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The stimulatory effect of silica nanoparticles on osteogenic differentiation of human mesenchymal stem cells

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Keywords: silica nanoparticles, human mesenchymal stem cells, osteogenic differentiation, cellular uptake, bone tissue engineering

Supplementary material for this article is available online

Abstract
Silica-based materials with favourable biocompatibility are generally considered as excellent candidates for applications in biomedical fields. However, previous researches mainly focused on the safety of silica-based materials, their effects on osteogenic differentiation of human mesenchymal stem cells (hMSCs) still need further investigations. In this study, core–shell fluorescent silica nanoparticles (silica NPs) with three different sizes (S1 ~ 50 nm, S2 ~ 200 nm, S3 ~ 400 nm, respectively) were prepared according to the Stöber method. The silica NPs with different sizes did not affect the cell viability (even up to a concentration of 500 µg ml⁻¹), showing size- and dose-independent cytocompatibility of silica NPs on hMSCs. Uptake of silica NPs significantly enhanced the activity of alkaline phosphatase (ALP) and the formation of bone-like nodules of hMSCs after osteogenic induction. At the concentration of 10 µg ml⁻¹, after treating hMSCs with larger sized silica NPs (S2 and S3), higher ALP activity of hMSCs was measured and larger sized bone-like nodules were formed by hMSCs compared with that treated with smaller sized silica NPs (S1). The enhanced osteogenic potential of hMSCs treated with silica NPs may be attributed to the Si released from silica NPs due to the lysosomal degradation inside hMSCs. These results demonstrate the stimulatory effect of silica NPs on osteogenic differentiation of hMSCs and the application potential of silica NPs in bone tissue engineering.

1. Introduction
In the 1970s, silicon (Si) was reported to play a significant role in bone repair and skeletal development [1, 2]. As an important trace element in human body, Si has been found to be beneficial to bone health and defined as a safe agent by the US Food and Drug Administration (FDA) [3, 4]. Si, located at the active calcification sites of bone, plays an important role in the mineralization process during bone growth [4]. Due to the biological function of Si, Si-containing biomaterials such as Si-substituted calcium phosphates [5], Si-based bioceramics [6, 7] and bioglasses [8] have been widely investigated in the past few decades. These materials have been reported to have positive effects on the proliferation and differentiation of osteoblasts.

Recently, thanks to the advances in fabrication methods, silica nanoparticles have emerged as a novel biomaterial for the applications in bone tissue engineering and drug delivery. Neumann et al found that BMP2-loaded silica NPs could promote osteogenic differentiation of adipose tissue-derived mesenchymal stem cells (adMSC) via enhancing the mineralization [3]. Shi et al investigated the effect of dimethyloxaloylglycerine- (DMOG, a hypoxia-inducing therapeutic drug) loaded silica NPs on osteogenesis and angiogenesis of hMSCs, their results showed that DMOG-loaded silica NPs stimulated both osteogenic and angiogenic
differences of hMSCs as indicated by the enhanced ALP activity, expression of bone-related genes/proteins and vascular endothelial growth factor (VEGF) secretion [9]. Similarly, BMP7-loaded silica NPs facilitated the proliferation and enhanced the osteogenesis-related proteins expression of hMSCs [10]. Collectively, these studies indicate that the combination of silica NPs with some bioactive factors or functional drugs could stimulate the osteogenic differentiation of stem cells. However, silica NPs were used only as carriers for biological factors, it is difficult to elucidate the exact role of silica NPs in the process of osteogenesis. It is therefore of great interest to specifically investigate the osteogenic effect induced by silica NPs themselves. Understanding the effect of silica NPs on osteogenic differentiation of stem cells is of great significance for their applications in bone tissue engineering.

Silica can be easily prepared with different sizes and shapes in both nano- and submicron-scales. Previous studies have found that the biocompatibility of silica NPs is related to their physical characteristics such as shape and size [11, 12]. Ye et al. evaluated the cytotoxicity of silica NPs with the sizes of 21 and 48 nm using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays. The results showed that silica NPs could be cytotoxic to myocardial cells in size and dose (0.1–1.6 mg ml$^{-1}$) dependent manners [13]. Jin et al. systematically investigated the cytotoxicity and genotoxicity of 50 nm silica NPs to human lung epithelial cells (A549 cells) by detecting the integrity of the DNA and the cell proliferation. The uptake of silica NPs did not cause significant cytotoxicity below a concentration of 0.1 mg ml$^{-1}$ [14]. Huang et al. found that exposure to 110 nm silica NPs at the concentration range of 4–200 $\mu$g ml$^{-1}$ had no effect on the cell viability, proliferation and osteogenic differentiation of hMSCs as analyzed by MTT and ALP activity assays [15]. Additionally, it has been reported that 50 nm silica NPs could promote differentiation and mineralization of osteoblasts [16–18]. Taken together, the current data related to biological effect of silica NPs are controversial, the different effects of silica NPs on cellular response in these studies may be caused by the difference in particle sizes. To the best of our knowledge, there are few studies investigating the effect of silica NPs with different concentrations and sizes on the cell viability and mineralization of hMSCs. For these reasons, the objective of this study is to investigate the effect of silica NPs on osteogenic differentiation of hMSCs and the dose- and size-effect on the in vitro performance of silica NPs.

In this study, the core-shell fluorescent silica NPs with three different sizes were successfully prepared. We investigated the effect of silica NPs with different sizes and concentrations on cell viability and osteogenic differentiation of hMSCs by staining the mineralized nodules and measuring ALP activity. The intracellular Si ions concentration in silica NPs-treated hMSCs was measured. It is expected that this study will provide a reference for the application of silica NPs combined with stem cells in bone tissue engineering.

2. Materials and methods

2.1. Preparation and characterization of silica NPs

Core–shell fluorescent silica NPs with three different sizes (~50 nm, ~200 nm, ~400 nm, designated herein as S1, S2 and S3, respectively) were synthesized according to the Stöber method [19, 20]. The nanoparticles comprise a solid silica shell (SiO$_2$) doped with the fluorescein isothiocyanate (FITC) as described before [21–23]. Briefly, 2 ml of absolute ethanol, 24 $\mu$l of (3-aminopropyl)triethoxysilane (APS) and 10.6 mg of FITC were mixed firstly with stirring for 16 h to get solution A. Afterwards, 0.5 ml of solution A and 1.65 ml of tetraethyl orthosilicate (TEOS) were added to solution B (premixed ethanol and ammonia) with slow stirring for 24 h. Finally, the nanoparticles were collected by centrifugation, washed twice with deionized water and dried at 50 °C overnight. In this study, the silica NPs with three different sizes were prepared by changing the volume of absolute ethanol and aqueous ammonia (Table 1). As the amount of ammonia increased, the nucleation number in the system reduced, followed by an increase of the particle size [24].

The size and morphology of silica NPs were characterized by transmission electron microscopy (TEM, Tecnai G20, FEI, USA). The specific surface area measurement of silica NPs was performed on Quantachrome Instruments Autosorb-IQ2-MP (USA) using the Brunauer–Emmett–Teller method. To further determine the hydrodynamic diameter of S1, S2 and S3 in basal medium (Cyagen Biosciences Inc, USA) with 10% fetal bovine serum (FBS), dynamic light scattering (DLS) measurement was performed on Malvern Instruments Zetasizer Nano ZS90 (UK). The zeta potential of S1, S2 and S3 was measured using the same device.

2.2. Cell culture and silica NPs treatment

hMSCs, purchased from Cyagen Biosciences Inc (USA), were expanded in a basal medium (Cyagen Biosciences Inc, USA) supplemented with 10% FBS, 1% glutamine and 1% penicillin-streptomycin (growth medium, GM) at 5% CO$_2$, 37 °C. The medium was changed every 2 d. Upon reaching approximately 80–90% confluence, the cells were passaged and further expanded. The cells at 4–6th passage were used in this study.

Before incubated with the cells, a certain weight of silica NPs were sterilized by $^{60}$Co γ-irradiation at a dose of 110 nm silica NPs at the concentration range of 4–200 µg ml$^{-1}$ had no effect on the cell viability, proliferation and osteogenic differentiation of hMSCs as analyzed by MTT and ALP activity assays [15]. Additionally, it has been reported that 50 nm silica NPs could promote differentiation and mineralization of osteoblasts [16–18]. Taken together, the current data related to biological effect of silica NPs are controversial, the different effects of silica NPs on cellular response in these studies may be caused by the difference in particle sizes. To the best of our knowledge, there are few studies investigating the effect of silica NPs with different concentrations and sizes on the cell viability and mineralization of hMSCs. For these reasons, the objective of this study is to investigate the effect of silica NPs on osteogenic differentiation of hMSCs and the dose- and size-effect on the in vitro performance of silica NPs.

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<table>
<thead>
<tr>
<th>Particle</th>
<th>TEOS (ml)</th>
<th>EtOH (ml)</th>
<th>NH$_3$ (ml)</th>
<th>APS-FITC (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>1.65</td>
<td>0.5</td>
<td>39.625</td>
<td>2.125</td>
</tr>
<tr>
<td>S2</td>
<td>1.65</td>
<td>0.5</td>
<td>38.55</td>
<td>3.2</td>
</tr>
<tr>
<td>S3</td>
<td>1.65</td>
<td>0.5</td>
<td>33.23</td>
<td>8.3</td>
</tr>
</tbody>
</table>

The volume of chemical reagents used for preparing silica NPs.
of 20 kGy. After sterilization, silica NPs were firstly dispersed in GM at a concentration of 2.5 mg ml$^{-1}$, then the suspension was diluted to the tested concentrations (10, 50, 100, 250 and 500 µg ml$^{-1}$).

### 2.3. Assessment of the internalization of silica NPs

TEM and confocal-laser scanning microscopy (CLSM) were employed to observe the internalization of silica NPs. For TEM observation, the cells were seeded in a six-well plate at a density of 5 × 10^4 cells per well. After incubated with 100 µg ml$^{-1}$ of NPs for 24 h, hMSCs were washed three times with phosphate buffered saline (PBS, 0.01 M). Then they were harvested with trypsin and fixed in 2.5% glutaraldehyde at 4 °C for 24 h. The post-fixation was performed using 1% osmium tetroxide in 0.1 M cacodylate buffer at 4 °C for 1 h. After that, hMSCs were dehydrated stepwise in ethanol and then embedded in epoxy resin. Ultrathin sections of 70 nm were sectioned with a microtome. The cells were then washed three times with PBS, permeabilized with 0.1% Triton X-100 (Sigma) and blocked for non-specific stain with 1% bovine serum albumin (BSA). Thereafter, the cells were stained with 5 µg ml$^{-1}$ of 4′, 6-diamidino-2-phenylindole (DAPI, Roche) at room temperature for 10 min. The stained samples were visualized and photographed with a CLSM (LSM710, CarlZeiss, Germany).

For CLSM observation, the cells were seeded on glass coverslips in a 24-well plate at a density of 1 × 10^4 cells per well and incubated with 100 µg ml$^{-1}$ of silica NPs for 24 h. After washed three times with PBS, hMSCs were fixed in 4% paraformaldehyde for 15 min at room temperature. The cells were then washed twice with PBS, permeabilized with 0.1% Triton X-100 (Sigma) for 5 min and blocked for non-specific stain with 1% bovine serum albumin (BSA). Thereafter, the cells were stained with 5 µg ml$^{-1}$ of phalloidine-TRITC (Sigma) for 40 min, and cell nuclei were stained with 4 µg ml$^{-1}$ of 4′, 6-diamidino-2-phenylindole (DAPI, Roche) at room temperature for 10 min. The stained samples were visualized and photographed with a CLSM (LSM710, CarlZeiss, Germany).

### 2.4. Cytotoxicity assays

The in vitro acute cytotoxicity of silica NPs was assessed using the LDH and Cell Count Kit-8 (CCK-8, Dojindo) assays. The cells were seeded in 48-well plates at a density of 5 × 10^4 cells per well and incubated with different concentrations (10, 50, 100, 250 and 500 µg ml$^{-1}$) of silica NPs for 24 h. LDH testing kit (Jiancheng, China) was used to detect the amount of released LDH. Following the protocol, 20 µl of culture medium from each well and the LDH reaction solution were transferred into a new 96-well plate. After incubation, the absorbance of the solution was measured at 450 nm using a microplate reader (PerkinElmer, USA). For CCK-8 assay, briefly, 200 µl fresh culture medium containing 20 µl CCK-8 was added to each well and then incubated at 37 °C for 3 h. Afterwards, 100 µl of the solution was transferred into a new 96-well plate and the absorbance (OD) was measured at 450 nm using the same microplate reader.

On day 7 and 14 after osteogenic induction, CCK8 assay was also conducted to evaluate the effect of silica NPs on cell viability of hMSCs.

### 2.5. Osteogenic induction

The osteogenic medium (OM) consists of growth medium (GM), 10^{-7} M dexamethasone, 10 mM β-glycerophosphate disodium and 50 µg ml$^{-1}$ ascorbic acid. After the cells were treated with silica NPs for 24 h, the culture medium was changed to osteogenic medium, which was changed every 3 d.

### 2.6. ALP activity assay

ALP activity of hMSCs was assessed using a kit purchased from Jiancheng (China). The cells were seeded in 48-well plates with a density of 5 × 10^3 cells per well. On day 7, 14 and 21 after osteogenic induction, ALP activity was measured using the method as described before [25]. Briefly, 1 µl of 1 mM phenylmethanesulfonyl fluoride (PMSF) in 100 µl RIPA lysis buffer (Beyotime, China) was added to each well for 15 min. The lysate was pre-centrifuged at 12000 rpm for 10 min at 4 °C and analyzed following the manufacturer’s protocol. For normalization, the total content of cell lysate protein was measured by a bicinchoninic acid (BCA) assay kit (Aidlab, China).

### 2.7. Alizarin Red S Staining

To identify the mineralized nodule formation, Alizarin Red S Staining was performed at day 21 after osteogenic induction. The cells were fixed using 4% paraformaldehyde for 30 min, washed twice with PBS and stained with the solution of 1% Alizarin Red S (AR-S) at pH 4.2 for 15 min, the stained cells were photographed under a phase-contrast microscope (Leica, Germany). In order to quantify the orange–red complex of AR-S, 500 µl of 10% (w/v) cetylpyridinium chloride monohydrate dissolved in sodium phosphate buffer (10 mM, pH = 7) was added to each well. After 15 min of incubation at 37 °C, 100 µl of each sample was transferred to a new 96-well plate and the absorbance (OD) of the solution was measured using a microplate reader (PerkinElmer, USA) at 570 nm.

### 2.8. The degradation of silica NPs in hMSCs

To investigate the intracellular degradation behavior, the Si ions concentration of cell lysate was measured by inductively coupled plasma atomic emission spectrometer (ICP-AES). The cells were seeded in a 48-well plate with a density of 5 × 10^3 cells per well and incubated with 100 µg ml$^{-1}$ of silica NPs for 24 h. On day 7, 14 and 21 after osteogenic induction, the cells were lysed using the same method mentioned at section 2.6. The lysate was centrifuged at 12000 rpm for 20 min to get supernatant for ICP-AES measurement. Moreover, on day 14, the Si concentration in the lysate of hMSCs cultured in GM without osteogenic induction was also measured.

### 2.9. Statistical analysis

All results were expressed as the mean ± standard deviation (SD). Statistical analysis of the data was performed using SPSS 15.0.1 by one-way analysis of
variance (ANOVA) followed by the least significant difference (LSD) method and the $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Characterization of silica NPs

The size and uniformity of silica NPs are two important factors for biological applications [21]. In this study, silica NPs with three different sizes (S1, S2 and S3) were synthesized and characterized. The size and morphology of silica NPs were exhibited by TEM images. As shown in figures 1(A), (C) and (E), all the silica NPs are spherical in shape and the average diameter is ~50 nm (S1), ~200 nm (S2), ~400 nm (S3), respectively. The core–shell structure can be clearly observed in figures 1(B), (D) and (F). The average diameter of the core and shell of silica NPs was shown in table 2. The hydrodynamic diameter of S1, S2 and S3 was 164 ± 76, 269 ± 73 and 388 ± 18 nm, respectively, showing good monodisperse stability of S2 and S3 in culture medium. The zeta potential and specific surface area of silica NPs were also shown in table 2.
3.2. Cellular uptake of silica NPs

The internalization of silica NPs in hMSCs was visualized by TEM and CLSM. Figure 2 presents the TEM images of hMSCs after exposure to 100 µg ml⁻¹ silica NPs in GM for 24 h. It can be seen that the silica NPs were aggregated and existed in some vesicles, possibly the endosomes or lysosomes (figures 2(D)–(F)). After internalized through endocytosis, the silica NPs were finally transported into the lysosomes where the degradation happened [26, 27]. The diameter of the S1, S2 and S3 is further confirmed in higher magnification in figures 2(D)–(F). Ha et al also observed the cellular uptake of 50 nm silica NPs by TEM and found that the silica NPs existed in autophagosomes and autolysosomes [17]. In addition, the aggregation size of S1 is generally larger than that of S2 and S3 (figures 2(A)–(C)), which may be related to the smaller size of S1.

In this study, the silica NPs were labeled with FITC, so CLSM was used to observe the internalization of silica NPs. As shown in figure 3, the internalization of the labeled silica NPs is confirmed by the CLSM images. At the concentration of 100 µg ml⁻¹, the silica NPs with green fluorescence were internalized, accumulated along the F-actin fibers and widely distributed in cytoplasm around the cell nucleus. The result is similar to
Luo’s work, in which the internalization and the distribution of 400 nm mesoporous silica nanoparticles (MSNs) were also clearly observed by CLSM at the concentration of 100 µg ml\(^{-1}\) [10]. Furthermore, from the CLSM images, it could be seen that the hMSCs displayed a healthy morphology, i.e. spindle shape, indicating internalization of silica NPs did not affect the spreading of hMSCs.

### 3.3. The degradation behaviour of silica NPs in hMSCs

The degradation behaviour of silica NPs was investigated using TEM and ICP-AES (figure 4). As shown in figures 4(A)–(D), S3 were internalized and finally transported into the lysosomes where they were degraded. The outline of the S3 became rougher in lysosome (figures 4(B)–(D)) compared with that of original S3 (figures 1(E) and (F)). As a major degradative organelle, the lysosome contains a series of hydrolases, which are adequate to degrade the endocytosed substrates at the acidic environment inside lysosome [28]. The Si ions concentration of cell lysate was measured by ICP-AES (figures 4(E) and (F)). In OM, the Si ions concentration decreased significantly over time from 7.55 ± 0.66 (day 7) to 2.11 ± 0.56 µg ml\(^{-1}\) (day 21) when S1 was added to the cells. While, the Si ions concentration maintained a high level (>7 µg ml\(^{-1}\)) in the lysate of S2 and S3 treated groups. On day 21, the Si ions concentration even reached 12.65 ± 1.13 µg ml\(^{-1}\) (12.65 ± 1.13% of the initial silica NPs concentration) in the lysate of S2 treated group. There was significant difference in Si ions concentration between larger sized particles (S2 and S3) and smaller sized particles (S1) on day 14 and 21. Furthermore, the concentration of Si ions released from S2 and S3 was also higher than that released from S1 in GM on day 14. All these findings demonstrate that silica NPs degrade slowly over time in intracellular environment. Shi et al investigated the in vitro degradation behaviour and the uptaken amount of 90 nm MSNs at concentrations of 31.25, 125 and 500 µg ml\(^{-1}\). The Si concentration in the medium reached about 40% of the initial MSNs concentration.
after 7 d soaking. While, the uptaken percentage of MSNs was very low (only 0.932% at the concentration of 500 µg ml$^{-1}$ and 2.4% at 31.25 µg ml$^{-1}$) [9]. In present study, according to the Si ions concentration in cell body on day 7, the uptaken percentage of silica NPs is higher than 7% at the concentration of 100 µg ml$^{-1}$. The different uptaken percentage of MSNs and silica NPs may be caused by their different physicochemical properties, including size, specific surface area, aggregation, etc. The intracellular concentration of Si ions is related to uptaken amount and degradation rate of silica NPs. Larger aggregation size of nanoparticles can result in lower uptaken amount and degradation rate. This may be the reason to explain the pronounced difference in concentration of Si ions between larger sized particles (S2 and S3) and smaller sized particles (S1) treated hMSCs.

3.4. The cytotoxicity of silica NPs

The biocompatibility of nanoparticles is the prerequisite for their biomedical applications. In order to determine the in vitro acute cytotoxicity of silica NPs, both CCK-8 (figure 5(A)) and LDH (figure 5(B)) testing were used after hMSCs were treated with different concentrations (0, 10, 50, 100, and 500 µg ml$^{-1}$) of silica NPs for 24 h. The cells cultured in GM without particle treatment were considered as control (100% cell viability for CCK-8 assay, 100% LDH release for LDH assay). Values are expressed as mean ± SD. (n = 3 for each sample). No significant differences ($p > 0.05$) compared with control group were found for all the tested conditions.

Figure 5. Cell viability detected by CCK-8 assay (A), the amount of released LDH detected by LDH assay (B). The hMSCs were treated with different concentrations (0, 10, 50, 100, 250 and 500 µg ml$^{-1}$) of silica NPs for 24 h. Values are expressed as mean ± SD. (n = 3 for each sample). No significant differences ($p > 0.05$) compared with control group were found for all the tested conditions.

In present study, the data of CCK-8 and LDH leakage in present study. LDH, a stable enzyme present in all cells, is released after the plasma membrane is damaged [31, 32]. As an important assay for cytotoxicity, LDH assay is used to indirectly quantify plasma membrane damage [31, 33]. Figure 5(B) indicates that the LDH level in media remains unaffected after silica NPs treatment in all groups. The LDH release result is in accordance with the cell viability data determined by CCK-8, providing another evidence of cytocompatibility of silica NPs in the concentration and size range used in this study. Overall, the used silica NPs showed no cytotoxicity on hMSCs at concentrations lower than 500 µg ml$^{-1}$.

Many previous reports have shown that several factors of nanoparticles may induce cytotoxicity, including the inherent chemical composition, size, shape and location of nanoparticles [15]. For example, the silver NPs can cause generation of intracellular reactive oxygen species, which may result in the cytotoxicity of silver NPs, even at a very low concentration [34, 35]. Napierska et al investigated the cytotoxicity of silica NPs with different sizes (~16, ~19, ~60, ~104 and ~335 nm) on human endothelial cells (EAHY926 cell line) and found that bigger particles showed significantly lower cytotoxicity than the smaller particles [36]. Huang et al showed that three types of silica NPs (sphere, short rod, long rod) were nontoxic even at concentration of 1 mg ml$^{-1}$ and the spherical particles had lower cytotoxicity than the other two shapes [30]. Furthermore, the sterilization method may influence the cytotoxicity of NPs. The γ-irradiation is one of the most effective and common choices employed for biomaterial sterilization [37]. In present study, the data of CCK-8 and LDH leakage showed that the silica NPs is noncytotoxic even up to 500 µg ml$^{-1}$, proving the cytocompatibility of silica NPs in the given size and dose range on hMSCs. Based on the result of cytotoxicity, the concentrations of 0, 10, 100 and 250 µg ml$^{-1}$ were selected for further experiments.

The hMSCs were exposed to the selected concentrations (0, 10, 100 and 250 µg ml$^{-1}$) of silica NPs for 24 h and then induced into osteogenic differentiation. The
cell viability on day 7 and 14 are shown in online supplementary figure S1 (stacks.iop.org/BMM/12/015001/mmedia). The cell viability is higher than 95% at the selected concentrations. It can be seen that S1, S2 and S3 have no effect on the cell viability of hMSCs at 10, 100 and 250 µg ml⁻¹ after long time incubation.

3.5. The effect of Silica NPs on cell differentiation

Two methods (ALP activity assay and Alizarin Red S Staining) were employed to estimate the influence of silica NPs on the osteogenic differentiation of hMSCs. The cells without particles treatment were considered as control.

ALP is an early marker for osteogenic differentiation. ALP, osteocalcin (OCN) and osteopontin (OPN) have been reported to support the formation of bone-like nodules which are used for further matrix mineralization during osteogenesis process [4, 31, 38]. The main physiological function of ALP in the body is the hydrolysis of phosphate esters in the process of osteogenesis, which provides the necessary phosphate groups for the deposition of hydroxypatite. In general, an increase in ALP activity will be detected during the early stage of osteogenic differentiation. The ALP activity on day 7, 14 and 21 after osteogenic induction is shown in figure 6. It could be seen that ALP activity increased from day 7 to 14 and then decreased at 21 d. The upregulation of ALP activity was significantly enhanced due to the addition of silica NPs, indicating that silica NPs had positive effect on the osteogenic differentiation of hMSCs. The same trend of ALP activity was also reported in Gaharwar’s work [31], an increase in ALP activity of hMSCs incubated with silicate nanoplates (1, 10 and 100 µg ml⁻¹) was observed with a peak at day 14. Moreover, at concentration of 10 µg ml⁻¹, higher ALP activity of hMSCs was measured after the treatment with larger sized NPs (S2 and S3) compared with the smaller sized group (S1). This indicates that the addition of silica NPs increases ALP activity.

Then, we investigated the mineralization of hMSCs after 21 d of osteoinduction using the Alizarin Red S Staining, which is generally employed to confirm the presence of nodules and calcium deposition [3, 10]. At the late stage of osteogenic differentiation, another critical indicator for osteogenic efficiency of hMSCs is the formation of calcium nodules, which are the basis for further matrix mineralization [9]. Alizarin Red stain can specifically bind to highly enriched calcium deposits to form salt and chelate [39]. The colour of the production is orange red, which is easily distinguished. The hMSCs incubated with S1, S2 and S3 at 10, 100 and 250 µg ml⁻¹ formed large amount of bone-like nodules in OM (figure 7(A) and supporting information, figure S2). While, although with S1–S3 treatment, there were almost no bone-like nodules formed by the cells in GM without osteoinduction (figures not shown). Interestingly, at relatively lower concentration (10 and 100 µg ml⁻¹), the cells formed larger sized bone nodules with S2, S3 treatment compared with that treated with S1 group. The quantitative result of the orange–red colouration of Alizarin Red S is shown in figure 7(B). The silica NPs significantly enhanced the production of mineralized matrix of hMSCs in OM, but had no effect on that in GM (figure 7(B) and supporting information, figure S2). These results indicate that the presence of silica NPs can enhance the formation of bone-like nodules of hMSCs only after osteoinduction and it has no effect on the differentiation of hMSCs into osteoblast lineage.

As mentioned above, we analyzed the effect of silica NPs on both early and late marker of osteogenic
differentiation of hMSCs. The data clearly show that silica NPs can promote osteogenic differentiation of hMSCs in OM with the presence of dexamethasone, β-glycerophosphate disodium and ascorbic acid.

As a trace element, Si is beneficial to bone health and plays an important role in the growth of human bone [40]. Si has been widely incorporated into biomaterials to enhance their osteogenic potential. Many previous studies have focused on investigating the osteogenic potential of Si-containing biomaterials [3, 4, 9, 17, 41]. Gaharwar et al reported that synthetic silicate nanoplatelets (~25 nm in diameter) based on Laponite was cytocompatible at concentrations lower than 1 mg ml⁻¹, the addition of silicate nanoplatelets significantly increased the ALP activity and the production of the osteocalcin (OCN), osteopontin (OPN), runt-related transcription factor 2 (RUNX2) and mineralized nodules of hMSCs [31]. Reffitt et al found that orthosilicic acid (H₄SiO₄) could stimulate the synthesis of collagen type I (Col I) and improve ALP activity of human osteoblast-like cells [42]. Similarly, Zhou et al investigated the effect of ionic products from Ca₇Si₂P₂O₁₆ ceramic on proliferation and differentiation of periodontal ligament cells (PDLCs) and found that the ionic products significantly stimulated the proliferation, ALP activity, Ca deposition and osteogenesis related gene expression (ALP, Col I, RUNX2) of PDLCs [41]. These results prove that Si ions may play a key role in the osteogenic process. Several studies have investigated possible mechanisms for the positive effect of Si ions on differentiation of bone-related cells. For instance, Gu et al found that the ionic products from akermanite (Ca₂MgSi₂O₇) significantly enhanced the osteogenic differentiation of human adipose-derived stem cells (hASCs) via activation of the ERK pathway [43]. Similarly, it has been reported that Si ions at the concentration of 4 mM can activate the ERK pathway in MG63 cells [44] and the ionic products of Ca₂SiO₄ coating enhanced the differentiation and collagen production of MG63 via the TGF-β1 pathway [45]. Another possible explanation might be that Si ions significantly enhanced the mineralization, proliferation and differentiation of hMSCs through WNT and SHH signaling pathways [4]. Silica NPs have been shown to stimulate

![Figure 7](image-url)
osteogenic differentiation of hMSCs through enhancing mineralization and increasing the expression of ALP, bone sialoprotein (BSP) and CoI [3, 16, 46]. It has been reported that the internalization of MSNs into hMSCs induced actin polymerization and activated the small GTP-bound protein RhoA, which was believed to cause osteogenesis of hMSCs [15]. Similarly, Ha et al found that 50 nm silica NPs entered the osteoblast through a caveolae-mediated endocytosis and promoted mineralization and differentiation of osteoblast through activating the mitogen activated protein kinase ERK1/2 (a protein associated with cell growth and proliferation) and stimulating autophagy by directly binding with LC3 and p62 (two key proteins involved in autophagosome formation) [17]. In present study, we studied the degradation behaviour and observed the internalization of silica NPs. Considering the high uptaked percentage of silica NPs and the high concentration of Si ions in intracellular matrix, we believed that both Si ions and silica NPs had beneficial influence on the osteogenic differentiation of hMSCs which were committed to osteoblast. The internalization of silica NPs caused actin fiber stress and affected the cellular mechanical properties, which stimulated the osteogenic differentiation of hMSCs [15, 47]. Cellular mechanical properties have been shown to regulate cell growth, proliferation and differentiation, playing a very important role in cellular metabolism [47]. After internalization, the silica NPs degraded and released Si ions, which might enhance the mineralization and differentiation of hMSCs through WNT and SHH signaling pathways [4]. Taken together, these may be possible explanations for the obtained results in this study. The positive effect of silica NPs on hMSCs was clearly confirmed in this study, offering additional evidence to explain the osteostimulation properties of silica and Si-containing biomaterials. However, the exact mechanism of silica NPs to promote osteogenesis still requires further research. In any case, silica NPs combined with stem cells may be a suitable tool for bone regeneration.

4. Conclusions

In summary, the core–shell fluorescent silica NPs with three sizes (~50 nm, ~200 nm, ~400 nm) were successfully prepared and the effect of silica NPs on cell viability and osteogenic differentiation of hMSCs was investigated. TEM and CLSM results confirmed the cellular uptake of silica NPs. At the given concentrations (0, 10, 50, 100, and 500 μg m⁻²), the silica NPs with three different sizes did not cause cytotoxicity and significantly enhanced the bone-related protein (ALP) expression and bone-like nodule formation of hMSCs. Results presented herein suggest the stimulatory effect of silica NPs themselves on hMSCs differentiation. Based on the positive results, silica NPs can promote osteogenic differentiation of hMSCs and may be applied in the form of silica NPs/polymer composite scaffolds in bone tissue engineering.

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