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Investigation of luminescent dye-doped or rare-earth-doped monodisperse silica nanospheres for DNA microarray labelling

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ABSTRACT

DNA microarray is a high-throughput technology used for detection and quantification of nucleic acid molecules and others of biological interest. The analysis is resulting by specific hybridization between probe sequences deposited in array and a target ss-DNA usually functionalized by a luminescent dye. These organic labels have well known disadvantages like photobleaching and limited sensitivity. Therefore in this work we investigate a different strategy, based on the use of inorganic silica nanospheres incorporating standard luminescent dyes or rare earth ions.

The synthesis and characterization of these biomarkers is reported and their application to the DNA microarray technology in comparison to the use of standard molecular fluorophores or commercial quantum dots is discussed. We show that dye doped silica spheres provides a significant increase of the optical emission signal with respect to the use of free dyes, while rare earth doped silica spheres allow reducing or completely avoiding the background noise. These aspects, together with their cheap and easy synthesis, stability in water, easy surface functionalization and bio-compatibility makes them very promising for present and future applications in bio-labelling and bio-imaging.

1. Introduction

The DNA microarray technology is a powerful tool for the parallel, high-throughput detection and quantification of many nucleic acid molecules and other molecules of biological interest [1–5]. Originally developed for the analysis of whole genome gene expression as a cDNA microarray, it is nowadays used also as oligonucleotide microarray, protein microarray and tissue microarray [6,7]. Moreover, the applications have been expanded from gene expression analysis and profiling to genotyping, genetic screening, microbial diagnosis, environmental monitoring, immunoassay and protein assay [8,9].

A DNA microarray consists of an arrayed series of DNA molecules (cDNA or oligonucleotides), containing specific nucleotide sequences, which are used as probes to evaluate the presence of a target sequence in the sample, thanks to single strand DNA main feature to hybridize its complementary sequence under proper conditions.

The hybridization event (probe–target interaction) is usually identified and quantified using optical methods, by fluorescence-based detection: microarray sensitivity can be therefore improved by increasing the optical emission signal of the target and by reducing the background noise, which is mainly related to the intrinsic fluorescence of biological material.

Target molecules can be labeled in different ways (i.e. direct and indirect labelling) and with different fluorophores. Luminescent dyes commonly used for DNA labelling are cyanines, especially Cy3, which has a maximum fluorescence emission wavelength of 570 nm, and Cy5 with a maximum fluorescence emission wavelength of 670 nm. A new generation of fluorophores such as Alexa has been developed and widely used [10], providing similar spectra with higher luminescence emission and better photostability. Although the better performances of these new generation luminescent dyes, the demand for higher and higher sensitivity and for the detection of trace materials still need further improvements.

Strong efforts have been made for the development of new and more efficient nanomaterial-based biolabels with controlled size and shape as effective alternatives to organic fluorophores, to improve the sensitivity of the microarray assay and to avoid some limitations related to common fluorophores. Among them, quantum dots have been widely explored for biological applications and are commercially available [11]. QDs used for bio-labelling have a semiconductor nanocrystal core (CdSe or CdSeTe) that determines their colour, an inorganic shell (ZnS) that improves brightness and stability, an organic coating that provides water solubility, functional groups for conjugation and finally a biomolecules layer (i.e. immunoglobulins, streptavidin, ligands, peptides. . .) covalently

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attached to the shell, that ensure bio-labelling applications, for example with streptavidin–biotin interactions or with specific antigen–antibody interactions. QD has successfully been used for micro-array studies [12–14], as well as for protein immunoassays [15].

QDs present three main disadvantages: (1) they suffer from "blinking" effect (i.e. the photoluminescence intensity of single QD fluctuates), lowering the effective quantum efficiency, (2) they contain heavy metals, and therefore can be toxic, (3) their excitation wavelength maximum is typically in the UV, but most of the biological and bioanalytical instruments are, at present time, based on wavelength belonging to the visible part of the electromagnetic spectrum (especially 532 nm and 633 nm), so they cannot ensure the best excitation efficiency. Other luminescent tools could therefore be more suitable candidates for biological applications such as DNA microarray.

In this paper we report the use of luminescent dye doped silica nanoparticles and rare earth doped silica nanoparticles as biomarkers for DNA microarray, following our previous results on this subject [16–18]. Using appropriate synthetic conditions, a large number of dye molecules (either organic or inorganic) can be incorporated inside a single silica particle, producing a higher optical signal compared with a single dye molecule. Moreover, the dye is trapped inside the silica matrix, which provides an effective barrier keeping the dye from the surrounding environment. In this way both photobleaching and chemical degradation phenomena, that often affect conventional dyes, can be minimized. Finally, silica NPs can be easily functionalized and conjugated to appropriate biomolecules, providing a great improvement in analytical sensitivity [19]. Luminescent dye doped silica NPs have been used with RuBpy molecules [20], in an Affimetrix GeneChips application: the improved photostability makes dye doped silica NPs suitable for applications in bioanalysis and bio-imaging applications.

On the other hand, organic dyes suffer also other disadvantages besides photobleaching such as a broad emission band, superposition of excitation and emission spectra (small Stokes shift) and short luminescent lifetimes. In contrast, rare earth ions have narrow emission bands, large difference between excitation and emission, long luminescence lifetimes and in some cases up-conversion [21]. All these aspects can be used for increasing the signal to noise ratio in biological samples, where self-fluorescence is an important background noise and can overcome the signal under investigation, limiting the detection capability. As an example, the europium ions used for silica doping in this work can be efficiently excited at 393 nm, while their emission is far away peaked at 612 nm. This large difference between excitation and emission allows an easy filtering of the light scattered by the sample and at the same time the narrow emission can be easily discriminated from the broad emission typical of self-fluorescence. Furthermore, since rare earth luminescence is related to forbidden f–f transitions, their lifetime is very large compared to other processes. For Eu^{3+} ions, the 612 nm emission is due to the $^5D_0$–$^7F_2$ transition and its lifetime is around one millisecond, much longer than self-fluorescence from biological samples which is of few nanoseconds. This makes possible time-delayed analysis, which removes completely the biological noise [22].

In this work we present the synthesis of dye doped and rare earth doped silica nanoparticles as suitable labels in glass slide microarray for detection of DNA from biological samples, using the classical linkage system constituted by biotin-streptavidin conjugation.

2. Experimental

Reagents were purchased from Aldrich and Invitrogen and used without further purification.

2.1. Chemical-Physical section

Silica nanoparticles were synthesized following a standard Stöber synthesis [23]. Doping of these nanoparticles with luminescent dyes or with europium rare earth was obtained by adding to the previous synthesis solution one of the following:

(A) Fluorophore (Alexa Fluor 555, 647 and mixture of the two) dissolved in dimethylsulfoxide (DMSO) solution in a concentration 0.1 M.
(B) $Eu(NO_3)_3$ dissolved in ethanol solution in a calculated amount of 0.2%, 0.5%, 1% and 2% Eu/Si atoms with respect to the silicon coming from TEOS in the total synthesis solution.

The final synthesis solution was stirred overnight until formation of silica nanoparticles, centrifuged and washed with ethanol at least three times, then dried at ambient temperature.

In order to obtain surface functionalization with amino groups, a nanoparticles sample of 50 mg was re-suspended in ethanol and reacted with 3-aminopropyl-triethoxysilane (APTES) overnight, then washed and dried as reported.

The morphology of the synthesized particles was investigated in terms of dimension, shape and aggregation state by means of Scanning Electron Microscopy, performed in a VEGA TS 5130 LM (Tescan) microscope under 30 kV acceleration conditions. For this analysis particles were dispersed in ethanol and dip-coated on a silicon wafer.

The optical mapping of the nanoparticles was performed by an Alpha SNOM (Witec) instrument in transmission mode. The correlation of the morphological shape and the optical near field was obtained using a 514 nm Ar^+ laser focused by a 10× objective into a <100 nm holed-tip cantilever, controlled by AFM feedback. For this analysis particles were dispersed in ethanol and dip-coated on a 170 μm thick glass substrate.

Finally, the photoluminescence excitation and emission properties of the nanoparticles in the UV and visible regions (350–750 nm) have been measured in a Fluorolog3–21 system (Horiba Jobin Yvon). The system is equipped with a 450 W excitation lamp for PL excitation and emission measurements.

2.2. Biological section

The selected DNA probe was purchased from Operon, with a 5′ amino-modification to ensure the binding of the probes on the slide. A 15 μM probe concentration solution was loaded into micro-plates and submitted to the spotter for printing an array of 16 identical spots (4 × 4).

LifeLine slides (LifeLineLab, Italy) were printed using Spotter Arrayer (Versarray Chip Writer Pro System, BioRad) instrument, using Telechem SMP3 microspotting pins.

Target DNA sequence was amplified through Polymerase Chain Reaction (PCR) with specific primers, allowing the incorporation of biotin modified dCPT. The amount, integrity and dimension of the amplicons were verified with capillary gel electrophoresis with Agilent 2100 Bioanalyzer technology, using DNA electrophoresis chips. The efficiency of DNA biotinylation was estimated comparing the gel electro mobility of the DNA amplicons with the ones amplified with a standard dNTP mix (without dCTP-biotin). These biotin-labeled DNA samples were denatured and finally hybridized on the microarray slides (same amount of DNA per slide) in the Array Booster AB410 (Advalytix), using Advacard (Advalytix).

The slides washed in the Advawash Station AW400 (Advalytix) and spin-dried were ready for reaction with streptavidin-conjugated luminescent nanoparticles or Quantum Dots.