugates, the aforementioned nonspecific binding, especially the hydrophobic-hydrophobic interaction, is decreased significantly. The complex is unable to bind the double-strand DNA without sufficient energy when mismatched nucleotides are present on the sequence. As a result, off-target effects can be reduced significantly. In addition, benefiting from the super-hydrophilicity of the polymer, a tightly bound water layer is formed around and the nonspecific interactions between RNPs and zwitterionic polymers can be minimized as we

observed before.[25] As a result, the bioactivity of Cas9 can be retained after the polymer conjugation, which is very important to keep the on-target efficiency.

To examine whether pCB-conjugated CRISPR/Cas9 RNPs can reduce off-target effects on other DNA domains in human cells, we selected three new genomic loci in the VEGFA, EMX and CLTA genes due to their potential biomedical relevance and widely use in Cas9 off-target studies.[3, 14] As presented in Figure 3.7, for all three targets, CRISPR/pCB-Cas9 mediated indels at their endogenous loci were detected using the T7 endonuclease I (T7EI) assay. For each of these three target sequences, we examined the editing efficiencies of several potential off-target sites which have been observed in other studies.[3, 30] In this study, we observed similar trends as we showed in the previous examples. The rates of mutation at the selected off-target sites were very high, ranging from 9.4% to 93.6% when the cells were edited using native CRISPR/Cas9. In contrast, for the cells edited using pCB-conjugated CRISPR/Cas9, the off-target mutation rates were observed at a much lower level, ranging from 2.4% to 10.5%. It is noticeable that the editing efficiency of the pCB-Cas9 conjugate is slightly higher than that of native Cas9. This confirms our hypothesis that the bioactivity of Cas9 is reserved after conjugation.

After pCB conjugation is demonstrated to reduce the off-target mutations of CRISPR/CAS9 in HEK293-GFP cells, we proceed to evaluate this zwitterlated Cas9 in other types of human cells. Here, we employed U2OS and K562 cell lines as they were also widely used to test the on-/off-target activity of CRISPR/Cas9. We also explored the editing efficiencies on three targets using either native Cas9 or pCB-Cas9 in U2OS and K562 cell editing. As expected, pCB-Cas9 conjugates generate similar or slightly higher editing efficiency when compared with native Cas9 at the target genomic locus (Figure 3).

For the off-target examinations using variant mismatches, the pCB-Cas9 conjugate groups produced less than 4% indels rates at 20 out of 22 off-target sites. In contrast, native Cas9 generated 2.9% - 24.7% off-target indels, of which six were higher than 10%. These results further demonstrated that pCB-conjugated CRISPR/Cas9 showed reduced off-target effects in different cell lines.

3.4 Conclusions

In summary, a protein-polymer conjugation technology has been proposed, developed and demonstrated in this study to address the “off-target” concern faced by the CRISPR/Cas9 system. After being modified with a super-hydrophilic zwitterionic polymer, the CRISPR/Cas9 sys-tem shows significantly reduced “off-target” efficiency. More importantly, no reduced “on-target” frequency, which is usually noticed in many strategies, was observed. This technology will potentially provide a simple and ro-bust strategy to improve the efficiency and safety of a wide range of CRISPR/Cas9-based biological and clinical applications.

3.5 Tables

Table 3.1 Activity of CRISPR/Cas9 system harboring variant mismatched sgRNAs in GFP disruption assay.

Target Site	GGGCACGGGCAGCTTGCCGGTGG	% indel	
		Native Cas9	pCB-Cas9 conjugate
Perfectly matched sgRNA	GGGCACGGGCAGCTTGCCGG	61.1 ± 4.5	66.3 ± 3.8
Mismatched sgRNAs	GGGCTCGGGCAGCTTGCCGG	57.4 ± 2.6	14.2 ± 1.5
	GGGCACGGCCAGCTTGCCGG	60.6 ± 3.7	12.6 ± 0.8
	GGGCACGGGCAGCTTGGCGG	49.3 ± 2.9	6.8 ± 0.7
	GGGCAGCGGCAGCTTGCCGG	42.6 ± 3.3	10.4 ± 0.9
	GGGCAGGGGCAGCATGCCGG	39.4 ± 4.1	8.9 ± 1.1
	GGGCACGCGCTGCTTGCCGG	44.6 ± 1.9	7.0 ± 0.4
	GGGGACGGCCAGCATGCCGG	12.4 ± 0.7	NA
	GGGCACGGCGAGCTAGCCGG	7.9 ± 0.9	NA
	GGGCTGCGGCAGCTTGCCGG	9.3 ± 1.0	NA

Table 3.2 On-target and known off-target substrates of Cas9:sgRNAs that target sites in *GFP*, *EMX*, *VEGF*, and *CLTA*. List of genomic on-target and off-targets sites for *GFP*, *EMX*, *VEGF*, and *CLTA* are shown with mutations from the on-target sequence shown in lower case and red.

Target	Site name	Sequence
Target 1 (GFP)	GFP-On	GGGCACGGGCAGCTTGCCGG
Target 2 (VEGF)	VEGF-On	GGGTGGGGGGAGTTTGCTCC
	VEGF-Off1	GGaTGGaGGGAGTTTGCTCC
	VEGF-Off2	GGGaGGGtGGAGTTTGCTCC
	VEGF-Off3	cGGgGGaGGGAGTTTGCTCC
	VEGF-Off4	GGGgaGGGGaAGTTTGCTCC
Target 3 (EMX)	EMX-On	GAGTCCGAGCAGAAGAAGAA
	EMX-Off1	GAGgCCGAGCAGAAGAAagA
	EMX-Off2	GAGTCCtAGCAGgAGAAGAA
	EMX-Off3	GAGTCtaAGCAGAAGAAGAA
	EMX-Off4	GAGTtaGAGCAGAAGAAGAA
Targrt 4 (CLTA)	CLTA-On	GCAGATGTAGTGTTTCCACA
	CLTA-Off1	aCAtATGTAGTaTTTCCACA
	CLTA-Off2	cCAGATGTAGTaTTcCCACA
	CLTA-Off3	ctAGATGaAGTGcTTCCACA
	CLTA-Off4	ctAGATGaAGTGcTTCCACA

3.6 Figures

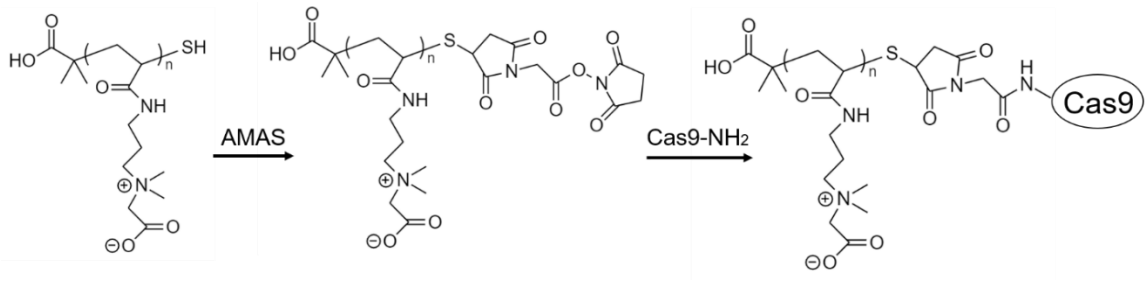


Figure 3.1 Synthetic route of pCB-Cas9 conjugates.

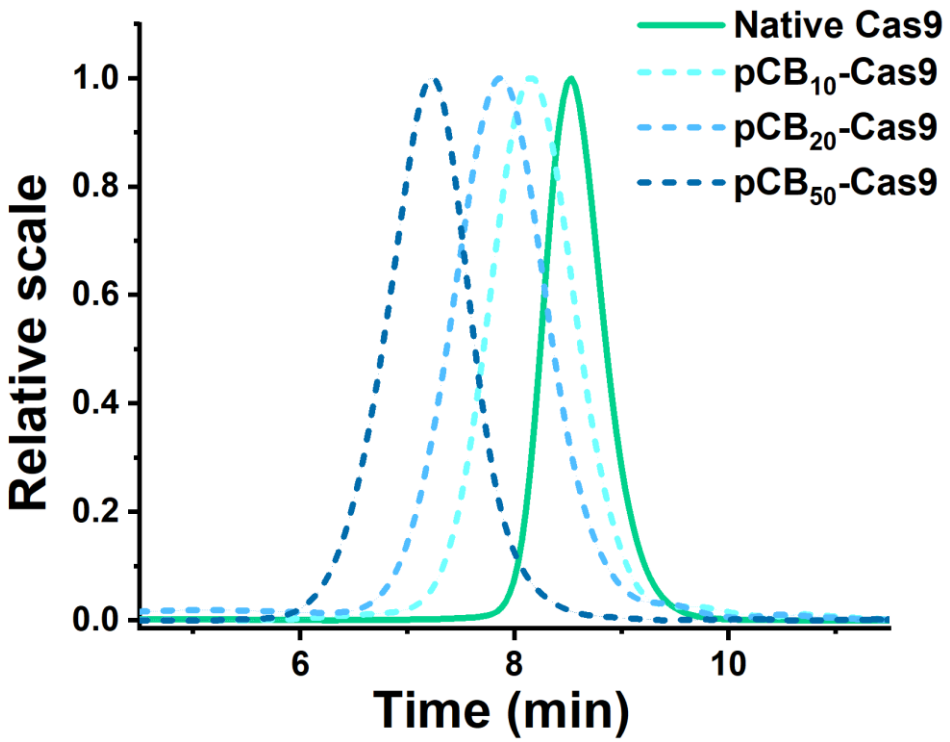


Figure 3.2 Size-exclusion chromatogram of native Cas9, and pCB_{10}-Cas9, pCB_{20}-Cas9, pCB_{50}-Cas9 conjugates.

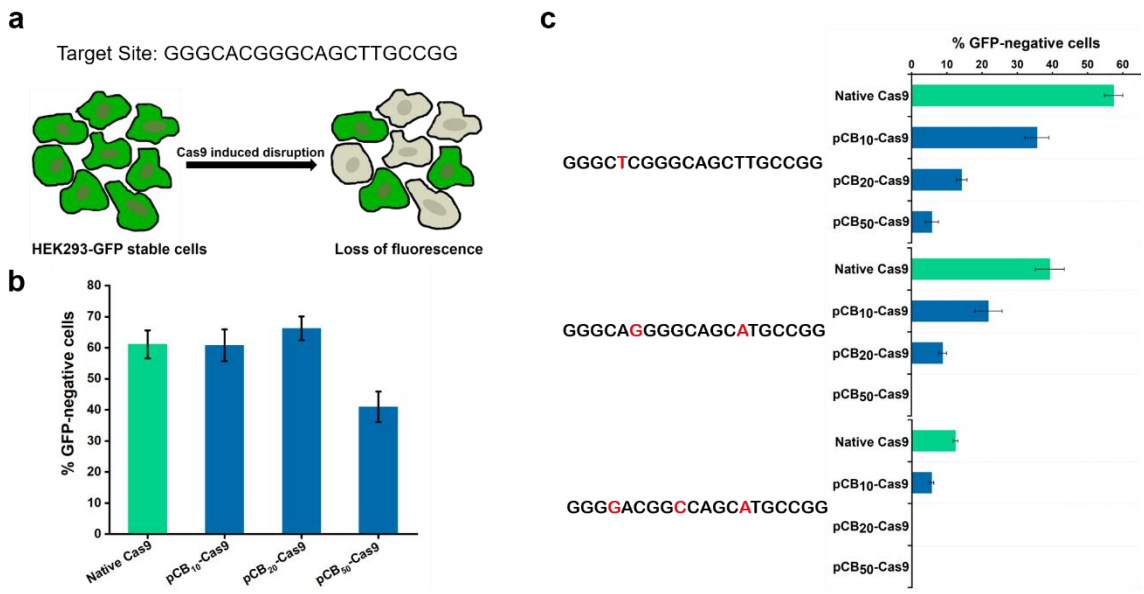


Figure 3.3 a) Schematic overview of the GFP disruption assay and the target site used in the GFP gene; b) Efficiency of GFP disruption in HEK293-GFP cells mediated by native Cas9 and pCB-Cas9 conjugates; c) Off-target editing efficiency of native Cas9 and pCB-Cas9 conjugates with mismatched sgRNA harboring one, two, or three nucleotide mutations in GFP disruption assay. Mutated nucleotides are colored red.

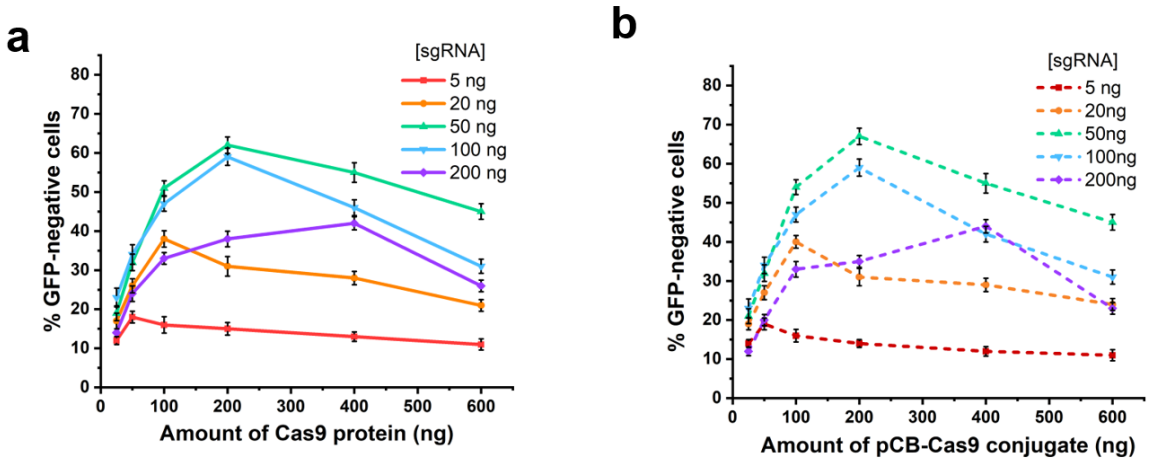


Figure 3.4 Optimal sgRNA to protein ratio for native Cas9 (a) and pCB-Cas9(b). All experiments were performed in a 96-well plate using a volume of 110 ul.

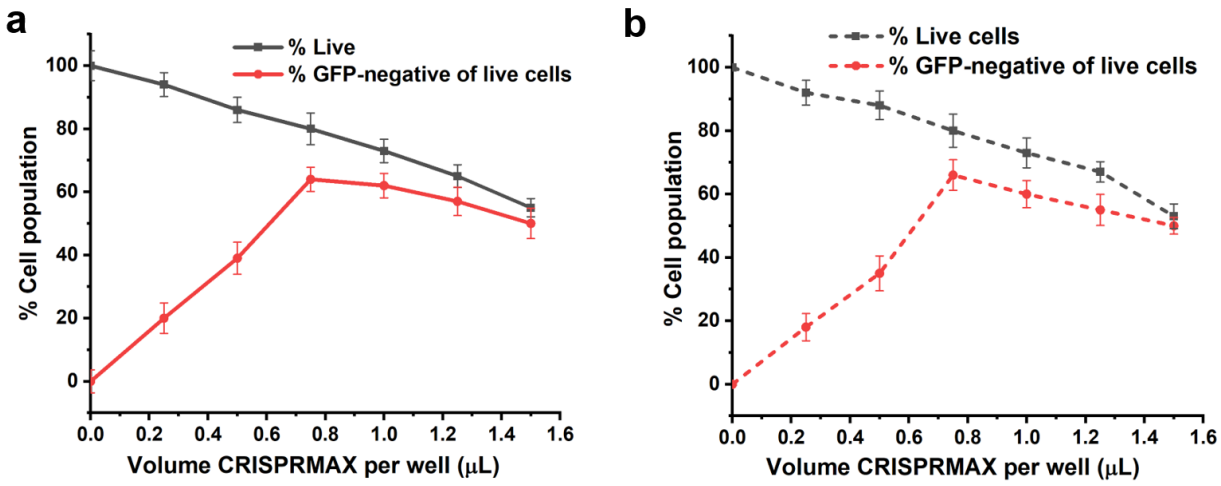


Figure 3.5 Effect of CRISPRMAX dose on the delivery efficiency and cellular toxicity of Cas9/sgRNA (a) and pCB-Cas9/sgRNA (b).

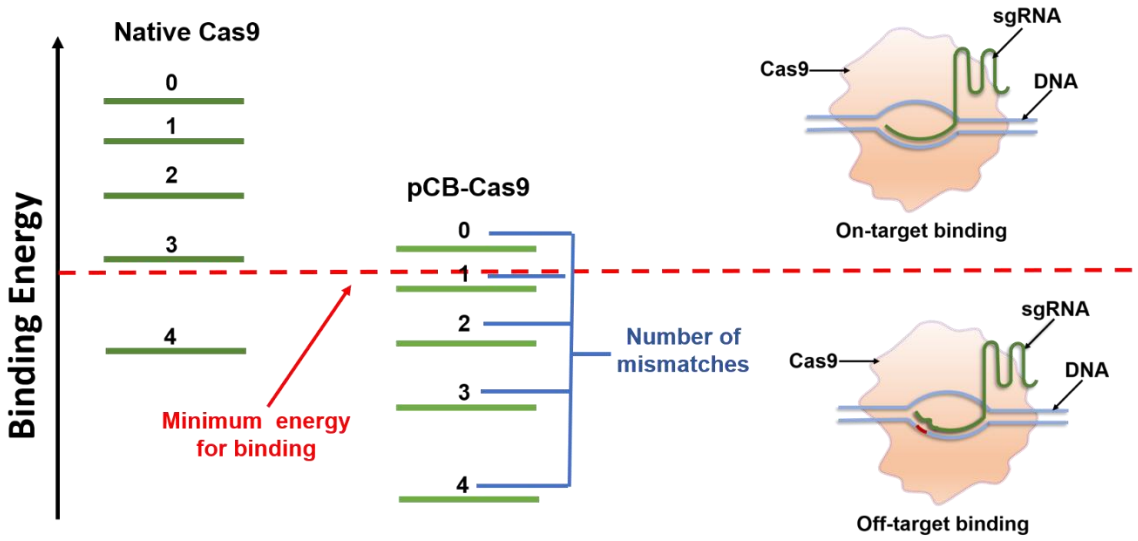


Figure 3.6 Mechanism of pCB conjugation in reducing the off-target efficiency of the CRISPR/Cas9 system. The Cas9/sgRNA complex possesses more energy than what is needed for optimal recognition of its target DNA site, leading to the cleavage of mismatched off-target sites. pCB polymer conjugation eliminates the non-specific binding between Cas9/sgRNA complex and double-strand DNA, thereby decreasing the binding energy. The remained energy is strong enough for on-target binding, but not enough for mismatched binding.

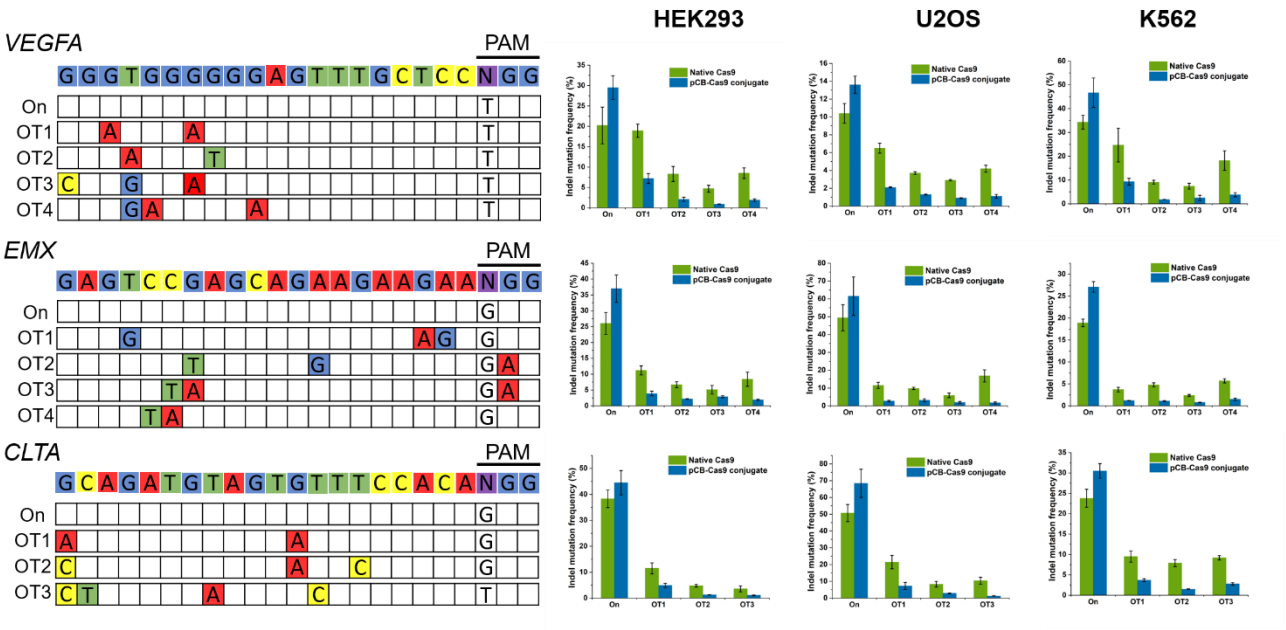


Figure 3.7 On-target and off-target DNA editing efficiencies resulting from native Cas9 and pCB-Cas9 in three different cell lines.

Chapter 4 Exploring Genetically Fused Zwitterionic Peptide on the On/off target Effect of CRISPR/Cas9 System Using mRNA Delivery

In Chapter 3, we have demonstrated that the off-target effect of CRISPR/Cas9 system can be significantly reduced after the zwitterionic conjugation to Cas9 protein. In this chapter, besides pCB chemical conjugation, zwitterionic $(EK)_n$ peptides were fused to Cas9. A 'one-step' strategy called 'EKylation' was employed to genetically fuse zwitterionic poly(EK) peptide to Cas9 protein at the mRNA level. The zwitterionic peptide tails can be considered as a peptide analogue of the zwitterionic pCB that exhibits similar resistance to non-specific mismatch between gRNA and DNA. Poly(EK) is both biocompatible and degradable. We appended poly(EK) tails of well-defined lengths to the C-terminus and N-terminus of the Cas9 protein and study the on/off target effect of these constructs. To avoid the constitutive presence of the plasmids and transcripts which can result in high levels of undesired off-target gene editing, we turned to DNA-free CRISPR gene-editing systems by transfecting both *in vitro* transcribed sgRNA and Cas9 mRNA to achieve their desired gene editing effect.

4.1 Introduction

Successful delivery of Cas9 nuclease to the right tissues and cells at the right time is the key to transforming the genome-editing technology into clinical application[86]. Currently, genome-editing nucleases can be introduced into the system in three platforms: DNA, mRNA, and protein. Each of these delivery strategies has advantages and shortcomings in effectiveness. The delivery of native Cas9 protein is the most

straightforward approach, as it provides the best control over nuclease dosage without any signal amplification.[87] Protein delivery enables the fastest genomic editing as it obviates the transcriptional and/or translational processes. It also offers the most transient genome-editing cassette with reduced off-target effects and toxicity, and, thus, it is perfect for ex vivo cell therapy. However, protein delivery of nucleases has several limitations for in vivo therapy. First, the effective delivery of Cas9/sgRNA ribobucleoprotein is a challange due to the large zise (~160 kD) and positive charge of Cas9 protein, and the strong negative charge of sgRNA. [88-91] Meanwhile, different from the delivery of Cas9 in the mRNA or DNA format, there is no signal amplification for protein format. Thus a sufficient amount of Cas9 protein is required. Second, direct protein delivery of Cas9 might trigger a cellular and humoral immune response.[92] A Cas9-specific T cell receptor has been identified in mice and humans.[93] Since CRISPR/Cas9 system is derived from bacteria, it is not surprising that 79% and 65% of the human population have preexisting antibodies against Staphylococcus aureus Cas9 (SaCas9) and spCas9, respectively.[94] Third, the cost of the complicated protein purification process for large nucleases and the potential bacteria endotoxin contamination during manufacture also need to be carefully addressed.[95]

Another alternative option is the plasmid-based CRISPR/Cas9 system.[96] DNA delivery of programmable nucleases is widely used in basic research owing to the simplicity and low cost of manipulation. Both Cas9 and sgRNA can be packed in the same plasmid, which usually render more sustained expression than protein delivery.[97] However, there are several drawbacks of DNA delivery that limit its applications in clinical use. First, the persistent nuclease expression, may result in high off-target effects and genome instability.[95] Second, the giant size of Cas9 (~4.5 kb) and the total plasmid size

(>7 kb) greatly increase the difficulty of delivery and expression of Cas9. [98]The third obstacle for DNA delivery is the requirement of nuclear entry for DNA transcription, which may affect the gene-editing efficiency and induce the delay in therapeutic efficacy.[99] Finally, viral DNA delivery could trigger strong immune responses and increase risks of integration of plasmid into the host genome.[100]

The third option is the in vitro-transcribed (IVT) mRNA delivery system for nucleases.[101, 102] IVT mRNA minimizes the risk of genome insertion, and it bypasses the requirement of nuclear entry for transcription, resulting in quick onset of genome editing.[103] In addition, Cas9 mRNA delivery provides transient expression of Cas9 protein, which may potentially decrease off-target effects.[104] However, the short half-life of Cas9 mRNA may also lead to low efficiency and the relatively poor stability is an important obstacle of for the mRNA delivery strategy. To overcome this drawback, various strategies have been developed on the structural elements of mRNA. The incorporation of anti-reverse m^7GpppG cap analog,[106] poly(A) tail, modified nucleotides,[107] 5′ and 3′ UTRs containing regulatory elements,[108] and synonymously frequent codons into mRNA[109] have exhibited enhanced stability and translational efficiency in IVT mRNA. Therefore, mRNA delivery of nucleases holds great potential for therapeutic application.

Bioinformatics studies of over 1000 proteins have revealed the predominance of negatively charged glutamic acid (E) and positively charged lysine (K) residues on protein surfaces at a balanced ratio[110]. It is approved that the distribution of equal amounts of oppositely charged E and K residues provides a zwitterionic layer to stabilize protein and resist non-specific adsorptions. Both alternating and mixed EK sequences have been shown to confer nonfouling zwitterionic characteristics to surfaces[25] and nanoparticles[23]. A

zwitterionic $(EK)_n$ peptides would provide protection to the protein in a similar way as a zwitterionic pCB polymer. Building on poly(zwitterionic) protein conjugation, we have developed a method to produce poly(EK)-protein constructs through biosynthesis. This 'EKylation' strategy allows for one-step production of recombinant conjugates with perfect structural homogeneity and eliminates the need for secondary chemical conjugation steps that generate products with different isoforms[111]. Based on the finding in Chapter 3, it is expected that $(EK)_n$ peptides conjugated Cas9 is able to reduce the DNA cleavages at off-target site. One major advantage of this strategy is that a simple alternating $(EK)_n$ peptides can be attached to a protein via genetic fusion in addition to chemical conjugation. This 'one-step' strategy to produce conjugate or fused protein provides a broadly applicable alternative to synthetic polymer conjugation. Poly(EK) is both biocompatible and degradable. Here we developed a new strategy for reducing off-target effect of CRISPR/Cas9 systems that relies on mRNA delivery of Cas9 with poly(EK) extensions to its termini via genetic engineering.

4.2 Experimental Section

4.2.1 Construction of Cas9-EK plasmids and sgRNA

Human codon-optimized DNA encoding Cas9 nuclease from Streptococcus pyogenes with an N and C terminal nuclear localization signal (NLS) was cloned into a pcDNA3.1 vector (GenScript). DNA encoding a poly(EK) with various length were commercially synthesized and appended to the C-terminal or both C- and N-terminal of the Cas9 gene to generate a Cas9-EK constructs (Figure 4.1). The Cas9 sequence without poly(EK) sequence was used as the control sequence. The sgRNAs that target GFP

(GGGCACGGGCAGCTTGCCGG), VEGF (GGGTGGGGGGAGTTTGCTCC) and EMX (GAGTCCGAGCAGAAGAAGAA) were purchased from Synthego. The potential off-target sites listed in Table 1 were used to measure the off-target efficiency in human cells.

4.2.2 Mammalian cell culture

HEK 293-GFP cells were maintained in DMEM medium supplemented with 10% FBS. K562 cells were propagated in RPMI 1640 medium containing 10% FBS. After thawing, cells were passaged 4–5 times before using for transfection. When setting up the experiments for transfections, cultured cells were plated in 24-well format (500 µl volume) in complete growth medium at a cell density necessary to reach ~70% confluence the next day. Full serum media was replaced with the same media but containing no antibiotics at least 1 h before delivery. All cultures were maintained in 5% CO_2 at 37◦C in a humidified incubator.

4.2.3 *In vitro* transcription of Cas9/Cas9-EK mRNA

The Cas9 and Cas9-EK plasmids were linearized using BbsI (New England Biolabs) according to manufacturer's instructions. Following purification, the Cas9 and Cas9-EK mRNAs were transcribed using mMESSAGE mMACHINE T7 Ultra Transcription Kit (ThermoFisher) according to manufacturer's instructions with a 2 hours incubation time at 37℃. TURBO DNase was added to stop transcription. Prior to polyadenylation, a 2 µl aliquot was removed and diluted into 10 µl of nuclease free water. This aliquot was used as an untailed control in the RNA gel. The polyadenylation reaction was started with the addition of the E-PAP enzyme and incubated for 30 mins at 37℃.

4.2.4 In vitro delivery of Cas9/Cas9-EK mRNA

One day prior to transfection, the cells were seeded in a 24-well plate at a cell density of 1–2 × 10^5 cells per well. 0.5 μg Cas9 or Cas9-EK mRNA was added to 25 μL of Opti-MEM, followed by the addition of 50–100 ng gRNA. Meanwhile, 2 μL of Lipofectamine MessengerMax (ThermoFisher) was diluted into 25 μL of Opti-MEM and then mixed with mRNA/gRNA sample. The mixture was incubated for 15 min prior to addition to the cells. Then the entire solution was added to the cells and mixed by gently swirling the plate. The plate was incubated at 37 ◦C for 48 h in a 5% CO_2 incubator. Genomic DNA was extracted from cells using the Quick-DNA Miniprep Plus Kit (Zymo Research). The cut efficiency is assessed by T7E1 assays and site-specific Sanger sequencing of on-target and putative off-target sites.

4.2.5 Determination of on- and off-target mutation frequencies in human cells.

Genomic DNA was harvested 3 d after transfection from HEK293 or K562 cells using the Quick-DNA Miniprep (Zymo Research), according to the manufacturer's instructions. 100 ng of isolated genomic DNA will be used as template to PCR amplify the targeted genomic sites with primer pairs. PCR products were purified with a PureLink™ PCR Purification Kit (Thermo Fisher) and quantified on a microplate reader. 250 ng of purified PCR DNA will be combined with 2 μl of NEBuffer 2 (NEB) in a total volume of 19 μl and denatured then re-annealed with thermocycling at 95 °C for 5 min, 95–85 °C at 2 °C/s; 85–20 °C at 0.2 °C/s. The re-annealed DNA will be incubated with 1 μl of T7 Endonuclease I (10 U/μl, NEB) at 37 °C for 30 min. Cas9-induced cleavage bands and the uncleaved band will be visualized under UV light and quantified using ImageJ software30. The peak intensities of the cleaved bands will be divided by the total intensity of all bands (uncleaved

+ cleaved bands) to determine the fraction cleaved, which will be used to estimate gene modification levels. For each sample, transfections and subsequent modification measurements will be performed in triplicate on different days. Off-target analysis will be performed using a bioinformatics based search tool to select potential off-target sites, which will also be evaluated using the T7E1 mutation detection assay.

4.2.6 Sanger Sequencing

To better determine the mutation rate, the same purified PCR products used for T7EI assay were sequenced to observe the individual mutations and determine the mutational spectra. Sanger sequencing was used to confirm the gene modification frequencies for the modified and unmodified CRISPR/Cas9 systems. The results were analyzed by ICE Analysis (Synthego)

4.3 Results and Discussion

In this study, human codon-optimized DNA encoding Cas9 nuclease from Streptococcus pyogenes with N and C terminal nuclear localization signal (NLS) was cloned into a pcDNA3.1 vector. DNA encoding poly(EK) with 10 KDa or 30 KDa length were commercially synthesized and appended to the C-terminal or both C- and N-terminals of the Cas9 gene to generate Cas9-EK constructs (Figure 4.1). We constructed 3 Cas9-EK plasmids, Cas9-$(EK)_{10}$, $(EK)_{10}$-Cas9-$(EK)_{10}$, and Cas9-$(EK)_{30}$, based on the length and fusion position of poly(EK). The Cas9 sequence without poly(EK) sequence was used as the control sequence. Since the constitutive presence of the plasmids and transcripts could result in high levels of undesired off-target gene editing, we turned to DNA-free CRISPR gene-editing systems by transfecting both *in vitro* transcribed sgRNA and Cas9 mRNA to

achieve their desired gene editing effect. This system relies on the translation of Cas9 mRNA in cells, so polyadenylation (poly(A)) of Cas9 and Cas9-EK mRNA prior to transfection is required to prevent Cas9 mRNA from degradation before i*n vivo* translation occurs. The polyadenylation reaction was started with the addition of the E-PAP enzyme and incubated for 30 mins at 37℃. The band shift after polyadenylation in the electrophoresis image (Figure 4.2) confirmed the presence of poly(A) tails. Smeared bands indicate degradation.

After getting the mRNA, we first confirmed the activity of lab-prepared Cas9 mRNA in mammalian cells by comparing it with the commercialized Cas9 mRNA. The gRNA that target GFP (GGGCACGGGCAGCTTGCCGG) was selected for this analysis. Two days after co-transfecting HEK293-GFP cells with mRNA expressing either commercialized Cas9 or lab-prepared Cas9 together with the GFP gRNA, the percentage of indel mutations was quantified by the T7EI assay. As shown in Figure 4.3, the lab-prepared Cas9 mRNA possesses similar on-target editing efficiency to the commercial Cas9 mRNA, which indicates the successful synthesis of the *in vitro* transcribed Cas9 mRNA.

To investigated whether the Cas9-EK mRNAs could be programmed by gRNAs to cleave chromosomal DNA in mammalian cells, we used the same assay to test the gene-editing efficiency of Cas9-$(EK)_{10}$, $(EK)_{10}$-Cas9-$(EK)_{10}$, and Cas9-$(EK)_{30}$. As shown in Figure 4.4a, all three Cas9-EK mRNAs show a similar editing level to the Cas9 mRNA. The presence of poly(EK) did not compromise the on-target gene editing efficiency on the selected on-target site. To approve that this effect is not target site-specific, the similar editing frequency was further verified with genomic loci VEGF(GGGTGGGGGGAGTTTGCTCC) (Figure 4.4b) and EMX

(GAGTCCGAGCAGAAGAAGAA) (Figure 4.4c). The quantified data are summarized and presented in Figure 4.4d.

To examine whether the presence of poly(EK) can reduce the off-target effects in human cells, we selected several potential off-target sites for VEGF and EMA target loci, which have been observed in other studies[29, 112]. The results are shown in Table 4.2. For Cas9-$(EK)_{10}$, the off-target activity was similar or slightly reduced for some off-target sites compared to the native Cas9. We believe this is due to the insufficient length of poly(EK) that can't reduce the nonspecific force between gRNA and double-strand DNA. However, both $(EK)_{10}$-Cas9-$(EK)_{10}$, and Cas9-$(EK)_{30}$ showed a significantly reduced off-target editing efficiency, ranging from 1.2% to 3.5%. These results demonstrated that Cas9-EK showed reduced off-target effects on different genomic loci.

4.4 Conclusions

In summary, the author reported a 'one-step' strategy called 'EKylation' to genetically fuse zwitterionic poly(EK) peptide to Cas9 protein inside cells using mRNA delivery. After being fused with this super-hydrophilic zwitterionic peptide, CRISPR/Cas9 system showed significantly reduced "off-target" efficiency. Compared with zwitterionic conjugation method, the poly(EK) peptide is biodegradable and potentially safer for clinical applications. This technology was realized through mRNA delivery and will provide a robust and easily used strategy which is able to reduce "off-target" mutations on a genome-wide scale and to improve the efficiency of a wide range of CRISPR/Cas9-based biological and clinical applications.

4.5 Tables

Table 4.1. On-target and known off-target substrates of Cas9:sgRNAs that target sites in *GFP*, *EMX*, *VEGF*. List of genomic on-target and off-targets sites for *GFP*, *EMX*, *VEGF* are shown with mutations from the on-target sequence shown in lower case and red.

Target	Site name	Sequence
Target 1 (GFP)	GFP-On	GGGCACGGGCAGCTTGCCGG
Target 2 (VEGF)	VEGF-On	GGGTGGGGGGAGTTTGCTCC
	VEGF-Off1	GGaTGGaGGGAGTTTGCTCC
	VEGF-Off2	GGGaGGGtGGAGTTTGCTCC
	VEGF-Off3	cGGgGGaGGGAGTTTGCTCC
	VEGF-Off4	GGGgaGGGGaAGTTTGCTCC
Target 3 (EMX)	EMX-On	GAGTCCGAGCAGAAGAAGAA
	EMX-Off1	GAGgCCGAGCAGAAGAAagA
	EMX-Off2	GAGTCCtAGCAGgAGAAGAA

Table 4.2. On-target and off-target DNA modification resulting from CRISPR/Cas9 designed to endogenous human genes in HEK293-GFP cells.

		Cas9 (%)	Cas9-$(EK)_{10}$ (%)	$(EK)_{10}$-Cas9-$(EK)_{10}$ (%)	Cas9-$(EK)_{30}$ (%)
GFP-On	GGGCACGGGCAGCTTGCCGG	38.1	36.4	37.2	35.7
VEGF-On	GGGTGGGGGGAGTTTGCTCCTGG	26.9	27.3	24.8	25.3
VEGF-Off1	GGaTGGaGGGAGTTTGCTCC	7.4	7.0	3.5	1.7
VEGF-Off2	GGGaGGGtGGAGTTTGCTCCTGG	8.5	7.6	2.6	3.7
VEGF-Off3	cGGgGGaGGGAGTTTGCTCCTGG	6.8	4.9	2.1	1.9
VEGF-Off4	GGGgaGGGGaAGTTTGCTCC	4.5	4.8	1.2	1.5
EMX-On	GAGTCCGAGCAGAAGAAGAAGGG	16.9	16.5	17.2	15.8
EMX-Off1	GAGgCCGAGCAGAAGAAagACGG	7.5	7.0	2.4	2.1
EMX-Off2	GAGTCCtAGCAGgAGAAGAAGaG	5.7	5.9	1.9	2.1

4.6 Figures

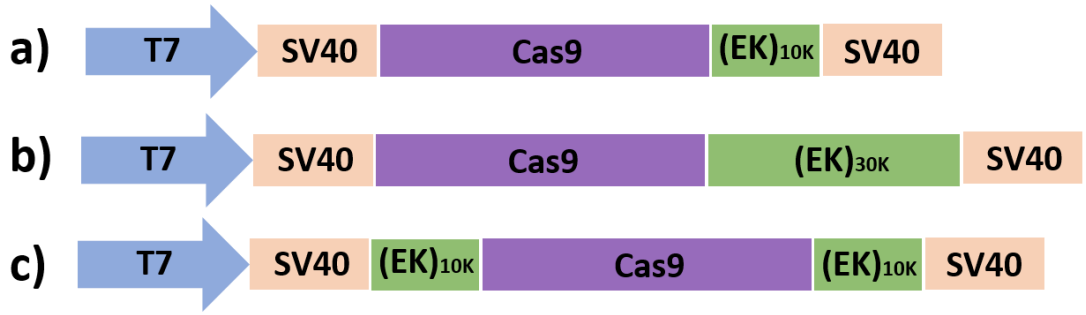


Figure 4.1. Construction of expression plasmid encoding Cas9-(EK)n.

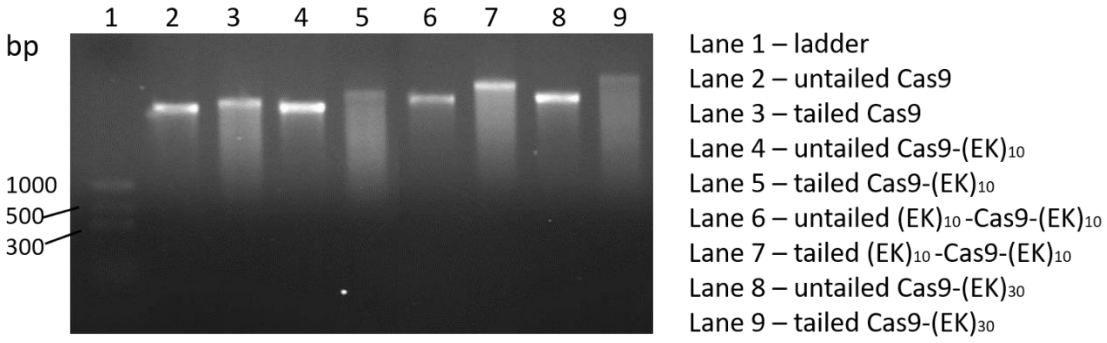


Figure 4.2. Electrophoresis of in vitro transcribed Cas9 and Cas9-EK mRNA pre- and post-polyadenylation.

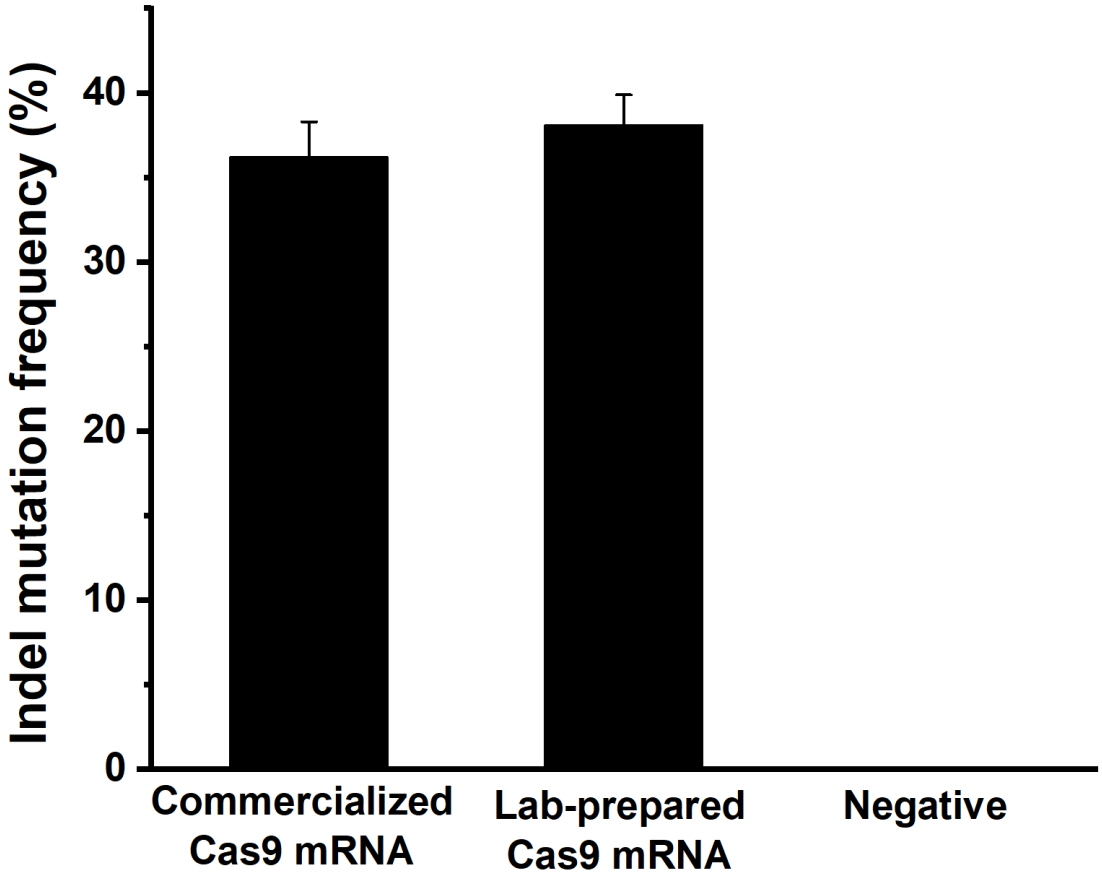


Figure 4.3. Gene editing efficacy of Cas9 using commercialized Cas9 mRNA and lab-prepared Cas9 mRNA.

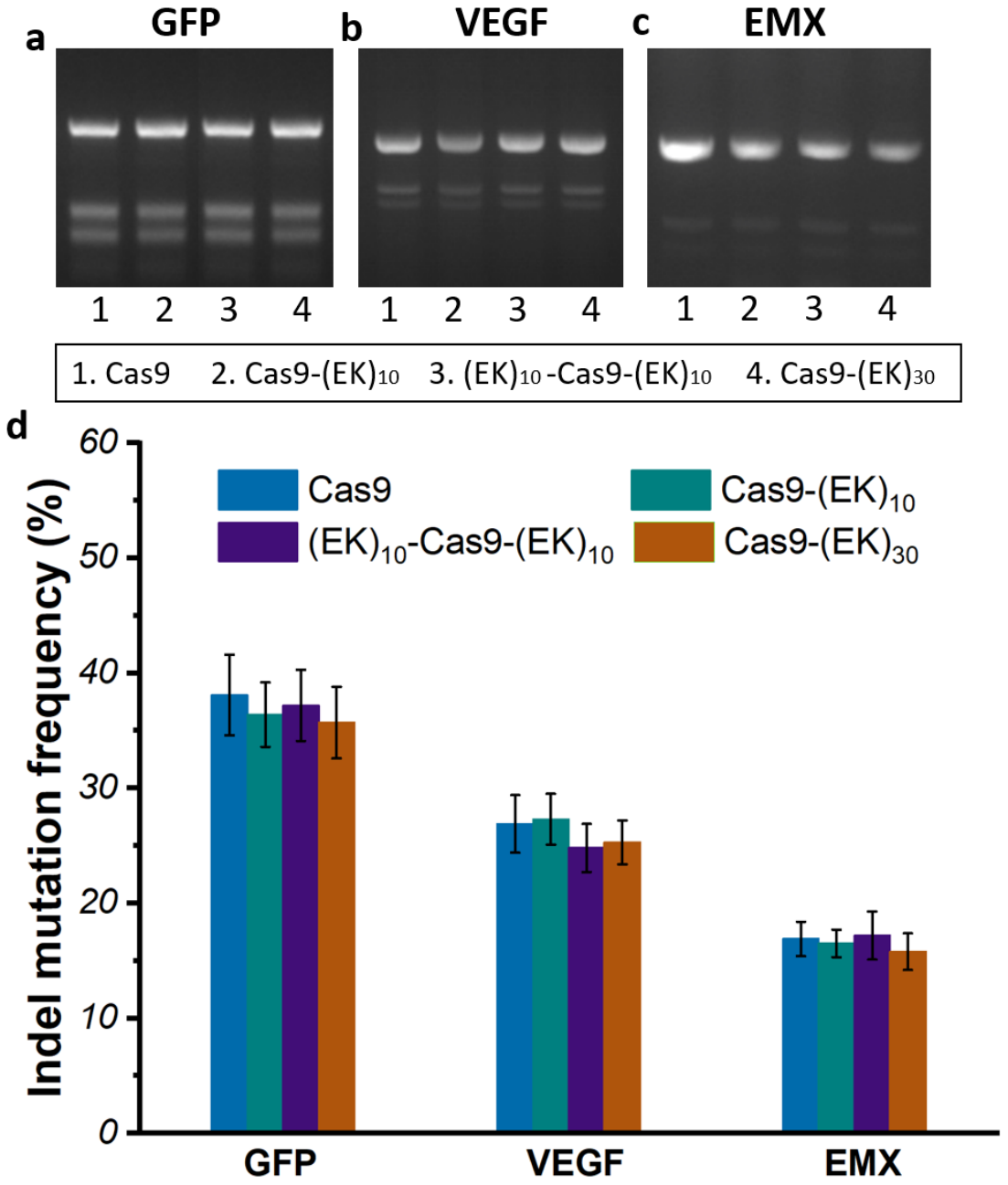


Figure 4.4 Electrophoresis of on-target DNA editing resulting from native Cas9, Cas9-$(EK)_{10}$, $(EK)_{10}$-Cas9-$(EK)_{10}$, and Cas9-$(EK)_{30}$ for target sites GFP (a), VEGF (b), and EMA (c) in HEK293-GFP cells and quantified data (d).

Chapter 5 Conclusions

In this thesis, the author aims to develop new methods to address the efficacy and safety concerns of using engineered proteins as biopharmaceutical drugs and gene editing tools.

In Chapter 2, the author discusses the tactics to improve the efficacy and safety of protein therapeutics. Learning lessons from PEGylation, the author explores zwitterionic conjugation as an alternative protein modification technology. To protect the protein from body clearance and immune system, a poly(carboxybetaine) (PCB) polymer is conjugated to the protein surface. Compared with PEG conjugation, the zwitterionic coating more effectively shields protein epitopes, rendering the whole protein invisible to the body immune system. This strategy is proved to be effective in increasing protein stability, prolonging in vivo circulation half-life and reducing immunogenicity, and is successfully applied to interferon-alpha.

In Chapter 3, the author further discusses the tactics to improve the efficacy and safety of CRISPR/Cas9 for gene editing. To improve the safety of CRISPR/Cas9 system, the author discusses a novel method to reduce the off-target editing concern of this system via conjugating CRISPR/Cas9 with zwitterionic pCB polymer or poly(EK) peptide. The author demonstrates that the conjugation of CRISPR/Cas9 system with pCB polymer can both increase the efficacy of Cas9 nuclease and reduce non-specific interactions between sgRNA and DNA (off-target DNA break).

In Chapter 4, the author further converts the strategy in Chapter 3 to mRNA version via a 'one-step' strategy called 'EKylation' to genetically fuse zwitterionic poly(EK) peptide

to Cas9 protein at mRNA level. The zwitterionic peptide poly(EK) is a peptide analog of the zwitterionic pCB that exhibits similar resistance to non-specific mismatch between gRNA and DNA. After being fused with this super-hydrophilic zwitterionic peptide, CRISPR/Cas9 system showed significantly reduced "off-target" efficiency. Compared with the zwitterionic conjugation method, the poly(EK) peptide is biodegradable and potentially safer for clinical applications.

In summary, by tailoring the material and chemistry, the author explores strategies to improve the efficacy and safety of protein and gene therapeutics. New materials and methods are developed to overcome current challenges facing PEGylation. These new technologies can greatly improve the pharmacokinetics and immunogenicity of currently marketed biopharmaceutics, and unlock the possibility of adopting highly specified gene-editing tools for gene therapy.

References

[1] Leader B, Baca QJ, Golan DE. Protein therapeutics: a summary and pharmacological classification. Nature reviews Drug discovery. 2008;7:21-39.

[2] Dewan S. Global Markets for Bioengineered Protein Drugs. BCC Research. 2017.

[3] Mitragotri S, Burke PA, Langer R. Overcoming the challenges in administering biopharmaceuticals: formulation and delivery strategies. Nature reviews Drug discovery. 2014;13:655-72.

[4] Chirino AJ, Ary ML, Marshall SA. Minimizing the immunogenicity of protein therapeutics. Drug discovery today. 2004;9:82-90.

[5] Veronese FM, Pasut G. PEGylation, successful approach to drug delivery. Drug discovery today. 2005;10:1451-8.

[6] Shankar G, Pendley C, Stein KE. A risk-based bioanalytical strategy for the assessment of antibody immune responses against biological drugs. Nature biotechnology. 2007;25:555-61.

[7] Veronese FM, Pasut G. PEGylation, successful approach to drug delivery. Drug discovery today. 2005;10:1451-8.

[8] Veronese FM, Mero A. The impact of PEGylation on biological therapies. BioDrugs. 2008;22:315-29.

[9] Fishburn CS. The pharmacology of PEGylation: balancing PD with PK to generate novel therapeutics. Journal of pharmaceutical sciences. 2008;97:4167-83.

[10] Harris JM, Chess RB. Effect of pegylation on pharmaceuticals. Nature Reviews Drug Discovery. 2003;2:214-21.

[11] Molineux G. Pegylation: engineering improved pharmaceuticals for enhanced therapy. Cancer treatment reviews. 2002;28:13-6.

[12] Turecek PL, Bossard MJ, Schoetens F, Ivens IA. PEGylation of biopharmaceuticals: a review of chemistry and nonclinical safety information of approved drugs. Journal of pharmaceutical sciences. 2016;105:460-75.

[13] Podobnik B, Helk B, Smilovic V, Skrajnar S, Fidler K, Jevsevar S, et al. Conjugation of PolyPEG to interferon alpha extends serum half-life while maintaining low viscosity of the conjugate. Bioconjugate chemistry. 2015;26:452-9.

[14] Hershfield MS, Buckley RH, Greenberg ML, Melton AL, Schiff R, Hatem C, et al. Treatment of adenosine deaminase deficiency with polyethylene glycol–modified adenosine deaminase. New England Journal of Medicine. 1987;316:589-96.

[15] Keefe AJ, Jiang S. Poly(zwitterionic)protein conjugates offer increased stability without sacrificing binding affinity or bioactivity. Nature chemistry. 2012;4:59-63.

[16] Leng C, Hung H-C, Sun S, Wang D, Li Y, Jiang S, et al. Probing the surface hydration of nonfouling zwitterionic and PEG materials in contact with proteins. ACS applied materials & interfaces. 2015;7:16881-8.

[17] Shao Q, Jiang S. Molecular understanding and design of zwitterionic materials. Advanced Materials. 2015;27:15-26.

[18] Chen S, Li L, Zhao C, Zheng J. Surface hydration: Principles and applications toward low-fouling/nonfouling biomaterials. Polymer. 2010;51:5283-93.

[19] García KP, Zarschler K, Barbaro L, Barreto JA, O'Malley W, Spiccia L, et al. Zwitterionic Coatings: Zwitterionic-Coated “Stealth” Nanoparticles for Biomedical

Applications: Recent Advances in Countering Biomolecular Corona Formation and Uptake by the Mononuclear Phagocyte System (Small 13/2014). Small. 2014;10:2505-.

[20] White AD, Nowinski AK, Huang W, Keefe AJ, Sun F, Jiang S. Decoding nonspecific interactions from nature. Chemical Science. 2012;3:3488-94.

[21] Liu EJ, Sinclair A, Keefe AJ, Nannenga BL, Coyle BL, Baneyx F, et al. EKylation: addition of an alternating-charge peptide stabilizes proteins. Biomacromolecules. 2015;16:3357-61.

[22] Nowinski AK, White AD, Keefe AJ, Jiang S. Biologically inspired stealth peptide-capped gold nanoparticles. Langmuir. 2014;30:1864-70.

[23] Keefe AJ, Caldwell KB, Nowinski AK, White AD, Thakkar A, Jiang S. Screening nonspecific interactions of peptides without background interference. Biomaterials. 2013;34:1871-7.

[24] Nowinski AK, Sun F, White AD, Keefe AJ, Jiang S. Sequence, structure, and function of peptide self-assembled monolayers. Journal of the American Chemical Society. 2012;134:6000-5.

[25] Chen S, Cao Z, Jiang S. Ultra-low fouling peptide surfaces derived from natural amino acids. Biomaterials. 2009;30:5892-6.

[26] Cao ZQ, Yu QM, Xue H, Cheng G, Jiang SY. Nanoparticles for Drug Delivery Prepared from Amphiphilic PLGA Zwitterionic Block Copolymers with Sharp Contrast in Polarity between Two Blocks. Angewandte Chemie-International Edition. 2010;49:3771-6.

[27] Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. Nature biotechnology. 2014;32:347-55.

[28] Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, et al. Multiplex genome engineering using CRISPR/Cas systems. Science. 2013;339:819-23.

[29] Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, et al. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nature biotechnology. 2013;31:822-6.

[30] Cho SW, Kim S, Kim Y, Kweon J, Kim HS, Bae S, et al. Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. Genome research. 2014;24:132-41.

[31] Brunet E, Simsek D, Tomishima M, DeKelver R, Choi VM, Gregory P, et al. Chromosomal translocations induced at specified loci in human stem cells. Proceedings of the National Academy of Sciences. 2009;106:10620-5.

[32] Kim Y, Kweon J, Kim A, Chon JK, Yoo JY, Kim HJ, et al. A library of TAL effector nucleases spanning the human genome. Nature biotechnology. 2013;31:251-8.

[33] Lee HJ, Kim E, Kim J-S. Targeted chromosomal deletions in human cells using zinc finger nucleases. Genome research. 2010;20:81-9.

[34] Lee HJ, Kweon J, Kim E, Kim S, Kim J-S. Targeted chromosomal duplications and inversions in the human genome using zinc finger nucleases. Genome research. 2012;22:539-48.

[35] Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, et al. DNA targeting specificity of RNA-guided Cas9 nucleases. Nature biotechnology. 2013;31:827.

[36] Hasty P, Montagna C. Chromosomal Rearrangements in Cancer: Detection and potential causal mechanisms. Molecular & cellular oncology. 2014;1:e29904.

[37] Tsai SQ, Zheng Z, Nguyen NT, Liebers M, Topkar VV, Thapar V, et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. Nature biotechnology. 2015;33:187-97.

[38] Fu Y, Sander JD, Reyon D, Cascio VM, Joung JK. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. Nature biotechnology. 2014;32:279-84.

[39] Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, Zheng Z, et al. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature. 2015;523:481-5.

[40] Mali P, Aach J, Stranges PB, Esvelt KM, Moosburner M, Kosuri S, et al. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. Nature biotechnology. 2013;31:833-8.

[41] Ran FA, Hsu PD, Lin C-Y, Gootenberg JS, Konermann S, Trevino AE, et al. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell. 2013;154:1380-9.

[42] Tsai SQ, Wyvekens N, Khayter C, Foden JA, Thapar V, Reyon D, et al. Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing. Nature biotechnology. 2014;32:569-76.

[43] Guilinger JP, Thompson DB, Liu DR. Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification. Nature biotechnology. 2014;32:577-82.

[44] Wyvekens N, Topkar VV, Khayter C, Joung JK, Tsai SQ. Dimeric CRISPR RNA-guided FokI-dCas9 nucleases directed by truncated gRNAs for highly specific genome editing. Human gene therapy. 2015;26:425-31.

[45] Jiang S, Cao Z. Ultralow-fouling, functionalizable, and hydrolyzable zwitterionic materials and their derivatives for biological applications. Advanced Materials. 2010;22:920-32.

[46] Keefe AJ, Jiang S. Poly (zwitterionic) protein conjugates offer increased stability without sacrificing binding affinity or bioactivity. Nature chemistry. 2012;4:59-63.

[47] Keefe AJ, Jiang S. Poly (zwitterionic) protein conjugates offer increased stability without sacrificing binding affinity or bioactivity. Nature chemistry. 2012;4:59.

[48] Frokjaer S, Otzen DE. Protein drug stability: a formulation challenge. Nature Reviews Drug Discovery. 2005;4:298-306.

[49] Dougherty TJ, Pucci MJ. Antibiotic discovery and development: Springer Science & Business Media; 2011.

[50] Shaunak S, Godwin A, Choi J-W, Balan S, Pedone E, Vijayarangam D, et al. Site-specific PEGylation of native disulfide bonds in therapeutic proteins. Nature chemical biology. 2006;2:312.

[51] Lee BK, Kwon JS, Kim HJ, Yamamoto S, Lee E. Solid-phase PEGylation of recombinant interferon α-2a for site-specific modification: process performance, characterization, and in vitro bioactivity. Bioconjugate chemistry. 2007;18:1728-34.

[52] Qi Y, Chilkoti A. Protein–polymer conjugation—moving beyond PEGylation. Current opinion in chemical biology. 2015;28:181-93.

[53] Zhang L, Cao Z, Bai T, Carr L, Ella-Menye J-R, Irvin C, et al. Zwitterionic hydrogels implanted in mice resist the foreign-body reaction. Nature biotechnology. 2013;31:553.

[54] Bai T, Sun F, Zhang L, Sinclair A, Liu S, Ella-Menye JR, et al. Restraint of the differentiation of mesenchymal stem cells by a nonfouling zwitterionic hydrogel. Angewandte Chemie. 2014;126:12943-8.

[55] Sener G, Krebs MD. Zwitterionic cryogels for sustained release of proteins. RSC Advances. 2016;6:29608-11.

[56] Rajan R, Matsumura K. Inhibition of protein aggregation by zwitterionic polymer-based core-shell nanogels. Scientific reports. 2017;7:45777.

[57] Seo J-H, Matsuno R, Lee Y, Takai M, Ishihara K. Conformational recovery and preservation of protein nature from heat-induced denaturation by water-soluble phospholipid polymer conjugation. Biomaterials. 2009;30:4859-67.

[58] Bhattacharjee S, Liu W, Wang WH, Weitzhandler I, Li X, Qi Y, et al. Site-Specific Zwitterionic Polymer Conjugates of a Protein Have Long Plasma Circulation. ChemBioChem. 2015;16:2451-5.

[59] Lewis A, Tang Y, Brocchini S, Choi J-w, Godwin A. Poly (2-methacryloyloxyethyl phosphorylcholine) for protein conjugation. Bioconjugate chemistry. 2008;19:2144-55.

[60] Zhang P, Sun F, Tsao C, Liu S, Jain P, Sinclair A, et al. Zwitterionic gel encapsulation promotes protein stability, enhances pharmacokinetics, and reduces immunogenicity. Proceedings of the National Academy of Sciences. 2015;112:12046-51.

[61] Xie J, Lu Y, Wang W, Zhu H, Wang Z, Cao Z. Simple protein modification using zwitterionic polymer to mitigate the bioactivity loss of conjugated insulin. Advanced healthcare materials. 2017;6:1601428.

[62] Liu S, Jiang S. Zwitterionic polymer-protein conjugates reduce polymer-specific antibody response. Nano Today. 2016;11:285-91.

[63] Walter MR, Bordens R, Nagabhushan TL, Williams BR, Herberman RB, Dinarello CA, et al. Review of recent developments in the molecular characterization of recombinant alfa interferons on the 40th anniversary of the discovery of interferon. Cancer biotherapy & radiopharmaceuticals. 1998;13:143-54.

[64] Hu X, Olivier K, Polack E, Crossman M, Zokowski K, Gronke RS, et al. In vivo pharmacology and toxicology evaluation of polyethylene glycol-conjugated interferon β-1a. Journal of Pharmacology and Experimental Therapeutics. 2011;338:984-96.

[65] Yang Q, Lai SK. Anti-PEG immunity: emergence, characteristics, and unaddressed questions. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology. 2015;7:655-77.

[66] Garay RP, El-Gewely R, Armstrong JK, Garratty G, Richette P. Antibodies against polyethylene glycol in healthy subjects and in patients treated with PEG-conjugated agents. Taylor & Francis; 2012.

[67] Richter A, Åkerblom E. Antibodies against polyethylene glycol produced in animals by immunization with monomethoxy polyethylene glycol modified proteins. International Archives of Allergy and Immunology. 1983;70:124-31.

[68] Hershfield MS, Ganson NJ, Kelly SJ, Scarlett EL, Jaggers DA, Sundy JS. Induced and pre-existing anti-polyethylene glycol antibody in a trial of every 3-week dosing of pegloticase for refractory gout, including in organ transplant recipients. Arthritis research & therapy. 2014;16:R63.

[69] Lipsky PE, Calabrese LH, Kavanaugh A, Sundy JS, Wright D, Wolfson M, et al. Pegloticase immunogenicity: the relationship between efficacy and antibody development in patients treated for refractory chronic gout. Arthritis research & therapy. 2014;16:R60.

[70] Armstrong JK, Hempel G, Koling S, Chan LS, Fisher T, Meiselman HJ, et al. Antibody against poly (ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. Cancer. 2007;110:103-11.

[71] Ishida T, Kiwada H. Accelerated blood clearance (ABC) phenomenon upon repeated injection of PEGylated liposomes. International journal of pharmaceutics. 2008;354:56-62.

[72] Shiraishi K, Hamano M, Ma H, Kawano K, Maitani Y, Aoshi T, et al. Hydrophobic blocks of PEG-conjugates play a significant role in the accelerated blood clearance (ABC) phenomenon. Journal of controlled release. 2013;165:183-90.

[73] Yang Q, Jacobs TM, McCallen JD, Moore DT, Huckaby JT, Edelstein JN, et al. Analysis of pre-existing IgG and IgM antibodies against polyethylene glycol (PEG) in the general population. Analytical chemistry. 2016;88:11804-12.

[74] van der Eijk AA, Vrolijk JM, Haagmans BL. Antibodies neutralizing peginterferon alfa during retreatment of hepatitis C. New England Journal of Medicine. 2006;354:1323-4.

[75] Pelegri-O'Day EM, Lin E-W, Maynard HD. Therapeutic protein–polymer conjugates: advancing beyond PEGylation. Journal of the American Chemical Society. 2014;136:14323-32.

[76] Styslinger TJ, Zhang N, Bhatt VS, Pettit N, Palmer AF, Wang PG. Site-selective glycosylation of hemoglobin with variable molecular weight oligosaccharides: potential alternative to PEGylation. Journal of the American Chemical Society. 2012;134:7507-15.

[77] Saifer MG, Williams LD, Sobczyk MA, Michaels SJ, Sherman MR. Selectivity of binding of PEGs and PEG-like oligomers to anti-PEG antibodies induced by methoxyPEG-proteins. Molecular immunology. 2014;57:236-46.

[78] Schneck E, Berts I, Halperin A, Daillant J, Fragneto G. Neutron reflectometry from poly (ethylene-glycol) brushes binding anti-PEG antibodies: Evidence of ternary adsorption. Biomaterials. 2015;46:95-104.

[79] Sherman MR, Williams LD, Sobczyk MA, Michaels SJ, Saifer MG. Role of the methoxy group in immune responses to mPEG-protein conjugates. Bioconjugate chemistry. 2012;23:485-99.

[80] Koo T, Lee J, Kim J-S. Measuring and reducing off-target activities of programmable nucleases including CRISPR-Cas9. Molecules and cells. 2015;38:475.

[81] Lin Y, Cradick TJ, Brown MT, Deshmukh H, Ranjan P, Sarode N, et al. CRISPR/Cas9 systems have off-target activity with insertions or deletions between target DNA and guide RNA sequences. Nucleic acids research. 2014;42:7473-85.

[82] Fu Y, Sander JD, Reyon D, Cascio VM, Joung JK. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. Nature biotechnology. 2014;32:279.

[83] Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, et al. High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects. Nature. 2016;529:490-5.

[84] Liu S, Jiang S. Chemical conjugation of zwitterionic polymers protects immunogenic enzyme and preserves bioactivity without polymer-specific antibody response. Nano Today. 2016;11:285-91.

[85] Han Y, Yuan Z, Zhang P, Jiang S. Zwitterlation mitigates protein bioactivity loss in vitro over PEGylation. Chemical science. 2018;9:8561-6.

[86] Yin H, Kanasty RL, Eltoukhy AA, Vegas AJ, Dorkin JR, Anderson DGJNRG. Non-viral vectors for gene-based therapy. 2014;15:541-55.

[87] Liu J, Gaj T, Yang Y, Wang N, Shui S, Kim S, et al. Efficient delivery of nuclease proteins for genome editing in human stem cells and primary cells. 2015;10:1842.

[88] Zuris JA, Thompson DB, Shu Y, Guilinger JP, Bessen JL, Hu JH, et al. Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. Nature biotechnology. 2015;33:73-80.

[89] Subburaj S, Chung SJ, Lee C, Ryu S-M, Kim DH, Kim J-S, et al. Site-directed mutagenesis in Petunia× hybrida protoplast system using direct delivery of purified recombinant Cas9 ribonucleoproteins. Plant cell reports. 2016;35:1535-44.

[90] Woo JW, Kim J, Kwon SI, Corvalán C, Cho SW, Kim H, et al. DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. Nature biotechnology. 2015;33:1162-4.

[91] Schumann K, Lin S, Boyer E, Simeonov DR, Subramaniam M, Gate RE, et al. Generation of knock-in primary human T cells using Cas9 ribonucleoproteins. Proceedings of the National Academy of Sciences. 2015;112:10437-42.

[92] Chew WLJWIRSB, Medicine. Immunity to CRISPR Cas9 and Cas12a therapeutics. 2018;10:e1408.

[93] Chew WL, Tabebordbar M, Cheng JK, Mali P, Wu EY, Ng AH, et al. A multifunctional AAV–CRISPR–Cas9 and its host response. 2016;13:868-74.

[94] Simhadri VL, McGill J, McMahon S, Wang J, Jiang H, Sauna ZEJMT-M, et al. Prevalence of pre-existing antibodies to CRISPR-associated nuclease Cas9 in the USA population. 2018;10:105-12.

[95] Wang H-X, Li M, Lee CM, Chakraborty S, Kim H-W, Bao G, et al. CRISPR/Cas9-based genome editing for disease modeling and therapy: challenges and opportunities for nonviral delivery. 2017;117:9874-906.

[96] Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. Nature protocols. 2013;8:2281-308.

[97] Ortinski PI, O'Donovan B, Dong X, Kantor BJMT-M, Development C. Integrase-deficient lentiviral vector as an all-in-one platform for highly efficient CRISPR/Cas9-mediated gene editing. 2017;5:153-64.

[98] Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, et al. In vivo genome editing using Staphylococcus aureus Cas9. Nature. 2015;520:186-91.

[99] Yin H, Kauffman KJ, Anderson DGJNRDD. Delivery technologies for genome editing. 2017;16:387.

[100] Bessis N, GarciaCozar F, Boissier MJGt. Immune responses to gene therapy vectors: influence on vector function and effector mechanisms. 2004;11:S10-S7.

[101] Shen B, Zhang W, Zhang J, Zhou J, Wang J, Chen L, et al. Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. Nature methods. 2014;11:399-402.

[102] Chang N, Sun C, Gao L, Zhu D, Xu X, Zhu X, et al. Genome editing with RNA-guided Cas9 nuclease in zebrafish embryos. Cell research. 2013;23:465-72.

[103] Leonhardt C, Schwake G, Stögbauer TR, Rappl S, Kuhr J-T, Ligon TS, et al. Single-cell mRNA transfection studies: delivery, kinetics and statistics by numbers. 2014;10:679-88.

[104] Pruett-Miller SM, Reading DW, Porter SN, Porteus MHJPg. Attenuation of zinc finger nuclease toxicity by small-molecule regulation of protein levels. 2009;5.

[105] Sahin U, Karikó K, Türeci ÖJNrDd. mRNA-based therapeutics—developing a new class of drugs. 2014;13:759.

[106] JEMIELITY J, FOWLER T, ZUBEREK J, STEPINSKI J, LEWDOROWICZ M, NIEDZWIECKA A, et al. Novel "anti-reverse" cap analogs with superior translational properties. 2003;9:1108-22.

[107] Körner CG, Wahle EJJoBC. Poly (A) tail shortening by a mammalian poly (A)-specific 3′-exoribonuclease. 1997;272:10448-56.

[108] Kwon H, Kim M, Seo Y, Moon YS, Lee HJ, Lee K, et al. Emergence of synthetic mRNA: in vitro synthesis of mRNA and its applications in regenerative medicine. 2018;156:172-93.

[109] Gustafsson C, Govindarajan S, Minshull JJTib. Codon bias and heterologous protein expression. 2004;22:346-53.

[110] Sternberg SH, Redding S, Jinek M, Greene EC, Doudna JAJN. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. 2014;507:62-7.

[111] van Hest JC, Tirrell DA. Protein-based materials, toward a new level of structural control. Chemical communications. 2001:1897-904.

[112] Zuris JA, Thompson DB, Shu Y, Guilinger JP, Bessen JL, Hu JH, et al. Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. Nature biotechnology. 2015;33:73.

Curriculum Vitae

Yanjiao Han was born and raised in Shijiazhuang, Hebei Province, China. She received her Bachelor degree (2011) in Materials Science and Engineering and her Master degree (2014) in Material Science from Tianjin university, China. In 2020, she is expected to receive her Doctor of Philosophy in Molecular Engineering & Science from the University of Washington.

Publications:

1. **Yanjiao Han**, Zhefan Yuan, Shaoyi Jiang. Minimizing off-target frequency of CRISPR/Cas9 system via zwitterionic polymer conjugation. ***Advanced Materials***, under review.
2. **Yanjiao Han**, Zhefan Yuan, Peng Zhang, Shaoyi Jiang. Zwitterlation Mitigates *in vitro* Protein Bioactivity Loss over PEGylation. ***Chemical Science*** 2018, 45, 8561-8566
3. **Yanjiao Han**, Tao Bai, Yuan Liu, Xinyun Zhai, Wenguang Liu. Zinc ion uniquely induced triple shape memory effect of dipole-dipole reinforced ultra-high strength hydrogels. ***Macromolecular rapid communications*** 2012, 33, 225-231
4. **Yanjiao Han,** Tao Bai, Wenguang Liu. Controlled heterogeneous stem cell differentiation on a shape memory hydrogel surface. ***Scientific Reports*** 2014, 5815
5. Tao Bai*, **Yanjiao Han***, Peng Zhang, Wei Wang, Wenguang Liu. Zinc ion-triggered two-way macro-/microscopic shape changing and memory effects in high strength hydrogels with pre-programmed unilateral patterned surfaces. ***Soft Matter*** 2012, 8, 6846-6852
6. Ning Wang, **Yanjiao Han**, Yuan Liu, Tao Bai, Han Gao, Peng Zhang, Wei Wang, Wenguang Liu. High-strength hydrogel as a reusable adsorbent of copper ions. ***Journal of hazardous materials*** 2012, 213, 258-264
7. Tao Bai, Peng Zhang, **Yanjiao Han**, Yuan Liu, Wenguang Liu, Xiaoli Zhao, William Lu. Construction of an ultrahigh strength hydrogel with excellent fatigue resistance based on strong dipole–dipole interaction. ***Soft Matter*** 2011, 7, 2825-2831
8. Han Gao, Ning Wang, Xiufeng Hu, Wenjing Nan, **Yanjiao Han**, Wenguang Liu.

Double hydrogen-bonding pH-sensitive hydrogels retaining high-strengths over a wide pH range. ***Macromolecular rapid communications*** 2013, 34, 63-68

9. Lu Liu, Ning Wang, **Yanjiao Han**, Yongmao Li, Wenguang Liu. Redox-triggered self-rolling robust hydrogel tubes for cell encapsulation. ***Macromolecular rapid communications*** 2013, 35, 344–349