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Supramolecular Spectrally Encoded Microgels with Double Strand Probes for Absolute and Direct miRNA Fluorescence Detection at High Sensitivity

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Supporting Information

ABSTRACT: We present novel microgels as a particle-based suspension array for direct and absolute microRNA (miRNA) detection. The microgels feature a flexible molecular architecture, antifouling properties, and enhanced sensitivity with a large dynamic range of detection. Specifically, they possess a core−shell molecular architecture with two different fluorescent dyes for multiplex spectral analyses and are endowed with a fluorescent probe for miRNA detection. Encoding and detection fluorescence signals are distinguishable by nonoverlapping emission spectra. Tunable fluorescence probe conjugation and emission confinement on single microgels allow for ultrasensitive miRNA detection. Indeed, the suspension array has high selectivity and sensitivity with absolute quantification, a detection limit of 10^{-15} M, a dynamic range from 10^{-9} to 10^{-15} M, and higher accuracy than qRT-PCR. The antifouling properties of the microgels also permit the direct measurement of miRNAs in serum, without sample pretreatment or target amplification. A multiplexed assay has been tested for a set of miRNAs chosen as cancer biomarkers.

Recent studies have demonstrated that the signature of microRNAs (miRNAs) in blood, in particular changes in their concentration, predicts development and prognosis of several tumor types.[1,2,3] However, direct and absolute quantification of miRNAs in body fluids is particularly challenging because of their small size, high levels of sequence homology, complex secondary structures, demand of high sensitivity in a range between atto- (aM) to femtomolar (fM), specificity,[2] and multiplexing.[4]

Well-known technologies for the detection of oligonucleotides, based on hybridization to complementary probes, are represented by polymerase chain reaction (PCR, qRT-PCR)[5] and oligonucleotide probes such as molecular beacons (MB)[6] and double strands (ds).[7] In particular, qRT-PCR shows high sensitivity (down to 10 fM) and high-throughput ability. However, extraction, amplification, and calibration steps are time-consuming but always necessary, while preliminary amplification may compromise the assay accuracy.[5] Furthermore, in the case of short nucleic acids, primer design may reduce specificity.[8] Several alternatives include thermodynamic,[9] enzymatic,[10] and electrochemical methods[11] combined with electronic,[12] fluorescence, and colorimetric detection.[13] Recently, among those, particle-based suspension arrays[4] have been attracting increasing interest for the multiplexed detection of nucleic acids, offering high flexibility, easy probe-set modification, efficient mixing steps, and high degrees of reproducibility. Fluorescent molecules, vibrational signatures, quantum dots, photonic crystals, shape, and photo pattern have been used as encoding elements for the particles.[14,15] Although suspension arrays show a number of significant advantages, certain drawbacks related to the material remain, such as limited binding kinetics, reduced activity of surface-bound biomolecules, low sensitivity, limited range of detection, and, mainly, nonspecific surface adsorption.

In contrast, soft materials such as hydrogels show high ability for biomolecule immobilization, solution-like binding kinetics, and antifouling properties to limit nonspecific interactions.[16,17] Smart molecular building blocks can be indeed easily incorporated into the polymer network providing multivariable control over swelling, mechanical and interface properties, and their hydrophilic characteristics and encoding ability. However, even though they have already demonstrated the possibility to be decorated with unique and decodable identities by small molecular footprints for multiplex detection,[18] their chemical potentials in highly sensitive diagnoses have not been fully exploited yet.

Herein we introduce a particle suspension array based on multifunctional microgels for an ultrasensitive, direct, multiplex, and absolute quantification of miRNA in fluorescence. In this frame, we have evolved the oligonucleotide-based recognition concept by introducing flexible and sensitive probes in a tridimensional hydrophilic polymer network with modular architecture (Figure 1)

In particular we developed probes for a ds displacement assay and synthesized core−shell microgels. The probes were then integrated into the microgel molecular network.

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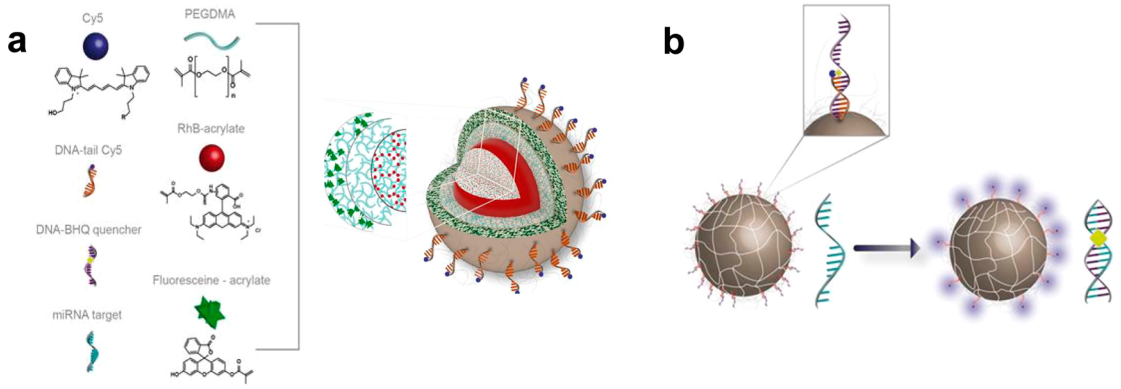

Figure 1. Sketch of engineered core-double shell microgel and its mechanism of miRNA detection. (a) Graphical representation of core double shell microgels. (b) Scheme showing the mechanism of miRNA detection on microgel.

In particular, based on the miRNA sequence (23 nucleotides), we designed the ds probes formed by (i) a tail (12 nt), labeled with Cy5 at the 5′ end, modified with an amine group on the 3′ position for covalent immobilization on the microgel and (ii) a quencher strand (23 nt) internally modified with a Black Hole Quencher (BHQ),[19] partially complementary to the tail and fully complementary to the target. The probe design implies that, when the quencher strand and tail partially hybridize, the BHQ comes in close proximity and fluorescence quenching occurs. In presence of the target, the quencher and the target hybridize so that the Cy5 and BHQ are no longer in close proximity and, therefore, Cy5 fluorescence emission is recovered. The length of the tail was optimized to obtain an appropriate difference in free energy (~20 kcal mol^{-1}) between the tail-quencher and the target-quencher duplex (see Scheme S1 and Table S1, Supporting Information). The probe design was then validated assessing the selectivity and the specificity in miRNA detection. As shown in Figure 2a the probes are not affected by the presence of noncomplementary miRNA sequences or by human serum. The limit of detection (LOD) of the probes in solution is about 172 pM (Figure 2b).

For the assay setup, we engineered microgels with fluorescence encoding and surface chemistry for conjugation of nucleic acid probes (Figure 1a).[20] Briefly, core microgels based on a copolymer of poly(ethylene glycol) (PEG) and Rhodamine B methacrylate monomer were prepared by free-radical precipitation polymerization. Then, core microgels were used as seeds in two subsequent polymerization reactions to obtain a first shell of PEG and a second shell of a copolymer of PEG-*co*-Acrylic acid doped by different amounts of a Fluoresceine O-methacrylate monomer (Figure S1, Tables S2 and S3, Supporting Information). The ratio between fluorescence emission of fluoresceine (490−550 nm) and rhodamine (580−640 nm) (Fluo/Rhod) represents a robust spectral encoding (Figure 3). Chemical, physical, and morphological characterization of microgels is reported in Figure S2, Supporting Information.

Furthermore, we functionalized the spectrally encoded microgels with nucleic acids probes for selective and specific miRNA fluorescence detection according to a double strand (ds) displacement scheme (Figure 1b).

We optimized the covalent coupling of the 3′-NH2-DNA-tail Cy5 probe on microgels in order to obtain a probe density in a range of 6.2×10^{12} probes/cm^2 (~4.7×10^4 DNA probes/microgel) at which point the repulsive electrostatic interactions

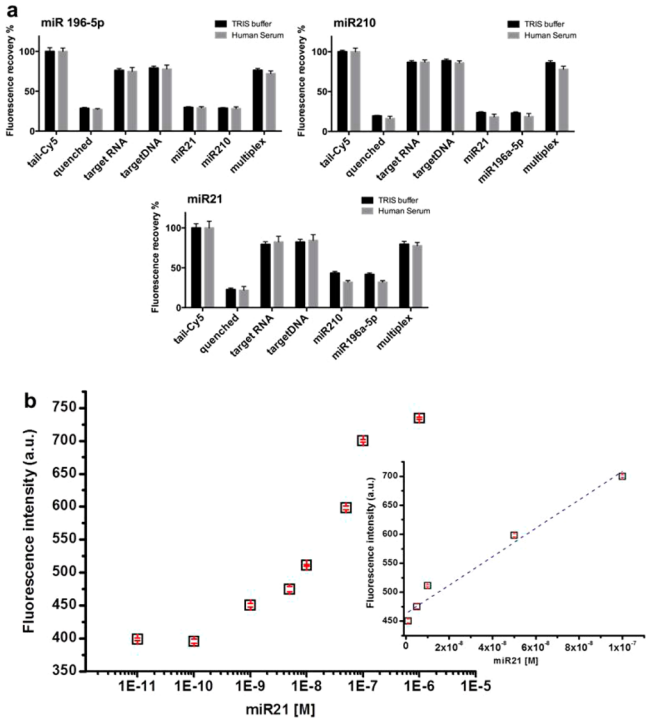

Figure 2. Performance of double strand probes for miRNA target detection. (a) Specificity and selectivity for miR21, miR210, miR196a-5p targets of double strand probes in solution by spectrofluorometer measurements. (b) Plot of fluorescence recovery after double strand displacement for the detection of miR21 RNA target over a dynamic range concentration of 10^{-11}−10^{-6} M and fluorescence recovery response in linear−linear plot over 10^{-9}−10^{-7} M range.

and steric hindrance between oligonucleotides are minimized (Supporting Information).[2122]

In order to validate the microgel in detection applications, the miR21 sequence was chosen as an miRNA target (Table S1, Supporting Information). After miR21 target incubation (in a concentration range between 10^{-9} and 10^{-15} M), the microgels were loaded onto μ-slide channels. A fixed number of microgels were selected for each sample (i.e., different miRNA target concentrations), and their fluorescence images were analyzed (Figure 4a and Supporting Information).

The fluorescence recovery for the miR21 was measured on the microgels in a dynamic range of 10^{-9} −10^{-15} M with an LOD of 2.6 fM (Figure 4b). This result demonstrates that the assay

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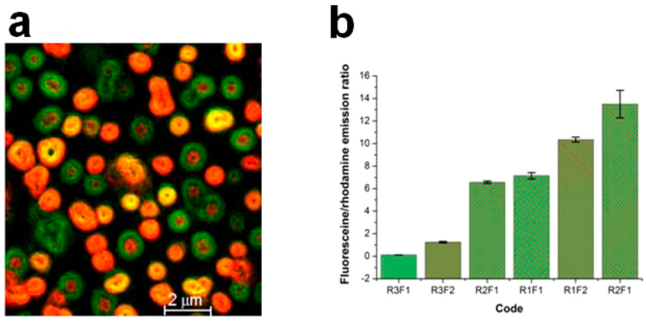

Figure 3. Core double shell microgel characterization. (a) Fluorescence images of the three sets of core-double shell microgels excited by 488 and 543 nm wavelength lasers. (b) Column diagram of the different Fluo/Rhod fluorescence emission related to six microgel codes.

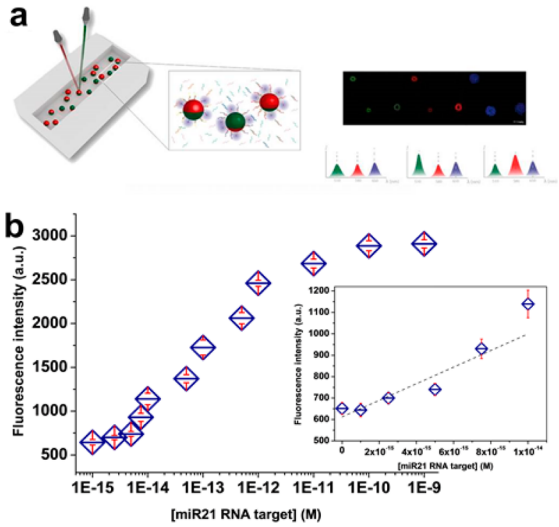

Figure 4. Performance of multifunctional microgels for miR21 fluorescence detection. (a) Sketch of encoded microgel assay for miRNA fluorescence quantification. (b) Plot of fluorescence recovery of miR21 over a concentration range 10^{-9} to 10^{-15} M and linear regression applied to the collected data in a dynamic range of concentration of 10^{-14} to 10^{-15} M for the limit of detection (LOD) determination.

performed with microgels allows lowering the LOD to 5 orders of magnitude if compared with the probes alone. Such enhancement is ascribable to the spatial confinement on a single microgel of the fluorescence emission upon detection event.

The selective detection of a triplex miRNA panel represented by miR21, mivR210, and miR196a-5p was achieved by conjugating the corresponding DNA tail Cy5 probes on three different encoded microgels (Figure 5, left panel). Such targets were chosen as they are biomarkers for pancreatic cancer diagnosis, when simultaneously overexpressed.[23] The quenched microgels were mixed and incubated with a solution containing synthetic miR21 and mir196a-5p targets (3×10^9 copies of each; see Supporting Information). The Cy5 fluorescence recovery occurred only on miR21 and 196a-5p specifically encoded microgels. The ratio Fluo/Rhod indeed correlates with the specific code corresponding to miR21 and 196a-5p identity (Figure 5, right panel).

To compare our approach with the gold standard technique, we measured the endogenous content of miR21 in healthy human serum extracts measured by the microgels as well as by the qRT-PCR (Figure S5, Supporting Information). The concentration of endogenous miR21 detected in human serum

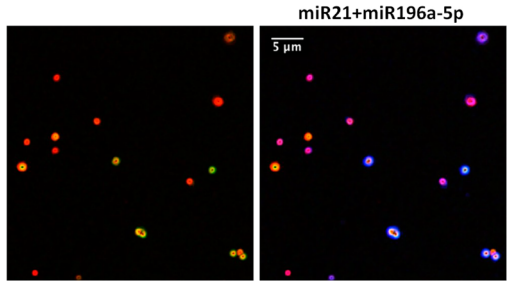

Figure 5. Microgel based assay for miR21 and miR196a-5p target detection in multiplex. Synthetic targets (1.0×10^{-11} M) were added to Tris buffer solution containing three different quenched encoded microgels specific for miR21, miR210, miR196a-5p, respectively (left panel). Upon miR21/miR196a-5p detection, the Cy5 fluorescence recovery was observed only for the corresponding encoded microgels (right panel).

by the microgel corresponds to $(4.3 \pm 0.59) \times 10^3$ copies/μL and exhibits no statistical differences (ANOVA and Tukey test, 95% confidence level) when compared with the miR21 concentration measured by qRT-PCR ($(5.1 \pm 2.94) \times 10^3$ copies/μL). Noticeably, it shows a higher precision than the qRT-PCR (Figure 6a).

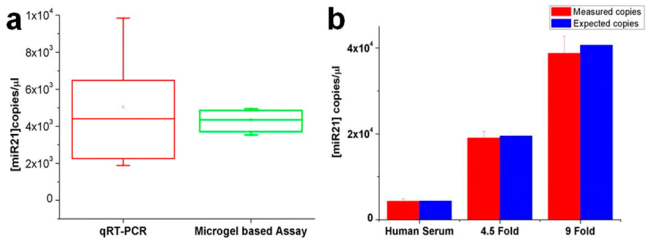

Figure 6. Direct quantification of miR21 by microgels. (a) Mean of miR21 concentration in healthy human serum measured by qRT-PCR is 5.04×10^3 vs 4.33×10^3 copies /μL measured by microgel based assay. (b) Different amounts of miR21 RNA target were spiked into human serum in order to obtain increasing concentrations ranging from a 4.5- to 9-fold increase.

The last step was to evaluate the accuracy of the microgel based assay directly in human serum (see Figure S6, Supporting Information). The serum miR21 concentration was adjusted in order to obtain 4.5- and 9-fold increases in miR21 amount (corresponding theoretically to 1.95×10^4 and 4.06×10^4 copies/μL respectively). Those amounts were selected as representative of pathological overexpressed levels of miRNA in relevant diseases.[23] Figure 6b shows that the miR21 quantified by microgels corresponds to the expected amounts ($(1.90 \pm 0.15) \times 10^4$ copies/μL and $(3.88 \pm 0.40) \times 10^4$ copies/μL) after spiking into the serum (reduced chi square test, $\chi^2 = 0.96$). Furthermore, the miR21 endogenous amount detected directly in serum (Figure 6b) is fully in agreement with that measured in serum extracts (Figure 6a). Such unmet performance is mainly ascribable to PEG interfaces, because of their resistance to protein adsorption, which can be attributed to their strong interactions with water, charge neutrality, and absence of hydrogen donors.[24,25]

In conclusion, we report on an innovative assay based on molecular probes for fluorescence detection mounted on spectrally encoded microgels.

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Recently, ds displacement probes have evolved for stability and pH control; however, the requirement of additional sources limits their use in biosensing applications.[2627]

Our probe design is flexible toward the detection of different miRNAs, can be used at room temperature, does not require additional reagents, is stable in biological fluids, and is conceived for solid suspension arrays.[20]Unmet performances in miRNA detection have been achieved by coupling those probes on engineered multifunctional microgels.[20] The use of the microgel platform corresponds to a 10^5-fold enhancement in sensitivity (2.6 fM) compared to what is achieved by the same probes not conjugated to microgels (172 pM) because of the confinement of the fluorescence emission in a small volume. The use of microgels leads to a simple and absolute quantification in serum extracts without target amplification steps, internal normalization, or primer optimization with a higher precision than qRT-PCR. Furthermore, our work shows that the microgel performance is not significantly affected by the complexity of the serum. The microgel, indeed, recapitulates a highly hydrated bioinspired environment, provides an enhancement in nucleic acid hybridization, when compared to other solid surfaces, offers antifouling properties, and, thus, decreases the background interference due to nonspecific interactions.[1617]

The flexibility and specificity of the molecular probe, combined with the three-dimensional hydrophilic polymer networks, represent a modular platform that can be generalized for any direct biodetection in whole serum and, thus, applied to a wide spectrum of biomedical applications.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Materials; Probe design; Double strand assay specificity; ds displacement assay; Microgels synthesis; Chemical, physical, and morphological characterization of microgels; Microgel based assay setup; Encoded microgel specificity; RT-PCR versus microgel-based assay in miR21 quantification; Direct quantification of miR21 in serum by microgel-based assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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