Selective Protein Recognition

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Tuning Linear Copolymers into Protein-Specific Hosts**

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The selective protein recognition by synthetic receptor molecules remains an important challenge for supramolecular and bioorganic chemistry. To distinguish between proteins of similar sizes and pI values (pI = isoelectric point), but of different biological function, an artificial host must be able to recognize the pattern of amino acid residues, that is, the topology, polarity, as well as the electrostatic potential of a protein surface. From protein–protein interactions, we can learn that nature prefers large contact areas (approximately 1500 $Å^2$) with an unusual amount of arginine and aromatic amino acid residues involved in polar interactions (Coulomb, π-cation). These interactions are complemented and enforced by hydrophobic attraction between approaching nonpolar domains or patches.[1] This combination is partly reflected in recent attempts to produce artificial protein binders that include secondary-structure mimetics,[2] multiplication of single specific[3] or unspecific binding sites,[4] evolutive optimization of biomacromolecular scaffolds,[5] and molecular

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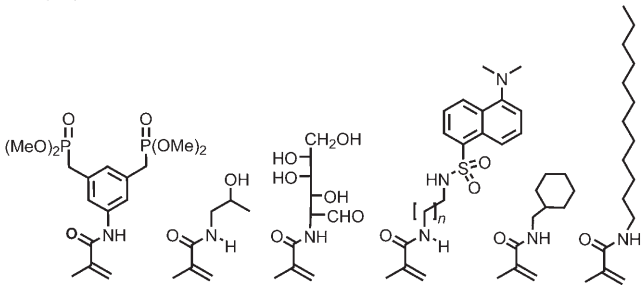
imprinting.[6] Herein, we introduce a new chemical concept that involves statistical copolymerization of simple binding monomers in defined stoichiometric ratios.[7]

The underlying idea is the following: for a given protein target, the chemist chooses from a preformed set of polymerizable binding sites those that match the majority of amino acid residues dominating the surface character of the protein. By statistical free-radical copolymerization, a flexible copolymer is produced that is capable of performing an extensive "induced-fit" procedure on top of the respective protein surface, and therefore reaching maximal attractive noncovalent interactions. No cross-linking is employed to guarantee perfect water solubility; no imprinting technique is used because the linear polymer is expected to adapt its shape to flat and rugged surface topologies. Thus, technical simplicity is maintained to accomplish a complex task. This modular approach deliberately avoids extensive design, requiring exact knowledge of protein-surface topology from crystal structures. Herein, we disclose preliminary, but highly promising, results.

According to the above-outlined concept, the whole procedure can be subdivided into three main steps: 1) A set of simple methacrylamide-based co-monomers is prepared; this ensemble is by itself capable of recognizing all proteinogenic basic, acidic, polar, aromatic, and nonpolar amino acid residues. 2) From this set, the co-monomers that are complementary to the most characteristic protein surface residues are copolymerized in an estimated appropriate stoichiometric ratio, with simultaneous incorporation of a small amount of polymerizable fluorophors (see Table 1). The new polymeric materials are conveniently characterized by ^{1}H and ^{31}P NMR spectroscopy, gel permeation chromatography, elemental analysis, as well as fluorescence properties. 3) In the last phase, affinities to very similar proteins are evaluated, at best in mixtures.

To demonstrate the feasibility of this concept, we faced the task of distinguishing between various basic proteins of similar size and pI value. To this end, we prepared four different binding monomers, tailored for basic, aliphatic, and aromatic residues; an amino sugar based component was added for increased water solubility. Further to the simple polymers **1** and **2**, four copolymers (**3**–**6**) were synthesized, each of which contained three different binding monomers in a 1:1:1 or 3:1:1 ratio and 10% of a dansyl fluorescence label. Free-radical copolymerization was conducted in *N*,*N*-dimethylformamide (DMF) and initiated by 0.5–0.7 mol% of azobisisobutyronitrile. Subsequent polymer-analogous reactions with LiBr led to mild dealkylation of the bisphosphonates and afforded the final receptor polymers. Their composition is depicted in Table 1. Molecular-weight determinations for **1** and **2** in DMF and water produced comparable results, excluding a potential ion-pair induced aggregation. For copolymer **5** with hydrophobic residues, very similar hydrodynamic radii were calculated from NMR spectroscopic diffusion experiments at varying concentrations (50 μM to 1.6 mM). These hydrodynamic radii also suggest monomeric species as do the symmetric M_w/M_n curves. Moreover, this copolymer displays a very compact nature—resembling a protein—most likely resulting from burial of dodecyl tails in its inner core while exposing the polar bisphosphonate head groups into the bulk solvent. Copolymerization parameters, determined for **2**, indicate a perfect statistical copolymerization, ruling out the formation of hydrophobic blocks or aggregated micelles.

Table 1: Modular set of methacrylamide-based co-monomers ($n = 1$ or 7) and polymers **1**–**6** derived thereof.[a]



a **b** **c** **d** **e** **f**

Polymer	Bisphosphonate **a**	Alcohol **b**, **c**	Dansyl **d**	Cyclohexyl **e**	Dodecyl **f**
1	1	–	0.1	–	–
2	1	3[b]	0.4	–	–
3	1	1[c]	1	–	–
4	3	1[c]	0.5	1	–
5	1	1[c]	0.3	–	1
6	3	1[c]	0.5	–	1

[a] Each row indicates the relative ratio of co-monomers for a given copolymer. [b] Alcohol monomer from 2-hydroxypropylamine **b**. [c] Alcohol monomer from 2-glucosamine **c**.

In a systematic study, each fluorescent polymer was titrated with the same series of neutral and basic proteins.[8] In most cases, fluorescence emission intensities increased markedly on protein binding, presumably owing to reorientation of the polymer's binding sites towards the protein surface with concomitant deshielding of the fluorophor. Unfortunately, the most basic protein histone H1 formed insoluble complexes even at 10 nM concentrations, precluding the exact determination of its free binding energies. All of the other examined protein complexes, however, furnished binding isotherms with a perfect fit by nonlinear regression methods.[9] The respective association constants (K_a) are summarized in Table 2; they cover a relatively broad range from $10^4\,M^{-1}$ up to over $10^7\,M^{-1}$.

It is instructive to compare K_a values for a given protein among the various copolymers because they reveal the contribution of additional noncovalent interactions, especially in relation to pure polybisphosphonates. Thus, bovine serum albumin (BSA), a blood lipid carrier, is bound much more strongly if the polymer (**5**) also contains long aliphatic side chains for additional hydrophobic attraction (Figure 1). Most likely these imitate the natural binding mode inside the nonpolar cleft. Similarly, for all copolymers, the dansyl-containing **3** displays superior affinity towards trypsin with its unusually high content of aromatic residues (dissociation constant, $K_d = 170$ nM).[10] Clearly, additional π stacking renders complex formation even 100-times more efficient than the parent bisphosphonate copolymer **2**. Experimentally determined stoichiometries (Job plots) remain around 1:1

Table 2: Protein affinities to hosts **1**–**6**, determined as $K_{1:1}$ values by fluorescence titrations in 30 mM phosphate buffer at pH 7.0.[a]

Polymer	Cytochrome c		Trypsin		Lysozyme		BSA	
	K_a [M^{-1}]	Ratio[b]	K_a [M^{-1}]	Ratio[b]	K_a [M^{-1}]	Ratio[b]	K_a [M^{-1}]	Ratio[b]
1	6×10^5	1:2.0	$>10^5$	–	$>10^5$	–	2×10^5	1:2.5
2	2×10^5	1:3.0	4×10^4	1:4.0	4×10^6	1:4.0	2×10^5	1:1.5
3	5×10^5	1.0:1	6×10^6	1.5:1	7×10^6	1:2.0	2×10^5	1:1.5
4	3×10^5	1.5:1	–	–	9×10^5	2.0:1	4×10^5	1.5:1
5	2×10^6	1.7:1	–	–	–	–	7×10^5	1.5:1
6	4×10^5	2.0:1	6×10^5	1.5:1	4×10^7	2.0:1	6×10^5	1.5:1

[a] Complex formation was assumed to occur without cooperativity and with uniform binding constants for each step. Standard deviations from the nonlinear regressions were calculated at 2–17%. [b] Polymer/protein stoichiometry.

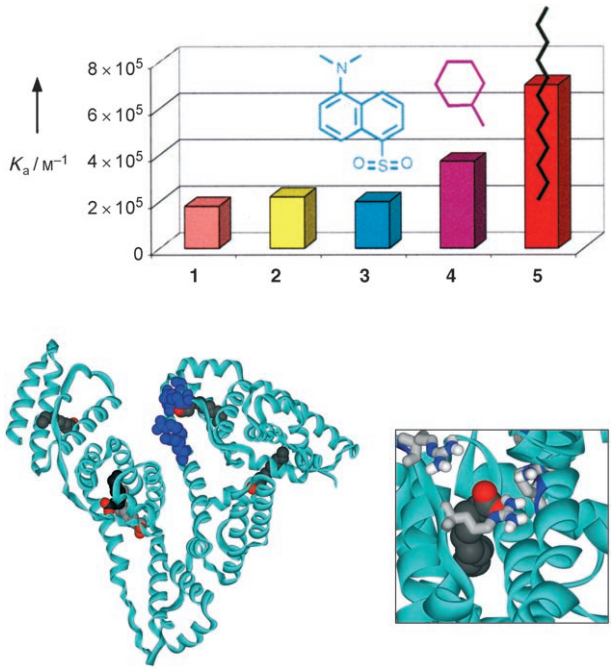


Figure 1. Top: Target optimization of homopolymer **1** into an efficient BSA binder **5** by incorporation of dodecyl co-monomers for additional hydrophobic interactions. Bottom: Crystal structure of human serum albumin with complexed lipids.[12] Note the basic residues (blue) at the entrance of the clefts, holding the myristic acid guest (black) in place (see zoom).

throughout the whole series, indicating optimal surface coverage of the protein.[11] To prove that cooperative effects are negligible, two more copolymers, **7** and **8**, were synthesized that were comprised of the same monomer composition but different molecular weights (see Supporting Information). Their bisphosphonate (BP) content (2:1:1 BP/dodecyl/sugar) lies between that of polymer **5** (1:1:1) and polymer **6** (3:1:1). This also implies that the amount of hydrophobic dodecylmethacrylamide co-monomers steadily increases from **6** over **7** and **8** to **5**. Intriguingly, the binding constants with cytochrome c follow the very same trend, indicating that, in this series, the number of hydrophobic interactions and not electrostatics determines the relative polymer affinities (**6**: 4×10^5; **8**: 6×10^5; **7**: 8×10^5; **5**: 2×10^6).

In our systematic binding study for representative basic proteins, we identified among polymers **1**–**6** a candidate with pronounced protein selectivity (Figure 2). Arginine-rich lysozyme is recognized by bisphosphonate-rich copolymer **6** with a remarkable K_d value of 25 nM, which is 100-times superior to cytochrome c (comparable in size and pI; Table 2). Close inspection of the electrostatic potential surface[13] reveals numerous hydrophobic patches between the Arg residues, ideal for van der Waals interactions with the copolymer's long aliphatic tails. Compared to protein complexes of **1** or **2**, the glucosamine content in **3**–**6** leads to markedly improved water solubilities.

Preliminary results from an enzyme assay measuring the kinetics of bacterial cell wall degradation by lysozyme (*Micrococcus lysodeicticus*) indicate a competitive inhibition of enzyme activity by bisphosphonate-containing copolymers. Intriguingly, the corresponding IC_{50} values follow the same order as their binding constants determined by fluorescence titration. Thus, polymers **4** ($K_a = 9\times10^5\,M^{-1}$), **3** ($K_a = 7\times10^6\,M^{-1}$), and **6** ($K_a = 4\times10^7\,M^{-1}$) produced IC_{50} values of roughly 20, 10, and 1 μM for enzymatic bacterial degradation, indicating, that 2 equivalents of polymer **6** suffice to completely shut down lysozyme activity, whereas polymer **4** needs about 50 equivalents for the same task (≈1 μM protein concentration).

Reflectometric interference spectroscopy (RIfS) measurements record the change of optical thickness for compound layers deposited successively on glass chips. A first layer of cationic polyethyleneimine (PEI) was coated with negatively charged copolymers **3** and **5**, followed by noncovalent immobilization of various proteins. The binding constants and dissociation rates derived thereof confirm the affinity and selectivity trends observed in solution (see Supporting Information for details).[14] More importantly, however, the amount of irreversible binding contributions compared to the parent polybisphosphonates **1** and **2** increased drastically (Figure 3). As the latter rely mostly on electrostatic interactions, this change strongly indicates

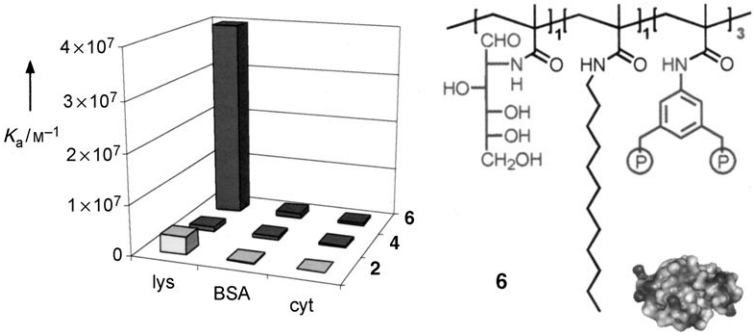


Figure 2. Drastic selectivity increase from copolymer **2** to lysozyme binder **6**. lys = lysozyme, cyt = cytochrome c, P = phosphonate.

essential hydrophobic, van der Waals, or π-stacking interactions between the aliphatic and aromatic co-monomer residues and nonpolar protein surface areas. For a kinetically stable protein immobilization, low off-rates (dissociation rates) are mandatory; the new copolymers thus offer the

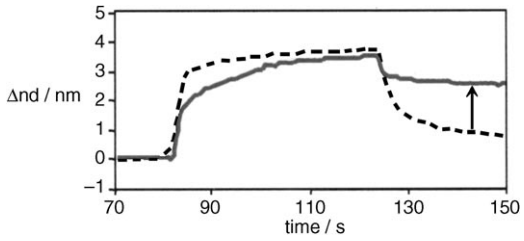


Figure 3. RIfS curves for lysozyme recognition on a polymer-coated PEI surface (solid line: homopolymer **1**; dashed line: dansyl copolymer **3**). Note the elevated level of irreversible binding with **3**. nd = optical thickness.

unique option of mild noncovalent protein attachment to the chip surface and its potential observation in a functional state (e.g., in new enzyme-sensor systems).

We are currently optimizing the above-introduced modular concept by creating diverse copolymer libraries with a representative pool of binding monomers for all important noncovalent interactions occurring in protein recognition events. A fast RIfS protocol will be useful for efficient screening. Optimized binding monomers for each class of amino acid residues may pave the way towards the ultimate goal—artificial antibodies.

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