Molecular Recognition

DOI: 10.1002/anie.201406852

# Conjugated-Protein Mimics with Molecularly Imprinted Reconstructible and Transformable Regions that are Assembled Using Space-Filling Prosthetic Groups**

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***Abstract:*** *Conjugated-protein mimics were obtained using a new molecular imprinting strategy combined with post-imprinting modifications. An antibiotic was employed as a model template molecule, and a polymerizable template molecule was designed, which was composed of the antibiotic and two different prosthetic groups attached through a disulfide bond and Schiff base formation. After co-polymerization with a cross-linker, the template molecule was removed together with the prosthetic groups, yielding the apo-type scaffold. Through conjugation of the two different prosthetic groups at pre-determined positions within the apo-type scaffold, the apo cavity was transformed into a functionalized holo cavity, which enables the on/off switching of the molecular recognition ability, signal transduction activity for binding events, and photoresponsive activity.*

The biological functions of proteins in living cells are commonly provided by enzymatic and/or non-enzymatic post-translational modifications after biosynthesis in ribosomes, and further modifications are sometimes carried out by forming adducts with non-protein prosthetic groups and cofactors, yielding conjugated proteins, to acquire more specific functions.[1–4] These chemical modifications allow proteins to develop a diverse range of biofunctions, which play important roles in biosystems. Such post-biosynthetic processing through a chemical-modification strategy can inspire the creation of new classes of synthetic multifunctional materials, where common polymer scaffolds are first constructed according to pre-determined designs involving orderly oriented binding sites for subsequent chemical modification within well-defined reaction fields, after which further functionalities are acquired by specific post-polymerization modifications within the reaction fields, including: 1) the addition of new functional groups, 2) transformations and rearrangements of intrinsic functional groups into more favorable ones, and 3) complexation with different macromolecular and/or inorganic substances from the scaffold matrices.

Herein, we demonstrate that such materials with versatile built-in functionalities, which can be viewed as mimics of naturally occurring conjugated proteins, can be realized using a newly developed molecular imprinting technique involving post-imprinting modifications. Molecular imprinting,[5–13] which is a template polymerization technique, has a reputation as a promising strategy for the preparation of tailor-made synthetic functional polymer receptors for target molecules. In the presence of conjugates of template molecules (target molecules or their derivatives) covalently linked or non-covalently bound to functional monomers that can assemble around the template molecules into complementary orientations, co-polymerization is performed with cross-linkers. After removal of the template molecules, tailor-made molecular recognition cavities, with orderly assembled binding sites that are complementary in size, shape, and chemical properties to the template molecules used, are left behind in the resultant polymer matrices, yielding molecularly imprinted polymers (MIPs).

For MIP modifications after polymerization through post-imprinting modifications (PIMs) to introduce desired functions, predetermined specific reactive sites with suitable spaces for further modifications are necessary at desired positions of the resultant molecularly imprinted reaction fields.[14–26] Owing to the intrinsic properties of MIPs, functional-group residues remain within the cavities after removal of the template molecules, meaning that alignment of designed moieties and/or functional groups allows the resulting cavities in MIPs to be capable of PIMs with prosthetic groups and cofactors at pre-determined positions.

In previous work, simple on/off switching of molecular recognition was successfully achieved by the insertion and removal of covalently coupled prosthetic groups and non-covalently attached cofactors, to switch between the inactive apo-type binding scaffolds (without prosthetic groups) and the active holo-type binding sites (with prosthetic groups).[20] Site-directed introduction of signaling functions into protein-MIPs was also achieved.[23–26] However, the effectiveness of PIMs was only partially demonstrated in previous reports, and they have limitations with regard to multifunctional materials owing to the use of single prosthetic groups or cofactors. Naturally, plural reactive sites that work independently for PIMs within a molecularly imprinted cavity are required, and

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[**] We thank Dr. Hideyuki Shinmori (Yamanashi University, Japan) for his comments at an early stage of this work. This work was supported in part by a grant from PRESTO (Creation of Innovative Technology by Integration of Nanotechnology with Information, Biological, and Environmental Technologies), Japan Science and Technology Agency (JST).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201406852.

  

they should be able to undergo coupling reactions under different conditions, making MIPs very feasible and versatile multi-functional materials.

Herein, we synthesized a newly designed template molecule to obtain apo-type binding scaffolds towards antibiotics with two variable regions that are capable of independent conjugation with different prosthetic groups. Versatile functional MIPs with such binding sites that mimic conjugated proteins were obtained by the assembly of several prosthetic groups within the apo-type scaffold by PIMs, making use of the pre-determined alignment to transform the common apo-type scaffold into multi-functionalized holotype MIPs with completed recognition fields. This transformation enables the switching of molecular recognition activity, the introduction of fluorescence signaling for specific binding events, and a photoresponsive switching function. These modifications are comparable to the post-translational protein modifications and activations that are normally achieved by complexation of prosthetic groups and cofactors to yield conjugated proteins in nature.

β-Lactam antibiotics were used as model targets, and a designed template molecule, which comprises cephalexin as the core unit and two different prosthetic groups attached through methacrylamide moieties, was synthesized (Figure 1). The prosthetic group 1,4-mercaptoaniline (AN) was connected to *N*-(4-mercaptophenyl)methacrylamide through a disulfide bond to yield *N*-methacryl-bis(4-anilinyl)disulfide (FM1), and the second prosthetic group, 4-formylbenzoic acid (BA), was connected to *N*-(4-aminophenyl)methacrylamide by Schiff base formation to obtain 4-(4-methacrylamido)phenyliminomethyl benzoic acid (FM2). Next, FM1 and FM2 underwent condensation reactions with the carboxylic acid and the amine moieties of cephalexin, respectively, for the preparation of the designed template molecule (TEMPLATE 1). Disulfide and imine bonds were selected for the generation of new molecular recognition fields in synthetic polymers that are coupled to several prosthetic groups, as the two different reversibly formed linkages can be formed and broken under different conditions.

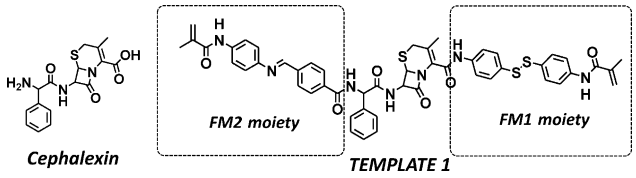


***Figure 1.*** Chemical structure of cephalexin and design of TEMPLATE 1 for the preparation of molecular recognition fields mimicking conjugated proteins.

For the preparation of an apo-type scaffold, molecular imprinting was conducted using TEMPLATE 1 as shown in Figure 2 (step A). After co-polymerization with triethyleneglycol dimethacrylate as a cross-linker (94% conversion, as measured by gravimetry, see the Supporting Information), followed by removal of the target-molecule moiety from the resulting polymer, the molecularly imprinted apo-type scaffold (PRECURSOR) was obtained with a template removal rate of approximately 100% (as measured by gravimetry, see the Supporting Information), which may have sufficient space

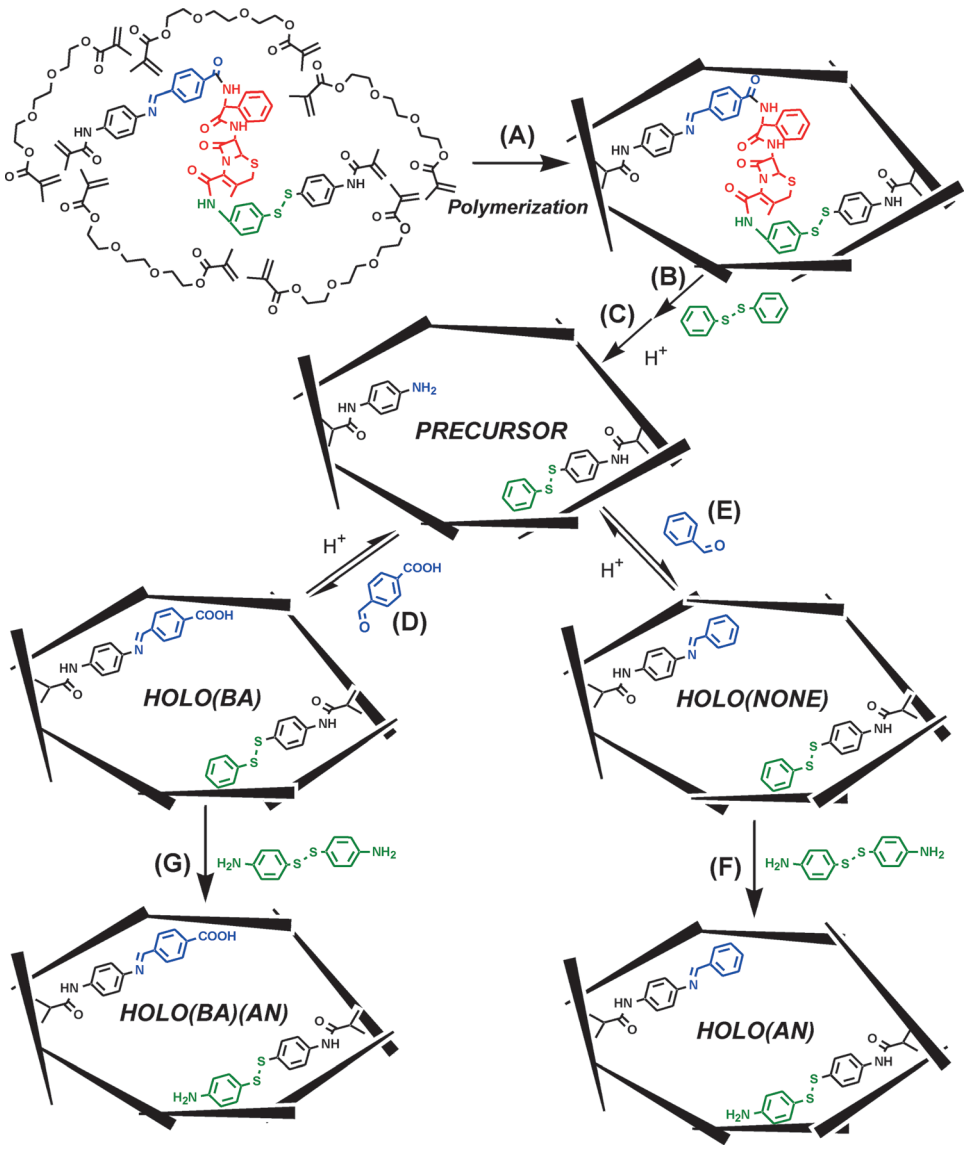


***Figure 2.*** Preparation of PRECURSOR and a series of HOLO polymers.

for the assembly of prosthetic groups (steps B and C). PRECURSOR was then transformed into the programmed binding cavity (holo-type cavity) by assembling the prosthetic groups to fill the extra space (PIMs), making the cavity in which suitable interactive functional groups were located for target binding suitable in size for the target.

When the prosthetic group 4-formylbenzoic acid (BA) was conjugated with PRECURSOR by Schiff base formation (step D), HOLO(BA) was obtained with a yield of approximately 90% (measured by spectrophotometry; see the Supporting Information, Table S1), which only featured a benzoic acid residue in the cavity as a binding site. A cavity with no functional group, HOLO(NONE), was obtained by adding benzaldehyde to PRECURSOR instead of BA (step E), and the only 4-mercaptoaniline (AN)-conjugated binding cavity HOLO(AN) was obtained by a disulfide exchange reaction of the 4,4′-diphenyldisulfide residue in HOLO(NONE) with 4,4′-dithiodianiline (step F). The yield of the disulfide exchange reaction to yield HOLO(AN) was estimated to be approximately 75% (as measured by HPLC, see Table S2). The binding cavity bearing both amino and carboxylic acid groups, HOLO(BA)(AN), was obtained by conjugation of the prosthetic group AN to HOLO(BA) through a disulfide exchange reaction with 4,4′-dithiodianiline (step G) with a high residual amount of the Schiff base (93%, measured by HPLC, Table S3).

To confirm the enhancement of molecular recognition by conjugation of the two prosthetic groups, the binding activities of HOLO(BA)(AN) and other HOLO polymers, including HOLO(NONE), HOLO(AN), and HOLO(BA), towards ampicillin were examined in dimethyl sulfoxide (DMSO; Figure 3a). Ampicillin was used instead of cephalexin, because free cephalexin appeared to be unstable under the binding conditions employed (Figure S1). HOLO(BA)(AN) showed a significantly higher binding activity, with an apparent binding constant estimated to be $2.42 \times 10^6\,\text{M}^{-1}$ by a Langmuir plot, and the other HOLO polymers showed poor ampicillin adsorption, thus suggesting that the bidentate ligand ampicillin is recognized by the synergetic action of the carboxy and amine groups in the HOLO(BA)(AN) cavity (Figure 3b). This also indicates that the prosthetic groups BA and AN were assembled in the cavity as originally designed.

Reusability was examined by conducting repetitive constructions of the conjugated binding cavity (Figure 3c). Ampicillin binding experiments were performed with HOLO(BA)(AN) by successively attaching and detaching AN and BA to/from PRECURSOR. The reconstructed HOLO(BA)(AN) binding cavities showed almost the same binding activity as the original HOLO(BA)(AN), even after several repetitions. These results reveal that the prosthetic-group conjugation process is reversible, and that PRECURSOR is reusable as a common source for versatile and robust artificial polymer receptor preparation.

Further characterization of the HOLO(BA)(AN)-type binding cavity was carried out by examining the cross-reactivity towards various structurally related compounds, including a bidentate ligand (ampicillin), monodentate ligands [cephalexin isopropyl amide (Ceph-*i*Pr) and *N*-*tert*-butoxycarbonyl cephalexin (Boc-Ceph)], an ambilaterally protected ligand (Boc-Ceph-*i*Pr), and a smaller bidentate ligand (*para*-aminobenzoic acid, PABA). As shown in Figure 4, the HOLO(BA)(AN) binding cavity showed

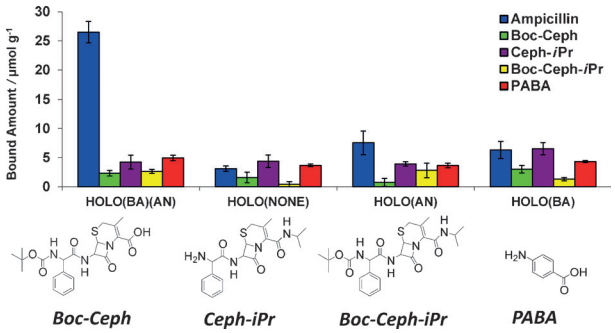


***Figure 4.*** Selectivity of HOLO polymers towards structurally related compounds.

strong binding of the bidentate ligand ampicillin. In contrast, poor adsorption was observed for the monodentate and smaller ligands. These results confirm that transformable binding sites similar to those of conjugated proteins in nature can be artificially constructed to enable molecular recognition by the molecularly imprinted cavity, with PIMs attaching and detaching prosthetic groups to the common apo-type scaffold.

To explore another emerging function introduced by PIMs, a fluorescent prosthetic group was introduced into PRECURSOR to obtain fluorescence-signaling properties that are responsive to the binding of target molecules (Figure 5a, step H and I). 5-Formylsalicylic acid (SA) was selected as the fluorescent prosthetic group instead of BA, as it is based on benzoic acid and similar in size to the original prosthetic group BA. We constructed HOLO(SA), in which BA was replaced by SA as an alternative prosthetic group. Furthermore, instead of the original group

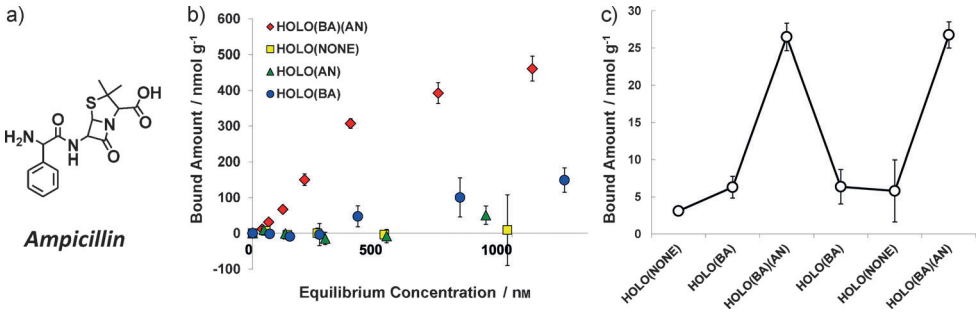


***Figure 3.*** Binding experiments with HOLO polymers towards ampicillin. a) Chemical structure of ampicillin. b) Binding isotherms of HOLO(BA)(AN), HOLO(NONE), HOLO(AN), and HOLO(BA). c) Amount of ampicillin bound to HOLO polymers with sequential attaching/detaching of prosthetic groups.

  

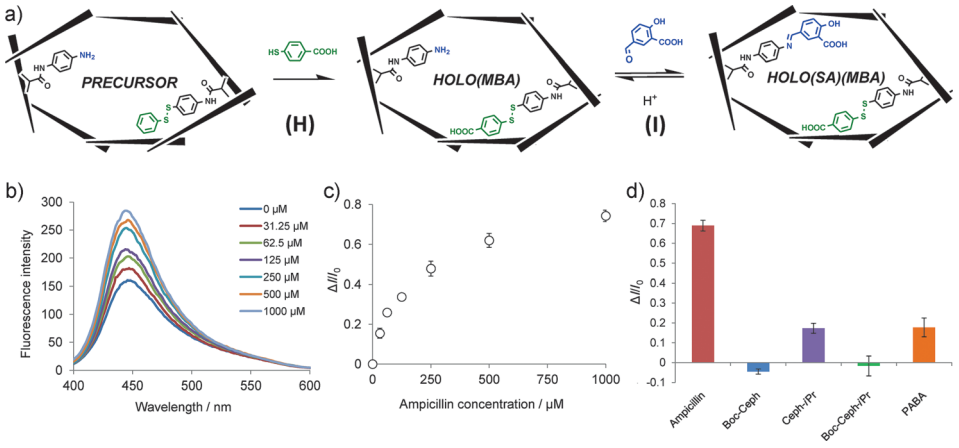


***Figure 5.*** Introduction of fluorescence-signaling functionality within the imprinted cavity. a) Preparation of fluorescent HOLO polymers. b) Changes in the fluorescence spectrum of HOLO(SA)(MBA) on addition of ampicillin ($\lambda_{ex} = 365$ nm). c) Relative fluorescence intensity changes at 446 nm with increasing ampicillin concentration. d) Relative fluorescence intensity changes after addition of structurally related compounds.

AN, 4-mercaptobenzoic acid (MBA) was attached as a new prosthetic group yielding HOLO(SA)(MBA), which had two carboxylic acid moieties within the HOLO cavity that were well positioned to interact with the amino and carboxylic acid groups of ampicillin by synergetic hydrogen bond formation.

The ampicillin adsorption activity of HOLO(SA)(MBA) was examined by measuring the fluorescence change in the presence of different amounts of ampicillin ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 446$ nm). Clear, concentration-dependent, and saturable fluorescence changes were observed, indicating that binding events could be transduced by the fluorescence changes (Figure 5b,c). The fluorescence intensity was enhanced when ampicillin was bound to HOLO(SA)(MBA), thus suggesting that the amino group of ampicillin interacts with SA, as SA itself possesses intrinsic fluorescence, showing strong fluorescence in the presence of amines in DMSO (see Figure S2).

Among the structurally related compounds, significantly higher fluorescence intensity was only observed for ampicillin, suggesting that the conjugated binding cavity in HOLO(SA)(MBA) recalls the shape and size of the original template molecule when the corresponding HOLO-type conjugated binding cavity was constructed similarly to natural apo/holo proteins. Furthermore, this memory can be preserved, even when the prosthetic groups are replaced with different prosthetic groups, provided that they are similar in shape and size and have similar chemical binding properties (Figure 5d).

Photoresponsive materials are of great importance as advanced materials. The present PIM strategy can be applied to the transformation of simple molecularly imprinted cavities into photoresponsive cavities by introducing a prosthetic group with a phototunable binding domain. The cavity in PRECURSOR has an aniline residue for the conjugation of BA; therefore, it can be transformed into an azobenzene-4-carboxylic acid (Az-BA) moiety by treatment with 4-nitrosobenzoic acid as an irreversibly attached prosthetic group (Figure 6, step J); the Az-BA moiety has a similar size and shape to the original binding site with a conjugated prosthetic group. After introduction of the Az-BA moiety into PRECURSOR, which resulted in the formation of HOLO(Az-BA), there was an increase in absorbance at 365 nm, which should be due to the π–π* transition of the *trans* azobenzene moiety, confirming the existence of the *trans* azobenzene moiety in HOLO(Az-BA) (see Figure S3). By NMR analysis of the *trans*/*cis* isomerization of 4-carboxy-4′-(methacrylamido)azobenzene (monomer), it could be determined that approximately 80% of the monomer is initially present as the *trans* isomer, thus the Az-BA moiety in the HOLO polymer may have a similar *trans*/*cis* ratio (see Figure S4).

Subsequently, the conjugation of AN to HOLO(Az-BA) was carried out to attach the second prosthetic group, yielding HOLO(Az-BA)(AN) (step K in Figure 6a), which features both carboxylic acid and amine groups within the imprinted cavity for possible synergetic binding of the target antibiotic. As a reference, HOLO(Az) was prepared by using 4-nitrosobenzene instead of 4-nitrosobenzoic acid in order to obtain an azobenzene moiety without a carboxylic acid, in which the extra space was filled by the azobenzene prosthetic group, but no interactive groups were available (step L). HOLO(Az)-(AN) was also prepared from HOLO(Az), which has a thioaniline moiety and an azobenzene moiety without the carboxylic acid (step M).

When compared to the binding activities of the four HOLO polymers, namely HOLO(Az), HOLO(Az)(AN), HOLO(Az-BA), and HOLO(Az-BA)(AN), the highest binding activity towards ampicillin was observed for HOLO(Az-BA)(AN), as was the case for HOLO(BA)(AN) and HOLO(SA)(MBA), confirming that the template effect is again maintained after removal of the template molecule followed by reconstruction of the binding cavity by conjugating structurally similar prosthetic groups, and the two binding sites work synergistically for ampicillin binding (Figure 6b). Under light irradiation for ten minutes at 365 nm with an LED lamp, the amount of bound ampicillin decreased, whereas visible-light irradiation with fluorescent light for one hour led to recovery of the binding activity. This observation clearly demonstrates the transformation of the HOLO-type binding cavity into a photoresponsive one on PIM treatment (Figure 6c).

In conclusion, for the first time, we have used MIP-based conjugated protein mimics for the exploration of rational designs towards the next generation of multifunctional materials using molecular imprinting with a circumspectly designed template molecule and post-imprinting modifications. Common molecularly imprinted apo-type scaffolds

  

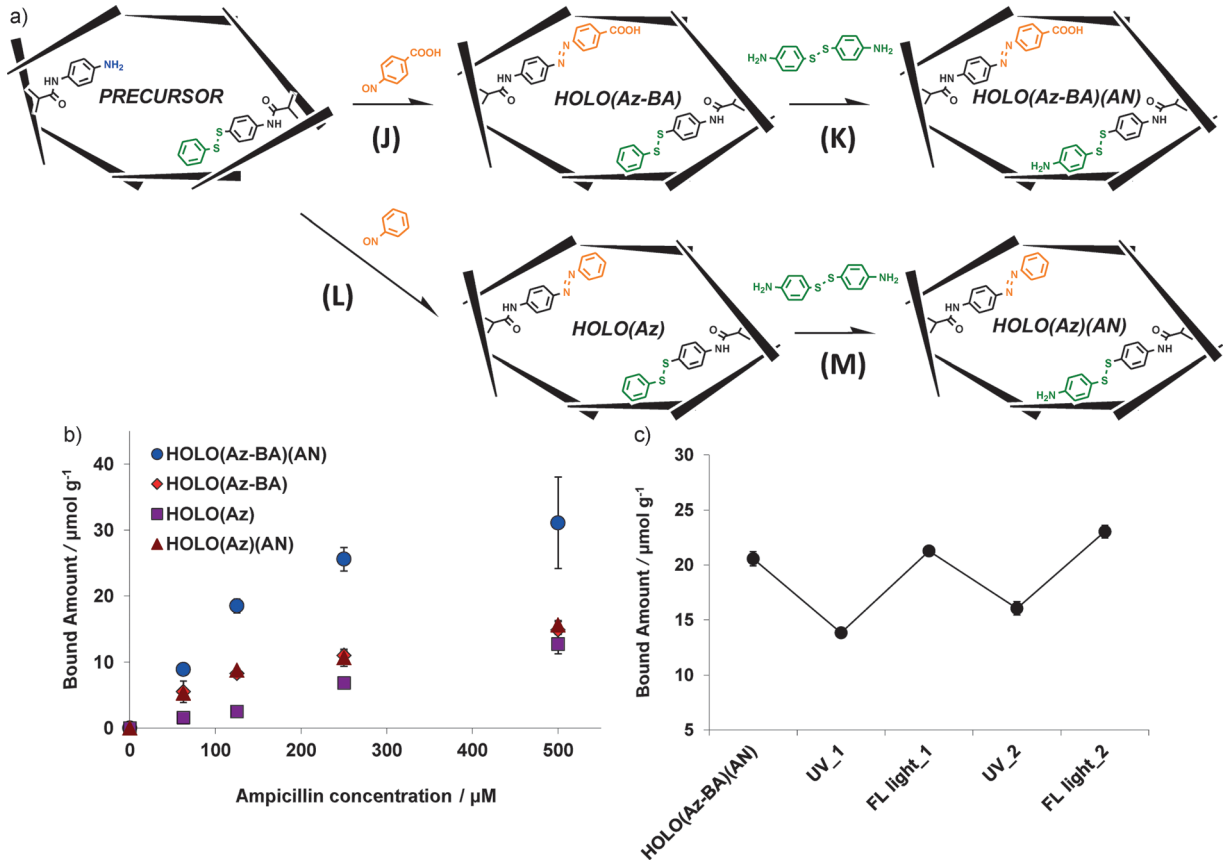


***Figure 6.*** Introduction of photoresponsive properties into the apo-type scaffold (PRECURSOR). a) Preparation of a series of HOLO polymers with a photoresponsive azobenzene moiety. b) Binding isotherms of HOLO(Az-BA)(AN), HOLO(Az-BA), HOLO(Az), and HOLO(Az)(AN). c) Amount of ampicillin bound on HOLO(Az-BA)(AN) on alternating irradiation with UV light ($\lambda = 365$ nm) and fluorescent light.

capable of independent conjugation of two different prosthetic groups that were pre-determined according to the design of the template molecules used can recall their molecular recognition abilities on conjugation of the original prosthetic groups or structurally similar compounds. Depending on the reactions and the prosthetic groups employed in PIMs, a diverse range of functions can be introduced into the common apo-type scaffold, including site-specific addition, transformation, rearrangement, and other available alterations of functionalities within molecularly imprinted cavities. Furthermore, on/off switching of functions can be achieved by changing between the apo- and holo-type structures.

The present strategy can be used to provide new classes of synthetic materials with various functions, such as artificial enzymes, signaling plastic antibodies, drug-delivery systems, antidotes, restoration materials, stimuli-responsive materials, and tools for logic circuits. The development of specific reaction fields within artificial materials that mimic conjugated proteins has rarely been seen to date; therefore, we believe that the present findings could lead to a paradigm-shift in the fundamental aspects of functional materials.

Received: July 3, 2014
Published online: September 24, 2014

**Keywords:** imprinting · molecular recognition · polymers · protein mimics

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