A Gold@Polydopamine Core–Shell Nanoprobe for Long-Term Intracellular Detection of MicroRNAs in Differentiating Stem Cells

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EXPERIMENTAL SECTION

1. Analysis of Osteogenic Marker Gene Expression

For gene expression analysis, hMSCs were grown in a 6-well plate (SPL Lifescience). Upon reaching confluence, cells were harvested and homogenized in Trizol Reagent (Invitrogen) with a tissue grinder. Total RNA in cells was extracted according to the manufacturer's instructions, and the RNA concentration was determined by a UV spectrometer (Nanodrop Technologies). 500 ng of the extracted RNA was reverse-transcribed into cDNA with the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) for each sample. Real-time polymerase chain reaction (RT-PCR) was performed using Taqman primers and probes specific for *RUNX2* and *ALP* (early marker genes for osteogenesis) and *GAPDH* (a housekeeping gene) with an Applied Biosystems Step One Plus real-time PCR system. The relative gene expression of each marker gene was calculated by the quantitation-comparative CT ($\Delta\Delta_{CT}$) method. Fold difference between different groups was calculated using the expression $2^{-\Delta\Delta_{CT}}$. Each sample expression was internally normalized to the *GAPDH* data and each group expression was normalized to the expression level of control group of the same culture time. Sequence information of the primer and probe of the marker *ALP* used in the analysis are listed in Table S4. Note that the respective sequences for the *RUNX2* marker are proprietary and not disclosed by the company (Applied Biosystem).

2. Immunofluorescence Staining of RUNX2 and ALP Staining

hMSCs were grown in a coverglass bottom dish and allowed to differentiate in osteogenic induction medium. At each selected time point (i.e., 1 d, 3 d, 5 d, and 7 d), cells were fixed with 4% formalin for 15 min at room temperature, rinsed with DPBS (Invitrogen) for three times, permeabilized with 2% triton-X 100 (Sigma Aldrich) in DPBS for 10 min at room temperature, and then blocked with 1% bovine serum albumin (BSA) (Sigma Aldrich) at 37 °C for 1 h. To visualize the change in the nuclear RUNX2 level, cells were immuno-stained with 1:100 primary antibodies (Santa Cruz Biotechnology, sc-101145) against RUNX2 in 0.5% BSA in PBS for overnight at 4 °C. Following that, secondary antibody labeling was performed by incubation in 1:200 labeled goat anti-mouse or anti-rabbit IgG (Life Technologies, A-11001) containing Alexa488-phalloidin in 0.5% BSA in PBS for 2 h at room temperature. The stained cells were thoroughly rinsed with DPBS and imaged under a Nikon Eclipse TI microscope (Nikon).

To stain ALP, cells were incubated in the staining solution containing 4% of naphthol AS-MX phosphate (Sigma Aldrich) in Fast Blue RR Salt solution (Sigma Aldrich) in deionized water for 20 min at room temperature in dark. Next, hMSCs were rinsed in PBS and imaged under microscopy with constant illumination.

3. Quantification of Fluorescently-labeled Oligonucleotides Loading

The concentration of the AuNP stock solution was first measured by using a Cary 5000 UV-vis spectrophotometer (Agilent). Triplicate measurements were performed to obtain the data. These absorbance values were then related to the nanoparticle concentration via Beer's Law ($A = \epsilon bc$). The wavelength of the absorbance maximum (λ_{max}) was determined as 530 nm and the respective extinction coefficient (ϵ) used for the calculation was extracted from previous report.¹ To determine the concentration of immobilized fluorescently-labeled oligonucleotides, fluorescence measurement was performed as depicted in Scheme S1. Triplicate measurements were performed to obtain the data. The number of the oligonucleotide strands per particle is calculated by dividing the concentration of loaded fluorescently-labeled hpDNAs by the concentration of nanoparticles.

4. Inductively Coupled Plasma Optical Emission Spectrometry

To quantify the amount of nanoparticles (based on the Au content) inside the treated hMSCs after initial cellular entry, cells were grown in a 24-well plate (SPL Lifescience) to 80–90% confluence and incubated with ~0.025 nM of either Au@PDA NPs or Au@PDA–hpDNA NPs for 24 h. At each selected time point (i.e., 1 d, 3 d, and 5 d), cells were washed thoroughly with PBS. Following that, cell pellets were obtained and then digested in 0.25 mL of freshly prepared aqua regia ($\text{HCl}:\text{HNO}_3 = 1:3$, v/v) at 55 °C for 30 min. After adding 0.08 mL of 1000 ppm indium (internal standard; AccuStandard) and 3.52 mL of matrix solution (2% HCl and 2% HNO_3), the atomic Au content in the resultant solution was determined by an Optima 4300DV inductively coupled plasma optical emission spectrometer (PerkinElmer). The data obtained from untreated hMSCs were used for background correction.

Table S1

Name	Sequence (5' → 3')
hsa-miR-29b	UAG CAC CAU UUG AAA UCA GUG UU
hsa-miR-31	AGG CAA GAU GCU GGC AUA GCU
DNA analog of hsa-miR-29b	TAG CAC CAT TTG AAA TCA GTG TT
FITC-labeled hpDNA-29b	FITC- <u>CCG GGT</u> AAC ACT GAT TTC AAA TGG TGC TA <u>ACC CGG</u>
Cy3-labeled hpDNA-31	Cy3- <u>CCG GGT</u> AGC TAT GCC AGC ATC TTG CCT <u>ACC</u> <u>CGG</u>
FITC-labeled scrambled DNA	FITC-GCT GCT TTA TAG AGC CTG TTG A
Random DNA	CAG TGG TTT TAC CCT ATG GTA G

Table S1. DNA sequences employed for the extra- and intracellular detection of miRNAs. All nanoprobe are prepared by immobilizing the hpDNA strands onto the surface of Au@PDA NPs upon 1 h of incubation. The scrambled DNA sequence refers to a DNA sequence with the following properties: (i) bears the same nucleotide composition as the probe sequence, (ii) has no complex sequence, and (iii) shows the weakest (or no) matches with any mRNA in the mRNA pool for human. Base pairs in the stem region of the hpDNA probes are underlined for clarity. Cy3 = cyanine 3, FITC = fluorescein isothiocyanate.

Table S2

Sample	Hydrodynamic size [nm] (PDI)	Zeta potential [mV]	Number of oligonucleotides per particle
Au@PDA NPs (Before the DNA immobilization)	56.1 ± 1.6 (0.19)	-39.0 ± 1.5	N. A. ^a
Au@PDA–hpDNA NPs (After the DNA immobilization)	68.5 ± 1.2 (0.14)	-28.3 ± 2.3	256 ± 16

Table S2. Physiochemical properties of Au@PDA–hpDNA NPs. For comparison, characterization data of the bare Au@PDA NPs are shown in the upper row. Results are given in mean $\pm$ SD of 10 measurements. PDI = poly-dispersity index.

^aMeasurement is not applicable to the sample.

Table S3

Methods	Minimal Time for Distinguishing the Differentiation Status of hMSCs	Is Cell Lysis Required?	Live Cell Monitoring?	Long-term Monitoring?
qRT-PCR of Genes	3 d	Yes	No	No
Staining of Proteins	5–7 d	No	No	No
Commercial RNA Detection Probe e.g., SmartFlare	1 d	No	Yes	Up to 3 d if single administration is applied to cells
Au@PDA–hpDNA Nanoprobes	1 d	No	Yes	Up to 5 d if single administration is applied to cells

Table S3. Summary of the performance of conventional methods and nanoparticle-based methods used for the monitoring of the differentiation status of hMSCs.

Table S4

Gene	Forward Primer	Reverse Primer	Probe
<i>ALP</i>	CGGAACTCCTGAC CCTTGAC	TG TTCAGCTCGTACTG CATGTC	TCGAAGAGACCCA ATAGGT

Table S4. Sequence information of the primers and probe of osteogenic marker gene employed in the RT-PCR analysis. *ALP* = alkaline phosphatase.

Figure S1

Figure S1. qRT-PCR measurements of two early osteogenic marker genes, namely (A) *RUNX2* and (B) *ALP*, in hMSCs. Our results show that both the *RUNX2* and *ALP* show differential gene expression between the differentiating and undifferentiated hMSCs upon 3 d of osteogenic induction. qRT-PCR is performed using an off-target gene *GAPDH* as the internal control. Data obtained from three independent measurements are presented as mean $\pm$ SD. * = $p < 0.05$; ** = $p < 0.005$. *RUNX2* = Runt-related transcription factor 2. *ALP* = alkaline phosphatase. *GAPDH* = glyceraldehyde 3-phosphate dehydrogenase.

Figure S2

Figure S2. Conventional methods of monitoring the differentiation progress of hMSCs. (A) Results of immunofluorescence staining against RUNX2. Significant difference of the nuclear fluorescence (green) between the differentiating and undifferentiated hMSCs is observed upon 5 d of osteogenic induction. (B) Results of histochemical staining of ALP. Significant difference between the differentiating and undifferentiated hMSCs is observed upon 7 d of osteogenic induction. Positively stained cells appear as purple in color. Scale bar is 200 μm . Note that one cannot observe major differences between the differentiating and undifferentiated hMSCs in the early stage of osteogenesis by using these two conventional methods.

Figure S3

Figure S3. Dynamic light scattering (DLS) measurements of (A) AuNPs and (B) Au@PDA NPs in water at 25 °C. The hydrodynamic size of AuNPs increases by around 12 nm after coating with the thin PDA shell, which is consistent with the TEM measurements (see Figure 1B).

Figure S4

Figure S4. Representative TEM images of Au@PDA NPs with different PDA shell thicknesses. (A) AuNPs are coated with a PDA shell of ~10 nm thickness in 0.1 mg/mL of dopamine solution. (B) AuNPs are coated with a PDA shell of ~35 nm thickness in 0.4 mg/mL of dopamine solution. The PDA shell thickness can be tuned by simply varying the dopamine concentration while keeping the reaction time constant at 1 h.

Figure S5

Figure S5. FT-IR spectra of AuNPs (red) and Au@PDA NPs (purple). Newly emerged absorption bands at 3410 cm⁻¹ (stretching vibration of phenolic O–H and N–H), 1605 cm⁻¹ (stretching vibration of aromatic ring and bending vibration of N–H), 1510 cm⁻¹ (shearing vibration of N–H), and 1295 cm⁻¹ (stretching vibration of phenolic C–O) are observed after coating AuNPs with PDA, indicating the presence of the PDA shell on the surface of AuNPs.

Figure S6

Figure S6. Stability test of Au@PDA NPs in water, PBS, and basal medium (α -MEM + 10% FBS). (A) Photographs of Au@PDA NPs before and after incubation in different solutions for 24 h. The solutions remain clear without obvious aggregation. α -MEM = alpha minimum essential medium, FBS = fetal bovine serum. (B) UV-vis spectra of the solutions shown in (A). No significant red-shift of the $\lambda_{\max}$ is observed in all the solutions with respect to the peak of the stock prepared in water (indicated by the black line), supporting our observation that no obvious aggregation happens.

Figure S7

Figure S7. Cytotoxicity of hMSCs evaluated by the Alamar blue assay after incubation with AuNPs or Au@PDA NPs at different concentrations (expressed as Au content obtained from ICP-OES measurement) for 24 h. Results are normalized by using the cytotoxicity data of untreated hMSCs. Data obtained from six independent measurements are presented as mean $\pm$ SD.

Figure S8

Figure S8. Standard linear calibration curve used for the estimation of % release of immobilized fluorescently-labeled hpDNA strands from the surface of Au@PDA NPs as shown in the inset of Figure 2B. Data are recorded from fluorescence measurements of known concentrations of FITC-labeled hpDNA against miR-29b. Data obtained from three independent measurements are presented as mean $\pm$ SD.

Figure S9

Figure S9. Release assay of Au@PDA–hpDNA nanoprobes in a buffer system. 100 nM of FITC-labeled hpDNA strands that specifically recognize miR-29b are first immobilized onto Au@PDA NPs to form the nanoprobes against miR-29b. Red trace shows the fluorescence signals of the nanoprobes upon 2 h of incubation with 500 nM of DNA analog of miR-29b (target). Blue trace shows the fluorescence signals of the nanoprobes upon 2 h of incubation with 500 nM of random DNA sequence as control. Purple trace shows the fluorescence signal of the nanoprobes itself. These data support that our nanoprobes show reasonable specificity against miR-29b.

Figure S10

Figure S10. Representative TEM images of hMSCs incubated with Au@PDA NPs for 24 h. Magnified images show that most uptaken Au@PDA NPs are located in the perinuclear region inside hMSCs. Cy = cytoplasm, Nu = nucleus.

Figure S11

Figure S11. Examination of the Au content inside hMSCs 1 d, 3 d, and 5 d after initial cellular uptake of nanoparticles. hMSCs are treated with nanoparticles for 24 h before rinsing with PBS. (A) Photographs of cell pellet collected for (i) untreated hMSCs, (ii) Au@PDA NP-treated hMSCs, and (iii) Au@PDA–hpDNA nanoprobe-treated hMSCs. Color of cell pellets for both the Au@PDA NP-treated and nanoprobe-treated hMSCs remain intense as purple in color within the observation time window. (B) ICP-OES measurements show that there is no obvious reduction in the Au content of cell-associated nanoparticles for both the Au@PDA NP-treated and nanoprobe-treated hMSCs even after 5 d of incubation, suggesting minimal exocytosis of the nanoparticles. Data obtained from three independent measurements are presented as mean $\pm$ SD.

Figure S12

Figure S12. Standard linear calibration curve obtained from the ICP-OES measurements of varying concentration of gold standard solutions. This calibration curve is used to determine the concentration of the stock solution of AuNPs and the Au content per well as shown in Figure 3C. Data obtained from three independent measurements are presented as mean $\pm$ SD.

Figure S13

Figure S13. Quantification of intracellular fluorescence of Au@PDA–hpDNA nanoprobe-treated hMSCs upon different days of osteogenic induction. Green trace shows the plot of cell fluorescence associated with the detection of miR-29b as a function of day of osteogenic differentiation. Red trace shows the corresponding plot for the detection of miR-31. A monotonic increasing trend of the intracellular fluorescence along with the osteogenic induction time is observed for both osteogenic marker miRNAs, consistent with our qRT-PCR results of these two miRNAs as shown in Figure 4. Cell fluorescence signals are presented as I/I_0 , where I_0 represents the mean fluorescence of hMSCs upon 1 d of osteogenic induction. Data obtained from over 50 cells per sample are presented as mean $\pm$ SD.

Figure S14

Figure S14. Comparison of cellular uptake property of our Au@PDA–hpDNA nanoprobe (nanoprobes) with the commercial RNA detection probe SmartFlare in hMSCs. hMSCs are treated with nanoparticles for 24 h. Concentrations of the nanoparticles are adjusted to be equivalent at ~ 0.025 nM. (A) Photographs of cell pellets collected for (i) untreated hMSCs, (ii) SmartFlare-treated hMSCs, and (iii) nanoprobe-treated hMSCs. Intense color is observed only for the nanoprobe-treated cell pellet but not the SmartFlare-treated cell pellet. (B) ICP-OES measurements show that the Au content in the nanoprobe-treated cell samples is ~ 7 -fold higher than that in the SmartFlare-treated cell samples, indicating that our nanoprobe is more efficiently internalized by hMSCs. Data obtained from three independent measurements are presented as mean $\pm$ SD.

Figure S15

Figure S15. Intracellular specificity and stability of Au@PDA-hpDNA nanoprobe. 500 nM of the FITC-labeled scrambled DNA strands are immobilized onto Au@PDA NPs and then incubated with (A) hMSCs and (B) primary osteoblasts for 24 h. Cells are imaged at the selected time points after incubation. Results show negligible fluorescence responses in both the differentiating hMSCs and osteoblasts, indicating that our nanoprobe is specific and resistant to nuclease degradation for at least 5 days of culture. Nuclei were counter-stained with Hoechst 33342. Scale bar is 100 μm . DAPI = 4,6-diamidino-2-phenylindole.

Scheme S1

Scheme S1. Monitoring of the immobilization of fluorescently-labeled hpDNA strands onto the surface of Au@PDA NPs. All nanoprobe s used in this study are prepared in the same manner as described.

REFERENCES

- (1) Khlebtsov, N. G. *Anal. Chem.* **2008**, *80*, 6620.