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Straightforward production of encoded microbeads by Flow Focusing: Potential applications for biomolecule detection

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Abstract

Fluorescently encoded polymeric microparticles are acquiring great importance in the development of simultaneous multianalyte screening assays. We have developed a very versatile and straightforward method for the production of dye-labeled microparticles with a very reproducible size distribution and freely-chosen and discernible fluorescent properties. Our method combines Flow Focusing technology with a solvent evaporation/extraction procedure in a single step, yielding spherical, non-aggregate and non-porous particles. We have designed a multi-coloured bead array which includes the possibility of modifying the surface properties of the microparticles, which offer excellent properties for covalent attachment of biomolecules such as peptides, oligonucleotides, proteins, etc. We also show the potential of the fluorescently labeled microspheres for the detection of biomolecule (peptides and oligonucelotides) interactions using flow cytometry.

Keywords: Multiplex flow cytometric analysis; Microbeads; Flow Focusing

1. Introduction

Microarray technology has been used for the design, synthesis, analysis and management of large chemical libraries with many important applications in genomics, proteomics and drug discovery (Howbrook et al., 2003). Some of the major applications include identification of disease-related targets for therapeutic and diagnostic applications, correlation of changes in gene expression with disease, and determination of the relationship between genetic variation and disease susceptibility (Meloni et al., 2004; Sebat et al., 2004). In spite of the great impact of microarrays on high-throughput genomic screening applications, the cost of such devices has remained relatively high and they are currently restricted to a "small" library size because of their limited pixel size and their inherent two-dimensional geometry (Marshall, 2004).

Dye-labeled fluorescent microspheres have emerged as a very important alternative to the traditional microarrays. They present the possibility of multiplex colour detection and are expected to be more flexible in target selection, faster in binding, and less expensive in production, everything with a very small sample volume with a 3D configuration (Battersby et al., 2001; Chan et al., 2002; Xu et al., 2003; Seideman and Peritt, 2002; Lu et al., 2005). Other advantage of using fluidic arrays of fluorescent microparticles is the possibility of using flow cytometry as a fast, sensitive and accurate detection technique of biomolecule interactions (Kellar and Iannone, 2002; Kettman et al., 1998; Fuja et al., 2004; Morgan et al., 2004).

In general, the most widely used microsphere system is the one constituted by a fluorescent dye combination surrounded by the polymeric material. In this configuration, the matrix has a multiple function. In one hand, it protects the embedded dyes from external quenching agents, photobleaching, and effects of solvent polarity, pH and ionic strength. On the other hand, the surface reactive groups are available for conjugation with different compounds without affecting the fluorescent properties of the microsphere.

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The advantages of these microbead-based assays strongly involve the possibility of using microparticles of different sizes or colours, that permits identify different bead populations individually linked to specific biomolecules. Therefore, the functionality of the fluidic arrays strongly relies on the properties of the microspheres, such as their range of sizes, uniformity and capacity to retain the fluorescent dye. Despite all the efforts employed in the preparation of labeled functionalized polymeric beads (Stsiapura et al., 2004; Yang et al., 2001; Han et al., 2001; Bradley et al., 2003, 2005; Sosnowski et al., 1994) it is necessary to develop and optimize a methodology that permits the preparation of high quantities of fluorescent encoded microparticles with a uniform shape, homogeneous distribution and controlled fluorescent properties in a simple manner.

Here we report a very versatile and controlled procedure for the production of dye-labeled solid polymeric microparticles, yielding remarkable size accuracy with negligible size dispersion and allowing surface design and internal composition selection. We have developed a combined technique using the Flow Focusing technology (Gañán-Calvo, 1998; Gañán-Calvo and Barrero, 1999; Martín-Banderas et al., 2005) followed by a solvent evaporation/extraction procedure (O'Donell and McGinity, 1998) to afford the solid fluorescent beads. In this situation, we have employed a standard Flow Focusing nozzle to produce drops of the disperse phase inside the continuous phase of the future emulsion. The oil drops are generated with very controlled and narrow size distributions that are preserved during the drying process to afford the final microparticles in a single step, without any external excitation source or additional purification steps (Martín-Banderas et al., 2006). We have used this approach to produce fluorescent encoded beads with a uniform morphology, narrow diameter distribution and controlled and suitable fluorescent properties in an easy way. The dye-labeled microspheres properties were evaluated as a function of fluorescent probe content, fluorescent probe type and microparticle size. The effectiveness of the microbead array for covalent attachment of biomolecules was tested using oligonucleotides, simple proteins and more complex antibodies. Finally, the ability of the fluorescently labeled microspheres for the detection of biomolecule interactions using flow cytometry was also tested.

2. Materials and methods

2.1. Materials

Polystyrene (PS, Mw 4000–200,000), poly(styrene-co-maleic acid), partial isobutyl/methyl esther (PSMA, Mw 180,000), dichloromethane (DCM), ethyl acetate (EA), rhodamine B (Rh B), fluorescein (I) (Fl), bovine serum albumin (BSA), fluorescein isothiocyanate (FITC)-labeled rabbit antibody specific for mouse anti-IgG, *N*-hydroxysulfosuccinimide (NHSS), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC), SYBR-Green I and all other media components were purchased from Sigma–Aldrich. Nile Blue A (NB) was from Panreac. Polyvinyl alcohol (PVA, Mw 72,000) was from Fluka. Green fluorescent protein (GFP), IgG mouse antibody and peptide 33-mer were obtained from Biomedal S.L. Amino and fluorescently modified oligonucleotides (probes based on $ACT1_{low}$: 5′-AAATGGGATGGTGCAAGCGCTAGAACATA-CCAGAATCCAT$(T)_{20}$-NH_2-3′, $ACT1_{up}$: 5′-ATGGATTCT-GGTATGTTCTAGCGCTTGCACCATCCCATTT-3′, $GAL1_{up}$: 5′-ATGACTAAATCTCATTCAGAAGAAGTGATTGTACCT-GAGT-3′) were from Stab Vida.

2.2. Microparticle preparation

The preparation of PS microparticles involves the formation of an oil-in-water emulsion using a standard Flow Focusing nozzle in a liquid–liquid configuration (Martín-Banderas et al., 2006). The Flow Focusing device is immersed into the final continuous phase. Therefore, the formation of the oil drops was carried out inside the carrier liquid phase without any shape deformation. The resulting o/w emulsion was continuously stirred at room temperature until most of the organic solvent was evaporated leaving solid microparticles.

As the oil phase, we used a homogeneous polystyrene solution of (4% (w/v) of different polystyrenes) in the organic solvent containing or not the fluorophore. The disperse phase is injected through a capillary tube (150 μm) using a syringe pump, and is focused and pressed out of the device through the orifice (100 μm) using distilled water. To avoid the formation of aggregates, the production of the oil drops was carried out inside a 1% (w/v) PVA solution. The final PVA concentration of the continuous phase was 0.25% (w/v). The o/w emulsion was stirred for 16 h at room temperature for solvent removal to harden the oil droplets. After this, microparticles were collected by centrifuge (4000 rpm, 10 min), washed three times with distilled water and fully dried either by lyophilization or at bain-marie (65 °C, 4 h) and stored at 4 °C; or storage in a solution of NaN_3 0.1% (w/v).

To produce fluorescent microparticles, Rh B, Fl and NB at different concentrations, or mixtures of them were homogeneously co-dissolved in polystyrene solutions.

Different flow rates for polystyrenes solutions (oil phase) and distilled water (aqueous phase) were assayed.

2.3. Microparticle characterization

Diameters from non-lyophilized samples were determined by using an optical microscope and an image processing program (Image J, 1.30v). Diameters were measured for 500–1000 microparticles from various micrograph images and statistical data calculated. The shape and surface characteristics of microspheres were determined by scanning electron microscopy (SEM) (Philips XL-30).

MPs fluorescence properties were determined with a fluorescence microscope and a flow cytometer. The microscope is fitted with a UV lamp (OSRAM mercury short arc photo optic lamp HBO 103 W/2). The images were obtained with a camera Leica DC 350F. Mixtures of fluorescent beads were also analyzed in a Becton Dickinson FACScalibur flow cytometer equipped with an argon-ion laser (15 mW, 488 nm). Detectors used to discriminate different bead populations were Fl1, Fl2 and Fl3, detecting fluorescence at different wavelength (λ) ranges 515–545, 564–606 and >661 nm, respectively.

2.4. Conjugation of ligands to microparticle surface

We have used carbodiimide methodology for covalent immobilization of ligands on microparticle surface. GFP and fluorescently modified 3′-amino oligonucleotides (60 bp) and antibodies were linked to the carboxyl groups of microparticles prepared with poly(styrene-co-maleic acid), partial isobutyl/methyl esther using optimized protocols for each type of analyte.

For oligonucleotide coupling, microspheres were washed three times in MES hydrate buffer 50 mM, pH adjusted to 5.4. The microspheres were then incubated in a 10 mg/mL EDC and 10 mg/mL NHS solution in 2(*N*-morpholin)ethane sulfonic acid hydrate (MES hydrate) buffer in a final volume of 150 μl/mg of microspheres. Amino-modified oligonucleotide was also added in a final concentration of 1 nmol/mg of microsphere. After 30 min, another amount of the coupling solution was added. Incubation was performed under agitation using a Labnet VX-100 vortex mixer (Woodbridge, NJ, USA). Finally, the coupling mix was washed three times with 0.02% (v/v) Tween20 and 2 M NaCl in MES buffer and stored in 0.2% (w/v) sodium azide until use.

For protein conjugation, microspheres were washed three times with MES hydrate buffer 50 mM, pH adjusted to 5.4. After that, microspheres were incubated for 20 min in a 10 mg/mL EDC solution in MES hydrate buffer, pH 5.4 in a final volume of 300 μl/mg of microspheres. Microparticles were then centrifuged and resuspended in the desired protein solution in a concentration of 10 μg protein/mg of microspheres in phosphate buffered saline (PBS) buffer pH 7.4 and incubated for 2 h. After that the protein coupled microspheres were washed three times with Tween20 0.02% (v/v), NaCl 2 M, MES hydrate buffer 50 mM, pH 5.4 and storaged in sodium azide 0.2% (w/v) until use.

The results were evaluated analyzing the fluorescence intensity on a fluorescent microscope.

2.5. Detection of biomolecule interactions

Oligonucleotide hybridization assays were performed as follows: 0.5 mg samples of oligonucleotide coupled microspheres were incubated in hybridization buffer (SCC 6×, Denhart 1× (potassium inorganic pyrophosphate) KPPi 0.05% (w/v), pH 8.0) and 1 nmol of sense or antisense oligo was added in a final volume of 100 μl. The samples were maintained in an hybridization oven for 3 h at 45 and 55 °C. After that, the samples where washed twice with 1 mL of hybridation buffer. For the fluorescent analysis, the microspheres where immerse in Tris–EDTA buffer (tris[tris(hydroxymethyl)aminomethane]–(disodium ethylenediaminetetraacetate) buffer) with SYBR-Green I (1×) and measured by flow cytometry.

For the analysis of protein–protein interaction, 0.3 mg of microparticles conjugated to the analyte were incubated for an hour in 100 μl of a BSA solution in PBS buffer, pH 7.4. After that, samples were washed three times with Tween20 0.02% (v/v), NaCl 2 M, in MES hydrate buffer 50 mM solution, pH 5.4 and then analyzed using flow cytometry.

3. Results and discussion

3.1. Microparticle preparation

The preparation of PS microparticles involves the used of a standard Flow Focusing nozzle (Fig. 1a) to produce drops of the disperse phase inside the continuous phase of the future emulsion (Fig. 1b). The oil drops are generated with very controlled and narrow size distributions that are preserved during the solvent evaporation/extraction procedure used for drying. As it is

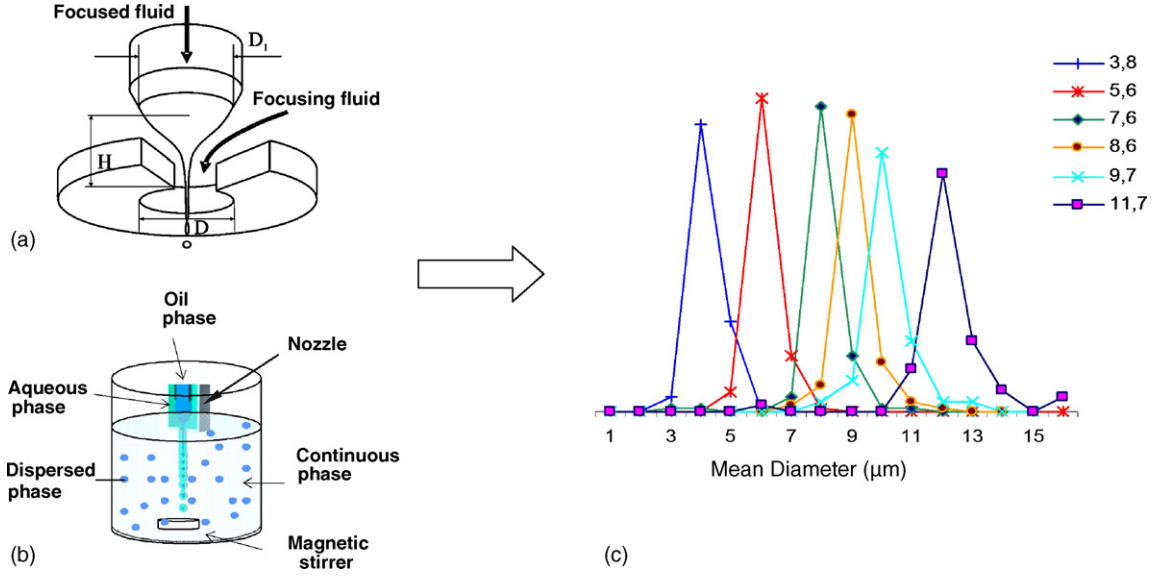

Fig. 1. (a) Flow Focusing atomizer, (b) polystyrene microparticles production using a Flow Focusing nozzle combined with a solvent evaporation/extraction process and (c) some examples of particle size distributions obtained using the same nozzle just changing experimental conditions.

shown in Fig. 1a, the Flow Focusing device used consists of a chamber pressurized with a continuous *focusing* water supply. Inside, a *focused* homogeneous polymeric solution is injected through a capillary feed tube whose extremity opens up in front of a small orifice linking the chamber with the exterior ambient. The *focusing* water stream moulds the meniscus of the polymeric solution into a cusp which gives rise to a microjet exiting the chamber through the orifice; the jet diameter is much smaller than the exit orifice diameter, thus precluding any contact. The stationary jet breaks up by capillary instability into homogeneous droplets (Rayleigh, 1879; Rutland and Jameson, 1970, 1971; Chandrasekhar, 1961; Tomotika, 1935) which are continuously stirred at room temperature in order to evaporate the organic solvent.

A theoretical and experimental study of this process was carried out by our group with good conclusions (Gañán-Calvo, 1998; Martín-Banderas et al., 2006). When viscosity of the liquids involved is sufficiently small (Reynolds numbers based on the jet diameter $Re = \rho Q \mu^{-1} d_j^{-1}$, larger than about 10), the jet diameter, d_j, is approximately given by $d_j = (8\pi^{-2}\rho\Delta P^{-1})^{1/4} Q^{1/2} + 2^{-1}\gamma\Delta P^{-1}$, where γ is the interfacial tension between both *focusing* and *focused* fluids, ρ the focused liquid density, Q the flow rate of the focused liquid, and ΔP is the pressure drop. In general, the system will be steady when the pressure drop is sufficiently higher than the maximum surface tension stress, of the order of γ/d_j. Another simpler expression for the jet diameter can be derived for small to moderate Reynolds numbers, assuming that both focusing and focused phases reach the same velocity after a certain initial short distance downstream of the meniscus apex, which reads: $d_j = D(Q/Q_F)^{1/2}$. Here, D is the orifice diameter and Q_F is the focusing fluid flow rate through the orifice.

In first place, we explored the best conditions for the production of certain microparticle patterns by means of Flow Focusing technology using blank polystyrene (PS) microparticles. Thus, we have performed several experiments using single nozzles with the same geometrical parameters varying both focusing and focused fluid rates. The influence of the drying method was also evaluated without finding relevant differences. Results of some of the experiment performed to obtain particles with 5–15 μm in diameter are listed in Table 1. We have obtained different sized microparticles with very homogeneous distribution without using neither external excitation force nor further purification step.

As it is shown in Table 1, using the same polymer solution, given a certain polymer flow, an increase of focusing water flow causes a decrease of the microparticle diameter. In contrast, when the external flow remains constant, as the polymer flow grows, the particle diameter increases. Desired microparticle size can be achieved simply by the adjustment of spray conditions (see Fig. 1c), obtaining very reproducible data for each experimental conditions (see entry $Q_{polymer}$ 1 mL/h, Q_{water} 3 mL/min in Table 1). In this system, the *focused* fluid composition has to bear in mind to predict the final microparticle size. The diameter of the solid particles that are thereby produced can be calculated using equation for d_j to obtain the drop size d from Rayleigh's relationship $d = 1.89 d_j$, and then, take into account the diameter reduction due to solvent evaporation. Therefore, the concentration of the polymer solution becomes another versatile parameter to determine the final particle size.

It is worth noting the fact that, due to the combination of the focusing effect and the result of the solvent evaporation procedure, we were able to obtain particles with a final size much smaller than either the feeding tube or the exit hole of the nozzle. The possibility to reduce and control the particle size just using hydrodynamic forces and, the fact that the jet diameter exited the orifice surrounded by the focusing fluid precluding any clogging, turn this technique into a valuable procedure for efficient nano- and microencapsulation and particle design.

The shape and surface characteristics of freeze-dried microparticles were determined by scanning electron microscopy (Fig. 2). The microparticles appeared to be spherical, non-aggregate and non-porous.

In order to obtain fluorescent polystyrene microparticles, we prepared some homogeneous solutions with different concentrations of fluorescein, rhodamine B and Nile Blue A or mixtures of them dissolved along with polystyrene 4% (w/v) in ethyl acetate. Microparticles were produced as described previously to obtained spheres with 5 μm in diameter (see Table 1), with freely-chosen and discernible fluorescent properties. The procedure presented high reproducibility without any significant

Table 1
Blank polystyrene microparticles size data obtained using CH_2Cl_2 polymer solutions

Experimental conditions		Microparticle size				
$Q_{polymer}$ (mL/h)	Q_{water} (mL/min)	Mean diameter (μm)	d_{50} (μm)	d_{85} (μm)	S.D. (μm)	%VC
0.5	4	3.78	3.90	4.37	0.41	10.78
1[a]	3	5.29	5.45	5.97	0.51	9.64
	3	5.41			0.60	11.09
	2	6.65	7.00	7.88	0.84	12.63
	1.5	8.08	8.51	9.63	1.04	12.87
2	3	7.59	7.79	8.99	0.82	10.80
	2.5	8.56	8.76	9.48	0.69	8.01
	2	9.67	9.91	11.84	0.87	8.95
	1.5	11.72	12.07	13.71	1.27	10.81

[a] EtOAc polymer solutions used.

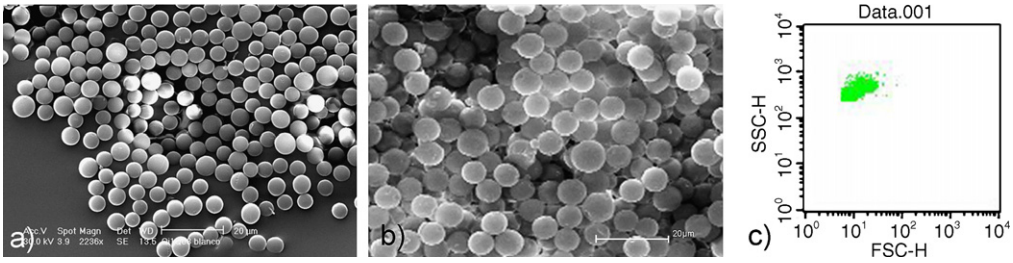

Fig. 2. SEM microphotographs of: (a) 5 μm; (b) 9 μm PS microparticles; (c) cytometry dot plot of FSC *vs.* FSC of 5 μm microparticles showing homogeneous shape and low complexity.

differences in the particle size distribution. All the samples had regular spherical shape with a smooth surface.

In a first step, we determined the fluorescence properties of polystyrene microparticles with a fluorescence microscope, and we checked the internal structure with a scanning confocal microscope. As shown in Fig. 3, the microparticles were constituted by a single phase with the organic dye, homogeneously distributed all over the continuous polystyrene matrix. This result indicates that the microparticles preserve the properties of the initial fluorescent solutions, without any aggregation or diffusion processes being involved in the drying procedure.

Due to its great importance in clinical diagnostics and basic research, we have also used flow cytometry for the analysis of the labeled microparticles produced using the Flow Focusing technology. In order to validate the procedure for the differentiation of multiplex encoded microparticles and the detection of biomolecule interactions, we have designed the coloured combination as a result of an initial division of the spectra in two regions. Green colour has been selected for measurement of analyte interactions and the rest of the spectra was to identify different internally labeled microparticle populations. All microparticle populations were prepared with the same size in diameter, 5 μm, therefore differentiation was only due to their unique fluorescent signal achieved by combination of organic fluorochromes. With the aim of simulating the detection of green-marked conjugates using the same set of beads, some microparticles were marked adding some amount of fluorescein to the initial particular dyed composition. Therefore, for some populations we had both, microparticles with the corresponding pre-adjudicated colour and the same internal labeling plus the effect of fluorescein. Following these guidelines, the fluorescent profile resulting from the analysis of a mixed sample of 12 different sets of stained microparticles in a flow cytometer is shown in Fig. 4. Different detection channels were used for differentiation of microparticles populations (Fig. 4a) and, detection of beads encoded with fluorescein in which analyte interaction was supposed to occur (Fig. 4b). Moreover, the analysis of bead arrays using flow cytometry allows direct quantification of the analytes of interest present in the test sample, just by establishing the specific calibration curves for the analyte concentration. The amount of molecule of interest present in the sample would result in different signal intensities coming from the specifically designed capture bead (show red arrows in Fig. 4b). Fig. 4 suggested that higher levels of complexity could be possible by making use of additional colours, as well as additional intensity levels.

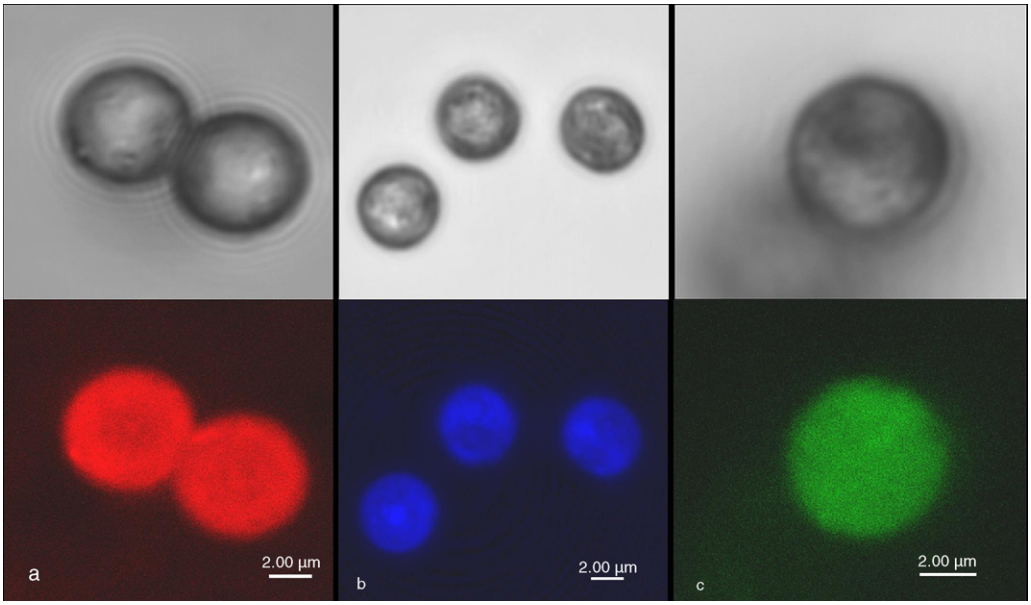

Fig. 3. Visible and confocal fluorescence microscopic images of 5 μm fluorescent PS microparticles produce by Flow Focusing. The images were produced by projecting multiple layers of picture on top of each other: (a) rhodamine B; (b) Nile Blue A; (c) fluorescein.

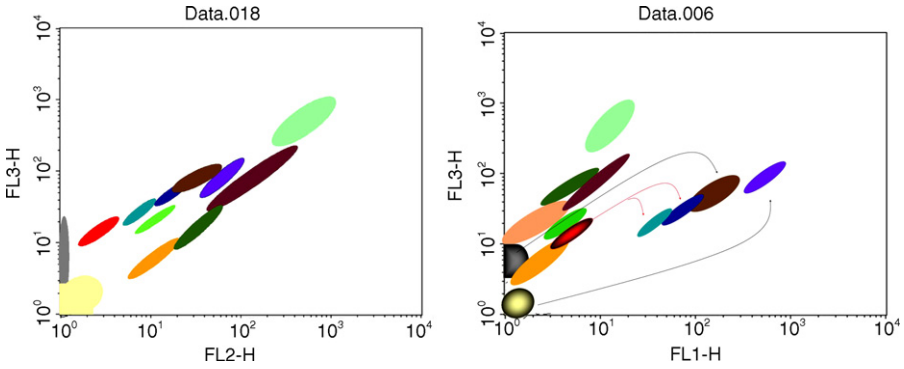

Fig. 4. Dot plots showing fluorescent profiles resulting from the analysis of a mixed sample of 5 μm fluorescently encoded polystyrene microparticles using flow cytometry: (a) blank NB 0.2 mM NB 0.6 mM/Rh B 0.0004 mM NB 0.6 mM/Rh B 0.004 mM NB 0.2 mM/Rh B 0.003 mM NB 0.6 mM/Rh B 0.0004 mM/Fl 0.2 mM NB 0.6 mM/Rh B 0.0004 mM Fl 0.4 mM NB 0.2 mM/Fl 0.2 mM Fl 0.4 mM Rh B 0.2 mM Rh B 0.05 mM Rh B 0.01 mM Rh B 0.003 mM; (b) dot plot of the fluorescent patterns of the same microparticles obtained from different channels (Fl1–Fl2). Differentiation of microparticles in which analyte interaction was supposed to occur (arrows) is shown. Red arrows show the possibility of analyte quantification depending on the amount of green fluorescence detected. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

3.2. Conjugation of ligands to microparticle surface

Bead-based array technology has become a very important tool for multiplex evaluation of soluble analytes in many different fields, presenting the ability to obtain rapid access to such diverse information as genetic evaluation or simultaneous immunoassays. In order to validate the functionality of the microparticles obtained by Flow Focusing for different biomolecules assays, we have extended the experiments to the conjugation of the main important ligands related to biological systems, oligonucleotides and proteins.

Conjugation of ligands to microparticle surface was performed with microparticles obtained using modified polystyrene with carboxylic acid groups along the polymeric chain (PSMA). We have produced the microparticles by the simple procedure described above, using a combination of Flow Focusing and solvent extraction/evaporation process to obtain bead populations with the same morphological and fluorescent properties than the ones described previously prepared with plain polystyrene. Analyte conjugation was carried out using modified carbodiimide methodology adapted to the type of biomolecule to be linked to the surface. The assays were performed with fluorescently modified molecules (FITC-5′Gal1$_{low}$3′-NH_2 and anti-mouse IgG antibody-FITC conjugated) and GFP. Controls for non-specific adsorption of biomolecules to microparticle surface were also run without coupling reagent. The presence of the ligands covalently attached to the surface was evaluated analyzing the fluorescence intensity with a fluorescent microscope (Fig. 5), yielding to successful coupling for each type of conjugation.

3.3. Detection of biomolecule interactions

Once we have optimized covalent biomolecule conjugation to microparticle surface, we have explored the ability of the

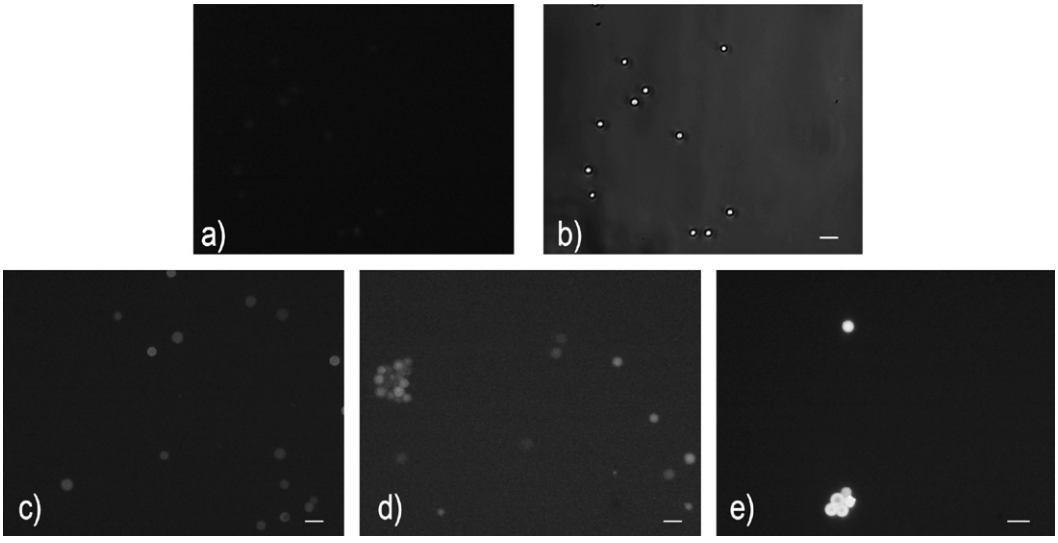

Fig. 5. (a) Fluorescent and (b) visible images of the same sample of blank PSMA microparticles without analyte conjugated on the surface; fluorescent microscopic images of 5 μm polystyrene microparticles with different analytes covalently attached to the surface; (c) fluorescein-oligo probe (FITC-5′Gal1$_{low}$3′-NH_2); (d) GFP; (e) anti-mouse IgG antibody-FITC conjugated. Scale bar corresponds to 10 μm.

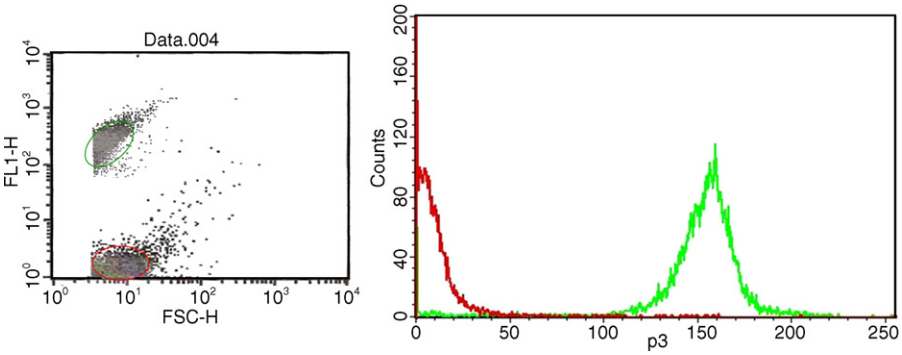

Fig. 6. Two different representations of flow cytometry analysis of $Act1_{low}$-PSMA microparticles incubated with: the not complementary oligonucleotide $Gal1_{up}$ (marked in red), where the absence of fluorescence indicates that hybridization has not occurred and the antisense complementary oligonucleotide $Act1_{up}$ (marked in green) where the fluorescence enhancement indicates that hybridization has taken place. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

bead-array system for hybridization analysis and immunoassays using flow cytometry. Hybridization assays have been carried out with fluorescent microparticles (NB 0.2 mM/Rh B 0.003 mM) with a 60-mer 3′ amino modified oligonucleotide conjugated to the surface ($ACT1_{low}3'$-NH_2) and the complementary antisense 40-mer oligonucleotides $ACT1_{up}$. As a control, we also performed hybridation assays with the non-complementary 40-mer oligonucleotide $GAL1_{up}$. Several experimental conditions have been assayed testing different temperatures, concentrations, buffer solutions, etc., in order to improve hybridization preserving microparticle stability. The detection of the hybridize probes was performed by the addition of a fluorescent stain, SYBR-Green I, specific for its ability to intercalate double chains of DNA. Once the complementary interaction between probes has occurred, the fluorescent intensity of SYBR-Green is enhanced over 100-fold allowing obtaining strong fluorescence signals easily appreciable using flow cytometry as it is shown in Fig. 6. When PSMA-$ACT1_{low}$ microparticles are incubated with the non-complementary oligonucleotide, the fluorescence intensity detected using specific green channel Fl1 remained constant. On the contrary, for microparticles incubated with the complementary probe, an enhancement of fluorescence is observed due to the interaction of SYBR-Green with the double chain formed.

As an additional application, the evaluation of ligand-conjugated microspheres for immuno-related assays was also performed. Taking into account that the detection of biomolecule interactions by means of indirect immunofluorescence is one of the most common techniques for immunoassay analysis, we have selected antibody–antibody interaction as a preliminary model system that would allow detecting preceding antigen–antibody interactions by fluorescence enhancement. Considering this, in order to assess the specificity of the system, we have conjugated in parallel an IgG mouse antibody and a model small peptide (33-mer) to different batches of PSMA microparticles. Both bead populations were incubated with an anti-mouse IgG antibody-FITC conjugated and the fluorescence analysis was performed. The dot plot presented in Fig. 7, shows the enhancement of fluorescence intensity when the stained antibody is conjugated with the microparticles conjugated to its specific ligand allowing differentiation of both microparticle populations. These promising results allow considering developed polystyrene microparticles as a very useful support for more complex simultaneous measurements of biomolecule interactions.

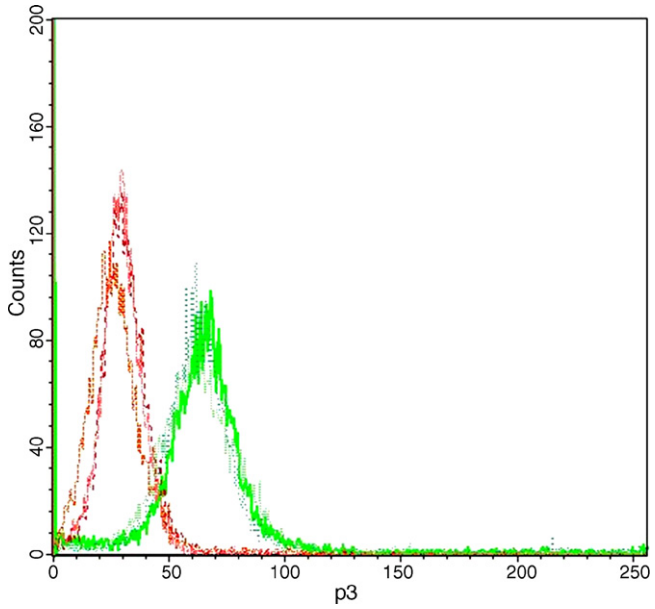

Fig. 7. Flow cytometry analysis of the specificity of the interaction of the anti-mouse IgG antibody-FITC conjugated with: PSMA-peptide microparticles (red) and PSMA-IgG mouse antibody microparticles (green) where the fluorescence intensity enhancement indicated successful detection of interaction. Duplicate analysis (dotted and full lines) showed the reproducibility of the assay. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

4. Conclusions

We have presented a straightforward method for the controllable production of functionalized polystyrene microparticles using a combination between Flow Focusing technology and solvent extraction/evaporation technique. This robust and controllable process has become a very valuable procedure for efficient microparticle design leading to monodisperse microbeads

with diameters of a few microns with the desired internal composition. We have also produced encoded microparticles with different and discernible fluorescent properties. The dye-labeled beads functionality has been evaluated allowing conjugation of biomolecules of different nature, including nucleic acids and proteins. Conjugated microparticles present the ability to be a valuable tool for the detection of analyte interactions using flow cytometry as one of the most the accurate and simple techniques for analysis. Therefore, Flow Focusing could be considered as a suitable low cost alternative for bead array production, dealing to dye labeled microspheres that have been validated for its useful properties for biomolecule analysis.

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