Poly(zwitterionic)protein conjugates offer increased stability without sacrificing binding affinity or bioactivity

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Abstract

Treatment with therapeutic proteins is an attractive approach to targeting a number of challenging diseases. Unfortunately, the native proteins themselves are often unstable in physiological conditions, reducing bioavailability and therefore increasing the dose that is required. Conjugation with poly(ethylene glycol) (PEG) is often used to increase stability, but this has a detrimental effect on bioactivity. Here, we introduce conjugation with zwitterionic polymers such as poly(carboxybetaine). We show that poly(carboxybetaine) conjugation improves stability in a manner similar to PEGylation, but that the new conjugates retain or even improve the binding affinity as a result of enhanced protein–substrate hydrophobic interactions. This chemistry opens a new avenue for the development of protein therapeutics by avoiding the need to compromise between stability and affinity.

For decades, the attachment of poly(ethylene glycol) (PEG)—known as PEGylation—has been the benchmark for achieving protein stabilization and increased body circulation times[1,2]. Although greatly beneficial as a protein stabilizer, PEGylation is also known to diminish binding affinity, thereby reducing overall bioactivity[3,4]. Efforts have been made to control the location of PEG attachment so as to minimize this effect[5], but the material problem has remained unresolved for the last 30 years. For example, the activity of marketed PEGylated interferon-α2a (Pegasys, Roche) has been seen to drop to 7% compared to its native form[6]. When PEG is attached to monoclonal antibodies, large reductions in activity are found[7], and the effect is also observed for PEGylated enzymes[8]. For this reason, the current pool of available therapeutic proteins has been limited to either humanized proteins, or proteins with small-molecule substrates that are able to resist inhibition from PEGylation, such as Pegaspargase (Oncaspar) and Pegademase Bovine (Adagen)[6]. These are just a few of many examples.

Over the last several years, we have demonstrated how poly(zwitterions) act as super non-fouling surfaces that prevent protein adsorption from complex biological media[9]. We have

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also shown how poly(zwitterions) such as poly(carboxybetaine) (pCB) outperform PEG in terms of resistance to non-specific protein adsorption from blood serum and plasma[10]. This is noteworthy given that PEG has been universally used as a highly resistant non-fouling surface in many applications[11,12]. PEG obtains its non-fouling surface characteristics from its ability to bind large amounts of water by means of hydrogen bonding among its many ether groups. PEG therefore takes on a ballooning-type conformation that increases solution viscosity and acts as a water barrier that prevents proteins from approaching the surface[13]. PEG, although able to form many bonds with water, is also known to have amphiphilic character, being soluble in both polar and nonpolar organic solvents (such as toluene and dichloromethane). Zwitterions, on the other hand, are polyelectrolytes that have both positively and negatively charged groups, but have an overall neutral charge. Poly(zwitterions) obtain their non-fouling characteristics through strong ionic structuring of water, which creates a super-hydrophilic surface[14]. The properties through which ions such as those found with pCB affect water structure are described in what is referred to as the Hofmeister series[15].

Results and discussion

In this work, pCB was conjugated to a model protein to demonstrate its stabilizing properties, and PEG was used for comparison. Ideally, PEG and pCB polymers of similar size would be chosen. However, poly(zwitterions) are known to have strong intramolecular interactions through ionic binding, and this gives pCB a higher density in solution than predicted. This is referred to as the anti-polyelectrolyte effect[16]. In contrast, PEG, which binds to a large number of water molecules, has an artificially larger size in solution than would be expected based on its molecular weight. For this reason, two differently sized pCB polymers were synthesized for comparison with PEG: pCB M_n (equivalent molecular weight to 5 kDa PEG, determined by NMR) and pCB R_h (equivalent hydrodynamic size to 5 kDa PEG determined by size exclusion chromatography, SEC) (Supplementary Table S1). Details of synthesis and conjugation can be found in Supplementary Fig. S1. The well studied and characterized enzyme α-chymotrypsin (CT) was chosen as the model protein. The second parameter adjusted, after polymer size, was the number of polymers per protein. This parameter was varied by adjusting the feed ratio to give three degrees of conjugation for each polymer conjugate. The degree of modification (number of polymers per CT protein) was determined by an amine sensitive assay. Conjugation was also confirmed from the SEC chromatograms (Supplementary Fig. S2).

To stress the stability of the prepared conjugates, activities were tested in 5 M urea (Fig. 1a). Values are represented as percentages relative to their activities before urea was added. As anticipated, PEG was seen to provide a stabilizing effect that correlated with increasing degree of conjugation, with the least conjugated showing no improvement over the bare enzyme. An increase in stability was also observed for the pCB conjugates, but with little dependence on polymer size or number of polymers; all conjugates had a stability that was comparable to the most stable PEG conjugate. A thermal stability test was also performed, measuring the effect of temperature on enzyme activity. It was observed that both pCB M_n and PEG conjugates had stabilizing effects at elevated temperatures, resisting a loss in activity compared to the unconjugated enzyme (Fig. 1b). Even though PEG is a known

thermal stabilizing agent[17], pCB M_n was seen to provide increased stability when compared to PEG conjugates with a comparable number of polymers/protein. Figure 1c shows that pCB R_h conjugates provided a significant improvement over the PEG samples. These results indicate that pCB has a strong stabilizing effect against chemical and thermal denaturation. It should be noted that, although both polymers have stabilizing properties, the proposed mechanisms by which they function are very different. PEG, as previously mentioned, acts as a protective barrier around the protein. This phenomenon results in an increased local viscosity, thereby reducing the structural dynamics of the protein, making it more difficult to unfold[18]. pCB, on the other hand, stabilizes globular proteins by strengthening the hydrophobic interactions holding the protein together by reducing the interaction of water with the surface[19]. By reducing the degree to which water can interfere with the structure of the protein, the conjugates become less likely to unfold. These different mechanisms become important when evaluating their effect on binding affinity.

The affinities of the prepared conjugates were determined by measuring their Michaelis constants, K_m. N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide was used as a substrate. This is a peptide-based substrate with an explicit binding region for cleavage by CT. This system was chosen because substrates of this size are affected by polymer inhibition[8]. Small-molecule substrates are known to have high affinities (small values of K_m) as a result of their high diffusivities. As expected, PEG conjugates were seen to have a decreasing affinity for the peptide substrate as the number of polymers per protein increased (Fig. 2a). Lower affinities are indicated by an increase in K_m, where K_m is the concentration of substrate required to achieve 50% full activity (k_{cat}). For pCB conjugates of both polymer sizes, substrate inhibition was seen to be absent. Most notably, for the larger pCB R_h conjugates, a negative correlation was observed between the amount of conjugated pCB and K_m. This suggested that the addition of pCB polymer assisted in binding.

A general mechanism for molecular binding has two species with higher solubility for each other than their environment (Fig. 3a). For proteins, hydrophobic–hydrophobic interactions are significant[20]. Attaching polymers such as PEG to the protein surface imposes steric hindrances as well as competitive interactions with the substrate and binding site, thereby reducing the binding affinity (Fig. 3b). With PEG, amphiphilicity reduces the hydrophobic–hydrophobic driving force of the substrate–binding site interaction while also imposing a steric hindrance. In contrast, with the pCB conjugates, the binding affinity was either unaltered or 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 (Fig. 3c). To support this model, the solubility of the peptide substrate was measured in solutions of PEG and pCB polymers. This was carried out by measuring the partitioning of the substrate molecule into free polymer solutions of PEG and pCB through a semi-permeable membrane (Supplementary Fig. S3a). These results showed that the substrate had higher solubility in PEG, and lower solubility in pCB compared to the buffer control (Supplementary Fig. S3b). These results support how the amphiphilic PEG polymer can interfere with substrate binding, and also how the super-hydrophilicity of the pCB polymer has low affinity for hydrophobic materials.

To confirm that the changes in affinity originated from differences in the local hydrophilicity, K_m values of the bare enzyme were measured in solutions of ammonium acetate (NH$_4$OAc) salt and 650 M_n PEG. The same improvement in binding affinity was observed with ammonium acetate as with the pCB conjugates (Fig. 2b). The effect of adding 650 M_n PEG into the solution was also seen to correlate with the observations for the conjugated PEG form—leading to significant losses in affinity. This provides further evidence that the results of enhanced affinity obtained for the pCB conjugates originated from the ionic characteristics of the polymer. Ions, and their effects on protein stability and activity, follow a principle referred to as the Hofmeister series (Supplementary Fig. S4). The trend seen in the series correlates greater enzyme performance (stability, activity and affinity) with high surface charge density (kosmotropic) anions and low surface charge density (chaotropic) cations. These are referred to as hard anions and soft cations, respectively. This effect has been shown for several enzyme and substrate pairs, demonstrating trends of increasing values of k_{cat} and decreasing values of K_m and resulting in overall greater activities[21], and also for non-enzyme-based binding[22]. Unfortunately, this type of stabilization and increased activity, achieved by altering the environment with salts, is lost when considering applications *in vivo*. However, the phenomenon can be retained by covalently attaching a zwitterionic polymer such as pCB to the protein surface. The monomer unit itself is a derivative of glycine betaine, which is a natural molecule found in many living systems as a stabilizing agent. On examining the molecular structure of pCB (Fig. 3d), the betaine monomer is seen to correspond to the most stabilizing and active monovalent ions in the series, ammonium and acetate, thereby explaining the mechanism by which pCB enhances binding affinity (Fig. 3c).

To confirm that the observed effects of the polymer conjugates originated from the attached polymer, and to show the sensitivity of substrate size for polymer inhibition, a small-molecule control substrate was also evaluated (resorufin bromoacetate, Fig. 2c). It can be seen that, compared to the larger peptide-based substrate, the presence of conjugated polymer had no observable effect on affinity. The accessibility of the active site by the control substrate can be seen by noting the overall lower values of K_m relative to the larger peptide-based substrate. Values of k_{cat} were also calculated for all kinetic experiments and can be seen in Supplementary Fig. S5. For most conjugates there was no significant effect, but pCB R_h conjugates had an improved activity (k_{cat}) at higher degrees of conjugation.

Conclusions

It has been demonstrated in this work that zwitterionic pCB polymers are able to maintain the stability of proteins without sacrificing binding affinity, a combination never achieved before. This finding represents a major improvement over the current PEGylation technique, which maintains the stability of proteins at the expense of their binding affinity. Zwitterionic pCB polymers derive their unique properties by incorporating the most stabilizing and bio-active monovalent ions (as defined in the Hofmeister series), resulting in pCB both strengthening the stability of the protein folded structure while also encouraging specific binding at the hydrophobic binding site. The mechanism is believed to mimic the action of protein stabilizing ions (kosmotropic anions and chaotropic cations). This work represents a new avenue for protein therapeutics in that it provides accessibility to a pool of potential

protein therapeutics that was originally thought impossible. Other limitations of PEG have been addressed, including oxidative degradation, nonbiodegradability and the toxicity of side products[23]. These are issues beyond what is seen with proteins, suggesting the potential for zwitterionic pCB polymers to be used with nanoparticles[24]. Furthermore, the acting monomer unit of pCB is composed of the naturally occurring glycine betaine. Thus, it is expected that zwitterionic polymers can substitute the current benchmark, PEG, and take a key role in the future of protein therapeutics.

Methods

Materials

The following were used: CT from bovine pancreas type II, lyophilized powder,   40 units/mg protein (Sigma), copper(I) bromide (CuBr, 99.999%, Aldrich), copper(II) bromide (CuBr$_2$, 99.999%, Aldrich), 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA 97%, Aldrich), N-succinyl-Ala-Ala-Pro-Phe p-nitroanilide (Sigma) and methoxy PEG succinimidyl carboxymethyl ester, $M_w = 5,000$ (Jenkem Technology, China).

N-Hydroxysuccinimide terminated poly(carboxybetaine methacrylate) polymer synthesis

Synthesis of the N-hydroxysuccinimide (NHS) ATRP initiator[24] and poly(carboxybetaine) methacrylate with tert-butyl protected ester (CBMA-1-*t*-But)[23] were performed as described previously. Atom transfer radical polymerization was carried out in anhydrous dimethylformamide (DMF) using a Cu(I)Br/HMTETA catalyst (Supplementary Fig. S1a). In a typical polymerization, DMF and HMTETA were separately purged of oxygen by bubbling with nitrogen. CBMA-1-*t*-But (1 g, 3.67 mmol) monomer and 125 mg (0.5 mmol) of NHS-initiator were added to a Schlenk tube. To a second Schlenk tube was added 71.7 mg (0.5 mmol) of Cu(I)Br. Both tubes were deoxygenated by cycling between nitrogen and vacuum three times. Deoxygenated DMF in volumes of 8 and 2 ml was added to the monomer/initiator and Cu(I)Br tubes, respectively. Deoxygenated HMTETA (136 µl, 0.5 mmol) was added to the Cu(I)Br-containing solution and was stirred for 30 min under nitrogen. The catalyst solution (Cu(I)/HMTETA) was then added to the monomer/initiator solution to start the reaction. The reaction was run to completion at room temperature while being monitored using NMR. After polymerization, the reaction was fully precipitated in ethyl ether. The precipitate was then dried under vacuum and redissolved in minimal DMF (3–5 ml). The solution was reprecipitated in anhydrous acetone to remove the soluble catalyst and trace monomer. This was repeated a total of three times to fully remove the catalyst. The remaining ester polymer was dried overnight under vacuum and analysed by NMR. To hydrolyze the tert-butal group, 500 mg NHS-pCBMA-1-*t*-But was dissolved in 5 ml trifluoroacetic acid (Supplementary Fig. S1b). This was allowed to sit for 2 h. The solution was then precipitated in ethyl ether, dried overnight under vacuum and subsequently analysed by NMR ($M_w = 4.2$ kDa) and gel permeation chromatography ($M_w = 2.5$ kDa, polydispersion index = 1.05).

CT polymer conjugation and purification (Supplementary Fig. S1c)

CT conjugates were prepared in 200 mM HEPES buffer (pH 8.5). The α-chymotrypsin concentration was fixed at 4 mg ml^{-1}, and the polymer solutions were 4, 8 and 16 mg ml^{-1}.

This was done for all three polymers: PEG, pCB M_n and pCB R_h. Solutions were mixed with a stir bar for 30 min, then incubated at 4 °C for 3 h. The conjugates were purified and concentrated by ultrafiltration using a 30 kDa molecular weight cutoff membrane. The pCB R_h conjugates were further purified using a size exclusion column with a Superdex 200 10/300 column. These samples were then re-concentrated using ultrafiltration. The number of polymers per protein for each sample was determined using a trinitrobenzene sulfonate (TNBS) assay to determine the number of unreacted surface amines, together with area under the curve of SEC chromatograms.

Urea stability

Conjugates were incubated in 0.1 M Tris at pH 8.0 for 8 h at 0.5 μg ml^{-1}, with and without 5 M urea. Measurements were performed in triplicate. Solutions were also incubated with 0.1 mg ml^{-1} bovine serum albumin (BSA) as a blocking agent to prevent depletion of enzyme from surface adsorption. After 8.0 h, activities were measured for all samples by adding *N*-succinyl-Ala-Ala-Pro-Phe p-nitroanilide at 667 μg ml^{-1}. Activities were measured as the increase in absorbance at 412 nm with time. Values were recorded as percentage of activity with urea, relative to without urea.

Thermal stability

Conjugates were incubated in 0.1 M Tris, pH 8.0, at temperatures of 40, 45, 50 and 55 °C and a concentration of 5.0 μg ml^{-1}. After 10 min at each temperature, each conjugate was diluted into refrigerated buffer to a concentration of 0.5 μg ml^{-1}. Solutions were also incubated with 0.1 mg ml^{-1} BSA as a blocking agent. Activities were measured for all samples by adding *N*-succinyl-Ala-Ala-Pro-Phe p-nitroanilide to a final concentration of 667 μg ml^{-1}. Activities were measured as the increase in absorbance at 412 nm with time. Values were recorded as percentage of each conjugate's activity at 40 °C.

Conjugate kinetics with *N*-succinyl-Ala-Ala-Pro-Phe p-nitroanilide

Conjugates were incubated at 20 pM in 0.1 M Tris, pH 8.0 with 0.1 mg ml^{-1} BSA as a blocking agent. Low enzyme concentrations were required to achieve accurate activity measurements at low substrate concentrations. *N*-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide was added at varying concentrations from 0 to 667 μg ml^{-1} (0–1,070 μM). Concentrations of each substrate were prepared in triplicate and activity was measured as an increase in absorbance with time at 412 nm.

Conjugate kinetics with resorufin bromoacetate

Conjugates were incubated at 20 pM in 0.1 M MES, pH 6.0, with 0.1 mg ml^{-1} BSA as a blocking agent. A lower pH was required to retain the stability of the base-labile substrate. Resorufin bromoacetate was added at varying concentrations from 0 to 50 μg ml^{-1} (0–150 μM). Concentrations of each substrate were prepared in triplicate and activity was measured as an increase in absorbance with time at 571 nm.

Evaluation of polymer–substrate solubility

Dialysis containers containing either buffer (control), 5 mg ml^{-1} PEG or 5 mg ml^{-1} pCB were exposed to a large bath in excess volume containing 2.5 μg ml^{-1} of peptide substrate (*N*-succinyl-Ala-Ala-Pro-Phe p-nitroanilide). A dialysis membrane separated the polymers from the substrate, which allowed the substrate to pass freely, but not to the polymers. After 3 days, the sample solutions were measured by high-performance liquid chromatography for peptide concentration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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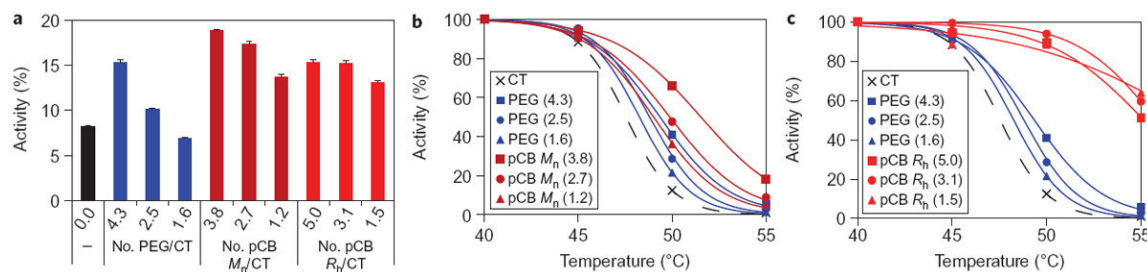
Figure 1. Stability of PEG, pCB M_n (similar molecular weight) and pCB R_h (similar hydrodynamic size) conjugated with enzyme CT

a, Activity of conjugates after 8 h incubation in 5 M urea. Values are calculated as percentages of conjugate activity in buffer without urea. Error bars represent standard deviation. For each polymer tested, a trend is observed in which conjugates with a higher degree of conjugation show increased stability. PEG and pCB polymers both showed stabilizing effects, but pCB to a high degree. **b,c**, Relative activity of PEG and pCB M_n conjugates (**b**) and PEG and pCB R_h conjugates (**c**) after 10 min incubation at different temperatures. The optimum operating temperature of the enzyme is known to be 40 °C. Here, a trend is also observed in which conjugates with a higher degree of conjugation show increased stability. The larger pCB polymers (pCB R_h) show significant stabilizing characteristics.
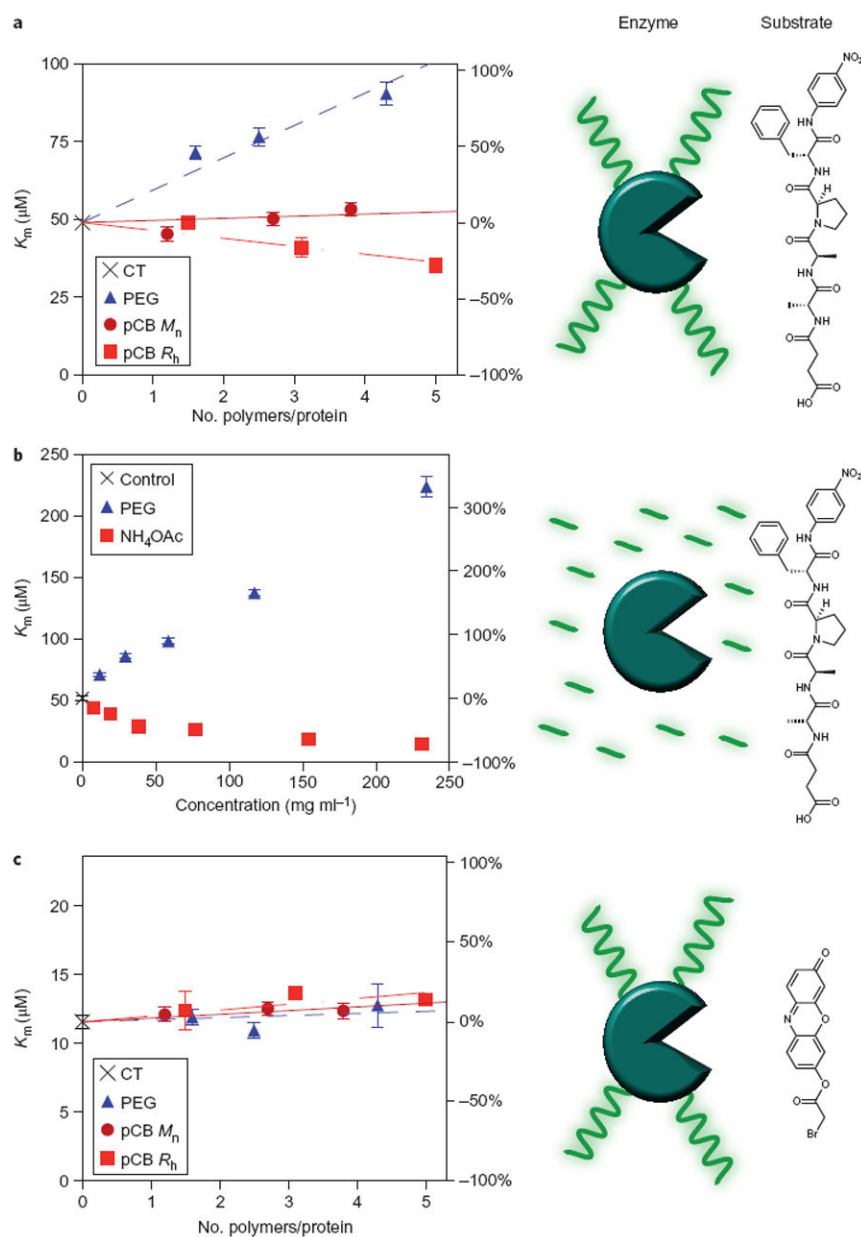
Figure 2. Affinities of prepared conjugates measured as Michaelis constants K_m

Error bars are values of standard error (s.e.m.). **a**, K_m of prepared conjugates to the large peptide substrate. K_m represents the concentration of substrate required to achieve 50% maximum activity. A decrease in K_m represents an increase in binding affinity. The increase in K_m with an increase in the number of PEG polymers per protein shows the inhibitory effects of PEG. In contrast, pCB M_n conjugates (similar molecular weight) showed no significant effect on binding affinity, whereas pCB R_h conjugates (similar hydrodynamic size) showed an increase in substrate affinity. **b**, K_m values of native enzymes for the large peptide substrate in the presence of 650 M_n PEG and ammonium acetate solutions. These data support the effects observed with analogous polymers with the same chemistry. **c**, K_m

values of prepared conjugates for the small-molecule substrate. This supports the claim that K_m is dependent on substrate size.
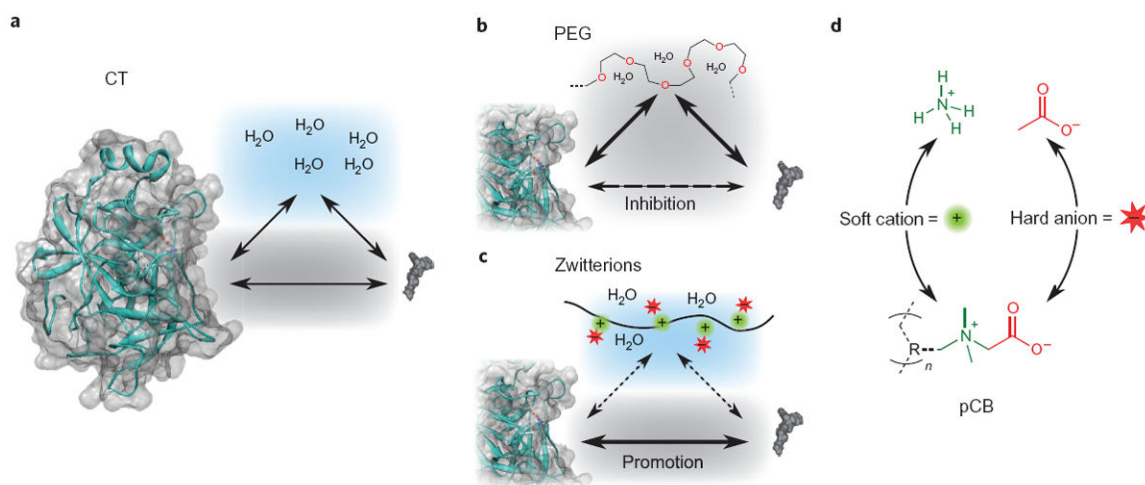
Figure 3. Mechanism of how PEG and pCB polymers influence binding affinity
a, Relationship between enzyme and substrate without polymer. **b**, PEG impedes affinity by reducing enzyme–substrate hydrophobic–hydrophobic interactions as a result of its amphiphilic characteristics. **c**, Super-hydrophilic pCB has a strong effect on the structure of water, creating a local environment that increases enzyme–substrate hydrophobic–hydrophobic interactions, thereby increasing the substrate's affinity for the binding pocket. **d**, The structural relationship between pCB and ammonium acetate, both of which contain protein-stabilizing ions found in the Hofmeister series. The R group represents a methacrylate backbone.