Research Article

Polymethacrylate Sphere-Based Assay for Ultrasensitive miRNA Detection

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1. Introduction

MicroRNAs (miRNAs) are short regulatory ribonucleic acids (RNAs) ranging from 18 to 25 nucleotides in length [1]. Overexpression or underexpression of miRNAs is associated with various diseases and with distinct stages of specific illnesses [2–6]. Monitoring changes in expression levels of miRNAs is invaluable for timely initiation of treatment and/or for monitoring the effectiveness of an ongoing treatment [7–9]. MiRNAs are challenging targets for biorecognition due to their small size, high levels of sequence homology, and semistable secondary structures [10]. Routinely applied strategies for detection of miRNAs including northern blot, microarrays, or real-time polymerase chain reaction (PCR) require complex equipment and data analysis, while they are costly and not always available in every clinical setup [8, 11]. Moreover, several reports indicate insufficient specificity and sensitivity...
when dealing with these techniques which add to the existing challenges of miRNA detection [12].

Nano/microspheres have drawn a great deal of interest in immunoassays due to their key advantageous features including (i) amenability to screening and multiplexing; (ii) significantly larger specific surface area in comparison with two-dimensional (2D) platforms which facilitate higher analyte-surface interaction; and (iii) high spatial freedom for interaction with biomolecules of interest [13–21]. miRNA detection using different particle types are reported in the literature [22, 23]. A wide range of particles were used for the purpose of miRNA detection including magnetic, carbon, graphene oxide, silver, and copper particles [24–30]. Quantum dots-encoded microbeads (Qbeads) introduce another strategy for detection of miRNAs [31, 32]. While these technologies opened windows of opportunity for effective detection of miRNAs, a vast majority of them involve time-consuming functionalization steps, expensive reagents, complicated procedures, and sophisticated laboratory setups [12]. Even then, the stability of the modified bioreceptive surfaces is not guaranteed, as they might lose their functionality over time [33, 34].

In this work, we describe a proof-of-concept strategy that involves cross-linked polymethacrylate microspheres of different sizes as bioreceptor surfaces for miRNA detection based on nucleotide hybridization. The polymer-based microspheres possess tailored physical and chemical properties. While offering a large surface area for analyte-surface interaction, the spheres are benefited from the inherent presence of carbonyl (-C=O), hydroxyl (-OH), and aromatic groups that further promotes biomolecular interactions. This makes the functionalization and surface activation steps unnecessary. Microspheres were integrated into a conventional 96-well plate for a one-step hybridization assay for biorecognition of Cy3-labeled miR159 as the target analyte, using a complementary amino-modified DNA capture probe. Moreover, synthetic unlabeled miR159 was detected in a competitive assay as a further proof-of-concept since the concentration of miR159 in blood serum is inversely correlated to the breast cancer incidence and progression in humans [35]. This straightforward strategy for the first time allows a routine analytical assay to detect microRNAs in the picomolar (pM) range without any amplification.

2. Materials and Methods

2.1. Chemicals and Reagents. Sodium citrate (Na3C6H5O7), sodium chloride (NaCl), hydrochloric acid (HCl), sodium dodecyl sulfate (SDS), polyborate 20 (Tween 20), toluidine blue (TB), sodium hydroxide (NaOH), acetic acid (AcOH), nuclease-free water, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Capture probe, amino-modified DNA (5’-TTTAAGGAGGCTCataccgggccc-3’/amino modifier/), target Cy3-labeled analyte miR159 (5’-GAGCUCUCCUAAGuuaaaac-3’/Cy3/), synthetic target miRNA159 (5’-GAGCUCUCCUAAGuuaacca-3’) and the noncomplementary negative control, Cy3-tagged miR-lin4 (5’-acaccgggcuucggguac-3’/Cy3/), were purchased from Integrated DNA Technologies (Coralville, IA, USA). The uppercase and lowercase letters in the biomolecules represent complementary and noncomplementary nucleotides, respectively (amino modifier is a primary amine with no spacer arm).

2.2. Microsphere Synthesis. Polymethacrylate microspheres were synthesized in a suspension by polymerization (Geleen, the Netherlands) with monomers methyl methacrylate (MMA), 2-hydroxylethylmethacrylate (HEMA), 4-iodo-benzoyoethyl methacrylate (4-IEMA), and tetraethylene glycol dimethacrylate (TEGDMA). Details of particle synthesis and characterization (storage stability, hemo- and cyto-compatibility, structure, and absence of leachable components) have previously been reported [36, 37]. Spheres were sieved and size-sorted as follows: MMS-1 (200–400 μm); MMS-2 (400–600 μm); MMS-3 (600–700 μm); and MMS-4 (700–900 μm) (Figure 1). The spheres are slightly hydrophilic and relatively dense (~1.3 g/mL) which are advantageous features for biorecognition applications, as the conjugated particles will sink in aqueous media without clustering. This, in turn, maximizes the contact with the analyte of interest.

2.3. Morphology, Size Distribution, Surface Area, and Raman Spectroscopy Analyses. A scanning electron microscope equipped with a field emission gun (FESEM, JEOL, JSM7600F, USA) was used for the morphological analysis of platinum-coated spheres from different size categories. The acceleration voltage of the instrument was 0.5 kV. The size distribution of each size category was calculated from optical microscope images of 500 ± 5 randomly selected spheres from each group (OLYMPUS, BX5iTRF, Japan). The specific surface area of each size category (per 10 mg) was calculated from the size distribution analysis (Figure 1) [36]. Raman spectra were recorded on a Raman Spectrometer, LabRAM HR Evolution (Horiba, Japan), coupled to an Olympus BX-4 microscope. The wavelength used to excite the sample was 532 nm, which was provided with a Nd:YAG laser as an irradiation source. The specific conditions were as follows: laser ND filter 25%, accumulation time of 3 s, 6 accumulations, 600 lines/mm grating (500 nm), a hole of 50 micrometers.

2.4. Topography Analysis of Microspheres before and after miRNA Immobilization. The surface of the microspheres before and after miRNA immobilization was analyzed by atomic force microscopy (AFM, Asylum Research MFP3D SA) in the tapping mode in air. An Asylum Research model AC240TS-R3 rectangular tip was used to analyze the surface. The scans covered areas of 60 × 60 μm and 1 × 1 μm with a speed interval from 0.20 to 0.50 Hz. The frequency of the first nominal resonance was 70 kHz, the nominal spring constant was 2 N/m, and the nominal curvature radius was 9 ± 2 nm. Prior to AFM analysis, samples of microspheres were incubated in hybridization solution (1 μM of capture probe and 1 μM of the Cy3-miRNA analyte) for 2 hours followed by washing two times with SSC and 0.01X SSC, respectively.
2.5. Oligonucleotide Immobilization and Toluidine Blue Titration. The ability of the microspheres in accommodating miRNAs on their surface was assessed via a toluidine blue assay. The assay was calibrated prior to readout using calibration solutions of 2 μM, 4 μM, 6 μM, 8 μM, and 10 μM TB. Microspheres (10 mg) were incubated in a 1 μM solution of the capture probe (200 μL, 37°C, 2 h) followed by thorough washing with sodium saline citrate (SSC). This method was previously reported in the literature and was thoroughly tested [38–40]. Toluidine blue (TB) titration was used to

Figure 1: SEM images of the spheres from different size categories along with their size distributions: (a) MMS-1 (200–400 μm); (b) MMS-2 (400–600 μm); (c) MMS-3 (600–700 μm); (d) MMS-4 (700–900 μm). Specific surface area measurement for 10 mg of each size group is presented in the center.
confirm the presence of the oligonucleotide capture probes bonded to the surface of the microspheres. This technique relies on the pH-dependent electrostatic interaction between TB dyes and nucleotide phosphate groups (Figure 2(a)) [38]. Spheres of different size categories (10 mg) were immersed in 8 mL of 0.5 mM TB and 0.1 mM NaOH solution for 2 hr, followed by washing the spheres in 0.1 mM NaOH for the complete removal of noncomplexed TB dye. The spheres were subsequently incubated in 3 mL of acidic solution (50% AcOH in distilled water) for 45 minutes in order to strip the complexed TB molecules into the acid solution for measurement at 635 nm by using a Jenway (Stone, Staffordshire, UK) spectrophotometer.

2.6. Buffer Preparation for the Analytical Assay. The stock solution of 20 times concentrated SSC (20X, 3 M NaCl, 0.3 M Na₂C₆H₅O₇, pH adjusted to 7 using HCl) and subsequent dilutions of this buffer (5X and 0.01X) were prepared with nuclease-free water (Sigma Aldrich, St. Louis, MO, USA). All dilutions were filtered with sterile 0.2 μm syringe filters (Corning, Corning, NY, USA). The capture probe solution was initially prepared in nuclease-free water and was diluted in SSC buffer to 10 μM. This concentration was utilized as the stock solution for further dilutions of the capture probe. The solutions of SSC and 0.01X SSC have been used as the washing buffers after incubation with the capture probe and hybridization miRNA, respectively. A blocking buffer containing (1%, w/v) BSA, (0.02%, w/v) SDS, and (0.05%, w/v) Tween 20 in 5X SSC was used to reduce the chance of nonspecific binding.

2.7. Indirect and Competitive Hybridization Assays. A predetermined amount of the spheres (10 mg) was loaded into the wells of conventional 96-well plates (Corning, NY, USA). As control, the indirect assay was also conducted in the conventional 96-well plate without spheres. To minimize experimental variability, all the assays were performed under the exact same conditions and by using the same batch of buffers. The miRNA probes were physically immobilized on the surface of the spheres. The spheres were loaded inside a 96-well plate, and each well was charged with 200 μL solution of capture probe (1 μM). The incubation was carried out for 2 hr at 37°C. All the wells were thoroughly washed with SSC buffer (3 times, 200 μL) and were charged with blocking buffer (200 μL) to avoid nonspecific binding. The incubation was carried out for 1 hr at 37°C followed by a complete washing process with SSC buffer. Each well has subsequently received 200 μL of the analyte solution (2 hr at 37°C). Analyte solutions (Cy3-miR159) were prepared by diluting the original concentration (100 μM) with 0.01X SSC buffer in order to achieve a concentration range as follows: 1000 nM, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM, and 1 pM. To avoid fluorescent bleaching, from this step onwards, the assay was performed in a dark room. The assay was finalized by another round of washing (0.01X SSC) before readout. The fluorescence intensity of the Cy3 label was measured with 530/25 nm and 590/35 nm excitation and emission filters, respectively. The readout was performed with an integration time of 0.1 s and 120% sensitivity in a Synergy 2 microplate reader (BioTek Instruments, Inc., Winooskin, VT, USA).

Since the concentration of the target analyte is inversely correlated with breast cancer, a competitive hybridization assay was additionally performed to assess the potentials of the developed assay. Synthetic miR159 was detected in competition with Cy3-labeled miR159. In this procedure, 10 mg of microspheres (MMS-3 as the representative group) was loaded in each well of a 96-well plate and coated with capture probe at 1 μM. The fluorescence-labeled miRNA (Cy3-miR159) was diluted to 100 nM and 1000 nM, while synthetic miR159 was diluted to 0 nM, 10 nM, 100 nM, and 1000 nM. Competitive binding was performed using the same buffers and at the same incubation time and temperatures as reported above. Relative fluorescent intensity was calculated by dividing intensity (for each concentration of the untagged miRNA) by the negative outcomes (calculated in the absence of untagged miRNA).

2.8. Calibration and Evaluation of the Assay. Calibration of the assay was performed with capture probe (1 μM) and different concentrations of the target analyte (1000 nM, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM, and 1 pM). Calibration curves were plotted by conversion of the data to the logarithmic scale. Negative replicates were conducted with the noncomplementary Cy3-labeled miR-lin4 as hybridizing miRNA (n = 10). Cutoff values for each individual size category of the microspheres were calculated as twice that of the mean values of the negative controls [41]. Only readouts with intensity outcomes above cutoff values were interpreted as positives.

A total number of 80 positive and 40 negative replicates were conducted to obtain important parameters such as analytical sensitivity, specificity, and accuracy of the assay. Calculations were performed following the equations below considering the negative/positive readouts in comparison with the total number of the conducted replicates [42]:

\[
\text{sensitivity} = \frac{TP}{TP + FN} \times 100, \\
\text{specificity} = \frac{TN}{TN + FP} \times 100, \\
\text{accuracy} = \frac{TP + TN}{\text{total replicates}} \times 100.
\] (1)

Variables in these equations are as follows:

- True positive (TP)
- True negative (TN)
- False positive (FP)
- False negative (FN)

Limit of detection (LOD) for each size category was determined as 3 times the average standard deviation (s, in the case of lowest miRNA concentration) divided by the slope of the calibration curve (m) following the given equation [34, 43–48]:
3. Results and Discussion

3.1. Morphology, Size Distribution, and Surface Area of the Spheres. Microspheres of different size groups were imaged by SEM, and representative morphologies are presented in Figures 1(a)–1(d). The recorded morphologies were found to be smooth, and the dimensions of the spheres were within the expected range as the sieved groups. Uniform spheres with consistent surface morphologies allow the interpretation to be focused on the effects of specific surface area and chemistry of the spheres for biomolecule immobilization.

Diameter range and size distribution (graphs in Figures 1(a)–1(d)) were calculated for each size group of the spheres by using optical images. Subsequently, the specific surface area for each size category was carefully calculated (for 10 mg of the microsphere) from the respective size distributions. The surface area ranged from $6 \times 10^7$ to $17 \times 10^7 \mu m^2$ (Figure 1, the central pie chart). As expected, the highest surface area per mass was offered by the smallest spheres (MMS-1), and the lowest specific surface area per mass was measured for the largest spheres (MMS-4). In principle, the higher specific surface area enhances the analyte-surface interaction resulting in higher probability of the biomolecular coupling and subsequent biorecognition [33, 49].

3.2. Raman Spectroscopy Analyses of the Spheres. Figure 2 shows the Raman spectrum of the microspheres. It should be noted that there is no signal from the C=O stretching mode from the methacrylic group, normally around 1640 cm$^{-1}$, indicative of successful copolymerization (an evidence for no free monomers) [50, 51]. Several signals were assigned to functional groups of the different polymers involved in synthesis of the spheres. The signal at 597 cm$^{-1}$ may correspond to PMMA polymer (ν(C-COO), ν(C=O)) [52] as well as to p-HEMA (δ(O=C=O)) [53]. The peak at 811.8 cm$^{-1}$ can be assigned to several signals from p-HEMA (ν(C=O), ν(C-CH3)) [4]. Also arising from p-HEMA, the signal at 848 cm$^{-1}$ can be assigned to γ(CH2) or ν(C=C) [53]. The deformation localized on the OCH2, CH2OH part of the p-HEMA molecule can be seen at 955 cm$^{-1}$ [53]. The signal at 1280.5 cm$^{-1}$ can be assigned to ν(C=C) and ν(C=O) from PMMA. At 1448.8 cm$^{-1}$ [52], deformation from (C-CH3) of p-HEMA can be observed [53]. The aromatic ring stretch from p-LEMA is shown at 1586.41 cm$^{-1}$ [54]. Another signal from the carbonyl is observed at 1719.8 cm$^{-1}$ (ν(C=O) H bonded from p-LEMA or p-TEGDMA) [55]. The wide band around 2949 cm$^{-1}$ arises from several signals from PMMA (ν(C=O) of O=CH with ν2(C-H) of α-CH3 and ν(C=O) C-O) [56]. Finally, the shoulder at 3065 cm$^{-1}$ may arise from the iodo benzyl part of p-LEMA or various signals from PMMA and p-TEGDMA [52, 55].

3.3. Toluidine Blue (TB) Titration. The presence of negatively charged nucleotide strands on the surface of the spheres was investigated by a TB assay. As described before, each TB molecule contains an aromatic cation segment and a chloride anion (Figure 3(a)). A pH-sensitive adsorption/desorption mechanism leads to the ionization of the TB dye in the alkaline environment (Figure 3(a), step 1). Positively charged TB then binds to the negative –PO4 groups of the miRNAs (Figure 3(a), step 2) and desorbs upon subsequent lowering of the pH (Figure 3(a), step 3). Concentration of the TB dyes measured by UV-Vis is expected to be proportional to the concentration of the capture probes on the surface of the microspheres.

Figure 3(b) shows a highly linear calibration plot for TB assay with predetermined concentrations of TB in acidic

\[
\text{LoD} = \frac{3 \times s}{m}
\]
Figure 3: TB analysis for indirect confirmation of the presence of capture probe on the surface of the spheres: (a) adsorption and desorption mechanisms of the TB molecules to the −PO₄ groups of the nucleotide available on the surface of the spheres via alteration of the pH from the alkaline to acidic environment; (b) calibration curve plotted by titration of the TB assay using different concentrations of the calibration solutions (2 µM, 4 µM, 6 µM, 8 µM, and 10 µM); (c) TB absorbance per mm² of the surface measured by spectroscopic UV-Vis (635 nm); and (d) breakdown calculations of the TB assay. AFM analysis ((e) 60 µm) and zoomed-in view ((f) 10 µm) of the microspheres prior to immobilization and ((g) 10 µm) after surface attachment of the hybrid strands which shows the clear alteration of the surface roughness.
solution. While the concentration of TB is considered proportional to the concentration of the capture probe on the surface of the spheres, it is important to note that the results of TB assay is a comparative means for such correlation. Figure 3(c) represents the TB absorbance on 10 mg of the spheres from different size groups. As can be observed, the TB absorbance increases as the size of the spheres increases, which demonstrates that a higher particle size encourages a higher number of TB dye molecules to bind to the captured strands on the surface even though a lower total surface area is offered by the larger sizes. The latter is explained by the fact that spatial freedom for an efficient interaction between capture probes and TB molecules increases as the dimension of the spheres increases [49, 56, 57]. The approximate size of the TB molecules (≈0.7 to 1.1 nm) is in the range of the length of a horizontally oriented single miRNA strand (≈1 nm) on the beads so the more the space for maneuvering between the strands, the easier it is for TB to bind [58, 59]. Presumably available surface functionalities reacted with miRNA strands; thus, the inter-miRNA distance on the smaller particle size microspheres would make the TB diffusion rather difficult which is not the case when the larger sphere size is applied. In Figure 3(d), a breakdown calculation of the TB assay is provided. This analysis provides an approximate concentration of TB dyes per mm² of the spheres’ surface. Every strand of the capture probe consists of 25 nucleotides with individual PO₄ groups. It is known that TB is highly interactive towards anionic sulfates, carboxylates, and phosphate groups [60]. If the interaction between positively charged TB and negative PO₄ groups occurs in a 1:1 ratio, TB absorbance would be proportional to the capture probes present on each mm² of the spheres’ surface (Figure 3(d)); thus the number of capture probes that are immobilized on the total surface of the spheres can be indirectly calculated (Figure 3(d)).

3.4. Topography Analysis of the Spheres before and after miRNA Immobilization. Surface topography of the microspheres was analyzed by AFM to study surface changes (roughness and surface area) before and after miRNA immobilization. AFM analysis (presented in Figures 3(e) and 3(f), before miRNA immobilization, and Figures 3(g), after immobilization) shows a clear alteration in the topography of the surface between pristine surfaces (Figures 3(e) and 3(f), zoomed-in view) and the surfaces after miRNA immobilization (Figure 3(g)). An increased surface roughness (from 6.8 nm to 41.2 nm) was recorded for surfaces of the spheres before and after miRNA immobilization, respectively. As a result of coupling in the hybrid strand, a capture probe shares 12 nucleotides with tagged miRNA analyte. Therefore, the coupled strand contains a total of 33 nucleotides. Knowing the approximate size of each nucleotide (~1 nm), the size of the hybrid strand can be roughly calculated (~33 nm). This number closely corresponds to the improved surface roughness analyzed by AFM (34.4 nm). Additionally, the recorded surface area was found to be ~100-fold greater when comparing surface-immobilized spheres with those before immobilization (from 1 μm² to 100.6 μm²). Such enhancement in the surface area is a direct function of the hybrid strands present on the surface of the bioreceptive microspheres.

3.5. Performance Analysis of the Microspheres in Indirect Detection of miR159. Figure 4(a) shows the calibration curves plotted in a logarithm scale for each microsphere size group conducted in varied concentrations of the labeled miRNA. Increasing correlation coefficient values are observed for MMS-1, MMS-3, and MMS-4, respectively. Figures 4(b) and 4(c) depict fluorescence images of the spheres after detection of 1 nM and 100 nM of the labeled analyte, respectively. According to these images, partial clustering of the spheres has seemingly limited complete analyte-surface interaction. Incorporation of gentle mixing/shaking systems could be a suitable method to allow better accessibility of the spheres to the entire sample volume and to enhance the detection signal further.

Figure 4(d) provides a detection performance comparison among different size groups of the spheres in contrast to their cutoff values (twice the mean values of the negative outcomes, presented in red). The detection signals in this chart are plotted with their original values without subtracting the cutoff values for detailed performance comparison among the size groups. As can be seen, the MMS-1, MMS-2, and MMS-3 groups of the microspheres offered higher detection signal in comparison with the largest size category (MMS-4), when subtracting the cutoff values from the actual detection signals. The overall signal intensity obtained from the developed assay in this study falls within the previously reported values for successful detection of miRNAs using a solid-phase hybridization assay [7, 61]. None of the previously reported detection methods, however, rely on conventional ELISA for the detection miRNAs, which is the point of the current study.

3.6. Performance of the Microspheres in a Competitive Assay. The performance of the spheres was further assessed in a competitive assay. In this protocol, concentration of the fluorescently tagged Cy3-miR159 remained constant, while the concentration of the untagged synthetic miR159 (target analyte) was varied, resulting in decreased fluorescence intensity as the concentration of the target analyte increased. Figure 4(e) represents the result of the competitive assay, which was conducted with the MMS-3 category of the spheres as representative. Concentrations of the untagged miRNA (x-axis) are presented in the logarithm scale, while the y-axis depicts the relative fluorescence intensity. The systematic decrease in fluorescence intensity as a result of increased concentration of the untagged miRNAs provides a clear proof-of-concept that the proposed hybridization assay is an easy and reliable method for quantification of miR159 within this conventional platform.

3.7. Possible Analyte-Surface Interactions. The surface of the microspheres promotes a variety of interaction types between the analyte of interest and the surface. Figure 4(f) schematizes
Figure 4: Continued.
the possible physical interactions between the engineered surface of the spheres and the nucleotide strands. Hydrogen bonding (H-bond) between the $-\text{PO}_4^-$ and the $-\text{NH}_2$ groups of the capture probe and $-\text{OH}$ groups of the spheres has the highest likelihood. The H-bond occurs between the H atoms of the spheres’ $-\text{OH}$ groups (O and H are covalently bound) and the N and O atoms of the miRNAs with lone-pair electrons. Furthermore, aromatic rings of the spheres can involve negatively charged $-\text{PO}_4^-$ groups of the biomolecules in ionic attraction (electrostatic interaction) [62]. Moreover, carbonyl groups (\text{C}=\text{O}) of the spheres could promote van der Waals forces in interaction with the biomolecules [63].

**Figure 4:** Performance analysis of the microspheres for detection of miR159: (a) calibration curves plotted for different size categories of the spheres in varied concentrations of the labeled miRNA (1 nM, 10 nM, 100 nM and 1000 nM, 100 pM, 10 pM, and 1 pM); (b, c) fluorescence images of the spheres (MMS-3 as the representative) after detection of 1 nM and 100 nM of the labeled miRNA, respectively; (d) performance analysis of the spheres via indirect detection of miRNA in comparison to the cutoff values, which are twice that of the average negative controls calculated for different size categories (concentration of the labeled miRNA = 1 nM); (e) relative fluorescence signal resulting from competitive assay conducted with a mixture of fluorescent Cy3-miR159 (concentration: 1000 nM or 100 nM) and non-conjugated miR159 at different concentrations (1000, 100, and 10 nM); and (f) schematic representation of the possible physical interactions between the sphere’s surface and a capture probe.

**Figure 5:** Performance evaluation of conventional 96-well plates: (a) performance analysis of the conventional well plate in miRNA detection (concentration of the labeled miRNA = 1 nM); (b) ELISA calibration curve plotted for varied concentrations of the labeled miRNA (1 nM, 10 nM, 100 nM, and 1000 nM); (c) evaluation of the assay and calculated analytical sensitivity, specificity, accuracy, and LOD.
Additionally, hydrophobic interaction can play a major role in attracting miRNAs to the surface [62, 64], since the monomers involved in chemical synthesis of the spheres are mostly hydrophobic in their nature [37]. This multitude of physical attraction can strongly influence the biomolecular immobilization and subsequent detection of the miRNAs. Noteworthy that the inherent presence of the surface functional groups on the spheres also promotes covalent attachment of biomolecules to the surface via application of zero-length cross-linking agents or spacer [49, 56].

3.8. Evaluation of the Assay. The assay conducted in a conventional 96-well plate without microspheres was conducted and evaluated for its performance in miRNA detection. Figure 5 provides a detailed analysis of the conventional assay in detection of miR159: (A) detection performance; (B) calibration analysis; and (C) evaluation of the assay. The conventional assay in a 96-well plate shows a rather low detection ability in comparison with the obtained cutoff values that correspond to the negative controls (Figure 5(a)). While the calibration plot refers to a standard linearity level, the assay has proven to suffer from low analytical sensitivity (50%), low accuracy (40%), and unacceptable LOD (Figure 5(c)). A comparison between performance of the 96-well plate with and without microspheres shows a significant detection enhancement due to the presence of microspheres (Figure 5(c)). In particular, size 3 group of the spheres marks a 10-fold higher fluorescence intensity in comparison with that of conventional assay.

| Table 1: Calculated analytical sensitivity, specificity, accuracy, and LOD of the microspheres in miRNA detection. |
| Detection status | MMS-1 | MMS-2 | MMS-3 | MMS-4 |
|---|---|---|---|---|
| Positive (TP, FP) | + | − | + | − |
| − | + | − | + | − |
| − | + | − | + | − |
| Negative (FN, TN) | 0 | 9 | 0 | 8 |
| 0 | 7 | 0 | 7 |
| Total | 20 | 10 | 20 | 10 |
| Sensitivity (%) | 100 | 100 | 100 | 100 |
| Specificity (%) | 90 | 80 | 70 | 70 |
| Accuracy (%) | 93 | 90 | 86 | 90 |
| LOD (pM) | 90 | 40 | 50 | 2 × 10^6 |

| Table 2: Comparison of the current method with the commercial technologies for miRNA detection. |
| Platform | Time (min) | Complexity | Accessibility | Specificity | Sensitivity | LOD | References |
|---|---|---|---|---|---|---|---|
| Gel electrophoresis assays | ~280 | Moderately complex depending on the assay type | Highly accessible in regular laboratory setups | Standard procedure may induce a chance for nonspecific binding; DNA fragments of interest must be gel-purified and verified to avoid nonspecific bindings | Not suitable for detecting low concentrations | Depends on the assay type (picomolar range) | [80, 81] |
| Polymerase chain reaction (PCR) | ~35 | Complex, expensive, time-consuming, and labor-intensive | Not amenable to many laboratory setups | Highly specific | Accuracy can be compromised by contamination causing amplification of spurious DNA products | Femtomolar range | [65] |
| Real time polymerase chain reaction (RT-PCR/ qRT-PCR) | ~120 | Primer design, normalization, and optimization techniques are complex | Involves challenging and individualized processes | Highly specific | High-throughput quantification of miRNAs is error prone | | [65, 82] |
| Polymethacrylate sphere-based assay | ~240 | Simple and straightforward | Highly accessible in regular laboratory setups | Moderately specific | Highly sensitive | Picomolar range | Current study |
which enables the detection of challenging biomolecules as miRNAs by gold standard technique.

Table 1 summarizes the evaluation parameters for the assay conducted with integrated microspheres. As can be seen, application of the spheres within the conventional assay resulted in 100% analytical sensitivity regardless of the size category. MMS-1, in particular, improved the analytical specificity considerably. Except for MMS-4, all size categories have shown LODs within a picomolar range which is highly desirable for miRNA detection. In the case of MMS-4, recorded standard deviation for lowest concentration of the analyte contributed to the LOD outcome which is not as favorable as those of other size groups. It is noteworthy that the high TB absorption by this size category (MMS-4) does not guarantee its better performance in the analytical assay since the evaluation parameters are also the function of key elements such as the negative control, the calibration curve’s slope, and the standard deviation.

While a vast number of reports in the literature provides insight into biodiagnosis strategies, sensitive, selective, accessible, and cost-effective miRNA detection remains a challenge [65]. Circulating miRNAs are present in blood at ng/mL levels. This, according to the length of a fragment, corresponds to a molecular concentration within a picomolar range [66]. Taking the abundance of miRNAs into account, a high level of sensitivity and selectivity is required to detect these challenging bioentities in an effective manner. While several efforts have introduced new methodologies or modified strategies for ultrasensitive targeting miRNAs [67–79], the conventional detection is currently performed by gel electrophoresis assays, polymerase chain reaction (PCR), and real-time polymerase chain reaction (RT-PCR/ qRT-PCR). Table 2 provides a comparison between different aspects of commercially applied techniques in comparison with the proposed method here. This table summarizes time, complexity, accessibility, specificity, sensitivity, and LOD for these techniques. While the presented strategy in this study is not as powerful as PCR or qRT-PCR (comparison of the LOD in Table 2), it can be widely strategy in any laboratory setup. This, however, is not the case for PCR/RT-PCR, which is typically operated with highly sophisticated machinery. Further LOD enhancement of the presented strategy can be achieved by incorporating shaking/mixing techniques that would allow spheres to have higher chance of interaction with biomolecules. Comparatively, the proposed strategy offers the least complexity of operation. 96-well plates are available almost in any laboratory setup, and lab technicians familiar with gold standard detection methods could carry out the assay protocols without further training. Most importantly, PCR/RT-PCR is far more inexpensive when compared to the proposed assay while its accuracy might be compromised by contamination.

4. Conclusion

In this work, we have demonstrated a proof-of concept methodology for miRNA detection. Methacrylate microspheres were integrated into a 96-well plate, and immobilized DNA probes were used to capture and detect miR159 within a picomolar range. All important parameters of the assay including analytical sensitivity, specificity, accuracy, and the limit of detection were improved due to the presence of the spheres. This is particularly promising as this simple integration offers the chance of biorecognition for challenging biomolecules including miRNAs within a conventional platform that is typically available in any laboratory setup. Application of the polymer microspheres hold a great potential as they are cost-effective bioreceptive platforms that can be mass-produced in desirable size ranges and with controlled properties depending on the type of desired biorecognition.

Data Availability

All data are presented within the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors’ Contributions

Samira Hosseini and Patricia Vázquez-Villegas contributed equally to the work. Marc Madou was responsible for conceptualization. Samira Hosseini, Patricia Vázquez-Villegas, and Margarita Sanchez-Dominguez were involved in formal analysis. Sergio Martínez-Chapa carried out funding acquisition. Samira Hosseini, Patricia Vázquez-Villegas, and Leo Koole performed methodology. Marco Rito-Palomares was responsible for project administration. Marco Rito-Palomares and Leo Koole provided resources. Samira Hosseini, Richard Willson, Leo Koole, Marc Madou, and Sergio Martínez-Chapa supervised the study. Samira Hosseini and Marc Madou wrote the original draft. Margarita Sanchez-Dominguez wrote and edited the manuscript.

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