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RAPID COMMUNICATION

Chemical conjugation of zwitterionic polymers protects immunogenic enzyme and preserves bioactivity without polymer-specific antibody response

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Summary Proteins are promising therapeutics with several design challenges, such as their inherent immunogenicity due to their exogenous source or short circulation time. The common solution to such issues is the chemical conjugation of the amphiphilic polymer poly(ethylene glycol) (PEG), a process known as PEGylation. However, several studies demonstrated a decrease in protein bioactivity or an increase in the presence of specific antibodies post PEGylation, highlighting the importance of an alternative strategy. Here we compare the performance of a superhydrophilic zwitterionic polymer in protecting an immunogenic protein to PEG in (i) the maintenance of enzyme activity and stability post modification, (ii) mitigation of the antibody response elicited by the polymer and any subsequent effect on pharmacokinetics, and (iii) minimization of host response toward the underlying protein. In contrast to PEG, zwitterionic conjugation decreases host antibody response without sacrificing bioactivity or generating polymer-specific antibodies.

Introduction

Proteins play critical and diverse roles in the normal function of the body, which makes them excellent candidates as therapeutics to alleviate diseases not easily treated with simple chemical drugs. However, this line of treatment is not without its shortcomings. Proteins as a macromolecule present many stability issues, both during their

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manufacturing and subsequent usage in the body: their aggregation is often a problem and various host defense mechanisms usually rapidly remove them from circulation. In some cases of exogenous protein therapeutics, the body even mounts an efficient immune response to cleanse them prematurely from the system and thus lowering the efficacy or increasing the dosage necessary to achieve the desired response [1—3].

The chemical conjugation of the synthetic polymer polyethylene glycol (PEG) has been the solution of the industry to these challenges, albeit an imperfect one. The addition of PEG, or PEGylation, provides bulk while conferring a protective layer: this often mitigates clearance mechanisms that rely on filtering of smaller sizes or recognition due to nonspecific adsorption of serum proteins [4]. However, PEGylation adds another complexity, as multiple studies demonstrated a decrease in protein activity [5—7], as well as an apparent antigenicity [8—12] of PEG. Much like a hapten, PEG may induce antibody generation once conjugated to a large protein, which was demonstrated in many animal models as well as humans [8—11,13—15].

The PEG-specific antibodies are reported in two cases: pre-existing and study-induced. In one clinical trial, 36% of enrolled patients were positive for anti-PEG antibodies before treatment and three of them then suffered severe allergic reactions minutes after the injection of the PEGylated therapeutic [16]. Other researchers have detected PEG-specific antibodies in the blood of healthy donors, anywhere from 25% to 63% of unique samples tested, and have hypothesized that the incidence may be due to exposure to increasing amount of commercial products that contain PEG, such as cosmetics and food additives [17—19]. In clinical trials for another PEGylated protein, 19% of patients presented with PEG-specific antibodies prior to the start of the studies and later reported more frequent and severe infusion reactions when compared to the initially anti-PEG-negative patients [20]. However, the patients without pre-existing anti-PEG antibodies then developed them as these trials progressed [20] — this induction of anti-PEG antibodies due to repeated administration of the PEGylated therapeutic is also observed in other clinical studies, where 32—46% of patients became anti-PEG-positive [15,21]. This translates to a compromise between the favorable protection afforded by PEG and the increased immunogenicity of the final overall therapeutic product. In certain systems, the former may not outweigh the problems introduced by the latter.

In contrast to the amphiphilic PEG, zwitterionic polymers based on naturally occurring betaines are entirely hydrophilic and extend the same protection without the complicating negative traits. Each polymer contains the same number of positively and negatively charged groups, thus maintaining an overall neutral charge, much like many proteins. While many types of polyzwitterions exist, such as poly(phosphorylcholine), poly(sulfobetaine), and poly(carboxybetaine), we focus on the last group as it contains the naturally occurring glycine betaine and was shown to maintain protein stability and bioactivity as demonstrated in our previous studies [22]. These polymers as components of nanoparticles (NPs) demonstrated excellent long-circulation abilities, often yielding half-lives multiple times longer than the equivalent PEG NP [23—28]. At the same time, polymer-specific antibodies were absent, while anti-PEG antibodies were clearly detected and quantified [27,28]. Furthermore, protein conjugates studied *in vitro* showed maintenance or improved stability of the underlying protein without reducing bioactivity or binding affinity [22]. Combining these encouraging results, we designed a new protein conjugate system to study the effect of zwitterionic polymers on enzyme activity, pharmacokinetics, and immunogenicity, with native and PEGylated proteins as comparison.

The model protein chosen for this study is uricase, an FDA-approved treatment for refractory gout. However, uricase is by definition a foreign protein and thus highly immunogenic. While PEGylated uricase reduced host response toward the protein, it instead raised antibodies toward PEG, which still negatively affected the *in vivo* pharmacokinetics of the conjugate [29,30]. Thus, this protein system presents an area where we can directly compare to a commercial conjugate that improved upon the performance of the bare protein, but is an insufficient solution to the problem.

We aim to address three questions regarding protein conjugation with zwitterionic poly(carboxybetaine acrylamide) (PCB): (i) can it retain native activity post protein modification, (ii) does the superhydrophilic polymer generate any specific antibody response toward itself like PEG does, and (iii) can polyzwitterions protect the underlying enzyme as well as the PEG counterpart? An extensive study in the rat animal model provides antisera generated in response to bare protein, PEGylated protein, and polyzwitter-conjugated protein, which yields both antibody data and enzyme activity measurements. The collected findings offer encouraging support for polyzwitterions as the next generation of non-immunogenic polymers for the protection of exogenous protein therapeutics via chemical conjugation.

Materials and methods

Materials

All chemicals (unless otherwise specified) and recombinant uricase from *Candida* sp. expressed in *Escherichia coli* were purchased from Sigma—Aldrich (St. Louis, MO). Methoxy-PEG—COO—NHS (NHS-mPEG, 10-kDa MW) was purchased from NOF America Corporation (White Plains, NY). N-α-maleimidoacet-oxysuccinimide ester (AMAS), Amplex Red Uricase Assay kits, Costar 96-well EIA/RIA plates, and Pierce Protein Concentrators (150 kDa MWCO) were purchased from Thermo Fisher Scientific (Waltham, MA). Male and female Sprague Dawley rats were purchased from Charles River Laboratories (Burlington, MA). Sterile 0.22 μm Millex-GP syringe filters and Amicon Ultra centrifugal filter units were purchased from EMD Millipore (Billerica, MA). Spectra/Por regenerated cellulose dialysis membranes were purchased from Spectrum Laboratories, Inc (Rancho Dominguez, CA). Antibodies were purchased from Abcam (Cambridge, MA). 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution was purchased from eBioscience (San Diego, CA).