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Smart Magnetic Nanosensors Synthesized through Layer-by-Layer Deposition of Molecular Beacons for Non-Invasive and Longitudinal Monitoring of Cellular mRNA

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ABSTRACT: Non-invasive and longitudinal monitoring of gene expression in living cells is essential for understanding and monitoring cellular activities. Herein, a smart magnetic nanosensor is constructed for the real-time, non-invasive and longitudinal monitoring of cellular mRNA expression through the layer-by-layer deposition of molecular beacons (MBs) and polyethylenimine on the iron oxide nanoparticles. The loading of MBs, responsible for the signal intensity and the tracking time, was easily tuned with the number of layers incorporated. The idea was firstly demonstrated with the magnetic nanosensors for Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) mRNA, which was efficiently internalized into the cells under the influence of magnetic field. This nanosensor allowed the continuous monitoring of the cellular GAPDH mRNA expression for one month. Then, this platform was further utilized to incorporate two kinds of MBs for Alkaline Phosphatase (ALP) and GAPDH mRNAs respectively. The multifunctional nanosensors permitted the simultaneous monitoring of the reference gene (GAPDH mRNA) and the early osteogenic differentiation marker (ALP mRNA) expression. By taking the fluorescence signal ratio between ALP mRNA MBs and GAPDH mRNA MBs, the dynamic osteogenic differentiation process of MSCs was accurately monitored.

KEYWORDS: Magnetic nanoparticle, nanosensor, molecular beacon, layer-by-layer deposition, mRNA detection, osteogenic differentiation

INTRODUCTION

Quantitative, non-invasive and longitudinal analysis of messenger RNA (mRNA) in living cells has attracted great interest due to its importance for cell biology, drug discovery, disease diagnosis and treatment.¹ Unfortunately, conventional methods for monitoring cellular mRNA expression, including northern blotting, ribonuclease protection assays, complementary DNA microarrays and *in situ* hybridization, suffer from low sensitivity, high cost, and the time-consuming procedures.² Real-time reverse-transcription quantitative polymerase chain reaction (RT-qPCR) is often taken as the standard protocol, but this sensitive method requires the extraction of mRNA by disrupting cells and cannot provide the spatial distribution of mRNA in cellular level.³ One solution is to use molecular beacons (MBs), the hairpin-structured and dual-labelled oligonucleotide probes, which can enter the cytoplasm under the help of chemical/physical methods and recognize the presence of specific nucleic acids in a quantitative way.⁴ However, the biggest challenge for this methodology is that free MBs cannot enter cells themselves and are easily degraded by the intracellular nucleases,⁵ which significantly limits their wide applications.

Recently, studies showed that the immobilization of nucleic acids on nanoparticles could improve the delivery efficiency and cellular stability of the nucleic acids.^{6,7} With the rational design of sizes, morphologies and surface coating, many types of nanoparticles such as polymeric nanoparticles^{8,9}, micelles¹⁰, gold nanoparticles¹¹, quantum dots¹² and magnetic nanoparticles¹³ have been proposed as the potential carriers of MBs for efficient detection of intracellular mRNA and microRNA. In comparison to others, magnetic nanoparticles can enhance the cellular uptake of the loaded MBs and target the interested tissue/organ under the magnetic guidance while they act as contrast agents in magnetic resonance imaging.¹⁴ For

example, Kim et al conjugated MBs onto the fluorescent magnetic nanoparticles (MNP@SiO₂(RITC)-PEG/COOH) through amidation reaction for detecting and monitoring the intracellular microRNA during neurogenesis (6 days).^{15,16} In those studies, the binding between magnetic nanosensors and target microRNA caused the distance change between nanoparticles and allowed the detection of this binding through measuring T2 relaxation. However, these nanosensors could not allow the simultaneous detection of multiple mRNAs in living cells. Chen et al attached the DNA probes onto Fe₃O₄@polydopamine core-shell nanoparticles through the π - π stacking interactions to construct a multifunctional platform for intracellular mRNA detection and imaging-guided photothermal therapy.¹³ However, these magnetic nanosensors didn't show the potential for longitudinally monitoring of the cellular gene expression, probably due to the poor loading of probes in the nanosensors.

It is well-known that electrostatic layer-by-layer assembly of oppositely charged species allows the control of the molecular loading on nanoparticles by tuning the deposited layers.¹⁷ And layer-by-layer deposition also permits the incorporation of multiple molecules onto the same nanoassembly. Therefore, this work utilized the layer-by-layer deposition technology to construct a smart mRNA magnetic nanosensor for non-invasively and longitudinally monitoring the cellular expression of multiple mRNAs. This nanosensor platform developed here was synthesized through the layer-by-layer deposition of the negatively charged probe MBs and the positively charged polyethylenimine (PEI). The loading of MBs was controlled by the deposited MB layers while multiple types of MBs could be incorporated to detect two or more kinds of mRNA. During the construction of nanosensors, there was an alternation of the zeta potential between +47 mV and +40 mV, which corresponded to the PEI and MB coating respectively. And the hydrodynamic diameter increased from 42 nm to 67 nm to 119 nm and to 195 nm when there

were 1, 3, 7, and 11 layers. More layers meant slower signal attenuation and longer signal lasting. By taking this platform, the GAPDH mRNA magnetic nanosensors were firstly synthesized. They were efficiently internalized into the cells under the influence of magnetic field and allowed the continuous monitoring of the cellular GAPDH mRNA expression for one month. Finally, ALP mRNA MBs (the probe MBs) and GAPDH mRNA MBs (the internal reference MBs) were incorporated onto the same magnetic nanoparticles for the real-time and *in situ* monitoring of the osteogenic differentiation of mesenchymal stem cells (MSCs). Normalized by the GAPDH mRNA expression level, the ALP mRNA exhibited a well-defined dynamic expression with a peak expression around day 7 during the osteogenic differentiation, which matches well with the RT-qPCR results.

EXPERIMENTAL SECTION

Materials. Iron acetylacetonate ($\text{Fe}(\text{acac})_3$), benzyl ether, oleylamine, branched polyethylenimine (PEI, 25 KDa), molecular beacons and primers for GAPDH mRNA and ALP mRNA (purified by HPLC and characterized by mass spectra) were purchased from Sigma-Aldrich (USA). Dulbecco's modified eagle medium (DMEM) with high glucose (4.5 g/L) and L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin (10,000 U/ml), and trypsin-EDTA (0.5 %, 10x) were obtained from Gibco Life Technologies (USA). MSC Osteogenic Differentiation BulletKit™ Medium was purchased from Lonza (Singapore). DNase I (RQ1, RNase-free, 1,000 U/mL) was obtained from Promega (USA). AlamarBlue® cell viability reagent and TRIzol® reagent were purchased from Thermo Fisher Scientific (USA).

Characterization. Zeta potential and dynamic light scattering (DLS) measurements were performed on a Zetasizer Nano ZS (Malvern Instruments, UK). All the fluorescence imaging was performed on a LX71 inverted fluorescence microscope (Olympus, Japan) equipped with a Retiga-2000R CCD camera by using an Image-Pro Insight software (Media Cybernetics). The fluorescence intensity of individual cells in fluorescence images was obtained by using Image J. Transmission electron microscopy (TEM) images were acquired by JEM-2100 high-resolution transmission electron microscope (JEOL, Japan). Flow cytometry data was collected on an LSR Fortessa™ X-20 flow cytometer (BD Biosciences, USA) and analyzed with FlowJo (Tree Star). At least 100,000 gated events were collected per sample.

Synthesis of PEI coated iron oxide nanoparticles (PEI-IONPs). The oleylamine coated iron oxide nanoparticles (IONPs) with *ca.* 7 nm in diameter were prepared according to the previous reports.¹⁸ To coat PEI, IONPs solution (2 mg/mL in chloroform) was added into PEI solution (2-12 mg/mL in chloroform) under stirring. After removing chloroform, a PEI-IONPs thin film was obtained and subsequently dispersed in sterile deionized water. The resulted dispersion was centrifuged at low speed (i.e. 3,000 rpm) to remove the uncoated IONPs sediment, centrifuged at high speed (i.e. 12,000 rpm) to remove the residual PEI, and finally redispersed in sterile deionized water.

Synthesis of the mRNA magnetic nanosensors (PEI-(MB-PEI)_n-IONPs). The mRNA magnetic nanosensors were synthesized through the layer-by-layer deposition of MBs and PEI. The coating of MBs or PEI was performed by mixing 10 μ L of MBs (10 μ M) or PEI (5 mg/mL) with 100 μ L of magnetic nanoparticles solution (3 mg/mL) for 30 min at room temperature. After each coating, the resulting mixture was centrifuged for 20 min at 8000 rpm and washed repeatedly to remove the unbound PEI or MBs. This process was repeated several times for

constructing the PEI-(MB-PEI)_n-IONPs nanosensors loaded with 1, 3 and 5 layers of MBs (i.e. n=1, 3 and 5, respectively). In addition, after each coating of MBs, the supernatant was collected and treated with DNase I (20 U/mL) at 37 °C for 20 min. Then the fluorescence intensity at 570 nm was recorded through a spectromax M5 plate reader (Molecular Devices, USA) and the amounts of MBs were calculated based on a calibration curve obtained from standard solutions containing known MB concentrations (0 nM-100 nM, Figure S1).

For the osteogenic differentiation, 100 µL of ALP MBs (10 µM) and 100 µL of GAPDH MBs (10 µM) were mixed together. Then the osteogenic nanosensors with 5 layers of the mixed MBs could be constructed by alternatively coating the MBs mixture and PEI onto magnetic nanoparticles for 5 cycles according to the above protocol.

Nuclease resistance. For testing the nuclease resistance of the as-prepared nanosensors, DNase I was chosen as the model nuclease. PEI-(MB-PEI)_n-IONPs containing 30 µg/mL IONPs (n=1, 3 and 5) and free GAPDH MBs at the desired concentrations (10, 30 and 50 nM) in PBS were incubated at 37 °C in the absence and presence of 2 U/mL DNase I. In addition, the nuclease resistance of PEI/MBs complex was also checked. PEI/MBs complex was prepared by mixing 6 µL PEI solution (50 µM) with 50 µL GAPDH MB solution (1 µM) for 1 h. Then the resulting PEI/MBs containing 50 nM MBs was incubated at 37 °C in the absence and presence of 2 U/mL DNase I. At different time points, the fluorescence intensity of samples at 570 nm was recorded upon the excitation at 550 nm by using the spectromax M5 plate reader.

MB release from nanosensors. 300 µL of PEI-(MB-PEI)_n-IONPs nanocomplex in PBS (n=1, 3 and 5) was transferred into a wet dialysis tubing (Spectra/Por® membrane, MWCO 20 kDa, Spectrum Lab, USA) which was placed into 10 mL PBS at 37 °C. Later, 150 µL of dialysate was

collected, aliquoted into three wells of 96-well plates at various time points (i.e. day 1, 2, 4, 7, 10, 14, 17, 21, 25 and 30) and then kept at -20 °C. The same amount of fresh PBS was replenished to keep the total volume of dialysate unchanged. After collected for eight time points, the samples were treated with DNase I (20 U/mL) at 37 °C for 20 min and their fluorescence intensity at 570 nm was recorded upon the excitation at 550 nm by using the spectromax M5 plate reader. The amounts of MBs were calculated based on a calibration curve obtained from standard solutions containing known MB concentrations (0 nM-100 nM).

Stability of nanosensors in cell culture medium. PEI-(MB-PEI)_n-IONPs nanocomplex (n=1, 3 and 5) containing 1 mg/mL IONPs was dispersed in normal cell culture medium (high glucose DMEM supplemented with 10 % FBS and 1 % penicillin-streptomycin). The hydrodynamic sizes of the suspension were collected at different time points (i.e. 0, 1, 5 and 12 h) through dynamic light scattering measurements (Zetasizer Nano ZS, Malvern Instruments, UK).

Monitoring the expression of GAPDH mRNA in MSCs. MSCs harvested from normal human bone marrow (Lonza, Singapore) were cultured in DMEM (high glucose) supplemented with 10 % FBS and 1 % penicillin-streptomycin at 37 °C with 5 % CO₂. MSCs in all assays were used by passage 5. Before the experiment, MSCs were seeded onto 6-well plates until the confluency reaches 80 %. The culture medium was changed every three days. Then the cells were incubated with PEI-(MB-PEI)_n-IONPs containing 30 µg/mL IONPs (n=0, 1, 3 and 5) in normal culture medium with and without magnetic field. After incubation for 1 h, the labelled cells were washed with PBS three times to remove free nanoparticles and replenished with fresh medium. In addition, free MBs and PEI conjugated MBs (PEI/MB_n) with the similar concentrations of MBs in PEI-(MB-PEI)_n-IONPs nanosensors were used as the control. 5 µL GAPDH MBs with different concentrations (2 µM, 6 µM and 10 µM) was mixed with 5 µL 5 mg/mL PEI solution

for 30 min to construct the PEI/MB₁, PEI/MB₃ and PEI/MB₅, respectively. Then MSCs was incubated with 1 mL normal culture medium containing 10 µL obtained PEI/MB complex (PEI/MB₁, PEI/MB₃ and PEI/MB₅), 10 nM, 30 nM and 50 nM free MBs. After 1 h incubation, the labelled cells were washed with PBS three times. At various time points post labeling, the expression of GAPDH mRNA was examined by fluorescence microscopy (Olympus IX71, Japan, capture setting: exposure time 500 ms, gain 7, offset -600).

Monitoring the osteogenic differentiation in MSCs. MSCs on 6-well plates (confluency > 90 %) were labelled with osteogenic nanosensors containing 30 µg/mL IONPs in normal culture medium according to the same labeling protocol in the above section. After incubation overnight, the osteogenic differentiation of the MSCs was induced by replacing the normal culture medium with osteogenic differentiation bulletKit™ medium (Lonza, Singapore) supplemented with 1 % penicillin-streptomycin. At various time points (i.e. day 0, 3, 5, 7, 9, 11 and 13), the expression of ALP mRNA and GAPDH mRNA was observed by fluorescence microscopy (capture setting: green channel, exposure time 500 ms, gain 4, offset -1000; red channel, exposure time 500 ms, gain 4, offset -600) and RT-qPCR according to the following procedures. The statistical significance of ALP mRNA expression between different time points was determined by calculating the *p* value using one way analysis of variance (ANOVA).

Cell viability assay. MSCs were seeded onto a 96-well plate at a density of 15,000 cells per cm² and cultured for 24 h. To test the cytotoxicity of PEI-IONPs, MSCs were labelled with 50 µg/mL PEI-IONPs with different mass ratios for 24 h. To test the cytotoxicity of PEI-(MB-PEI)_n-IONPs, MSCs were labelled with PEI-(MB-PEI)_n-IONPs with different layers of MBs (i.e. 0, 1, 3 and 5 layers) at different concentrations (i. e. 2, 5, 10, 20 and 30 µg/mL) for 24 h. The free nanoparticles were removed with PBS and then 100 µL culture medium containing 10 %

AlamarBlue was added into each well. After incubation for 4 h at 37 °C in the incubator, their fluorescence intensity was recorded at 590 nm upon the excitation at 545 nm by using a SpectroMax M5 plate reader. The experiment was performed three times with five replicates per group. To test the long-term cytotoxicity of PEI-(MB-PEI)_n-IONPs, MSCs were labelled with 30 µg/mL PEI-(MB-PEI)_n-IONPs (n=1, 3 and 5) for 24 h and washed with PBS. At different time points post exposure to the nanosensors (i.e. day 1, 2, 3, 5 and 7), 100 µL culture medium containing 10 % AlamarBlue was added into each well. After 4 h incubation, their fluorescence intensity was also recorded through the same protocol described above. The unlabelled MSCs at the same time points were used as the control to evaluate the cell viability.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR). After initiating the osteogenic differentiation, MSCs were collected at various time points (i.e. day 0, 3, 5, 7, 9, 11 and 13) and subsequently lysed with TRIzol® reagent to extract the cellular RNA. cDNA conversion was performed with the reverse transcription kit containing M-MLV RNase H (-) Mutant and dNTP (Promega, USA). qPCR analysis of the obtained cDNA was performed on a CFX Connect™ Real-Time PCR System (Bio-Rad, USA). The reaction solution contained 10 µL iQ SYBR Green Supermix (Bio-Rad, USA), 7.8 µL RNase-free water, 1.2 µL primer mixture (the primer sequences of ALP mRNA and GAPDH mRNA were listed in Table S1) and 1 µL cDNA. The values of $2^{-\Delta(C_T(Alp) - C_T(GAPDH))}$ for all the samples were used for evaluating the fold change of ALP mRNA relative to GAPDH mRNA by normalizing to the expression of differentiated MSCs on day 3.

RESULTS AND DISCUSSION

Synthesis and characterization of the magnetic nanosensors. The magnetic nanosensors were synthesized through the layer-by-layer deposition process (Scheme 1). Briefly, oleylamine coated IONPs were firstly rendered water-soluble by grafting the cationic branched PEI. Then negatively charged MBs were absorbed onto the PEI-IONPs under the electrostatic interactions as the second layer. Subsequently, the third layer of PEI and the fourth layer of MBs could be grafted alternatively. The outmost layer of magnetic nanosensors was PEI for preventing the burst release of the coated MBs.¹⁹ All the sequences of MBs and primers used are listed in Table S1.

Scheme 1. Illustration of the synthesis of magnetic mRNA nanosensors through the layer-by-layer deposition

The first important factor during the synthesis is the mass ratio between PEI and IONPs. PEI is known to be cytotoxic but is critical for the assembly of the MBs and construction of the layer-by-layer structure. An optimal PEI concentration should allow the formation of the cationic layer on nanoparticles while presenting minimal cytotoxicity. Here, a series of PEI/IONP mass ratios were screened for their influence over the surface charges, hydrodynamic size and cytotoxicity of the PEI-IONP complexes. The studies showed that both the surface charge and

the hydrodynamic size of the as-prepared PEI-IONPs decreased with the increase of the mass ratios (R) of PEI to IONPs and reached a plateau at $R=3$ (Figure 1A). These results may be ascribed to the different surface coverage of PEI on IONPs at different R. The as-prepared PEI-IONPs may be not fully covered with branched PEI at $R=1$, resulting in the weak electrostatic repulsion between the PEI-IONPs in water. Therefore, the PEI-IONPs may be close to each other to exhibit the “false aggregates” in water. With the increase of the mass ratio, more PEI attached onto the IONPs, resulting in higher surface coverage of PEI, the stronger electrostatic repulsion between the PEI-IONPs, and thus smaller “false aggregates”. Therefore, the obtained PEI-IONPs would show smaller hydrodynamic size and lower surface charges. At $R \geq 3$, PEI-IONPs would be fully covered by branched PEI and then the PEI amount per nanoparticle reached a plateau at $R \geq 3$. And the hydrodynamic size and surface charges kept unchanged.

Although PEI is known as one of the most common polymeric carriers for non-viral gene vectors due to its good pH buffering capacity and high transfection efficiency, its high cytotoxicity is still a major concern.²⁰ Therefore, the cytotoxicity of the as-prepared PEI-IONPs was investigated. Figure 1B shows the cell viability for PEI-IONPs with different mass ratios of PEI to IONPs. With the increase of the mass ratios, the PEI-IONPs showed higher cytotoxicity. Considering the surface coverage of PEI as well as the cytotoxicity, the mass ratios of PEI to IONPs was fixed at $R=3$ for the subsequent electrostatic layer-by-layer assembly of MBs and PEI to construct the mRNA magnetic nanosensors.

Figure 1. The influence of the mass ratio between PEI and IONPs over the physical and biological properties of PEI-IONPs: **(A)** A correlation plot between the mass ratios of PEI to IONPs, versus zeta potential/hydrodynamic size of PEI-IONPs. **(B)** Cytotoxicity induced by PEI-IONPs with different mass ratios of PEI to IONPs in terms of cell viability. The concentration of the PEI-IONPs was 50 $\mu\text{g/mL}$.

The layer-by-layer deposition of PEI and MB on the IONPs was confirmed by the zeta potential measurement (Figure 2A). The initial PEI coating endowed the IONPs with a positive surface charge (+45 mV). After coating with negatively charged MBs, the zeta potential of hybrid nanoparticles decreased to +40 mV. The zeta potentials of the PEI-(MBs-PEI)_n-IONPs exhibited a zigzag change between +47 $\pm$ 2 mV and +40 $\pm$ 2 mV when alternately coating PEI and MBs, demonstrating the successful construction of layer-by-layer assembled nanosensors. In addition, the zeta potential of the as-prepared PEI-(MBs-PEI)_n-IONPs nanosensors (n=1, 3 and 5) in cell culture medium (DMEM containing 10 % FBS and 1 % penicillin-streptomycin) were also studied (Figure S2A). It is found that the zeta potential values were switched from positive (+47 $\pm$ 2 mV) to negative (-4.5 $\pm$ 2 mV) for all three kinds of nanosensors after transferring from

water to cell culture medium, which could be ascribed to the absorption of the negatively charged FBS on the surface of PEI-(MBs-PEI)_n-IONPs.¹⁹ The loading of MBs on the magnetic nanoparticles could be derived by qualifying the unbound MBs in the supernatants during the synthesis. As shown in Figure S3, the loading of MBs increased linearly with the number of the incorporated layers. Similar phenomenon was also observed in the case of the layer-by-layer assembly of inactivated polio vaccine on the microneedles.²¹

The hydrodynamic sizes of the nanosensors with different layers of MBs were examined by dynamic light scattering (DLS) (Figure 2B). The average hydrodynamic diameter of PEI-IONPs (42±7 nm, Figure 2B, red curve) was much larger than the size of IONPs core (7±3 nm, Figure 2B, black curve), which is ascribed to the coating layer of hydrophilic and positively charged PEI (25 KDa). Later, the average hydrodynamic diameters of PEI-(MBs-PEI)_n-IONPs nanosensors increased with the increase of layer numbers from 42±7 nm (n=0) to 67±15 nm, 119±24 nm and 195±29 nm for n=1, 3 and 5, respectively. It deserves to point out that the size increase became larger and larger when there were more layers of PEI/MB. For example, the addition of two more layers over PEI-(MB-PEI)-IONPs resulted in a 52 nm increase of the hydrodynamic diameter. However, the addition of two more layers over PEI-(MB-PEI)₃-IONPs provided a 76 nm increase. This may be attributed to the weakened interactions between PEI and MB in the outer layers of the PEI-(MB-PEI)_n-IONPs complex. The TEM study of the nanoparticles with 5 layers of MBs revealed that the coating didn't cause any significant damage over the morphology of IONPs in comparison to PEI-IONPs (Figures 2C and 2D). For the subsequent intracellular application, we compared the hydrodynamic size of the PEI-(MB-PEI)_n-IONPs (n=1, 3 and 5) in water and cell culture medium and investigated the stability of nanosensors incubated in cell culture medium within 12 h. PEI-(MB-PEI)_n-IONPs nanosensors

showed the increased hydrodynamic size (217 ± 23 nm, 330 ± 9 nm and 335 ± 40 nm for $n=1, 3$ and 5 , respectively) in cell culture medium compared that in water (Figure S2B), which may be attributed to the absorption of large FBS and slight aggregation. It was also found that the hydrodynamic size of nanosensors slight decreased (ca. 50 nm) after 12 h incubation in culture medium due to the possible disassembly of the outer layer of the nanosensors (Figure S2C), which suggested the high stability of the constructed nanosensors under cell culture conditions.

Figure 2. Insight of the deposition process of PEI-(MB-PEI)_n-IONPs and their morphology characterization. (A) Zeta potentials of PEI-(MB-PEI)_n-IONPs as a function of total layer numbers. (B) Hydrodynamic diameter of IONPs and PEI-(MB-PEI)_n-IONPs with $n=0, 1, 3, 5$. (C, D) The representative TEM images of PEI-(MB-PEI)_n-IONPs with $n= 0$ (C) and 5 (D).

The cytotoxicity of PEI-(MB-PEI)_n-IONPs was revealed by examining the viability of MSCs when being exposed to PEI-(MB-PEI)_n-IONPs under different concentrations for 24 h (Figure S4A). When the nanoparticle concentrations were $\leq 20 \mu\text{g/mL}$, all four kinds of nanoparticles didn't show noticeable cytotoxicity. However, $20.6 \pm 5.4 \%$ of MSCs were dead when treated with $30 \mu\text{g/mL}$ PEI-IONPs. Interestingly, under the same concentration ($30 \mu\text{g/mL}$), PEI-(MB-PEI)-IONPs, PEI-(MB-PEI)₃-IONPs, PEI-(MB-PEI)₅-IONPs didn't cause similar cytotoxicity. Clearly the complexation of the negatively charged MBs decreased the cytotoxicity of PEI-IONPs, which might be explained by the increased stability of PEI on IONPs after complexation.^{22,23} Considering the long-term intracellular application of the constructed nanosensors (like longitudinally monitoring the osteogenic differentiation of MSCs), their long-term cytotoxicity during one week was also investigated. As shown in Figure S4B, regardless of the number of MB layers, these nanosensors showed high cell viability one week post labeling ($> 85 \%$), suggesting that the slow release of PEI from internalized nanosensors didn't cause significant damage to cells.

In addition to the low cytotoxicity, a good stability of the complexed MBs against the nuclease is also essential. Previous studies showed that the immobilization of nucleic acids on various nanoparticles could prevent the nucleic acids from the degradation of nuclease.^{6,24,5} Here, PEI-(MB-PEI)_n-IONPs were treated with 2 U/mL of DNase I (an endonuclease capable of nonspecifically degrading both single- and double-stranded DNA) to examine the nuclease resistance of MBs. Free MBs with the similar concentrations in the PEI-(MB-PEI)_n-IONPs were used as the control. Any degradation of MBs could be detected from the fluorescence restoration which is caused by the efficient separation of fluorescein from the quencher BHQ1 (Figure 3A). As shown in Figure 3B-D, regardless of the concentrations, free MBs treated with DNase I

showed a gradual increase in fluorescence signal with the increasing incubation time and reached a plateau at *ca.* 40 min. However, almost no fluorescence enhancement could be observed for PEI-(MB-PEI)-IONPs, PEI-(MB-PEI)₃-IONPs, PEI-(MB-PEI)₅-IONPs, which reveals that the complexation of MBs with magnetic nanoparticles could efficiently protect MBs from the nuclease degradation. The nuclease resistance may be explained by that the encapsulated MBs in the constructed magnetic nanosensors were totally embedded in the interlayers of nanocomplex and thus nucleases cannot physically access the MBs.²⁵ However, the complex of MBs with PEI in the absence of magnetic cores could not completely protect the MBs from nuclease degradation. Small part of MBs may be still recognized by nuclease and subjected to slow degradation although the PEI complex may change the MBs to be unrecognizable to enzyme binding pockets (Figure S5).

Figure 3. Fluorescence assay for the stability of free MBs and PEI-(MB-PEI)_n-IONPs nanosensors against DNase I digestion: **(A)** Illustration of the cleavage of free MBs and PEI-(MB-PEI)_n-IONPs nanosensors in the presence of DNase I; **(B)** PEI-(MB-PEI)-IONPs and 10 nM free MBs; **(C)** PEI-(MB-PEI)₃-IONPs and 30 nM free MBs; **(D)** PEI-(MB-PEI)₅-IONPs and 50 nM free MBs. The fluorescence intensity of the samples at 570 nm was monitored in the absence (-) or presence (+) of 2 U/mL DNase I during a period of 120 min.

The release profile of MBs from nanosensors was examined by immersing the nanosensors in PBS at 37 °C and determining the amounts of MBs in the dialysate at different time points. The cumulative release of MBs from PEI-(MB-PEI)-IONPs, PEI-(MB-PEI)₃-IONPs, PEI-(MB-PEI)₅-IONPs nanosensors during a period of 40 days (Figure S6A) was dependent on the layer number of MBs on nanosensors. More layers of MBs means more released MBs. For example, 6, 10 and 12 pmol of MBs were released from nanosensors with 1, 3 and 5 layers of MBs on day 1, respectively. On day 21, the cumulative release amounts of MBs from nanosensors with 1, 3 and 5 layers of MBs were 64, 73 and 91 pmol, respectively. However, the higher MB amount released from nanosensors with more layers of MB didn't mean the higher release percentage of MBs (Figure S6B). The nanosensors with 5 layers of MBs, which had the highest loading of MBs, exhibited the highest release amounts but the slowest release rate of the encapsulated MBs. These results suggest that all three nanosensors showed a controlled release during 40 days, especially for nanosensors with 5 layers of MBs, which allow us to longitudinally monitor the gene expression.

Cell labeling with mRNA magnetic nanosensors. For cell labeling, MSCs were treated with PEI-(MB-PEI)_n-IONPs (n=0, 1, 3 and 5) for 1 h with or without the magnet (MBs for targeting GAPDH mRNA was used). After removing the free nanoparticles with PBS, labelled MSCs were cultured in fresh medium for 24 h to allow the intracellular release of MBs from the internalized nanosensors. The released MBs then bound to GAPDH mRNA and restored the pre-quenched fluorescence. As shown in Figure 4A, regardless of the number of MB layers and magnetic field, all the labelled cell groups showed the red fluorescence, suggesting the successful cell labeling with the constructed nanosensors and the successful detection of cellular GAPDH mRNA. To investigate the internalization efficiency of encapsulated MBs, MSCs labelled with free MBs and PEI/MB_n with similar concentration of MBs in the nanosensors were used as the control. Compared with free MBs and PEI/MB complex, the fluorescence signals of MSCs labelled with PEI-(MB-PEI)_n-IONPs were much stronger (Figure S7A). Specifically, quantified by Image J, the MSCs labelled with PEI-(MB-PEI)_n-IONPs showed 3.4, 4.4 and 4.0 times GAPDH mRNA signals of those with corresponding free MB_n for n=1, 3 and 5, respectively. In the case of PEI/MBs, the MSCs labelled with magnetic nanosensors exhibited 3.4, 3.1 and 2.6 times GAPDH mRNA signals of those with corresponding PEI/MB_n for n=1, 3 and 5, respectively (Figure S7B). The good linear correlation between the normalized intensity from Image J and mean intensity from flow cytometry confirmed the improved signals with the constructed nanosensors (Figure S7C-D). These results suggested the higher internalization efficiency of MBs in the presence of magnetic IONP cores compared with free MBs and PEI/MB complex.

It is well-known that magnetofection certainly facilitates the accumulation of the magnetic vectors at the cell surface and thus improve the internalization efficiency.²⁶ Herein, the

effect of magnetic field on the GAPDH mRNA signals was also studied. In the presence of magnetic field, the signals of the MSCs labelled with PEI-(MB-PEI)-IONPs and PEI-(MB-PEI)₃-IONPs are stronger than those without magnet (Figure 4A). Quantified the intensity by using Image J, MSCs labelled with PEI-(MB-PEI)-IONPs and PEI-(MB-PEI)₃-IONPs in the presence of magnetic field exhibited 2.0 and 1.3 times GAPDH mRNA signals of those without magnet, respectively, whereas no significant differences in the GAPDH mRNA signals in MSCs labelled with PEI-(MB-PEI)₅-IONPs (Figure 4B). More importantly, MSCs labelled with PEI-(MB-PEI)₅-IONPs showed the strongest red fluorescence in the presence of magnetic field. Through quantifying the average cellular fluorescence intensity, MSCs labelled with PEI-(MB-PEI)₃-IONPs and PEI-(MB-PEI)₅-IONPs in the presence of magnetic field exhibited 2.3 and 2.7 times GAPDH mRNA signals of those labelled with PEI-(MB-PEI)-IONPs (Figure 4B), which could be ascribed to the higher MB amounts released from nanosensors with more layers of MBs (Figure S6A). Similar phenomena were observed from the MSCs labelled with nanosensors in the absence of magnetic field. Flow cytometry analysis further confirmed the enhanced signals from labelled MSCs in the presence of magnetic field and the higher fluorescence intensity from nanosensors with more layers of MBs regardless of magnetic field (Figure 4C and Table S2). The mean fluorescence intensities obtained from flow cytometry linearly correlated with the normalized intensities from Image J ($R^2=0.974$, Figure 4D). These results suggest that the mRNA magnetic nanosensors could be efficiently uptaken by MSCs under magnetic field regardless of layer numbers of nanosensors and the intracellular signal intensity was dependent on the layer numbers of the assembled MBs.

Figure 4. Fluorescence imaging of MSCs labelled with PEI-(MB-PEI)_n-IONPs nanosensors: (A) Representative fluorescence images of MSCs 24 hours post the treatment with PEI-(MB-PEI)_n-IONPs in the absence and presence of magnetic field. Scale bar: 100 μ m. (B) Normalized Intensity of GAPDH-Cy3 signals from MSCs labelled with PEI-(MB-PEI)_n-IONPs in the absence and presence of magnetic field. (C) Flow cytometry analysis of MSCs treated with PEI-(MB-PEI)_n-IONPs in the absence and presence of magnetic field: n=0 (grey curve), n=1 (green curve), n=3 (blue curve) and n=5 (red curve). (D) A correlation plot between mean intensity of labelled MSCs obtained from flow cytometry and normalized intensity obtained from Image J.

Longitudinal monitoring of the cellular expression of housekeeping genes. To demonstrate the capability of the layer-by-layer based magnetic nanosensors for longitudinally monitoring of the cellular gene expression, the house-keeping gene GAPDH mRNA was chosen as the model target mRNA. MSCs were treated with 30 μ g/mL GAPDH MBs-coated magnetic nanoparticles with different layer number of MBs for 1 hour under the magnetic field and the fluorescence images at different time points were taken. Figure 5A showed the longitudinal monitoring of

GAPDH mRNA in MSCs in one month period. As seen, the mRNA nanosensors with different layer numbers of MBs exhibited different signal persistence. The nanosensors with single-layer loading of MBs ($n=1$) remained only 52 % and 13 % of the initial signal on day 7 and day 21, respectively (Figure 5B). There was almost no signal on day 28 for the nanosensors with $n=1$. For the nanosensors with three layers of MBs ($n=3$), the signal dropped from 80 % on day 7 to 50 % on day 21, 35 % on day 28. Furthermore, it is exciting that the magnetic nanosensors assembled with five layers of MBs ($n=5$) exhibited the most stable signal and remained 97 %, 85 % and 69 % of the initial signal on day 7, day 14 and day 21, respectively. Even on day 28 and 31, there was still 50 % and 37 % of the initial signal remaining. Therefore, the half-life (the time it takes for half of the signal of internalized nanosensors to decay) of the layer-by-layer assembled nanosensors was estimated as to be one, three and four weeks for $n=1$, 3 and 5, respectively. The rate of signal attenuation should be: $n=1 > n=3 > n=5$. These results indicate that more layers of MB coating on the nanosensors enable slower signal attenuation and longer cell tracking.

Figure 5. (A) Representative bright-field and fluorescence images of MSCs labelled with GAPDH MBs-loaded magnetic nanosensors with different layers of GAPDH MBs (n=1, 3 and 5) at different time points. Scale bar: 100 μ m. (B) Normalized intensity of GAPDH-Cy3 signals

from MSCs labelled with GAPDH MBs-loaded magnetic nanosensors with different layers of MBs at different time points.

Magnetic nanosensors for monitoring the early osteogenic differentiation of MSCs. The layer-by-layer deposition methodology allows the incorporation of two or more kinds of MBs that target different mRNAs. As a proof-of-concept, MBs targeting ALP mRNA (early osteogenic biomarker) and GAPDH mRNA (the housekeeping gene) were mixed equally and assembled onto nanoparticles through the above method. The as-synthesized nanosensors with 5 layers of mixed MBs were then loaded into MSCs. In the following two weeks, MSCs showed a strong and stable red fluorescence signal from GAPDH MBs, representing the stable expression of GAPDH mRNA (Figure S6). However, the negligible green fluorescence signal from fluorescein-labelled ALP MBs could be observed, demonstrating that the level of ALP mRNA in undifferentiated MSCs was very low and the constructed MBs-based nanosensors showed the high specificity to the target mRNA.

When the osteogenic differentiation of the labelled MSCs was induced, the expression of ALP mRNA would be significantly changed compared with that in undifferentiated MSCs. As shown in Figure 6A, the green signals from MBs targeting ALP mRNA changed continuously during the 13 days while the red signals remained unchanged. The signal from ALP mRNA MBs was normalized based on that of GAPDH mRNA MBs to avoid the possible false positive signals from the non-specific intracellular degradation of MBs, although our previous studies have demonstrated that the non-specific MBs-loaded nanosensors generated negligible signals.^{27,9} As shown in Figure 6B (black squares), the normalized signal of ALP mRNA MBs

increased from day 3, reached a peak on day 9 with 1.8 times the intensity of day 3 and then declined after the peak, which was consistent with the character of the typical osteogenic differentiation in previous reports.²⁸ In addition, RT-qPCR was performed to confirm this observation (Figure 6B, blue columns). Fold change of ALP mRNA expression relative to GAPDH mRNA showed the similar fluctuation to the intensity ratios obtained from fluorescent images. ALP mRNA exhibited an expression peak on day 9 with 2.2 times the value of day 3. In addition, there was a good linear correlation ($R^2=0.877$) between the intensity ratios of ALP to GAPDH mRNA and the fold change obtained from RT-qPCR (Figure 6C). These results suggest that magnetic nanosensors could achieve the real-time, non-invasive and longitudinal monitoring of ALP mRNA expression during osteogenic differentiation with good sensitivity and specificity.

Figure 6. (A) Representative bright-field and fluorescent images of MSCs during the early stages of osteogenic differentiation at different time points. Green channel is the level of ALP mRNA detected by fluorescein-labelled ALP MBs signals and red channel presents the level of GAPDH mRNA detected by Cy3-labelled GAPDH MBs. Scale bar: 100 μ m. (B) Normalized intensity ratio of ALP-Flc signal to GAPDH-Cy3 signal (black squares, left axis) and corresponding fold change of ALP mRNA relative to GAPDH mRNA (blue columns, right axis) at different time points during the differentiation. *** $p < 0.0005$, ** $p < 0.001$ and * $p < 0.02$. (C) A correlation plot between normalized intensity ratio of ALP-Flc signal to GAPDH-Cy3 signal and fold change of ALP mRNA relative to GAPDH mRNA at different time points during the differentiation.

CONCLUSION

In summary, a smart magnetic nanosensor, based on the electrostatic interactions of MBs and PEI, has been constructed for non-invasive and longitudinal monitoring the cellular mRNA expression. The example of cellular GAPDH mRNA monitoring reveals that both intracellular signal intensity and tracking time are dependent on the layers of MBs on nanoparticles. More layers of MBs on nanosensors means higher loading of MBs, slower signal attenuation and eventually longer cell tracking period. In addition, this strategy allowed the incorporation of multiple types of MBs within the same nanosensors. For example, two kinds of MBs for targeting osteogenic biomarker ALP mRNA and housekeeping gene GAPDH mRNA respectively were embedded within nanosensors for monitoring the osteogenic differentiation of

MSCs. By quantifying the ratio of ALP mRNA MBs' fluorescence intensity to GAPDH mRNA MBs' fluorescence intensity, we could judge the status of MSC differentiation.

ASSOCIATED CONTENT

Supporting Information. Sequences of the involved MBs and primers in Table S1, flow cytometry analysis in Table S2, the calibration curve of MBs, analysis of cumulative loading amounts of MBs on nanosensors, cell viability testing of MSCs exposed to nanosensors, nuclease resistance testing of PEI/MBs, release profiles of MBs from nanosensors, and representative images of undifferentiated MSCs. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Specifically, M.W. and C.J.X. conceived the project and wrote the manuscript. M.W., X.C.H, L.S, C.W, and Z.J.X performed the experiments. M.W prepared the figures.

Notes

The authors declare no conflict of interest.

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REFERENCES

- (1) Santangelo, P. J.; Nix, B.; Tsourkas, A.; Bao, G. Dual FRET Molecular Beacons for mRNA Detection in Living Cells, *Nucleic Acids Res.* **2004**, *32*, e57-e57.
- (2) Peng, Z.; Young, B.; Baird, A. E.; Soper, S. A. Single-Pair Fluorescence Resonance Energy Transfer Analysis of mRNA Transcripts for Highly Sensitive Gene Expression Profiling in Near Real Time, *Anal. Chem.* **2013**, *85*, 7851-7858.
- (3) Nolan, T.; Hands, R. E.; Bustin, S. A. Quantification of mRNA Using Real-Time RT-PCR, *Nat. Protoc.* **2006**, *1*, 1559-1582.
- (4) Kang, W. J.; Cho, Y. L.; Chae, J. R.; Lee, J. D.; Choi, K. J.; Kim, S. Molecular Beacon-Based Bioimaging of Multiple MicroRNAs during Myogenesis, *Biomaterials* **2011**, *32*, 1915-1922.
- (5) Tay, C. Y.; Yuan, L.; Leong, D. T. Nature-Inspired DNA Nanosensor for Real Time In Situ Detection of mRNA in Living Cells, *ACS Nano* **2015**, *9*, 5609-5617.
- (6) Hong, B. J.; Eryazici, I.; Bleher, R.; Thaner, R. V.; Mirkin, C. A.; Nguyen, S. T. Directed Assembly of Nucleic Acid-Based Polymeric Nanoparticles from Molecular Tetravalent Cores, *J. Am. Chem. Soc.* **2015**, *137*, 8184-8191.
- (7) Qian, R.; Ding, L.; Yan, L.; Lin, M.; Ju, H. A Robust Probe for Lighting Up Intracellular Telomerase via Primer Extension To Open a Nicked Molecular Beacon, *J. Am. Chem. Soc.* **2014**, *136*, 8205-8208.
- (8) Wiraja, C.; Yeo, D. C.; Chew, S. Y.; Xu, C. Molecular Beacon-Loaded Polymeric Nanoparticles for Non-Invasive Imaging of mRNA Expression, *J. Mater. Chem. B* **2015**, *3*, 6148-6156.
- (9) Yeo, D.; Wiraja, C.; Chuah, Y. J.; Gao, Y.; Xu, C. A Nanoparticle-Based Sensor Platform for Cell Tracking and Status/Function Assessment, *Sci. Rep.* **2015**, *5*, 14768.

- (10) Chen, T.; Wu, C. S.; Jimenez, E.; Zhu, Z.; Dajac, J. G.; You, M.; Han, D.; Zhang, X.; Tan, W. DNA Micelle Flares for Intracellular mRNA Imaging and Gene Therapy, *Angew. Chem.* **2013**, *125*, 2066-2070.
- (11) Wu, Z.; Liu, G. Q.; Yang, X. L.; Jiang, J. H. Electrostatic Nucleic Acid Nanoassembly Enables Hybridization Chain Reaction in Living Cells for Ultrasensitive mRNA Imaging, *J. Am. Chem. Soc.* **2015**, *137*, 6829-6836.
- (12) Lee, J.; Moon, S. U.; Lee, Y. S.; Ali, B. A.; Al-Khedhairi, A. A.; Ali, D.; Ahmed, J.; Al Salem, A. M.; Kim, S. Quantum Dot-Based Molecular Beacon to Monitor Intracellular MicroRNAs, *Sensors* **2015**, *15*, 12872-12883.
- (13) Lin, L. S.; Cong, Z. X.; Cao, J. B.; Ke, K. M.; Peng, Q. L.; Gao, J. H.; Yang, H. H.; Liu, G.; Chen, X. Y. Multifunctional Fe₃O₄@ Polydopamine Core-Shell Nanocomposites for Intracellular mRNA Detection and Imaging-Guided Photothermal Therapy, *ACS Nano* **2014**, *8*, 3876-3883.
- (14) Dobson, J. Magnetic Nanoparticles for Drug Delivery, *Drug Dev. Res.* **2006**, *67*, 55-60.
- (15) Lee, J.; Jin, Y. A.; Ko, H. Y.; Lee, Y. S.; Heo, H.; Cho, S.; Kim, S. Magnetic Resonance Beacon to Detect Intracellular MicroRNA during Neurogenesis, *Biomaterials* **2015**, *41*, 69-78.
- (16) Lee, J.; Kang, H. J.; Lee, Y. S.; Heo, H.; Gu, H. N.; Cho, S.; Kim, S. A Self-Assembling Magnetic Resonance Beacon for the Detection of MicroRNA-1, *Chem. Commun.* **2015**, *51*, 7199-7202.
- (17) Min, J.; Braatz, R. D.; Hammond, P. T. Tunable Staged Release of Therapeutics from Layer-by-Layer Coating with Clay Barrier Interlayer, *Biomaterials* **2014**, *35*, 2507-2517.
- (18) Xu, Z.; Shen, C.; Hou, Y.; Gao, H.; Sun, S. Oleylamine as Both Reducing Agent and Stabilizer in a Facile Synthesis of Magnetite Nanoparticles, *Chem. Mater.* **2009**, *21*, 1778-1780.
- (19) Arsianti, M.; Lim, M.; Marquis, C. P.; Amal, R. Assembly of Polyethylenimine-Based Magnetic Iron Oxide Vectors: Insights into Gene Delivery, *Langmuir* **2010**, *26*, 7314-7326.
- (20) Cho, C. S. Design and Development of Degradable Polyethylenimines for Delivery of DNA and Small Interfering RNA: An Updated Review, *ISRN Mater. Sci.* **2012**, *2012*, 1-24.
- (21) van der Maaden, K.; Sekerdag, E.; Schipper, P.; Kersten, G.; Jiskoot, W.; Bouwstra, J. Layer-by-Layer Assembly of Inactivated Poliovirus and N-trimethyl Chitosan on pH-Sensitive Microneedles for Dermal Vaccination, *Langmuir* **2015**, *31*, 8654-8660.

- (22) Moghimi, S. M.; Symonds, P.; Murray, J. C.; Hunter, A. C.; Debska, G.; Szewczyk, A. A Two-Stage Poly(ethylenimine)-Mediated Cytotoxicity: Implications for Gene Transfer/Therapy, *Mol. Ther.* **2005**, *11*, 990-995.
- (23) Zintchenko, A.; Philipp, A.; Dehshahri, A.; Wagner, E. Simple Modifications of Branched PEI Lead to Highly Efficient siRNA Carriers with Low Toxicity, *Bioconjugate Chem.* **2008**, *19*, 1448-1455.
- (24) Rush, A. M.; Thompson, M. P.; Tatro, E. T.; Gianneschi, N. C. Nuclease-Resistant DNA via High-Density Packing in Polymeric Micellar Nanoparticle Coronas, *ACS Nano* **2013**, *7*, 1379-1387.
- (25) Wu, Y.; Phillips, J. A.; Liu, H.; Yang, R.; Tan, W. Carbon Nanotubes Protect DNA Strands During Cellular Delivery, *ACS Nano* **2008**, *2*, 2023-2028.
- (26) Shi, W.; Liu, X.; Wei, C.; Xu, Z. J.; Sim, S. S. W.; Liu, L.; Xu, C. Micro-Optical Coherence Tomography Tracking of Magnetic Gene Transfection via Au-Fe₃O₄ Dumbbell Nanoparticles, *Nanoscale* **2015**, *7*, 17249-17253.
- (27) Wiraja, C.; Yeo, D. C.; Chong, M. S. K.; Xu, C. Nanosensors for Continuous and Non-invasive Monitoring of Osteogenic Differentiation of Mesenchymal Stem Cells, *Small* **2016**, DOI: 10.1002/smll.201502047.
- (28) Birmingham, E.; Niebur, G.; McHugh, P. Osteogenic Differentiation of Mesenchymal Stem Cells is Regulated by Osteocyte and Osteoblast Cells in a Simplified Bone Niche, *Eur. Cells Mater.* **2012**, *23*, 13-27.

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