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High-throughput optimization of surfaces for antibody immobilization using metal complexes

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Abstract

Using a high-throughput surface discovery approach, we have generated a 1600-member library of metal-containing surfaces and screened them for antibody binding potential. The surface library assembly involved graft modification of argon plasma-treated polyvinylidenedifluoride (PVDF) membranes with alternating maleic anhydride-styrene copolymer followed by anhydride ring opening with a range of secondary amines and microarray contact printing of transition metal complexes. The microarrays of metal-containing surfaces were then tested for their antibody binding capacity by incubation with a biotinylated mouse antibody in a chemiluminescence assay. A total of 11 leads were identified from the first screen, constituting a "hit" rate of 0.7%. A smaller 135-member surface library was then synthesized and screened to optimize existing hits and generate additional leads. To demonstrate the applicability of these surfaces to other formats, high-binding surface leads were then transferred onto Luminex beads for use in a bead flow cytometric immunoassay. The novel one-step antibody coupling process increased assay sensitivity of a Luminex tumor necrosis factor immunoassay. These high-binding surfaces do not require prior incorporation of polyhistidine tags or posttreatments such as oxidation to achieve essentially irreversible binding of immunoglobulin G.

Keywords: High throughput; Surface discovery; ELISA; Antibody immobilization; Metal complexes

Many high-throughput screening studies in drug discovery and diagnostics are performed with one of the biological components immobilized on artificial substrates such as microtiter plates, microarrays, and microbeads [1–5]. Similarly, affinity separations, biosensors, and other biotechnological applications also rely on the specificity of biomolecular interactions on artificial substrates [6]. In all such cases, it is important that immobilized proteins maintain their native functional state on the surface [5] and, preferably, are in an orientation to maximize interaction with their complementary binding partner [6]. In this study, a high-throughput surface discovery approach was used to identify optimal surface coatings.

Many different methods of immobilizing proteins have been developed, but the number of commonly used and robust coupling chemistries is limited [7]. For example, the most commonly used covalent coupling method for attaching proteins such as antibodies to surfaces is the well-known carbodiimide reaction. This reaction forms an amide bond between an amine on a protein and an activated carboxylic acid on a surface [7]. Here the key advantage is its simplicity, but there are many amine (and carboxylic acid) side chains on proteins, leading to random orientations on a surface and potential problems with reproducibility [8]. Modification of critical amine side chains can lead to loss of functional activity that is protein dependent. Other conjugation methods include thioether formation using a thiol with a maleimide or bromoacetamido group and disulfide formation using reagents such as pyridiyldisulfide [7]. Although selective thioether or

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disulfide interaction is possible, procedures to reduce disulfides in proteins or add either thiol or their reactive counterparts are labor intensive and can also lead to damage of protein function. A number of other site-selective protein modification procedures exist [9]. Depending on the exact method, selective binding through one site in a protein is possible, but procedures can be labor intensive and coupling of these "selective" crosslinking agents to proteins is dependent on other coupling methods (e.g., amide formation) that are not selective.

Rather than covalent interactions, a preferred strategy may be the use of strong biomolecule affinities such as biotin-streptavidin [10,11]. Assuming that the biotin is coupled to the nonbinding region of a protein and there are no other surface–protein interactions, this approach is more likely to maintain the native functional state of a protein. However, the dominant method of biotinylation is amide coupling, which may result in the aforementioned problems.

One commonly used affinity-based method for binding proteins is the use of metal complexes [12,13]. Immobilized metal ion affinity chromatography (IMAC),[1] a highly reliable purification procedure, employs metal–protein interactions for retaining proteins on a column [14]. Subsequently, the proteins are eluted from the IMAC column using a metal chelating buffer. Most often, IMAC is used for purification or crosslinking of recombinant proteins containing polyhistidine tags that form complexes with metals [12,14]. However, in certain cases, unmodified proteins have also been purified by IMAC [15–17]. In these latter cases, where the metal is interacting with existing metal chelating groups on proteins, the strength of binding is relatively weak for other applications such as immunoassays. In these cases, treatments such as oxidative transformations can help to stabilize the metal–protein interactions [17–19]. Chromium chloride is one metal complex that can form stable interactions without the need for recombinant tags [20] or posttreatment steps such as oxidation [21,22]. However, common reagents used in protein chemistry such as phosphate, acetate, and carbonate buffers; preservatives such as sodium azide; and any other potential chelating reagents cannot be used. The relative concentrations of chromium chloride with protein must be carefully controlled, and minor variations can lead to poor and highly variable binding efficiency. Chromium chloride solutions need to be "aged" for efficient and improved reproducibility of ligation [23]. Even small variations in any of the parameters, including the type of target protein, can give nonoptimal results.

To improve on existing methods and develop simple protein binding procedures, we have generated novel arrays of polymer metal chelating-based surfaces for protein binding studies initially based on metal salt libraries derived from chromium(III) salts. Using an antibody as the target protein, this study exemplifies rapid optimization of all binding parameters to identify high-binding surfaces as well as a range of intermediate-binding surfaces for applications where subsequent protein release is required.

Materials and methods

Instrumentation

The plasma source was an M4L radio frequency glow discharge plasma system (TePla America) with automated gas flow and power delivery. The microarray contact printer was a VersArray Chip Writer Compact System with solid spotting pins 300 microns in diameter (Bio-Rad). The Bio-Rad ChemiDoc XRS system was used for chemiluminescence imaging. An attenuated total reflection–infrared (ATR–IR) spectrometer from Thermo Nicolet was used. Luminex 100 was used for reading multiplexed beads in enzyme-linked immunosorbent assays (ELISAs).

Reagents

Ultra high-purity argon and oxygen (BOC Gases) were used as monomer gases in the plasma system. Polyvinylidenedifluoride (PVDF) membranes (Biotrace, Pall) were used as substrates for surface libraries. Styrene monomer (Sigma) was purified from the inhibitor prior to use in polymerization. Maleic anhydride, dimethylformamide (DMF), tetrahydrofuran (THF), ethyl acetate (EtOAc), all secondary amine reagents, and metal salts were purchased from Sigma and used as received. Polyethylenimine (PEI, Polysciences, MW = 1800) was dissolved in deionized water to 1 wt% and used for coating Luminex beads. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was obtained from Aldrich, and sulfo-*N*-hydroxysuccinimide (S-NHS) was obtained from Fluka. Deionized water was used unless stated otherwise.

Biotinylated mouse anti-rat interleukin 2 (IL-2, ARC0829, Biosource) was used to screen the surface libraries for antibody binding. Horseradish peroxidase (HRP)-labeled streptavidin (SNN1007, Biosource), hydrogen peroxide (SuperSignal West Pico Stable Peroxide Solution, Pierce), and luminol (SuperSignal West Pico Luminol Enhancer Solution, Pierce) were used to generate a chemiluminescence signal. Anti-tumor necrosis factor (anti-TNF) monoclonal antibody (cat. no. 551225, BD Pharmingen G281-2626 antibody) was used as a capture antibody in the TNF assay. A biotinylated rat anti-mouse TNF monoclonal antibody (MP6-XT3, BD Pharmingen)

[1] *Abbreviations used:* IMAC, immobilized metal affinity chromatography; ELISA, enzyme-linked immunosorbent assay; ATR–IR, attenuated total reflection–infrared; Boc, butyloxycarbonyl; PVDF, polyvinylidenedifluoride; DMF, dimethylformamide; THF, tetrahydrofuran; EtOAc, ethyl acetate; PEI, polyethylenimine; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; S-NHS, sulfo-*N*-hydroxysuccinimide; IL-2, interleukin 2; HRP, horseradish peroxidase; TNF, tumor necrosis factor; PBS, phosphate buffered saline; BSA, bovine serum albumin; sccm, standard cubic centimeters per minute; LbL, layer-by-layer; MAn, maleic anhydride; IgG, immunoglobulin G; NTA nitrilotriacetic acid; IDA, iminodiacetic acid.

was used for detection. Recombinant mouse TNF standard (cat. no. 554589, BD Pharmingen) was prepared in 10 mM phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). Streptavidin-*R*-phycoerythrin (Molecular Probes) was used at a concentration of 20 μg/ml in 10 mM PBS containing 1% BSA. Also, 10 mM PBS containing 1% BSA and 10 mM PBS containing 4% BSA were used as wash buffer and assay buffer, respectively. A chemiluminescent screen was chosen over a fluorescence-based assay due to strong variations in the fluorescent properties of the metal library screened.

Membrane plasma treatment

The PVDF membranes were placed in the plasma chamber. The plasma system was brought down to base pressure followed by three argon purges before plasma deposition. The plasma treatment was performed at a power of 40 W for 30 s at an argon flow rate of 200 standard cubic centimeters per minute (sccm) and a chamber pressure of 0.2 Pa. After argon plasma treatment, the system was evacuated to base pressure, ultra high-purity oxygen was fed into the chamber for 3 min to a pressure of 0.2 Pa, and the system was held under oxygen for 5 min to generate peroxide groups on the membrane surface. After this time, the treated membranes were removed from the system and placed in a reaction vessel ready for polymer grafting [24].

Graft polymerization

Plasma-treated PVDF membranes were placed in a reaction vessel containing maleic anhydride and styrene (200 mM in DMF). Polymerization solutions were purged with nitrogen, and polymer grafting was carried out at 75 °C for 3 h. All polymer-grafted membranes were washed thoroughly in THF until no further drop in the anhydride signal was detected by ATR–IR analysis.

Generating and screening a library of 1600 surfaces containing chromium(III) complexes on polymer-coated PVDF membranes

The poly(maleic anhydride/styrene)-coated PVDF membranes (Biotrace) were cut into 200 smaller sheets of 46×26 mm^2 (microscope slide dimensions) and were divided into 40 sets of 5 sheets each. Each of the 40 membrane sets was treated with 1 of 40 different secondary amines (Table 1) at 100 mM concentration in THF, DMF, or EtOAc depending on the amine solubility to give the ring opened mixed phenyl-amide-carboxylic acid system. The membranes were washed thoroughly in the corresponding solvent to remove unbound amines and were analyzed by ATR–IR to determine whether the chemical transformation was complete. Different chromium(III) formulations (Table 2) were robotically arrayed at sixfold redundancy (6×40 spots) onto each membrane sheet (spot size ~300 μm in diameter).

After surface assembly, the library of arrayed polymer-coated membranes was prewetted with methanol followed by deionized water and was treated with biotinylated mouse anti-rat IL-2 antibody (100 μg/ml) for 1 h. A solution containing HRP-labeled streptavidin (300 μl, 100-fold diluted from the original kit concentration) was incubated over the membrane for 1 h using a cover slip. The membrane was then washed for 1 h in 10 mM PBS and was treated with a mixture of hydrogen peroxide and luminol before chemiluminescence imaging.

Generating and screening a library of 135 surfaces containing different metal complexes on PVDF membranes

Libraries of polymer chelating surfaces containing different metal ions were generated as described above at 20-fold redundancy (for metal–ligand combinations, see Table 3). Antibody binding surface based on $Cr(ClO_4)_3.6H_2O$ identified in the first screen was used as a positive control. After surface assembly, this new surface library was treated as described above.

Transfer of lead surfaces from PVDF membrane to polystyrene Luminex beads

Lead metal complex combinations identified on PVDF membranes were coated onto polystyrene microbeads (Luminex) using layer-by-layer (LbL) techniques [25,26]. The beads (1.25×10^7 beads/ml, 500 μl) were washed with water once. The bead plugs were resuspended into 500-μl aliquots of a freshly made PEI solution (1 wt% in water).

Table 1
List of secondary amines reacted with maleic anhydride/styrene-coated PVDF membranes

Dimethylamine	*N*-Methylhomoveratry amine	2-(2-Methylaminoethyl) pyridine	Bis(2-Methyloxyethyl)amine
N-Ethylmethylamine	Piperazine	*N*-Methyloctylamine	1-(2-Hydroxyethyl)- piperazine
Dipropylamine	1,2,3,4-Tetrahydro isoquinoline	Thiazolidine	1-(2-(2-Hydroxyethoxy) ethylpiperizine
N-Methyl propargylamine	1-Acetylpiperazine	*N*-Pectamide	*N*-Omega- methyltryptamine
Diethylamine	1-Piperonyl piperazine	3,3′-Iminodipropionitrile	2-(Methylamino) ethanol
N-Methyl allylamine	Benzylmethylamine	*N*-Methylphenethylamine	*N*-Methyl-β-alaninenitrile
Dibutylamine	*N*-Methyl furfurylamine	Thiomorpholine	4-Piperidine ethanol
Pyrrolidine	1-Methylpiperazine	*N*,*N*,*N*′-Trimethyl ethylenediamine	Diethanolamine
Piperidine	*N*-Methyl butylamine	4-Piperidone monohydrate.HCl	*N*-Methyl-3-(aminoethyl)indole HCl
Morpholine	3,5-Dimethyl piperidine	2-Methoxyethylamine	*N*-Methyl-2-amino-(2- methoxyethoxy)ethane HCl

Table 2
List of chromium solutions arrayed onto the secondary amine-reacted membranes in the initial screen

Number	Chromium salt	Concentration (mM)	Chelating ligand	Concentration (mM)
1	Chromium(III) chloride	100	Water	100
2	Chromium(III) chloride	100	HCl	100
3	Chromium(III) chloride	100	Ethylenediamine	100
4	Chromium(III) chloride	100	Tetramethyl ethylenediamine	100
5	Chromium(III) acetate	100	Water	100
6	Chromium(III) acetate	100	HCl	100
7	Chromium(III) acetate	100	Ethylenediamine	100
8	Chromium(III) acetate	100	Tetramethyl ethylenediamine	100
9	Chromium(III) bromide	100	Water	100
10	Chromium(III) bromide	100	HCl	100
11	Chromium(III) bromide	100	Ethylenediamine	100
12	Chromium(III) bromide	100	Tetramethyl ethylenediamine	100
13	Chromium(III) nitrate	100	Water	100
14	Chromium(III) nitrate	100	HCl	100
15	Chromium(III) nitrate	100	Ethylenediamine	100
16	Chromium(III) nitrate	100	Tetramethyl ethylenediamine	100
17	Chromium(III) perchlorate	100	Water	100
18	Chromium(III) perchlorate	100	HCl	100
19	Chromium(III) perchlorate	100	Ethylenediamine	100
20	Chromium(III) perchlorate	100	Tetramethyl ethylenediamine	100
21	Chrome Alum	100	Water	100
22	Chrome Alum	100	HCl	100
23	Chrome Alum	100	Ethylenediamine	100
24	Chrome Alum	100	Tetramethyl ethylenediamine	100
25	Chromium(III) sulfate	100	Water	100
26	Chromium(III) sulfate	100	HCl	100
27	Chromium(III) sulfate	100	Ethylenediamine	100
28	Chromium(III) sulfate	100	Tetramethyl ethylenediamine	100
29	Cr(III) AAICP[a]	100	Water	100
30	Cr(III) AAICP[a]	100	HCl	100
31	Cr(III) AAICP[a]	100	Ethylenediamine	100
32	Cr(III) AAICP[a]	100	Tetramethyl ethylenediamine	100
33	Acidified chromium(III) chloride[b]	4	Water	4
34	Acidified chromium(III) chloride[b]	4	HCl	4
35	Acidified chromium(III) chloride[b]	4	Ethylenediamine	4
36	Acidified chromium(III) chloride[b]	4	Tetramethyl ethylenediamine	4
37	Acidified chromium(III) chloride[b]	0.4	Water	0.4
38	Acidified chromium(III) chloride[b]	0.4	HCl	0.4
39	Acidified chromium(III) chloride[b]	0.4	Ethylenediamine	0.4
40	Acidified chromium(III) chloride[b]	0.4	Tetramethyl ethylenediamine	0.4

[a] Cr(III) AAICP is an atomic absorption standard of chromium(III) chloride.
[b] Produced by the method detailed in Golding [23].

Table 3
List of metal ions and chelating ligands arrayed onto the secondary amine-reacted membranes in the second screen

Metal ions		
Chromium(III) perchlorate hexahydrate	Cobalt(III) perchlorate hexahydrate	Titanium(IV) bromide
Nickel(II) perchlorate hexahydrate	Nickel(II) bromide hydrate	Titanium(IV) iodide
Copper(II) perchlorate hexahydrate	Manganese(II) perchlorate	Ruthenium(III) bromide hydrate
Ruthenium(III) chloride	Platinum(II) iodide	Molybdenum(III) bromide
Iron(III) bromide	Iron(III) chloride	Zinc(II) perchlorate hexahydrate
Chelating ligands		
Water	Iminodiacetic acid	Nitrilotriacetic acid
Oxalic acid	Ethylene diamine	1,10-Phenanthroline
Salicylic acid	8-Hydroxyquinoline	
Tetramethylethylenediamine		

Coating was allowed to proceed for 30 min. After this time, the samples were washed with water four times.

Three different approaches to coating PEI beads with the desired secondary amine were tested.

Using poly(maleic anhydride/styrene) copolymer prereacted with a selected secondary amine

To a poly(maleic anhydride/styrene) copolymer solution (0.2 wt% in THF or DMF) was added an excess of the desired secondary amine (250 mM). The reaction mixture was left overnight at room temperature, the solvent was removed, and the residue was redissolved in water to a final concentration of 0.2 wt% of the derivatized polymer. The polymer was coated to PEI-treated Luminex beads following the same procedure as described above for PEI coating.

Coating beads with the hydrolyzed poly(maleic anhydride/styrene) copolymer followed by coupling of the secondary amine

Poly(maleic anhydride/styrene) copolymer (1 wt% in water) was left at 70 °C for 3 days. The hydrolyzed copolymer was further diluted in water to 0.2 wt% and coated to PEI-treated Luminex beads following the same procedure as described above for PEI coating. The poly(maleic acid/styrene)-coated beads (1.25×10^7 beads/ml, 500 μl) were treated with EDC and S-NHS (250 μl of 50 mg/ml in water each) and left at room temperature for 20 min with occasional agitation. The beads were then washed twice with water (500 μl) The secondary amine solution (60 mM in water, 50 μl) was added to the bead suspension, and the solution was mixed every 30 min for 2 h. The amine-coupled beads were then washed three times with water.

Coupling the secondary amine directly onto carboxylic acid groups on the uncoated Luminex beads

Unmodified Luminex beads (1.25×10^7 beads/ml, 500 μl) were treated with EDC and S-NHS (250 μl of 50 mg/ml in water each) and left at room temperature for 20 min with occasional agitation. The beads were then washed twice with 500 μl water. The secondary amine solution (60 mM in water, 50 μl) was added to the bead suspension, and the solution was agitated every 30 min for 2 h. The amine-coupled beads were washed three times with water. A 1:1 solution of $Cr(ClO_4)_3.6H_2O$ and EDA (40 mM in water) was used for incubation with beads coated with modified polymers using the three different methods described above. The beads were incubated with the metal complex for 60 min with occasional mixing. The beads were then washed three times in water.

TNF immunoassays on surface-modified beads

Beads conjugated to the capture antibody following the Luminex recommended amide-coupling protocol were used as a control [27]. Antibody conjugation using $CrCl_3$ stock solution was prepared as described previously [21] and diluted to 0.1 wt% with 100 mM saline solution prior to use. The anti-TNF monoclonal capture antibody was desalted by passing through an Amersham PD-10 column. The desalted capture antibody (200 μl, 100 μg/ml) was then combined with the $CrCl_3$ solution (2 μl) and mixed thoroughly over 30 min. Luminex beads (1.25×10^7 beads/ml, 100 μl) were washed with water, and supernatant was removed. The resulting bead pellet was combined with the antibody/$CrCl_3$ solution (100 μl). The coupling was allowed to proceed for 30 min with occasional mixing. After this time, the beads were washed with PBS three times and stored in 100 μl of 10 mM PBS with 1% BSA and 0.05% sodium azide.

Antibody conjugation using $Cr(ClO_4)_3$

A pellet of Luminex beads (from 1.25×10^7 beads/ml, 500 μl) coated with chromium perchlorate as described above was combined with the anti-TNF capture antibody (50 μg/ml, 500 μl). The solution was mixed and left to stand for 1 h with occasional agitation. After this time, the solution was washed once with 100 mM PBS. The antibody-coupled beads were stored in PBS containing 1% BSA and 0.05% sodium azide at 4 °C.

Assay procedure

A 96-well filter plate was prewetted with the wash buffer (100 μl/well). TNF standards and assay buffer for blanks (20 μl) were dispensed in corresponding wells. Capture antibody-coupled Luminex beads were then added to the wells (10 μl, 2000 beads/well). The filter plate was shaken at room temperature at 500 rpm for 1 h in the dark, and then the anti-TNF detection antibody solution was added to each well (20 μl, 1 μg/ml). The filter plate was shaken at room temperature at 500 rpm for 30 min in the dark. Then streptavidin-*R*-phycoerythrin solution (20 μl, 20 μg/ml) was added to each well. The filter plate was shaken at room temperature at 500 rpm for 15 min in the dark. The wells were emptied by applying vacuum and were washed with the wash buffer (100 μl) twice. At the end, 100 μl of the wash buffer was added to each well, and the plate was shaken for 60 s and loaded into the Luminex XYP instrument for reading.

Results

Generating and screening a library of 1600 surfaces containing chromium complexes on PVDF membranes

Fig. 1 shows the assembly of the combined PVDF sheets of 1600 different surfaces with six replicates for each arrayed metal complex surface. ATR–IR analysis shows the presence of considerable copolymer coating as indicated by the presence and intensity of the anhydride asymmetric doublet at 1858 and 1778 cm^{-1} (Fig. 2). The relative amount of grafted polymer can be estimated by the ratio of peak areas for $MAn_{1818cm^{-1}}/PVDF_{764cm^{-1}}$ (where MAn is maleic anhydride). In this study, a polymeric coating

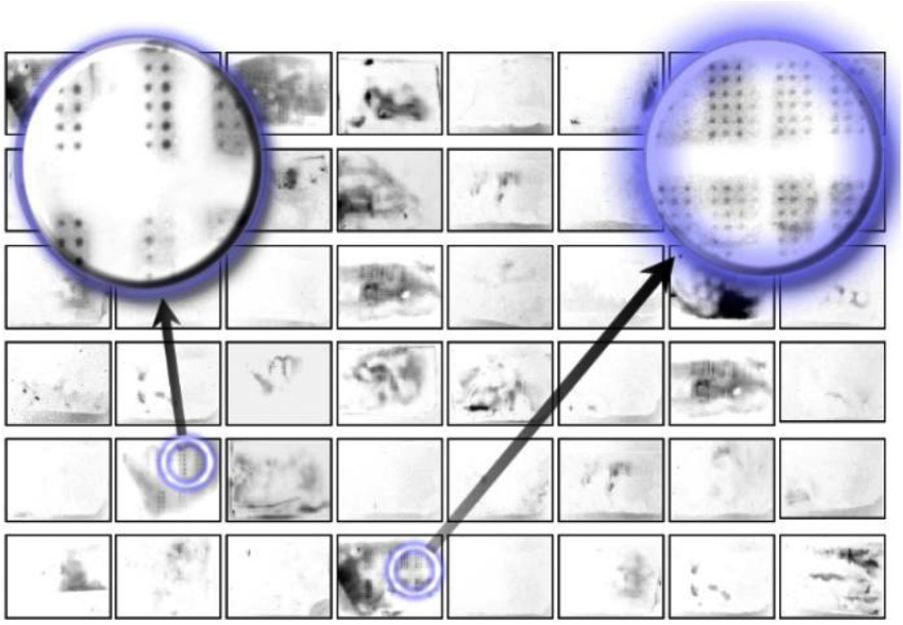

Fig. 1. Antibody binding on coated membranes arrayed with the metal–ligand library from Tables 1 and 2 at sixfold redundancy. The hits from the chemiluminescent screen using a biotinylated mouse anti-rat IL-2 IgG and subsequent incubation with HRP-labeled streptavidin can be seen as dark spots.

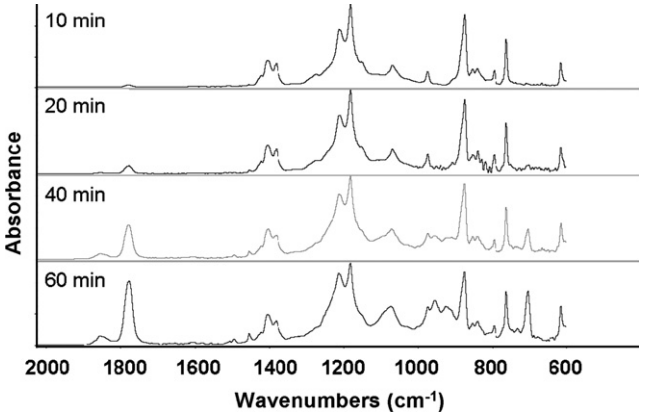

Fig. 2. ATR–IR analysis showing the presence of maleic anhydride-styrene copolymer coating, as indicated by the presence and intensity of the asymmetric doublet at 1858 and 1778 cm^{-1} The relative amount of grafted polymer can be estimated by the ratio of peak areas for $MAn_{1818cm^{-1}}/PVDF_{764cm^{-1}}$.

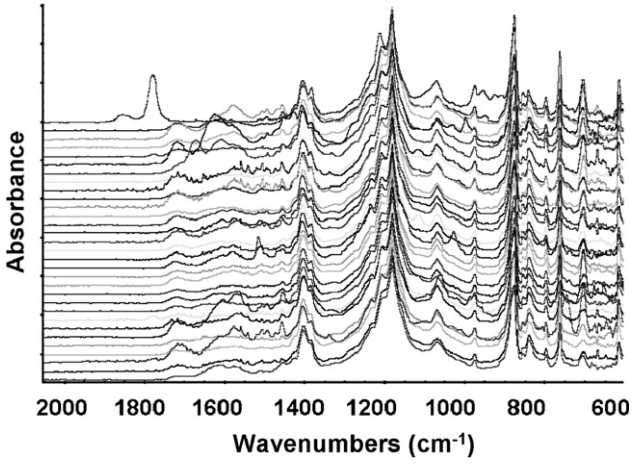

Fig. 3. ATR–IR spectra of the poly(maleic anhydride-styrene)-coated membranes after reaction with the library of secondary amines. The anhydride was seen to react to full conversion with the exception of 3,3′-iminodipropionitrile, as observed by the complete loss in the anhydride asymmetric doublet.

$MAn_{1818cm^{-1}}/PVDF_{764cm^{-1}}$ of 7.5 was used. At this polymer graft level, the membrane maintained the structural integrity of the nonmodified PVDF membrane. Reaction of the secondary amines with the MAn-coated membrane was also followed by ATR–IR. With the exception of 3,3′-iminodipropionitrile, all reactions were shown to be complete, as reflected in the loss of the asymmetric doublet (Fig. 3). Uncoated PVDF membranes have some potential for nonspecific protein adsorption, and after coating there are variations in this nonspecific adsorption potential according to the different coating chemistries. Consequently, individual sheets in this microarray give variations in assay background due to a combination of nonspecific adsorption of protein and a generalized washing protocol that is not ideal for each of the different coated membranes. However, under the described washing protocol, leads in which all of the replicates gave uniform signal over background and were reproducible on resynthesis of the same surface were identified. Table 4 shows 11 different surface coatings on PVDF membranes that were identified as antibody binding surfaces under the experimental assay conditions. Of note is that all surfaces based on chromium chloride [21] were not identified as potential leads. This is not surprising given that the antibody was suspended in 10 mM PBS containing sodium azide and the working solution concentration of the chromium chloride was rather low [28]. Both buffer and azide are known to inhibit chromium chloride ligation

Table 4
List of chromium metal ions and chelating ligand hits that displayed IgG binding

Number	Amine	Chromium complex	Chelating ligand
1	Piperidine	Chromium(III) sulfate	Ethylenediamine
2	Piperidine	Chromium(III) perchlorate	Ethylenediamine
3	Piperidine	Chromium(III) perchlorate	HCl
4	3,5- Dimethylpiperidine	Chromium(III) sulfate	Ethylenediamine
5	2-(2- Methylaminoethyl) pyridine	Chromium(III) bromide	Ethylenediamine
6	Piperazine	Chromium(III) bromide	Ethylenediamine
7	2-(Methylamino) ethanol	Chromium(III) perchlorate	Tetramethyl ethylenediamine
8	2-(Methylamino) ethanol	Chromium(III) bromide	Tetramethyl ethylenediamine
9	2-(Methylamino) ethanol	Chromium(III) sulfate	Tetramethyl ethylenediamine
10	*N*-Methylbutylamine	Chromium(III) bromide	Tetramethyl ethylenediamine
11	*N*-Methylbutylamine	Chromium(III) sulfate	Water

[21–23]. The 11 lead surfaces indicate that many variables are involved in obtaining efficient binding of antibody to this grafted PVDF membrane. The largest cluster included 2-(methylamino)ethanol as the secondary amine on the poly(maleic anhydride) copolymer with ethylenediamine or tetramethyl ethylenediamine as the additive. Even within this subset of surfaces, the counterions and specific metal binding ligands made a dramatic difference in binding efficiency. Screening the surfaces in this manner has resulted in the discovery of a range of new coordination polymers.

To validate the importance of the metal ion–chelating ligand combination, such as chromium perchlorate–EDA "lead," solutions of chromium perchlorate were arrayed with and without EDA at various concentrations (Fig. 4). In addition, EDA without chromium perchlorate and water without either of the two active constituents were arrayed onto a MAn membrane reacted with 2-methylamino ethanol. It can be seen that no hits are detected when the metal salts of the chelating ligand are arrayed separately, indicating that once the chromium perchlorate salt is partially complexed by EDA, the metal center becomes activated, allowing it to chelate to the coordination polymer and subsequently chelate antibody.

Generating and screening a library of 135 surfaces containing metal complexes on PVDF membranes

To test the efficacy of other metal–ligand complexes on the hit secondary amine-treated surface of 2-methylamino ethanol, 15 different metal salts complexed with 9 different

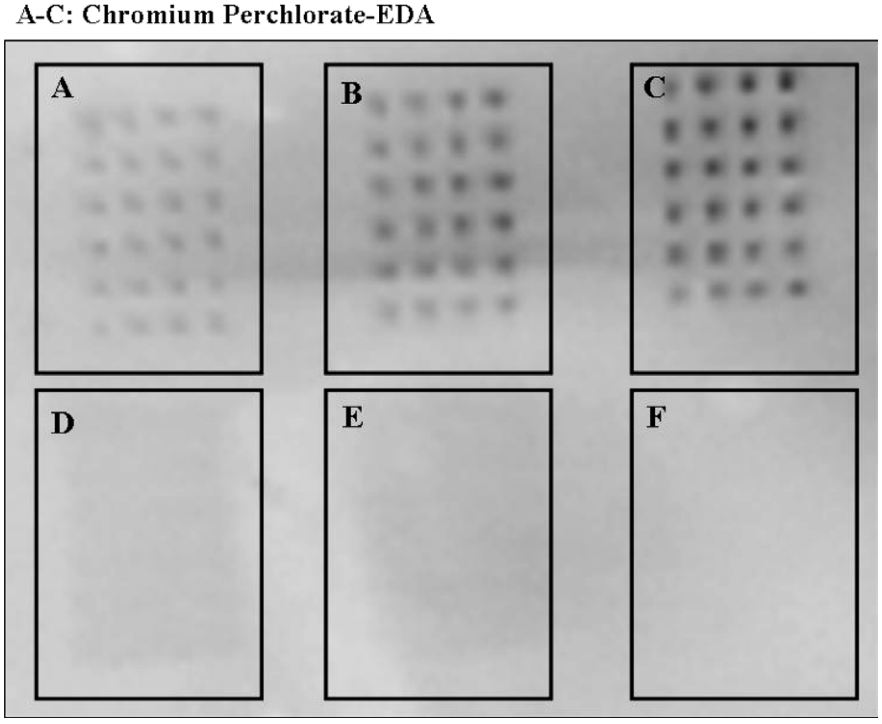

Fig. 4. Validation of a hit metal ion–chelating ligand pair on coated membranes. Chromium perchlorate solution complexed with EDA was arrayed at 1 mM (A), 10 mM (B), and 100 mM (C). As controls, 100 mM chromium perchlorate without the chelating ligand EDA was arrayed (D), and the ligand EDA (100 mM) without chromium perchlorate (E) and water (F) was also arrayed. With this particular hit, the importance of the chelating ligand EDA is evident.

additives (135 different surfaces) were tested at sixfold redundancy (Table 3). Fig. 5 shows the duplicate experiments of 4 metal ions, each tested with 9 different chelating ligands (including water), and the results show good reproducibility between the duplicate arrayed metals. For each metal grouping, a known lead surface from the previous chromium library (chromium perchlorate with EDA) was used as a positive control. As shown in Table 5, 21 different metal–additive combinations were identified as potential antibody binding surfaces. The data suggest that it is not difficult to identify metal complex surfaces for a particular target protein if all of the variables are tuned for that particular target protein. This experiment was performed on one particular precursor surface, namely, poly(maleic anhydride/styrene) copolymer ring opened with 2-methylamino ethanol. The experiment could have been performed on the 39 other precursor surfaces ring opened with other amines, but this surface gave the strongest hits in the initial screen and was chosen for a focused study. Far larger libraries can easily be assembled and screened using such methods to expand the chemical space of known coordination polymers [28].

Table 5
List of metal ions and chelating ligand hits that displayed IgG binding on coated PVDF membranes reacted with 2-methylaminoethanol

Number	Metal ion	Chelating ligand
1	Chromium(III) perchlorate	Iminodiacetic acid
2	Chromium(III) perchlorate	Oxalic acid
3	Zinc(II) perchlorate	Iminodiacetic acid
4	Zinc(II) perchlorate	Oxalic acid
5	Zinc(II) perchlorate	Ethylenediamine
6	Zinc(II) perchlorate	Tetramethyl ethylenediamine
7	Cobalt(III) perchlorate	Oxalic acid
8	Cobalt(III) perchlorate	Tetramethyl ethylenediamine
9	Iron(III) chloride	Oxalic acid
10	Iron(III) chloride	Ethylenediamine
11	Ruthenium(III) bromide	Iminodiacetic acid
12	Titanium(IV) iodide	Water
13	Titanium(IV) iodide	1,10-Phenanthroline
14	Titanium(IV) iodide	8-Hydroxyquinoline
	Titanium (IV) iodide	Oxalic acid
15	Nickel(II) perchlorate	Oxalic acid
16	Nickel(II) perchlorate	Tetramethyl ethylenediamine
17	Copper(II) perchlorate	Water
18	Copper(II) perchlorate	Oxalic acid
19	Copper(II) perchlorate	Tetramethyl ethylenediamine
20	Ruthenium(III) chloride	Water
21	Ruthenium(III) chloride	Salicylic acid

Transfer of a lead surface from PVDF membrane to polystyrene beads

Fig. 6 shows the outcome of a TNF-α sandwich immunoassay using a chromium perchlorate–EDA complex to couple the anti-TNF capture antibody to the bead surface using LbL techniques as discussed in Materials and methods. In this example, the chromium perchlorate–EDA complex gave dramatically improved sensitivity compared with the benchmark using conventional carbodiimide-mediated protein coupling as described by Luminex [29]. Unlike the original chromium chloride method that does not work in PBS [21–23], the surfaces based on chromium perchlorate–ethylene diamine gave similar results in saline, 10 mM and 100 mM PBS (data not shown). In addition,

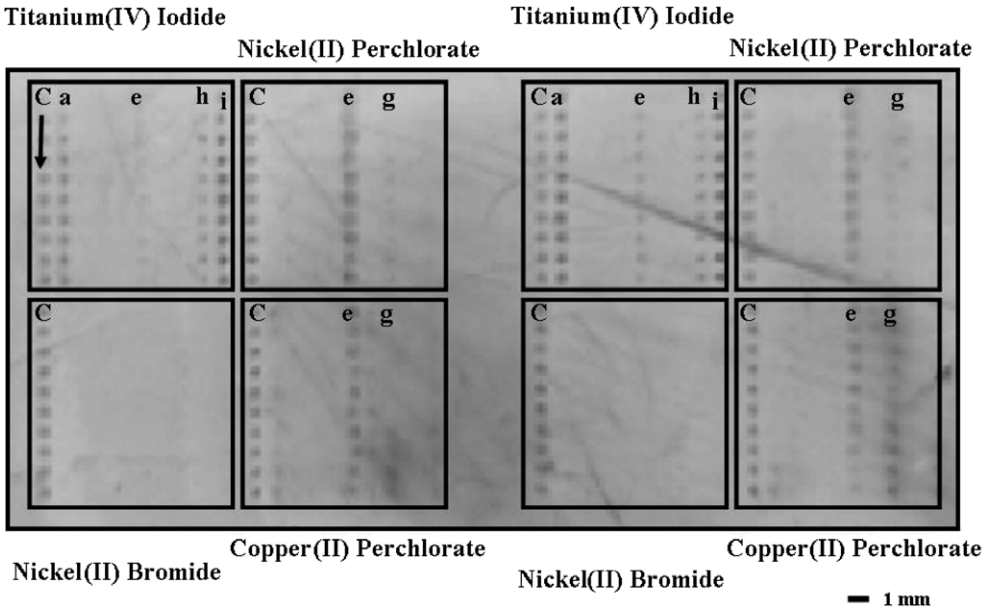

Fig. 5. Characterization of a mixed phenyl-amide-carboxylic acid coated membrane reacted with 2-methylamino ethanol. Within each square, metals were arrayed at 10-fold redundancy down the slide, and different metal–ligand combinations (a–i) were spotted from left to right across the slide. Each metal was arrayed with an equimolar amount or saturated solution of water (a), iminodiacetic acid (b), salicylic acid (c), nitrilotriacetic acid (d), oxalic acid (e), ethylene diamine (f), tetramethyl ethylenediamine (g), 1,10-phenanthroline (h), or 8-hydroxyquinoline (i). As a control (C), chromium perchlorate/EDA was used. The hits from the chemiluminescent screen can be seen as dark spots.

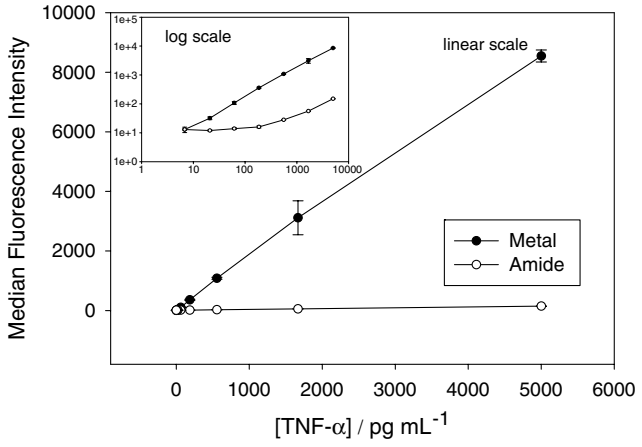

Fig. 6. TNF sandwich assay on Luminex beads using chromium perchlorate–EDA ligation of capture IgG. As a control, the standard amide coupling procedure was used. The experiment was performed in triplicate.

the coupling step is essentially a one-pot synthesis whereby the immunoglobulin G (IgG) of choice is incubated with the metal coordination polymer-coated bead followed by a final blocking step.

Discussion

In this study, we have demonstrated that surfaces can be tuned to optimize the binding of a target protein. Using an antibody as an example, libraries of surfaces composed of different metal ions, with different counterions, ligands, additives, and other surface components on chelating polymer-coated PVDF membranes, were screened for antibody binding efficiency. From the library of 1600 surfaces, 11 leads were identified, giving a hit rate of 0.7% (Table 4). Because of the variations in background, the 11 leads are not rank-ordered, but they are easily differentiated from the nonbinding metal combinations. The data clearly indicate that the secondary amine, the metal counterion, and chelating ligand all were variables in determining antibody binding. For example, 3 of the 11 leads were identified on the 2-methylamino ethanol-reacted polymer-coated membrane. This amine is expected to form a tridentate chelating structure along the backbone of the surface-immobilized polymers that is similar to the well-known nitrilotriacetic acid chelating molecule. Also, 9 of the 11 leads included a diamine chelating ligand (EDA or tetramethylethylene diamine) in preference to just water or HCl. As demonstrated by the repeat experiment of chromium perchlorate with and without EDA (Fig. 5), it is clear that these ligands can determine whether a metal complex becomes reactive to protein conjugation and is defined as a lead or not.

To further explore the metal and chelating ligand variables for antibody binding, another library (Fig. 6 and Table 5) of 135 metal–chelating ligand combinations was screened on the 2-methylamino ethanol-reacted polymer-coated membrane surface to give 21 leads and a hit rate of 15.5%. The high hit rates suggest that surface–antibody binding via metal complex interactions is a relatively common event that is not unexpected considering the chelating potential of various amino acid side chains [12,13]. However, there was no clear pattern suggesting that a particular metal, counterion, or chelating ligand was preferable in protein binding from this library. The data suggest that combinations of the component variables create the optimal conditions for potential protein binding. From studies on antibody purification by IMAC, it is believed that the metal complexes bind certain histidine-rich regions in the third constant domain of heavy chain (CH_3) of IgG [24,26]. A more recent study [30] of four different transition metals [copper(II), nickel(II), zinc(II), and cobalt(II)], chelated to iminodiacetate–sepharose for the purification of antibodies by IMAC, indicated that only two surface-accessible histidines were enough to provide significant preferential binding and retention of IgG. If this were the case, the pattern of binding observed in this study should have been less complex than that observed. Instead, surfaces identified in this study ranged from some leads having essentially irreversible binding to the vast majority not binding IgG at all. The surface components, the metal chelating ligands, and the counterions were just as important as the metal itself in surface immobilization of antibody.

Although it is accepted that metal binding affinity is determined primarily by individual amino acids such as histidine, tryptophan, and cysteine, it is known that amino acid sequence, folding, and their surface properties also affect overall affinities [12,15,17–19,31–34]. It is reasonable to assume that a number of metal complex interactions may exist per antibody and that effective binding between the antibody and the surface is a result of multivalent interactions. Surface variations affecting protein binding via metal chelation are consistent with earlier studies that found differences in copper(II) binding [32] of antibodies, presumably due to the differences in the chelating properties of nitrilotriacetic acid (NTA) and iminodiacetic acid (IDA) [31]. Our data imply that the surface needs to be tuned to the target protein for effective binding and that such surfaces will be difficult to identify without some high-throughput surface discovery system in place. Depending on the assay conditions, there is clear differentiation between binding and nonbinding surfaces. For the particular precursor surfaces used in our second library, transition group metal salts of chromium, cobalt, zinc, iron, ruthenium, titanium, nickel, and copper were clearly identified (Table 4). The discovery process may have identified other metal salts if the precursor surface or target protein had been different. The efficacy of a particular metal ion varied with its counterion, with examples of chromium sulfate, perchlorate, and bromide giving effective binding, whereas the chloride, nitrate, and acetate counterions did not lead to any appreciable binding in these experiments. These parameters also varied with the nature of the additives or coordinate ligands that were "available" for displacement by the antibody. The additives included

electron-rich donors such as EDA, tetramethyl ethylenediamine, IDA, and oxalic acid, which are multidentate ligands. Depending on the metal and counterion, it was observed that different additives were preferred. Effective binding was also influenced by the assay conditions showing variations in spot intensities among the lead surfaces. More than 1600 variant surface coatings were prepared, and a far larger number can easily be assembled and screened. Expansion and/or variation of the amines, metal salt, pH, solvent, additive, substrate material, backbone polymer structure, and composition all are plausible changes that can be made to screen binding efficiency, and such libraries are an efficient platform to rapidly customize surfaces for any target protein. The data suggest that strong and tuneable binding can be achieved in the absence of recombinant polyhistidine tags or posttreatments such as oxidation.

The precursor polymer coating used in this study was a poly(maleic anhydride/styrene) copolymer that was plasma coated to a PVDF membrane. The basic backbone structure is composed of repeat units of an unreactive monomer (styrene) in combination with a reactive monomer (MAn) that was ring opened with secondary amines. Secondary amines, as opposed to primary amines, were used to eliminate the possibility of the ring closing imide side reaction. Similar anhydride-based copolymers grafted to polyethylene have been reported for the selective removal of heavy metals from solution [35]. However, the concept of incorporating secondary amine chelating structures and subsequent protein binding studies has not been considered. The discovery concept is not restricted to this copolymer because there are many other combinations of monomers where one or both monomers can be replaced to give alternative background surface characteristics [36,37].

The patterns of binding or nonbinding between the antibody and different surfaces were reproducible and are indicative of binding to a specific region(s) of the antibody. To investigate whether these lead surfaces improved the effectiveness of immobilized antibody to interact with their complementary antigen, and to demonstrate translation of surfaces from a planer (microarray) to a bead-based format, surfaces based on chromium perchlorate/EDA were transferred to a Luminex bead and tested in triplicate for a TNF-α immunoassay using commercially available antibody pairs and recommended protocols [29]. These preliminary studies resulted in dramatically improved immunoassay sensitivity in comparison with the amide control. The TNF antigen and antibody pairs were recommended by BD Pharmingen for sandwich ELISA [38,39], and the assay data suggest that this sensitivity improvement is due more to a lesser degree of antibody damage/crosslinking when compared with conventional amide coupling methods. The assay data were also dependent on specific precursor polymeric coatings and different chromium perchlorate/EDA ratios, suggesting that further optimization of assay sensitivity is possible through surface design. In comparison, the original chromium chloride method of ligation [21] is a solution-based ligation method where the protein and the metal ion are first combined to form a metal–protein complex that is then immobilized onto a surface. The chromium chloride method did not bind antibodies when the metal was first immobilized on the surface. Repeated testing of this solution ligation technique indicates problems with reproducibility due to highly variable immunoassay results. There were also variations in the dropoff in assay signal with storage (as much as 20% within a few days), confirming the difficulties of achieving any reproducibility over time using the chromium chloride technique (data not shown). The experimental data are consistent with a ligation system having a very narrow optimal range. Although it is effective, even slight deviations from the optimum result in significant variations in assay signal and storage performance. In essence, the metal is important, but by tuning other variables, it is possible to manipulate binding strength to develop simple and robust immobilization protocols.

Immobilization of antibodies (or antigens) lies at the heart of any immunodiagnostic test, but the performance of existing methods varies widely according to many factors, including the antibody itself. Existing approaches often damage protein conformation, giving heterogeneous binding characteristics after immobilization [8]. Rather than accepting that this is an unavoidable consequence of immobilization, a high-throughput surface discovery approach can systematically tune key variables to minimize potential protein immobilization challenges. The discovery approach can quickly identify nonbinding surfaces and other surfaces ranging from weak (nonspecific) to those having essentially irreversible binding affinities according to the need. One example of a high-binding lead surface was successfully transferred to a TNF-α immunoassay in a bead-based format.

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