ONE-STEP PRODUCTION OF FLUORESCENT ENCODED POLYSTYRENE MICROPARTICLES USING FLOW FOCUSING. POTENCIAL APPLICATIONS IN HIGH-THROUGHPUT SCREENING

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INTRODUCTION

Spherical particles conjugated to molecules with affinity or able to interact with an analyte, have demonstrated to be one of the most convenient tools for parallel multiplex analysis in research and diagnostic (1). They present the possibility of multiplex colour detection and are expected to be highly flexible in target selection, fast in binding, and cheap in production, everything with a very small sample volume with a 3D configuration. One of the major advantages 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 (2).
We report here, a very versatile and controlled procedure for the production of dye-labelled 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 (3,4) followed by a solvent evaporation/extraction procedure to obtain the solid fluorescent beads. In this process we have employed a standard Flow Focusing nozzle (Figure 1a) to produce drops of the disperse phase inside the continuous phase of the future emulsion (Figure 1b). 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.

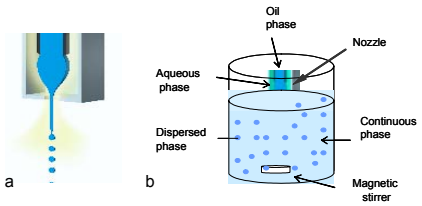

Figure 1. a) Flow Focusing atomizer. b) Polystyrene microparticles production using a Flow Focusing nozzle.

The dye-labelled microspheres properties were evaluated as a function of fluorescent probe content, fluorescent probe type and microparticle size. Covalent immobilization and detection of oligonucleotide probes to the microparticle surfaces were performed.

EXPERIMENTAL METHODS

As the oil phase, we used different homogeneous polystyrene solutions (4%; polystyrene Mw: 4000-200000; polystyrene monocarboxy terminated, Mw: 200000; poly(styrene-co-maleic acid), partial isobutyl/methyl esther, Mw: 180000) in the organic solvent (CH_2Cl_2 or EtOAc) containing or not the fluorophores. The disperse phase is focused and pressed out of the camera by distilled water. The oil droplets were hardened by stirring at room temperature, collected by centrifuge and stored at 4ºC.
To produce fluorescent microparticles, different amounts of Rhodamine B, Fluorescein (I) or Nile Blue A were homogeneously co-dissolved in PS solutions.
Amino modified oligonucleotide probes (Stab Vida, Portugal) were covalently attached using the carbodiimide methodology.
MPs diameters were determined by using an optical microscope (Leica DM LS) and an image-processing program (Image J. 1.30v). The shape and surface characteristics of microspheres were determined by scanning electron microscopy (SEM) (Philips XL-30).
Microparticles fluorescence properties were studied employing a Fluorescence Microscope (Leica DM R) and Laser Scanning Confocal Microscope (Leica TCS-SP2). Mixtures of fluorescent beads where analyzed in a Becton Dickinson FACScalibur flow cytometer.

RESULTS AND DISCUSSION

In first place, we explored the better conditions for the production of 5 μm microparticle by means of Flow Focusing technology, and we used these conditions (external flow 3mL/m, internal flow 1mL/h) for the main experiments with fluorescent polystyrene solutions. The procedure presented high reproducibility without any significant differences in the particle size distribution. All the samples have regular spherical shape with a smooth surface (Figure 2).
The fluorescence properties of polystyrene microparticles were determined employing a fluorescence microscope. We also used a scanning confocal microscope in order to check the internal structure of the polymeric microparticles produced by Flow Focusing. The microparticles were constituted by only one phase with the organic dye homogeneously distributed all over the continuous polystyrene matrix (Figure 3). This result indicates that the microparticles

obtained by this method, maintain the properties of the initial fluorescent solutions, without any aggregation or diffusion processes being involve in the drying procedure.

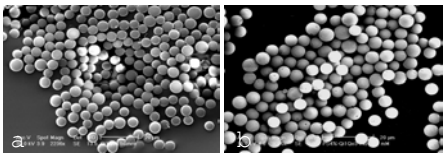

Figure 2. SEM images of freeze-dried 5 µm microparticles; a) blank PS (5.42 ± 0.61µm), b) Fluorescein 0.01 mM (5.21 ± 0.52µm)

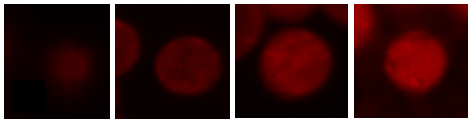

Figure 3. Confocal fluorescence microscopic images of 5 µm Rhodamine B/PS microparticles: Sequence of different sections from a xyz scan.

The fluorescence was evaluated as a function of the fluorescent probe content, the fluorescent probe type and the microparticle size. The resulted fluorescence microscope images are summarized in Figure 4. In every case it was possible to discriminate different microparticle populations using a standard technique like a fluorescent microscope.

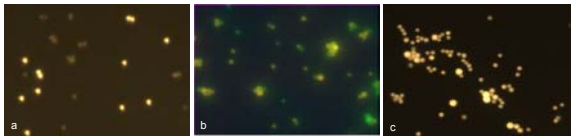

Figure 4. Discrimination of different microparticle populations as a function of: a) fluorescent probe content (Rh B, 5 µm), 0.006/0.6 mM; b) fluorescent probe type (5 µm), Rh B 0.06 mM/Flu 1 mM; c) sizes (Rh B 0.6 mM), 5 µm/9 µm.

We have also used flow cytometry for the analysis of the labelled microparticles produced using the Flow Focusing technology (Figure 5).

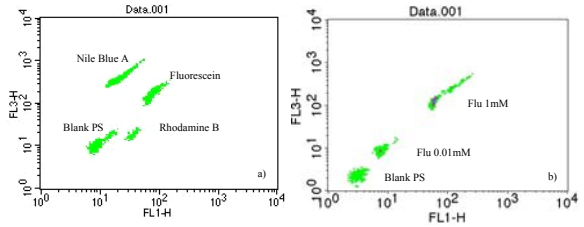

Figure 5. Dot plots showing fluorescent profiles resulting from the analysis of a mixed sample in a flow cytometer, of: a) four microparticle populations with different fluorophores; b) three microparticle populations with different amount of fluorescein.

We analyzed microparticle populations with 5 µm in diameter labelled with different fluorophores (Fig. 5a) and microparticles with different amounts of the same dye (Fig. 5b). The image suggested that higher levels of complexity could be possible by making use of additional colours, as well as additional intensity levels.

To demonstrate the possibility of using these microparticles for multiplex analysis of biomolecules, we have covalently immobilized on their surface some oligonucleotide probes with a 5'-fluorescein moiety. 3'-amino oligonucleotides (60 bp) were linked to the carboxyl groups of microparticles prepared with poly(styrene-co-maleic acid), partial isobutyl/methyl esther. The results were evaluated analyzing the fluorescence intensity (Figure 6).

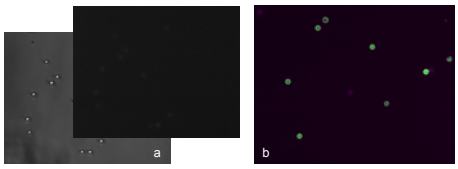

Figure 6. a) Visible (back) and fluorescent (front) images of the same sample of blank PS microparticles without oligo probe; b) fluorescent image of blank PS microparticles with the fluorescein-oligo probe linked.

CONCLUSIONS

We have demonstrated that the Flow Focusing microfluidic technique is amenable to design the size, surface modifications and internal composition of the particles in just one step. We have developed a very versatile and straightforward method for dye-labelled microparticles production, that can be consider as a suitable low cost alternative for mass fabrication of microspheres for multiplex biomolecule analysis.

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