Poly(m-Phenylenediamine) Nanospheres and Nanorods: Selective Synthesis and Their Application for Multiplex Nucleic Acid Detection

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Abstract

In this paper, we demonstrate for the first time that poly(m-phenylenediamine) (PMPD) nanospheres and nanorods can be selectively synthesized via chemical oxidation polymerization of m-phenylenediamine (MPD) monomers using ammonium persulfate (APS) as an oxidant at room temperature. It suggests that the pH value plays a critical role in controlling the the morphology of the nanostructures and fast polymerization rate favors the anisotropic growth of PMPD under homogeneous nucleation condition. We further demonstrate that such PMPD nanostructures can be used as an effective fluorescent sensing platform for multiplex nucleic acid detection. A detection limit as low as 50 pM and a high selectivity down to single-base mismatch could be achieved. The fluorescence quenching is attributed to photoinduced electron transfer from nitrogen atom in PMPD to excited fluorophore. Most importantly, the successful use of this sensing platform in human blood serum system is also demonstrated.

Introduction

During the past decades, conducting polymers (CPs) have constituted a subject of research for their unique properties and important application potential [1]. Polyaniline is one of the most studied CPs due to its chemical stability and relative high conductivity [2] and, at the same time, polymers based on aniline derivatives have also been widely investigated [3]. Among them, poly(phenylenediamine) (PPD) homopolymer is a highly aromatic polymer containing 2,3-diaminophenazine or quinoraline repeating unit and exhibiting high thermostability and has found important applications in sensor designing, immunospecies detection, and as component of rechargeable cells etc [4–13]. PPD is usually prepared by electrochemical [14] and chemical oxidation polymerization [15]. Although we [16] and other researchers [17–19] have successfully prepared poly(o-phenylenediamine) nanobelts and microparticles by chemical oxidation polymerization method, respectively, the selective synthesis of PPD with different morphologies has not been addressed so far.

On the other hand, it is vitally important to develop rapid, cost-effective, sensitive and specific methods for the detection of nucleic acid due to their various applications in gene expression profiling, clinical disease diagnostics and treatment [20]. The increasing availability of nanostructures has created widespread interest in their use in biotechnological system for diagnostic application [21]. Indeed, the use of a variety of nanostructures for this purpose has been well-demonstrated [22]. Recently, there have been many efforts toward developing homogeneous fluorescence assays based on fluorescence resonance energy transfer (FRET) or quenching mechanism for nucleic acid detection [23]. The use of nanostructures as a “nanoquencher” has a remarkable advantage in that the same nanostructure has the ability to quench dyes of different emission frequencies and thus the selection issue of a fluorophore-quencher pair is eliminated from the nanostructure-involved system [23,24]. Up to now, a number of structures have been successfully used by us and other researchers in this assay, including gold nanoparticles, single-walled carbon nanotubes (SWCNTs), multi-walled carbon nanotubes, carbon nanoparticles, carbon nanospheres, nano-C60, mesoporous carbon microparticles, graphene oxide (GO), polyaniline nanofibres, poly(o-phenylenediamine) colloids, poly(2,3-diaminonaphthalene) microspheres, coordination polymer colloids and nanobelts, Ag@poly(o-phenylenediamine) core-shell nanoparticles, tetracyanoquinodimethane nanoparticles, and supramolecular microparticles [23–46]. For the SWCNT or GO system, it has drawbacks: (1) several hours’ sonication is required to disperse SWCNT in an organic solvent like N,N-dimethylformamide (DMF) [30]; (2) the GO preparation by the Hummer’s method is time-consuming and labor-intensive [47]. We have also found that conjugation polymer poly(o-phenylenediamine) nanobelts (PNs) can serve as an effective fluorescent sensing platform for multiplex nucleic acid detection [48]; however, this system still has two serious drawbacks which limit its practical use: (1) the nanobelts are tens of micrometers in length and thus tend to sink in aqueous solution due to the gravity,
causing the problem of stability in detection; (2) it has poor
discrimination ability in that it gives only 8.8% difference of
detection signal between single-base mismatched and complemen-
tary sequences [48]. Accordingly, developing new nanostructure-
based fluorescent sensing platform to overcome all these
drawbacks is highly desired.

In this paper, we report on the selective synthesis of poly(m-
phenylenediamine) (PMPD) nanospheres and nanorods by chem-
ical oxidation polymerization of MPD monomers using ammoni-
um persulfate (APS) as an oxidant at room temperature for the first
time. It is found that the pH value is key to controlling the
morphology of the nanostructures and fast polymerization rate
favors the anisotropic growth of PMPD under homogeneous
nucleation condition. We further demonstrate that such PMPD
nanostructures can serve as an effective fluorescent sensing
platform for multiplex nucleic acid detection. A detection limit
as low as 50 pM and a high selectivity down to single-base
mismatch could be achieved. The fluorescence quenching is
attributed to photoinduced electron transfer from nitrogen atom in
PMPD to excited fluorophore. Most importantly, the successful
use of this sensing platform in human blood serum system is also
demonstrated.

Results and Discussion

Figure 1A shows low magnification SEM image of the products
thus formed in water (sample 1, see Materials and Methods for
preparation details), indicating that they consist exclusively of a
large amount of nanoparticles. A close view of such nanoparticles
further reveals that they are nearly spherical in shape and have size
ranging from 300 to 600 nm, as shown in Figure 1B. The chemical
composition of the nanospheres was determined by the energy-
dispersed spectrum (EDS), as shown in Figure S1. The peaks of C
and N elements are observed, indicating the nanospheres are
formed from MPD. The presence of the peaks of S and O
elements can be attributed to the fact that the polymerization of
MPD by APS yields cationic polymer PMPD due to the proton
doping effect, the SO_4^{2-} (the reduced product of APS) and
excessive S_2O_8^{2-} as counter-ions, however, will diffuse into the
PMPD nanostructures for charge compensation [49,50]. Very
interestingly, it is found that the PMPD morphology can be
changed by simply varying the reaction solvent used. Figure 1C
and 1D shows typical SEM images of the products obtained with
the use of N-methylpyrrolidone (NMPD) as reaction solvent, under
otherwise identical conditions used for preparing sample 1. It is
clearly seen that a large quantity of nanorods are produced as the
main products. It was found that the use of N,N-dimethylforma-
mide (DMF) and ethanol as the reaction solvent lead to nanorods
(Figure S2A) and nanospheres (Figure S2B), respectively. The
possible mechanism of the effect of solvent in controlling the
PMPD morphology is proposed as follows: The polymerization of
MPD monomers by APS leads to a decrease of pH value of the
system. Given the weak basic nature of NMPD and DMF, they are
expected to neutralize the protons generated and thus the rate of
polymerization of MPD is accelerated, which may favor the
anisotropic growth of PMPD under homogeneous nucleation
condition [51,52]. It was found that polymerization of MPD
monomers using water as reaction solvent but at basic condition
also produced rod-like products. (Figure S2C). All these observa-
tions indicate that the pH value has played a critical role in
controlling the morphology of the nanostructures. It is important
to mention that these PMPD nanospheres and nanorods have

Figure 1. Instrumental analysis of the precipitate thus formed. Low magnification SEM images of the PMPD nanostructures formed using (A)
water and (C) NMPD as reaction solvent, (B) and (D) corresponding to the high magnification SEM images.
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smaller sizes and higher zeta potential (5 mV) and thus their dispersion exhibits better stability than PNs [40]. Indeed, we have found that such PMPD nanostructures are well-dispersed in water or buffer solutions. The resultant dispersions are very stable and no precipitation is observed within a couple of days.

To test the feasibility of using PMPD nanostructures as an effective fluorescent sensing platform for nucleic acid detection, we chose PMPD nanorods and an oligonucleotide sequence associated with human immunodeficiency virus (HIV) as a model system. Figure 2A shows the fluorescence emission spectra of PHIV, the FAM-labeled probe, at different conditions. In the absence of PMPD, the fluorescence spectrum of PHIV exhibits strong fluorescence emission due to the presence of the fluorescein-based dye (curve a). However, the presence of PMPD results in about 96% quenching of the fluorescence emission (curve c), revealing that PMPD can strongly adsorb ssDNA and quench the fluorescent dye very effectively. However, the PHIV–PMPD complex exhibits significant fluorescence enhancement upon its incubation with complementary target T1 over a 1-h period, leading to a 77% fluorescence recovery (curve d). Note that the fluorescence of the free PHIV was, however, scarcely influenced by the addition of T1 in the absence of PMPD (curve b). It should be mentioned that the PMPD sample exhibits weak fluorescence emission (curve c) which contributes a little to the whole fluorescence intensity of each sample measured. Hence, a background fluorescence subtraction is performed for all PMPD-involved measurements. Figure 2A inset illustrates the fluorescence intensity changes (F/F0−1) of PHIV–PMPD complex upon addition of different concentrations of T1, where F0 and F are FAM fluorescence intensities at 522 nm in the absence and presence of T1, respectively. In the DNA concentration range of 5–300 nM, a dramatic increase of FAM fluorescence intensity was observed, which suggests that the nanorod/DNA assembly approach is effective in probing biomolecular interactions due to the excellent signalling process. It is worthwhile mentioning that optimal signal-to-noise ratio of 3.8:1 and thus low detection limit can be achieved by decreasing the amount of PMPD and PHIV used. A detection limit as low as 50 pM can be achieved when 1-μL PMPD sample and 500 pM PHIV are used in this system (Figure 2B).

Because PMPD is a π-rich polymer, it can strongly and effectively adsorb single-stranded DNA (ssDNA) on its surface via π–π stacking between unpaired DNA bases and PMPD [53]. The zeta potential of the nanorods was measured to be about 5 mV in water, indicating that the nanorod has a low positively charged surface. However, the electrostatic attractive interactions between nanorod and negatively charged backbone of ssDNA contribute little to the adsorption of ssDNA on nanorod in the presence of a large amount of salt in buffer [44]. In contrast, the PMPD nanorod should have weak or even no binding with double-stranded DNA (dsDNA) due to the absence of unpaired DNA bases and the rigid conformation of dsDNA. Figure 3A presents a schematic to illustrate the fluorescence-enhanced nucleic acid detection using PMPD nanorod as a sensing platform. The DNA detection is accomplished by the following two steps: (1) PMPD binds dye-labeled ssDNA and quenches the fluorescence of the dye when they are brought into close proximity. (2) The subsequent hybridization of the probe with its target produces dsDNA which detaches from PMPD, leading to fluorescence recovery. The release of the resultant dsDNA from PMPD can be supported by the following experimental observation that there is no obvious fluorescence change observed after the removal of the PMPD nanorods from the hybridized solution by centrifugation, as shown in Figure S3. The observed fluorescence quenching in our present study could be attributed to photoinduced electron transfer (PET) from nitrogen atom in PMPD to excited fluorophore FAM when they are brought into close proximity [54]. Figure 3B illustrates the quenching mechanism involved. When the fluorophore FAM is excited, an electron from the highest occupied molecular orbital (HOMO) is promoted to the lowest unoccupied molecular orbital (LUMO), leaving an electronic vacancy in the fluorophore HOMO, which is filled by transfer of an electron from the higher energy HOMO of the nitrogen atom in PMPD serving as a donor. The overall effect of PET process is that the excited state life time is shortened and little fluorescence occurs, leading to fluorescence quenching. Upon protonation of the donor, however, the redox potential of the protonated donor is raised and its HOMO becomes lower in energy than that of the fluorophore. Consequently, electron transfer is hindered and fluorescence quenching is suppressed. This PET-based fluorescence quenching is confirmed by the experimental observation that the quenching is suppressed with the decrease of pH value and thus the increase of protonation degree of donor [18], as shown in Figure 4.

It should be noted that the amount of PMPD nanorods used in this system has profound effect on the efficiency of the fluorescence quenching and the subsequent recovery. Figure S4 shows the fluorescence intensity histograms of five samples with the use of 0, 5, 10, 15, and 20-μL PMPD nanorods sample, respectively. It suggested that the use of increased amount of nanorods leads to an increase in quenching efficiency but a decrease in recovery efficiency. The above observations can be reasoned as follows: When the ssDNA probe molecules are mixed with nanorods, they will adsorb on the nanorod surface. Obviously, the use of more nanorods leads to more efficient adsorption of ssDNA and thus higher quenching efficiency. But at the same time, the possibility of direct surface adsorption of target molecules on those excess nanorods increases during the following hybridization process. As a result, decreased hybridization efficiency and thus lower recovery efficiency is observed. Based on these observations, an optimal volume of 10 μL was chosen in our present study if not specified. Figure S5 shows a Stern–Volmer quenching curve describing F0/F as a function of MPD concentration, where F0 and F are FAM fluorescence intensities at 522 nm in the absence and the presence of PMPD nanorods, respectively. The plot is linear in the concentration range of 0 to 5 μM and the Stern–Volmer quenching constant (KSV) is calculated to be 3.768×10^4 M^{-1} [55].

The kinetic behaviors of PHIV and PMPD, as well as of the PHIV–PMPD complex incubated with T1, were also studied by collecting the time-dependent fluorescence emission spectra. Curve a in Figure 5A shows the fluorescence quenching of PHIV in the presence of PMPD as a function of incubation time at room temperature of 25°C. In the absence of the target, the curve exhibits a rapid reduction in the first 20 min and a slow decrease over a period of 40 min. Curve b in Figure 5A shows the subsequent fluorescence recovery of PHIV–PMPD by T1 in Tris-HCl buffer as a function of incubation time. In the presence of the target T1, the curve shows a fast increase in the first 10 min, followed by a slow process over a period of 50 min. The best fluorescence response was obtained after 1 h of incubation time. All above observations indicate that both ssDNA–PMPD association and dsDNA–PMPD dissociation occur faster than SWCNT but slower than GO system [24,30,36,37]. These results are quite similar to those obtained from PN system [48]. We also investigated the influence of temperature on the kinetic behaviors of these two processes. Figure 3B shows the corresponding results obtained at 50°C, indicating that the time required to reach equilibrium is greatly shortened for both the quenching and the subsequent recovery process. It should be noted the decrease of
fluorescence recovery in intensity at elevated temperature is observed in our present study, which can be attributed to that hybridization temperature close to the melting temperature does not favor duplex formation [56], leading to decreased hybridization and thus decreased fluorescence recovery efficiency.

It is worthwhile mentioning that this sensing platform can well differentiate complementary and mismatched sequences. Figure 6A shows the fluorescence responses of PHIV–PMPD complex toward complementary target T1, single, two, and three-base mismatched target (T2, T3, and T4, respectively). The fluorescence intensity change \( (F/F_0-1) \) value obtained upon addition of 300 nM of T2, T3, and T4 is about 46%, 33%, and 14% of the value obtained upon addition of 300 nM of T1 into PHIV–PMPD complex at room temperature of 25°C, respectively (where \( F_0 \) and \( F \) are the fluorescence intensity without and with the presence of T1, respectively) plotted against the logarithm of the concentration of T1. (B) (a) Fluorescence emission spectra of PHIV (500 pM), (b) fluorescence quenching of PHIV (500 pM) by 1-μL PMPD nanorods, and (c) fluorescence recovery of (b) by T1 (50 pM). Inset in Figure 2B: the corresponding fluorescence intensity histograms with error bar. Excitation was at 480 nm, and the emission was monitored at 522 nm. All measurements were done in Tris-HCl buffer in the presence of 5 mM Mg2+ (pH: 7.4). 10-μL PMPD nanorods were used in each measurement. doi:10.1371/journal.pone.0020569.g002

**Figure 2. Performance of target DNA detection and determination of detection limit.** (A) Fluorescence emission spectra of PHIV (50 nM) at different conditions: (a) PHIV; (b) PHIV+300 nM T1; (c) PHIV+PMPD nanorods; (d) PHIV+PMPD nanorods+300 nM T1. Curve e is the emission spectra of PMPD nanorods. Inset: fluorescence intensity change \( (F/F_0-1) \) of PHIV–PMPD nanorods complex (where \( F_0 \) and \( F \) are the fluorescence intensity without and with the presence of T1, respectively) plotted against the logarithm of the concentration of T1. (B) (a) Fluorescence emission spectra of PHIV (500 pM), (b) fluorescence quenching of PHIV (500 pM) by 1-μL PMPD nanorods, and (c) fluorescence recovery of (b) by T1 (50 pM). Inset in Figure 2B: the corresponding fluorescence intensity histograms with error bar. Excitation was at 480 nm, and the emission was monitored at 522 nm. All measurements were done in Tris-HCl buffer in the presence of 5 mM Mg2+ (pH: 7.4). 10-μL PMPD nanorods were used in each measurement. doi:10.1371/journal.pone.0020569.g002
Figure 3. Illustration of the sensing process and fluorescence quenching mechanism. (A) A schematic (not to scale) illustrating the fluorescence-enhanced nucleic acid detection using PMPD nanorod as a sensing platform and (B) a schematic illustrating the photo-induced electron transfer (PET) process to explain the mechanism of fluorescence quenching.

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decreased fluorescence recovery efficiency. The inset is the corresponding fluorescence intensity histograms with error bar. It is worthwhile mentioning that, for the PN system, the addition of 300 nM of T₂ only leads to a 8.8% difference between complementary and single-base mismatched target under otherwise identical conditions [48], but our new platform described herein gives a 49% difference which is about 5.6 times that of PN system, suggesting this sensing platform can greatly improve discrimination ability. We also performed hybridization experiments at an elevated temperature of 50°C and compared the fluorescence signal enhancement of PHIV–PMPD complex upon incubation with T₁ and T₂ at 25 and 50°C, respectively, as shown in Figure 6B. We found that the fluorescence intensity change (F/F₀–1) value obtained upon addition of T₂ is about 33% of the value obtained upon addition of T₁ into PHIV–PMPD complex at 50°C. All the above observations indicate that the present nucleic acid detection system can distinguish complementary and mismatched nucleic acid sequences and its discriminating ability increases with increased temperature which makes the hybridization harder for probe and mismatched target due to that hybridization temperature close to the melting temperature does not favour duplex formation.

It is important to note that the use of shorter oligonucleotide can improve the mismatch discrimination ability of our present sensing system. Figure 7A shows the fluorescence responses of FAM-labeled, 9-nucleotide ssDNA probe Pₛ (50 nM) toward complementary target Tₛ₁, single-base mismatched target Tₛ₂, and non-complementary target Tₛ₃ at room temperature, in the presence of PMPD. The (F/F₀–1) value obtained upon addition of 300 nM of Tₛ₂ is about 38% of the value obtained upon addition of 300 nM of Tₛ₁ into Pₛ–PMPD complex. We further evaluated its ability of this sensing platform to distinguish single-base mismatch by mimicking the realistic situations, where a short oligonucleotide probe binds a small loci of a large DNA strand. Two long DNA strands were chosen as model systems: the middle part of Tₐ₁ is complementary target sequence to PHIV and the middle part of Tₐ₂ is single-base-mismatched target sequence to PHIV. Figure 7B shows the fluorescence responses of PHIV toward Tₐ₁ and Tₐ₂ in the presence of PMPD at room temperature. The addition of 300 nM of Tₐ₁ to PHIV–PMPD complex leads to about 41% fluorescence recovery which is much lower than 77% observed when 300 nM of Tₐ₁ was used as the target. Such observation is not surprising given that PHIV–Tₐ₁ is a complex with a duplex DNA in the middle and two single strands on both ends and thus there are unpaired DNA bases for binding to PMPD. The (F/F₀–1) value obtained upon addition of 300 nM of Tₐ₂ is about 58% of the value obtained upon addition of 300 nM of Tₐ₁ into PHIV–PMPD complex, indicating that this sensing platform is still able to discriminate complementary and mismatched target sequences embedded in a large DNA strand with a short oligonucleotide probe.

We also performed DNA detection in human blood serum. Figure S6 shows the fluorescence emission spectra of PHIV in the presence of 5% blood serum (volume ratio) in Tris-HCl buffer at different conditions. This system exhibits 86% fluorescence quenching and 50% fluorescence recovery and the difference of detection signal between single-base mismatched and complemen-
tary sequences is 40%. We further examined the influence of the amount of blood serum on the discrimination ability of this sensing system. Figure 8 shows the corresponding histograms of fluorescence intensity ratio of $F(T_2)/F(T_1)$, where $F(T_1)$ and $F(T_2)$ are the fluorescence intensities in the presence of human blood serum spiked with $T_1$ and $T_2$, respectively. It suggests that the increase of blood serum in amount leads to decreased discrimination ability. Note that, in the presence of different amount of blood serum, this system is still able to discriminate complementary and single-base mismatched sequences with good reproducibility. All the above observations indicate that there is no heavy interference from blood serum components on our measurements and thus this sensing system is promising for practically useful mismatch detection upon further development.

Multiplex detection of nucleic acid sequences is a challenge for many assays because of the need of eliminating probe set/target set cross-reactivity, minimizing nonspecific binding, and designing spectroscopically and chemically unique probes [57], which motivated us to explore the feasibility of using the platform described herein to detect multiple DNA targets simultaneously.

Figure 6. Evaluation of discrimination ability at different temperatures. (A) Fluorescence emission spectra of $P_{400}$ (50 nM) at different conditions: (a) $P_{400}$–PMPD complex; (b) $P_{400}$–PMPD complex+300 nM $T_1$; (c) $P_{400}$–PMPD complex+300 nM $T_2$; (d) $P_{400}$–PMPD complex+300 nM $T_3$; (e) $P_{400}$–PMPD complex+300 nM $T_4$; (f) $P_{400}$–PMPD complex+300 nM $T_5$. Inset: fluorescence intensity histogram with error bar. (B) Fluorescence signal enhancement of $P_{400}$–PMPD complex upon incubation with 300 nM $T_1$ and 300 nM $T_2$ at 25 and 50°C, respectively. 10-μL PMPD nanorods were used in each measurement.

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To do this, we chose three probes (PHIV, HBV, and PK167) labeled with FAM, ROX, and Cy5 (cyanine 5), respectively, as model systems. Because these three dyes are individually excited at 480, 587, and 643 nm to emit at 522, 601, and 660 nm, respectively, significant dye-to-dye energy transfer is avoided. It is found that the presence of PMPD leads to dramatic quenching of all dyes in the probe mixture, indicating that PMPD can effectively quench dyes of different emission frequencies. The absorption spectrum of the aqueous dispersion of PMPD nanorods shown in Figure S7 exhibits one strong peak at 206 nm and another pretty weak peak at 307 nm, suggesting there is no spectra overlap and thus no FRET occurs between PMPD and all the fluorescent dyes used. Figure 9 shows the fluorescence intensity histograms of the probe mixture toward different target combinations in the presence of PMPD under excitation/emission wavelengths of 480/522, 587/601, and 643/660 nm, respectively. It is clearly seen that

Figure 7. Evaluation of discrimination ability using (A) shorter probe or (B) longer target. (A) Fluorescence emission spectra of \( P_s \) (50 nM) at different conditions: (a) \( P_s \)–PMPD complex; (b) \( P_s \)–PMPD complex+300 nM \( T_{S3} \); (c) \( P_s \)–PMPD complex+300 nM \( T_{S2} \); (d) \( P_s \)–PMPD complex+300 nM \( T_{S1} \). (B) Fluorescence emission spectra of PHIV (50 nM) in the presence of PMPD nanorods at different conditions: (a) PHIV–PMPD complex; (b) PHIV–PMPD complex+300 nM TL1; (c) PHIV–PMPD complex+300 nM TL2. Inset: fluorescence intensity histograms with error bar. Excitation was at 480 nm, and the emission was monitored at 522 nm. All measurements were done in Tris-HCl buffer in the presence of 5 mM Mg\(^{2+}\) (pH: 7.4). 10-μL PMPD nanorods were used in each measurement.

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there is only one strong emission peak at 522 nm when excited at 480 nm in the presence of T₁ only. However, the target combination of T₁+T₆ gives two strong emission peaks at 522 and 601 nm when excited at 480 and 587 nm, respectively. As expected, three strong emission peaks are observed for the T₁+T₆+T₇ target combination at 522, 601, and 660 nm when excited at 480, 587, and 643 nm, respectively. Other target combinations also give similar results. All these observations indicate that this sensing platform can be used to detect multiple DNA targets with high selectivity.

In summary, PMPD nanospheres and nanorods can be selectively synthesized via the chemical oxidation polymerization of MPD monomers by APS with the use of different reaction solvent at room temperature. We demonstrate that such PMPD nanostructures can be used as an effective fluorescent sensing platform for multiplex nucleic acid detection with high sensitivity and selectivity. The fluorescence quenching mechanism involved is also studied and the application of this sensing platform in human blood serum system is also demonstrated. Our present observations are significant for the following three reasons: (1) It provides us a facile method for the selective synthesis of PMPD nanostructures for multiplex and single-base mismatch detection of nucleic acid; (2) This PMPD-based assay holds great promise for practical application in clinical sample analysis; (3) It provides us a promising, universal and effective sensing platform for a fluorescence-enhanced detection sensitive and selective to the target molecule studied.

Materials and Methods

All chemically synthesized oligonucleotides were purchased from Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). DNA concentration was estimated by measuring the absorbance at 260 nm. All the other chemicals were purchased from Aladin Ltd. (Shanghai, China) and used as received without further purification. The water used throughout all experiments was purified through a Millipore system. The PMPD nanospheres (sample 1) were prepared as follows: In a typical experiment, 0.06 mL of 0.5 M APS aqueous solution was diluted with 0.84 mL water at room temperature, followed by the addition of 0.1 mL of 0.1 M MPD aqueous solution under shaking. After that, a large amount of precipitates were observed. The resulting precipitates were washed with water by centrifugation twice first, and then redispersed in water to give a 8.6-μg/mL suspension and stored at 4°C for characterization and further use. The PMPD nanorods were similarly prepared except that an equivalent aliquot of N-
methylpyrrolidone (NMPD) was substituted for the 0.84 mL water. The volume of each sample for fluorescence measurement is 300 μL in 20 mM Tris-HCl buffer containing 100 mM NaCl, 5 mM KCl, and 5 mM MgCl₂ (pH 7.4).

The experiments of multiplex detection were performed as follows: In a typical multiplex assay, P_HIV, P_HBV, and P_K167 were added into Tris-HCl buffer (containing 20 mM Tris-HCl, 100 mM NaCl, 5 mM KCl and 5 mM MgCl₂, pH 7.4) to give a mixture of probes ([P_HIV] = [P_K167] = 50 nM). After that, PMPD nanorods were added and the resultant mixture was incubated over a 1-h period, and then the fluorescence emission spectra were collected. Different target combinations were then added and the fluorescence emission spectra were collected after incubation over a 1-h period.

Scanning electron microscopy (SEM) measurements were made on a XL30 ESEM FEG scanning electron microscope at an accelerating voltage of 20 kV. Fluorescent emission spectra were recorded on a PerkinElmer LS55 Luminescence Spectrometer (PerkinElmer Instruments, U.K.). Zeta potential measurement was performed on a Nano-ZS Zetasizer ZEN3600 (Malvern Instruments Ltd., U.K.). An energy-dispersive X-ray spectroscopic detecting unit was used to collect the energy-dispersed spectrum (EDS) for elemental analysis.

Oligonucleotide sequences are listed as follows (mismatch underlined):

1. [PHIV] (FAM dye-labeled ssDNA):
2. 5'-FAM-AGT CAG TGT GGA AAA TCT CTA GC-3'
3. T1 (complementary target to PHIV):
4. 5'-GCT AGA GAT TTT CCA CAC TGA CT-3'
5. T2 (single-base mismatched target to PHIV):
6. 5'-GCT AGA GAT TTT CCA CAC TGA CT-3'
7. T3 (two-base mismatched target to PHIV):
8. 5'-GCT AGA GAT TTT ACA CAC TGA CT-3'
9. T4 (three-base mismatched target to PHIV):
10. 5'-GCT AGA GAT TTT ACA CAC TGA CT-3'
11. P_HBV (ROX dye-labeled ssDNA):
12. 5'-ROX-TTT TTT TTT TTT TTT TTT TTT TT-3'
13. T5 (non-complementary target to PHIV):
14. 5'-TTT TTT TTT TTT TTT TTT TTT TT-3'
15. T6 (non-complementary target to PHIV):
16. 5'-TTT TTT TTT TTT TTT TTT TTT TT-3'

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