

Protein microarrays enhanced performance using nanobeads arraying and polymer coating

C.A. Marquette^{a,*}, M. Cretich^b, L.J. Blum^a, M. Chiari^b

^a *Laboratoire de Génie Enzymatique et Biomoléculaire, UMR CNRS 5013 Bat. CPE, Université Claude Bernard Lyon 1, 43 Bd. du 11 Nov. 1918, 69622 Villeurbanne Cedex, France*

^b *Istituto di Chimica del Riconoscimento Molecolare, CNR, Via Mario Bianco 9, 20131 Milano, Italy*

Received 8 March 2006; received in revised form 8 June 2006; accepted 30 June 2006

Available online 14 August 2006

Abstract

A nanosize material composed of 330 nm glass beads coated with a copolymer of *N,N*-dimethylacrylamide (DMA), *N,N*-acryloyloxysuccinimide (NAS) and [3-(methacryloyl-oxy)propyl]trimethoxysilane (MAPS) was developed to improve the protein immobilization on biochips. The developed material, bearing rabbit-IgG proteins, was arrayed as 150 μm spots trapped at the surface of a poly(dimethylsiloxane) elastomer (PDMS), and compared to copoly(DMA-NAS-MAPS)-coated glass slides and latex beads based biochips. Evidences were made through scanning electron microscopy that the newly developed material based microarray exhibited surface irregularities at the submicron level leading to high specific area.

The combination of such large immobilization area with the highly efficient protein immobilization of the copoly(DMA-NAS-MAPS) polymer, enabled the achievement of microarrays exhibiting good performances both in pure media and complex samples (human sera). Indeed, high specific/non-specific signal ratio was found using this optimized immobilization procedure.

Chemiluminescent detection of anti-rabbit-IgG was obtained through peroxidase labeled antibodies in the 5 $\mu\text{g/l}$ to 10 mg/l range. Application of the developed system to real samples was achieved for the detection of rheumatoid factor (RF) through a capture assay. Interesting results were obtained, with a RF detection over the 5.3–485 IU/ml range and without measurable matrix effect or non-specific signal.

© 2006 Elsevier B.V. All rights reserved.

Keywords: Biochip; Chemiluminescence; Microarray; PDMS; Rheumatoid factor

1. Introduction

Protein microarrays are becoming important tools in proteomics [1,2], drug discovery programs [3], and diagnostics [4,5]. The amount of information obtained from small quantities of biological samples is significantly increased in the microarray format. This feature is extremely valuable in protein profiling, where samples are often limited in supply and unlike DNA, cannot be amplified [6]. However, protein microarrays are more challenging to prepare than are DNA chips [5] because several technical hurdles hamper their application. The surfaces typically used with DNA [7–10] are not easily adaptable to proteins, owing to the biophysical differences between the two classes of bio-molecules [11–14]. Arrayed proteins must be immobilized

in a native conformation to maintain their biological functions. Unfortunately, proteins tend to unfold when immobilized onto a support so as to allow internal hydrophobic side chains to form hydrophobic bonds with the solid surface [15]. The accessibility of the protein is also of crucial importance to achieve proper recognition since the interactions between proteins and the immobilization support reduce the accessibility of the target, leading to false negative results [11].

The present study was aimed at evaluating the analytical performances of protein microarrays produced through different immobilization procedures. The proteins probes were grafted either to: (i) polymer-coated glass slides [16,17], (ii) latex microbeads immobilized at the surface of a PDMS (poly(dimethylsiloxane)) elastomer [18–20] or (iii) polymer-coated glass nanobeads entrapped at the surface of a PDMS substrate (Fig. 1).

The polymer coating was achieved by combining the adsorptive properties of poly(dimethylacrylamide) onto glass

* Corresponding author. Tel.: +33 4 72 44 82 14; fax: +33 4 72 44 79 70.
E-mail address: christophe.marquette@univ-lyon1.fr (C.A. Marquette).

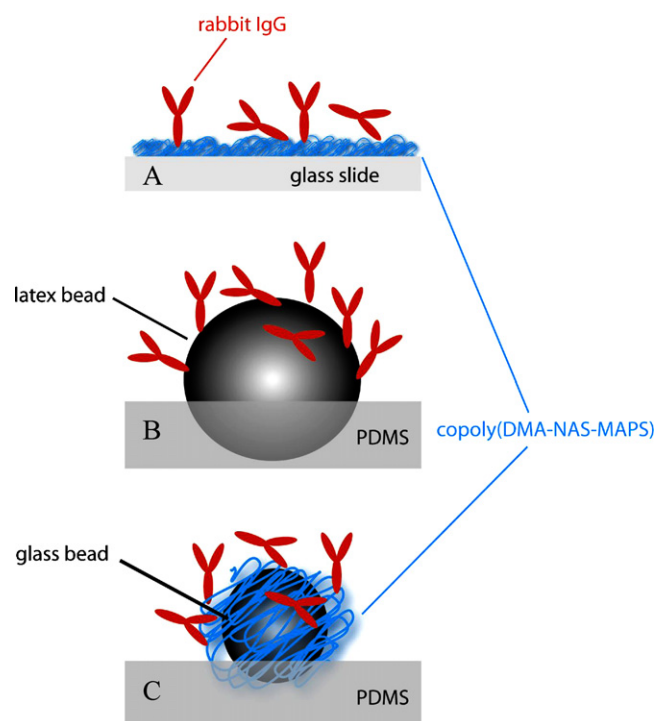


Fig. 1. Graphical representation of the three immobilization systems. (A) Copoly(DMA-NAS-MAPS)-coated glass slide; (B) latex based PDMS biochip; (C) copoly(DMA-NAS-MAPS)-coated glass nanobeads based PDMS biochip.

with a reaction between the silanol groups and electrophilic groups, pending from the polymer backbone. The copolymer of *N,N*-dimethylacrylamide (DMA), *N,N*-acryloyloxysuccinimide (NAS) and [3-(methacryloyl-oxy)propyl]trimethoxysilane (MAPS) formed then a highly hydrophilic coating with accessible functionalities to which modified DNA [21], proteins [17] or peptides can be covalently grafted. In all cases copoly(DMA-NAS-MAPS)-coated surfaces were shown to immobilize probes in a random conformation and to create an aqueous micro-environment in which linear epitopes or Fc and Fab domains of a capture antibody are freely exposed.

Protein immobilization on latex microbeads was obtained through the formation of covalent bonds between carboxylate residues present on the surface of the beads and protein amino residues. Those modified beads were trapped at the surface of a PDMS elastomer and generated spots of high specific surface [18]. Such system was previously shown to enable the successful “on chip” immobilization of various biological molecules such as inactive proteins (antigen), enzymes [20] and DNA strands [18]. However, a lack of protein accessibility was found in a particular case (P24 capsid protein from HIV-1) [19] leading to poor biochip performances.

As an original solution to this problem, the present study proposes a new hybrid immobilization technique taking advantages of both the high quality grafting of proteins onto copoly(DMA-NAS-MAPS) and the high specific surface generated by bead spotting.

The above immobilization systems will be herein used as a microarray support for protein–protein interaction experiments

and compared regarding their performances in the assessment of rheumatoid factor (RF) in human serum samples.

2. Experimental

2.1. Reagents

Ammonium sulfate, goat anti-rabbit-IgG peroxidase labeled (whole molecule), *N,N*-dimethylacrylamide (DMA), 3-(trimethoxysilane) propyl methacrylate (MAPS) were obtained from Sigma (France). *N*-Acryloyloxysuccinimide was supplied by Polysciences (Warrington, PA). Immunoglobulins from rabbit serum (rabbit-IgG) were obtained from Life Line Lab (Pomezia, Italy). Goat anti-human Ig(G + A + M) peroxidase labeled antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

PDMS precursor and curing agent (Sylgard 184) were supplied by Dow Corning. All buffers and aqueous solutions were made with distilled, demineralised water.

2.2. Synthesis of copoly(DMA-NAS-MAPS)

The monomers (20.17 mmol DMA, 0.42 mmol NAS, 0.21 mmol MAPS) were dissolved in dried tetrahydrofuran (THF) in a 50-ml, round-bottomed flask, equipped with condenser, magnetic stirrer, and nitrogen connection. The solution was degassed by alternating a nitrogen purge with a vacuum connection over a 30-min period. Azoisobutyronitrile (AIBN) was added to the solution, which was then warmed to 50 °C and maintained at this temperature under a slightly positive pressure of nitrogen for 24 h. After the polymerization was completed, the solution was precipitated by adding petroleum ether. The supernatant was eliminated, and the whole procedure was repeated two times. The polymer was dried under vacuum for 4 h at room temperature.

2.3. Glass slide coating and protein immobilization

Untreated microscope glass slides (Sigma) were pretreated with 1 M NaOH for 30 min and 1 M HCl for 1 h, washed with water, and dried. After the pre-treatment, glass slides were immersed for 30 min in a copoly(DMA-NAS-MAPS) solution (1%, w/v in water solution of ammonium sulfate at 20% saturation level), then rinsed with water and dried under vacuum at 80 °C.

Rabbit-IgG were dissolved in a PBS buffer (100 mM phosphate, 150 mM NaCl, 100 mM KCl, pH 7.4) at a 1 mg/ml concentration and spotted as 1.2 nl drop with a BioChip Arrayer BCA1 (Perkin-Elmer) on the copoly(DMA-NAS-MAPS) slides. Each array was composed of eight spots (four specifics and four non-specifics) where the non-specific ones were obtained by spotting bovine serum albumin.

After an overnight binding in a 30 °C thermostated water saturated chamber, the obtained microarrays were rinsed and blocked with BSA (1%, w/v) in PBS for 1 h.

2.4. Coating of silica nanospheres and protein immobilization

Silica nanospheres were coated with a copolymer of *N,N*-dimethylacrylamide (DMA), *N,N*-acryloyloxysuccinimide (NAS) and [3-(methacryloyl-oxy)propyl]trimethoxysilane (MAPS) using a fast, inexpensive and robust procedure consisting in simply dispersing nanobeads in a dilute aqueous polymer solution. Coating of nanospheres required then only two steps: pre-treatment and adsorption of polymer.

Silica nanospheres (non-porous, 330 nm from Bangs Laboratories Inc.) were pretreated with 1 M NaOH for 15 min, washed with water, treated with HCl 1 M for 1 h and extensively rinsed with water. In the second step, pretreated nanospheres were dispersed for 30 min in a solution of copoly(DMA-NAS-MAPS) (1%, w/v in a water solution of ammonium sulfate at a 20% saturation level). The nanobeads were then washed extensively with water and dried under vacuum at 80 °C for 30 min.

For protein immobilization, 45 mg of polymer-coated nanobeads were dissolved in 400 µl of a 10 mg/ml Rabbit-IgG solution in PBS (optimized concentration), sonicated for 2 min and stand to react under stirring during 2 h. At that time the reaction was completed and the non-grafted protein was washed out through four cycles of centrifugation/re-suspension in 500 µl of PBS and stored at 4 °C.

A similar protocol was applied to bovine serum albumin (BSA) to obtain non-specific beads used as negative control during the biochip experiments.

2.5. Protein immobilization onto latex microbeads

About 100 µl of commercialized latex beads suspension in water (1 µm of diameter from Polyscience, Inc. (France)) were mixed with 100 µl of 0.1 M 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide and 100 µl of 0.1 M *N*-hydroxysuccinimide and incubated under stirring for 1 h. The excess of reagents was then removed by centrifugation, and the activated beads were re-suspended in 400 µl of a 5 mg/ml rabbit-IgG solution in PBS (optimized concentration). The latex beads were incubated under stirring for 2 h, then washed in distilled water by successive centrifugation/re-suspension cycles and stored at 4 °C.

A similar protocol was applied to bovine serum albumin (BSA) to obtain non-specific beads used as negative control during the biochip experiments.

2.6. Micro- and nano-beads based biochip preparation

The biochips were prepared by arraying 1.2-nl drops of spotting solutions with a BioChip Arrayer BCA1 (Perkin-Elmer). The spotting solutions were prepared by mixing 100 µl of either spotting buffer A (glycerol 2.5%, NaCl 0.05 M in water) or spotting buffer B (30 mM Veronal buffer, pH 8.5 containing 30 mM KCl, 0.2 M NaCl, 0.1% Tween and 1% BSA) with 10 µl of an aqueous suspension (1%, w/v) of micro- or nano-beads bearing rabbit-IgG, respectively.

Each array was composed of 8 spots (4 specifics and 4 non-specific) that were spotted on the surface of a 3D PVC master composed of 24 rectangular structures ($w = 5$ mm, $l = 5$ mm, $h = 1$ mm). After spotting, the spots were dried and the arrays were transferred to a PDMS interface, by pouring a mixture of precursor and curing agent (10:1) onto the PVC substrate, and cured for 5 min at 90 °C. Peeling off the PDMS polymer then terminated the biochip preparation.

The 3D structure resulting from this process was then a 24-well PDMS microarray (inner volume 25 µl), with 16-spot arrays at the bottom of each well. All incubation or washing steps for a particular array were then performed by depositing a 20 µl solution volume into each well.

Before performing immunoassays, the micro- and nano-beads based biochips were saturated with PBSA (PBS containing BSA 1%, w/v), for 20 min.

2.7. Polyclonal anti-rabbit antibody-binding tests

The different systems – glass slide microarrays, microbeads and nanobeads biochips – already saturated with BSA were incubated at 37 °C for 1 h in the presence of increasing concentrations (from 5 ng/ml to 10 µg/ml) of peroxidase labeled anti-rabbit-IgG antibodies in PBSA. The biochips and microarrays were then washed 20 min with VBS (30 mM Veronal, 0.2 M NaCl, pH 8.5) and were ready to be measured.

2.8. Rheumatoid factor (RF) detection in human sera

The different systems – glass slide microarrays, microbeads and nanobeads biochips – already saturated were incubated at 37 °C for 1 h with standardized human serum samples (a gift from Life Line Lab) containing different amounts of RF (0–485 IU/ml, determined by RF ELISA Auraflex, Alfa Biotech, Pomezia, Italy). For glass slide microarrays, the sera were diluted 1:100 in PBSA, whereas they were diluted 1:10 in PBSA for microbeads biochips and 1:100 in PBSTA (PBS containing BSA 1%, w/v and Tween-20 0.1%, v/v) for nanobeads biochips.

The glass microarrays and the biochips were then washed 10 min with PBS, saturated with PBSA for 20 min and incubated for 1 h at 37 °C in the presence of peroxidase labeled anti-human IgG antibodies at a concentration of 26.6 µg/ml in PBSA for glass slide microarrays and microbeads biochips and at a concentration of 8 µg/ml in PBSTA for nanobeads biochips. The biochips and microarrays were then washed 20 min with VBS and were ready to be measured.

2.9. Microarrays and biochips measurements

The ready-to-measure biochips and microarrays were covered with VBS containing 200 µM of luminol, 500 µM of hydrogen peroxide and 20 µM of *p*-iodophenol. The arrays were introduced into the CCD light measurement system (Intelligent Dark Box II, Fuji Film) and the emitted light integrated for 10 min for binding tests and RF detection, respectively. The obtained biochip pictures were quantified with a FUJIFILM image analysis program (Image Gauge 3.12).

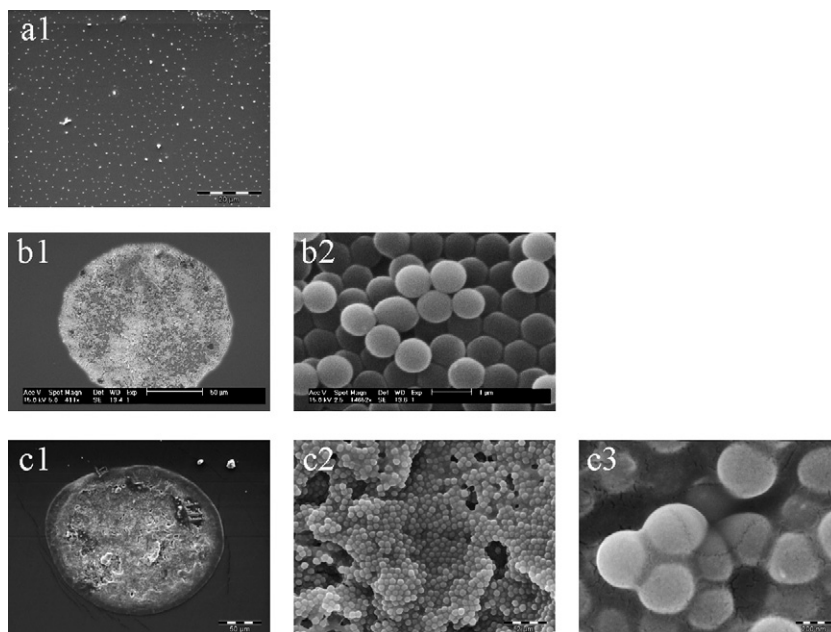


Fig. 2. Scanning electron microscopy images (15 kV) of the three developed systems based on: (a) coated glass slide; (b) 1 μm latex beads; (c) 330 nm coated glass beads.

3. Results and discussion

Surface irregularity is an important factor in sensing layer development since for equivalent geometrical surfaces, really different specific surfaces – i.e. real developed surfaces – could be obtained. Thus, using equivalent area of the microarray, different amounts of bio-molecules could be immobilized depending on the “roughness” of the surface.

The present study aims at evaluating the analytical performances of protein microarrays produced by three different immobilization procedures generating different levels of local specific surface and different protein immobilization efficiencies. These procedures are based on the grafting of protein probes to: (i) polymer-coated glass slides, (ii) latex microbeads immobilized at the surface of a PDMS (poly(dimethylsiloxane)) elastomer and (iii) polymer-coated glass nanobeads entrapped at the surface of a PDMS substrate.

These three different surfaces were first imaged through scanning electron microscopy (SEM). Fig. 2a1, b1 and c1 show the 150 μm spots obtained on copoly(DMA-NAS-MAPS)-coated glass slide, latex biochip and copoly(DMA-NAS-MAPS)-coated glass beads biochip, respectively. As expected, no irregularity at all could be observed from the coated glass slide spot, evidencing a low specific area. On the contrary, both latex and coated glass beads based spots exhibit nicely defined 150 μm spots with irregular surface.

Higher resolution images (Fig. 2b2 and c2) revealed differences between latex and coated glass beads based spots. The use of 330 nm glass beads instead of 1 μm latex beads generated the higher specific surface accompanied of a clear entrapment of the glass beads in the copoly(DMA-NAS-MAPS). This last point is made obvious with the highest resolution image (Fig. 2c3) where the nanobeads appeared to be entrapped together in polymer shells.

These morphological characterizations suggest differences in the organization of the microarrays surface, leading to increasing specific surfaces from coated glass slides to latex beads and finally coated glass beads based microarrays. Differences in the microarrays performances are then expected since more biological material will be immobilized onto highly irregular surfaces.

The present analytical system – RF detection – is an interesting model for studying protein immobilization on novel

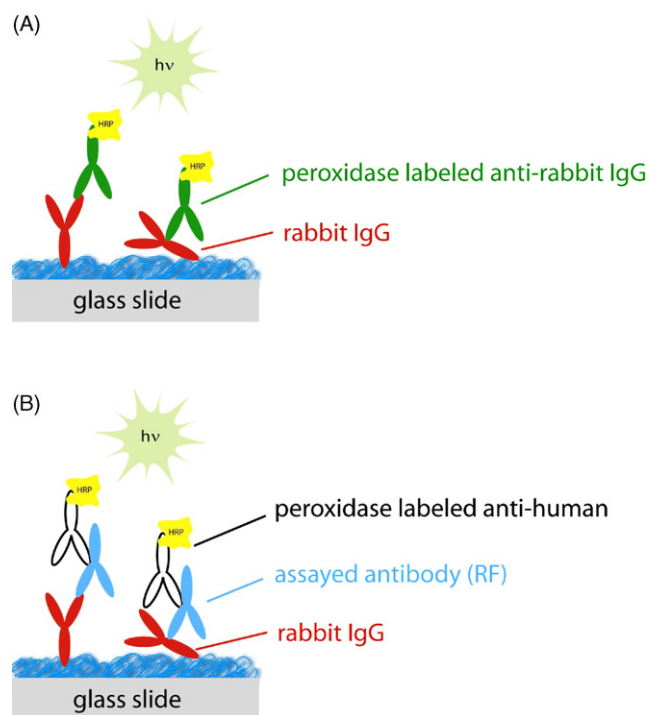


Fig. 3. Schematic view of the different immunoassays performed. Only the coated glass slide is shown. (A) Polyclonal anti-rabbit antibody-binding tests; (B) RF detection in human sera.

supports since it requires immobilizing the antigen within a highly accessible conformation. Indeed, because this test is based upon the recognition of rabbit-IgG by polyclonal antibodies from human serum [22–24], the sensitivity of the test depends on the number of available epitopes on the immobilized probe.

The three different immobilization procedures studied herein were first tested through their ability to generate sensing elements (spots of immobilized protein) on which polyclonal antibodies could bind specifically and extensively (Fig. 3A). For this purpose, glass slide microarrays, microbeads biochips and nanobeads biochips were incubated with peroxidase labeled anti-rabbit polyclonal antibodies from goat at different concentrations and the chemiluminescent signals obtained in each condition were quantified and plot to build the binding diagrams of Fig. 4.

As shown by Fig. 4 micrographs, the three systems are highly specific to polyclonal anti-IgG antibodies from purified solution, with no measurable non-specific binding on the immobilized BSA spots (negative control). Nevertheless, interesting differences in the three binding diagrams could be observed. As expected from previous studies [17] the copoly(DMA-NAS-MAPS)-coated glass slide provided a relatively good response

to the presence of polyclonal anti-rabbit-IgG antibodies through the entire range of concentration tested (from 5 $\mu\text{g/l}$ to 5 mg/l) whereas a saturation plateau was found for concentrations higher than 5 mg/l with a maximum chemiluminescent signal of 30 000 a.u. As expected [19], the latex microbeads based biochip exhibited a shifted detection range (from 50 $\mu\text{g/l}$ to 10 mg/l). Two important observations must be pointed out. The first is that, no saturation of the binding sites at high antibody concentrations was observed with the latex microbeads biochip. The second is that, the maximum signal obtained with this system (50 000 a.u.) was higher than that provided by the coated microarray slides. This suggests that using latex beads enabled the immobilization of a higher amount of rabbit-IgG in the sensing layer. However, the detection limit of the slides was 10 times lower than that obtained with the latex microchip. This difference reveals a lack of accessibility of one or more epitopes of the protein directly immobilized on latex beads, which could lead to an underestimated titration.

Finally, a mixed behavior could be found in the diagram of the coated beads biochip. Indeed, it could be seen that this last system produced no saturation at high concentrations, a maximum chemiluminescent signal of 35 000 a.u. and a detection range from 10 $\mu\text{g/l}$ to 10 mg/l.

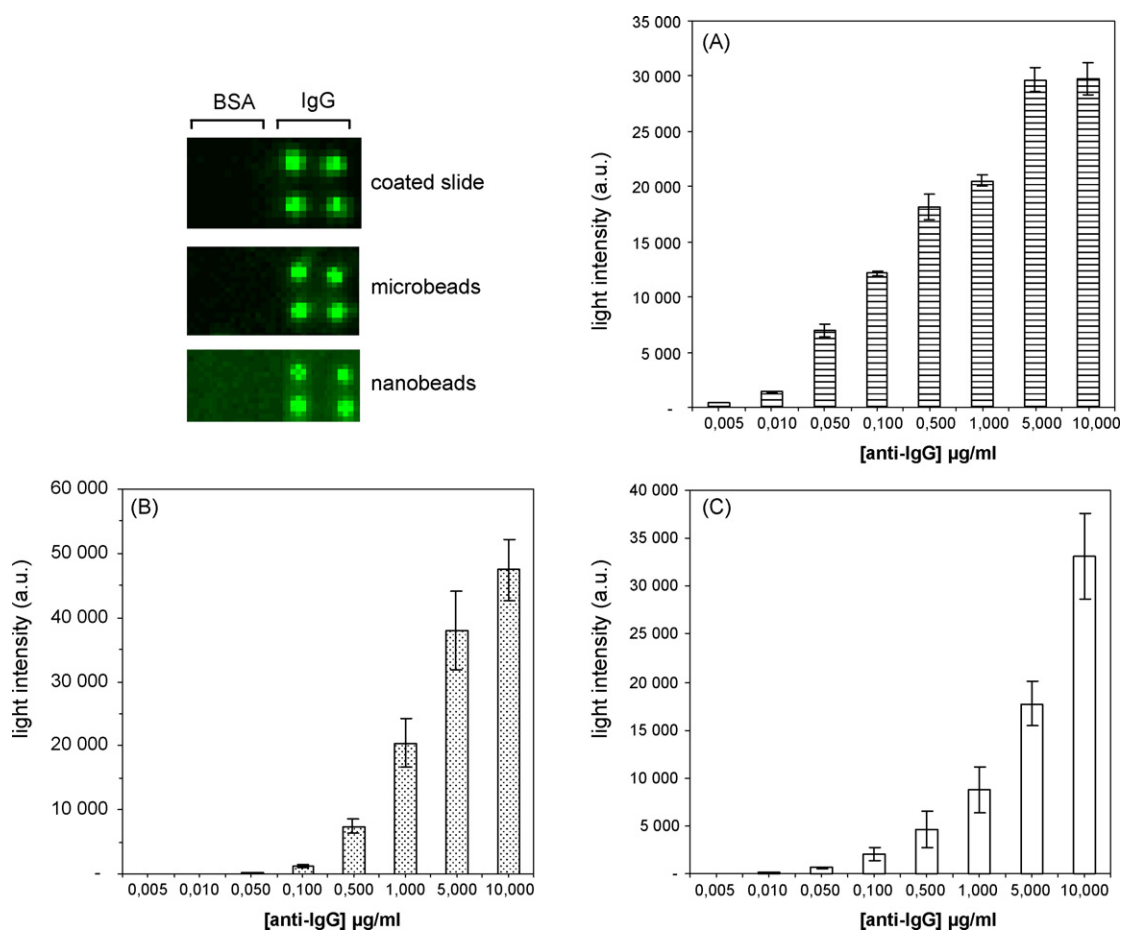


Fig. 4. Polyclonal anti-rabbit antibody-binding tests on the different analytical systems. The micrograph shows typical chemiluminescent images from each system. (A) Binding diagram from the copoly(DMA-NAS-MAPS)-coated glass slide microarray; (B) binding diagram from the latex beads based biochip; (C) binding diagram from the copoly(DMA-NAS-MAPS)-coated nanobeads based biochip. Error bars are the S.D. of three replicates.

Coating 330 nm beads with copoly(DMA-NAS-MAPS), grafting rabbit-IgG on it and using these hybrid beads to generate high specific surface spots at the surface of a PDMS elastomer combines then the advantages of polymer-coated glass slides with those of beads use, providing both high sensitivity and linearity in a broad range of concentrations.

As shown above, the three systems exhibit a different behavior against polyclonal anti-IgG antibodies from purified solution in which no interfering proteins or antibodies are present. The aim of this second study was to test the performances of each system in real micro-analytical experiments in the presence of high concentrations of background proteins (e.g., albumin, fibrinogen, antibodies).

RFs are immunoglobulins of any isotype with activity directed against the antigenic sites on the Fc region of human or animal IgG (Fig. 3B). Seventy to eighty percent of patients with confirmed rheumatoid arthritis show an increase in the RF [22]; therefore, RF assay is a serological test widely used for the diagnosis of rheumatoid arthritis. Conventional serological tests for the measurement of RF rely on enzyme-linked immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA). Test-

ing the present systems according to their ability to detect RF imply the use of human sera.

Thus, standardized human serum samples containing different amounts of RF (0–485 IU/ml) were incubated on the different microarrays and biochips. Fig. 5 presents the results obtained from each analytical system and each sample, with dataset standardized regarding the maximum chemiluminescent signal of each plot:

$$I = \frac{I_i}{I_{\max}}; \quad i = 0-485$$

For each particular system, the RF detection conditions were optimized in order to obtain the best analytical performances. Glass slide based microarrays and nanobeads biochips were incubated with sera diluted 1:100 whereas latex bead biochips were used on 10 times less diluted samples (1:10).

This first remark points out one of the major problems of the latex beads biochips (Fig. 5B), the lack of reactivity of the immobilized antigen against a multiple epitope binding antibody solution, which could be due to a non-adapted immobilization chemistry. In order to detect RF on a large concentration range,

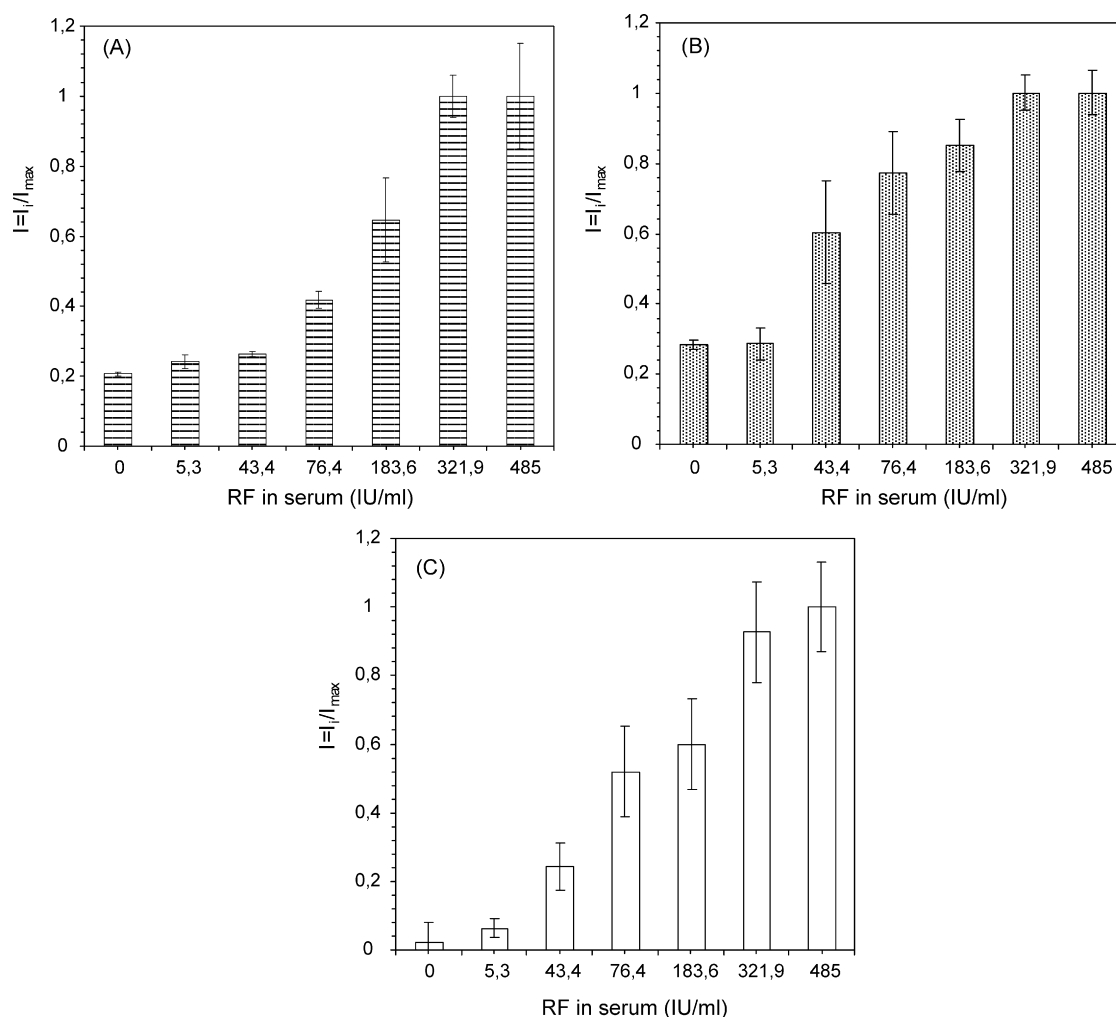


Fig. 5. RF detection in human sera using: (A) the copoly(DMA-NAS-MAPS)-coated glass slide microarray; (B) the latex beads based biochip; (C) the copoly(DMA-NAS-MAPS)-coated nanobeads based biochip. Error bars are the S.D. of three replicates.

the biochip requires highly concentrated samples, leading to significant non-specific signals at low RF values (0 and 5.3 IU/ml). The direct consequence of the presence of a strong background signal is a significant limitation in the detection range obtained.

For glass slide system, the copoly(DMA-NAS-MAPS) coating enables, as discussed before, the immobilization of freely accessible IgG which appeared to be a good sensing element for RF detection in diluted samples (Fig. 5A). Indeed, the entire range of tested RF was detected, except for the 321.9 IU/ml value which was not distinguished from the 485 IU/ml. Moreover, this system generated a non-specific signal for the 0 IU/ml sample, resulting in a poor reliability for the analytical results obtained at low RF concentrations.

Finally, coated nanobeads biochips provides the most interesting results. Indeed, the full range of RF concentrations tested in complex samples could be detected with no doubt and on diluted samples. Moreover, as shown in Fig. 5C, a very low signal was recorded from the sensing layers incubated with the 0 IU/ml sample, contrary to what was obtained with microbeads or glass slide biochips. This latter point evidences an interesting low non-specific binding of the hybrid sensing layer made of inorganic nanobeads coated with copoly(DMA-NAS-MAPS).

4. Conclusions

In this study we investigated the suitability, as a protein immobilization, of a new submicron material composed of glass nanometric beads coated with a copolymer of *N,N*-dimethylacrylamide (DMA), *N,N*-acryloyloxysuccinimide (NAS) and [3-(methacryloyl-oxy)propyl]trimethoxysilane (MAPS) [16]. Coating of glass nanobeads with copoly(DMA-NAS-MAPS) was produced using a fast, inexpensive and robust procedure consisting in simply dispersing nanospheres in a dilute aqueous polymer solution.

The new material, obtained as a suspension, was subsequently derivatized with an antigen (rabbit-IgG) and used to produce PDMS elastomer based biochips according to a well established previously described procedure [18–20]. The analytical performances of the novel biochip were compared to those obtained, either with copoly(DMA-NAS-MAPS)-coated glass slides or with a latex microbeads based systems. Scanning electron microscopy was used to characterize the surface of the different systems. An increasing surface irregularity was found from coated glass slides to latex beads and finally coated glass beads based microarrays.

Evidences were made that the new material exhibited interesting characteristics in term of protein immobilization, acces-

sibility and specific/non-specific signal ratio. The immobilized protein, used as antigen, was shown to enable the specific binding of large amount of polyclonal antibodies either in pure media or complex matrices. Thus, microarrays were developed, displaying mixed behavior combining the highly efficient protein immobilization of the copoly(DMA-NAS-MAPS) and the high specific surface of the beads based spotting procedure. Such novel nanosized structures could help increasing the performances of analytical microsystems.

Acknowledgment

Published with the support of the European Commission, Sixth Framework Program, Information Society Technologies. NANOSPAD (no. 016610).

References

- [1] J.M. Boutell, D.J. Hart, B.L.J. Godber, R.Z. Kozlowski, J.M. Blackburn, *Proteomics* 4 (2004) 1950.
- [2] R.B. Jones, A. Gordus, J.A. Krall, G. MacBeath, *Nature* 439 (2006) 168.
- [3] S. Bodovitz, T. Joos, J. Bachmann, *Drug Discov. Today* 10 (2005) 283.
- [4] Y.-S. Lee, M. Mrksich, *Trends Biotechnol.* 20 (2002) s14.
- [5] H. Zhu, M. Snyder, *Curr. Opin. Chem. Biol.* 7 (2003) 55.
- [6] D.S. Wilson, S. Nock, *Angew. Chem. Int. Ed.* 42 (2003) 494.
- [7] H. Salin, T. Vujanovic, A. Mazurie, S. Maitrejean, C. Menini, J. Mallet, S. Dumas, *Nucl. Acids Res.* 30 (2002) e17.
- [8] H.T. Ng, A. Fang, L. Huang, S.F.Y. Li, *Langmuir* 18 (2002) 6324.
- [9] O. Melnyk, X. Duburcq, C. Olivier, F. Urbès, C. Aurialt, H. Gras-Masse, *Bioconjugate Chem.* 13 (2002) 713.
- [10] S. Venkatasubbarao, *Trends Biotechnol.* 22 (2004) 630.
- [11] W. Kusnezow, J.D. Hoheisel, *J. Mol. Recognit.* 16 (2003) 165.
- [12] D. Stoll, M.F. Templin, J. Bachmann, T.O. Joos, *Curr. Opin. Drug Discov. Dev.* 8 (2005) 239.
- [13] J. LaBaer, N. Ramachandran, *Curr. Opin. Chem. Biol.* 9 (2005) 14.
- [14] K.Y. Tomizaki, K. Usui, H. Mihara, *ChemBioChem* 6 (2005) 782.
- [15] J.E. Butler, *Methods* 22 (2000) 4.
- [16] M. Chiari, M. Cretich, A. Corti, F. Damin, G. Pirri, R. Longhi, *Proteomics* 5 (2005) 3600.
- [17] M. Cretich, G. Pirri, F. Damin, I. Solinas, M. Chiari, *Anal. Biochem.* 332 (2004) 67.
- [18] C.A. Marquette, L.J. Blum, *Anal. Chim. Acta* 506 (2004) 127.
- [19] C.A. Marquette, A. Degiuli, E. Imbert-Laurenceau, F. Mallet, C. Chaix, B. Mandrand, L.J. Blum, *Anal. Bioanal. Chem.* 381 (2005) 1019.
- [20] C.A. Marquette, L.J. Blum, *Biosens. Bioelectron.* 20 (2004) 197.
- [21] G. Pirri, F. Damin, M. Chiari, E. Bontempi, L.E. Depero, *Anal. Chem.* 76 (2004) 1352.
- [22] E.S. Cathcart, *Rheumatoid Factors Laboratory Diagnostics Procedure in the Rheumatic Diseases*, 2nd ed., Little Brown, Boston, 1975.
- [23] T.L. Moore, R.W. Dorner, *Clin. Biochem.* 26 (1993) 75.
- [24] T. Ota, Rinsho Byori: Jpn. J. Clin. Pathol. 51 (2003) 649.