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Bioactive Silicate Nanoplatelets for Osteogenic Differentiation of Human Mesenchymal Stem Cells

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With an aging U.S. population, the occurrence of injuries and degenerative conditions are subsequently on the rise. As a direct result, there is an increase in demand for therapies that are able to repair damaged tissues and produce replacement organs.^[1] In particular, there is a great need for new bioactive materials that can direct stem cell differentiation and facilitate the formation of functional tissues.^[2] Several types of bioactive material that have clinical relevance have been reported for musculoskeletal tissue engineering in the last few years, including bioactive glasses ($\text{Na}_2\text{O}-\text{CaO}-\text{SiO}_2-\text{P}_2\text{O}_5$), hydroxyapatite (HA) ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), β -tricalcium phosphate (TCP) ($\text{Ca}_3(\text{PO}_4)_2$), β -wollastonite ($\text{CaO}-\text{SiO}_2$), and A-W (Apatite-Wollastonite) glass ceramic.^[3] Difficulties persisting with many of these known materials include lack of osteoinductive properties, poor processing abilities, and insufficient degradation.

Although, demineralized bone matrix (DBM) has been identified as an alternative to autografts (gold standard), its clinical application has been limited by its batch to batch variability due to its biological nature, immunogenicity, and high production costs.^[4]

Recently, bioactive nanomaterials have emerged as the next generation of advanced materials for biotechnological and biomedical applications due to their enhanced surface interactions. Synthetic silicate nanoplatelets have shown promise in developing a strong matrix,^[5] high-performance elastomers,^[6] superhydrophobic surfaces,^[7] super-barrier thin films,^[8] flame-retardant materials,^[9] mouldable hydrogels,^[10] hierarchical structures,^[11] and drug delivery devices.^[12] Although the above-mentioned reports have investigated synthetic silicates for a range of applications; the interaction of synthetic silicate nanomaterial with biological tissue at cellular levels has not yet been taken into consideration. Here, we present bioactive nanoparticles based on synthetic silicate (Laponite ($\text{Na}^+_{0.7}[(\text{Mg}_{5.5}\text{Li}_{0.3})\text{Si}_8\text{O}_{20}(\text{OH})_4]^-_{0.7})$), which is cytocompatible and promotes *in vitro* osteogenic differentiation of human mesenchymal stem cells (hMSCs) in the absence of any osteoinductive factor such as bone morphogenetic proteins-2 (BMP-2) or dexamethasone. To our knowledge, this is the first study showing that silicate nanoplatelets can induce osteogenic differentiation of hMSCs. The impetus for introducing this material for biological applications is due to the urgent unmet needs for bioactive materials for therapeutic applications, in the field of regenerative medicine.

Synthetic silicates are plate-like polyions composed of simple or complex salts of silicic acids with a heterogeneous charge distribution and patchy interactions.^[13] Synthetic silicate forms a physical gel at higher concentrations (40 mg/mL) due to electrostatic and van der Waals interactions, which result in the formation of a “house of cards” structure.^[14] This is attributed to the discotic charged nature and patchy interactions of the silicate nanoplatelets.^[15] Due to the strong physical interaction between silicate nanoplatelets, they have been extensively used for various commercial and industrial applications such as food additives, filler materials (glass, ceramics, refractories), catalysts, adsorbents and anticaking agents, but have not been previously considered for biological and medical applications.^[16]

We are interested in introducing these silicates to the field of regenerative medicine, as the dissolution products of synthetic silicate have properties that are useful for tissue engineering applications.^[17] Synthetic silicates (such as Laponite) dissociate into nontoxic products (Na^+ , Mg^{2+} , $\text{Si}(\text{OH})_4$, Li^+) in aqueous solution.^[14] It is reported that magnesium ions play

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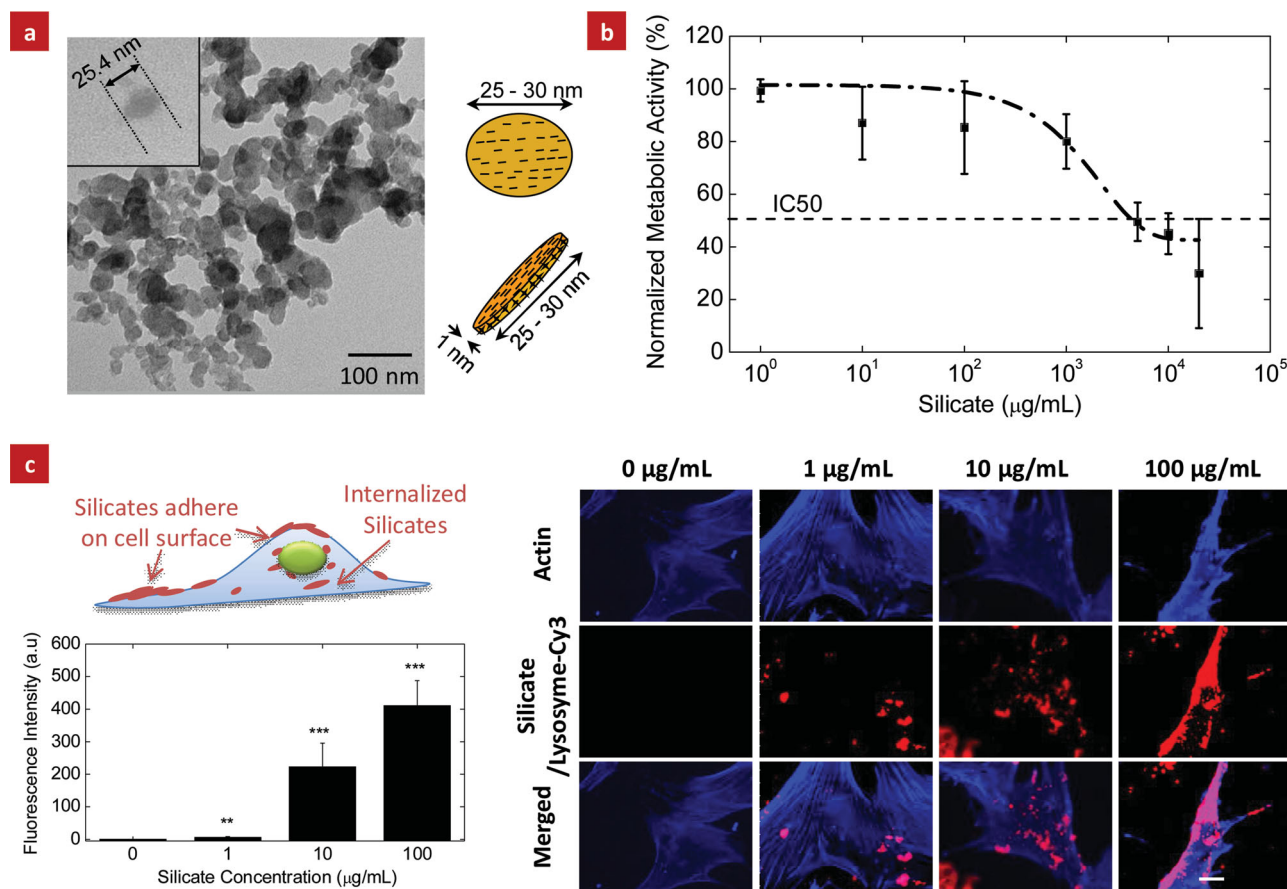


Figure 1. Cytotoxicity and cellular evaluation of silicate nanoplatelets. a) TEM image showing the size and morphology of silicate nanoplatelets. The inset shows the image of a single silicate platelet with 25.4 nm in diameter. b) The half maximal inhibitory concentration (IC_{50}) of the nanoplatelets was assessed by evaluating the metabolic activity of hMSCs in the presence of silicate nanoplatelets in the media using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 48 hours of post seeding. The metabolic activity was normalized with the control (without any silicate nanoplatelets). The dotted line shows fitted dose response curve, and the IC_{50} was found at a silicate concentration of 4 mg/mL. c) Internalization of silicate nanoplatelets were determined by incubating hMSCs with different concentrations of silicate nanoplatelets (0, 1, 10 and 100 $\mu\text{g/mL}$). Cells cytoskeleton was stained for actin filaments (blue) and the silicates were tagged with Cy3 labeled Lysozyme (red). Fluorescence images depicting cells fibroblast-like morphology, specific for hMSCs, as noticed by the stretch of actin filaments (blue), which also indicates that the addition of silicate nanoplatelets does not interfere with the adhesion properties of the cells. The overlapping of these actin filaments with the silicate nanoplatelets, suggests the high interaction between cells and particles both at membrane and cytoplasmic levels (scale bar = 20 μm). The quantification of the fluorescence intensity indicates significant uptake of silicate nanoplatelets was observed by hMSCs due to the increase in silicate concentrations, confirming the strong affinity of the nanoplatelets towards hMSCs (one-way analysis of variance (ANOVA) followed by Tukey posthoc, $*P < 0.05$, $***P < 0.001$).

a significant role in cellular adhesion to biomaterial surfaces mediated by the adhesion proteins of the integrin family.^[18] Orthosilicic acid ($\text{Si}(\text{OH})_4$), another dissolution product of silicate, promotes collagen type I synthesis^[19] and has been shown to be absorbed by the human body.^[20] Additionally, lithium activates Wnt-responsive genes by inhibiting the glycogen synthase kinase-3[beta] activity that controls osteogenesis via regulating Runt-related transcription factor-2 (RUNX2) activity.^[21] Thus, we believe that silicate nanoplatelets (such as Laponite) may have potential in triggering specific cellular responses towards bone-related tissue engineering approaches.

Intrinsic and extrinsic properties of nanoparticles such as specific surface areas, surface charge, functionality, size and shape, play direct roles in determining specific

cellular responses.^[22] Synthetic silicate nanoplatelets show a disc-shaped morphology, 20–30 nm in diameter (Figure 1a and Supporting Information, Figure S1). The effect of silicate nanoplatelets on cellular metabolism was investigated by monitoring the metabolic activity of adhered hMSCs (PT-2501, Lonza). The metabolic activity was normalized to the hMSCs control (without any silicate nanoplatelets) to determine the dose response. The addition of silicate nanoplatelets did not significantly affect the metabolic activity of hMSCs until the concentration of silicate reached 1 mg/mL (Figure 1b). However, at higher silicate concentrations (5 mg/mL) the metabolic activity dropped drastically. The concentration at which the metabolic activity of hMSCs was reduced to 50% was regarded as the half maximal inhibitory concentration (IC_{50}). The IC_{50}

for silicate nanoplatelets was determined by fitting the dose response curve and was obtained as 4 mg/mL of silicate concentration.

A possible explanation to the decrease in the metabolic activity of hMSCs could be that, at a high silicate concentration, a significant amount of nanoplatelets adhere to the cell surface and are internalized, which restricts the cellular functionality. The confocal imaging confirmed the attachment of the nanoplatelets to the cells and also showed the internalization of the silicate within the cell body as seen by the middle section of the *z*-stacked images of the cells (Figure 1c). Additionally, the charged silicate nanoplatelets also interact with the media proteins and results in the formation of aggregates that cannot be engulfed by the cells. This might also contribute to decrease in metabolic activity at higher silicate concentrations. Contrary to other bioactive materials such as hydroxyapatite nanoparticles (size = 50 nm, $IC_{50} \approx 250 \mu\text{g/mL}$),^[23] and silica nanoparticles (size = 30 nm, $IC_{50} \approx 400\text{--}500 \mu\text{g/mL}$),^[24] synthetic silicates show cytotoxicity at ten-fold higher concentration ($IC_{50} \approx 4 \text{ mg/mL}$).

The high surface area of silicate nanoplatelets, due to their disc shaped morphology, provides them with a high chemical reactivity as well as high biological activity. The chemical reactivity and cytotoxicity of silicate nanoplatelets can also be determined by monitoring the generation of intracellular reactive oxygen species (ROS), such as super oxide (SOx) and reactive nitrogen species (RNS), such as nitric oxide (NOx), as an evaluation of the immediate responses of cells when put in contact with the particles. An appropriate amount of ROS and RNS plays a vital role in the functionality of stem cells, which directly affects their self-renewal capacity, and differentiation potential.^[25] However, an excessive amount of ROS and RNS results in increased oxidative stress, inflammation and consequent damage to proteins, membranes, and DNA, triggering a series of events that can lead to senescence, apoptosis and/or stem cell transformation.^[25]

To investigate the interaction of silicate nanoplatelets with hMSCs, pre-seeded hMSCs were exposed to several silicate concentrations (0–20 mg/mL). **Figure 2** indicates that the addition of silicate to hMSCs results in the enhanced production of ROS and RNS. At a low silicate concentration (<1 mg/mL), hMSCs were able to effectively manage oxidative stress, but at higher silicate concentrations (>1 mg/mL), a significant increase in ROS and RNS production was observed (Figure 2). These results are in agreement with the cytotoxicity findings that highlight that the cellular metabolic activity was affected at high concentrations of silicates (>1 mg/mL). The high concentration of particles enables the formation of large aggregates that can jeopardize the internalization efficiency of the particles, while at the same time, generating external stress to the cell cytoskeleton.

We further investigated the cytotoxicity of these silicate particles by quantifying plasma membrane damage using lactate dehydrogenase (LDH) assays. It was also observed that the addition of silicate below the IC_{50} values did not change the LDH level in media, compared with the control (without nanoplatelets), providing additional evidence of cytocompatibility of silicate nanoplatelets in the given concentration range (Supporting Information, Figure S2). Furthermore, attachment

and internalization of silicate nanoplatelets did not alter cellular morphology and the function of the hMSCs, as we did not observe any alterations in cell-proliferation profiles over the period of 28 days (Supporting Information, Figure S3). Based on the cell-nanoplatelets interactions, cytotoxicity, and proliferation studies, we selected silicate concentrations of 0, 1, 10 and 100 $\mu\text{g/mL}$ for further long-term studies. Overall, it can be concluded that silicate nanoplatelets show nearly no cytotoxicity at concentrations lower than 1 mg/mL and can potentially be used for biomedical applications.

We hypothesize that these nanoplatelets possess a bioactive feature that promotes and further enhances the osteogenic phenotype of hMSCs. Furthermore, we believe that these silicate nanoplatelets could act as an osteoinductive agent when cells are cultured in normal media, by triggering the formation of mineralized matrix. The bioactivity of silicate nanoplatelets was investigated by monitoring the alkaline phosphatase (ALP) activity of hMSCs and the production of mineralized matrix in normal, osteoconductive and osteoinductive media. We had chosen different culture media to investigate the effect of silicate nanoplatelets on the osteogenic differentiation of hMSCs in the absence or presence of osteoinducing factors (such as dexamethasone). The normal growth medium is a maintenance medium that does not trigger the osteogenic differentiation of hMSCs.^[26] On the other hand, an osteoconductive (OC) medium (normal medium supplemented with β -glycerophosphate and ascorbic acid salts) supports the osteogenic differentiation of hMSCs and also promotes the formation of mineralized matrix in presence of bioactive materials.^[26] In contrast, the osteoinductive medium (OI) (normal medium supplemented with β -glycerophosphate, ascorbic acid, and dexamethasone) induces the osteogenic differentiation of hMSCs.^[26]

Amongst the major osteogenic hallmarks, the upregulation of ALP activity is a key event occurring during the early time points of osteogenesis.^[27] In normal medium (N), residual ALP activity was observed that was stable during the culture period (Supporting Information, Figure S4), which was considered as a residual activity of non-differentiated hMSCs, we did not observe any significant increase in ALP activity due to the addition of silicate nanoplatelets in normal media. On the other hand, an increase in ALP activity was observed in OC media with a peak at day 21 (**Figure 3a**). The addition of silicate nanoplatelets significantly enhances the upregulation of ALP activity, indicating that the presence of these particles within the intra- and extracellular environment, can trigger an upregulation of ALP, correlated with the first check-point for osteogenic differentiation. More than a three-fold increase in peak ALP activity was observed due to the addition of a small amount of silicates (1 and 10 $\mu\text{g/mL}$) in OC media compared to the control (without nanoplatelets in OC media). As expected, in OI media, ALP peak was shifted to day 14, due to the addition of dexamethasone, known for triggering the osteogenic differentiation. An almost two-fold increase in peak ALP activity was observed due to the addition of silicate nanoplatelets (1 $\mu\text{g/mL}$). Moreover, it is important to note that the addition of silicates (1 $\mu\text{g/mL}$) has a similar effect in ALP peak activity and cellular organization (Supporting Information, Figure S5) when compared to the positive control (hMSCs in osteoinductive media).

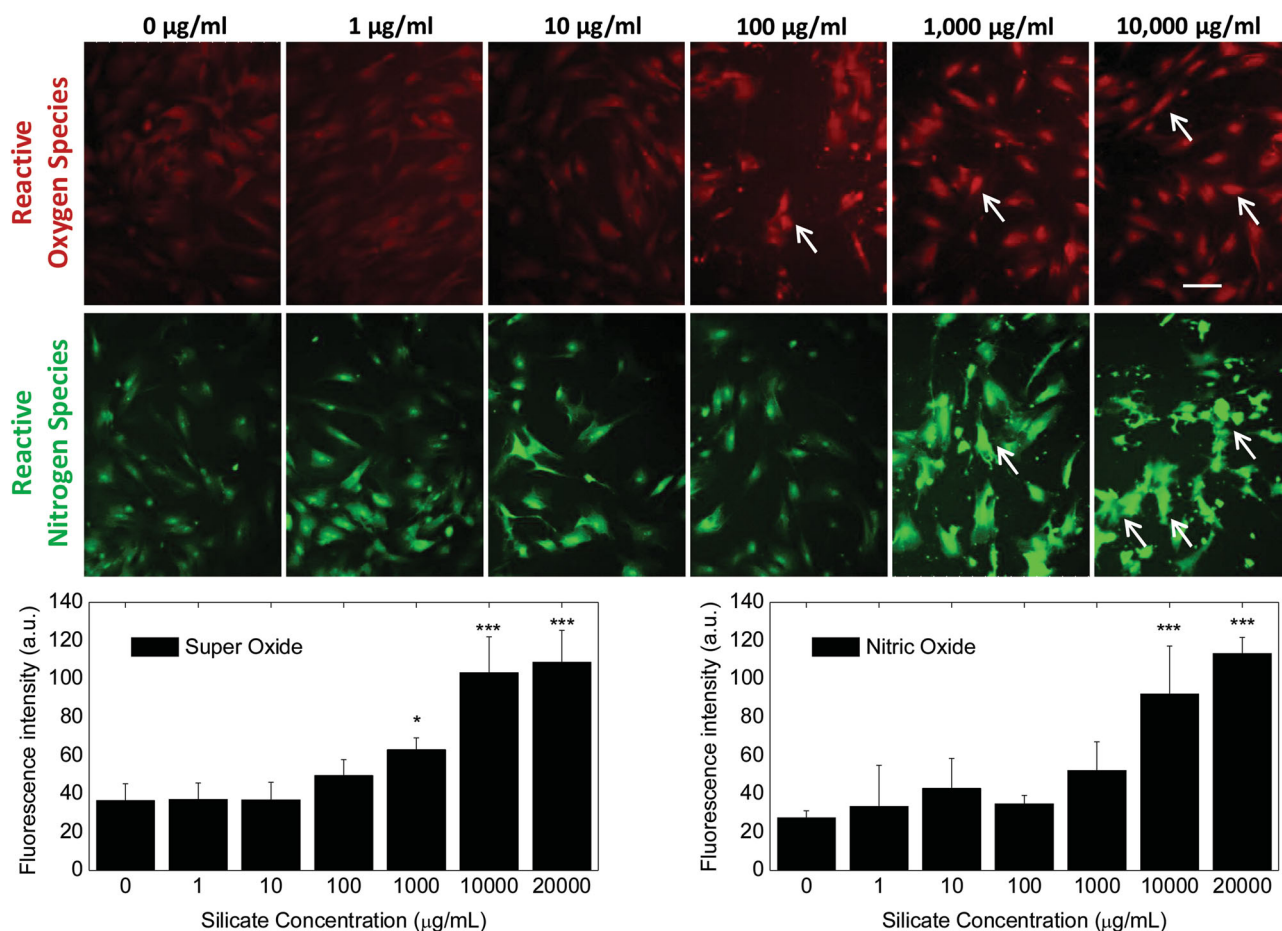


Figure 2. The formation of radicals as a measure of intracellular stress that usually generates a cytotoxic response was determined. The intracellular production of two reactive species, reactive oxide species (ROS) and reactive nitrogen species (RNS), was evaluated after hMSCs incubation in the presence of different silicate nanoplatelets concentrations. As the silicate concentration increased, no intracellular oxidative stress (ROS) was noticed until 100 µg/mL. However, at higher silicate concentrations (>1 mg/mL), a significant increase in ROS was observed as quantified using ImageJ. Similarly, at higher silicate concentrations (>10 mg/mL), a significantly higher amount of RNS was observed. These results are in agreement with the IC50 results (determined by MTT assay) that indicated that particle concentration >1 mg/mL affects cellular function. Therefore, further studies were carried out with the nanoplatelets concentration below 1 mg/mL (scale bar 100 µm) (one-way analysis of variance (ANOVA) followed by Tukey posthoc, * $P < 0.05$, *** $P < 0.001$).

Overall, the results indicate that silicate nanoplatelets can support and sustain the upregulation of ALP activity without the addition of dexamethasone.

At the same time, it is equally important for the cells to produce extracellular matrix (ECM), followed by a subsequent mineralization. The effect of silicate nanoplatelets on the production of ECM was investigated by determining the amount of insoluble proteins after 21 days. We show that the addition of silicate significantly enhanced the ECM production (Figure 3a). Further evaluation of ECM indicates that cells subjected to silicate nanoplatelets are characterized by an increase in the RUNX2 and an enhanced production of osteo-related proteins, like osteocalcin (OCN) and osteopontin (OPN) (Figure 3b,c). RUNX2 belongs to the RUNX family of transcription factors and is exclusively expressed in mineralized tissues.^[28] It is considered as a focal point for integration of a variety of signals affecting osteogenesis as it stimulates osteo-related genes that encode type I collagen, OCN, and OPN.^[28] We observed that the

addition of silicate significantly promotes RUNX2 expression. In the OC media condition, the addition of particles significantly promotes an increase of RUNX2 when compared with the OC media without particles. On the other hand, in the OI condition, the effect is similar.

Concomitantly, we also evaluated the presence of osteo-related proteins (OCN, OPN) that constitute the ECM produced by osteoblast-like cells. OCN is the most abundant bone-specific non-collagenous protein synthesized by osteoblasts and serves as a marker to evaluate osteogenic maturation and bone formation.^[29] OPN is a structural protein synthesized by pre-osteoblasts, osteoblasts, and osteocytes, and is considered an important factor in bone remodeling.^[30] The presence of these two proteins sets the basis for the upcoming mineralization as they sustain the formation of bone-like nodules that can further develop into complex 3D mineralized structures. Due to the addition of silicate, an enhancement in the deposition of these bone-related proteins was observed (Figure 3b,c). The presence

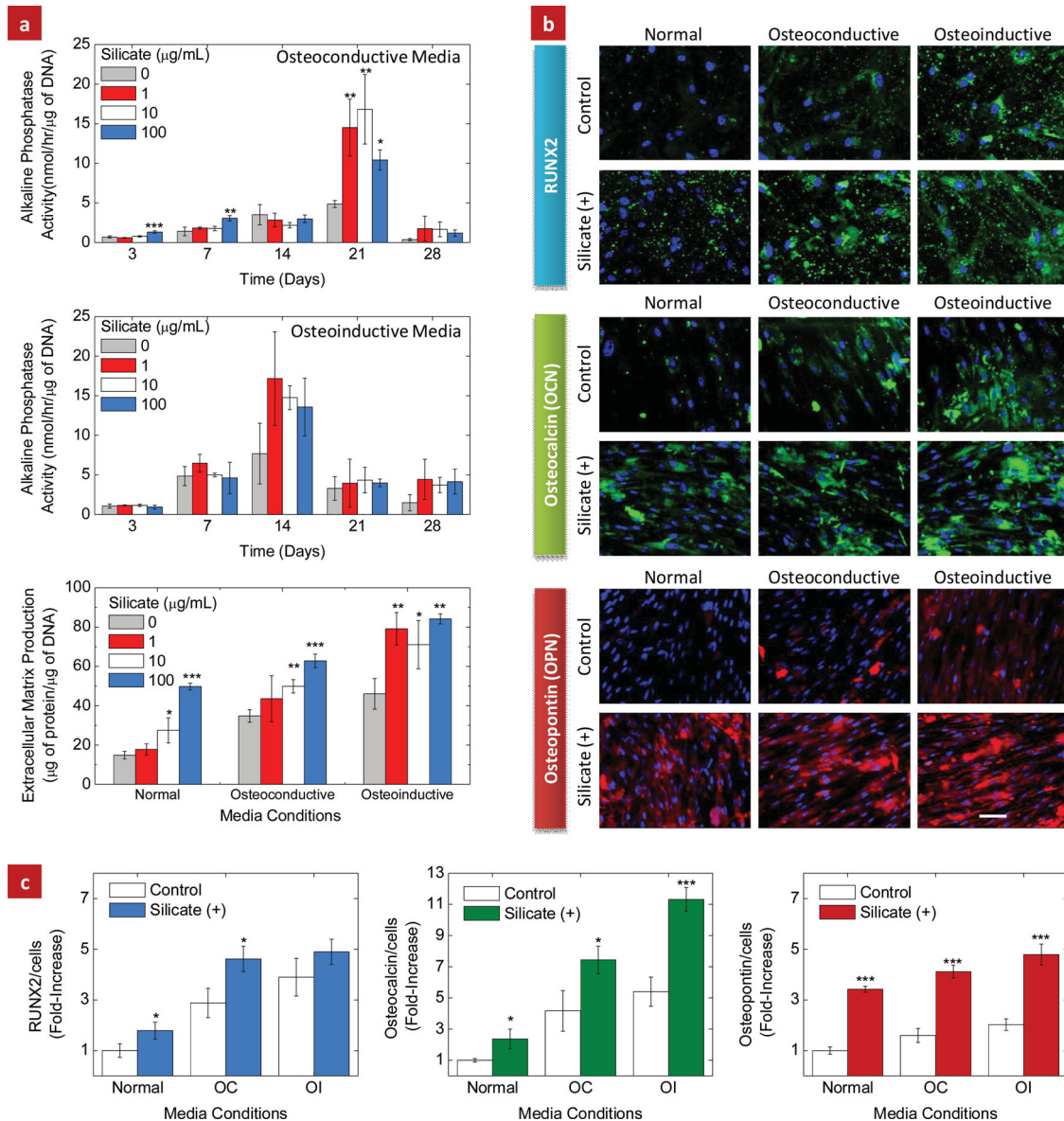


Figure 3. Effect of silicate nanoplatelets on hMSCs differentiation. a) The addition of silicate nanoplatelets upregulate alkaline phosphatase (ALP) activity of hMSCs. Cells grown in normal media, show a residual ALP activity, which is stable during the considered cell culture period (Supporting Information, Figure S3). In osteoconductive (OC) media, an upregulation of ALP activity was observed and the ALP peak was obtained on day 21. The addition of small amounts of silicate nanoplatelets (1 and 10 mg/mL) resulted in an almost 3-fold increase in ALP activity. On the other side, for the cells grown in osteoinductive (OI) medium, the ALP activity peaks shifts to an earlier time point (day 14). These data suggest that silicate nanoplatelets enhance the ALP activity and results in osteogenesis. Addition of silicate significantly enhances production of extracellular matrix (ECM) after 21 days. A similar trend was observed in OC and OI media. b) The increase in the RUNX2 (green) and production of bone-related proteins such as osteocalcin (OCN, green), and osteopontin (OPN, red) was observed due to the addition of silicates (scale bar = 200 μm). Cells in normal media without silicate particles act as a negative control whereas cells in OI serve as a positive control. Cell nuclei were counterstained with DAPI (blue). c) The protein production was quantified using image analysis from the fluorescence images. The intensity of protein per cell was quantified and later normalized by the control (hMSCs in normal growth media with no silicate particles) to obtain fold increase in the production of protein. The addition of silicate (100 $\mu\text{g/mL}$) results in the production of RUNX2, OCN, and OPN in normal media, which indicates a strong bioactive character of silicate nanoplatelets. The addition of OC and OI media further enhances the production of bone-related proteins. The results indicate that the silicate nanoplatelets promote an increase in the production of essential proteins for the osteogenesis of hMSCs (one-way ANOVA followed by Tukey post-hoc, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

of the silicate nanoplatelets results in two-fold increase in bone-related matrix proteins deposition (OCN and OPN) when compared to hMSCs subjected to dexamethasone. Taken together, we believe that the nanoplatelets act as a trigger from both the

exterior (by acting as growth nuclei for matrix deposition), as well as from the interior of the cells (by proving the chemical cues to the cells organelle to shift their metabolism towards the osteogenic differentiation). The concentration of the

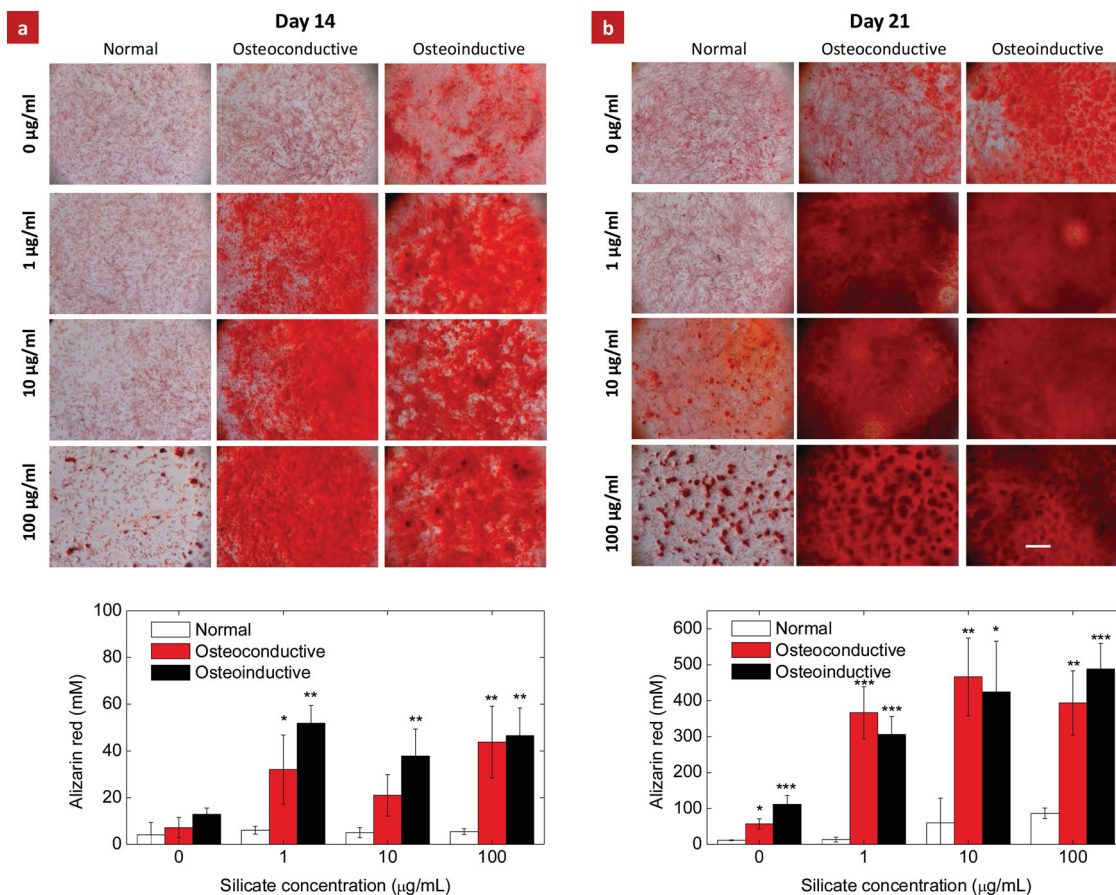


Figure 4. Effect of silicate on production of mineralized extracellular matrix. The mineralized matrix was stained with Alizarin Red S on days 14 and 21. a) At day 14, hMSCs were not able to produce any mineralized matrix in normal media (negative control). Similarly, in osteoconductive (OC) media, no obvious mineralization was observed, however the presence of silicate triggered the formation of large mineralized depots. In osteoinductive media (positive control), formation of mineralized nodules was observed. Addition of silicate nanoplatelets significantly enhances the production of mineralized matrix as determined by quantifying the amount of Alizarin Red S that stained the mineralized matrix. b) Similar behavior was observed on day 21. In normal media, mineralized matrix was not observed, but the addition of silicate significantly enhanced the ability of hMSCs to promote the matrix mineralization. This indicates that the nanoplatelets induce the reorganization and remodeling of the ECM towards osteogenesis. On the other hand, in OC and OI media, with the increase in nanoparticle composition, an enhanced calcification response was observed (scale bar = 1 mm) (one-way ANOVA followed by Tukey post-hoc, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

nanoplatelets directly affects the intensity of this outcome and the further mineralization process.

We analyzed the efficiency of the mineralization stage by using the Alizarin Red S staining as a marker for the inorganic calcium, a characteristic common to bone-like structures. **Figure 4**, Supporting Information, Figure S6 and S7 show the effect of silicate concentration on the formation of mineralized matrix in normal (N), osteoconductive (OC), and osteoinductive (OI) media on days 14 and 21. The effect of silicate on nodule formation was evident at higher silicate concentrations (100 µg/mL) in normal media. These findings reinforce the statement that the nanoplatelets can act as a promoter of osteogenic differentiation, by enhancing the protein production and its subsequent mineralization. On the other hand, in osteoconductive media, a significant increase in the number of nodules and the amount of mineralized matrix was observed due to addition of silicate. The negative controls (normal and osteoconductive media without silicate nanoplatelets) did not

show any nodules formation. These results indicate that silicate nanoplatelets are bioactive and promote osteogenic differentiation of hMSCs in the absence of osteoinductive factors. Similar results were obtained in osteoinductive media, where samples containing small amounts of silicate had much higher amount of mineralized matrix compared to the positive control (osteoinductive media without silicate nanoplatelets) underlining the role of silicates in the enhancement of osteogenic differentiation efficiency.

Taken together, the data presented here clearly showcases that synthetic silicate nanoplatelets can induce osteogenic differentiation of stem cells in the absence of any external osteoinductive factors (e.g., dexamethasone). These synthetic silicate nanoplatelets are cytocompatible and strongly interact with the cells. The presence of the silicate triggers a set of events that follow the temporal pattern of osteogenic differentiation (ALP/RUNX2 transcripts upregulation, bone-related matrix protein deposition (OCN and OPN), followed by matrix mineralization).

The advantage of using these particles as osteoinductive agents is that they are applied in a single dose, while other agents (dexamethasone and BMP-2) have to be added when changing the culture media (every 3–5 days). To our knowledge, this is the first study showing that synthetic silicate nanoplatelets alone can induce osteogenic differentiation of hMSCs. This unique bioactive property of silicate nanoplatelets may be processed to construct devices such as injectable tissue repair matrixes, bioactive fillers, or therapeutic agent for triggering specific cellular responses towards bone-related tissue engineering approaches.

Experimental Section

Materials and Methods are available online as Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

A.K.G. and A.K. designed the study. A.K.G., S.M.M., A.S., A.P., and S.S. performed the experiments. A.K.G. and S.M.M. analyzed the results. A.K.G. and A.S. investigated cell-nanoparticles interactions. R.L.R., A.P.M., and M.G. provided technical support. A.K.G. and A.K. wrote the paper. The manuscript was revised by all the authors and was approved to the final version of the manuscript. A.K.G. would like to thank Prof. Robert S. Langer for his technical insight and feedback throughout the project and acknowledges financial support from MIT-Portugal Program (MPP-09Call-Langer-47). S.M.M. thanks the Portuguese Foundation for Science and Technology (FCT) for the personal grant SFRH/BD/42968/2008 (MIT-Portugal Program) and the financial support of MIT/ECE/0047/2009 project. A.S. would like to acknowledge the National Institutes of Health (NIH) (CA151884) and the David Koch-Prostate Cancer Foundation Award in Nanotherapeutics. A.P. is supported by postdoctoral fellowship by Natural Science and Engineering Research Council (NSERC), Canada. S.S. acknowledges the postdoctoral fellowship awarded by Le Fonds Quebecois de la Recherche sur la Nature et les Technologies (FQRNT), Quebec, Canada and interdisciplinary training fellowship awarded by System-based Consortium for Organ Design and Engineering (SysCODE). This research was funded by the US Army Engineer Research and Development Center, the Institute for Soldier Nanotechnology, the NIH (EB009196; DE019024; EB007249; HL099073; AR057837), and the National Science Foundation CAREER award (A.K.).

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