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**Cancer Biomarkers Detection Using 3D Microstructured Protein Chip:
Implementation of Customized Multiplex Immunoassay**

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Abstract: Protein chips have demonstrated to be a sensitive and low cost solution to identify and detect tumor markers. However, efficient multiparametric analysis remains a challenge due to protein variability. Crucial parameters are the design of stable and reproducible surfaces which maintain biological activity of immobilized proteins. These parameters relate to surface chemistry and to immobilization conditions (printing buffers, washing etc). In this study, we have developed and characterized various surface chemistries for the immobilization of anti-tumor antigen antibodies onto microstructured glass slides. The effect of surface properties and antibody immobilization conditions were evaluated for the detection of tumor antigens involved in colorectal cancer. Experimental results demonstrated that the biological activities of the immobilized antibodies were dependent on the surface chemistry and on the immobilization procedure. Optimal immobilization conditions were different for each antibody. Limit of detection in tumor antigen as low as 10 pM can reach under optimized conditions. Our 3D microstructured chip offers the possibility to implement a customized multiplex immunoassay combining optimal immobilization condition for each antibody-antigen system on the same chip.

Key words: Protein chip; Cancer biomarkers; Surface chemistry; Immobilization, Diagnosis

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1. Introduction

Cancer is among the first leading causes of death in the world, but the possibility of cure would substantially be increased if diagnosis could be established at earlier stages. A plethora of serological tumor markers is available for clinical diagnosis, and they can be detected in the sera of cancer patients before clinical symptoms [1-4]. However, the low frequency and heterogeneity of these biomarkers in patient sera brings challenges to classical testing technique for detection of cancer, especially due to the lack of sensitivity and specificity of individual markers. High throughput technology such as protein chip gives the possibility to identify and detect sets of relevant biomarkers in a single assay, with miniaturized sample requirement and significant cost reduction.

Crucial parameters in the elaboration of sensitive protein chips are optimal surface properties such as geometrical consideration of the spots, spot uniformity/homogeneity, probe surface density and surface stability. Furthermore the biological activity of immobilized proteins has to be preserved [5-7]. It is essential to keep high binding capacity for proteins without changing their biological active (three-dimensional structure, functionality and binding sites) conformation as well as to generate a high signal to noise ratio to increase analytical sensitivity.

Various surface chemistries have been developed for the elaboration of protein chip, including epoxide, isothiocyanate, N-hydroxysuccinimide (NHS) ester, amine, semicarbazide, and aldehyde-derivatized surfaces, often introduced by silanization of glass slide as solid support [5, 7-9]. In a recent study, Seurynck-Servoss et al. [10] used a sandwich enzyme-linked immunosorbent assay (ELISA) microarray platform to analyze 17 different commercially available glass slide types. It was demonstrated that the properties of the slide surface affect not only the activity of immobilized antibodies and the quality of data produced, but also parameters such as spot size and morphology, slide noise, spot background, lower limit of detection, and reproducibility. Glass slides coated with aldehyde silane, poly-L-lysine, or

aminosilane (with or without activation with a crosslinker) consistently produce superior results in the sandwich ELISA microarray analyses. Functionalization of the solid support with biopolymers or with synthetic copolymers could also improve protein immobilization and its biological activity. Perrin and co-workers [11] immobilized the recombinant protein derived from the viral capsid p24 of the human immunodeficiency virus (HIV) on maleic anhydride and methyl vinyl ether copolymer (MAMVE) coated surface to elaborate array detecting infected human sera. The conjugated copolymer largely improved detection sensitivity of anti-p24 antibodies in infected human sera. In a previous work [12], we developed silanized glass slides functionalized with N-hydroxysuccinimide (NHS) ester and with MAMVE copolymer for immunofluorescent assays. Analytical performances of these microarrays were evaluated for the detection of anti-histone autoantibodies present in the sera of patients suffering from systemic lupus erythematosus. The detection limit of our MAMVE copolymer microarrays was 50-fold lower than that of the classical ELISA, indicating that MAMVE functionalization is an efficient surface chemistry for protein. Carboxy Methyl Dextran (CMD) was also reported as an efficient surface chemistry for manufacturing biosensor/biochip and it has been reported to be excellent immobilization of both monoclonal antibody and polyclonal antibodies [13, 14] [15]. Alternatively, specific affinity, such as protein A or G with Fc part of an antibody [16] and biotin–avidin/streptavidin interactions [17], are employed for site specific protein immobilization. The effects of the orientation of antibodies and Fab were also observed by Peluso and colleagues [18], indicating that an up to 10-fold increase could be detected in analyte-binding capacity of slide surfaces that promoted oriented immobilisation.

Herein, five anti-tumor antigen antibodies involved in colorectal cancer (anti-CEA, anti-CA19-9, anti-HSP60, anti-PDI, and anti-DEFA6) either were immobilized by covalent linking on three different reactive surfaces (NHS, MAMVE, CMD) or by physical adsorption on an aminated surface (chitosan) and on a carboxylic surface. Firstly, functionalized surfaces were characterized with contact

angle measurements and with the immobilization of fluorescent labeled proteins (IgG, streptavidin, BSA). Then, biological activity of the immobilized antibodies was evaluated by recognition of tumor antigens detected using fluorescent labeled detection anti-tumor antigen antibodies.

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, 4-chloro-1-naphthol (30 mg tablets), dimethyl sulfoxide (DMSO, anhydrous, 99.9%), 0.01 M phosphate-buffered saline (PBS, pH 7.4) at 25 °C (0.0027 M potassium chloride and 0.138 M sodium chloride), sodium dodecyl sulfate (SDS), sodium bicarbonate NaHCO_3 ($M_r = 84.01$ g/mol), sodium carbonate Na_2CO_3 ($M_r = 105.99$ g/mol), N-Hydroxysuccinimide (NHS), Jeffamine D-230 (polyoxypropylenediamine), N, N'-diisopropylcarbodiimide (DIC), tetrahydrofuran (THF) (purum grade), and maleic anhydride-alt-methyl vinyl ether (MAMVE, $M_w = 216000$ g/mol) were all obtained from Sigma (St. Quentin Fallavier, France). Dextran ($M_w = 40000$ g/mol) was purchased from Pharmacosmos. Tween 20 was purchased from Roth-Sochiel (Lauterbourg, France). Chitosan ($M_w=470000$ g/mol, degree of deacetylation (DD) 94 %) was kindly provided by Dr. T. Delair (Polymer Materials and Biomaterials Laboratory (LMPB), Université Claude Bernard Lyon 1).

Borosilicate flat glass slides (76 x 26 x 1 cm) were purchased from Schott (Mainz, Germany). Anti-tumor antibodies and tumor antigens were provided by Y. Ataman-Önal (bioMarkres Department, bioMérieux). Cy3-labeled goat anti-human antibody immunoglobulin G (IgG), Cy3-labeled streptavidin and bovine serum albumin (BSA) were purchased from Jackson ImmunoResearch and Sigma, respectively. Hydrogen peroxide (H_2O_2) solution (30 vol.) was obtained from Gilbert

Laboratories (Hérouville Saint-Claire, France). Ultrapure water (18.2 MΩ) was delivered by an Elga water system.

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. 0.02 M sodium carbonate buffers at pH 10.7 were prepared from 0.1 M NaHCO₃ and 0.1 M Na₂CO₃ solutions in ultrapure water. Blocking solution was prepared by dissolving 4 g of BSA in 100 ml of PBS 1X. Washing buffer contained PBS 1X and 0.05 % Tween 20 (PBS-T) at pH 7.4.

2.2. Surface functionalization of microstructured glass slide

Flat microscope glass slides were microstructured and silanized as described previously [19, 20]. Briefly, the microstructured slides were silanized with tert-butyl-11-(dimethylamino)silylundecanoate. The tert-butyl esters were then hydrolyzed with formic acid for 7 hrs 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.

Chitosan surface was obtained by functionalization of the NHS surface with chitosan solution at 5 mg/mL. Chitosan solution was prepared in acetic acid/DI-H₂O mixed solvents.

The NHS surface was treated in a 0.1 M solution of Jeffamine overnight at room temperature to generate aminated surface. The slides were then washed for 30 min with 0.1% SDS at 70 °C and rinsed with ultrapure water. The generated aminated surfaces are then incubated in 0.02 M sodium carbonate solution (pH 10.7) for 1 h at room temperature to deprotonate amine functions. Then they were incubated for 4hrs at room temperature with MAMVE (5 mg/ml) solubilized in DMSO to obtain MAMVE surface [12]. Slides were washed with PBS 1X and dried by centrifugation.

Carboxy Methyl Dextran (CMD) solution at 5 mg/mL (degree of substitution 63%, synthesized in our lab) was activated with EDC/NHS (molar ratio: 1:1) to react with the aminated surface to generate CMD surface. Prior to protein immobilization, CMD surface was activated with EDC/NHS. Scheme of these surface modifications were described in Figure 1.

2.3. Contact angle measurement of functionalized glass slide

The functionalized glass slides were characterized for surface energy by contact angle measurements (Digidrop Goniometer, GBX, France). De-ionized water, Ethylene-glycol and Diiodomethane were used in all measurements. To minimize the experimental error, the contact angle was measured at five random locations for each sample and the average value was reported. The surface tensions were determined according to Owens-Wendt model.

2.4. Protein chip manufacturing and multiplex immunoassays

Anti-CEA, anti-CA19-9, anti-HSP60, anti-PDI and anti-DEFA6 were spotted (Microgrid II, Biorobotics) into microwells of functionalized microstructured glass slides (Figure 2) (1 type of antibody per microwell) at different concentrations (0.1 μ M, 1 μ M, 5 μ M and 10 μ M) in PBS 1X/20% glycerol spotting buffer. IgG-Cy3 (0.1 μ M), Streptavidin-Cy3 (0.1 μ M), BSA-F647 (0.1 μ M, labeled in our lab) and buffer were spotted also as reference proteins for surface characterization and negative control, respectively. Proteins were allowed to react with functionalized surfaces under saturated water vapors overnight at 37 °C. Slides were washed sequentially for 2 x 5 min with PBS, for 5 min with PBS-T, and then dried by centrifugation for 3 min at 1300 rpm. The slides were blocked with 4% BSA/PBS solution to limit further non specific adsorption phenomena, left to incubate for 2 h at 37 °C, washed for 3 x 5 min with PBS-T and then dried.

Microwells were then incubated with antigens (CEA, CA19-9, HSP60, PDI, DEFA6 in 1% BSA/PBS) at different concentrations (one antigen concentration per microwell, CEA, PDI and DEFA6: 0.01 nM, 0.1 nM, 1 nM, 10 nM, 50 nM, 100 nM

and 500 nM; CA19-9: 10 U/ml, 30 U/ml, 50 U/ml, 100 U/ml, 250 U/ml, 500 U/ml, 1000 U/ml). The slides were left to react for 1 h at 37°C in a water-saturated atmosphere, thoroughly rinsed for 3 x 5 min with PBS-T and then dried.

Microwells were then incubated with 5 µM labelled detection antibodies (anti-CEA-DL647, anti-CA19-9-biot, HSP60-biot, PDI-biot, DEFA6-biot in 1% BSA/PBS-T 0.1%), for 1 h at 37 °C in a water-saturated atmosphere. After washing and drying, microwells were then incubated with streptavidin-Cy3 for 1 h at 37°C in a water-saturated atmosphere, except for the wells incubated with anti-CEA-DL647. The slides were washed for 3 x 5 min with PBS-T and for 1 min with water, followed by drying. The design of the array was presented in Figure 2.

2.5. Fluorescence scanning

After thoroughly washing, slides were scanned with the Microarray scanner, GenePix 4100A software package (Axon Instruments) at wavelengths of 532 and 635 nm with photomultiplier tube (PMT) 500. The fluorescence signal of each antibody was determined as the average of the median fluorescence signal of three spots as well as removing the signal of background. For the reference proteins, each microwell contains 3 spots and the whole slide contains 40 x 3 replicates. The threshold value (cut-off) for the determination of LOD (Limit of Detection) of antigen concentration was calculated as followed:

$$\text{Cut-off} = \text{Mean of blank fluorescence intensities} + 3 \text{ SD} \quad (1)$$

Where SD represents standard deviation. The dynamic range corresponded to the ratio of high detection limit over low detection limit of each immunoassay.

3. Results and discussions

3.1. Surface characterization of functionalized glass slides

The various surface chemistries were characterized by contact angle measurements to evaluate surface tension. The surface energies, viz., the total energy

(E_T), the dispersive energy (E_D) and the polar energy (E_P) are calculated from the wetting angle (θ) accordingly to the Owens–Wendt equations. As is shown in Table 1, the dispersive energy of the five functionalized surfaces almost keeps constant indicating that homogeneous silane layer on the surface was constructed [21]. Further cross-linking with polymers makes more contribution to polar tension. Indeed, surfaces functionalized only with silane, like COOH and NHS surfaces, display low polar surface energy. Functionalization of silanized surface with polymers leads to increase polar surface energy from 7.4 mJ/m² of COOH surface to 13.8 mJ/m² of chitosan surface. Moreover, polar surface energy increase with increasing polymer molecular weight as following: CMD (40000 g/mol) < MAMVE (216000 g/mol) < chitosan (470000 g/mol).

However, depending on the surface chemistry, the immobilization process of proteins is different. On COOH and chitosan surfaces, immobilization of proteins occur through physical adsorption, whereas on NHS, CMD and MAMVE surfaces covalent binding is achieved between activated carboxylic groups or anhydride moieties of the surface and amine groups of proteins.

The protein surface density and spot morphology for the five surface chemistries were evaluated with three fluorescent labeled proteins, also called reference proteins, displaying different molecular weights (M_w) and isoelectric points (pI): Streptavidin-Cy3 ($M_w = 52800$ g/mol; pI = 6.1), BSA-F647 ($M_w = 66433$ g/mol; pI = 5.6) and IgG-Cy3 ($M_w = 150000$ g/mol; pI = 4.4-10). Figure 3 shows the spot diameter measured by image analysis of scanning data for the three reference proteins (IgG, streptavidin and BSA) versus total surface energy. Two different behaviors were observed: for IgG and streptavidin the spot diameter increased with total surface energy whereas for BSA the spot diameter remained constant independently of the tested surface. These results could suggest that under our spotting condition, IgG and streptavidin interact with hydrophilic surfaces via hydrophilic domains while some of these domains remain available to the surrounding buffer. Consequently, their hydrophobic domains should be unexposed to the buffer, suggesting that the protein

retains its folding. On hydrophobic surface, interaction between their hydrophobic domain and the surface may remain limited compared to the one observed with hydrophilic surface. Conversely, BSA displays similar interactions towards all surfaces tested.

The relative immobilization rate of the 3 reference proteins was evaluated measuring the fluorescence intensity of immobilized fluorescent labeled proteins versus surface chemistry. As shown in Figure 4, the relative immobilization rate depended on the protein and on the surface chemistry. IgG is preferentially immobilized through covalent binding, the highest immobilization rate being obtained on MAMVE surface. BSA mostly immobilized on surfaces by physical adsorption. Indeed, high fluorescence intensity is observed on chitosan. Furthermore, the fluorescence signal is only weakly increased using NHS modified surfaces versus COOH surfaces. Covalent linking could be efficient on very reactive surfaces such as MAMVE surface. At least, the immobilization of streptavidin on surfaces was similarly efficient by covalent binding (highest fluorescence intensity on NHS and MAMVE surfaces) and by physical adsorption on chitosan surface.

These results clearly demonstrate that many parameters are involved in protein immobilization on a solid support: surface properties (composition, number and kind of reactive functions, surface tension) and protein characteristics (M_w , pI, 3D structure, and hydropathicity) would determine the interactions and protein conformation at the solid-liquid interface. Therefore, it is essential to screen various immobilization conditions (surface chemistry, spotting buffer, pH, protein concentration etc) in order to define the best one.

3.2. Multiplex sandwich immunoassays of colorectal cancer antigens on protein chip

The aim of this study was to determine optimal conditions (capture antibody concentration, surface chemistry) to implement sandwich based immunoassays for the detection of tumor antigens involved in colorectal cancer (CEA, HSP60, PDI, DEFA6 and CA19-9). Capture anti-tumor antigen antibodies were immobilized on chemically

functionalized microstructured glass slides at various concentrations (0.1 μM , 1 μM , 5 μM and 10 μM). They were allowed to interact with tumor antigens, then sandwiched using secondary biotinylated antibodies. Finally, incubation with Cy3 labeled streptavidine allowed the detection of the formed sandwich. The biological activities of immobilized antibodies and the analytical performances of the miniaturized immunoassay were tested by recognition of tumor antigens at various concentrations. Figure 5 illustrates the influence of capture anti-HSP60 antibody concentration in the immobilization buffer on NHS surface for the detection of HSP60 tumor antigen. All five anti-tumor antigen antibody/ tumor antigens tested displayed the same behavior on all the tested surfaces. Low capture antibody concentrations in the immobilization buffer typically 0.1 μM to 1 μM , were not sufficient to detect significant amount of tumor antigen in our miniaturized immunoassay (slope = 0). From 5 to 10 μM , fluorescence intensity increased with tumor antigen concentration, and the best dynamic range was obtained with 10 μM (slope = 5874, $R^2 = 0.96$). Therefore, the capture anti-tumor antigen antibody was optimized at 10 μM and the following results were all on the basis of this concentration.

Figure 6 shows results of multiplex immunoassays of the five tumor antigens tested on the various surface chemistries developed for protein chip implementation. Comparison of the graphs indicates that fluorescence intensity, which is proportional to the biological interaction between the anti-tumor antigen antibody and its tumor antigen, increases with increasing tumor antigen concentration and depends not only on the tumor antigen and but also on the surface chemistry. Indeed, fluorescence intensity obtained for CEA and HSP60 are hundreds times more than those for PDI and DEFA6. All five antibody/antigen systems display lower fluorescence intensity on COOH surface than on the other surfaces. This result suggests that the immobilization of antibodies by physical adsorption on COOH surface leads to low immobilization rate according to Figure 4 (IgG result), or to partial loss of biological activity. Although on chitosan surface, antibodies were also immobilized by physical adsorption with relatively lower immobilization capacity than covalent binding

(Figure 4), fluorescence intensity obtained from sandwich assay on this surface is significantly stronger than that on COOH surface, and in the same range for CEA, HSP60, CA19-9 and PDI antigens or even stronger for DEFA6 antigen than on covalent coupling surfaces (NHS, CMD and MAMVE). Because of its high molecular weight and its hydrophilic character, functionalization of glass slide with chitosan polymer increases the specific surface available for protein immobilization. This enables to maintain immobilized proteins away from the surface, in the aqueous solution, allowing preservation of biological activity. According to IgG results presented in Figure 4, covalent immobilization of anti-tumor antigen antibodies is more efficient than physical adsorption in most cases (CEA, HSP60, CA19-9, and PDI) to retain biological activity of immobilized proteins. Furthermore, surfaces functionalized with hydrophilic reactive polymers such as CMD or MAMVE, exhibit better tumor antigen detection probably because they display larger specific surface for protein immobilization. In a previous work, we demonstrated that MAMVE functionalized glass slide was a powerful surface to implement miniaturized immunoassays with better performances than classical ELISA [12].

The limit of detection (LOD) and the dynamic range were determined to evaluate analytical performances of our multiplex immunoassays (Table 2). The results clearly demonstrate that performances of the immunoassays depend on both the antibody to be immobilized and on the surface characteristics. Limit of detection as low as 10 pM (10 U/ml for CA19-9) and dynamic range as wide as 4.7 log (3.0 log for CA19-9) are obtained for tumor antigens on the optimal surfaces. In classical immunoassays such as ELISA, the limit of detection for CEA is about 1 ng/ml (5.5 nM) and that of CA19-9 is about 25 U/ml with a dynamic range around 2.0 log (CEA ELISA Catalog # EA-0104, CA 19-9 ELISA Catalog #EA-0102, Signosis Inc. CA, USA). Other research groups working on the development of a highly sensitive electrochemical immunosensor to quantify CEA reported a limit of detection at 0.01 ng/ml (55 pM) [22]. Table 2 presents optimal protein chip surfaces for the optimal detection of the five tumor antigens tested. Detection of HSP60, PDI and CA19-9 could be performed

on NHS surface, whereas detection of CEA should be performed on MAMVE surface and detection of DEFA6 on chitosan surface. These results demonstrate that it is important to adapt surface chemistry to the immobilized protein and to detection criteria. This is in agreement with the work of Angenendt et al [23] where they screened 11 different array surfaces of both types and compared them with respect to their detection limit, inter- and intra-chip variation, and storage characteristics. There is not a unique surface which suits all antibodies; surface modifications should be chosen according preliminary experimental data and may depend on the species of antibody to be immobilized. Although it is difficult to predict the suitability of microarray coatings for protein and antibody microarray technology using one protein and one antibody, this study points towards surface modifications that offer outstanding qualities for detection of serum tumor markers involved in colorectal cancer.

4. Conclusions

We developed and characterized various surface chemistries allowing the efficient immobilization of anti-tumor antigen antibodies. Fast screening and identification of optimal conditions for antigen/antibody recognition (surface chemistry, protein concentration) were performed using microstructured glass slides. Results indicated that surfaces functionalized with high molecular weight hydrophilic polymers such as chitosan or MAMVE exhibited excellent performances for the immobilization of anti-tumor antigen antibodies and the recognition of antigen-antibody. We also pointed out that physical adsorption could be better than covalent linking in some cases. However, since proteins (even IgG) display great variability it is essential to adjust the surface chemistry in each case. Our microstructured chips offer the possibility to test various immobilization conditions and to implement customized multiplex immunoassay combining optimal immobilization conditions for each protein on the same test. Analysis of performances

indicates the low limit of detection and the wide dynamic range obtained for each tumor antigen tested on optimal surfaces. Future work will focus on the validation of such a diagnosis test to detect a large panel of cancer biomarkers in serum from colorectal patients on the basis of the optimal surfaces. Another important point to be evaluated in future work is the storage (conditions and duration) of our protein chip. Indeed for medical application, the storage and stability of protein chips are essential parameters for commercial development.

Biographies

Zhugen Yang received his B.E. degree of Polymer Materials and Engineering from Harbin Institute of Technology in 2006 and M.Sc. degree of Polymer Chemistry and Physics from Sun Yat-sen University in 2009, in People's Republic of China. At present, he is working for his Ph.D. in the Department of Chemistry and Nanobiotechnology, Lyon Institute of Nanotechnology at Ecole Centrale de Lyon, France. His research interests include surface modification for biochip/biosensor, antibody microarray for diagnosis of cancer, modification of polymers and polymeric composites/blends.

Emmanuelle Laurenceau received PhD degree in Engineering Science (biological and biomedical specialities) from Paris XIII University in 1995. Between 1996 and 2001, she worked as associate professor at Paris XIII University on biomimetic and biospecific polymers for diagnosis and therapeutic applications. From 2001 to 2004, she joined CNRS-bioMérieux Lab at Ecole Normale Supérieure de Lyon (France) where she developed functionalized surfaces for HIV-1 capsid protein immobilisation. In 2004, she integrated the Institute of Nanotechnology of Lyon at Ecole Centrale de Lyon (France) as associate professor. Her current research concerns the development of protein and peptide chips for biomedical applications. She is also interested in biomaterials topic.

Yann Chevolot received the PhD degree in material science from the Ecole

Polytechnique Fédérale de Lausanne (EPFL) at Lausanne (Switzerland) in 1999. Between 1999 and 2001, he worked as assistant of Pr Mathieu at EPFL on bacterial adhesion to PVC endotracheal tubes and was in charge of ToF-SIMS analysis. From 2001 to 2004, he worked in the research department of Goemar SA, Saint Malo (France). Since 2004, he joined the CNRS as a senior scientist. He focuses on microfabrication, surface chemistry and characterisation, and biochips in particular glycoarrays.

Yasemin Ataman-Önal received her PhD degree in Engineering Science (biological and biomedical specialties) from Lyon I University in 1998. Between 1999 and 2000, she worked on HIV vaccines in CNRS-bioMérieux Lab. She joined bioMérieux in 2001, developing research activities in Vaccinology (viral & synthetic vectors, new adjuvants). In 2006, she joined bioMarkers department of bioMérieux and focused on discovery and validation of new biomarkers mainly in the oncology field.

Geneviève Choquet-Kastylevsky is a Medical doctor (thesis 1996) specialized in dermatology, immunology (Lyon and Paris). She obtained her PhD in Immunology in 2001(Lyon) and the Degree in pharmacology and toxicology. From 1996 to 2001, she was hospital assistant professor at Lyon hospital. From 2001 to 2008, she was in charge of proteomic research at bioMérieux, setting up the proteomic lab and program. The project aimed principally mass screening for colon protein markers identification (in tissue, blood), but also cancer monitoring. From 2008 to 2010, she became oncology program director at bioMérieux where she managed over 15 oncology research projects (colon, breast, prostate, liver), using various technologies (transcriptomics, molecular biology, proteomics, immunoassay, miRNA studies...). Since 2011, she is a scientific and medical advisor at bioMérieux.

Eliane Souteyrand received PhD degree (1979) from Paris VI University and the graduate of “Docteur d’Etat” in Materials Sciences (1985). She joined CNRS in 1981 for developing her research activities in Electrochemistry, Photovoltaic and Semiconductors. In 1991, she integrated the Ecole Centrale de Lyon and investigated

the field of Gas sensors, Biosensors and DNA chips. In 2000, she received the Award of Innovative Company Creation and found Rosatech S.A, a company devoted to the “Biochips for diagnostics”. Currently, she leads the “Biotechnology for Health” department and managed the “Chemistry and Nanobiotechnologies” team at the Institute of Nanotechnology of Lyon. She is the co-author of more than 80 international publications and scientific book chapters and holds 9 international patents.

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Captions:

Figure 1: Surface functionalization for 3D microstructured chip.

Figure 2: Scheme of protein chip design: For line 1 and 2, anti-CEA antibody was printed at 0.1, 1, 5 and 10 μM . Similarly, anti CA19-9 antibody, anti HSP 60 antibody and anti DEFA6 antibody were printed line 3 / 4, 5 / 6, 7 / 8, and line 9 / 10, respectively. The corresponding antigens were then incubated at concentrations ranking from C1 to C7 (0.01 nM to 500 nM for CEA, PDI and DEFA6; 10 U/ml to 1000 U/ml for CA19-9). Buff. stands for buffer. No antigen was added in that specific microwell. In each microwell, buffer and reference proteins were spotted at various locations (top, down, right, left, middle) in order to test the reproducibility of the surface chemistry and the repeatability of the spotting. Antibodies were spotted in the middle of the microwell in order to prevent side effects. There is an offset between two lines of spotted proteins in order to avoid mixing of the spots.

Figure 3: Spot diameters of reference proteins (IgG-Cy3, Strep-Cy3 and BSA-F647) varied with surface tensions of each surface

Figure 4: Fluorescence intensity of immobilized reference proteins versus surface chemistry.

Figure 5: Effects of various concentrations of capture anti-HSP60 antibody on the detection of HSP60 tumor antigen. For 0.1 μM and 1 μM of capture antibody concentrations, slope of curves = 0 until 100 nM of antigen concentration. For 5 μM of capture antibody concentration, slope of the curve = 1647 with $R^2 = 0.93$. For 10 μM of capture antibody concentration, slope of the curve = 5874 with $R^2 = 0.96$.

Figure 6: Multiplex immunoassays of tumor antigens on various functionalized protein chip surfaces (a) CEA, (b) HSP60, (c) PDI, (d) DEFA6 and (e) CA19-9; capture anti-tumor antigen antibody concentration is 10 μM .

Fig. 1 Surface functionalization for 3D microstructured chip.

Fig. 2 Scheme of protein chip design: For line 1 and 2, anti-CEA antibody was printed at 0.1, 1, 5 and 10 μ M. Similarly, anti CA19-9 antibody, anti HSP 60 antibody and anti DEFA6 antibody were printed line 3 / 4, 5 / 6, 7 / 8, and line 9 / 10, respectively. The corresponding antigens were then incubated at concentrations ranking from C1 to C7 (0.01 nM to 500 nM for CEA, PDI and DEFA6; 10 U/ml to 1000 U/ml for CA19-9). Buff. stands for buffer. No antigen was added in that specific microwell. In each microwell, buffer and reference proteins were spotted at various locations (top, down, right, left, middle) in order to test the reproducibility of the surface chemistry and the repeatability of the spotting. Antibodies were spotted in the middle of the microwell in order to prevent side effects. There is an offset between two lines of spotted proteins in order to avoid mixing of the spots.

Fig. 3 Spot diameters of reference proteins (IgG-Cy3, Strep-Cy3 and BSA-F647) varied with surface tensions of each surface

Fig. 4 Fluorescence intensity of immobilized reference proteins versus surface chemistry.

Fig. 5 Effects of various concentrations of capture anti-HSP60 antibody on the detection of HSP60 tumor antigen. For 0.1 μM and 1 μM of capture antibody concentrations, slope of curves = 0 until 100 nM of antigen concentration. For 5 μM of capture antibody concentration, slope of the curve = 1647 with $R^2 = 0.93$. For 10 μM of capture antibody concentration, slope of the curve = 5874 with $R^2 = 0.96$.

(a)

(b)

(c)

(d)

(e)

Fig. 6 Multiplex immunoassays of tumor antigens on various functionalized protein chip surfaces (a) CEA, (b) HSP60, (c) PDI, (d) DEFA6 and (e) CA19-9; capture anti-tumor antigen antibody concentration is 10 μ M.

Table 1 Wetting properties of each surface determined with Owens-Wendt model, E_T , E_P and E_D relative to the total, polar and dispersive energy, respectively.

Surfaces	E_T (mJ/m ²)	E_P (mJ/m ²)	E_D (mJ/m ²)	Contact angle (θ /°)		
				water	Ethylene- Glycol	Diiodo- methane
COOH	36.5	7.4	29.1	75.9±0.7	49.4±0.4	56.9±0.6
NHS	37.1	6.7	30.4	76.4±0.2	50.6±0.6	53.9±0.6
CMD	36.8	8.1	28.7	74.2±0.3	50.8±0.6	56.4±0.6
MAMVE	42.0	12.0	30.0	65.3±0.4	42.7±0.5	52.5±0.5
Chitosan	43.6	13.8	29.8	61.3±0.5	43.2±0.7	50.8±0.6

Table 2 Optimal analytical performances of tumor antigens immunoassays on functionalized protein chips.

Tumor antigens	Optimal surfaces	LOD	Dynamic range
CEA	MAMVE/CMD	10 pM	4.7 log/4.0 log
HSP60	NHS/Chitosan/MAMVE	10 pM	4.7 log/4.0 log
PDI	NHS	10 pM	4.7 log
DEFA6	Chitosan	10 pM	4.7 log
CA19-9	NHS/CMD	10 U/mL	3.0 log