Glycoprotein Assay

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A Boronate Affinity Sandwich Assay: An Appealing Alternative to Immunoassays for the Determination of Glycoproteins**

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Abstract: *Immunoassay has been an essential tool in many areas, including clinical diagnostics. However, it suffers from drawbacks, such as poor availability of high specificity antibodies, limited stability of biological reagents, as well as damage to health and susceptibility of chemical labels to the sample environment. Here we present a new approach, a boronate-affinity sandwich assay (BASA), for the specific and sensitive determination of trace glycoproteins in complex samples. BASA relies on the formation of sandwiches between boronate-affinity molecularly imprinted polymers (MIPs), target glycoproteins, and boronate-affinity surface-enhanced Raman scattering (SERS) probes. The MIP ensures the specificity, while the SERS detection provides the sensitivity. BASA overcomes the drawbacks of traditional immunoassays and offers a great prospect for application.*

Glycoproteins play vital roles in many biological processes, such as molecular recognition and immune response.[1] As the expression of glycoproteins is associated with the occurrence of diverse diseases, a large variety of glycoproteins have been employed as disease biomarkers for clinical diagnosis.[2] As a consequence of the limited concentration of glycoprotein biomarkers in biological samples as well as the presence of high-abundance interfering species in the sample matrix, analytical approaches for the specific detection of glycoprotein biomarkers in real-world applications require high-quality antibodies and high-sensitivity detection schemes.

Immunoassay has been an essential analytical tool for the analysis of proteins in many areas, such as clinical diagnostics,[3] biochemical research,[4] and antidoping analysis.[5] However, antibodies with high specificity toward their targets are difficult to prepare and thus costly. Antibodies also suffer from poor storage stability and loss of biological activity upon external treatment. Therefore, an antibody-free immunoassay-like assay is highly desirable.

Molecularly imprinted polymers (MIPs),[6] as economical and stable synthetic receptors with antibody-like binding properties, have found important applications from separation to sensing. Recently, general and facile imprinting approaches have been developed based on the reversible covalent binding between boronic acids and sugars[7] to prepare MIPs that can specifically recognize glycoproteins.[8] The boronate-affinity MIPs exhibited highly attractive characteristics, particularly high specificity, high affinity, and superb tolerance to interference, thus making them appealing alternatives to antibodies for applications in immunoassays.

To detect trace target antigens, immunoassays employ a variety of high sensitivity detection schemes and corresponding labels that are usually chemically conjugated to a desired antibody or antigen.[9] Widely used labels include enzymes, radioactive isotopes, DNA reporters, as well as fluorescence and chemiluminescence probes. However, these reagents are clearly associated with drawbacks. Enzymes and DNA reporters suffer from limited stability, radioactive isotopes are harmful to health, while fluorescence and chemiluminescence probes are susceptible to the sample environment. Compared with these detection schemes, surface-enhanced Raman scattering (SERS) exhibits several significant advantages, including ultrahigh sensitivity, less susceptibility to sample environment, rapid readout speed, and possibility for on-site or field detection.[10] SERS-based immunoassays have recently attracted great interest.[11]

Clearly, the combination of MIPs with SERS could generate new analytical approaches that outperform conventional immunoassays. Many attempts have been made in this direction.[12] However, most of them focused on small-molecule analytes and have not yet been applied to disease biomarkers. Here we present a boronate-affinity sandwich assay (BASA) for the specific and sensitive determination of trace glycoproteins in complex samples. The principle of BASA is shown in Scheme 1. BASA relies on the combination of a boronate-affinity MIP that functions similar to a capture antibody, as well as boronate-affinity-functionalized silver nanoparticles (AgNPs) that function as SERS probes. The boronate-affinity MIP ensures the specificity, while the SERS detection guarantees the sensitivity. A target glycoprotein is first specifically captured by a boronate-affinity MIP array from a sample under test. After unwanted species are washed away, the captured glycoprotein is labeled with boronate-affinity AgNPs. The formed MIP-target-AgNP sandwich is then subjected to SERS detection. As the use of undesirable biological and chemical reagents is avoided, BASA overcomes the drawbacks of conventional immunoassays. The procedure is straightforward, taking only 30 minutes in total.

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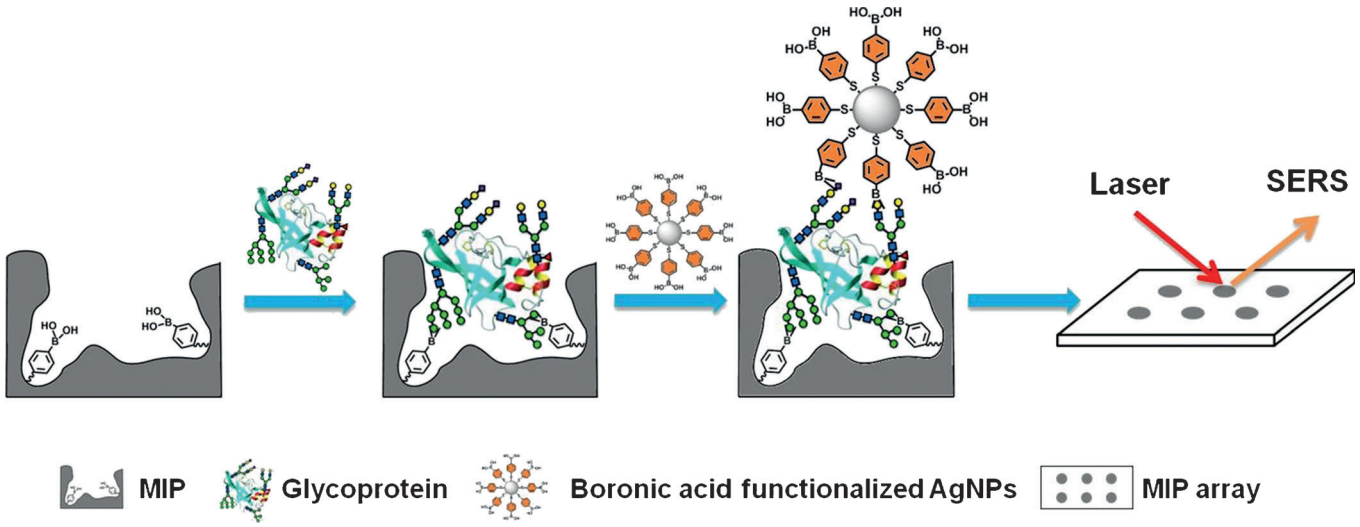

Scheme 1. Schematic representation of the boronate-affinity sandwich assay of glycoproteins.

The feasibility for real-world applications is demonstrated by the assay of trace α-fetoprotein (AFP) in human serum.

The boronate-affinity MIP arrays were prepared using the photolithographic boronate-affinity molecular imprinting[8a] approach, while the boronate-affinity SERS probe was prepared by modification of AgNPs with 4-mercaptophenylboronic acid (MPBA), which can function as a Raman reporter. Photo and electron microscopy images of a boronate-affinity MIP array, boronate-affinity AgNPs, and MIP-glycoprotein-AgNPs sandwiches are shown in Figure 1. As a proof-of-concept, an eight-spot array (Figure 1 a) was used in this study. However, MIP arrays can be easily fabricated into any format of larger numbers of spots for high-throughput assay, such as 96- and 384-spot arrays. The scanning electron microscopy (SEM) image (Figure 1 b) shows that the boronate-affinity MIP had a macroporous monolithic structure, while the transmission electron microscopy (TEM) image (Figure 1 c) indicates that the AgNPs were uniform-sized nanoparticles (diameter ca. 60 nm). The SEM image (Figure 1 d) shows that MIP-glycoprotein-AgNP sandwiches were formed by boronate-affinity binding between the MIP and the glycoprotein horseradish peroxidase (HRP) and between HRP and the SERS probes.

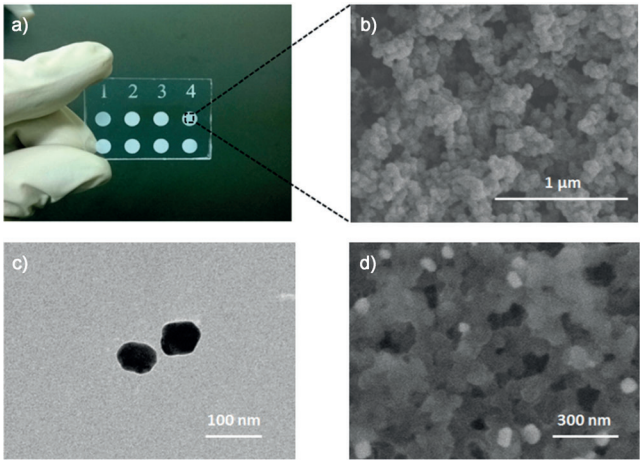

Figure 1. a) Photo image and b) SEM image of a boronate-affinity MIP array, c) TEM image of boronate-affinity AgNPs, and d) SEM image of MIP-glycoprotein-AgNP sandwiches (bright spots).

The TEM images (see Figure S1 in the Supporting Information) show that the AgNPs functionalized with boronic acid could disperse as well as the unfunctionalized AgNPs. The localized surface plasmon resonance (LSPR) of AgNPs before and after boronic acid modification was characterized by UV/Vis absorption spectrometry. The modification with MPBA did not alter the LSPR spectrum of AgNPs (see Figure S2 in the Supporting Information), which suggests a good dispersity of the MPBA-modified AgNPs. A comparison of the Raman spectrum of MPBA powder with the SERS spectrum of MPBA-modified AgNPs (see Figure S3 in the Supporting Information) confirms successful modification of the MPBA on the AgNPs. The MPBA concentration used for preparation of the SERS probe, the reaction time, and the incubation time for the reaction between the SERS probes and the captured glycoprotein were optimized (see Figures S4–S6 in the Supporting Information). The incubation time was extremely short, taking two minutes only. BASA is highly advantageous compared to an immunoassay, which needs hours or even overnight for incubation.

From the three different types of boronate-affinity MIPs that have been reported recently, including a nonporous MIP with poly(*m*-aminophenylboronic acid-*co*-dopamine) as an imprinting coating,[8b] a nonporous MIP with polyaniline as the imprinting coating,[8c] and a macroporous monolithic MIP,[8a] the last was selected for the development of the BASA because of its enhancement effect on the SERS signal. The macroporous monolithic MIP yielded the best SERS signal of the three MIP types under otherwise identical conditions (see Figure S7 in the Supporting Information), with the signal intensity 4.6 and 5.7 times higher than that of the two nonporous MIPs. To further confirm such an enhancement effect, the macroporous and nonporous MIPs were replaced by the boronate-affinity macroporous monolith

and nonporous layers while the target glycoprotein was substituted with glucose. Glucose can form 1:2 complexes with boronic acids, thus it can form a boronate-affinity sandwich with these boronate-affinity materials and MPBA-modified AgNPs. The SERS signal produced by the macroporous monolith was 2.6-fold higher than that of the nonporous layer (Figure 2a). Such a SERS enhancement is in agreement with a previous study,[13] in which the mechanism

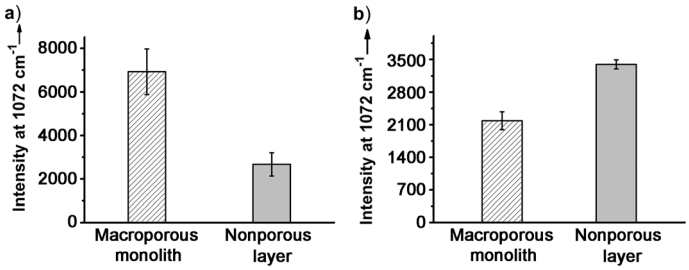

Figure 2. a) Comparison of the SERS intensity of MPBA-modified AgNPs absorbed on a glucose-saturated boronate-affinity macroporous monolith and nonporous layer, and b) the noise signals generated by the boronate-affinity macroporous monolith and nonporous layer.

was not clearly addressed. To rationalize this effect, the number of MPBA-modified AgNPs captured by the glucose-saturated macroporous monolith and nonporous layer was statistically analyzed by SEM (see Figures S8 and S9 in the Supporting Information). Although the macroporous monolith had a much larger surface area than the nonporous layer, the number of MPBA-modified AgNPs captured by the two materials was only slightly different (see Figure S10 in the Supporting Information). This finding suggests that the SERS enhancement was mainly due to another reason rather than the larger surface area of the macroporous monolith. Herein we propose a new mechanism called macroporous monolith-induced secondary Raman scattering (see Figure S11 in the Supporting Information). In addition to the normal Raman signal excited directly by incident laser, the macroporous monolith can reflect and scatter the laser light. The reflected and scattered laser light excites secondary Raman scattering, which enhances the overall SERS intensity. On the other hand, the noise produced by the macroporous monolith was lower than that by the nonporous layer by 35.6% (Figure 2b). This can be ascribed to the macroporous monolith having a lower signal-generating area.

To examine the performance of the BASA, HRP was first employed as a model glycoprotein. Figure 3a shows the Raman spectra for HRP with concentrations ranging from 0.1 ng mL^{-1} to 100 μg mL^{-1}. The results were further displayed by plotting the intensity at 1072 cm^{-1} against the logarithm of the HRP concentration, which generated a response curve (Figure 3b). The signal increased linearly with the logarithm of the protein concentration within the range of 1 ng mL^{-1} to 10 μg mL^{-1} ($r^2 = 0.99$). A response curve for a non-imprinted polymer (NIP) monolith under otherwise identical conditions is also shown in Figure 3b for comparison. The imprinting factor for the MIP was calculated to be 11.5 according to the maximum binding amounts of the MIP and NIP obtained from the two response curves, thereby confirming the significant binding affinity of the MIP toward the target glycoprotein. The binding isotherm for the MIP was further analyzed through data fitting according to the logistic function. The fitting yielded an excellent correlation ($r^2 = 0.99$; Figure 3c) and a dissociation constant (K_d) of 3.0 nM. The specificity of the boronate-affinity-sandwich approach was investigated using RNase B (glycoprotein), transferrin (TRF, glycoprotein), bovine serum albumin (BSA, nonglycoprotein), and glucose as interferants. The concentration of HRP was 1000- and 10000-fold lower than that of the interfering proteins and glucose, respectively. As shown in Figure 3d, all the interferants yielded slightly higher or comparable signals as the blank sample, while the signal for the target glycoprotein was significantly higher. The cross-reactivity of the HRP-imprinted MIP towards different proteins was measured. The highest cross-reactivity was for ovalbumin (9.7%; see Table S1 in the Supporting Information). As ovalbumin is a glycoprotein and its molecular weight (44.3 kDa) is very close to that of HRP (44.2 kDa), such cross-reactivity is acceptable. These results indicate the excellent specificity of the BASA approach. It benefited from the high specificity of the boronate-affinity MIP, which was further guaranteed by the boronate-affinity SERS probes. The reproducibility of the method was investigated. The relative standard deviation of the intensity for the concentration of 0, 1, and 1000 ng mL^{-1}

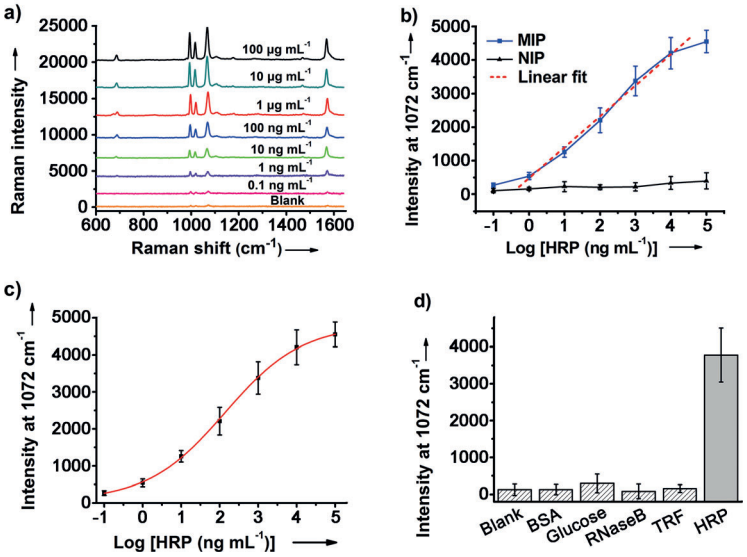

Figure 3. a) SERS spectra for HRP at different concentrations (containing 100 mM phosphate buffer, pH 7.4). b) Dependence of the SERS signal intensity detected on MIP and NIP arrays on the concentration of HRP. c) Logistic function fitting for determination of the binding constant. d) Interference test. Sample: 1 μg mL^{-1} HRP or 1 mg mL^{-1} interfering protein or 10 mg mL^{-1} glucose dissolved in 100 mM phosphate buffer, pH 7.4. The blank sample contained no protein or glucose.

was 28.8, 17.8, and 8.5%, respectively (see Figure S12 in the Supporting Information). Such reproducibility is highly acceptable for a SERS-based assay.

The feasibility of the BASA approach for real-world applications was demonstrated by an assay of the glycoprotein AFP in human serum. AFP has been routinely used as a biomarker in clinical screening for liver cancer. An AFP-imprinted MIP array was used to establish the assay. The MIP array exhibited a linear response toward AFP within the range of 1 ng mL^{-1} to 10 μg mL^{-1} (Figure 4a). The K_d value for the AFP-imprinted MIP was determined to be 0.7 nM (r^2 = 0.99; Figure 4b). To effectively reduce the influence of the

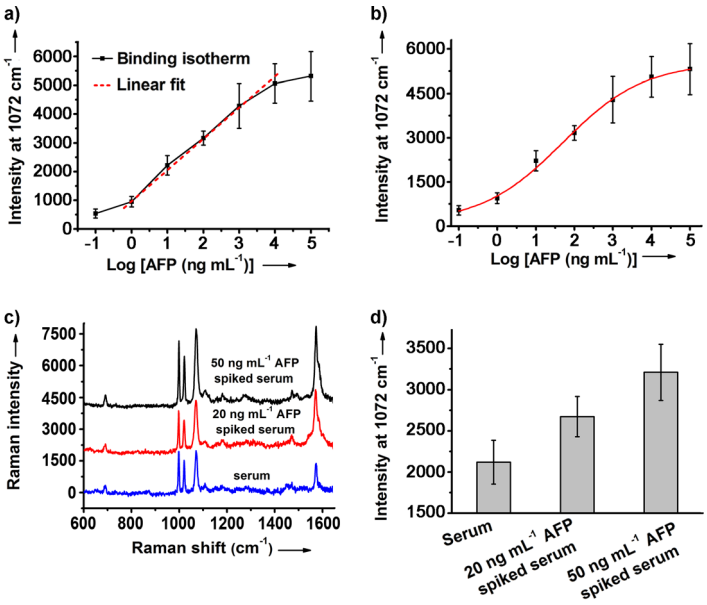

Figure 4. a) Dependence of the intensity of the SERS signal on the concentration of the AFP solution (containing 100 mM phosphate buffer, pH 7.4). b) Logistic function fitting for determination of the binding constant. c) SERS spectra for serum samples spiked with different AFP concentrations. d) SERS intensity for serum samples spiked with different AFP concentrations.

sample matrix, serum samples spiked with known AFP concentrations were measured. The AFP concentration in the serum from a healthy human was determined by the standard addition method to be 13.8 ± 3.3 ng mL^{-1}, which is in good agreement with the result obtained by another method (12.0 ± 2.0 ng mL^{-1}).[8c]

In summary, an appealing assay (BASA) has been developed through combining boronate-affinity MIP with boronate-affinity-based SERS detection. Its usefulness for real-world applications has been demonstrated. BASA exhibited significant advantages over a conventional immunoassay in terms of cost efficiency, stability, and speed. The method can be easily extended to other glycoprotein biomarkers. By using MIP arrays with larger numbers of spots, the BASA can be used for a high-throughput assay. The use of a portable Raman spectrograph allows point-of-care and on-site applications. We foresee the rapid development and promising applications of the approach in multiple areas, especially diagnostics.

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[1] P. M. Rudd, T. Elliott, P. Cresswell, I. A. Wilson, R. A. Dwek, *Science* **2001**, *291*, 2370–2376.
[2] H. Narimatsu, H. Sawaki, A. Kuno, H. Kaji, H. Ito, Y. Ikehara, *Febs. J.* **2010**, *277*, 95–105.
[3] J. Gosling, L. Basso, *Immunoassays*: *Laboratory Analysis and Clinical Applications*, CRC, London, **1994**.
[4] J. Vuori, S. Rasi, T. Takala, K. Vaananen, *Clin. Chem.* **1991**, *37*, 2087–2092.
[5] N. Robinson, P. E. Sottas, M. Saugy, *Clin. Lab.* **2010**, *56*, 197–206.
[6] a) G. Wulff, A. Sarhan, *Angew. Chem.* **1972**, *84*, 364–364; *Angew. Chem. Int. Ed. Engl.* **1972**, *11*, 341–341; b) G. Vlatakis, L. I. Andersson, R. Muller, K. Mosbach, *Nature* **1993**, *361*, 645–647; c) Y. Hoshino, H. Koide, T. Urakami, H. Kanazawa, T. Kodama, N. Oku, K. J. Shea, *J. Am. Chem. Soc.* **2010**, *132*, 6644–6645.
[7] a) R. Nishiyabu, Y. Kubo, T. D. James, J. S. Fossey, *Chem. Commun.* **2011**, *47*, 1106–1123; b) L. B. Ren, Z. Liu, Y. C. Liu, P. Dou, H. Y. Chen, *Angew. Chem.* **2009**, *121*, 6832–6835; *Angew. Chem. Int. Ed.* **2009**, *48*, 6704–6707; c) H. Y. Li, Z. Liu, *Trac-Trend. Anal. Chem.* **2012**, *37*, 148–161.
[8] a) L. Li, Y. Lu, Z. J. Bie, H. Y. Chen, Z. Liu, *Angew. Chem.* **2013**, *125*, 7599–7602; *Angew. Chem. Int. Ed.* **2013**, *52*, 7451–7454; b) S. S. Wang, J. Ye, Z. J. Bie, Z. Liu, *Chem. Sci.* **2014**, *5*, 1135–1140; c) X. D. Bi, Z. Liu, *Anal. Chem.* **2014**, *86*, 959–966.
[9] a) E. P. Diamandis, T. K. Christopoulos, *Immunoassay*, Academic Press, New York, **1996**; b) B. Schweitzer, S. Wiltshire, J. Lambert, S. O'Malley, K. Kukanskis, Z. R. Zhu, S. F. Kingsmore, P. M. Lizardi, D. C. Ward, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 10113–10119.
[10] a) X. M. Qian, S. M. Nie, *Chem. Soc. Rev.* **2008**, *37*, 912–920; b) M. D. Porter, R. J. Lipert, L. M. Siperko, G. F. Wang, R. Narayanan, *Chem. Soc. Rev.* **2008**, *37*, 1001–1011; c) J. F. Li, Y. F. Huang, Y. Ding, Z. L. Yang, S. B. Li, X. S. Zhou, F. R. Fan, W. Zhang, Z. Y. Zhou, D. Y. Wu, B. Ren, Z. L. Wang, Z. Q. Tian, *Nature* **2010**, *464*, 392–395.
[11] a) X. Dou, T. Takama, Y. Yamaguchi, H. Yamamoto, Y. Ozaki, *Anal. Chem.* **1997**, *69*, 1492–1495; b) J. Ni, R. J. Lipert, G. B. Dawson, M. D. Porter, *Anal. Chem.* **1999**, *71*, 4903–4908; c) J. D. Driskell, K. M. Kwarta, R. J. Lipert, M. D. Porter, J. D. Neill, J. F. Ridpath, *Anal. Chem.* **2005**, *77*, 6147–6154; d) X. X. Han, Y. Kitahama, Y. Tanaka, J. Guo, W. Q. Xu, B. Zhao, Y. Ozaki, *Anal. Chem.* **2008**, *80*, 6567–6572.
[12] a) M. Bompart, L. A. Gheber, Y. De Wilde, K. Haupt, *Biosens. Bioelectron.* **2009**, *25*, 568–571; b) K, Kantarovich, I, Tsarfati, L. A. Gheber, K. Haupt, I. Bar, *Anal. Chem.* **2009**, *81*, 5686–5690; c) M. Bompart, Y. De Wilde, K. Haupt, *Adv. Mater.* **2010**, *22*, 2343–2348.
[13] J. Liu, I. White, D. L. DeVoe, *Anal. Chem.* **2011**, *83*, 2119–2124.