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Danny Brouard, Olivier Ratelle, A. Guillermo Bracamonte, Maryse St-Louis and Denis Boudreau*

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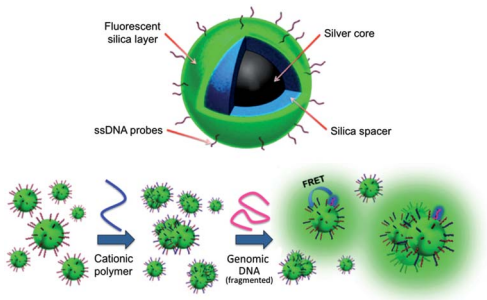

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A biosensor based on a cationic polymer and metal@silica core–shell nanoparticles is demonstrated for the rapid identification of the *SRY* gene from unamplified human genomic DNA. By amplifying the fluorescence signal triggered by the capture of target molecules, this biosensor avoids the added complexity of enzyme-based chemical amplification, makes genotyping assays faster and less costly and could have important applications in various areas of health sciences.

The identification of human genes and their alterations has important applications in various areas of health sciences including, among others, early cancer diagnosis and personalized cancer medicine,[1,2] genetic risk assessment[3] and blood group genotyping.[4] Because of the reduced amount of genetic material available and the limited sensitivity of conventional DNA detection techniques, genotyping protocols usually include target sequence amplification by PCR (polymerase chain reaction), which provides significant advantages in terms of detection accuracy, specificity and sensitivity.[5] Unfortunately, methods based on enzymatic amplification are prone to contamination or enzyme inhibition, and generally require skilled personnel to be performed properly. Furthermore, they are time-consuming and costly and, whereas high-throughput technology is available, the economy of scale and time expected is reached only for batches of hundreds of samples or more. There is therefore a need for a faster, simpler and more cost-effective genotyping technology.

Efforts have been made recently to develop new methods capable of identifying specific DNA sequences from unamplified genomic DNA, using a plurality of detection modalities. In particular, the remarkable properties of metallic nanostructures have been exploited to amplify the optical signal resulting from the capture of target DNA sequences.[6–9] We recently reported the development of a plasmonic biosensor combining the hybridization recognition capabilities of a cationic conjugated polymer (CCP) with the luminescent properties of Ag@silica multilayer dye-doped nanoparticles (NPs) grafted with single-stranded DNA (*ss*DNA) probes.[10] Upon hybridization of *ss*DNA targets to the probes, the CCP becomes luminescent,[11] allowing the optical transduction of hybridization events through a FRET-(Förster resonant energy transfer) mediated fluorescence signal by allowing the light from a suitable source to be coupled from the polymer to the acceptor molecules immobilized in the outer layer of the NPs (Fig. 1). The core–shell architecture features several advantages over conventional fluorescent biosensors leading to enhanced detectability. First of all, the

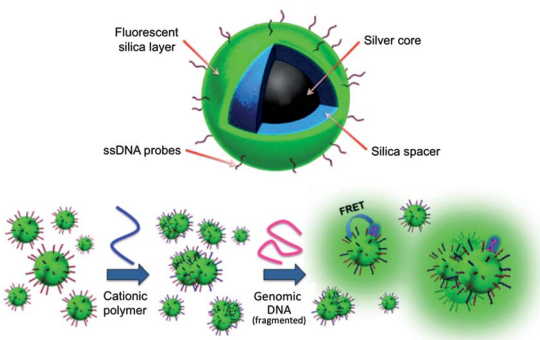

Fig. 1 Top: multi-layer core–shell nanoparticles (NPs) are made of an Ag core (~35 nm) separated from an outer fluorescent silica layer by a spacer also made of silica of a few nanometres in thickness. The outer silica surface is functionalised with oligonucleotide capture probes. Bottom: schematic description of the proposed detection scheme: (1) "target-ready" nanobiosensor is prepared by mixing the cationic polymer transducer with probe-grafted NPs and surface charge neutralization promotes formation of nanoparticle aggregates; (2) upon mixing the nanobiosensor with target DNA, hybridisation with oligonucleotide probes occurs, which activates the polymer transducer and amplifies the optical signal *via* energy transfer to the fluorescent NPs.

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interaction of the metallic core's plasmon with neighbouring fluorophore molecules greatly improves the excitation efficiency, emission rate and resistance to photobleaching, an effect called metal-enhanced fluorescence (MEF).[12,13] Moreover, the capacity of the polymer as an energy donor is increased by strong near-field interactions in this plasmon-enhanced FRET system and results in enhanced transfer efficiency, range and rate;[14–16] as a consequence, the strength of donor–acceptor interactions increases and allows the excitation of several dye acceptor molecules by a single CCP donor.[17] Furthermore, the binding of positively charged polymer chains partially neutralises the particles' surface charge and induces the formation of small NP aggregates; these networks allow collective interactions which, in turn, enhance plasmonic coupling and local electric field intensities and amplify the overall optical signal generated by DNA hybridization events. Excellent limits of detection in the femtomolar (10^{-15} mole L^{-1}) range were obtained with this biosensor using short synthetic oligonucleotides as target analytes; however the capabilities of this nanobiosensor had not until now been evaluated with real genomic DNA samples.

In this work we used the nanobiosensor to identify the *SRY* gene (*sex determining region of Y* chromosome) from unamplified human genomic DNA extracted from the blood collected from ten individuals. 22-mer oligonucleotide probes specific to the *SRY* gene were grafted to core–shell Ag–silica nanoparticles, a CCP was added and purified human DNA was allowed to hybridize to the nanobiosensor for 10 minutes. Fluorescence was measured by imaging flow cytometry (IFC) and the results were correlated with classic PCR and gel electrophoresis.

The Ag–silica core–shell nanoparticles were synthesized using a methodology described previously.[10] Since it is known that the nanoparticle's composition, size and geometry have a strong influence on the extent of MEF,[18] the synthesis methodology used in this work allows both the metal core size and the silica spacer thickness to be easily tuned so that the nanoparticle geometry provides maximum fluorescence enhancement and energy transfer from the CCP donor to the acceptor dye molecules. The synthesis methodology is simple, requires no complex apparatus and can be scaled up easily.

In the present case, Ag nanoparticles with an average diameter of 36 ± 7 nm were coated with a silica spacer shell 8 ± 1 nm in thickness (Fig. 2). This core size falls within the size range expected to provide optimal plasmonic enhancement.[19] Ag was preferred over other plasmonic metals such as Au or Cu in light of its stronger plasmonic properties[18] and also of the good overlap between the plasmon of Ag NPs and the absorption/emission spectra of the polymer transducer, leading to better fluorescence enhancement factors (EF).[17] The thickness of the silica spacer shell was adjusted to minimise fluorescence quenching of the acceptor dye molecules at very short distances and to maximize FRET between the polymer donors and the eosin acceptors. In addition, the silica surface also improves nanoparticle colloidal stability and shelf life while lending itself to a versatile surface chemistry for easy covalent linking to a wide range of molecules, extending the application field of the nanobiosensor.

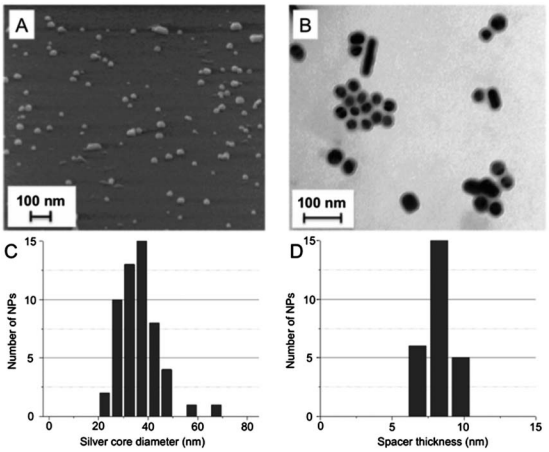

Fig. 2 Top: SEM images of Ag core NPs (A), TEM images of NPs with silica spacer (B). Bottom: size/thickness distribution histograms of silver core NPs (C) and silica spacers (D).

The outer fluorescent shell containing the acceptor molecules was obtained by mixing eosin isothiocyanate molecules with an amino-silane linker (aminopropyltriethoxysilane) and incorporating them into a thin (~3 nm) silica shell using the same sol–gel condensation process used to form the intermediate spacer layer.[10] By comparing the fluorescence intensity of eosin with and without the Ag core, we measured a fluorescence enhancement factor (EF) of ~8, indicative of an adequate silica spacer thickness.[14]

To generate probe-grafted NPs, 22-mer oligonucleotide probes specific to a region of the *SRY* gene were linked to NPs previously modified by a carboxylic acid surface using standard NHS cross-linking chemistry (oligonucleotide sequences used in this work are listed in Table S1†). The probe surface coverage on the nanoparticles was determined using 3′-Cy5.5- and 5′-amine-terminated probes by comparing the Cy5.5 fluorescence before and after the probe grafting step, and a surface coverage of $\sim 1.5 \times 10^3$ probes per NP was calculated, similar to surface coverage densities reported previously for similar nanoparticle surface chemistries.[20] Probed-grafted hybrid NPs can be stored for at least two months without any performance degradation if dispersed in ethanol and kept at 4 °C in the dark.

Prior to their use for analysis of human genomic DNA, so-called "target-ready" NPs were prepared by adding the CCP to the probe-grafted NPs, allowed to react overnight, then washed and resuspended in pure water. The CCP, by electrostatic binding to the negatively charged *ss*DNA probes, acts both as an optical transducer and as a counter-ion to allow the probes to hybridize to the targets. Hybridization efficiency was evaluated using an excess of 5′-Cy5.5-labeled complementary *ss*DNA target sequence. A fluorescence intensity ratio of 9 was recorded between the NPs signal and the supernatant after a 10 min hybridization step at 55 °C in pure water. No signal was observed when the same experiment was carried out without the polymer.

To evaluate the sensitivity and efficiency of the nanobiosensor for the detection of the chosen *SRY* sequence, a calibration curve in the femtomolar range (0 to 3×10^6

molecules per mL) was established using 22-mer synthetic oligonucleotides. Target-ready NPs were mixed with 22-mer *ss*DNA sequences that were perfectly matched to the *SRY* probe. After a 10 min hybridization period at 55 °C, 20 μL aliquots of each calibration standard were analysed in triplicate using an imaging flow cytometer where each nanoparticle aggregate was excited by a laser light sheet, imaged onto a CCD camera and counted.[10] The resulting calibration curve (Fig. 3) shows a linear range reaching 3×10^6 target sequences per mL (6 fM) and a 3σ detection limit of 1×10^5 *ss*DNA per mL (0.2 fM), whereas mixing target-ready NPs with non-complementary sequences led only to a slight increase in luminescence intensity. These figures of merit are adequate for the application at hand, given that the concentration of leukocytes in the blood of healthy individuals is *ca.* 10^7 cells per mL.

The nanobiosensor was then used to distinguish the *SRY* gene from genomic DNA extracted from unknown human blood samples without any prior enzymatic amplification. 10 mL blood samples were collected from ten individuals after informed consent. Genomic DNA was extracted using a well-established method (see ESI† for details) and resuspended in 300 μL of Tris–EDTA buffer. Because the concentration of leucocytes can vary substantially from person to person, the DNA concentration in each purified sample was measured by UV-vis (Table S2†). The DNA samples were then fragmented in an ultrasound bath, denaturated at 95 °C and diluted 10× in sterilized water. 100 μL aliquots of each sample were mixed in triplicate to target-ready NPs bearing *SRY* capture probes. After a hybridization step of 10 minutes at 55 °C, the samples were measured on the IFC apparatus in single blind fashion. The averaged signal intensity measured for each sample was normalized by the total DNA concentration as determined by UV-vis.

Since the DNA samples were anonymised, a classic PCR amplification was also performed to determine if they were obtained from female or male individuals. According to the result of the PCR assay, half of the samples came from female individuals and the other half from males (see Figure S5†). Comparing the PCR results with the PCR-free measurements (Fig. 4) shows that the nanobiosensor was able to detect the *SRY* gene from unamplified human genomic DNA samples in only a few minutes and using a very simple procedure. This result is remarkable considering the existence of several sites in the entire human genome displaying one or two base differences with the chosen *SRY* probe and the very low amount of DNA available (*ca.* 100 μg, or $\sim 10^8$ copies of the target sequence). Despite this, a clear signal contrast was found between the positive and negative samples, a consequence of the MEF- and FRET-enhanced response of the nanobiosensor and the ability of the polymer transducer to promote hybridization under very stringent conditions (*i.e.*, at 55 °C and in pure water, without other counter-ions to neutralise the negative charges of the DNA backbone phosphates).

One of the *SRY*-positive samples showed an abnormally low fluorescence signal which would have resulted in a false negative without the validation by PCR. Given that the total DNA concentration of this sample as determined by UV-vis was in the same range as the others and that the fluorescence signal intensity was actually lower than for the female (negative) samples for all 3 replicates, we theorize that an experimental error led to a lower concentration of target-ready nanoparticles brought in contact with the DNA material. For example, previous studies on the polymer transducer have shown that the fluorescence signal intensity varies for several minutes after initial contact with the target molecules.[21] Furthermore, the concentration of target-ready particles could have been affected by losses to gravitational settling. Since kinetics are expected to play an important role in the development of signal over time with this detection scheme, automation of the sample manipulation procedure would allow to minimise the impact of experimental variables on the analytical results by providing better timing of the different steps of the detection protocol. An internal standardisation procedure, using a ubiquitous housekeeping gene such as β-actin or 18S, could also be used to prevent the occurrence of false results and improve the assay's robustness.[22] This would be implemented by spectrally encoding nanobiosensors prepared with the different capture probes (*SRY* or internal standard), *i.e.*, using two different acceptor fluorophores spectrally separated from each other in the silica

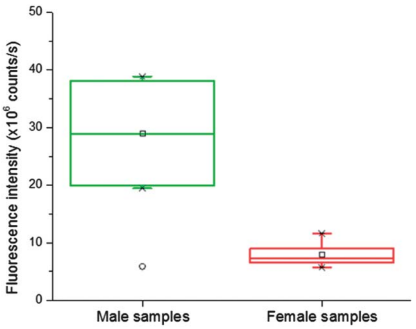

Fig. 4 *SRY* genotyping results for 10 unknown genomic DNA samples. DNA extracted from human blood samples was tested using a probe sequence specific to the *SRY* gene, and the fluorescence signals were correlated with PCR analysis. Five samples were female (negative) and the five others were male (positive). 9 out of 10 samples were correctly identified with the nanobiosensor. The 10th sample (false negative) is represented as an open circle.

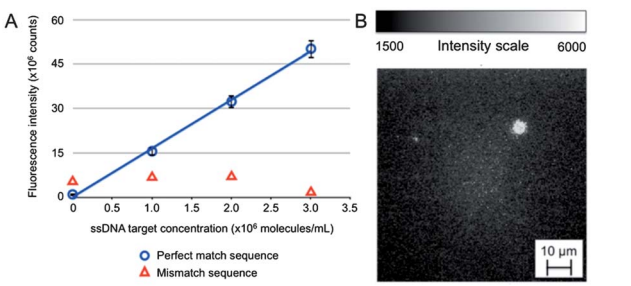

Fig. 3 Detection of synthetic 22-mer *ss*DNA targets with the nanobiosensor. Left: calibration curve for the detection of perfectly matched 22-mer target oligonucleotide targets (open circles) and non-complementary sequences (open triangles). The 3σ detection limit for perfectly matched targets is 1×10^5 molecules per mL, or 0.2 fM. Right: example of a fluorescence image captured by imaging flow cytometry.

shell.[23] This strategy could be extended further by incorporating multiple acceptor fluorophores and varying their doping ratio, and the fluorescence emitted in distinct spectral channels could be used as a barcoding scheme for multiplex genomic biomarker detection.

Conclusions

In conclusion, we reported the development of a nanoparticle-based biosensor and its use for the molecular identification of a gene in the unamplified human genomic DNA extracted from a blood sample. While the use of a polymer-based biosensor for the identification of a specific gene in unamplified DNA has been reported before,[24] this nanobiosensor offers the plasmon-enhanced fluorescence and the enhanced photostability that are characteristic of metal@silica fluorescent nanoparticles, and provides the ease of signal analysis offered by particle counting apparatus such as commercial flow cytometers commonly used in health science laboratories. In the present proof-of-concept, the nanobiosensor allowed the correct identification of 9 out of 10 unknown human DNA samples in only a few minutes and using a very simple procedure. Furthermore, since the capacity of the polymeric transducer to discriminate single-based mismatches (Single Nucleotide Polymorphism, SNP) has been demonstrated,[21] this nanobiosensor could in principle identify any known genetic variation, including SNPs, as long as a unique complementary oligonucleotide probe can be found. By avoiding the added complexity of enzyme-based chemical amplification approaches, it could make genotyping assays faster and less costly and have important applications in various areas of health sciences and, hopefully, contribute to bring personalized medicine to everyday life.

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