

Improvement of protein immobilization for the elaboration of tumor-associated antigen microarrays: Application to the sensitive and specific detection of tumor markers from breast cancer sera

Zhugen Yang^a, Yann Chevolot^a, Thomas Géhin^a, Jérôme Solassol^b, Alain Mange^b, Eliane Souteyrand^a, Emmanuelle Laurenceau^{a,*}

^a Université de Lyon, Institut des Nanotechnologies de Lyon (INL)—UMR CNRS 5270, Ecole Centrale de Lyon, 36 Avenue Guy de Collongue, 69134 Ecully cedex, France

^b CHU Montpellier, Laboratoire de biologie cellulaire et hormonale, Hôpital Arnaud de Villeneuve, 371 avenue du Doyen Giraud, Montpellier F-34295, France

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ABSTRACT

There is an urgent need to identify relevant tumor markers showing high sensitivity and specificity for early diagnosis and prognosis of breast cancer. Protein microarrays have demonstrated to be cost-effective, high through-put and powerful tools for screening and identifying tumor markers with only minute samples. Autoantibodies directed against tumor-associated antigens (TAAs) were shown to be relevant tumor markers. However, due to the variability of immune response from one individual to another and depending on the type of cancer, detection of only one type of anti-TAA autoantibody is not sufficient to give a reliable and precise diagnosis. It is necessary to use a set of several TAAs for determining specific autoimmune profiles. Therefore, combining various TAAs on different surfaces could improve sensitivity and specificity for anti-TAA autoantibody detection. Herein a panel of 10 proteins, including well-known tumor-associated antigens (TAAs) and potential new biomarkers of breast cancer, were immobilized onto microstructured microarray under optimized conditions (spotting pH buffer, surface chemistry, blocking procedure), in order to determine an autoimmune signature of breast cancer. Sera from 29 breast cancer patients and 28 healthy donors were screened in sandwich immunoassays on the miniaturized system to detect the eventual presence of anti-TAAs autoantibodies. Results indicated that the detection level of each anti-TAA autoantibody in a given serum sample was strongly dependant on the surface chemistry. Combining five TAAs (p53, Hsp60, Hsp70, Her2-Fc, NY-ESO-1) on two different surface chemistries (NHS and APDMES) allowed the significant detection of more than 82% breast cancer sera.

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1. Introduction

The global burden of cancer has more than doubled in the past 30 years and breast cancer is the most common cancer among women worldwide. It was reported by World Health Organization (WHO) that 636,000 and 514,000 incident cases occurred in developed and developing countries during 2008, respectively. Conventional techniques for breast cancer diagnosis include: mammography, ultrasonography, magnetic resonance imaging (MRI), and positron emission tomography (PET) (Lord et al., 2007). These techniques are often costly and actually not immediately available for many people. With the recent development of proteomic technologies, varieties of tumor markers were identified and employed for cancer detection with immunoassay

methods (Ludwig and Weinstein, 2005; Sidransky, 2002). However, the lack of sensitivity and specificity of these biomarkers tested individually and their low frequency and heterogeneity in patient sera are a challenge to testing techniques for cancer diagnosis.

Among the tremendous number of tumor markers relative to breast cancer, oncofoetal protein (CEA), oncoproteins (Her2, c-myc, p53) and carbohydrate antigens (CA15-3, CA27-29) are the most proposed. Besides, mammaglobin, survivin, livin, NY-ESO-1, Annexin XI-A, Endostatin, Hsp60 and p62 are well documented tumor markers (Duffy, 2006; Molina et al., 2005). Since breast cancer is a heterogeneous disease, these tumors express many aberrant proteins and diagnose based on single tumor marker detection of either antigen or antibody usually lack sensitivity and specificity as noticed in most studies. A research group reported that tumor marker guided PET scan in the follow-up of breast cancer patients has a sensitivity of 92%, specificity of 75% and a positive predictive value of 89% in the detection of

* Corresponding author. Tel.: +33 47 2186240; fax: +33 47 2186250.
E-mail address: emmanuelle.laurenceau@ec-lyon.fr (E. Laurenceau).

occult tumor recurrence (Suarez et al., 2002). However, PET could not identify low-grade lesions or tumors less than 5 mm in size. Cheung et al. (2000) summarized tumor marker measurements in the diagnosis and monitoring of breast cancer. CA15-3 has a higher sensitivity (73%) than CEA (50%) but with a similar specificity. However, combining several tumor markers demonstrates to be better than any single marker in the diagnosis and monitoring of breast cancer. The sensitivity could reach beyond 90% when the two markers (CEA and CA15-3) are used together.

As emphasized above, multiplex detection of a set of TAAs was shown to be more sensitive and specific than the detection of a single tumor marker (Cheung et al., 2000; Desmetz et al., 2009; Madoz-Gurpide et al., 2008). Thus, protein microarray technology has begun to play a significant role in the detection and quantification of proteins in complex biological samples. In particular such approach is high throughput and multiparametric assay for screening and identifying tumor markers from minute samples (Madoz-Gurpide et al., 2008; Robinson et al., 2002). Unlike traditional tumor markers, autoantibodies against tumor-associated antigens (TAAs) are found in serum from patients with different cancers, and may represent early indicators of tumor development (Desmetz et al., 2009; Liu et al., 2011). In a recent report (Desmet et al., 2011), a panel of 12 proteins involved in cancer pathology was immobilized within the nitrocellulose/cellulose acetate membrane of a 96-well filtering microtiter plate bottom via non-covalent interactions. The captured autoantibodies in cancer sera were detected using a staining approach based on alkaline phosphatase labeling. The results showed that five of these proteins (p53, NY-ESO-1, IMP1, cyclin B1 and c-myc) were able to detect 72.2% of the cancer patients tested (within a panel of 18 patients and 16 healthy donors). The authors demonstrated the advantage to use a multiple antigen panel for increasing the sensitivity of auto-antibody detection in cancer sera. To gain more diagnose efficiency of the immunoassay, it becomes essential to implement the TAA panel with complementary antigens. Moreover, testing a larger population of healthy sera would enable the determination of more accurate cut-off values for each probe in view to avoid false-positive results.

Although protein microarray can perform high throughput detection of tumor markers in single assay, efficient multiplex analysis remains challenge due to biomarkers variability and lack of sensitivity. One of the key parameters in protein microarray elaboration is to improve sensitivity is the surface chemistry. Indeed, immobilization of probe proteins on a surface leads to partial loss of their biological activity. Many efforts have been done to develop surfaces which retain biological activity of immobilized probes and limit non-specific adsorption of proteins (Olle et al., 2005; Lee and Shin, 2005; Monchaux and Vermette, 2007; Nijdam et al., 2009). However, because of the variability of protein structure and function, no universal surface chemistry meets this schedule of conditions. In a previous study, we demonstrated that the immobilization yield was dependent on the type of protein (bovine serum albumin, streptavidin, and immunoglobulin) and on surface properties (Laurenceau et al., 2011; Yang et al., in press). Moreover, regarding the biological activity of various immobilized antibodies for the detection of colorectal tumor markers, the analytical performances of the antibody microarrays were also dependent on surface chemistry. Thus, it is essential to optimize the surface chemistry to each type of immobilized proteins. Other parameters which could affect protein immobilization are the pH and ion composition of the spotting buffer (Cacace et al., 1997).

In this paper, we investigated both surface chemistry and protein immobilization conditions to improve the sensitivity of the detection of tumor autoantibodies based on TAAs microarray. Ten proteins including four well-known TAAs (CEA, p53, HER2,

NY-ESO-1), two heat shock proteins (Hsp60, Hsp70) and four potential new TAAs (MYCL1, CHEK2, HNRNPK, NME1), were immobilized onto microstructured glass slides functionalized with various surface chemistries. All the surface modifications were home-made according to well-established protocols. The presence and amount of autoantibodies directed against these proteins were evaluated in 29 sera from breast cancer patients and 28 healthy donors. Results evidenced that the sensitivity and specificity depends both on the surface chemistry, and on the panel of TAAs.

2. Experimental

2.1. Materials

All chemicals were of reagent grade or highest available commercial-grade quality and used as received unless otherwise stated. Bovine serum albumin (BSA) lyophilized powder, dimethyl sulfoxide (DMSO, anhydrous, P99.9%), 0.01 M phosphate-buffer saline (PBS, pH 7.4) at 25 °C (0.0027 M potassium chloride and 0.138 M sodium chloride), sodium bicarbonate NaHCO_3 ($M_r=84.01$ g/mol), sodium carbonate Na_2CO_3 ($M_r=105.99$ g/mol), N-Hydroxysuccinimide (NHS), N, N'-diisopropylcarbodiimide (DIC), 2-(N-morpholino) ethanesulfonic acid (MES), tetrahydrofuran (THF) (purum grade), poly vinylalcohol (PVA), (3-glycidoxypropyl) dimethylethoxysilane (APDMES), and maleic anhydride-alt-methyl vinyl ether (MAMVE, $M_w=216,000$ g/mol), all were obtained from Sigma-Aldrich (St. Quentin Fallavier, France). Tween 20 was purchased from Roth-Sochiel (Lauterbourg, France). Dextran ($M_w=40,000$ g/mol) was purchased from Pharmacosmos. Chitosan ($M_w=470,000$ g/mol, degree of deacetylation (DD) 94%) was kindly provided by Dr. T. Delair (Polymer Materials and Biomaterials Laboratory (LMPB), University Claude Bernard Lyon 1, Lyon, France).

Borosilicate flat glass slides ($76 \times 26 \times 1$ mm³) were purchased from Schott (Mainz, Germany). Hydrogen peroxide (H_2O_2) solution (30 vol) was obtained from Gilbert Laboratories (Hérouville Saint-Claire, France). Ultrapure water (18.2 M Ω) was delivered by an Elga water system.

Anti-tumor antibodies (anti-CEA, anti-Hsp60) and tumor antigens (CEA, Hsp60) were provided by bioMérieux. Other tumor markers and recombinant protein are commercial products. Myoglobin and p53 was obtained from Sigma-Aldrich. MYCL1, CHEK2, HNRNPK, NME1, glutathione S-transferase P1 (GSTP1), Transglutaminase 1 (TMG1), Epstein Bar Virus Nuclear Antigen 1 (EBV-NA) were supplied by Abnova Corporation (Taiwan). Hsp70 and Measle Hemagglutinin Protein (MHP) were provided by Abcam plc (UK). All the proteins were stored as aliquot at -20 °C or -80 °C following manufacturer specifications. Her2-Fc antigen was purchased from R&D system (Minneapolis, USA). NY-ESO-1 antigen and anti-Her2-Fc antibody was supplied by Thermo Scientific (USA). Cy3-labeled goat anti-human antibody (IgG+IgA+IgM) and Cy3-labeled streptavidin were purchased from Jackson ImmunoResearch and Sigma, respectively. Amersham dye of Cy3-NHS ester was purchased from GE healthcare (USA).

Myoglobin was labeled according to Alexa Fluor 647 Micro-scale Protein labeling Kit (A30009) (Molecular Probes, Inc. USA), and the dye/protein ratio was determined to 2.2.

0.01 M PBS or PBS 1X (pH 7.4) was prepared by dissolving the content of one pouch of dried powder in 1 L of ultrapure water. 0.1 M sodium acetate powder was dissolved to obtain the sodium acetate buffer, and pH was adjusted to 4.5. 0.02 M sodium carbonate buffers at pH 10.7 was prepared from 0.1 M sodium bicarbonate (NaHCO_3) NaHCO_3 and 0.1 M Na_2CO_3 solutions in ultrapure water. 0.1 M sodium bicarbonate (NaHCO_3) was

prepared to adjust pH at 8.3 for myoglobin labeling. 0.01 M 2-(N-morpholino) ethanesulfonic acid (MES) (pH 6.2) was prepared by dissolving the content of one pouch into 1 L ultrapure water and adjust pH up to 6.2. Washing buffer contained PBS 1× (pH 7.4) and 0.1% Tween 20 (PBS-T). Blocking solution was prepared by dissolving 10 g of BSA in 100 ml of PBS-T (10% BSA/PBS-T).

2.2. Serum samples

All human samples were prospectively collected between 2005 and 2007 at the CRLC Val d'Aurelle Cancer Institute, Montpellier, France, at the time of cancer diagnosis after obtaining written informed consent. Blood samples were centrifuged at 1250g for 5 min, and the serum was stored at -80°C . For the multiplex immunoassay, 57 serum samples were examined: 28 healthy controls with negative mammograms, negative physical breast exams for at least 4 years, and no history of prior malignancy, and 29 patients who underwent surgery and had a histopathologic diagnosis of early-stage breast cancer. This study was approved by the Montpellier University Hospital human research committee and the INSERM review board (RBM-03-63).

2.3. Surface modification of microstructured glass slides

Flat microscope glass slides were microstructured to 4×10 wells (Mazurczyk et al., 2008; Vieillard et al., 2007) and silanized as described previously (Dugas et al., 2004; Mazurczyk et al., 2008). Briefly, the slides were silanized with tert-butyl-11-(dimethylamino) silylundecanoate. The tert-butyl esters were then hydrolyzed with formic acid for 7 h at room temperature to convert into carboxylic group (COOH surface). The slides were washed with dichloromethane for 10 min in an ultrasonic bath followed by 10 min in deionized water (enough volume was added to completely immerse the slides). Activation of carboxylic acid was carried out with a mixture of NHS/DIC (molar ratio 1:1, 0.1 M) in THF overnight at room temperature to obtain NHS surface. Then slides were washed for 10 min in THF and 10 min in dichloromethane under ultrasound.

Alternatively, the microstructured slides were aminosilanized with (3-aminopropyl) dimethylethoxysilane (APDMES) as the following protocol: slides rinsed with fresh piranha ($\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$, v/v 70/30) for 30 min in ultrasonic bath and DI water were deposited in nitrogen stream at 150°C for 2 h. After the system cools down to room temperature, 0.1% of APDMES (v/v) in pentane were added and allowed to react at room temperature with stirring for 1 h. After evaporating pentane, incubation occurred overnight in nitrogen stream at 150°C . The silanized slides (APDMES surface) were rinsed with pentane, THF and DI water in ultrasonic bath for 10 min.

Chitosan surface was obtained by functionalization of the NHS surface with chitosan solution at 1 mg/mL prepared in acetic acid/DI- H_2O mixed solvents. The concentration of acetic acid was determined according to the degree of deacetylation (DD) and expected concentration of chitosan.

CMD surface was generated from the APDMES surface. 1 mg/mL CMD (degree of substitution (DS) 63%, synthesized in our lab) activated with EDC/NHS (CMD/EDC/NHS molar ratio: 1/4/1) in MES buffer (pH 6.2) was allowed to react with deprotonated APDMES surface (previously incubated in 0.02 M sodium carbonate solution pH 10.7 for 1 h at room temperature).

Deprotonated APDMES surface were incubated for 4 h at room temperature with MAMVE (1 mg/mL) solubilized in DMSO to obtain MAMVE surface. Slides were washed with THF and dichloromethane for 10 min, under ultrasonic bath.

All the surface chemistries of glass slides were illustrated in Supporting information (Fig. S1).

2.4. Tumor autoantigens microarray on functionalized surfaces

CEA, Hsp60, Hsp70, p53, Her2-Fc, NY-ESO-1, MYCL1, CHEK2, HNRNP, and NME1 were spotted at 0.1 mg/mL with sciFLEX-ARRAYER S3 (Scienion, Germany) into microwells of functionalized glass slides as described in Supporting information (Fig. S2). Each spot corresponded to a dispense volume of 400 pL. Spotting buffers were composed of sodium acetate (pH 4.5), PBS (1×, pH 7.4) and sodium carbonate (pH 9.6) with 0.05% PVA as additive. Three positive controls (EBV-NA, MHP, GSTP1) and one negative control (TMG1) were spotted at 0.1 mg/mL, as well as buffer and myoglobin-Cy3 at 0.6 μM as quality controls. Proteins were allowed to react with functionalized surfaces under water-saturated atmosphere overnight at 4°C . Slides were then washed for 2×5 min with PBS and for 5 min with PBS-T, and then dried by centrifugation for 3 min at 1300 rpm. To limit further non-specific adsorption, slides were blocked with 10% BSA/PBS-T solution for 2 h at room temperature, then washed for 3×5 min with PBS-T and dried.

Microwells were then incubated with biotinylated antibodies (anti-CEA, anti-Hsp60, anti-p53, and anti-Her2-Fc) and sera from breast cancer patients and healthy donors diluted at 1/250 in 4% BSA/PBS-T (Fig. S2). Each microwell was incubated with one sample (biotinylated antibody or serum) and left to react for 1 h at room temperature in a water-saturated atmosphere, thoroughly rinsed for 3×5 min with PBS-T and then dried. Microwells tested with sera were then incubated with Cy3-conjugated goat anti-human Ig(A+M+G) (0.015 mg/mL diluted in 1% BSA/PBS-T), and microwells tested with purified biotinylated antibodies were incubated with streptavidin-Cy3 (0.1 mg/mL in 1% BSA/PBS) for 1 h at room temperature in a water-saturated atmosphere, followed by washing for 3×5 min with PBS-T 0.1%, 1 min with DI water and dried by centrifugation.

2.5. Fluorescence scanning

After drying, slides were scanned with the Microarray scanner, GenePix 4100A software package (Axon Instruments) at 532 nm with photomultiplier tube (PMT) 400. The fluorescence signal of each antibody was determined as the average of the median fluorescence signal of six spots, and the value was divided by the signal of surface background to get the signal-to-noise ratio (SNR).

3. Results and discussion

3.1. Optimization of protein immobilization parameters

Many parameters such as surface chemistry, pH and ion composition of spotting buffer, drying effects, protein concentration, temperature, could affect the efficiency of protein immobilization following spotting. The use of hydroxylated molecules (glycerol, polyvinyl alcohol, trehalose) as additives in the spotting buffer was shown to prevent complete drying of the protein drop and to preserve protein structure and activity (Wu and Grainger, 2006). Best performances in terms of spotted protein distribution homogeneity, uniform spot morphology, immobilized protein bioactivity, and spot-to-spot variance, were obtained with polyvinyl alcohol (PVA). Taking into account these data, we introduced 0.05% PVA (m/v) as additive in spotting buffers. PBS pH 7.4 is mostly used as a spotting buffer in protein microarray technology because of its physiological pH value. However, during protein immobilization process on the surface, charge effects on both protein and the surface could influence protein loading capacity, as well as ion composition. Thus, we investigated the

effect of three commonly used buffers (sodium acetate (Ac) pH 4.5, PBS pH 7.4, and sodium carbonate (Car) pH 9.6) on protein immobilization capacity as a function of surface chemistry. Two fluorescent labeled proteins, also called reference proteins, streptavidin-Cy3 ($M_w=52,800$ g/mol; $pI=6.1$) and myoglobin-Cy3 ($M_w=17,083$ g/mol; $pI=7.29$) were used for this study because they displayed characteristics (M_w and pI) close to the immobilized TAAs. The surface chemistries employed were all based on silanization of glass slides introducing carboxylic groups (COOH surface) which could be activated with NHS/DCI (NHS surface), or amino groups (APDMES surface). These silane layers have already shown to be effective to immobilize proteins (El Khoury et al., 2010a, 2010b; Nijdam et al., 2007). In order to increase the specific surface area for protein immobilization, polymer layers were introduced on silane layers. Carboxymethyl dextran (CMD) and chitosan are two commonly used biopolymers (Monchaux and Vermette, 2007; Kim et al., 2004), and MAMVE copolymer contains amine reactive moiety for covalent coupling with proteins. In a recent study, we have shown that these three polymer layers allowed the efficient immobilization of antibodies for tumor markers detection (Yang et al., in press). In this paper, CMD and MAMVE were grafted on APDMES aminosilane layer instead of jeffamine crosslinker, to reduce the number of surface chemistry steps. Immobilization of proteins was achieved by covalent linking on NHS, CMD and MAMVE surfaces, whereas physisorption was involved on COOH, APDMES and chitosan surfaces.

The relative immobilization capacity of the reference proteins was evaluated by calculating the signal-to-noise ratio (SNR) of immobilized fluorescent labeled proteins versus surface chemistry. As shown in Fig. 1, SNR of myoglobin was overall higher than that of streptavidin. Firstly, the molecular weight of myoglobin is three times lower than that of streptavidin, involving that up to three times more myoglobin than streptavidin could bind to surfaces leading to higher fluorescent signal. Secondly, the degree of labeling of myoglobin and streptavidin was different, with about 2 and 3 to 9 dyes per molecule, respectively. Thus it is difficult to compare the immobilization level between the two proteins on the same surface. However, Fig. 1 indicates that both protein immobilization levels depended not only on the surface chemistry but also on the spotting buffer. Surfaces functionalized with carboxylic groups (COOH surface) or with amine reactive groups (NHS, CMD and MAMVE surfaces) displayed higher

protein immobilization levels with acetate buffer (pH 4.5) than that with the other two buffers. By contrast, on chitosan surface both proteins were more efficiently immobilized with carbonate buffer (pH 9.6). APDMES surface showed a middle behavior, with better protein immobilization using PBS (pH 7.4). These results pointed up the importance of electrostatic interactions between proteins and surfaces both for covalent linking and adsorption. Indeed, most proteins carry a net charge in physiological media, with the sign and magnitude depending on the isoelectric point (pI) of the protein. In PBS (pH 7.4), myoglobin carries neutral global charge because pH is very close to its isoelectric point $pI=7.29$ whereas in acetate buffer (pH 4.5) and in carbonate buffer (pH 9.6), myoglobin is positively and negatively charged, respectively. Immobilization of myoglobin was more effective when the protein was charged, i.e. using acetate or carbonate buffers than PBS (Fig. 1A). Concerning streptavidin ($pI=6.1$), in acetate buffer, the protein is positively charged whereas in PBS and carbonate buffer, it is negatively charged. Therefore, immobilization of streptavidin occurred efficiently with the three buffers. However, the level of adsorbed proteins also depends on the relative surface charge. Comparing various negatively charged surfaces (CMD coating) and non-charged surface, McArthur et al. (2000) have shown that electrostatic interactions dominate with highly charged CMD, while steric-entropic forces influence is increased on less charged surfaces. Moreover, they showed that wetting properties of the surface is also important.

In this study, adsorption of proteins occurred more efficiently when the surface was poorly or not charged (COOH surface in acetate buffer, APDMES surface in PBS, and chitosan surface in carbonate buffer) and protein carried a net charge. Indeed, COOH and APDMES surfaces are alkylsilane layers with either one carboxylic or one amine group per molecule, respectively. Both surfaces displayed low polar energy (~ 6 mJ/m²) (Yang et al., in press). This suggests that protein adsorption is mainly governed by Van der Waals forces rather than electrostatic interactions. However on chitosan surface, adsorption of proteins seems to be governed by hydrogen-bond. Indeed, the chitosan used in this study is highly deacetylated (DD=94%) and has a high molecular weight ($M_w=416,000$ g/mol). Chitosan amine functions were not charged at pH 9.6. Due to the polysaccharide backbone, chitosan surface displayed high polar energy (~ 14 mJ/m²) leading to hydrogen-bond with proteins.

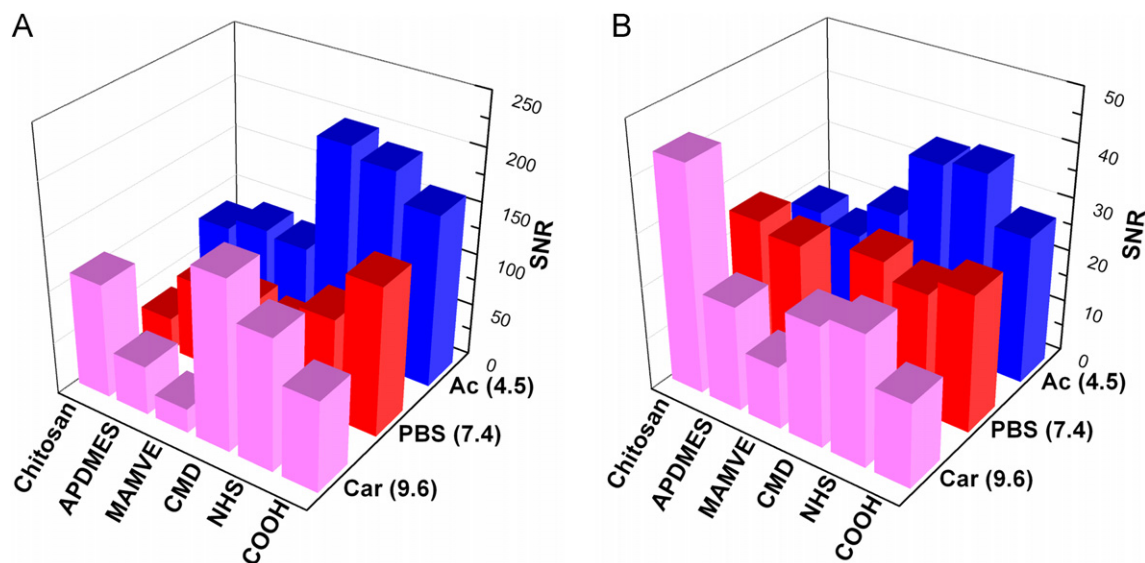


Fig. 1. Signal-to-noise ratio (SNR) of references protein (A) myoglobin-Cy3 and (B) streptavidin-Cy3 spotted at 0.6 μ M in three buffers with different pH values on various surface chemistries; standard deviation (SD) is in the range of 6%–18% of the mean value SNR.

The covalent coupling of protein on amine reactive surfaces (NHS, CMD, MAMVE) is also pH dependent: There is a balance between surface ionization, protein charge and amine protonation, and chemical reactivity of the surface. For example, it was reported that maleic anhydride copolymers could be negatively charged at low pH, due to partial hydrolysis of the anhydride moiety, and their swelling was not good (Pompe et al., 2005). In our case, for all amine reactive surfaces, the best coupling solution was the acetate buffer. According to the fluorescent signal, protein immobilization was more efficient on NHS and CMD surfaces than on MAMVE surface. As mentioned above, partial hydrolysis of anhydride moiety and bad swelling of MAMVE copolymer could occur at pH 4.5 reducing surface reactivity.

It is to note that buffers tested contain different ionic compositions which could also have an effect on protein–surface interactions (Cacace et al., 1997). Nevertheless, our experimental results demonstrated that myoglobin and streptavidin spotted in acetate buffer (pH 4.5) displayed good immobilization performance on the various surface chemistries tested. Therefore due to their similar molecular weights and *pI* with these proteins, acetate buffer would be employed to spot TAAs for microarray implementation.

3.2. Validation of TAAs immobilization and biological activity in purified conditions

The availability and reactivity of the immobilized antigens towards their human antibodies, and the spotting reproducibility

are crucial parameters in the implementation of TAA microarray to achieve the best analytical performances. As discussed above, good protein immobilization was obtained with acetate buffer. However, before testing our TAAs microarray on sera, we investigated the remaining biological activity of four immobilized proteins (CEA, Her2-Fc, p53, and Hsp60) representative of the overall panel used with sera. Commercial biotinylated antibodies (anti-CEA, anti-Her2-Fc, anti-p53, anti-Hsp60) were used for biological evaluation. As shown in Fig. 2, the range of SNR values depended on the TAA/anti-TAA system. CEA/anti-CEA recognition displayed very low SNR on all tested surfaces, compared to the other TAA/anti-TAA systems, while Hsp60/anti-Hsp60 exhibiting the highest ones. Thus the detection of anti-CEA autoantibody in patients' sera should not be sensitive enough and would require further implementation. For the other three pairs of antigens–antibodies, the biological recognition was well detected but SNR was dependent of surface chemistry.

Physical adsorption of Hsp60 on the silanized surfaces (COOH, APDMES) led to better immobilization and/or allowed better recognition of anti-Hsp60 antibody than covalent linking on amino-reactive surfaces (NHS, CMD, MAMVE). However, covalent coupling on polymer surfaces (CMD, MAMVE) displayed higher efficiency than on NHS surface, which is a monolayer reactive surface. p53 exhibited the same behavior with higher immobilization/recognition activities via physical adsorption on APDMES and COOH surfaces, and on amino-reactive polymer surface such as CMD. Hsp60 and p53 displayed similar molecular weight,

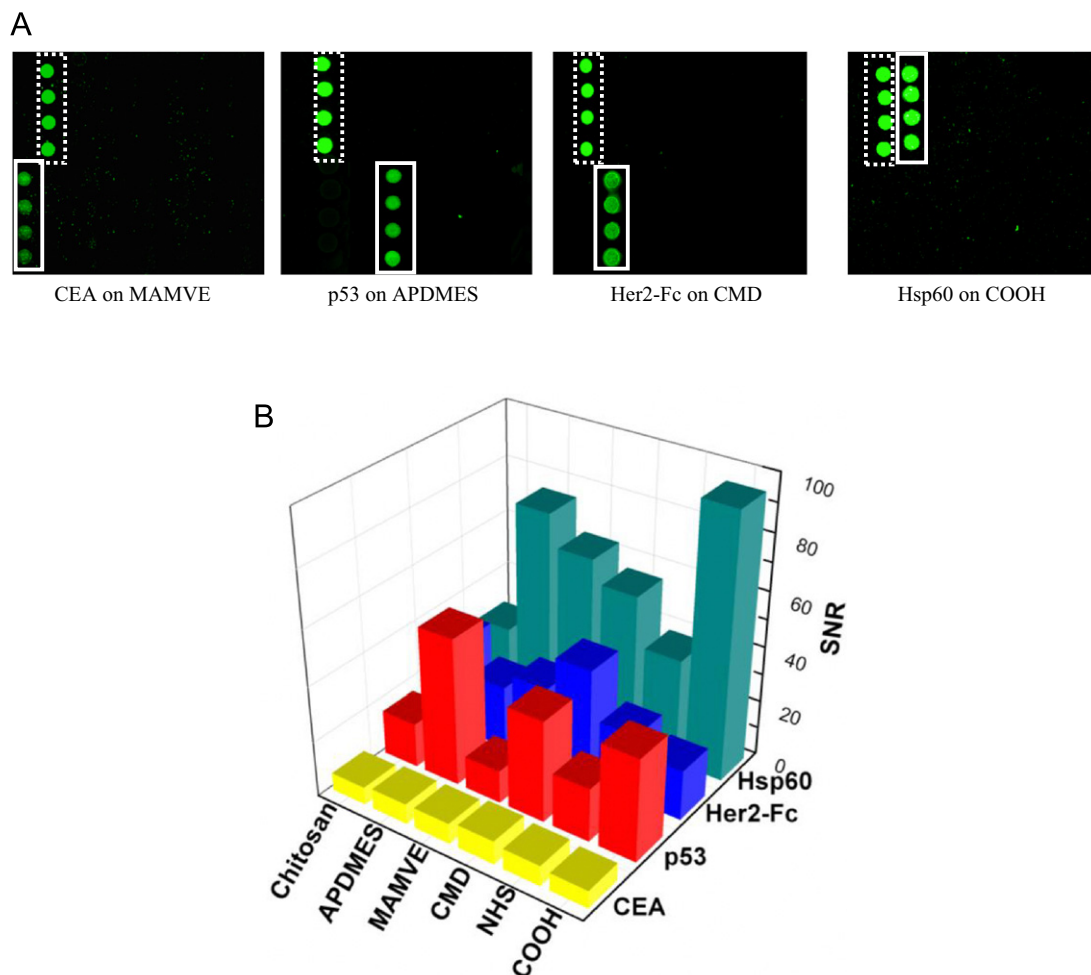


Fig. 2. Scanner images (A) and signal analysis (B) of the specific recognition of immobilized TAAs (CEA, p53, Her2-Fc and Hsp60) with biotinylated anti-TAAs antibodies on the different surface chemistries; all SD were in the range of 9%–20% of the mean value SNR. (A) Reference spots of myoglobin-Cy3 are framed by dash line; spots of TAA/anti-TAA recognition are framed by solid line.

60,000 g/mol and 53,000 g/mol, and similar isoelectric point (*pI*), 5.24 and 6.3 respectively. Therefore it is likely that these two proteins behave the same types of interactions towards our surfaces. Concerning the biological activity of Her2-Fc immobilized on the various surfaces, best results were obtained on polysaccharide functionalized surfaces, both through physical adsorption on chitosan surface and through covalent linking on CMD surface. It was previously suggested that immobilization of proteins by physical adsorption could be more efficient than covalent coupling but depends on probe proteins and surface chemistry (Angenendt et al., 2003; Kusnezov et al., 2003; Yang et al., in press).

Here, surfaces suitable for protein immobilization by physical adsorption (COOH and APDMES surfaces) were also shown to retain good biological activity. But with covalent immobilization of proteins, CMD and MAMVE surfaces were more suitable than NHS surface for maintaining good biological activity. Because there is not a unique surface which suits all proteins, surface modifications should be chosen after optimization of immobilization parameters and biological activity. However, the validation of the biological activity of immobilized proteins was performed with monoclonal antibodies which probably represent only part of the antibodies present in serum. Thus, the results obtained demonstrated that all developed surface chemistries were suitable for the immobilization of the tested tumor-associated antigens with respect to their biological activity. Thus, for a given TAA, two or three surface chemistries could be chosen and tested for the detection of auto-antibodies against TAAs in cancer patients' sera.

3.3. Detection of anti-TAA autoantibodies in breast cancer sera by multiplex immunoassay

Herein, microstructured glass slides functionalized with the six above described surface chemistries were spotted with 15 protein probes for the multiplex immunoassay approach. These probes included four well-known TAAs involved in breast and other cancers (CEA, Her2-Fc, NY-ESO-1, p53), two heat shock proteins (Hsp60, Hsp70), four potential new biomarkers of breast cancer (MYCL1, CHEK2, HNRNPK, NME1), three positive controls (GSTP1, MHP, EBV-NA), one negative control (TMG1) and Cy3-labeled myoglobin as quality control of protein immobilization (Fig. S2). The presence of anti-TAAs autoantibodies was evaluated from 29 breast cancer sera and 28 healthy donor sera collected by CHU Montpellier (France). According to preliminary results, the best serum dilution avoiding saturation and allowing the detection of low TAA concentration was 1/250 (data not shown). The response of some probes toward both normal and cancer sera are insufficient to be detected or distinguished from buffer spot, such as CEA, MYCL1, CHEK2, HNRNPK, NME1, GSTP1 and TMG1. These probe proteins displayed very low or even no signals probably due to weak immobilization/recognition on our microarray platform, and/or loss of biological activity after receipts of commercial products. In previous experiments, these probes were tested with five breast cancer sera and two healthy donor sera included in this study. Low or no responses were obtained (data not shown). Future work will focus on the purchase of these antigens in other commercial agent to repeat the experiments, as well as the incubation with available purified antibodies, in order to check the reasons of no responses and implement our detection system for such probes. Consequently, CEA, MYCL1, CHEK2, HNRNPK, NME1, GSTP1 and TMG1 were removed in the following discussion.

The reference protein Cy3-labeled myoglobin was highly reactive on all surfaces (mean SNR values between 33 and 81) confirming that our surface chemistries were suitable for efficient immobilization of proteins. Moreover, positive controls EBV-NA and MHP displayed significant signal ($1.5 < \text{mean SNR} < 13$) with all sera. As expected, no significant differences were obtained between breast cancer sera and

Table 1

Occurrence of positive sera from healthy donors and breast cancer patients for each TAA immobilized on various surface chemistries. Healthy donors data are indicated in bracket. Data in bold represent the occurrence upper than 10% in positive sera.

Surfaces/TAAs	p53	Hsp60	Hsp70	NY-ESO-1	Her2-Fc
COOH	6.9 (0)	6.9 (3.6)	0 (0)	0 (0)	3.4 (0)
NHS	3.4 (0)	41.4 (3.6)	13.8 (0)	20.7 (0)	31.0 (0)
CMD	10.3 (0)	0 (3.6)	0 (0)	13.8 (0)	3.4 (0)
MAMVE	3.4 (0)	0 (0)	0 (4.8)	6.9 (0)	0 (7.1)
APDMES	31.0 (0)	20.7 (0)	0 (0)	0 (0)	6.9 (7.1)
Chitosan	17.2 (0)	6.9 (0)	0 (0)	0 (0)	13.8 (0)

healthy donor sera. From these data, we can conclude that all tested sera were suitable for the immunoassay.

In the following, among the 10 tumor markers immobilized, only the five giving significant signals (Her2-Fc, p53, NY-ESO-1, Hsp60, and Hsp70) were analyzed. For each TAA immobilized on each surface, a cut-off value for the detection of the corresponding autoantibody was calculated from healthy donor sera as the mean of SNR + 3SD (Standard deviation). A serum was considered as positive toward a given TAA if the corresponding SNR value was higher than the cut-off. According to this criterion, the occurrence of positive sera from healthy donors and breast cancer patients were reported in Table 1.

Hsp60 on COOH surface, Hsp70 and Her2-Fc on MAMVE surface, and Her2-Fc on APDMES surface displayed false positive from the healthy donor samples. Moreover, depending on the surface chemistry, Hsp60, Hsp70, NY-ESO-1 and Her2-Fc did not show any positive response from breast cancer samples. However, each TAA were able to detect more than 10% of breast cancer patients when immobilized on optimal surface chemistry: p53 on CMD, APDMES and chitosan surfaces; Hsp60 on NHS and APDMES surfaces; Hsp70 on NHS surface; NY-ESO-1 on NHS and CMD surfaces; Her2-Fc on NHS and chitosan surfaces. As expected, the optimal surfaces with sera were not exactly the same as with monoclonal antibodies. For example, anti-p53 autoantibodies present in breast cancer sera were efficiently detected on CMD and APDMES surfaces as for monoclonal anti-p53 antibody. Chitosan surface was not a good surface with monoclonal antibody, but allowed good detection of anti-p53 autoantibodies in sera. Furthermore, whilst the biological activity of Hsp70 and NY-ESO-1 was not evaluated with monoclonal antibodies, detection of anti-Hsp70 and anti-NY-ESO-1 autoantibodies in sera was effective indicating that parameters used for protein immobilization were well defined. Thus it is essential to identify the best surface chemistry retaining the highest biological activity of the immobilized protein. Based on these results, we selected surfaces and TAAs which compiled the best predicting parameters to discriminate the healthy donor and breast cancer groups. Fig. 3 presented SNR values of healthy donor and breast cancer sera related to this selection: Hsp60, Her2-Fc, NY-ESO-1, and Hsp70 immobilized on NHS surface, and p53 immobilized on APDMES surface.

We can observe that the combination of several TAAs led to increase in TAA autoantibodies occurrence in breast cancer sera (Table 2). Indeed, combining Hsp60 (on NHS) and p53 (on APDMES) increased the predicting percentage of positive patients from 41.4% to 62%. Adding Her2-Fc (on NHS), NY-ESO-1 (on NHS) and Hsp70 (on NHS) allowed increasing more this predicting percentage to 68.9%, 75.8% and 82.7%, respectively. Thus, using these five TAAs, each of them immobilized on the best surface chemistry, 82.7% of breast cancer patients could be detected in one immunoassay thanks to our customized microstructured microarray. It is likely that the level of breast cancer sera detected could be improved using more than five TAAs. Moreover, notice that in addition to NHS and APDMES surfaces selected above, immobilization of p53 and Her2-Fc on chitosan surface, and of NY-ESO-1 on CMD surface could confirm the positivity of 37.9% of breast cancer sera.

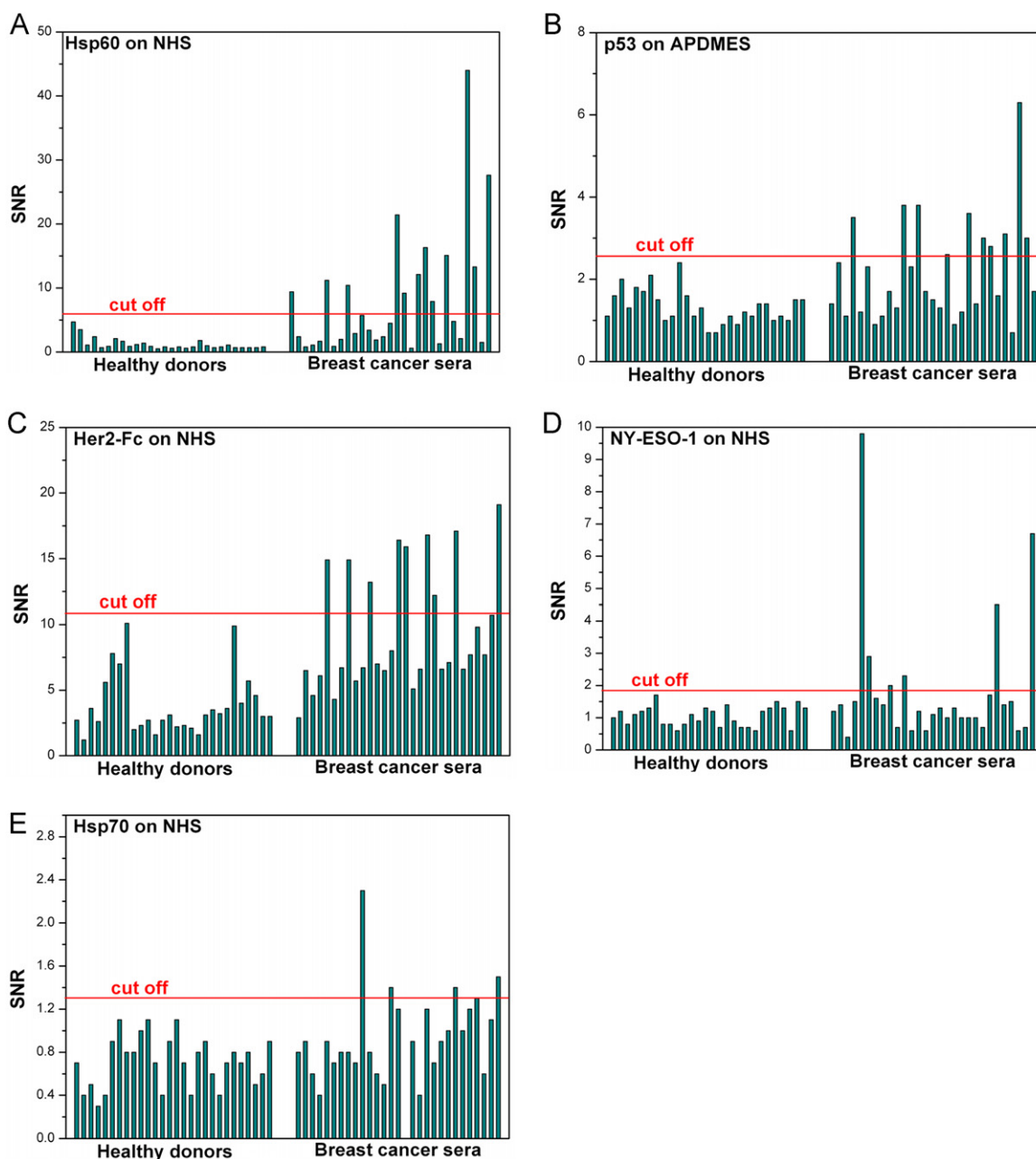


Fig. 3. Detection of autoantibodies directed against the five selected TAAs immobilized on the best surface chemistry, in healthy donor and breast cancer patient sera using multiplex immunoassay; for each TAA, the cut-off value was calculated as the mean SNR+3SD of healthy donor sera.

Table 2

Dependency of tumor-associated antigen autoantibodies occurrence in breast cancer patient sera (%) with combination of TAAs.

Selected TAAs (surface chemistry)	% Occurrence
Hsp60 (NHS)	41.4
Hsp60 (NHS)+p53 (APDMES)	62
Hsp60 (NHS)+p53 (APDMES)+Her2-Fc (NHS)	68.9
Hsp60 (NHS)+p53 (APDMES)+Her2-Fc (NHS)+NY-ESO-1 (NHS)	75.8
Hsp60 (NHS)+p53 (APDMES)+Her2-Fc (NHS)+NY-ESO-1 (NHS)+Hsp70 (NHS)	82.7

4. Conclusions

In this study, a panel of 10 tumor-associated antigens (TAAs) were immobilized onto microstructured glass slides functionalized with six different surface chemistries in order to detect

autoantibodies in breast cancer patients' sera. Surface chemistries included covalent binding (NHS, CMD and MAMVE surfaces) through protein amino groups or physical adsorption (COOH, APDMES and chitosan surfaces). The influence of spotting buffer (pH) was studied on the immobilization of two model proteins

(myoglobin and streptavidin) on the six surfaces. It was found that protein immobilization was partially triggered by electrostatic interactions as well as steric–entropic forces. Thus, protein properties (Mw, pI, structure, etc.) and surface properties (surface energy, number of functional groups, structure, etc.) have to be taken into account to define spotting conditions. Consequently, not a unique surface chemistry is suitable for all proteins and immobilization parameters have to be optimized for each protein.

Then, to validate our customized microarray, four TAAs (CEA, p53, Hsp60, and Her2-Fc) were used with purified antibodies on each surface chemistry. We have demonstrated the dependence of the detection signal of anti-TAA autoantibodies (SNR) on surface chemistry. It relates to the immobilization yield of TAA and to their remaining biological activity. Good relationship between best surfaces for protein immobilization and best surfaces for biological activity was found.

Next, 29 breast cancer patients and 28 healthy donors' sera were tested on our TAA microarrays. Thanks to a combination of five TAAs (Hsp60, p53, Her2-Fc, NY-ESO-1 and Hsp70) immobilized on the optimized surface chemistry, 82.7% of breast cancer patients have been specifically detected. Our results are comparable with other studies concerning anti-TAA autoantibodies detection for cancer diagnosis (Chapman et al., 2007, in press). Further works will focus on increasing the number of tested TAAs in order to identify other relevant TAAs for breast cancer. Moreover, the accuracy will be probably improved by increasing the number of immobilized TAAs on the microarray. Finally, larger cohort of healthy donors and cancer patients will be used to validate the diagnosis test. Thanks to the microstructuration, all optimal surface chemistries will be integrated on the same support leading to improve the accuracy of a cost-effective test.

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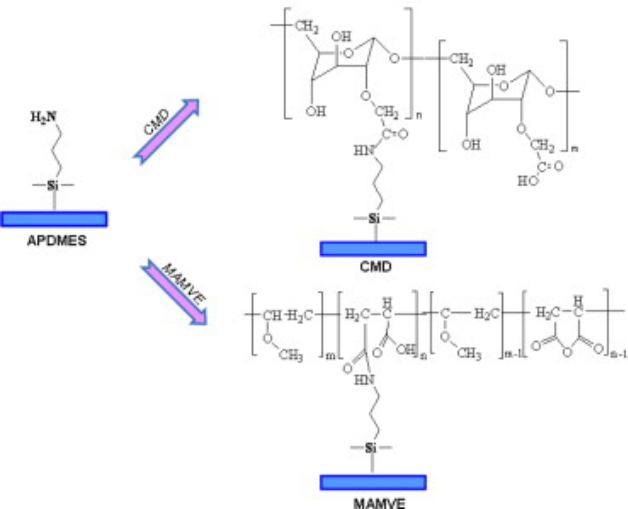
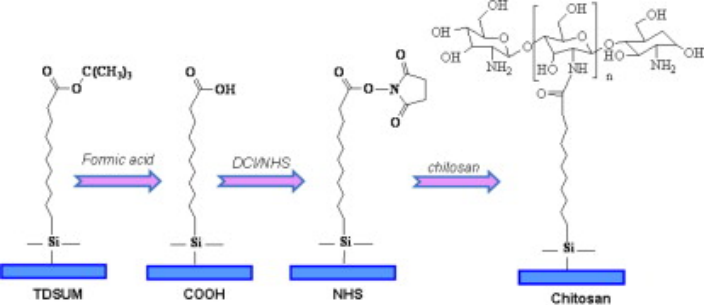
Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2012.08.019>.

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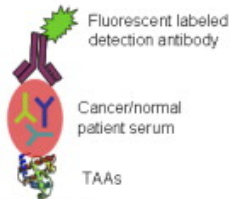




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9	10	11	12	13	14	15	16
9	10	11	12	13	14	15	16

well

1	Buffer	9	CEA
2	Myo-Cy3	10	NY-ESO-1
3	Hsp60	11	Her2-Fc
4	Hsp70	12	P53
5	MYCL1	13	EBVNA1
6	CHEK2	14	MHP
7	HNRNPK	15	GSTP1
8	NME1	16	TMG1



Surface chemistry

Immunoassay on each well