Cobalt-Directed Assembly of Antibodies onto Metal–Phenolic Networks for Enhanced Particle Targeting

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The orientation-specific immobilization of antibodies onto nanoparticles, to preserve antibody–antigen recognition, is a key challenge in developing targeted nanomedicines. Herein, we report the targeting ability of metal–phenolic network (MPN)-coated gold nanoparticles with surface-physisorbed antibodies against respective antigens. The MPN coatings were self-assembled from metal ions (Fe^{III} , Co^{II} , Cu^{II} , Ni^{II} , or Zn^{II}) crosslinked with tannic acid. Upon physisorption of antibodies, all particle systems exhibited enhanced association with target antigens, with Co^{II} systems demonstrating more than 2-fold greater association. These systems contained more metal atoms distributed in a way to specifically interact with antibodies, which were investigated by molecular dynamics simulations. A model antibody fragment crystallizable (Fc) region in solution with Co^{II} -tannic acid complexes revealed that the solvent-exposed Co^{II} can directly coordinate to the histidine-rich portion of the Fc region. This one-pot interaction therefore anchors the antibody Fc region to the MPN, allowing for enhanced targeting.

KEYWORDS: antibodies, targeting, self-assembly, metal–phenolic networks, molecular modeling

Antibodies (Abs) are glycoproteins that are key components of the immune system and important for exploiting target-specific treatments in bio-nanomedicine.¹⁻⁴ Ab-based targeting of tissues is an attractive concept owing to the unique in vivo properties and high target specificities of Abs when compared to small-molecule-based motifs,⁵ thus allowing for the specific recognition of complex cellular interfaces even in the presence of a protein corona (PC).⁶ A variety of Abs, mostly immunoglobulin G (IgG), have been used as protein drugs for targeted therapy (e.g., Herceptin (trastuzumab) for breast cancer and Rituxan (rituximab) for leukemia and lymphoma),⁷⁻⁹ as biomolecule detectors in western blotting and enzyme-linked immunosorbent assays, as well as for decorating motifs in targeted drug delivery.¹⁰⁻¹² Abs are composed of two fragment antigen binding (Fab) regions and one fragment crystallizable (Fc) region.¹³⁻¹⁵ The complementarity-determining regions with the Fab vary highly, whereas the Fc region is highly conserved between different Abs. When Abs are conjugated to a drug carrier, for example, the main challenge is to preserve the activity of the Fab regions, i.e., to specifically conjugate via the Fc region and keep the Fab regions available for target engagement,^{16,17} which requires control over the conjugation process.

The physical adsorption of Abs onto substrates, such as nanoparticles, often occurs via hydrophobic and/or electrostatic interactions. Although adsorption via such interactions offers simplicity, these interactions are difficult to control and Abs can be immobilized in a random orientation, denatured, or displaced in subsequent washing steps.¹⁷ Abs can also be covalently conjugated to carboxylate-decorated nanoparticles via amine–carboxylic acid coupling chemistry. However, such conjugation is not specific for the Fc region. The orientation in this case depends on the surface density of the Ab, for example.¹⁸ Importantly, the lack of control over the Ab orientation in these methods can result in the loss of the antigen-recognizing function of the Abs.¹⁹

More sophisticated strategies to specifically conjugate Abs in the Fc region include boronate ester covalent conjugation with vicinal diols in *N*-glycans present in the Fc region²⁰ and genetic modification of the Abs to include coupling sites, such as histidine tags, biotin, and “click” functionalities, but these can be relatively difficult to introduce.²¹⁻²⁵ Although effective, these strategies increase the complexity of synthesizing the conjugates by requiring advanced protein engineering.²⁶ As an alternative, some transition metal ions, including Co^{II}, Ni^{II}, Cu^{II}, and Zn^{II}, can bind to the histidine-rich domain in the Fc region via labile inorganic coordination bonding, which can be exploited for attaching Abs to a substrate in a favorable orientation.²⁷⁻³¹

We have previously reported a facile strategy to prepare metal–phenolic network (MPN) films comprising metal ions and chelated poly phenolic ligands.³² MPNs can coat various substrates to achieve different applications, e.g., medical imaging,^{33,34} modular assembly,³⁵ and cell targeting.^{36,37} For instance, we have previously demonstrated the use of cobalt(II)-tannic acid (Co-TA) networks in inhibiting amyloid fibril formation.³⁸ This distinct feature of the Co-TA networks was attributed to the strong interactions between the solvent-exposed cobalt metal center and histidine and/or methionine residues on the amyloid β peptide. Herein, we report the targeting efficiencies of Abs immobilized on MPN-coated gold nanoparticles (AuNP@MPNs) with different metal ions (Fe^{III}, Co^{II}, Ni^{II}, Cu^{II}, or Zn^{II}) and a PC (Figure 1a). Targeting was assessed against antigens and cells. The interaction between the MPNs and Abs was probed by molecular dynamics (MD) simulations, allowing direct inference of the specific metal–antibody interaction and subsequently the targeting efficiency toward antigens.

To determine MPN film thickness when different metals are used, scratch atomic force microscopy (scratch-AFM) was performed on planar silicon wafer substrates that were first sputter-coated with Au and subsequently coated with Fe^{III}, Co^{II}, Ni^{II}, Cu^{II}, or Zn^{II} MPNs. After

washing and drying, AFM measurements were performed on each system (Figure S1a). Among the MPN film systems examined, MPN films containing Fe^{III} were the thickest, ~ 8 nm dry height, which is similar to the thickness achieved for Fe^{III} -TA MPNs on other templates.³⁹ The other metal systems displayed film thicknesses of 2–4 nm. All films were reasonably smooth (Figure S1b). The surface morphology of the bare Au substrate, Au@Co-TA film, and Au@Co-TA film with immobilized Herceptin (Her), Au@Co-TA@Her, can be observed from the in situ (wet) AFM images in Figure S1c–e. The average surface roughness (R_a) of the bare surface was 1.18 nm, whereas the R_a values of the Au@Co-TA and Au@Co-TA@Her were 0.96 and 1.84 nm, respectively. Owing to the roughness of the surface, which masked the presence of small features, the individual location or alignment of Abs at the interface could not be determined. In general, these features are more readily observed when adsorbed on atomically smooth substrates;⁴⁰ however, distinct differences were noted among the three images, confirming the modification of the bare Au surface.

Figure 1. (a) Schematic of the formation of antibody and protein corona layers on AuNP@MPNs. Antibodies orient most favorably in the presence of Co^{II} MPNs. (b) Size distribution of AuNP@Co-TA@Ab without or with a protein corona determined by dynamic light scattering. (c) ζ -Potential of different AuNP@MPNs without or with an antibody and protein corona layer. Ab, antibody; PC, protein corona.

All AuNP@MPNs with different metal ions were prepared and characterized as previously described.³⁸ Briefly, TA-stabilized AuNPs of ~20 nm diameter were coated with different metal-TA networks to form AuNP@MPNs. Sheep serum IgG was used as a model Ab and incubated with AuNP@Co-TA to form AuNP@Co-TA@Ab. During the washing cycle by centrifugation to remove excess Abs, we observed aggregation of AuNP@Co-TA@Ab even with 20 min of sonication. To address this problem, a PC was used to stabilize the particles.⁴¹ After incubation with the Abs, full fetal bovine serum was added to the mixture to form the PC (Figure 1a). AuNP@Co-TA@Ab with the PC remained relatively monodisperse after intensive washing by centrifugation (Figure 1b). Notably, the presence of a PC has also been reported to reduce nonspecific cell association of MPN capsules.³⁷ Cell association of AuNPs was analyzed by cytometry by time-of-flight (CyTOF) by measuring the ¹⁹⁷Au mass intensity of single cells. The medium ¹⁹⁷Au intensity value was used to quantify the association level. Bare AuNP@Co-TA without a PC showed high cell association with RAW 264.7 cells in serum-free medium, whereas in the presence of a PC, cell association decreased significantly (Figure S2).

After the adsorption of Abs and PC, all AuNP@MPN@Ab systems, with different metals, showed similar UV-visible spectra (Figure S3). In addition, all AuNP@MPN@Ab systems showed similar ζ -potentials (approximately -10 mV). In contrast, the AuNP@MPNs systems featured ζ -potentials of approximately -35 mV (Figure 1c). As determined by dynamic light

scattering, AuNP@MPN@Ab were larger (~45 nm) than AuNP@MPNs (~20 nm), indicating the successful formation of PC and Ab coatings (Figure S4). However, owing to the low density of protein, the Ab and PC layers of AuNP@MPN@Ab could not be directly observed by transmission electron microscopy (TEM)—only the MPN coating was observed (Figure S5). The adsorbed Abs and PC on the AuNP@MPNs were eluted in loading buffer with heating and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Various protein bands were observed, including bands corresponding to the heavy and light chains of the Ab, and the patterns were similar for all AuNP@MPN@Ab systems, which suggests that PC formation is relatively independent of the incorporated metal ion (Figure S6).

Next, we determined the Ab loading amount on AuNP@MPNs with Alexa 647-labeled rabbit anti-goat IgG (647-RaG). As AuNPs may partly quench the fluorescence of 647-RaG, we used an indirect method to determine the loading amount by measuring the unbound 647-RaG in supernatants. Notably, we observed a relatively lower Ab loading on AuNP@Co-TA, whereas comparable higher loading amounts were observed for the other AuNP@MPNs (Figure 2a).

To explore the Ab presentation on the surface of AuNP@MPNs, we used a “secondary” Ab Alexa 488-labeled goat anti-rat IgG (488-GaR) as the antigen for 647-RaG. Immobilized 647-RaG Abs with accessible Fab regions on the AuNP@MPN surface can specifically bind to 488-GaR. Thus, from the on-particle fluorescence measurement of bound 488-GaR, the RaG Fab region presentation on AuNP@MPN@647-RaG nanoparticles with different incorporated metal ions was compared. The binding of the antibody–antigen complex introduces a larger distance between the Alexa 488 label and the Au NP,⁴² therefore we expect potential fluorescence quenching effects to be negated. The fluorescence intensity of the antigen, 488-GaR, bound to AuNP@Co-TA@647-RaG was ~1.7 times higher than the fluorescence intensities observed for the other

AuNP@MPN@647-RaG systems, which were comparable (Figure S7). As the 647-RaG loading amounts varied between the different systems, with AuNP@Co-TA@647-RaG showing the lowest loading amount, the fluorescence intensity of 488-GaR was normalized based on the 647-RaG loading amount to determine the actual 647-RaG Fab region presentation that is the most suitable for quantifying the specific interaction with the antigen. The normalized results showed that cobalt achieved a higher fluorescence intensity (>2.2-fold) than the other metal MPNs (Figure 2b). These results suggest that AuNP@Co-TA@647-RaG features more Fab-accessible 647-RaG than the other AuNP@MPN@647-RaG systems.

Figure 2. (a) Alexa 647-labeled rabbit anti-goat IgG (647-RaG) loading on different AuNP@MPNs (1 nM). Error bars represent standard deviations. Statistical significance was determined by a two-tailed *t*-test and is reported as $*p < 0.05$. (b) Normalized fluorescence intensity of bound Alexa 488-labeled goat anti-rat IgG on different AuNP@MPN@647-RaG systems based on 647-RaG loading amounts. Error bars represent standard deviations.

To further confirm the enhanced targeting in the presence of cobalt, the current system was examined toward in vitro cell targeting. Herceptin (Her) was used to target human epidermal growth factor receptor 2 positive (Her2+) cell line BT-474. AuNP@MPN@Her with different metal ions in the MPN film were added to a BT-474 cell culture medium at a particle-to-cell ratio of 10000:1 and incubated in full medium at 37 °C for 24 h. The cell viability test showed that at the working concentration (100 pM), AuNP@MPN@Her showed no cytotoxicity against BT-474 cells (Figure S8). Cell targeting was measured by CyToF using the ^{197}Au signal. All AuNP@MPN@Her particles showed targeting to BT-474 cells, thus demonstrating that the PC did not diminish the Ab targeting ability. Among all the particle systems, AuNP@Co-TA@Her showed the highest medium ^{197}Au intensity (~2.7-fold), whereas the other particle systems showed comparable medium ^{197}Au intensities (Figure 3). These results are consistent with the antigen targeting results (Figure 2b) and confirm the targeting enhancement by cobalt MPNs.

Figure 3. Cell targeting of different AuNP@MPN@Her particles to BT-474 (Her2+) cells after incubation for 24 h in full medium at 37 °C. The particle-to-cell ratio was set at 10000:1. Medium ¹⁹⁷Au intensity was used to quantify the targeting level. Error bars represent standard deviations. Statistical significance was determined by a two-tailed *t*-test and is reported as **p* < 0.05.

We additionally examined the targeting specificity of AuNP@Co-TA@Her. First, the cell targeting of AuNP@Co-TA@Her to BT-474 cells was found to be dose dependent (Figure S9). Next, AuNP@Co-TA@Her was treated to Her2+ BT-474 cells and Her2 negative (Her-) MDA-MB-468 cells with a particle-to-cell ratio of 10000:1 and incubated for 24 h in full medium at 37 °C. AuNP@Co-TA without Her was also treated to BT-474 cells as a control under the same conditions. BT-474 cells treated with AuNP@Co-TA@Her showed a higher ¹⁹⁷Au intensity than the MDA-MB-468 cells treated with AuNP@Co-TA@Her or the BT-474 cells treated with AuNP@Co-TA (Figure 4a and Figure S10). Targeting specificity was also observed for all AuNP@MPN@Her as the medium ¹⁹⁷Au intensity of the treated MDA-MB-468 cells was undetectable (i.e., at the instrument background) in contrast to that obtained for the treated BT-474 cells (Table S1). To confirm the specificity of AuNP@Co-TA@Her targeting, free Her was

used to pre-block Her2+ on the BT-474 cell membrane. When the BT-474 cells were pre-incubated with Her at different concentrations for 1 h, cell association of AuNP@Co-TA@Her was no longer dependent on dose (Figure 4b). When treated with $\geq 2 \mu\text{g mL}^{-1}$ free Herceptin, cell association was inhibited to a very low level. These results confirmed the specific targeting of AuNP@Co-TA@Her. The targeting ability of Co-TA@Her coating was not unique to AuNPs. Fluorescein isothiocyanate-labeled polystyrene (PS) particles (diameter: 288 nm) were also used as coating templates and the resulting PS@Co-TA@Her with a PC also showed enhanced targeting to Her2+ BT-474 cells (Figure S11).

Figure 4. (a) CyToF histograms of cell targeting of AuNP@Co-TA@Her and AuNP@Co-TA to BT-474 (Her2+) and MDA-MB-468 (Her2-) cells after incubation for 24 h in full medium at 37°C. (b) CyToF histograms of cell targeting of AuNP@Co-TA@Her to BT-474 (Her2+) cells pre-blocked with Her at different concentrations. The particle-to-cell ratio in (a) and (b) was set at 10000:1.

Figure 5. (a) Molecular model of the Her Fc (HerFc) fragment in aqueous solution. Subunits A and B are colored black and grey, and the CH2 and CH3 domains comprise residues 236–337 and

344–442, respectively. Histidine (HIS) and methionine (MET) residues are colored according to their solvent-accessible surface area (SASA) in the representative structure taken from molecular dynamics (MD) simulation in water. (b) SASA probability distributions for HIS and MET over the final 50 ns of MD simulation in water. (c) HerFc with seven Co^{II} -TA₂ complexes coordinated. For the tannic acid and HIS and MET sidechains, carbon, oxygen, nitrogen, and sulfur atoms are colored grey, red, dark blue, and yellow, respectively. The Co^{II} is colored pink. Other HIS and MET residues are colored according to their SASA as in (a). In (a) and (c), solvent molecules and hydrogen atoms are not shown for clarity. Glycan is shown in licorice representation, protein backbone secondary structure is highlighted in cartoon representation with all side-chains hidden except for HIS/MET residues, which are shown in a space-filling vdW representation.

To understand the fundamental differences between the Co-TA and other metal films, X-ray photoelectron spectroscopy (XPS) was used to examine the MPN films on planar Au surfaces. XPS analysis as a function of the platform tilt angle revealed that the Co^{II} concentration in the film was not uniform (Figure S12a), with a greater concentration ($\sim 3.8\%$ Co atoms) observed in the middle of the film than at the Co-TA surface ($\sim 2.5\%$). This gradient possibly arises due to the preferential interaction between the Co d electrons and Au, leading to a higher Co accumulation at the Au surface. This is contrary to Fe-TA films, which presented a lower ($\sim 1.8\%$ Fe atoms) but more uniform Fe^{III} atomic concentration throughout the film (Figure S12b). As Fe^{III} can interact with three gallol groups and Co^{II} can only chelate two gallol groups, the relative ratios of the atomic composition should be 2Fe:3Co, which matches our XPS results (average of $\sim 1.8\%$ Fe atoms vs $\sim 2.9\%$ Co atoms). The C1s spectra suggested that the TA concentration in both films was similar (Figure S12c,d). Compared to the Fe-TA film, the uneven, irregular Co-TA surface (Figure S1), along with its higher concentration of metal ions, provides a different presentation of metal

atoms for the Ab to potentially interact with beyond nonspecific adsorption. Such Co^{II} -Ab interactions can potentially orientate the Ab on (or within) the MPN film.³⁸

MD simulations were employed to explore the interactions between key residues on the Ab Fc region and Co^{II} -TA₂ complexes, with particular focus on HIS and MET as these residues were previously reported to coordinate to Co^{II} -TA complexes.³⁸ In total, there are six histidines (HIS268, HIS285, HIS310, HIS429, HIS433, and HIS435) on each Her Fc (HerFc) subunit and two methionines (MET252 and MET428) (Figure 5a). Aside from MET428, all HIS and MET residues of HerFc are found on flexible loops. In the X-ray crystal structure⁴³ and in MD simulations of HerFc in water, these residues displayed different solvent-exposure profiles (Figure 5b). Stepwise or simultaneous addition of Co^{II} -TA₂ complexes in proximity to exposed HIS or MET residues resulted in similar Co^{II} -TA₂@HerFc configurations; the HerFc alpha carbon root-mean-square deviation ($\text{C}\alpha$ RMSD) profiles are shown in Figure S13. In all cases, the HerFc $\text{C}\alpha$ RMSD remained close to the values found for free HerFc in water despite the addition of Co^{II} -TA₂, suggesting that the addition of Co^{II} -TA₂ does not distort the HerFc secondary structure. However, there was significant movement in the coordinated Co^{II} -TA₂ to maximize contact between adjacent Co^{II} -TA₂ complexes. While this resulted in relatively larger $\text{C}\alpha$ RMSDs, due to the flexible loop between the CH2 and CH3 domains of the HerFc, significant movements in the domains were observed, whereas the overall secondary structure remained largely unchanged (Figure S13g,i,j,k, Table S4). Moreover, movements in the Co^{II} -TA₂ coordinated to HIS433 and HIS435 induced a shift in the flexible loop comprising residues 429–435, which in turn increased the maximum solvent exposure of HIS429 from ~26 to ~41% (relative to the most solvent-exposed HIS, Figure S14) and enabled the addition of a seventh Co^{II} -TA₂ complex. This resulted in a configuration mimicking a fragment of a continuous Co^{II} -TA film along the CH2–CH3 domains

of the HerFc subunit (Figure 5c) and seven Co^{II}-HerFc coordinate bonds in total, where each bond has an interaction enthalpy of approximately $-23 \text{ kcal mol}^{-1}$ (Table S5).

Although flat-on and tail-on orientations cannot be ruled out, the MD simulations suggest that the HerFc orientation that maximizes the number of coordination bonds within the experimental constraints of $\sim 2 \text{ nm}$ Co-TA film thickness and $\sim 1\text{--}2 \text{ nm}^2$ film roughness is the side-on orientation with respect to the HerFc. In this orientation, both Fab regions, attached to the HerFc by a flexible linker, can extend away from the AuNP core, while the biomolecules of the PC pack nonspecifically around the HerFc, resulting in the observed AuNP@CoTA@Ab with a diameter of $\sim 45 \text{ nm}$ and cell targeting characteristics.

In conclusion, we have reported the functionalization of AuNPs with MPNs containing different d-block metals crosslinked by TA, followed by physisorption of antibodies and a study of their targeting abilities toward respective antigens. By changing the metal ion incorporated in the AuNP@MPNs, we investigated the effects of different metals (Fe^{III}, Co^{II}, Ni^{II}, Cu^{II}, Zn^{II}) on antibody-mediated particle targeting. All systems demonstrated enhanced targeting effects owing to the adsorbed antibodies, with systems containing Co^{II} showing the most significant increase in antigen recognition. The targeting improvement by Co^{II} MPNs was not due to a higher antibody loading, as the Co^{II} particle systems contained a lower antibody loading than the other metal systems. XPS characterization of the surface films demonstrated a greater concentration of solvent-exposed Co^{II} (compared to other metals), while MD results suggested that this exposure facilitates coordination binding to the histidine-rich domain of the antibody Fc region, thereby effectively anchoring the Fc region of the antibody to the particle. Moreover, a protein corona was introduced into the system to stabilize AuNP@MPNs after the adsorption of antibodies, without significantly diminishing the targeting affinity. Our methods are not limited to AuNPs—other substrates can be

coated with MPN films achieving similar targeting efficacies. With the benefits provided by the convenience of self-assembled MPN coatings, our study presents a facile system for particle functionalization with antibodies for both diagnosis and targeted drug delivery.

ASSOCIATED CONTENT

Supporting Information. Theoretical details, experimental results for AFM, cytometry, UV–vis spectroscopy, DLS, TEM, SDS-PAGE, fluorescence, cell viability, XPS, and theoretical results for RMSD profiles, SASA, and model structures.

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