VIP Molecular Imprinting Very Important Paper

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Boronate-Affinity Glycan-Oriented Surface Imprinting: A New Strategy to Mimic Lectins for the Recognition of an Intact Glycoprotein and Its Characteristic Fragments**

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Abstract: *Lectins possess unique binding properties and are of particular value in molecular recognition. However, lectins suffer from several disadvantages, such as being hard to prepare and showing poor storage stability. Boronate-affinity glycan-oriented surface imprinting was developed as a new strategy for the preparation of lectin-like molecularly imprinted polymers (MIPs). The prepared MIPs could specifically recognize an intact glycoprotein and its characteristic fragments, even within a complex sample matrix. Glycan-imprinted MIPs could thus prove to be powerful tools for important applications such as proteomics, glycomics, and diagnostics.*

Lectins are unique among biomolecules that can recognize a target molecule. A lectin recognizes a specific sugar moiety. It can thus also recognize a compound in the form of a glycoprotein, glycopeptide, glycan, or other structure, provided that the compound contains the sugar moiety. Such binding properties have particular value in molecular recognition since they enable the indirect detection or monitoring of an unstable glycoconjugate through detection of its characteristic fragments. So far, lectins have been useful tools in proteomics,[1] glycomics,[2] and diagnostics.[3] However, lectins suffer from several disadvantages, such as being hard to prepare, showing poor storage stability, and requiring solutions with high sugar content for target release. Therefore, alternatives that can overcome these drawbacks are highly desirable.

Boronic acids can covalently bind *cis*-diol-containing compounds such as sugars, glycoproteins, and glycopeptides at relatively high pH values, while the complexes dissociate reversibly when the environmental pH becomes acidic.[4] Boronate affinity has been an important means for mimicking the properties of lectins for a long time.[5] However, boronate-affinity materials usually exhibit class selectivity toward *cis*-diol-containing compounds[6] it is therefore difficult to provide exactly lectin-like binding properties.

Molecularly imprinted polymers (MIPs),[7] which are chemical receptors synthesized through polymerization in the presence of a template, have developed into important functional materials with antibody-like binding properties or enzyme-like catalytic activities, and they have found wide applications from sensing to separation to catalysis. Although MIPs specific to sugars, particularly glucose, have been widely reported,[8] to the best of our knowledge, MIPs that can recognize both an intact glycoprotein and its characteristic fragments have not been reported to date. Recently, by using boronic acids as functional moieties, MIPs with excellent binding properties have been developed for the specific recognition of glycoproteins, and these MIPs have exhibited great potential for real-world applications such as medical diagnostics.[9] These studies inspired us to further mimic lectins by combining boronate affinity and molecular imprinting.

In this study, we present a new strategy for producing lectin-like MIPs that can specifically recognize an intact glycoprotein and its characteristic fragments. The strategy is called boronate-affinity glycan-oriented surface imprinting. As a proof of principle, magnetic nanoparticles (MNPs) were used as a substrate material for the imprinting. The glycan preparation and imprinting procedures are illustrated in Scheme 1. Glycans (multiple or single) of a target glycoprotein are digested by a glycosidase and purified as templates by ultrafiltration. Boronic acid functionalized MNPs are prepared as nanoscale cores. The glycan templates are immobilized onto the boronic acid functionalized MNPs through boronate-affinity interactions. Then, a thin layer of polymer of appropriate nature is formed to cover the glycans to an appropriate thickness. After that, the templates are removed by washing with an acidic solution to disrupt the boronate-affinity interaction. The prepared MIPs can recognize not only the intact glycoprotein but also its characteristic fragments, including glycopeptides and glycans. The bound species can be readily released by eluting with an acidic solution. As a result of these unique features, glycan-imprinted MIPs could be powerful tools for monitoring unstable glycoproteins in biological samples, particularly in proteomics, glycomics, and medical diagnostics.

A detailed procedure for the synthesis of glycan-imprinted MNPs is shown in Figure S1 in the Supporting Information. Two issues are critical for the imprinting of glycan(s). On the one hand, an imprinting coating of an appropriate nature has to be available, since it will mainly determine the binding properties of the prepared MIPs

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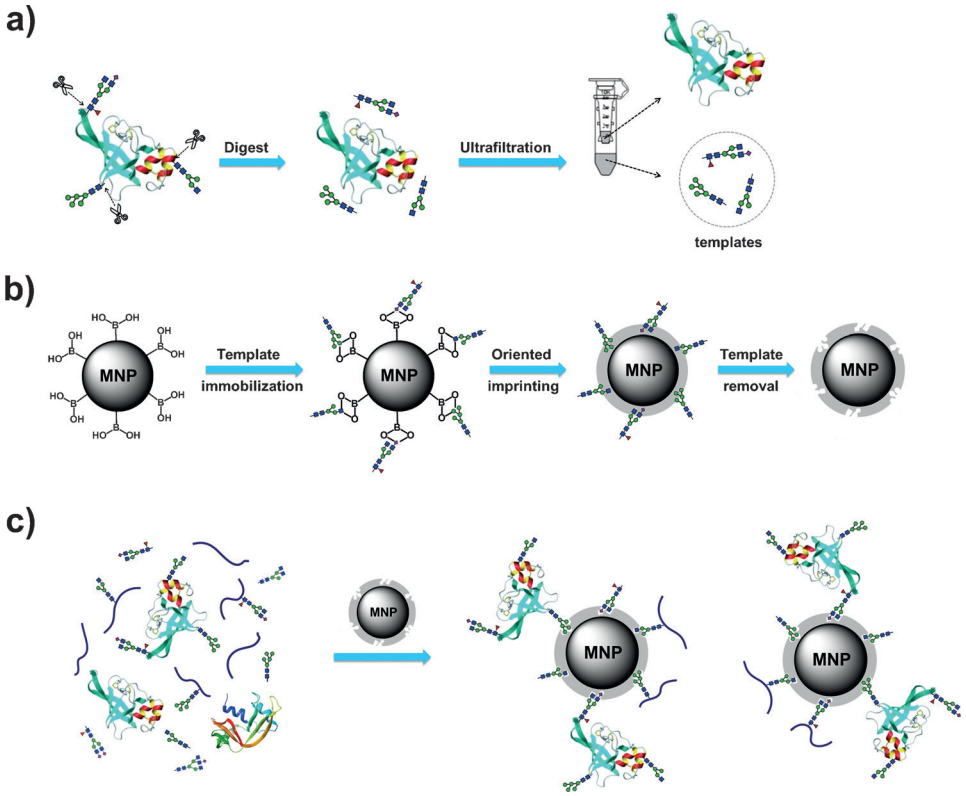

Scheme 1. a) Preparation of the glycan templates. b) Boronate-affinity glycan-oriented surface imprinting for producing glycan-imprinted MNPs. c) Recognition of a glycoprotein and its characteristic fragments by glycan-imprinted MNPs.

toward the templates. Furthermore, the thickness of the imprinting coating must be controlled to cover all of the immobilized glycan templates to an appropriate thickness. In this study, a silica thin layer formed by hydrolysis in ethanol of tetraethyl orthosilicate (TEOS) was employed as an imprinting coating. There are two reasons for the choice of TEOS. First, a silica layer formed by TEOS polycondensation is hydrophilic and thereby non-specific adsorption can effectively be avoided. Second, the thickness of the resulting silica layer can be controlled. As shown in Figure 1 a, the thickness of the silica layer increases linearly with increasing polymerization time ($R^2 = 0.994$, slope $= 0.04\ \text{nm min}^{-1}$). This indicates that the thickness of the silica layer can be controlled by simply adjusting the polymerization time.

In this study, RNase B, which possesses one glycosylation site, to which five high-mannose glycan isoforms may be attached, was first used as a target glycoprotein, and its glycans were used as the templates. The structures and estimated lengths of the glycan isoforms of RNase B are listed in Table S1 in the Supporting Information. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of the glycans digested from RNase B by PNGase F indicates that only four glycans (M5–M8) were present at detectable levels (Figure S3). Thus, only glycans M5–M8 were employed as eventual templates.

According to the dependence of the layer thickness on the polymerization time, the polymerization time for the imprinting was optimized by setting the polymerization time at 40, 50, and 60 min. The imprinting effect was evaluated in terms of imprinting factor (IF), which was calculated according to the ratio of the absorbance value of RNase B captured by glycan-imprinted MNPs over that for non-imprinted MNPs. As shown in Figure S6, glycan-imprinted MNPs prepared with 50 min polymerization time exhibited the best performance, with an IF value of 8.4. Actual binding affinity and specificity also confirmed the optimization result (Figure S7–S9). The RNase B glycan-imprinted MNPs prepared with the optimal polymerization time were thus used in the rest of the experiments.

The magnetization curves (Figure 1 b) show that the saturation magnetization of boronic acid functionalized MNPs, RNase B glycan-imprinted MNPs, and non-imprinted MNPs were about 46, 30, and 20 emu g^{-1}, respectively, which indicates superparamagnetic behavior. The glycan-imprinted MNPs dispersed homogeneously in water and were quickly attracted to the wall of the container by an external magnet (Figure 1 c). Upon removing the magnet, the aggregated particles could be redispersed by ultrasonication. The boronic acid functionalized MNPs, RNase B glycan-imprinted MNPs, and non-imprinted MNPs were all well shaped with a diameter of about 100 nm (Figure 1 d–f).

The binding isotherm of the RNase B glycan-imprinted MNPs toward RNase B was evaluated. As shown in Figure 2 a, the RNase B glycan-imprinted MNPs exhibited much stronger affinity toward RNase B compared to the non-imprinted MNPs. Scatchard plot analysis (Figure S10) gave a maximum absorption capacity of $0.097 \pm 0.009\ \mu\text{mol g}^{-1}$ and a dissociation constant (K_d) of $24.89 \pm 2.48\ \mu\text{M}$. Although the K_d value was much higher than for boronate-affinity glycoprotein-imprinted MIPs (10^{-8}–10^{-10} M),[9] this is still reasonably acceptable because the nature of the imprinting coating has not been optimized yet. The imprinting efficiency was calculated to be 44.8% (see the Supporting Information), thus indicating that the imprinting approach was highly efficient. The specificity toward intact proteins was examined by using RNase B as a target protein, with RNase A (a non-glycosylated protein that possesses an identical polypeptide chain to RNase B), transferrin (TRF, a sialylated glycoprotein containing two glycosylation sites for the attachment of two identical sialylated glycans), and horseradish peroxidase (HRP, a glycoprotein containing nine glycosylation sites occupied by eight or nine identical hybrid glycans) as

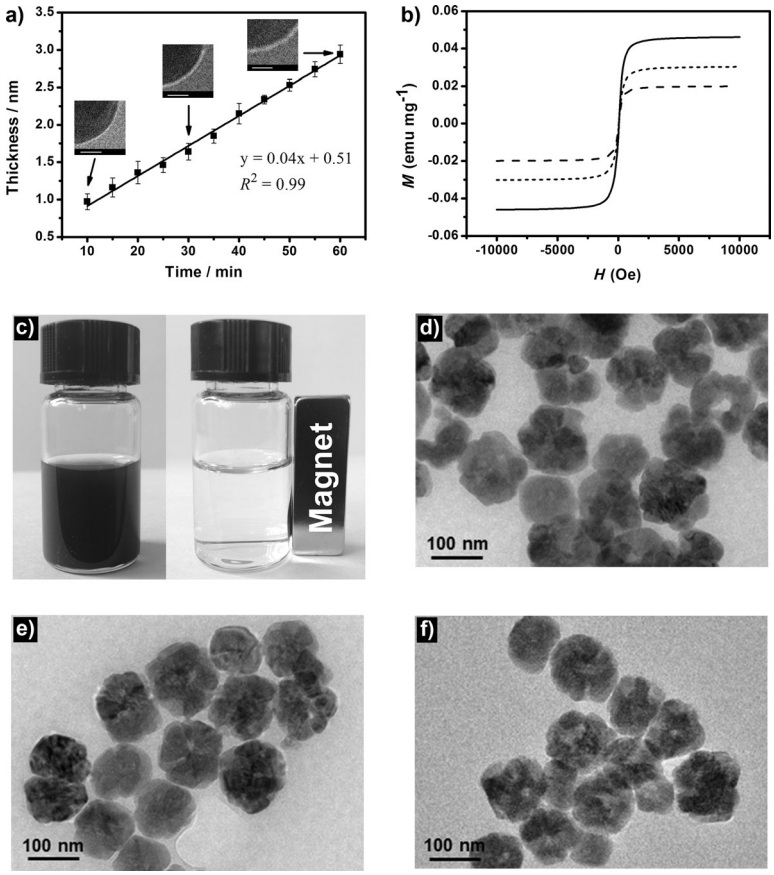

Figure 1. a) Dependence of the thickness of the silica thin layer on polymerization time. Insets: TEM images of silica coated Ag NPs at different polymerization times (scale bars: 10 nm). b) Magnetization curve of boronic acid functionalized MNPs (——), RNase B glycan-imprinted MNPs (-----), and non-imprinted MNPs (– – –). c) Photographs showing dispersion and magnetic separation of the RNase B glycan-imprinted MNPs. TEM images are shown for boronic acid-functionalized MNPs (d), RNase B glycan-imprinted MNPs (e), and non-imprinted MNPs (f).

competing proteins (see glycan structures in Figure S11). As shown in Figure 2b, the imprinted MNPs exhibited high specificity. This was further confirmed by the corresponding MALDI-TOF MS results (Figure S8). The imprinted MNPs could be used six times consecutively without apparent loss of specificity (Figure S12). Moreover, high-concentration free mannose neither significantly reduced the affinity toward RNase B nor released RNase B captured by the imprinted MNPs (Figure S13).

Next, the recognition specificity at the peptide level was investigated. Tryptic digest of HRP was employed to give competing species. A mixture of tryptic digests of RNase B and HRP (weight ratio, 1:1) was treated with the imprinted MNPs or non-imprinted MNPs. Before extraction, four glycopeptides from RNase B and four glycopeptides from HRP were detected (Figure 3). However, after extraction with the glycan-imprinted MNPs, only the four glycopeptides from RNase B were detected (See Table S2 for the identities of the glycopeptides). By contrast, nearly nothing was extracted by the non-imprinted MNPs. To further examine the specificity, mixtures of tryptic digest of RNase B and bovine serum albumin (BSA) in the weight ratios 1:1 and 1:50 were employed as samples. As shown in Figure S14, four glycopeptides from RNase B, as well as a few non-glycosylated peptides, were extracted by the glycan-imprinted MNPs. Although a few non-glycosylated peptides were also extracted, considering that a large number of non-glycosylated peptides were present in the samples, such specificity is well acceptable. By contrast, no glycopeptides were extracted by non-imprinted MNPs. These results suggest that the RNase B glycan-imprinted MNPs exhibit high specificity at the peptide level. Owing to difficulty in the detection of glycans, specificity toward the template glycans was not directly demonstrated. However, the specificity toward glycopeptides containing the template glycans indirectly confirms specificity toward the template glycans.

To further investigate the properties of RNase B glycan-imprinted MNPs, human serum samples spiked with RNase B and its tryptic digest were analyzed. Human serum is a complex biological sample containing a huge variety of compounds. The results indicate that the RNase B glycan-imprinted MNPs exhibited excellent specificity toward the intact target glycoprotein and its characteristic glycopeptides in human serum (Figure S15 and S16).

The generality of this new strategy was further demonstrated by applying this method to a different glycoprotein. TRF was chosen as another target because TRF contains a different type of glycan. More importantly, TRF exists in mammalian systems and its levels in human serum are associated with several diseases, such as iron deficiency anemia, protein malnutrition, and atransferrinemia.[10] TRF glycan-imprinted MNPs were easily prepared according to the method by simply setting the imprinting time to an appropriate value. The imprinting factor was 21.8 while the imprinting efficiency was 43.5% (see the Supporting Information), which further confirms the high efficiency of the imprinting approach. The specificity of TRF glycan-imprinted MNPs was investigated at the intact protein and peptide levels. At the intact protein level, only TRF was extracted by the TRF glycan-imprinted MNPs (Figure S17 and S18). At the peptide level, from the tryptic digest of a mixture of TRF, RNase B, and HRP, only the glycopeptides of TRF were extracted by the TRF glycan-imprinted MNPs (Figure S20). These results demonstrate the generality of the new strategy.

The potential of glycan-imprinted MNPs for real-world applications was demonstrated by the extraction of TRF directly from human serum. Human serum contains TRF at relatively low abundance, as well as many abundant interfering compounds, such as human serum albumin (HSA), immunoglobulin G (IgG), and sugars. TRF was purified and enriched from a human serum sample through extraction with the TRF glycan-imprinted MNPs (Figure 4).

In summary, we have proposed a new strategy to mimic lectins for the recognition of an intact glycoprotein and its

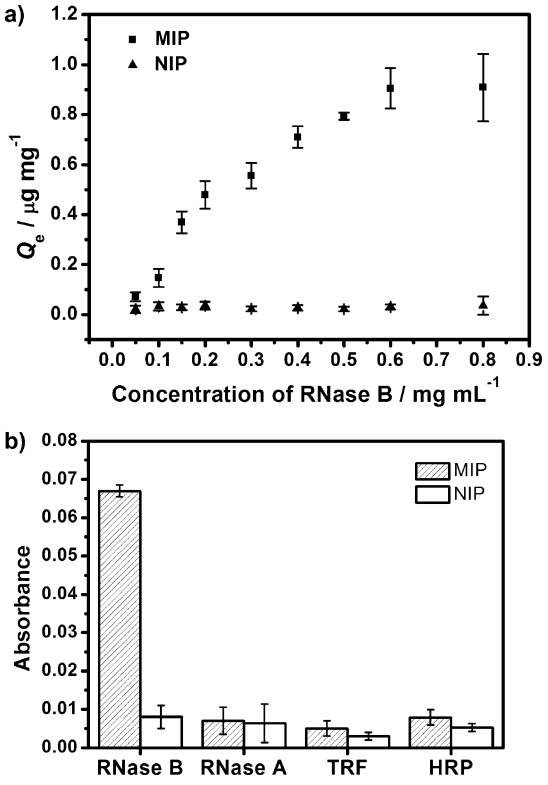

Figure 2. a) Binding isotherms for binding of the RNase B glycan-imprinted MNPs and non-imprinted MNPs to RNase B. b) Comparison of the amounts of different proteins captured by the imprinted MNPs and non-imprinted MNPs.

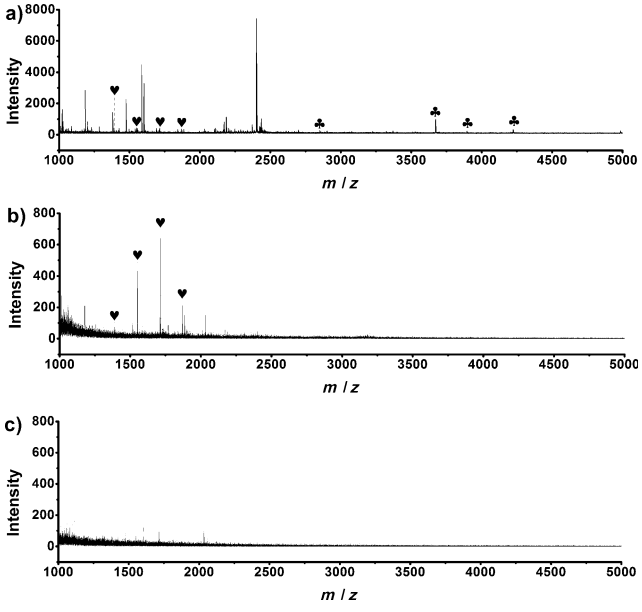

Figure 3. MALDI-TOF MS spectra for a) direct analysis of the mixture of tryptic digest of RNase B and HRP with the weight ratio 1:1, b) analysis after extraction by the RNase B glycan-imprinted MNPs, and c) analysis after extraction by the non-imprinted MNPs. Hearts = glycopeptides from tryptic digest of RNase B; clubs = glycopeptides from tryptic digest of HRP.

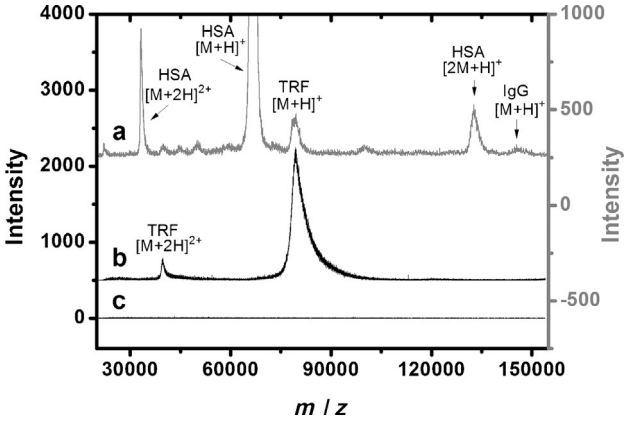

Figure 4. MALDI-TOF MS spectra for the analysis of human serum. a) Direct analysis. b) Analysis after extraction with TRF glycan-imprinted MNPs. c) Analysis after extraction with non-imprinted MNPs.

characteristic fragments. Compared to lectins, glycan-imprinted MIPs are much easier to prepare, more cost-efficient, and more stable. Furthermore, target release can be carried out under gentle conditions. The proposed strategy is applicable to a large variety of glycoproteins. The unique binding properties of this system could prove to be promising assets for important applications such as glycoproteomics and medical diagnostics.

Keywords: boronate affinity · glycans · glycoproteins · molecular imprinting · molecular recognition

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