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A Highly Selective, Biofunctional Surface for Molecule/Cell Sorting

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ABSTRACT We report in this paper an approach to the effective capture of IgM antibodies from antisera and solutions based on the formation of a carpet of molecules exposing thiols off a surface. Surfaces of different nature, such as OH-exposing (glass, SiO_2, metal oxides, etc.) and noble metal ones (Au, Ag, etc.), have been first functionalized in the liquid phase by suitable chemistry [3-(mercaptopropyl)trimethoxysilane or 1,4-benzenedimethanethiol]. The resulting exposed SH moieties have been further used for binding anti-A, -B, and -D IgM molecules from goat sera via a thiol exchange reaction involving the J chain and other disulfide bonds present in the IgM molecular structure. Antibodies preserve their functional activity at the surface and appear to be able to bind specifically erythrocytes of the proper group in a fast and reliable way. These results can be generalized to the use of any kind of IgM antibody and can be valuable in surface biofunctionalization in the fields of biosensors and immunoassays.

KEYWORDS: surface biofunctionalization • antibodies • scanning force microscopy

INTRODUCTION

In most applications and contexts involving functional biomolecules immobilized at surfaces and interfaces of different nature, such as in the case of biosensors, innovative biodiagnostic tools (e.g., laboratory on chip and high throughput cellular screening chips), in proteomics, as well as in bioinspired molecular architectures, there is an impellent need of biofunctionalized surfaces able to bind in a reliable, effective, and function-preserving way both molecules and cells (1−3).

Typically, such a goal is achieved by using suitable linkers that can both exploit functional groups at the surface of technologically relevant substrates and provide other selected chemical functionalities for anchoring the desired biomolecule (e.g., ssDNA, antibodies, enzymes, and other proteins) to the surface (4−7). In general, because biomolecules differ in chemical composition, structure, and functional behavior, the chemical functionalization needed to anchor them to a surface needs to be tailored for the specific molecule/application. Another important issue in the design of a functionalization approach is the preservation of the functional activity of the biomolecules at surfaces, which is needed to give rise to a biofunctional interface. Generally speaking, biomolecular structure, and hence functional activity, can be seriously affected by interaction with high-free-energy surfaces and interfaces due to a phenomenon such as surface-induced (partial) unfolding and conformational alterations or changes in the microenvironment due to the presence of a substrate (8−11). These problems can be often solved by using molecular spacers, which prevent direct biomolecule−surface interaction. Nevertheless, the functional activity of surface-immobilized biomolecules can be affected also by their orientation (e.g., in case of antibodies that have to interact with antigens in the liquid phase or enzymes that operate on soluble substrates). In order to avoid surface-induced hindrance, a careful choice of the functional groups exploitable in surface immobilization has to be made (e.g., either present naturally or induced artificially at the biomolecule surface).

Among the inorganic surfaces of (bio)technological relevance, those terminated in −OH and the noble metal ones represent important sets because glass, SiO_2, some polymeric materials, and metal oxides, on the one hand, and Au, Pt, Ag, and Cu, on the other hand, belong to such ensembles. In the case of −OH-terminated surfaces, silanes are often used for surface chemical functionalization (5, 12). When one deals with electronically soft metal surfaces, their strong reactivity toward thiols is usually the approach of choice for achieving stable functionalization. In those cases, the first functionalization step can accomplish both the task of introducing a spacer and that of supplying the surface with chemical functionalities suitable for binding biomolecules in the desired orientation.

We have already used 3-(mercaptopropyl)trimethoxysilane (3-MPTS) on glass and metal oxides (e.g., Al_2O_3) and 1,4-benzenedimethanethiol (*p*-BDMT) on Au in order to provide those surfaces with thiol moieties. The exposed thiols can then react with activated thiols or disulfides by a thiol-exchange mechanism that has been used for assembling molecular (sub)monolayers of both DNA (13) and proteins (14, 15).

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Here, we report the use of thiol-exposing molecular carpets for binding IgM antibodies at surfaces. IgMs are large (950 kDa) pentamers formed by five IgG-like monomers connected through a polypeptide chain rich in disulfide bonds and characterized by the presence of a disulfide-rich J chain (16). These molecules represent the first antibodies produced by the immunological system of an organism upon exposure to an antigen, and their presence in sera is a valuable indicator of running infections. Furthermore, naturally occurring IgMs against ABO blood group antigens (groups A, B, AB, and O) find application in biomedical tests such as blood grouping (17, 18), where they can induce agglutination of positive red blood cells (RBCs).

As for any antibody, the specificity toward a certain antigen is given only by the peculiar sequence of the antigen binding regions at the extremities of the Fab fragments. Therefore, any immobilization strategy not involving functional groups present in those regions will be generally applicable to any kind of IgMs.

Direct immobilization of IgMs has been obtained on dextran matrixes (19), on a protein A layer, even if the affinity of protein A for IgM is lower than that for IgG, and on a glutaraldehyde-activated surface exploiting primary amines at the IgM surface (20, 21).

The route suggested in this work relies on surfaces that have been functionalized in order to expose thiol groups and on the following thiol-exchange reaction between those surface mioeties and disulfide bonds, which are abundant in the IgM intersubunit connections. We will show that the proposed approach is suitable to capture effectively IgMs even if a nonpurified antiserum is used, giving rise to functionally active surfaces. Scanning force microscopy (SFM) in a liquid environment (22) and a quartz crystal microbalance (QCM) (23) will be used to image at submolecular resolution the captured IgMs and to monitor surface mass changes resulting from functionalization steps or the formation of biocomplexes, respectively. In fact, the resonance frequency of the QCM crystal plate decreases as the mass on its surface increases according to a direct proportionality discovered by Sauerbrey (24).

The choice of IgMs against antigens of the ABO and the Rhesus blood group (D antigen) systems present on the surface of human erythrocytes allowed us a simple and direct microscopic inspection of the retained functional activity of the surface-immobilized molecules. Furthermore, this antigen–antibody couple is of great interest for the development of immunobiosensors.

MATERIALS AND METHODS

Sample preparation consisted, first, of a chemical functionalization step involving substrate silylation/thiolation as described elsewhere (14). All substrates but mica were cleaned in a piranha solution (H_2SO_4/H_2O_2 70%/30%) and exposed to oxygen plasma for 5 min just before use. Mica (Muscovite Mica from SPI Supplies) was always cleaved just before functionalization. Both the functionalizing reagents, i.e., 3-mercaptopropyltrimethoxysilane (3-MPTS; purity >98%) and 1,4-benzenedimethanethiol (*p*-BDMT; purity >98%), were purchased from Sigma Co. and dissolved in toluene at 3% v/v and at 2×10^{-4} M concentration, respectively. Milli-Q-grade water (resistivity >18.2 MΩ cm) was used for the preparation of any water-based solution/suspension.

After this first step, IgM immobilization on thiol-exposing surfaces was achieved by incubating silylated/thiolated substrates for typically 10 min with goat antisera followed by a rinse in a physiologic buffer. Goat antisera (EuroBIO, Paris, France) containing anti-A, -B, and -D IgMs were used without further dilution or purification.

The reducing solution for testing the involvement of disulfides in binding IgMs on the functionalized surfaces was 10 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP · HCl) in a PBS buffer at pH 8. This reducing agent was preferred over thiol-based ones because it does not interfere with Au substrates and does not affect Au–S bonds (25).

In the case of the use of antigen-carrying erytrocytes, exposure to RBCs of different groups (A, B, Rh^+, Rh^-) was performed by incubating the aforementioned biofunctionalized surfaces for 5 min at room temperature with pure erythrocyte suspensions at a typical concentration of 5% (v/v) in a preservative solution. Test RBCs were kindly supplied by DiaMed Belgium and Transfusion Center of the General Hospital of Modena, Italy.

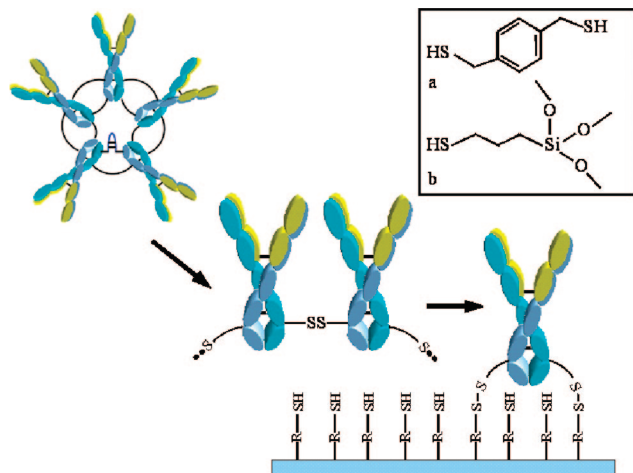
Tests and measurements were performed by QCM, optical microscopy, and SFM. QCM measurements were performed with a homemade device using 10 MHz resonators (typical quality factor in air $Q = 1 \times 10^6$) equipped with Ag or Au electrodes. Optical inspection was performed by light microscopy (IX70, Olympus Co., Tokyo, Japan) at 200× magnification. SFM imaging was performed with a PicoSPM (Molecular Imaging Co. AZ) equipped with a $6 \times 6\ \mu m^2$ scanner operated in magnetic alternating contact (MAC) mode. Samples were imaged in a physiologic buffer. MAC rectangular type II cantilevers with a nominal spring constant of 1.7 N/m were used. For SFM imaging, the dilution of the IgM antisera and incubation times were adjusted in order to obtain either an almost completely coated surface or a surface with a few IgM molecules. Images were elaborated with WSxM freeware software (26).

RESULTS AND DISCUSSION

First, surface biofunctionalization was achieved by thiolating the surface of Ag electrodes of 10 MHz quartz crystal oscillators with *p*-BDMT. This dithiol has a rigid structure, which helps to avoid both SH moieties in positions 1 and 4 binding to the substrate, as is the case of longer and more flexible dithiol molecules (4). We have chosen the QCM technique because, when operated in air, it enables one to follow the various reaction steps, from chemical modification of bare electrodes to the capture of antibodies and binding of test antigens, in a direct, easy, and quantitative way. In liquid, it allows one to retrieve kinetic information on adsorption/desorption phenomena. The exposed SH moieties were further used to capture IgMs from goat antisera by disulfide exchange reactions, as depicted in Scheme 1.

As such, our functionalization approach is not specific for a particular IgM. Indeed, we have successfully used it for capturing anti-A, -B, and -D IgMs. In Figure 1, examples of frequency shifts caused by exposure of different quartz to *p*-BDMT and antisera, followed by rinsing in pure water, are reported. These data show the variability in the captured IgM amount in 20–30 independent trials. Taking advantage of the calibration of the used resonators reported elsewhere (27), we estimated a surface coverage of $(1.24 \pm 0.29) \times 10^9$ molecules/mm^2, consistent with a dense IgM monolayer.

Scheme 1. Schematic Representation of the Immobilization Reaction for IgM Molecules[a]



[a] The IgM structure is rich in disulfide bonds. Once at the surface, these bonds can be involved in a thiol-exchange reaction that results in the covalent binding of the various IgG-like monomers at an SH-exposing surface. Such a reaction scheme appears to be the most likely in view also of other results vide infra. The inset reports the structures of (a) *p*-BDMT and (b) 3-MPTS, respectively.

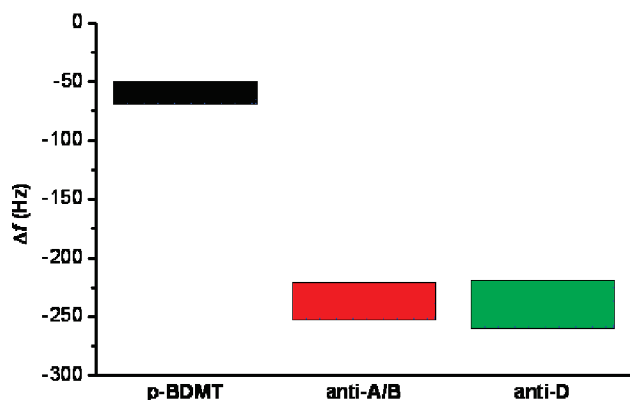


FIGURE 1. QCM monitoring of the various surface immobilization steps: *p*-BDMT and anti-A, -B, and -D IgMs from goat antisera. Each datum is the result of an average of 20−30 trials. The vertical widths of the bars correspond to the standard deviation of the data distributions.

A further insight into reaction mechanisms is provided by kinetic measurements by QCM in liquid. Figure 2 shows the adsorption kinetics of anti-A IgMs on a preformed *p*-BDMT layer on a transducer with Au electrodes. The adsorption was quite fast and reached completion on a time scale of typically 1000 s. Afterward, the liquid cell was perfused with 10 mM TCEP in PBS. This molecule is known to reduce effectively protein disulfides albeit neither affecting the S−Au bond nor adsorbing on Au surfaces (25). Interestingly, the QCM signal recovers at values close to those before IgM adsorption in a time period of about 7000 s, confirming that disulfide bonds are involved in IgM immobilization. Of course, it is possible that also other disulfide or activated thiol-bearing molecules chemisorb at the functionalized surface. Nonetheless, in antisera IgMs are overexpressed and possess many exposed disulfides, at variance with other molecules (e.g., albumin) that, albeit present in high concentration in serum, have less exposed disulfides and are not always available for surface reaction due to steric hindrance. That is likely why the absorbed molecules from antisera are mainly IgMs, as is confirmed by functional tests vide infra.

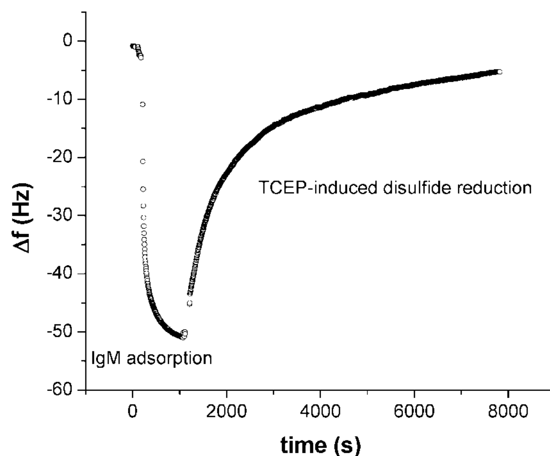


FIGURE 2. QCM kinetic measurement of IgM adsorption and subsequent desorption induced by TCEP via disulfide reduction.

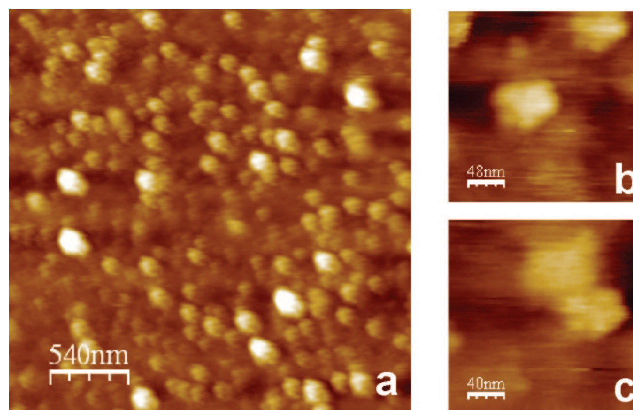


FIGURE 3. AFM imaging of IgMs adsorbed on muscovite mica functionalized with 3-MPTS: (a) low-resolution topography image; (b and c) higher magnification images showing single pentamer structures corresponding to IgM ones.

In order to inspect the status of the functionalized surface after adsorption of IgMs, SFM imaging was performed. Silylation with 3-MPTS was performed on freshly cleaved mica, which exposes −OH moieties and which has been reported to bind readily these kinds of silanes, providing a carpet of SH groups exposed off the surface (13−15). After exposure to anti-A antiserum, liquid SFM imaging in MAC mode was used to inspect the mica surface.

Figure 3 reports liquid SFM images of the surface. Figure 3a shows a low-resolution image of the silylated surface of mica covered with anti-A IgMs. The sample was prepared by incubating for 30 min the mica surface with the antiserum in order to get high coverage. In this case, a dense layer of rather uniform adsorbates is clearly visible. Parts b and c of Figure 3 show higher magnification images on a less dense sample. Adsorbates, shaped like flowers with five petals (the five monomers), are clearly visible on a clean background. Interestingly, the size of these adsorbates exceeds that (about 30 nm) estimated from a molecular model of an IgM as constituted by five IgG-like monomers

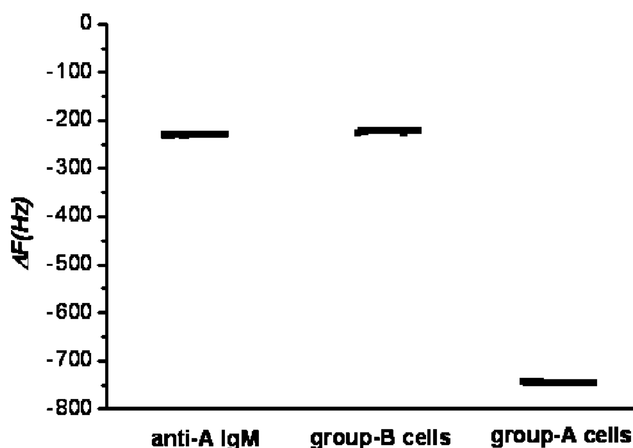


FIGURE 4. QCM assessment of the retained binding activity and selectivity of immobilized anti-A IgMs. Surface-immobilized anti-A IgMs have been first exposed to group-B RBC, which does not induce any appreciable signal difference. Further exposure to specific group-A RBC results in a marked frequency decrease, confirming binding. The frequency shift corresponding to each different sample has been measured for 5 min.

joined by a polypeptide chain. The size exceeds also that obtained from electron microscopy measurements performed with a negative contrast technique (28) and multifrequency atomic force microscopy (AFM) (29) on physisorbed IgM molecules. Even considering a convolution effect of the AFM tip on the imaged IgM moleclues, the obtained size is higher than previously obtained values.

This fact could be tentatively ascribed to a surface diffusion mechanism that the IgG-like monomers undergo when they interact with the surface. In fact, the presence of a carpet of exposed thiols at the substrate surface enables the reduction of the intramolecular disulfide bonds present in the polypeptide joint of IgMs and the consequent formation of intermolecular disulfides between the SH moieties of the silanes and those in the fragmented joint chain of IgMs. Such dissociation phenomena could, in principle, affect the IgM structure to such an extent that its functionality could be compromised. Therefore, to shed light on the retained functionality of the immobilized antibodies, we tested the biofunctionalized surfaces against their specific antigens.

The test was performed both by exploitation again of QCM and by optical microscopy inspection on standard glass slides. In both cases, we used again the aforementioned functionalization chemistry.

In Figure 4, a QCM experiment is reported for an anti-A IgM coated QCM incubated first with nonspecific group B erythrocytes and after with the specific group A ones.

The specific response of the biofunctionalized transducer is evident; whereas, exposure to group B cells does not cause any appreciable shift in the resonance frequency of the QCM, the subsequent exposure to group A erythrocytes induces a marked frequency shift, revealing cell immobilization. In this case, a quantitative interpretation of the QCM frequency shifts is complicated by the presence of viscous loads (30).

An even more direct proof of the retention of the functional activity of the immobilized IgMs was achieved by direct optical inspection via light microscopy on glass slides functionalized with 3-MPTS. Two functionalized slides were first exposed to anti-A IgMs from goat antiserum and subsequently incubated with a different kind of erythrocyte. Figure 5 reports the results of such a test. Just after incubation, the two slides, exposed to group A and B erythrocytes, respectively, appear to be covered by a dense layer of cells. Nevertheless, a simple rinse in a physiologic solution removes almost completely the B-type cells, which are just lying on the substrate. This is not the case with the group A cells, which instead remain at the surface as a result of their specific immobilization on the (anti-A) IgM layer.

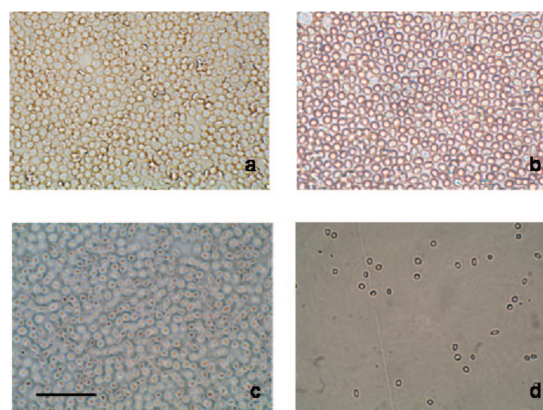


FIGURE 5. Results of optical inspection of microscopy slides functionalized with 3-MPTS and then exposed to goat antiserum containing anti-A IgMs: slides after exposure to (a) group-A RBC and (b) group-B RBC and before rinsing in PBS; (c and d) the two slides after the rinsing procedure. Bar = 50 μm.

This evidence, showing retention of the functional activity of the immobilized biomolecular layer, outlines also the high specificity of the realized biointerface. If, on the one hand, such a specificity is provided by the avidity that the selected antibodies display for their specific antigens, on the other hand, it is not a priori obvious that such an avidity is preserved after IgM binding to a solid surface. In fact, upon interaction with high-free-energy, chemically reactive surfaces, biomolecules can easily undergo a number of phenomena ranging from slight conformational alterations to partial or even total unfolding with a loss of functional activity. In the present case, instead, whereas the native molecular structure appears to be quite affected, the overall antigen recognition ability of IgMs is preserved. Functional activity preservation can likely be ascribed to the fact that the selected immobilization strategy targets chemical moieties (exposed S—S bonds), which are basically involved in keeping the Y-shaped functional monomers together but are far from the antigen binding regions, located on the two "arms" of each monomer (Fab fragments), that are involved in the specific binding of antigens.

Therefore, we can conclude that we have demonstrated a robust and simple method for the effective capture of IgMs from antisera and their immobilization on solid substrates by means of suitable molecules (silanes and dithiols in the present cases) bearing SH moieties. IgM antibodies, albeit likely affected in their structure by interaction with the surface, retain their functional activity as demonstrated by

ARTICLE

their preserved ability to bind selectively their specific antigens. We believe that the realized biomolecular functional interface can find useful applications in the field of biosensors and biomolecular devices in general.

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