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Cancer Biomarkers Detection Using 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, and immobilization conditions (buffer, pH, concentration). 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 was evaluated on the detection of tumor antigens involved in colorectal cancer. Experimental results demonstrated that each antibody displays variable biological activities depending on the surface chemistry and on the immobilization procedure. Under optimized conditions, we can reach a limit of detection in tumor antigen as low as 10 pM. Our microstructured chip offers the possibility to implement a customized multiplex immunoassay combining optimal immobilization condition for each antibody on the same chip.

Key words: cancer biomarkers; protein chip; surface chemistry; immobilization.

1. Introduction

A plethora of serological tumor markers is available for clinic diagnosis, but individually their lack of specificity decreases their diagnostic and prognostic values. High throughput technology as protein chip

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would give the possibility to identify and detect sets of relevant biomarkers in a single assay, with miniaturized sample requirement and significant cost reduction. One of the crucial parameters in the elaboration of sensitive protein chips is the design of stable and reproducible surfaces which will preserve the biological activity of immobilized proteins [1, 2]. In this study, we immobilized five anti-tumor antigen antibodies involved in colorectal cancer (anti-CEA, anti-CA19-9, anti-HSP60, anti-PDI, anti-DEFA6) by covalent linking on three different amino-reactive surfaces (NHS, MAMVE, CMD) and by physical adsorption on an aminated surface (chitosan) and on a carboxylic surface. Functionalized surfaces were characterized with contact angle measurements and XPS analysis, as well as with the immobilization of fluorescent labeled proteins (IgG, streptavidin, BSA). 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. Chitosan was kindly provided by Dr. T. Delair (Laboratoire des Matériaux Polymères et Biomatériaux, Université de Lyon). Dextran (Mw=40000 g/mol) was obtained from Pharmacosmos and maleic anhydride-alt-methyl vinyl ether (MAMVE, Mw=216000 g/mol) from Sigma-Aldrich. Borosilicate flat glass slide were purchased from Schott. Anti-tumor antibodies and tumor antigens were provided by bioMérieux. Cy3-labeled goat anti-mouse antibody (IgG), Cy3-labeled streptavidin and bovine serum albumin (BSA) were purchased from Jackson ImmunoResearch and Sigma, respectively.

2.2. Surface functionalization and characterization of glass slide

Flat microscope glass slides were microstructrued and silanised as described previously [3, 4]. The tert-butyl ester was then converted into carboxylic group (COOH surface) and activated with DIC/NHS (NHS surface). NHS surface was further functionalized with Jeffamine D-230 to generate aminated surface, followed by incubation with MAMVE solution at 5mg/mL to obtained MAMVE surface [5]. CMD solution at 5mg/mL (degree of substitution 63%, synthesized in our lab) was activated with EDC/NHS to react with the aminated surface to generate CMD surface. Prior to protein immobilization, CMD surface was activated with EDC/NHS. Chitosan surface was obtained by functionalization of the NHS surface with chitosan solution at 5 mg/mL (M_w=470000 g/mol, degree of deacetylation (DD) 94 %).

Functionalized glass slides were characterized for surface energy by contact angle measurements using Owens-Wendt model. Characterization for chemical composition was performed by XPS analysis.

2.3. Protein chip manufacturing and multiplex immunoassays

Anti-ACE, anti-CA19-9, anti-HSP60, anti-PDI and anti-DEFA6 were spotted into microwells of functionalized microstructured glass slides (fig.2) (1 kind of antibody per microwell) at different concentrations (0.1 μM, 1 μM, 5 μM and 10 μM) in PBS 1X/20% glycerol spotting buffer. IgG-Cy3 (0.1 μM), Streptavidin-Cy3 (0.1 μM) and BSA-F647 (0.1 μM, labeled in our lab) were spotted at the mean time as reference proteins for surface characterization. Proteins were allowed to react with functionalized surfaces under saturated water vapors overnight at 37 ^{o}C. Microwells were then incubated with tumor antigens (ACE, CA19-9, HSP60, PDI, DEFA6) at different concentrations (from 0.01nM to 500 nM, one

antigen concentration per microwell) after capping with 4% BSA/PBS 1X (pH=7.4), followed by incubation with labelled detection antibodies. After thoroughly washing, slides were scanned with the GenePix 4100A scanner (Axon Instruments) at wavelengths of 532 nm and 635 nm.

3. Results and discussion

3.1. Surface characterization of functionalized glass slides

The various surface chemistries developed were characterized by XPS analysis (data not shown) and by contact angle measurements to evaluate surface tension (Table 1). Surfaces functionalized with high molecular weight polymers such as MAMVE or chitosan, are the most hydrophilic ones displaying the highest polar and total surface energies. Although CMD surface is functionalized with a hydrophilic polymer, its total surface energy is equivalent to the one of NHS surface displaying the lowest polar surface energy. Moreover the spot diameter of immobilized IgG and streptavidin (Table 1) varies in the same way as the total surface energy. These results indicate that the behaviour of protein solutions on a surface is related to surface tension and surface composition. However, protein properties are also an important parameter. Indeed, unlike IgG and streptavidin, spot diameter of immobilized BSA doesn't vary with surface chemistry. As shown in Fig. 2, the relative immobilization rate of these 3 proteins depends not only on the protein but also on the surface chemistry.

Table1. Spot diameter of immobilized proteins on various surface chemistries and contact angle measurements of each surface.

Surfaces	Spot diameter (µm)			Contact angle	Total energy	Polar energy
	IgG	Streptavidin	BSA	with water (°)	(mJ/m²)	(mJ/m²)
COOH	126 ± 7	140 ± 10	128 ± 8	75.9±0.6	36.5	7.4
NHS	143 ± 6	164 ±10	129 ± 7	76.4±0.2	37.1	6.7
CMD	120 ± 6	125 ± 7	124 ± 11	74.2±0.3	36.8	8.1
MAMVE	146±8	166±6	129±10	61.3±0.4	42.0	12.0
Chitosan	153±9	172±8	119±6	61.3±0.5	43.6	13.8

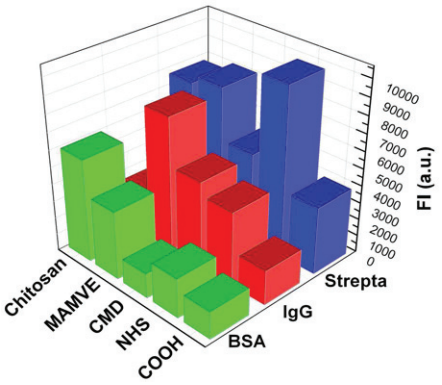

Fig. 1. Fluorescence intensity of immobilized fluorescent labeled protein versus surface chemistry.

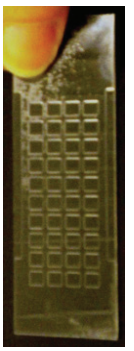

Fig. 2. Microstructured glass slide.

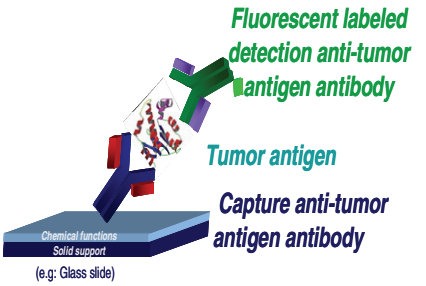

Fig. 3. Scheme of sandwich immunoassay on 3D-chip

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

The aim of this study was to determine optimal conditions (surface chemistry,concentration) to implement immunoassays for the detection of tumor antigens involved in colorectal cancer (CA19-9, CEA, HSP60, PDI and DEFA6). Capture anti-tumor antigen antibodies were immobilized on chemically functionalized microstructured glass slides. Biological activity of immobilized antibodies was tested by recognition of tumor antigens detected using fluorescent labeled detection anti-tumor antigen antibodies (Fig. 3). The lower limit of detection (LOD) and the dynamic range were determined to evaluate analytical performances of our protein chips. Results presented in Table 2 clearly demonstrate that performances of the immunoassays depend on the antibody to be immobilized and on the surface characteristics. Limit of detection as low as 10 pM and dynamic range as wide as 4.7 log are reached for each tumor antigen but on different surfaces.

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

Surface	Limit of dectection (LOD)					Dynamic Range				
	CEA	HSP60	PDI	DEFA6	CA19-9	CEA	HSP60	PDI	DEFA6	CA19-9
COOH	10 pM	10 pM	100 nM	10 nM	10 U/ml	4.0 log	3.0 log	3.0 log	4.7 log	3.0 log
NHS	250 pM	10 pM	10 pM	10 nM	10 U/ml	3.3 log	4.7 log	4.7 log	4.0 log	3.0 log
CMD	10 pM	20 pM	100 nM	n.d.	10 U/ml	4.0 log	2.7 log	2.0 log	n.d.	3.0 log
MAMVE	10 pM	10 pM	n.d.	n.d.	n.d.	4.7 log	4.0 log	n.d.	n.d.	2.4 log
Chitosan	40 pM	10 pM	25 pM	10 pM	10 U/ml	4.1 log	4.7 log	4.3log	4.7 log	n.d.

4. Conclusion

We have 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, spotting and capping buffer, etc) were performed using microstructured glass slides. These microstructured chips offer the possibility to implement customized multiplex immunoassay combining optimal immobilization conditions for each protein on the same test. Future work will focus on the validation of such a diagnosis test to detect a large panel of cancer biomarkers in serum.

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