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Aromatic Functionality of Target Proteins Influences Monomer Selection for Creating Artificial Antibodies on Plasmonic Biosensors

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Abstract

Natural antibodies used as biorecognition elements suffer from numerous shortcomings, such as limited chemical and environmental stability and cost. Artificial antibodies based on molecular imprinting are an attractive alternative to natural antibodies. We investigated the role of aromatic interactions in target recognition capabilities of artificial antibodies. Three proteins with different aromatic amino acid content were employed as model targets. Artificial antibodies were formed on nanostructures using combinations of silane monomers of varying aromatic functionality. We employed refractive index sensitivity of plasmonic nanostructures as a transduction platform for monitoring various steps in the imprinting process and to quantify the target recognition capabilities of the artificial antibodies. The sensitivity of the artificial antibodies with aromatic interactions exhibited a protein-dependent enhancement. Selectivity and sensitivity enhancement due to the presence of aromatic groups in imprinted polymer matrix was found to be higher for target proteins with higher aromatic amino acid content. Our results indicate that tailoring the

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Depicts LSPR shift spectra for PLIN2 and hemoglobin for each step of artificial antibody synthesis (PDF)

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monomer composition based on the amino acid content of the target protein can improve the sensitivity of plasmonic biosensors based on artificial antibodies without affecting the selectivity.

Graphical abstract

Keywords

artificial antibodies; plasmonic biosensors; molecular imprinting; aromatic interactions; synthetic biorecognition elements

Introduction

Localized surface plasmon resonance (LSPR), which involves the collective oscillation of dielectrically confined conduction electrons of metal nanostructures, is highly attractive for a number of applications including chemical and biological sensing, energy harvesting, bioimaging, and locoregional therapy.^{1–3} In particular, LSPR sensors based on the refractive index sensitivity of plasmonic nanostructures is considered to be highly attractive for on-chip and point-of-care biondiagnostics. Most early investigations related to plasmonic biosensors relied on natural antibodies as biorecognition elements, which exhibit excellent sensitivity and selectivity.^{4,5} However, natural antibodies exhibit limited chemical, thermal, and environmental stability, which makes them unsuitable for applications in point-of-care and resource-limited settings.^{6,7} For instance, natural antibodies would have to be repurchased with each new application which contributes to recurring cost. Additionally, natural antibodies lose their activity when stored at ambient or elevated temperatures.^{6,7} We recently demonstrated plasmonic biosensors based on artificial antibodies achieved through molecular imprinting on the nanotransducer surface.^{8–10} Artificial antibodies to a kidney injury biomarker had no change in analyte recognition over a pH range of 4.5–8.5 and specific gravity up to 1.03.⁹ Apart from significantly improving the stability and lowering the cost of the biosensors, use of artificial antibodies instead of the natural antibodies as biorecognition elements significantly shortens the bioassay development time as the synthetic imprinting approach can be rapidly applied to a broad class of biomolecules. For instance, it takes months to generate and characterize a natural antibody be it polyclonal or monoclonal versus the just days to a week to prepare and characterize an artificial antibody. However, the sensitivity and selectivity of the existing artificial antibody-based plasmonic biosensors is typically lower compared to those based on natural antibodies. Overcoming the limited sensitivity and selectivity of the artificial antibody-based plasmonic biosensors is critical to translate this powerful class of biosensors to real-world clinical applications.

Artificial antibodies based on molecular imprinting rely on complementarity in size, shape, and chemical functionality of the binding pocket to the target biomolecule.^{11,12} In a typical procedure, functional monomers are polymerized on the transducers in the presence of the template biomolecules. Subsequent removal of the template biomolecules leaves cavities that are complementary in size, shape, and chemical functionality to the target biomolecules. The choice of the monomers bearing complementary functional groups to the imprinted species is of great importance in realizing highly selective recognition cavities after removing template bio-molecules.¹³ Two different approaches have historically been explored for forming imprinted cavities, which rely on covalent and noncovalent interactions. Imprints relying on covalent interactions tend to exhibit slow binding and dissociation.¹⁴ Conversely, molecular imprints based on noncovalent interactions that exhibit faster binding and unbinding are more extensively employed for biondiagnostic applications. However, the noncovalent interactions between template biomolecules and polymer matrix tend to be weaker in aqueous media, thus lowering the target recognition capabilities of the imprints.¹¹ Since electrostatic, hydrogen bonding, and hydrophobic interactions are assumed to be the most prominent contacts between the imprint cavities and the target biomolecules, extensive efforts have been dedicated to strengthen these binding interactions to improve the sensitivity and specificity of the artificial antibodies to specific analytes.^{15–18} Previously, we employed two silane monomers, trimethoxypropylsilane (TMPS) and (3-aminopropyl) trimethoxysilane (APTMS), for molecular imprinting on plasmonic nanostructures.^{8,9} The siloxane copolymer is comprised of amine, methyl, and hydroxyl groups that provide electrostatic, hydrophobic, and hydrogen-bonding interactions. Apart from silane monomers, a wide variety of other hydrophilic or hydrophobic monomers have been investigated to maximize the recognition capabilities of the artificial antibodies.^{19–23}

Although extensive efforts have been dedicated to the synthesis of artificial antibodies with high sensitivity and selectivity, most of these efforts are generic and the composition of the monomers is not often tailored to the target analyte. In this work, we have investigated the influence of the presence of an aromatic monomer, which provides aromatic electron donor–acceptor interactions, on the recognition capabilities of artificial antibodies. Refractive index sensitivity of plasmonic nanostructures is utilized to monitor various steps of the molecular-imprinting process. We demonstrate that incorporation of an aromatic monomer significantly improves the sensitivity of artificial antibodies without sacrificing its selectivity.

Results and Discussion

Selective and sensitive recognition of target molecules by artificial antibodies can be achieved by optimizing the chemical functionality of the cavities in the polymer layer. As mentioned earlier, in addition to the size and shape complementarity, binding interactions between the walls of the cavities and the target biomolecules is critical in molecular imprinting.^{11,24} Analogous to binding interactions between protein and natural antibodies, the silane monomers bearing different functional groups interact with various chemical functionalities of the template protein and self-assemble during the formation of the polymer layer around the template protein. The concerted interactions between the chemical functional groups within the cavity and the target protein lead to sensitive and selective detection of the target biomolecules (Figure 1A). We hypothesize that inclusion of

trimethoxyphenylsilane (TMPhS), an organic monomer with aromatic functionality, can facilitate aromatic interactions between the cavity and electron-deficient aromatic ring of target molecules.^{25,26} The silane monomer with aromatic side chain is expected to result in aromatic interactions between the cavity and target biomolecule, thus improving the binding affinity and selectivity of the artificial recognition elements.^{8,27–30} To verify this hypothesis, we synthesized and tested artificial antibodies with three sets of monomers using three different proteins with different amino acid contents as template biomolecules. We used the combination of two silane monomers, APTMS and TMPS, as Composition 1. Composition 2 is comprised of a third monomer, TMPhS. We then attempted to eliminate TMPS or APTMS in the three monomer mixture to determine the optimal combination of different organosilane monomers. Preliminary experiments indicated that the TMPhS and APTMS pair could form a uniform polymerization layer, named Composition 3 (Figure 1A). However, polymerization could not be achieved with TMPhS and TMPS mixture and was not investigated further.

Gold nanorods (AuNRs) have been employed as plasmonic nanotransducers to probe the effect the monomer chemistry has on the affinity and selectivity of the artificial antibodies. AuNRs were synthesized using a seed-mediated approach (see Experimental Section for details). The TEM images revealed the diameter of AuNRs to be 13.6 ± 0.8 nm and the length to be 49.1 ± 2.8 nm, respectively (Figure 1B,C). The vis-NIR extinction spectrum of AuNRs exhibited characteristic extinction bands corresponding to transverse (515 nm) and longitudinal (750 nm) plasmon resonances (Figure 1C). AuNRs are known to exhibit significantly higher refractive index sensitivity compared to spherical nanoparticles, making them better candidates for optical transduction of refractive index changes and plasmonic biosensors.^{31,32} Apart from higher refractive index sensitivity, yet another advantage of using AuNR is the large surface-enhanced Raman scattering (SERS) activity of these nanostructures.^{33–35} We previously demonstrated that SERS can be employed to monitor various steps along the imprinting process including surface modification of AuNR to render amine functionality, template protein immobilization, polymerization (using APTMS and TMPS), and template removal.⁸ In the present study, we employed SERS to monitor the successful incorporation of TMPhS into the imprinted polymer layer. SERS spectra obtained from AuNR following the polymerization of TMPS and APTMS exhibited bands at 939 and 1205 cm^{-1} assigned to rocking vibration and twisting vibration of CH_2 in APTMS. After addition of TMPhS, the SERS spectra exhibited bands at 1003 and 1031 cm^{-1} , corresponding to $=\text{C}-\text{H}$ in-plane deformation modes and 1595 cm^{-1} corresponding to $\text{C}=\text{C}$ stretching vibration in TMPhS (Figure 2A,B).²⁴ The SERS spectra confirm the successful incorporation of aromatic functionality into the molecular imprints formed after the inclusion of TMPhS.

In this study, we have synthesized artificial antibodies for three proteins with different aromatic amino acid contents: lysozyme (8.8%), human hemoglobin (6.3%), and human PLIN2 (5.3%). We first describe the results using lysozyme as template and target protein. Refractive index sensitivity of LSPR wavelength of AuNRs (step 1, black spectra of Figure 3A) was employed to monitor each step along the molecular-imprinting procedure, including formation of cross-linker layer with *p*-aminothiophenol and glutaraldehyde (step 2, red spectra of Figure 3A), binding of template proteins (step 3, blue spectra of Figure 3A),

copolymerization of monomers (step 4, lavender spectra of Figure 3A). All spectra in Figure 3A were acquired in aqueous media. All of the spectra of Figure 3B were acquired in air and included step 4 (black spectra), the release of the template in step 5 (blue spectra), and the capture of target protein in step 6 (red spectra). By limiting the red shift in the LSPR wavelength to ~ 3 nm during polymerization step 4, we maintained similar thickness of polymer layer for each monomer mixture. After treatment with oxalic acid and sodium dodecyl sulfate, the template protein was removed by breaking the reversible imine bond and overcoming noncovalent interplays, respectively. Following the removal of the template protein, the AuNRs are left with polymer layer with cavities that are complementary in shape, size, and chemical functionality to lysozyme. Figure 3C shows the entire molecular-imprinting procedure and recapture of lysozyme (from phosphate buffered saline (PBS) spiked with lysozyme) using TMPS and APTMS (Composition 1) as the monomers for polymerization. The accumulated red shifts following completed steps of the imprinting process reached ~ 17 nm, while the blue shift corresponding to template protein removal was ~ 9 nm. After incubation in $5 \mu\text{g/mL}$ of lysozyme solution, an ~ 7.4 nm red shift in the LSPR wavelength indicated the successful recognition and rebinding of the target protein (Figure 3C).

The LSPR shift of AuNR imprinted with lysozyme with three different monomer compositions (Compositions 1, 2, and 3 described above) exhibited a monotonic increase with increase in the concentration of lysozyme in the analyte solution (Figure 3D,E). Composition 2 showed significantly larger ($P = 0.01$) LSPR shifts at each lysozyme concentration compared to that of the other two compositions. For the highest concentration tested here ($5 \mu\text{g/mL}$), the monomer mixture comprised of TMPS and APTMS (Composition 1) exhibited a red shift of 7.3 ± 0.2 nm. In contrast, for the same concentration of lysozyme, the three-monomer mixture (Composition 2) exhibited a red shift of 9.3 ± 0.3 nm, which represents a 40% improvement over Composition 1. This indicates that TMPhS significantly improved the sensitivity of the plasmonic biosensor to the target protein, possibly through the aromatic electron donor–acceptor interactions. Conversely, the average LSPR shift with Composition 3 was found to be 6.2 ± 0.2 nm, nearly $\sim 36\%$ lower compared to that in Composition 1 ($P = 0.03$). The result clearly demonstrates that TMPhS could not efficiently substitute for TMPS since the latter has hydroxyl and methyl functional groups that facilitate hydrogen-bonding and hydrophobic interactions, respectively.^{36,37} To investigate the selectivity of artificial antibodies, lysozyme-imprinted AuNRs were separately challenged with either human or bovine serum albumin at a higher concentration ($20 \mu\text{g/mL}$) compared to lysozyme ($5 \mu\text{g/mL}$). The LSPR shifts of this nontemplate protein in Compositions 1, 2, and 3 ranged from 0.9 ± 0.2 to 1.5 ± 0.3 nm, respectively, suggesting high selectivity of the artificial antibodies for the original template protein.

To further probe whether the fraction of aromatic amino acids in the target protein has a role in enhanced sensitivity with TMPhS, we chose two other proteins and implemented molecular imprinting with Compositions 1 and 2. PLIN2 is a protein with much larger molecular weight ($\text{MW} = 49.3$ kDa) but significantly less aromatic amino acid content (5.3%) compared to lysozyme (8.8%). We controlled the thickness of the artificial antibody polymer layer by limiting the LSPR shift of AuNR during polymerization to ~ 3 nm (Figure S1A). LSPR shifts for each step along the molecular imprinting procedure are depicted in

aqueous media (Figure S1A) or air (Figure S1B). Figure 4A shows the LSPR shift of AuNR imprinted with PLIN2 upon exposure to different concentrations of PLIN2. The average sensitivity enhancement of LSPR shift for PLIN2 under Composition 2 compared to that under Composition 1 was found to be 19%. This sensitivity enhancement is smaller than the enhancement noted for lysozyme (40%). The reason for the lower sensitivity enhancement may be due to the lower content of aromatic amino acid content in PLIN2 compared to that in lysozyme, which results in fewer aromatic binding sites. The PLIN2 artificial antibodies prepared using Compositions 1 and 2 exhibited excellent selectivity with LSPR shifts of only 0.9 ± 0.2 to 1.4 ± 0.2 nm upon exposure to either $20 \mu\text{g/mL}$ of human or bovine serum albumin as an interfering protein. This further demonstrates the specificity of the antibodies to the template protein.

To further investigate the effect of aromatic amino acid content on the sensitivity and specificity of the artificial antibodies, one more protein was included in this study. Hemoglobin contains a higher aromatic amino acid content (6.9%) than PLIN2, while its molecular weight is quite close to the former (MW = 64 kDa). We again optimized the thickness of polymer layers by controlling LSPR shift to about 4 nm (Figure S1C) shown in aqueous media or air (Figure S1D). Upon exposure to $20 \mu\text{g/mL}$ hemoglobin, AuNR imprinted with Composition 1 exhibited a red shift of 9.6 ± 0.3 nm (Figure 4C). The inclusion of TMPhS (i.e., Composition 2) significantly increased the LSPR shift to 11.2 ± 0.1 nm ($P = 0.007$) for $20 \mu\text{g/mL}$ of Hb. Overall, we note an $\sim 26\%$ enhancement in the sensitivity, which was 1.4 times higher than the sensitivity enhancement noted for PLIN2. This truly demonstrates that although the size of the template may also influence sensitivity enhancement obtained by including TMPhS,²⁵ the aromatic amino acid content in the protein played a dominant role in improving the sensitivity of artificial antibodies with aromatic electron donor–acceptor interactions facilitated by TMPhS. The artificial antibodies showed high selectivity as evidenced by only ~ 1.0 nm LSPR shift upon exposure to either human or bovine serum albumin ($20 \mu\text{g/mL}$) (Figure 4D). This suggests that the incorporation of aromatic functionality in the artificial antibodies significantly improves the sensitivity but does not alter selectivity for the target protein.

The sensitivity of the artificial antibodies in this proof of concept study using gold nanorods is on the order of 20–100 nM. Previously, we had shown that the sensitivity of the artificial antibodies for the same analyte could be decreased to the 1 nM range by using gold nanocages as the plasmonic nanotransducer.⁹ The sensitivity of DNA-driven, self-assembling nanopyramids with a Raman reporter or aptamer-based, surface-enhanced Raman scattering sensors can push the sensitivity of biomarker assays to an attomolar range.^{40–43} Basically, assays can be tailor-made to measure biomarkers in concentration ranges of biologic importance with suitably functionalized transducers.

Conclusions

In summary, we investigated the role of aromatic interactions in the biorecognition capabilities of artificial antibodies using three model target proteins with different aromatic amino acid content. Artificial antibodies with aromatic functionality exhibited a significant, albeit protein-dependent, improvement in sensitivity. The sensitivity enhancement was found

to be higher for proteins with higher aromatic amino acid content. Significantly, inclusion of aromatic interactions did not compromise the specificity of artificial recognition elements. Our results clearly highlight the need for fine-tuning the composition of functional monomers employed for molecular imprinting. Enhancement in the sensitivity of plasmonic biosensors based on artificial antibodies through rational choice of the functional monomer composition takes this plasmonic biosensing technology closer to real-world applications.

Experimental Section

Trimethoxyphenylsilane (TMPhS, 97%), trimethoxypropylsilane (TMPS, 97%), (3-aminopropyl) trimethoxysilane (APTMS, 99%), *p*-aminothiophenol (*p*-ATP), glutaraldehyde (Grade I, 25% in water), cetyltrimethylammonium bromide (CTAB), lysozyme, human serum albumin, and hemoglobin (all lyophilized powders) were purchased from Sigma-Aldrich (St. Louis). Recombinant human Perilipin-2 (PLIN2) (liquid in 20 mM Tris-HCl buffer, pH 8.0) was purchased from Creative BioMart (Shirley, NY).

Gold Nanorod Synthesis

Gold nanorods (AuNRs) were synthesized by using a seed-mediated method.^{38,39} After sequential addition of 10 mL of 0.1 M CTAB, 2.5×10^{-4} M HAuCl₄, and 0.6 mL of ice-cold 10 mM sodium borohydride under vigorous magnetic stirring, the seed solution color changed to brown. Growth solution was prepared by adding 38 mL of 0.1 M CTAB, 0.4 mL of 10 mM silver nitrate, 2 mL of 10 mM HAuCl₄, and 0.22 mL of 0.1 M ascorbic acid in order under gentle stirring. Then 0.6 mL seed solution was mixed into the above colorless solution and left undisturbed in the dark for 15 h. The reaction solution turned to violet brown in the first hour. The AuNR solution was centrifuged twice at 8000 rpm for 20 and 10 min, respectively, to remove excess CTAB. The particles were redispersed in nanopure water before modifying glass substrates. The AuNR showed a narrow size distribution, whose average edge length was 49.1 ± 2.8 nm ($n > 50$) and side wall thickness was 13.6 ± 0.8 nm ($n > 50$) (Figure 1B,C). The spectral peak of the AuNR solution was 770 nm (Figure 1C).

Adsorption of Au Nanorods on Glass

Before coating with nanorods, piranha-cleaned glass substrates were exposed to 1% (3-mercaptopropyl)triethoxysilane (MPTES) solution in ethanol for 1 h and then rinsed with ethanol. The glass substrates were modified with nanorod solution for 3 h. Finally, the glass substrates were rinsed with water and thoroughly dried under a nitrogen stream.

Target Protein-Imprinting Procedure

The nanorod-modified glass substrates were immersed into a freshly prepared 2 mL phosphate buffer solution (pH 8.0) with 3 μ L of glutaraldehyde (25%) and 3 μ L of pATP (4 mM in ethanol). The immersion time was controlled within 1 min to achieve a polymer thickness of 3 nm, providing a 2–3 nm LSPR shift.⁸ After the samples were rinsed with buffer gently, protein immobilization was performed by exposing samples in 10 μ g/mL lysozyme (MW = 14.3 kDa), 5 μ g/mL human recombinant perilipin 2 (PLIN2; MW = 49.3 kDa), or 25 μ g/mL hemoglobin (MW = 64 kDa) solution for 2 h at 4 °C, respectively. Preliminary studies had been performed by monitoring the red shift of the LSPR peak to

saturate the activated nanorods with template protein and the time at 4 °C to achieve saturation. After rinsing in the same phosphate buffer solution, the protein-immobilized glass substrates were incubated into 3 mL of phosphate buffered saline (pH 7.5) with mixtures of the following: 10 μL of TMPS and 10 μL of APTMS (Composition 1); 5 μL of TMPS, 5 μL of APTMS, and 0.5 μL of TPhS (Composition 2); 10 μL of APTMS and 1 μL of TPhS, respectively (Composition 3). A 3 min reaction lead to a 3–4 nm LSPR red shift for synthesizing the artificial antibodies to lysozyme. Meanwhile, the reaction time was controlled within 10 min when detecting human recombinant perilipin 2 (PLIN2) and hemoglobin, resulting in a 4–5 nm LSPR red shifts at the polymerization step. After the samples were gently rinsed with phosphate buffer, they were kept in the buffer solution at 4 °C overnight. The imprinted substrates were exposed to a mixed solution of 2% sodium dodecyl sulfate and 10 mM oxalic acid for 1 h for template release by breaking the reversible imine bond. After removal of the template, the AuNR were thus functionalized with complementary cavities with well-designed binding sites. Each step in the process was verified by monitoring the extinction spectrum.

Characterization Techniques

The processes of AuNRs synthesis and molecular imprinting were monitored through extinction spectra using a Shimadzu UV-1800 spectrophotometer. Transmission electron micrograph images of the AuNRs were collected by a field emission transmission electron microscope (JEM-2100F, JEOL) at a voltage of 200 kV. The chemical composition of imprints were detected by surface-enhanced Raman spectra obtained by using Renishaw inVia confocal Raman spectrometer installed on a Leica microscope with a 50 $\times$ objective in a range of 100-3200 cm^{-1} .

Protein-Binding Experiments

The molecularly imprinted (MIP) glass substrates were immersed in solutions of lysozyme, hemoglobin, or PLIN2 (0.5 to 20 $\mu\text{g}/\text{mL}$ in phosphate buffered saline) for 4 h at 4 °C. Preliminary studies had been performed by monitoring the red shift in the LSPR peak to achieve analyte binding, usually 2 h, so this time was doubled. The substrates were then rinsed and dried under a stream of nitrogen prior to spectral analysis in air media. The cross-binding experiments were performed by immersing imprinted sensors (MIP-lysozyme, MIP-PLIN2, and MIP-hemoglobin) in a solution with 20 $\mu\text{g}/\text{mL}$ human serum albumin for 4 h at 4 °C, rinsing, and drying prior to spectral analysis.

Statistical Analysis

Significance between conditions was determined by Analyze-it for Excel 2010 (Analyze-it Software Ltd., Leeds, U.K.). Significance was defined as $P > 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Schematic illustration showing the steps involved in molecular imprinting of gold nanorods with different monomers. (B) TEM image of gold nanorods (scale bar is 50 nm). (C) Vis-NIR extinction spectra of aqueous suspension of gold nanorods. Inset shows the histogram of long axis length of gold nanorods obtained from TEM images.

Figure 2. Surface-enhanced Raman scattering spectra with different amorphous polymers on gold nanorods reveal the Raman bands corresponding to trimethoxyphenylsilane.

Figure 3.

Extinction spectra and corresponding LSPR shift corresponding to each step in the molecular imprinting process when detecting $5 \mu\text{g/mL}$ lysozyme. (A) Spectra in aqueous media with step 1 (black spectra), step 2 (red spectra), step 3 (blue spectra), and step 4 (lavender spectra). (B) Spectra in air with step 4 (black spectra), step 5 (blue spectra), and step 6 (red spectra). (C) Summary of LSPR peak spectra shift at each step of the imprinting process. (D) Sensitivity of MIP-AuNR nanosensor expressed by the shift of LSPR wavelength when detecting lysozyme at different concentrations with polymers of different compositions. (E) Specificity of the lysozyme-imprinted artificial antibody compared with interference protein (HSA or BSA) at distinct concentrations with polymers of different compositions.

Figure 4.

(A) Sensitivity of MIP-AuNR nanosensor expressed by the shift of LSPR wavelength when detecting PLIN2 at different concentrations with polymers of different compositions. (B) Specificity of the PLIN2-imprinted artificial antibody compared with interference protein (HSA or BSA) at distinct concentrations with polymers of different compositions. (C) Sensitivity of MIP-AuNR nanosensor expressed by the shift of LSPR wavelength when detecting hemoglobin at different concentrations with polymers of different compositions. (D) LSPR shift of interference proteins (HSA or BSA) on hemoglobin-imprinted plasmonic artificial antibody with polymers of different compositions.