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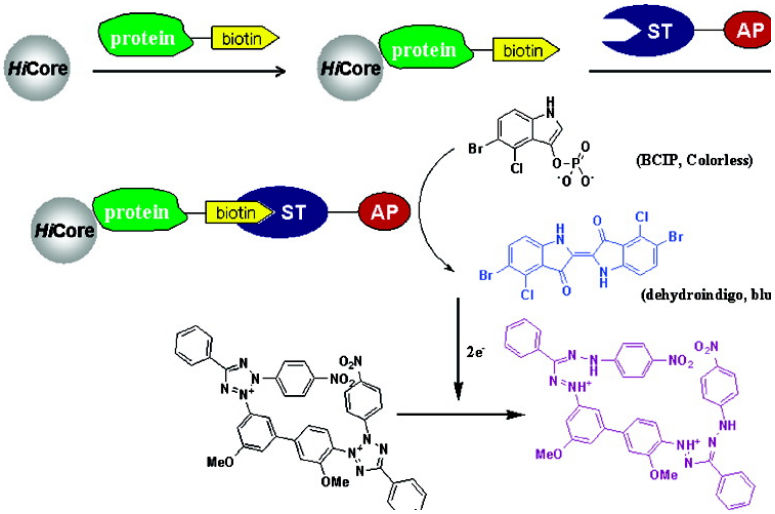

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Quantitative Evaluation of *Hi*Core Resin for the Nonspecific Binding of Proteins by On-Bead Colorimetric Assay

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Because of their numerous advantages over conventional solution phase methods, combinatorial strategies on solid supports[1] have been successfully applied to the discovery of novel therapeutic agents.[2] One of the continuing objectives in this area is the development of ideal solid supports for the rapid and reliable identification of bioactive small molecules using efficient high-throughput screening. In this study, our goal was the development of an ideal solid support which enhances the production of diverse small molecules with high purity and the rapid evaluation of the produced libraries without cleavage from the solid support. However, the biological evaluation of small-molecule libraries in terms of their specific interaction with biopolymers (i.e., protein, DNA, RNA) is limited due to the nonspecific adsorption of the biopolymers onto the polymeric solid supports.[3]

The solid supports which have so far been developed for the purpose of bioassays can be structurally classified into two types: sugar-based polymers, such as agarose[4] or sepharose,[5] of which a wide variety exist, and synthetic functional polymers. Even though the sugar-based supports are commonly used as bioseparators in the field of molecular biology, these supports are highly vulnerable to irreversible denaturation under general synthetic conditions in which organic solvents or strong acids or bases are present. In contrast, functional polymers, such as poly(methacrylate) derivatives or polystyrene derivatives, which are tolerant to various synthetic conditions, enable the more facile library synthesis of bioactive small molecules. However, synthetic polymer supports have a limited application in biological science due to their swelling property in water as well as their high levels of nonspecific binding with biomaterials[6] in comparison to sugar-based solid supports. Especially, nonspecific binding is thought to occur by hydrophobic interaction and constitutes a major limitation in bioapplications, such as affinity chromatography for target protein identification and the enzyme-linked on-bead colorimetric assay for specific substrate/ligand identification.[7] For these reasons, many researchers have attempted to address this issue by introducing hydrophilic spacers, such as poly(ethylene glycol) (PEG), onto the solid support to minimize the undesired nonspecific binding.[8] TentaGel resin[9] is the preferred choice for the synthesis of bioactive small molecules on solid supports associated with the enzyme-linked on-bead colorimetric assay[10] due to its superior swelling properties in aqueous media and minimal nonspecific binding with biopolymers.[11]

Table 1. Swelling Volume of the Polymer Supports in Various Solvents

resins	swelling volume (mL/g resin)					
	H_2O	CH_3CN	CH_3OH	CH_2Cl_2	THF	NMP
AM PS (1% DVB)		3.2	1.6	8.3	8.8	5.7
TentaGel S−NH_2	3.6	4.2	3.6	6.3	5.0	4.6
*Hi*Core	2.8	3.3	3.7	4.3	4.0	4.1

Recently, we reported a highly cross-linked core−shell-type (*Hi*Core) resin which was synthesized from aminomethyl polystyrene (AM PS) resin in two simple steps,[12] namely, the cross-linking of AM PS resin with 2,4,6-trichloro-1,3,5-triazine for the preparation of the rigid core (core region), followed by the functionalization of the triazine with a diamino PEG derivative (shell region). The resulting triazine cross-linked rigid core became very inert to chemical and physical changes, such as solvents, acidic/basic conditions, and temperature; therefore, *Hi*Core resin remained intact during various synthetic processes. The hydrophilic PEG chain in the surface region, which contains 13−14 ethylene glycol repeating units, serves as a reaction site with their amine functionalities and provides excellent biocompatibility in aqueous media. Most importantly, the PEG units provide significant reduction of the nonspecific bindings with biopolymers.

Due to the highly cross-linked core, the swelling properties of *Hi*Core resin are significantly different from those of other commercial polymers, such as PS resin or TentaGel, with relatively consistent swelling volumes in various solvents (Table 1). In contrast to TentaGel resin, which has a crinkled surface, the surface morphology of *Hi*Core resin is relatively smooth on the basis of the scanning electron microscopy (SEM) image shown in Figure 1. Therefore, we concluded that the PEG moieties on the surface of *Hi*Core resin are evenly distributed in the swollen state, which might minimize the nonspecific binding with biopolymers.

Unlike the gel-type supports, all of the available functional groups are located on PEG chains of the shell region of *Hi*Core resin, which provides superior reaction kinetic in various chemical transformations and eliminates the undesired carryover of reagents entrapped on the polymer matrix due to the diffusion barrier created by the rigid core[12] (Figure 2). In the case of bioapplications of solid supports loaded with druglike small molecules, *Hi*Core resin may have the additional advantage over gel-type resin by allowing the efficient display of small molecules on its surface. Thereby high molecular weight biopolymers can gain easy access to them with minimum nonspecific binding.

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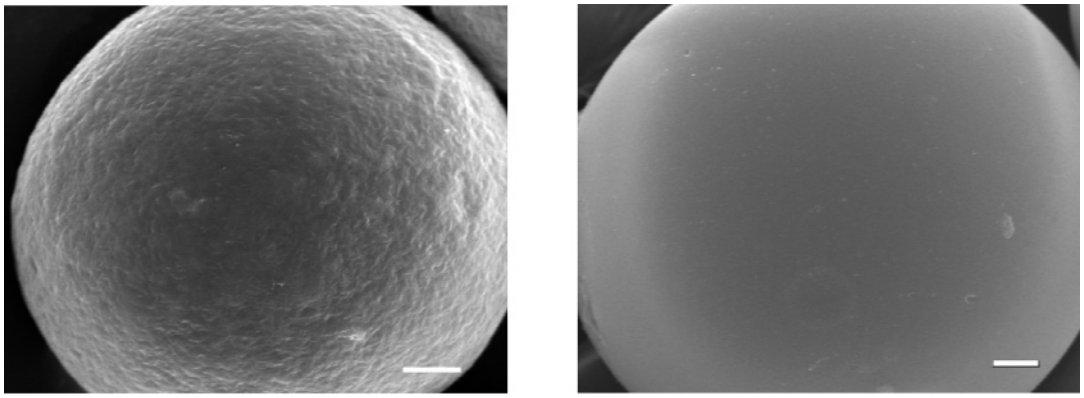
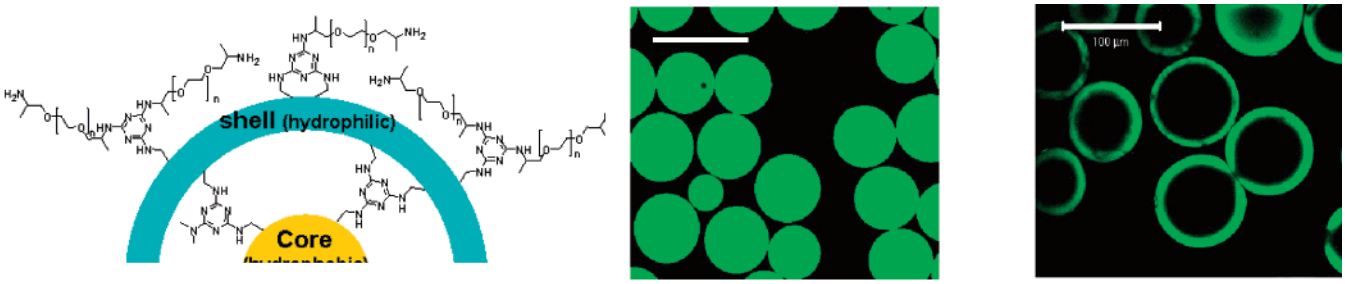


(a) (b)

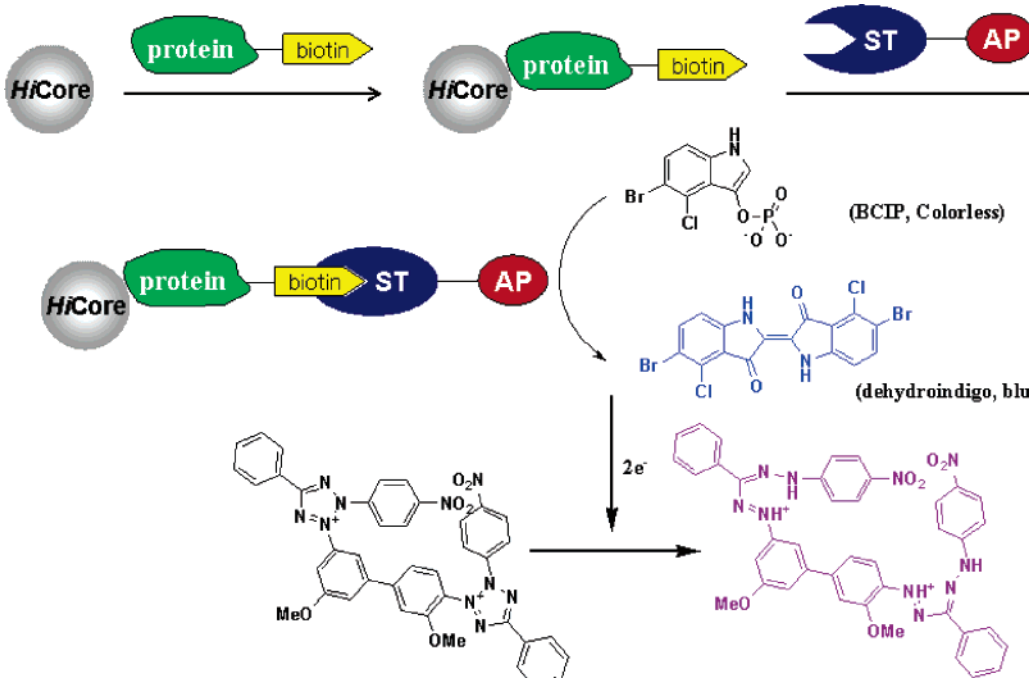
Figure 1. Scanning electron microscopy images of the resins: (a) TentaGel resin and (b) *Hi*Core resin. Each scale bar represents 10 μm.



(a) (b) (c)

Figure 2. Cross-sectional images of the resins: (a) schematic image of *Hi*Core resin, (b) FITC-coupled image of TentaGel resin (0.30 mmol NH_2/g), and (c) *Hi*Core resin (0.22 mmol NH_2/g) through a confocal laser scanning microscope. Each scale bar represents 100 μm.

Scheme 1. Enzyme-Linked On-Bead Colorimetric Assay for Nonspecific Binding of Biotinylated Proteins[a]



[a] AP, alkaline phosphatase; ST, streptavidin; BCIP, 5-bromo-4-chloroindolyl 3-phosphate; NBT, nitro blue tetrazolium.

To evaluate the physical properties of *Hi*Core resin, we employed a simple enzyme-linked, on-bead colorimetric assay to measure the nonspecific binding of biopolymers toward the various solid supports. As illustrated in Scheme 1, the assay is designed to detect the biotinylated protein nonspecifically bound to the solid support with alkaline−phosphatase-linked streptavidin, followed by colorization with the following reagents: 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT).[13]

To confirm the sensitivity and efficiency of the modified enzyme-linked, on-bead colorimetric assay using alkaline phosphatase and BCIP/NBT as well as the quantification of

Figure 3. Color development on various polymer supports after enzyme-linked, on-bead colorimetric assay with ST−AP followed by BCIP/NBT: (a) AM PS resin, (b) TentaGel resin, and (c) *Hi*Core resin.

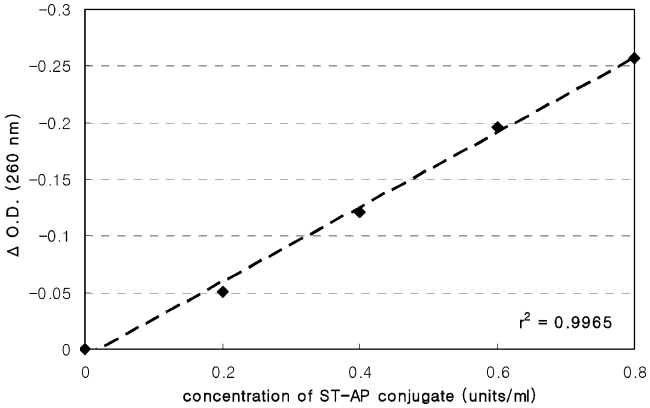


Figure 4. Correlation between the absorbance difference (260 nm) and the concentration of ST−AP conjugate.

Table 2. Physical Properties of Biotinylated Proteins

	MW kDa	pI value	no. of biotin per protein[a]
bovine serum albumin (BSA)	67	4.8	6.5
casein	24	5.0	2.0
FK506 binding protein/glutathione S-transferase (FKBP-GST)	40	7.0	13
hemoglobin	64	7.0	10
γ-globulin	150	7.4	15
lysozyme	14	10.0	3.1

[a] Numbers of biotin per protein were measured by the weight gain after biotinylation (see Supporting Information).

nonspecific binding events on solid supports, we first investigated the following solid supports: *Hi*Core resin, TentaGel resin, and hydrophobic AM PS resins. Three solid supports were acetylated, followed by extensive washing, and incubated with streptavidin−alkaline phosphatase conjugate (ST−AP) to observe the nonspecific binding of streptavidin on the surface of respective solid supports as a background. The sequential addition of NBT and BCIP resulted in a dark purple color development through the enzymatic reaction of alkaline phosphatase and the precipitation of diformazan on the bead surface. On the basis of this preliminary binding study with ST−AP, as shown in Figure 3, we found that *Hi*Core resin has minimal nonspecific binding of ST−AP on its surface, whereas there was a significant color development on the AM PS resin and a slight color development even on TentaGel resin. Therefore, we were confident that the enzyme-linked, on-bead colorimetric assay we developed is sensitive enough to clearly differentiate the degrees of nonspecific binding on various solid supports. Due to the prominent nonspecific binding on the AM PS resin, we concentrated our efforts on the comparison of the nonspecific binding of *Hi*Core resin with TentaGel resin.

The direct quantification of nonspecific binding through the color developments with the UV-spectrometric method, however, could not provide an accurate measurement, because when using any available solvents, it was impossible to completely extract the deposited diformazan precipitate on the polymer supports. Due to this limitation of the colorimetric assay, we instead measured the UV absorbance of unreacted NBT at 260 nm for the quantitative determination of the binding events of ST−AP. When an excess amount of NBT is put into the reaction mixture, the concentration of unreacted NBT will be inversely related to the amount of alkaline phosphatase,[13] which, in turn, can easily be converted to the amount of biotinylated proteins nonspecifically bound on the solid supports.

To determine the relationship between the absorbance difference of the unreacted NBT and the corresponding amount of immobilized ST−AP conjugate, we fitted a correlation curve (Figure 4) and observed good linearity up to 0.8 units/mL of ST−AP conjugate. Therefore, at a fixed concentration of ST−AP conjugate, the amount of biotinylated proteins nonspecifically bound on the solid supports could be calculated by measuring the UV absorption of remaining NBT at 260 nm.

With this quantification system, we exploited the nonspecific binding of various proteins labeled with a high-affinity natural ligand of streptavidin, biotin (Scheme 1). Proteins subjected to nonspecific binding on the solid supports were chosen on the basis of those physical properties which were capable of influencing their nonspecific binding, such as their molecular weight (size) and pI values (charge), as summarized in Table 2. We then biotinylated six different proteins using sulfo-NHS-LC-Biotin (Pierce, U.S.), and the number of biotins per protein was measured by SDS−PAGE and MALDI-TOF mass spectrometry. On the basis of our preliminary data, we focused our attention on a comparison of the nonspecific protein binding properties on TentaGel resin (gel-type, PEG-grafted PS resin) and *Hi*Core resin (core−shell-type, PEG-grafted PS resin).

To maximize the sensitivity for nonspecific binding events, the resins were treated with the optimized procedure described in the Supporting Information. After color development with BCIP/NBT, the resulting resins were stored at 0 °C for 24 h prior to the image capturing (Figure 5). On the basis of these results, we concluded qualitatively that, in all cases, TentaGel resin showed a higher nonspecific binding affinity toward proteins of interest than the *Hi*Core resin; the former provided the dark purple color development from diformazan precipitate on the solid support, whereas the latter exhibited only the yellow color resulting from the absorption of the unreacted NBT.

For the quantitative analysis of nonspecific binding, we measured the UV absorbance of unreacted NBT in solution after color development. The absorbance at 260 nm was

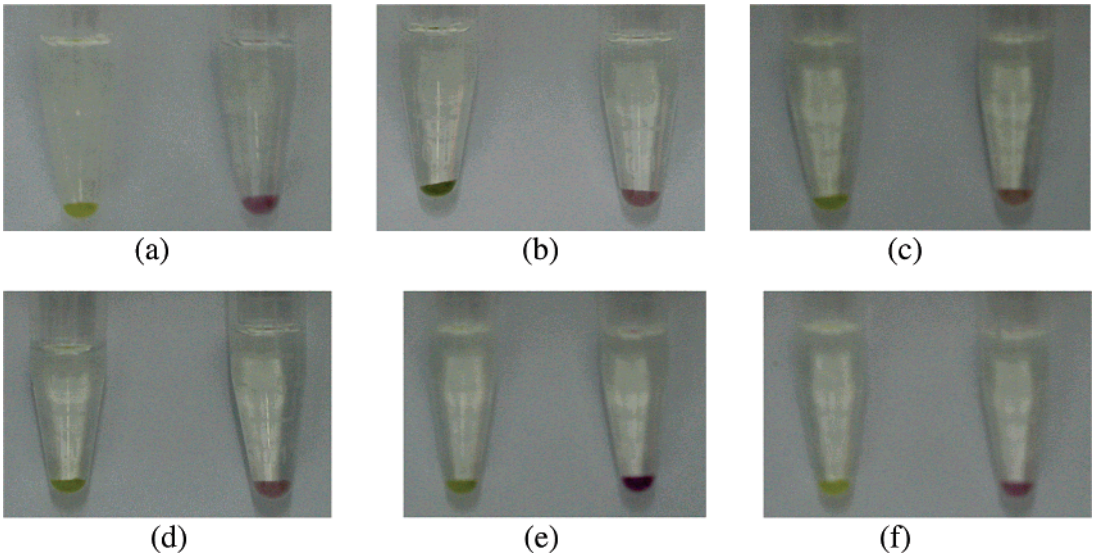


Figure 5. Images of the polymer supports after the BCIP/NBT color development through on-bead nonspecific protein binding assay. The left-hand side of each picture is *Hi*Core resin, and the right-hand side is TentaGel resin, both of which are treated with biotinylated (a) BSA, (b) casein, (c) FKBP-GST, (d) γ-globulin, (e) hemoglobin, and (f) lysozyme.

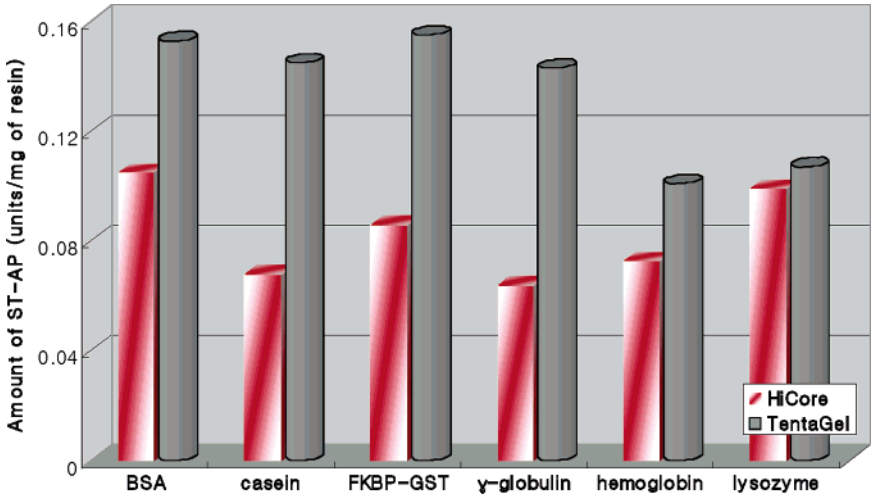


Figure 6. The amounts of the nonspecifically bound proteins on each polymer support, as determined by the quantitative enzyme-linked colorimetric assay.

Table 3. Amounts of the Biotinylated Proteins Nonspecifically Bound on the Polymer Support[a]

	biotin-labeled protein					
resin	BSA	casein	FKBP-GST	γ-globulin	hemo-globin	lysozyme
*Hi*Core	0.105	0.068	0.086	0.064	0.073	0.099
TentaGel	0.153	0.145	0.155	0.143	0.101	0.107

[a] Unit of ST−AP conjugate per milligram of resin.

converted to the amount of ST−AP on the solid supports by using the correlation curve; therefore, we could determine the amount of nonspecifically bound proteins of interest using the high affinity of biotin toward ST−AP. As shown in Table 3 and Figure 6, in all cases except lysozyme, the *Hi*Core resin shows improved repellency of 30−50% over the TentaGel resin toward the nonspecific adsorption of proteins with a wide range of physical properties. Lysozyme has a pI value of ~10.7, as shown in Table 2, which means that it is positively charged under the experimental condition used in this enzyme-linked, on-bead colorimetric assay (pH = 7.5), whereas the other proteins (pI range of 5−7) are neutral or negatively charged under the same conditions. We assume that the positive charges on the protein surface might reduce improved repellency of *Hi*Core resin over TentaGel resin, which was caused by the complex formation of PEG chains with ammonium groups,[14] and this property might induce the nonspecific binding of positively charged proteins on the PEG-grafted polymer supports.

In an effort to verify the bioapplication of *Hi*Core resin, we next sought to determine the specific interactions of resin-bound ligands to proteins of interest. Recognizing the high affinity of AP1497, a synthetic analogue of FK506, for FKBP12 (K_D = 8.9 nM),[15] we synthesized and immobilized AP1497 on TentaGel resin and *Hi*Core resin with an appropriate spacer to investigate the specific binding ability of the resins (see Supporting Information for details). The immobilization site of AP1497 has previously been shown to have no influence on the affinity toward FKBP12-GST,[16] which was also subject to nonspecific binding in this study (see Table 3 and Figure 6). The resins loaded with AP1497

Table 4. Amounts of the Biotinylated FKBP-GST Specifically/Nonspecifically Bound on the Polymer Supports[a]

resin	after nonspecific binding	after specific binding	S/N ratio
*Hi*Core	0.086	0.197	2.29
TentaGel	0.155	0.288	1.86

[a] Unit of ST−AP conjugate per milligram of resin.

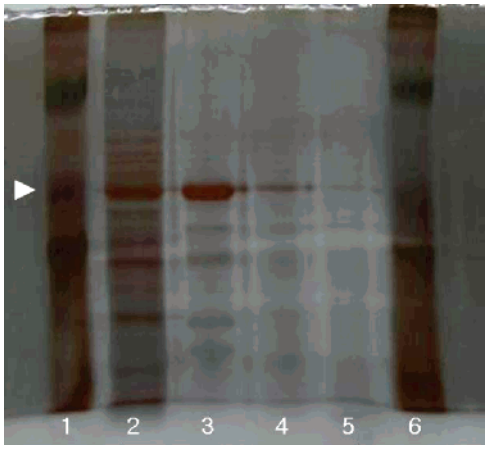


Figure 7. Detection of binding proteins for small-molecule-immobilized solid supports. Lanes 1 and 6, size marker; lane 2, FK lysate; lane 3, FKBP12-GST purified protein using GST-affinity chromatography; lane 4, specifically bound protein on AP1497-loaded *Hi*Core resin released by photocleavage; lane 5, specifically bound protein on AP-1497-loaded TentaGel resin released by photocleavage.

were incubated with biotinylated FKBP-GST to measure the specific binding of protein on the solid support through enzyme-linked, colorimetric assay. As shown in Table 4, the *Hi*Core resin shows a 23% higher specific/nonspecific binding ratio toward biotinylated FKBP-GST than does the TentaGel resin, which is a pronounced improvement of the signal-to-noise (S/N) ratio in the on-bead assay system. Both solid supports showed significant color developments through the specific binding event of AP1497, which indicates the recruitment of biotinylated FKBP12-GST on solid supports; however, TentaGel resins demonstrated higher specific binding on the basis of the quantitative binding assay data, probably due to the higher loading level of TentaGel resin than *Hi*Core resin. The improved specific/nonspecific binding ratio of *Hi*Core resin resulted from the specific binding events of proteins to their resin-bound ligands as well as superior repellency toward the nonspecific adsorption.

For final confirmation of the applicability of *Hi*Core resin in affinity chromatography, we loaded a high-affinity synthetic ligand, AP1497, with a photocleavable linker on both solid supports. The resulting resins (5 mg each) were incubated with cell lysate of *Escherichia coli* overexpressing FKBP12-GST (namely FK lysate). After the resins were washed with the incubation buffer, they were irradiated with 365-nm light to release FKBP12-GST bound on the solid supports through specific interaction with AP1497. They were suspended in sample loading buffer and subjected to SDS−PAGE. The gel was visualized by silver staining (Figure 7). On the basis of the gel image, we could identify a band of 40-kDa proteins from *Hi*Core resin which is matched with purified FKBP12-GST using agarose-based GST affinity chromatograpy from FK lysate; however, TentaGel resin showed significantly lower detection limits, which is probably due to the inefficient photocleavage process in gel-type, PEG-grafted TentaGel resin.[17] These results demonstrated that *Hi*Core resin can be applied in target identification using affinity chromatography through specific interaction of ligands with proteins of interest.

In summary, we modified the quantitative enzyme-linked, on-bead colorimetric assay to determine the nonspecific binding of proteins on the polymer supports. On the basis of a series of experiments, we confirmed that the core−shell-type *Hi*Core resin can efficiently reduce the nonspecific bindings of proteins with a wide spectrum of molecular weights and pI values without a significant loss of specific binding ability. The surface-grafted PEG chains and the smooth surface of the *Hi*Core resin may play an important role in reducing the nonspecific binding events. Moreover, the chemical and physical stability of *Hi*Core resin provides an additional advantage in the synthetic application of various bioactive molecules with higher efficiency while also being suitable for use in the on-bead bioassay as well as target identification using affinity chromatography. This study revealed that *Hi*Core resin could be used for the discovery of novel therapeutic agents and the identification of various biological mechanisms.

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Supporting Information Available. Detailed experimental procedures; MALDI-TOF spectral data of biotinylated proteins; and copies of ^{1}H, ^{13}C NMR, and mass spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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