

Nanomanipulation of Ligand Nanogeometry Modulates Integrin/Clathrin-Mediated Adhesion and Endocytosis of Stem Cells

Bohan Yin, Qin Zhang, Jiaxiang Yan, Yingying Huang, Chuanqi Li, Jiareng Chen, Chunyi Wen, Siu Hong Dexter Wong,* and Mo Yang*



Cite This: <https://doi.org/10.1021/acs.nanolett.3c01757>



Read Online

ACCESS |

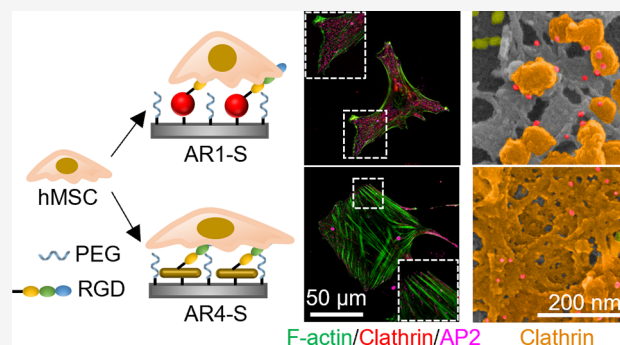
Metrics & More

Article Recommendations

Supporting Information

ABSTRACT: Nanosubstrate engineering can be a biomechanical approach for modulating stem cell differentiation in tissue engineering. However, the study of the effect of clathrin-mediated processes on manipulating this behavior is unexplored. Herein, we develop integrin-binding nanosubstrates with confined nanogeometries that regulate clathrin-mediated adhesion- or endocytosis-active signaling pathways for modulating stem fates. Isotropically presenting ligands on the nanoscale enhances the expression of clathrin in cells, thereby facilitating uptake of dexamethasone-loaded nanoparticles (NPs) to boost osteogenesis of stem cells. In contrast, anisotropic ligand nanogeometry suppresses this clathrin-mediated NP entry by strengthening the association between clathrin and adhesion spots to reinforce mechanotransduced signaling, which can be abrogated by the pharmacological inhibition of clathrin. Meanwhile, inhibiting focal adhesion formation hinders cell spreading and enables a higher endocytosis efficiency. Our findings reveal the crucial roles of clathrin in both endocytosis and mechanotransduction of stem cells and provide the parameter of ligand nanogeometry for the rational design of biomaterials for tissue engineering.

KEYWORDS: Nanosubstrate engineering, ligand nanogeometry, cell–substrate interaction, endocytosis, stem cell differentiation



Human mesenchymal stem cells (hMSCs) are a great potential source for regenerating damaged/injured tissue due to their self-renewal capability and multilineage differentiation potency.¹ Therefore, effectively guiding hMSCs toward specific lineage is of utmost importance in the field of tissue engineering.² In the past decades, methods for manipulating hMSC differentiation (e.g., osteogenic differentiation) have been intensively investigated. Nanomaterials have emerged as an effective tool to enter stem cells owing to their small size and regulate stem cell differentiation via particular signaling pathways.³ For instance, nanomaterials serve as nanocarriers with high loading capacity to deliver the potent inducer of osteogenesis [e.g., dexamethasone (Dex)] to augment the osteogenic capability of stem cells.^{4,5} Generally, clathrin-mediated endocytosis (CME) is one of the major pathways for nanoparticles (NPs) to enter the cells, with the formation of clathrin-coated pits and recruitment of related adaptor proteins [e.g., adaptor protein 2 (AP2)] on the cell membrane to form intracellular vesicles for delivering cargoes.^{6,7} Therefore, recent studies exploited substrate engineering, such as optimizing its surface roughness,⁸ topography,^{9–11} or stiffness,^{12,13} to promote the intracellular delivery of NPs, especially via CME pathway.

Besides mediating cellular uptake of NPs, clathrin also participates in integrin-associated adhesion independent of its

role in endocytosis.^{14,15} Previous studies showed that the effective engagement between integrins and ligands recruited clathrin via AP2 to regulate the associated signaling.¹⁶ Critically, this activation can be subjected to biophysical cues of the microenvironment. For example, large and flat clathrin-coated plaques are formed on the stiff substrate to upregulate extracellular signal-regulated kinase (ERK) activation, which is abrogated on the soft substrate.^{17,18} It is known that Arg-Gly-Asp (RGD), a bioactive ligand of extracellular matrix (ECM), can specifically activate integrin $\beta 3$ for cell adhesion.¹⁶ Sheetz et al. reported that cells cultured on a substrate with mobile RGD exhibited enhanced integrin-based CME activity, while this process was suppressed in cells on the fixed RGD substrate with mature focal adhesion (FA) formation.¹⁶ These studies suggest that the key roles of integrin and clathrin can be interchanged in response to various conditions of ligand presentations. Material surfaces with nano/micro features can modulate CME functions of cells.^{19–22} Hence, it is highly

Received: May 11, 2023

Revised: July 3, 2023

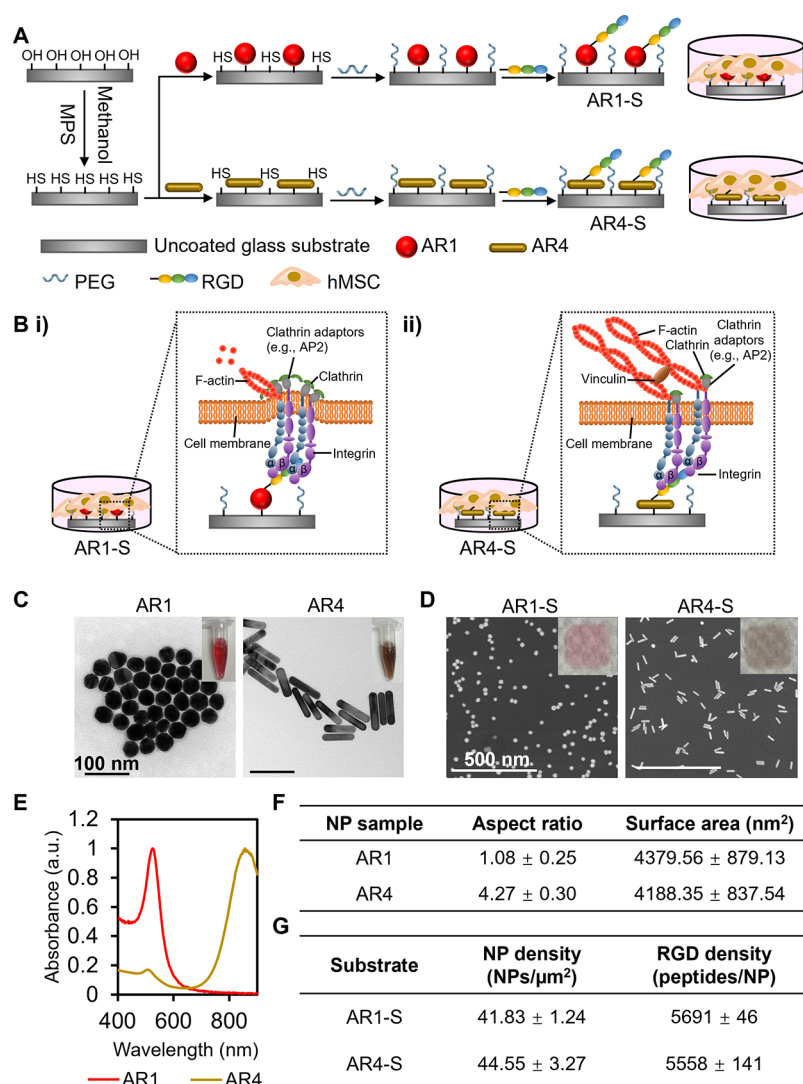


Figure 1. Characterization of glass substrates with ligand nanogeometry created by gold nanorods (AuNRs). (A) Schematic illustration of surface modification of a glass substrate with AuNRs with an aspect ratio (AR) of 1 or 4 (denoted AR1 or AR4) to form AR1-S or AR4-S, respectively. AR1-S and AR4-S refer to the insertion of an AR1 or AR4-coated glass substrate to a well of a conventional 24-well tissue culture plate (TCP), respectively. (B) Schematic postulation of how (i) AR1-S and (ii) AR4-S regulate cell endocytosis and adhesion structures. (C) Representative transmission electron microscopy (TEM) images reveal the morphology of AR1 and AR4 suspended in nanopure water. (D) Scanning electron microscopy (SEM) images reveal the morphology and coating density of AR1-S and AR4-S. (E) Ultraviolet–visible (UV–vis) spectra of AR1 and AR4 suspended in nanopure water with surface plasmon resonance (SPR) peaks at 530 and 866 nm, respectively. (F) Quantification of AR and surface area of AR1 and AR4 by counting at least 50 nanoparticles (NPs) in TEM images. (G) Quantification of NP density and Arg-Gly-Asp (RGD) density by analyzing at least 10 SEM images of 1 μm × 1 μm. Error bars denote the standard deviation resulting from three independent experiments.

possible that the subcellular level interactions between cell receptors and bioactive molecules can regulate the CME activity. Therefore, controlling the nanogeometric display of specific ligands may potentially unravel how integrin and clathrin are recruited to “turn on” the endocytosis- or adhesion-active state.

Integrin–ligand binding occurs on the nanoscale. Thus, precisely controlling the nanoscale display of ligands is critical to orthogonally investigate cell–material interactions.^{23–26} In particular, ligand clustering and nanospacing controlled by nanostructures are pivotal for activating integrin and the associated signaling.^{27–30} We have also demonstrated that manipulating local clustering,³¹ tether mobility,³² or physical caging³³ of RGD-displaying nanoparticles regulated cell adhesion and fates. A recent study revealed that MC3T3

preosteoblasts cultured on nanogrooved substrates showed enhanced cytoskeletal structures and mature FA but exhibited poor endocytic uptake of cell-penetrating peptides compared to those on flat control.³⁴ Nevertheless, how these nano-features directly regulate integrin and clathrin activation remains elusive. It is conducive to controlling the nanogeometric presentation but decoupling the other effects of the ligand arrangement on the nanoscale (e.g., ligand density) on cell adhesion or endocytosis.

Herein, we engineer cell seeding nanosubstrates by coating gold nanospheres (AuNPs) with an aspect ratio (AR) of 1 (AR1-S) or gold nanorods (AuNRs) with an AR of 4 (AR4-S). The random-oriented coating of AR4 ensures the anisotropy presentation of RGD peptides at the nanoscale instead of micro/macroscale to cell receptors.³⁵ The choice of these two

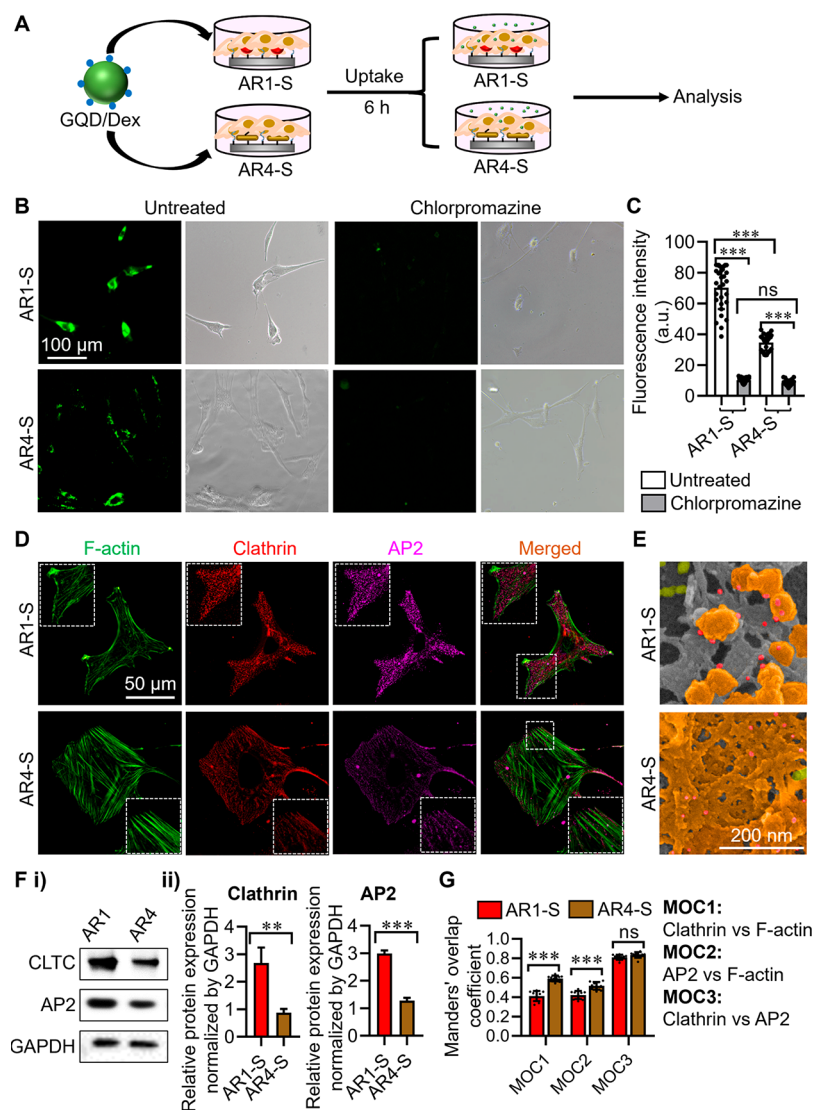


Figure 2. Examination of endocytic activity and uptake of dexamethasone (Dex)-loaded graphene quantum dots (GQDs; GQD/Dex) by hMSCs that were seeded on AR1-S or AR4-S. (A) Schematic illustration of cellular uptake experiment. The cells were preseeded on the AR1-S or AR4-S in a culture well and incubated with a growth medium for 24 h. During cellular uptake, the cells were incubated with GQD/Dex for 6 h. The relative amount of GQD/Dex internalized by cells was measured by fluorescence microscopy. (B) Representative fluorescence and optical images of hMSCs after endocytosis of GQD/Dex with or without pretreatment of 5 μ g/mL chlorpromazine (inhibitor of clathrin) in both AR1-S and AR4-S groups. (C) Quantitative analysis of fluorescence signals in cells from different groups corresponding to panel B. Averaged fluorescence signals of GQD/Dex were measured in each cell. Twenty cells were examined for each group. (D) Representative fluorescence images of cells that were seeded on AR1-S or AR4-S for 24 h, followed by immunostaining of the filamentous actin (F-actin, green), clathrin (red), and adaptor protein 2 (AP2, purple). (E) Representative SEM images show 13 nm AuNPs preadsorbed with goat antimouse (H+L) IgG, binding to cells that were cultured on the substrates after 24 h and further preincubated with mouse antihuman clathrin overnight. Clathrin, 13 nm AuNP, and the NP on the substrates (AR1 and AR4) were painted with the pseudocolors orange, red, and golden yellow, respectively. (F) Western blotting results of clathrin heavy chain (CLTC) and AP2 in cells seeded on AR1-S and AR4-S with (ii) statistical analysis ($n = 3$). (G) Quantitative analysis of colocalization measured between clathrin/AP2 and F-actin by MOC with Costes ($\geq 95\%$) involving at least 10 cells. Error bars denote the standard deviation. Statistical analysis of pairwise comparison was determined by one-way ANOVA. No significance (ns): $P > 0.05$; * $P < 0.01$; *** $P < 0.001$.

aspect ratios (AR1 and AR4) was based on the critical threshold of the effective development of traction forces in cells that were cultured on substrates with AuNRs of larger AR (≥ 4).³⁵ We aim to adopt the well-established anisotropic AuNRs to study the interplay among (1) nanogeometric ligand presentation, (2) integrin/clathrin-mediated adhesion, and (3) cell endocytosis of NPs that has not yet been explored. These two types of nanosubstrates present the same amount of RGD peptides of varying ligand nanogeometries for studying the dynamic changes of integrin-mediated adhesion and endocytic activity (Figure 1A). We postulate that the AR4-S with a large

AR presents a biomimetic hierarchical organization of polypeptides, such as collagen fibrils,³⁶ to promote cell adhesion behavior but does not favor endocytic uptake of NPs (Figure 1Bii). In contrast, we hypothesize that AR1-S disables the possibility of lateral clustering of integrins for FA maturation³⁵ but permits the development of endocytic activity (Figure 1Bi). In our findings, we demonstrated that hMSCs cultured on AR1-S showed enhanced expression of clathrin and AP2 for the formation of clathrin-coated vesicles that facilitate the cellular uptake of NPs. Conversely, AR4-S activated $\beta 3$ integrin and recruited FA proteins with promoted

mechanotransduced signaling, ERK and yes-associated protein (YAP), in hMSCs but much less endocytic uptake of NPs compared to that of the AR1-S group. We visualized that clathrin and AP2 spatially colocalized with FA in the AR4-S group with the formation of clathrin-coated plaques. Mechanistically, pharmacological inhibition of clathrin suppressed cellular uptake of NPs in both groups and substantially reduced nuclear localization of ERK in AR4-S. Meanwhile, the blocking of $\beta 3$ integrin and associated FA further boosted the level of NP uptake in both groups. More importantly, utilizing the promoted endocytosis capability of hMSCs on AR1-S by delivering Dex-loaded NPs induced a more robust osteogenic differentiation than that on AR4-S. This work reveals how these engineered nanosubstrates regulate the cellular uptake of suspended drug-loaded NPs. Moreover, our results not only highlight the altered roles of integrin/clathrin structures in response to nanogeometries of ligand display but also aid nanosubstrate and nanomedicine development to achieve the desired hMSC differentiation for modulating tissue-regenerative processes.

To construct AR1-S and AR4-S, we first synthesized citrate-capped nanospheres (AR1) with a diameter of 36.51 ± 3.85 nm³⁷ and cetyltrimethylammonium bromide (CTAB)-capped nanorods (AR4) with a length of 77.12 ± 8.67 nm, a width of 18.08 ± 2.13 nm, and an AR of 4.27 ± 0.30 ,³⁸ respectively (Figure 1C,D). Overall, both AR1 and AR4 possessed a similar surface area (~ 4000 nm²; Figure 1F). The surface plasmon resonance (SPR) peaks of AR1 and AR4 were 530 and 866 nm, respectively (Figure 1E). Before nanosubstrate fabrication, we replaced the CTAB surfactant of AR4 with citrate by ligand exchange (Figure S1).^{39,40} This successful modification was confirmed by ultraviolet–visible absorption spectroscopy, which showed similar SPR peaks (Figure S1B) and consecutive changes of zeta potentials throughout the exchange (Figure S1C). Next, we engineered the nanosubstrate by employing mercaptopropylsilatrane (MPS) for thiolation, followed by immobilizing AR1 or AR4 onto the thiolated substrate via Au-thiol linkages, forming AR1-S or AR4-S, respectively (Figure 1A). By optimizing the concentration and incubation time of the NP immobilization, the coating densities of AR1 and AR4 on the substrates were controlled to be comparable at 41.83 ± 1.24 and 44.55 ± 3.27 NPs/ μm^2 , respectively (Figure 1G). We verified that the high coating density (e.g., exceeding 100 NPs/ μm^2) of AR1 or AR4 would cancel out the local anisotropic effect to promote integrin-mediated adhesion (Figure S2). Furthermore, cyclic RGD peptides were conjugated onto the surface of AR1 and AR4 via Au-thiol reaction, resulting in a ligand density of 5691 ± 46 or 5558 ± 141 peptides/NP, respectively (Figure 1G), as quantified by Ellman's assay.⁴¹ Subsequently, the remaining space on the substrate was blocked with maleimide-terminated polyethylene glycol (PEG) strands via a thiol-maleimide Michael addition click reaction to prevent nonspecific cell adhesion.³⁵ Our previous studies proved that the covalent bonds (Au-thiol) between AR1 (or AR4) and the thiolated substrate were highly stable to avoid NP internalization by the cells seeded atop or detachment from the substrate throughout the cell culture.^{8,35} Hence, we fabricated nanosubstrates that present integrin-binding ligands of two distinguished nanogeometries, respectively.

Next, we examined the effect of diverse nanoscale displays of ligands on the endocytosis of drug-loaded NPs in hMSCs. We confirmed that the hMSCs cultured on AR1-S or AR4-S were highly viable ($>90\%$) over 10 days (Figure S3). Thereafter, we

employed graphene quantum dots (GQDs), a well-studied biocompatible zero-dimension NP with a large surface area for drug loading and high fluorescence quantum yield properties for its intracellular tracking.^{42,43} GQDs were loaded with Dex (GQD/Dex) to examine the endocytosis capability of hMSCs seeded on AR1-S or AR4-S (Figure 2A). The GQDs purchased from the company emitted a strong fluorescence signal at 450 nm under an excitation of 380 nm (Figure S4). Loading of Dex minimally affected the fluorescence spectrum of GQDs but exhibited the adsorption peak at 242 nm of Dex (Figure S5).⁴⁴ The slightly increased physical size of GQD/Dex (3.87 ± 1.04 nm) compared to that of unmodified GQD (1.96 ± 0.36 nm) also indicated the successful loading of Dex to GQDs via π - π stacking (Figure S4 and S5).⁴⁵ We verified that GQD/Dex was biocompatible with hMSCs and did not significantly reduce the viability of hMSCs upon incubation for 6 h (Figure S6). Before the uptake, we inserted AR1-S or AR4-S into a tissue culture plate (TCP) well and seeded the cells on top for 24 h. The fluorescence images showed that hMSCs cultured on AR1-S endocytosed more GQD/Dex than those cultured on AR4-S, evidenced by 1-fold higher cytoplasmic fluorescence signals (Figure 2B,C). As literature precedents reported that GQDs entered the cells through the CME pathway,^{46,47} we inhibited clathrin using chlorpromazine, a drug that can hinder the formation of clathrin-coated pits with the adapter proteins from the plasma membrane to intracellular vesicles and also suppress the expression of clathrin.^{8,48,49} This inhibition showed a profound decrease in clathrin expression and the intracellular GQD fluorescence signal and minimal effects on the cell viability (Figure 2B,C; Figures S7 and S8). These results demonstrate that AR1-S enhanced the cellular uptake of GQD/Dex by hMSCs via the CME pathway.

We investigated whether the nanogeometry of ligands regulates adhesion/endocytosis via integrin, as RGD is highly specific to integrin.^{50,51} In the nanoscale, our Au-immunolabeling results confirmed that integrin $\beta 3$ engaged with both AR1 and AR4 on the substrates (Figure S9). In the microscale, cells showed a higher spreading area with a polygonal shape, more intense stress fibers (F-actin), and larger FA (vinculin) size on AR4-S than those on AR1-S that showed a spindle shape (Figure 2D; Figures S10 and S11). These results suggest that the anisotropic display of ligands by AR4-S enables effective integrin activation for adhesion structure maturation.³⁵ As previous studies generally demonstrate the conflict between cell adhesion and endocytosis activity,¹⁶ we evaluated the expressions of clathrin and AP2 proteins in cells and their relationship with the adhesion structures in different conditions. Critically, cells expressed an intense dotted pattern of clathrin and AP2 on AR1-S, while these two proteins assembled as FA-like plaques on AR4-S (Figure 2D and Figure S12). Strikingly, more clathrin-coated vesicles on the cytosolic side of the plasma membrane were formed in cells on AR1-S, but plaque-like structures with little vesicles were observed in cells on AR4-S, as indicated by Au-immunolabeling results (Figure 2E and Figure S13). In addition, cells in the AR1-S group expressed higher protein levels of clathrin and AP2 than those in the AR4-S group (Figure 2F). These data indicate that hMSCs show endocytosis-active state on AR1-S and adhesion-active state on AR4-S. Although cells primarily interacted with the AR1 that were immobilized on the substrate surface through the basal membrane, the overall expression of clathrin and AP2 was upregulated in the cells (Figure 2D,F and Figure S12). Meanwhile, our pharmacological blockade of the clathrin

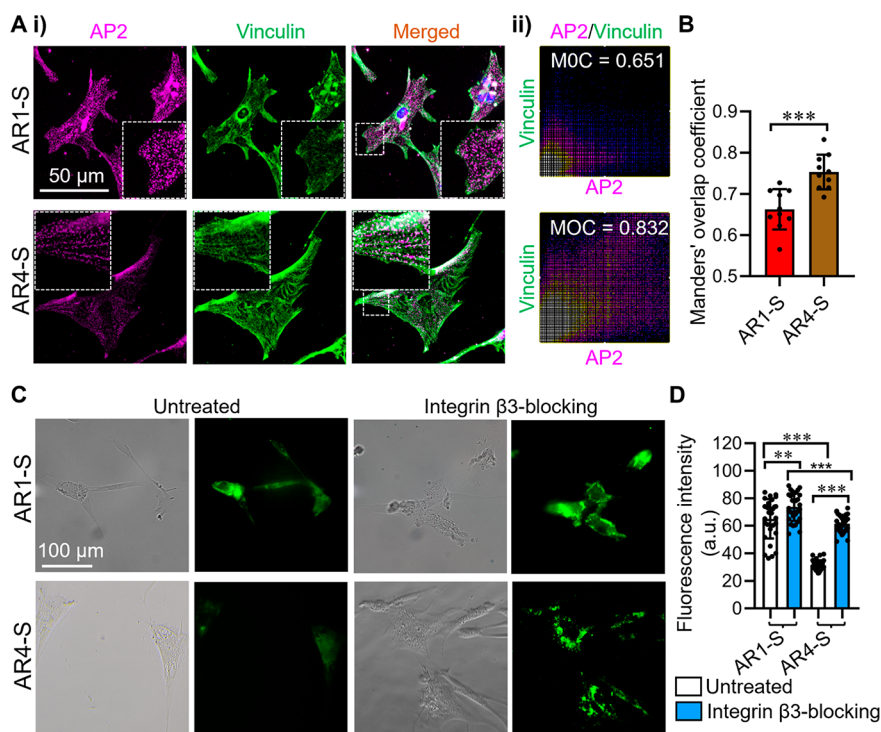


Figure 3. Evaluation of clathrin and AP2 in the role of endocytosis and integrin-mediated mechanosensing. (A) (i) Representative fluorescence images of cells that were seeded on AR1-S or AR4-S for 24 h, followed by immunostaining against AP2 (purple) and vinculin (green). (ii) Quantitative colocalization of AP2 and vinculin scatter plots of the boxed areas as shown in part i. The numbers indicate the MOC. (B) Quantitative analysis of colocalization measured by MOC with Costes ($\geq 95\%$) involving 10 cells cultured on AR1-S or AR4-S. (C) Representative fluorescence images of hMSCs after endocytosis of GQD/Dex with or without pretreatment of $5 \mu\text{g/mL}$ integrin $\beta 3$ in both AR1-S and AR4-S groups. (D) Quantitative analysis of fluorescence signals in cells from different groups corresponding to panel C. Averaged fluorescence signals of GQD/Dex were measured in each cell. Twenty cells were examined for each group. Error bars denote the standard deviation. Statistical analysis of pairwise comparison was determined by one-way ANOVA. ** $P < 0.01$; *** $P < 0.001$.

heavy chain markedly downregulated the expression of clathrin (Figure S8) and reduced the amount of endocytosed GQD/Dex (Figure 2B,C). We conclude that ARS-1 enhances the endocytic activity of the entire cells that were seeded atop, not restricted to the basal membrane area that interacts with the substrate nanostructures.

As cells seeded on AR4-S depicted FA-like clathrin/AP2 plaques, we question their association with adhesion structures. By analyzing Mander's overlap coefficient based on the immunofluorescence staining results, clathrin and AP2 in AR4-S showed significantly higher colocalization/alignment with the F-actin bundles than those in AR1-S (Figure 2D,G and Figure S14). Similar to F-actin, FA showed higher colocalization with AP2 in the AR4-S group than the AR1-S group (Figure 3A,B). In contrast, integrin $\beta 3$ highly colocalized with AP2 in both AR1-S and AR4-S groups (Figure S15). These data imply that the integrin $\beta 3$ in cells that were cultured on AR4-S may be associated with FA/clathrin structures. We suggest that the clathrin may participate in integrin-mediated mechanotransduced signaling in cells on AR4-S (anisotropic ligand presentation), while AR1-S (isotropic ligand presentation) does not support the maturation of FA and promotes CME function. To support this argument, we included two control groups, AR1-S without PEG blocking (Nonblocking AR1-S) and TCP as a blank control, which both provided a homogeneous presentation of cell adhesive peptides on the surface and should empower a well-developed integrin-mediated adhesion.^{35,52} As a result, both groups promoted cell spreading and development of FA

compared to the AR1-S group (Figure S16). In contrast, the immunofluorescence intensity level of AP2 and relative amounts of endocytosed GQD/Dex in cells were descending in the following order: AR1-S, Nonblocking AR1-S, and TCP (Figures S16 and S17). Thus, the homogeneous display of RGD reduces the confined nanogeometry effect of AR1-S on enhancing the CME activity.

Integrin $\beta 3$ is one of the cellular receptors to sense the surrounding microenvironment,⁵³ necessary for cell adhesion and spreading on the RGD-presenting nanosubstrates.⁵⁴ The cells in both AR1-S and AR4-S groups adopted integrin $\beta 3$ to interact with the immobilized RGD on the AuNRs (Figure S9). The inhibition of integrin $\beta 3$ weakened cell adhesion behaviors in both groups, as evidenced by the decreased cell spreading areas (Figure S18). More importantly, blocking integrin $\beta 3$ caused a 12% and 97% increase in endocytosed GQD/Dex signals in cells seeded on AR1-S and AR4-S when compared to their untreated counterparts (Figure 3C,D). This inhibition remarkably reversed this adhesion/endocytosis trend in AR4-S group by suppressing the integrin-mediated mechanosensing signaling that "relinquishes" the clathrin for more endocytic activities. Moreover, this blocking was also effective for the AR1-S group that showed a slight but statically significant (** $P < 0.01$) enhancement of GQD/Dex uptake (Figure 3D), suggesting a further suppression of the integrin $\beta 3$ -mediated mechanotransduction in the AR1-S group. This result indicates that integrin $\beta 3$ is a mediator that participates in cell adhesion and that the suppression of FA development rescues CME activity from the strong association with

adhesion structures. To further understand this pathway, we employed Y-27632, an inhibitor of Rho-associated protein kinase (ROCK), to suppress cell contractility and FA.⁵⁵ Critically, this pharmacological experiment remarkably promoted the uptake of GQD/Dex (>1 fold) by cells on AR4-S while slightly increasing the amount of endocytosed GQD/Dex (~0.5 fold) by cells on AR1-S (Figure S19), further revealing a potential competitive relationship between cell adhesion and endocytosis. To test the generality of our platform, we have added two more aspect ratio groups (AR2 and AR7) by constructing AR2-S and AR7-S (Figure S20) and studied the adhesion- (Figure S21) and endocytosis-related behaviors (Figure S22 and S23) of cells that were seeded atop. As expected, considering the results from AR1-S to AR7-S, the cells cultured on a substrate with AuNRs of large AR (≥ 4) displayed more mature cell adhesion behaviors (e.g., larger spreading area and FA size) but suppressed cell endocytosis behaviors (e.g., lower expressions of clathrin and AP2 as well as the endocytosed amount of GQD/Dex). In short, we validate the essential roles of nanoscale ligand geometry in regulating integrin-associated cell adhesion and endocytosis.

We previously showed that the enhanced cytoskeletal tension via mature FA activated the signaling of mechanosensitive transcriptional factor YAP, which potentiated osteogenic differentiation of hMSCs.⁵⁶ Hence, we analyzed the nuclear localization ratio of YAP in the cells primed by AR1-S and AR4-S. Our immunostaining results showed that the cells cultured on AR4-S displayed 43% higher nuclear localization ratio of YAP than those cultured on the AR1-S (Figure S24), confirming the elevated mechanosensing engagement between cells and AR4-S. Furthermore, integrin-mediated mechanotransduced signaling involves the activation of ERK, which can upregulate the transcription activity of runt-related transcription factor 2 (RUNX2), an osteogenic marker of stem cells at an early stage.⁵⁷ Consistently, the ERK nuclear/cytoplasmic fluorescence intensity ratio in the AR4-S group was ~2-fold higher than that in the AR1-S group (Figure 4), demonstrating the activation of diverse mechanosensing-dependent signalings. Intriguingly, clathrin also participates in modulating this integrin-mediated downstream ERK signaling pathway.¹⁷ Given the high spatial correlation between clathrin/AP2 and F-actin/FA in cells cultured on AR4-S (Figures 2G and 3A,B), we pharmaceutically inhibited clathrin signaling to study their relationship. Strikingly, this inhibition substantially downregulated the nuclear presence of ERK by 243% in the AR4-S group (Figure 4), due to the impediment to integrin/clathrin-mediated cell adhesion behavior. In contrast, cells cultured on AR1-S already showed 233% lower ERK activity than those on AR4-S, attributed to enhanced endocytosis activity mediated by clathrin. Inhibition of clathrin only slightly decreased the ERK activity by 9% in cells cultured on AR1-S (Figure 4), owing to the original weak role of clathrin in cell adhesion. These findings confirm that the anisotropic display of RGD peptides underpins the engagement between clathrin and adhesion structures for mechanotransduced signaling but refrains from endocytosis.

As there is an imbalanced biophysical environment between AR1-S and AR4-S that respectively influence differentiation potentials of hMSC cultured atop, we wonder whether leveraging the merit of endocytosis enhancement in the AR1-S group can alter its differentiation outcome. Hence, we designed two conditions for assessing the enhanced endocytic effect on osteogenic differentiation of hMSCs by AR1-S: (1)

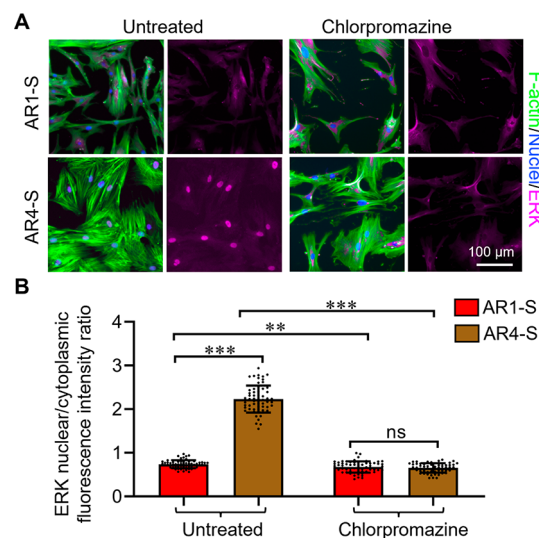


Figure 4. Evaluation of clathrin in the role of promoting mechanotransduction-based signaling. (A) Representative fluorescence images of cells that were seeded on AR1-S or AR4-S for 24 h, followed by immunostaining of the F-actin (green), nucleus (blue), and signal-regulated kinase (ERK, purple) with or without pretreatment of 5 $\mu\text{g}/\text{mL}$ chlorpromazine. (B) Quantitative analysis of fluorescence signals in cells from different groups corresponding to panel A. Sixty cells were examined for each group. Error bars denote the standard deviation. Statistical analysis of pairwise comparison was determined by one-way ANOVA. No significance (ns): $P > 0.05$; ** $P < 0.01$; *** $P < 0.001$.

cells cultured on the nanosubstrate under osteogenic induction medium with free Dex molecules over 7 days and (2) cells cultured on the nanosubstrate and incubated with Dex-delivering nanocarrier, GQD/Dex, under osteogenic induction medium without free Dex molecules (Figure 5A and Figure S25A). Note that the amount of loaded Dex by GQDs in condition (2) was equivalent to that of free Dex in condition (1). Also, cells were preseeded on the nanosubstrate for 24 h before any NP incubation or induced osteogenic differentiation. Besides, we also controlled the duration of the GQD/Dex incubation with hMSCs for 6 h, consistent with the cellular uptake experiment (Figure 2A). As exocytosis may affect the retention of GQD/Dex inside the cells, we first inspected the fluorescence signals of GQDs inside cells during the 7-day osteogenic differentiation after the 6 h of uptake. Interestingly, the AR1-S group outperformed the AR4-S group in both efficiency of cellular uptake and retention of NPs (Figure S26). In particular, GQD signals were still detectable in the AR1-S group 7 days post cellular uptake but not in the AR4-S group (Figure S26), indicating the privilege of AR1-S for acquiring Dex from GQDs. It is known that the acidic environment in organelle compartments post endocytosis, such as endosome and lysosome,⁵⁸ can elicit drug release via weakening the π - π stacking between drugs and GQDs.⁵⁹ We also demonstrated that the condition of pH 5 induced a robust cumulative Dex release from GQD/Dex (more than 50% within 1 day) and reached the maximum within 2 days compared to that at pH 7.4 with less than 20% cumulative Dex release over 7 days (Figure S27), confirming its release mechanism triggered by the intracellular microenvironment.

We assessed the activity of the early stage markers of hMSC osteogenesis, RUNX2 and alkaline phosphatase (ALP), in cells treated with various conditions after 7 days of the differ-

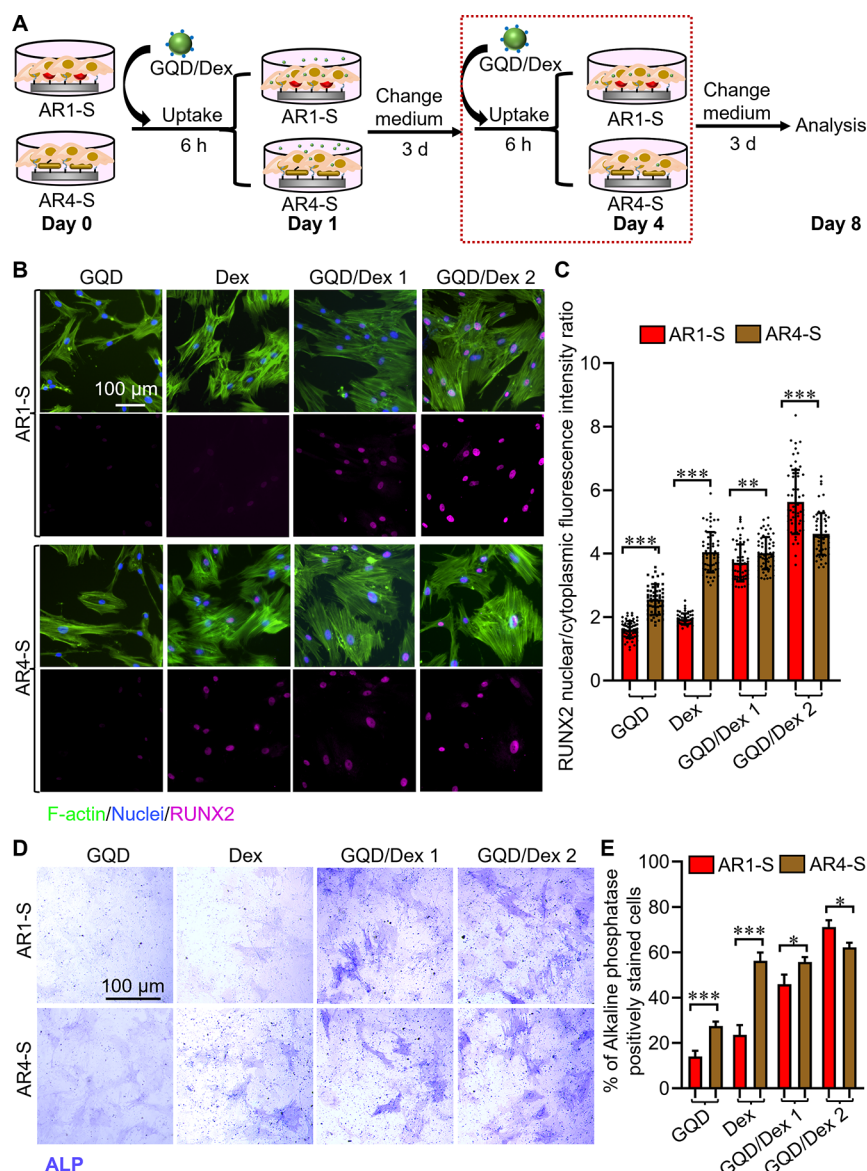


Figure 5. Osteogenesis differentiation of hMSCs induced by GQD/Dex when conditioned to AR1-S and AR4-S, respectively. (A) Schematic illustration of differentiation experiment. The cells were preseeded on the AR1-S or AR4-S in a culture well on Day 0 and incubated with growth medium for 24 h. On Day 1, the cells were incubated with or without GQD/Dex for 6 h, and the particle-containing medium was replaced by osteogenesis induction medium. On Day 4, the cells were or were not incubated with GQD/Dex as the second dose for 6 h, and the particle-containing medium was replaced by osteogenesis medium. The cell osteogenesis was analyzed on Day 8. (B) Representative fluorescence images of cells after different treatments on Day 8, followed by immunostaining of the F-actin (green), nucleus (blue), and Runt-related transcription factor 2 (RUNX2, purple). (C) Quantitative analysis of fluorescence signals in cells from different groups corresponding to panel B. Sixty cells were examined for each group. (D) Representative images of cells after different treatments on Day 8, followed by staining of alkaline phosphatase (ALP, purple). (E) Quantitative analysis of the percentage of ALP-positive cells from different groups corresponding to panel D ($n = 3$). Error bars denote the standard deviation. Statistical analysis of pairwise comparison was determined by one-way ANOVA. Significance difference: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

entiation assay.^{60,61} As expected, the AR4-S group showed a higher nuclear ratio of RUNX2 by 108% (Figure 5B,C) and a higher expression of ALP by 143% (Figure 5D,E and Figure S28) than those in the AR1-S group in condition (1), respectively. Strikingly, the relative expression levels of these two markers were significantly enhanced in the AR1-S group in condition (2), reaching levels similar to those in the AR4-S group (Figure 5B–E and Figure S28). Meanwhile, adding GQD alone did not influence these differentiation outcomes (Figure 5B–E). These results are highly consistent with the promoted GQD/Dex uptake via CME in the AR1-S group and

support the advantage of AR1-S for boosting stem cell osteogenesis. To achieve a synergistic effect of both AR1-S and AR4-S, we cultured the cells on AR1-S first for the uptake of GQD/Dex and next transferred the cells to AR4-S for osteogenic differentiation over 7 days (Figure S25A). Cells primed by this condition expressed a higher level of ALP and more intense nuclear localization of RUNX2 than those cultured on AR1-S or AR4-S only (Figure S25B–E). Hence, cells benefited from both initially enhanced cellular uptake of drug-loaded NPs and subsequently promoted mechanical feedback toward osteogenesis. Nevertheless, this method

requires cell harvesting and may disturb the continuity of the cell culture, which complicates the process of osteogenesis.

We question whether a second dose of GQD/Dex can further amplify the effect of drug-induced osteogenesis. Hence, we selected Day 4 for the second administration of GQD/Dex and assessed the results on Day 8. We showed that adding Dex molecules and GQD/Dex (1 or 2 doses) to the cells insignificantly affected their viability (Figure S29). Remarkably, the AR1-S group even showed ~22% more nuclear translocation of RUNX2 and ~15% higher percentage of ALP-expressing cells than that of the AR4-S group under the 2-dose treatment. We last verified that the nanogeometry of ligand presentation (AR1-S or AR4-S) and the GQD/Dex independently affected osteogenic differentiation of the stem cells, evidenced by the lower biomechanical feedback of cells in AR1-S group than those in AR4-S group even after the treatment of GQD/Dex over 7 days of differentiation (Figure S30). This probably stems from blocking the empty space of our platforms with PEG that prevents nonspecific attachment of proteins,⁶² decoupling the effect of nanogeometry of ligand presentation on stem cell adhesion from other confounding factors such as adhesion proteins in culture medium or secreted by cells. Hence, we confirm that Dex is critical for the osteogenic differentiation of hMSCs, and hMSCs cultured on AR1-S can benefit from the enhanced drug delivery for intensifying osteogenic differentiation outcomes over the enhanced mechanotransduction on AR4-S.

In conclusion, we elucidate the roles of clathrin in hMSCs for mediating endocytosis or participating in integrin-mediated mechanotransduction by nanosubstrate engineering. Our study modulates the anisotropy of ligand presentation on the nanoscale by Au nanostructures with various ARs (1 or 4) and strictly controlled surface areas, particle coating densities, and ligand densities. The cells cultured on AR4-S display higher levels of mechanical feedback in terms of cell adhesion, spreading, and osteogenesis. In contrast, cells on AR1-S show a reverse trend of adhesion-associated events but elevated expressions of clathrin and AP2 that facilitate the uptake of drug-loaded nanomaterials via the CME pathway. Furthermore, we demonstrate that endocytosis may be retarded by a strong association between clathrin and adhesion structures on AR4-S, as evidenced by the high colocalization between clathrin/AP2 and FA complexes/F-actin and the impeded mechanotransduced signaling ERK pathway by inhibiting clathrin. We manipulate the feature of ligand nanogeometry on AR1-S to maximize the osteogenic differentiation outcome by the enhanced uptake of GQD/Dex via robust endocytic activity, regardless of their weak mechanical engagement between integrin and ligand. Our findings not only highlight the dual functions of clathrin in CME and integrin-mediated mechanosensing by tuning the anisotropic ligand nanogeometry but also provide design rules of nanosubstrates for improving hMSC osteogenesis or other potential types of differentiations (e.g., adipogenesis) for tissue engineering.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.nanolett.3c01757>.

Experimental methods, characterization of ligand exchange of AR4, cell viability under different treatments, characterization of GQD and GQD/Dex, character-

ization of 13 nm AuNPs with antibody adsorption, additional fluorescence images with corresponding quantifications, additional Western blot data, additional SEM images, quantification of cell spreading area and FA sizes, quantification of clathrin and AP2 fluorescence intensity in cells, scatter plots of colocalization analysis, additional control experiments, the study of retention of NPs in cells, and Dex release profile (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Siu Hong Dexter Wong – Department of Biomedical Engineering, The Hong Kong Polytechnic University, Kowloon, Hong Kong 999077, China; Research Institute for Sports Science and Technology, The Hong Kong Polytechnic University, Kowloon, Hong Kong 999077, China; orcid.org/0000-0001-7920-4599; Email: shongwong@polyu.edu.hk

Mo Yang – Department of Biomedical Engineering, The Hong Kong Polytechnic University, Kowloon, Hong Kong 999077, China; Research Institute for Sports Science and Technology, The Hong Kong Polytechnic University, Kowloon, Hong Kong 999077, China; orcid.org/0000-0002-3863-8187; Email: mo.yang@polyu.edu.hk

Authors

Bohan Yin – Department of Biomedical Engineering, The Hong Kong Polytechnic University, Kowloon, Hong Kong 999077, China; orcid.org/0000-0002-3814-470X

Qin Zhang – Department of Biomedical Engineering, The Hong Kong Polytechnic University, Kowloon, Hong Kong 999077, China

Jiaxiang Yan – Department of Biomedical Engineering, The Hong Kong Polytechnic University, Kowloon, Hong Kong 999077, China

Yingying Huang – Department of Biomedical Engineering, The Hong Kong Polytechnic University, Kowloon, Hong Kong 999077, China

Chuanqi Li – Department of Biomedical Engineering, The Hong Kong Polytechnic University, Kowloon, Hong Kong 999077, China

Jiareng Chen – Department of Biomedical Engineering, The Hong Kong Polytechnic University, Kowloon, Hong Kong 999077, China

Chunyi Wen – Department of Biomedical Engineering, The Hong Kong Polytechnic University, Kowloon, Hong Kong 999077, China; orcid.org/0000-0003-1949-7822

Complete contact information is available at:

<https://pubs.acs.org/doi/10.1021/acs.nanolett.3c01757>

Author Contributions

B.Y. conceived the idea, performed experiments, analyzed the data, and drafted the paper. Q.Z., J.C., and C.W. contributed to fluorescence imaging data collection. J.Y., Y.H., and C.L. contributed to TEM imaging data collection. S.H.D.W. and M.Y. conceived the idea, contributed to scientific advice, resource for this study, editing of the paper, and supervising the whole project. All authors reviewed and approved the paper.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the Shenzhen Science and Technology Program-Basic Research Scheme (JCYJ20220531090808020), the Research Grants Council (RGC) of Hong Kong Collaborative Research Grant (C5078-21 EF), the Research Grants Council (RGC) of Hong Kong General Research Grant (PolyU 15217621 and PolyU 15210818), the Guangdong-Hong Kong Technology Cooperation Funding Scheme (GHP/032/20SZ and SGDX20201103095404018), the Hong Kong Polytechnic University Shenzhen Institute Bai Cheng Bai Yuan Fund (I2022A002), and the Hong Kong Polytechnic University Internal Fund (1-ZVVQ and 1-CD6J). S.H.D.W. would like to acknowledge the start-up funding (1-ZVRY) from the Department of Biomedical Engineering and Start-up Fund for RAPs under the Strategic Hiring Scheme (1-BD8Q), the Hong Kong Polytechnic University (PolyU, University Grant Council), and PolyU Projects of RISports (1-CD5P) for supporting this work. This work was also supported by the University Research Facility in Life Sciences of the Hong Kong Polytechnic University.

REFERENCES

- (1) Zhang, Y.; Fang, C.; Zhang, S.; Campbell, R. E.; Serpe, M. J. Controlled osteogenic differentiation of human mesenchymal stem cells using dexamethasone-loaded light-responsive microgels. *ACS Appl. Mater. Interfaces* **2021**, *13*, 7051–7059.
- (2) Wei, M.; Li, S.; Le, W. Nanomaterials modulate stem cell differentiation: biological interaction and underlying mechanisms. *J. Nanobiotechnol.* **2017**, *15*, 75.
- (3) Yi, C.; Liu, D.; Fong, C. C.; Zhang, J.; Yang, M. Gold nanoparticles promote osteogenic differentiation of mesenchymal stem cells through p38 MAPK pathway. *ACS Nano* **2010**, *4*, 6439–6448.
- (4) Madamsetty, V. S.; Mohammadinejad, R.; Uzielienė, I.; Nabavi, N.; Dehshahri, A.; García-Couce, J.; Tavakol, S.; Moghasssemi, S.; Dadashzadeh, A.; Makvandi, P.; et al. Dexamethasone: Insights into pharmacological aspects, therapeutic mechanisms, and delivery systems. *ACS Biomater. Sci. Eng.* **2022**, *8*, 1763–1790.
- (5) Bae, S. E.; Choi, D. H.; Han, D. K.; Park, K. Effect of temporally controlled release of dexamethasone on in vivo chondrogenic differentiation of mesenchymal stromal cells. *J. Controlled Release* **2010**, *143*, 23–30.
- (6) Wang, X.; Li, L.; Song, F. Interplay of nanoparticle properties during endocytosis. *Crystals* **2021**, *11*, 728.
- (7) Kovtun, O.; Dickson, V. K.; Kelly, B. T.; Owen, D. J.; Briggs, J. A. G. Architecture of the AP2/clathrin coat on the membranes of clathrin-coated vesicles. *Sci. Adv.* **2020**, *6*, No. eaba8381.
- (8) Yin, B.; Ho, L. W. C.; Liu, S.; Hong, H.; Tian, X. Y.; Li, H.; Choi, C. H. J. Sub-10 nm substrate roughness promotes the cellular uptake of nanoparticles by upregulating endocytosis-related genes. *Nano Lett.* **2021**, *21*, 1839–1847.
- (9) Li, X.; Klausen, L. H.; Zhang, W.; Jahed, Z.; Tsai, C. T.; Li, T. L.; Cui, B. Nanoscale surface topography reduces focal adhesions and cell stiffness by enhancing integrin endocytosis. *Nano Lett.* **2021**, *21*, 8518–8526.
- (10) Pennacchio, F. A.; Caliendo, F.; Iaccarino, G.; Langella, A.; Siciliano, V.; Santoro, F. Three-dimensionally patterned scaffolds modulate the biointerface at the nanoscale. *Nano Lett.* **2019**, *19*, 5118–5123.
- (11) Zhao, W.; Hanson, L.; Lou, H. Y.; Akamatsu, M.; Chowdary, P. D.; Santoro, F.; Marks, J. R.; Grassart, A.; Drubin, D. G.; Cui, Y.; Cui, B. Nanoscale manipulation of membrane curvature for probing endocytosis in live cells. *Nat. Nanotechnol.* **2017**, *12*, 750–756.
- (12) Huang, C.; Butler, P. J.; Tong, S.; Muddana, H. S.; Bao, G.; Zhang, S. Substrate stiffness regulates cellular uptake of nanoparticles. *Nano Lett.* **2013**, *13*, 1611–1615.
- (13) Lee, A.; Sousa de Almeida, M.; Milinkovic, D.; Septiadi, D.; Taladriz-Blanco, P.; Loussert-Fonta, C.; Balog, S.; Bazzoni, A.; Rothen-Rutishauser, B.; Petri-Fink, A. Substrate stiffness reduces particle uptake by epithelial cells and macrophages in a size-dependent manner through mechanoregulation. *Nanoscale* **2022**, *14*, 15141–15155.
- (14) Elkhatib, N.; Bresteau, E.; Baschieri, F.; Rioja, A. L.; van Niel, G.; Vassilopoulos, S.; Montagnac, G. Tubular clathrin/AP-2 lattices pinch collagen fibers to support 3D cell migration. *Science* **2017**, *356*, No. eaal4713.
- (15) Leyton-Puig, D.; Isogai, T.; Argenzio, E.; van den Broek, B.; Klarenbeek, J.; Janssen, H.; Jalink, K.; Innocenti, M. Flat clathrin lattices are dynamic actin-controlled hubs for clathrin-mediated endocytosis and signalling of specific receptors. *Nat. Commun.* **2017**, *8*, 16068.
- (16) Yu, C. H.; Rafiq, N. B.; Cao, F.; Zhou, Y.; Krishnasamy, A.; Biswas, K. H.; Ravasio, A.; Chen, Z.; Wang, Y. H.; Kawauchi, K.; Jones, G. E.; Sheetz, M. P. Integrin-beta3 clusters recruit clathrin-mediated endocytic machinery in the absence of traction force. *Nat. Commun.* **2015**, *6*, 8672.
- (17) Baschieri, F.; Dayot, S.; Elkhatib, N.; Ly, N.; Capmany, A.; Schauer, K.; Betz, T.; Vignjevic, D. M.; Poincloux, R.; Montagnac, G. Frustrated endocytosis controls contractility-independent mechanotransduction at clathrin-coated structures. *Nat. Commun.* **2018**, *9*, 3825.
- (18) Lock, J. G.; Baschieri, F.; Jones, M. C.; Humphries, J. D.; Montagnac, G.; Stromblad, S.; Humphries, M. J. Clathrin-containing adhesion complexes. *J. Cell Biol.* **2019**, *218*, 2086–2095.
- (19) Almalik, A.; Karimi, S.; Ouasti, S.; Donno, R.; Wandrey, C.; Day, P. J.; Tirelli, N. Hyaluronic acid (HA) presentation as a tool to modulate and control the receptor-mediated uptake of HA-coated nanoparticles. *Biomaterials* **2013**, *34*, 5369–5380.
- (20) Zhu, C.; Zhou, X.; Liu, Z.; Chen, H.; Wu, H.; Yang, X.; Zhu, X.; Ma, J.; Dong, H. The morphology of hydroxyapatite nanoparticles regulates cargo recognition in clathrin-mediated endocytosis. *Front. Mol. Biosci.* **2021**, *8*, 627015.
- (21) Adler, A. F.; Leong, K. W. Emerging links between surface nanotechnology and endocytosis: impact on nonviral gene delivery. *Nano today* **2010**, *5*, 553–569.
- (22) Teo, B. K.; Goh, S. H.; Kustandi, T. S.; Loh, W. W.; Low, H. Y.; Yim, E. K. The effect of micro and nanotopography on endocytosis in drug and gene delivery systems. *Biomaterials* **2011**, *32*, 9866–9875.
- (23) Karimi, F.; Thombare, V. J.; Hutton, C. A.; O'Connor, A. J.; Qiao, G. G.; Heath, D. E. Beyond RGD; nanoclusters of syndecan and integrin-binding ligands synergistically enhance cell/material interactions. *Biomaterials* **2018**, *187*, 81–92.
- (24) Ye, K.; Wang, X.; Cao, L.; Li, S.; Li, Z.; Yu, L.; Ding, J. Matrix stiffness and nanoscale spatial organization of cell-adhesive ligands direct stem cell fate. *Nano Lett.* **2015**, *15*, 4720–4729.
- (25) Kang, H.; Wong, S. H. D.; Pan, Q.; Li, G.; Bian, L. Anisotropic ligand nanogeometry modulates the adhesion and polarization state of macrophages. *Nano Lett.* **2019**, *19*, 1963–1975.
- (26) Liu, W.; Sun, Q.; Zheng, Z. L.; Gao, Y. T.; Zhu, G. Y.; Wei, Q.; Xu, J. Z.; Li, Z. M.; Zhao, C. S. Topographic cues guiding cell polarization via distinct cellular mechanosensing pathways. *Small* **2022**, *18*, No. 2104328.
- (27) Yao, X.; Peng, R.; Ding, J. Cell-material interactions revealed via material techniques of surface patterning. *Adv. Mater.* **2013**, *25*, 5257–5286.
- (28) Wang, X.; Yan, C.; Ye, K.; He, Y.; Li, Z.; Ding, J. Effect of RGD nanospacing on differentiation of stem cells. *Biomaterials* **2013**, *34*, 2865–2874.
- (29) Wang, X.; Li, S.; Yan, C.; Liu, P.; Ding, J. Fabrication of RGD micro/nanopattern and corresponding study of stem cell differentiation. *Nano Lett.* **2015**, *15*, 1457–1467.

- (30) Sun, Q.; Pei, F.; Zhang, M.; Zhang, B.; Jin, Y.; Zhao, Z.; Wei, Q. Curved nanofiber network induces cellular bridge formation to promote stem cell mechanotransduction. *Adv. Sci.* **2023**, *10*, No. 2204479.
- (31) Yin, B.; Yang, H.; Yang, M. Integrating soft hydrogel with nanostructures reinforces stem cell adhesion and differentiation. *J. Compos. Sci.* **2022**, *6*, 19.
- (32) Wong, D. S.; Li, J.; Yan, X.; Wang, B.; Li, R.; Zhang, L.; Bian, L. Magnetically tuning tether mobility of integrin ligand regulates adhesion, spreading, and differentiation of stem cells. *Nano Lett.* **2017**, *17*, 1685–1695.
- (33) Wong, S. H. D.; Wong, W. K. R.; Lai, C. H. N.; Oh, J.; Li, Z.; Chen, X.; Yuan, W.; Bian, L. Soft polymeric matrix as a macroscopic cage for magnetically modulating reversible nanoscale ligand presentation. *Nano Lett.* **2020**, *20*, 3207–3216.
- (34) Di Natale, C.; Natale, C. F.; Florio, D.; Netti, P. A.; Morelli, G.; Ventre, M.; Marasco, D. Effects of surface nanopatterning on internalization and amyloid aggregation of the fragment 264–277 of Nucleophosmin 1. *Colloids Surf., B* **2021**, *197*, 111439.
- (35) Wong, S. H. D.; Yin, B.; Yang, B.; Lin, S.; Li, R.; Feng, Q.; Yang, H.; Zhang, L.; Yang, Z.; Li, G.; et al. Anisotropic nanoscale presentation of cell adhesion ligand enhances the recruitment of diverse integrins in adhesion structures and mechanosensing-dependent differentiation of stem cells. *Adv. Funct. Mater.* **2019**, *29*, 1806822.
- (36) Gautieri, A.; Vesentini, S.; Redaelli, A.; Buehler, M. J. Hierarchical structure and nanomechanics of collagen microfibrils from the atomistic scale up. *Nano Lett.* **2011**, *11*, 757–766.
- (37) Bastus, N. G.; Comenge, J.; Puntès, V. Kinetically controlled seeded growth synthesis of citrate-stabilized gold nanoparticles of up to 200 nm: size focusing versus Ostwald ripening. *Langmuir* **2011**, *27*, 11098–110105.
- (38) Yang, H.; Chen, Z.; Zhang, L.; Yung, W. Y.; Leung, K. C.; Chan, H. Y.; Choi, C. H. Mechanism for the cellular uptake of targeted gold nanorods of defined aspect ratios. *Small* **2016**, *12*, 5178–5189.
- (39) Mehtala, J. G.; Zemlyanov, D. Y.; Max, J. P.; Kadasala, N.; Zhao, S.; Wei, A. Citrate-stabilized gold nanorods. *Langmuir* **2014**, *30*, 13727–13730.
- (40) Oh, J.; Xia, X.; Wong, W. K. R.; Wong, S. H. D.; Yuan, W.; Wang, H.; Lai, C. H. N.; Tian, Y.; Ho, Y. P.; Zhang, H.; Zhang, Y.; Li, G.; Lin, Y.; Bian, L. The effect of the nanoparticle shape on T cell activation. *Small* **2022**, *18*, No. 2107373.
- (41) Yin, B.; Chan, C. K. W.; Liu, S.; Hong, H.; Wong, S. H. D.; Lee, L. K. C.; Ho, L. W. C.; Zhang, L.; Leung, K. C.; Choi, P. C.; Bian, L.; Tian, X. Y.; Chan, M. N.; Choi, C. H. J. Intrapulmonary cellular-level distribution of inhaled nanoparticles with defined functional groups and its correlations with protein corona and inflammatory response. *ACS Nano* **2019**, *13*, 14048–14069.
- (42) Shang, W.; Zhang, X.; Zhang, M.; Fan, Z.; Sun, Y.; Han, M.; Fan, L. The uptake mechanism and biocompatibility of graphene quantum dots with human neural stem cells. *Nanoscale* **2014**, *6*, 5799–5806.
- (43) Ghaffarkhah, A.; Hosseini, E.; Kamkar, M.; Sehat, A. A.; Dordanihaghghi, S.; Allahbakhsh, A.; van der Kuur, C.; Arjmand, M. Synthesis, applications, and prospects of graphene quantum dots: A comprehensive review. *Small* **2022**, *18*, 2102683.
- (44) Devi, G. R.; Prathyusha, V.; Shanthakumari, K.; Rahaman, S. Development and validation of UV-Spectrophotometric method for the estimation of dexamethasone sodium phosphate in bulk and pharmaceutical dosage form. *Indo Am. J. Pharm. Res.* **2013**, *3*, 5055–5061.
- (45) Hao, Y.; Zhang, F.; Mo, S.; Zhao, J.; Wang, X.; Zhao, Y.; Zhang, L. Biomedical applications of supramolecular materials in the controllable delivery of steroids. *Front. Mol. Biosci.* **2021**, *8*, 700712.
- (46) Das, G.; Benyettou, F.; Sharama, S. K.; Prakasam, T.; Gandara, F.; de la Pena-O'Shea, V. A.; Saleh, N.; Pasricha, R.; Jagannathan, R.; Olson, M. A.; Trabolsi, A. Covalent organic nanosheets for bioimaging. *Chem. Sci.* **2018**, *9*, 8382–8387.
- (47) Chen, X.; Fan, Y.; Sun, J.; Zhang, Z.; Xin, Y.; Li, K.; Tang, K.; Du, P.; Liu, Y.; Wang, G.; Yang, M.; Tan, Y. Nanoparticle-mediated specific elimination of soft cancer stem cells by targeting low cell stiffness. *Acta Biomater.* **2021**, *135*, 493–505.
- (48) Vercauteren, D.; Vandenbroucke, R. E.; Jones, A. T.; Rejman, J.; Demeester, J.; De Smedt, S. C.; Sanders, N. N.; Braeckmans, K. The use of inhibitors to study endocytic pathways of gene carriers: optimization and pitfalls. *Mol. Ther.* **2010**, *18*, 561–569.
- (49) Zhang, Y.; Zhang, L.; Li, Y.; Sun, S.; Tan, H. Different contributions of clathrin- and caveolae-mediated endocytosis of vascular endothelial cadherin to lipopolysaccharide-induced vascular hyperpermeability. *PLoS One* **2014**, *9*, No. e106328.
- (50) Acharya, A. P.; Dolgova, N. V.; Moore, N. M.; Xia, C. Q.; Clare-Salzler, M. J.; Becker, M. L.; Gallant, N. D.; Keselowsky, B. G. The modulation of dendritic cell integrin binding and activation by RGD-peptide density gradient substrates. *Biomaterials* **2010**, *31*, 7444–7454.
- (51) Wirkner, M.; Weis, S.; San Miguel, V.; Alvarez, M.; Gropeanu, R. A.; Salierno, M.; Sartoris, A.; Unger, R. E.; Kirkpatrick, C. J.; del Campo, A. Photoactivatable caged cyclic RGD peptide for triggering integrin binding and cell adhesion to surfaces. *ChemBiochem* **2011**, *12*, 2623–2629.
- (52) Hou, Y.; Yu, L.; Xie, W.; Camacho, L. C.; Zhang, M.; Chu, Z.; Wei, Q.; Haag, R. Surface roughness and substrate stiffness synergize to drive cellular mechanoreponse. *Nano Lett.* **2020**, *20*, 748–757.
- (53) Xie, W.; Wei, X.; Kang, H.; Jiang, H.; Chu, Z.; Lin, Y.; Hou, Y.; Wei, Q. Static and dynamic: Evolving biomaterial mechanical properties to control cellular mechanotransduction. *Adv. Sci.* **2023**, *10*, No. 2204594.
- (54) Jeschke, B.; Meyer, J.; Jonczyk, A.; Kessler, H.; Adamietz, P.; Meenen, N. M.; Kantlehner, M.; Goepfert, C.; Nies, B. RGD-peptides for tissue engineering of articular cartilage. *Biomaterials* **2002**, *23*, 3455–3463.
- (55) Yang, B.; Wei, K.; Loebel, C.; Zhang, K.; Feng, Q.; Li, R.; Wong, S. H. D.; Xu, X.; Lau, C.; Chen, X.; Zhao, P.; Yin, C.; Burdick, J. A.; Wang, Y.; Bian, L. Enhanced mechanosensing of cells in synthetic 3D matrix with controlled biophysical dynamics. *Nat. Commun.* **2021**, *12*, 3514.
- (56) Zhang, C.; Zhu, H.; Ren, X.; Gao, B.; Cheng, B.; Liu, S.; Sha, B.; Li, Z.; Zhang, Z.; Lv, Y.; Wang, H.; Guo, H.; Lu, T. J.; Xu, F.; Genin, G. M.; Lin, M. Mechanics-driven nuclear localization of YAP can be reversed by N-cadherin ligation in mesenchymal stem cells. *Nat. Commun.* **2021**, *12*, 6229.
- (57) Eskildsen, T.; Taipaleenmäki, H.; Stenvang, J.; Abdallah, B. M.; Ditzel, N.; Nossent, A. Y.; Bak, M.; Kauppinen, S.; Kassem, M. MicroRNA-138 regulates osteogenic differentiation of human stromal (mesenchymal) stem cells in vivo. *Proc. Natl. Acad. Sci., U. S. A.* **2011**, *108*, 6139–6144.
- (58) Hu, Y. B.; Dammer, E. B.; Ren, R. J.; Wang, G. The endosomal-lysosomal system: from acidification and cargo sorting to neurodegeneration. *Transl. Neurodegener.* **2015**, *4*, 18.
- (59) Zhuang, W. R.; Wang, Y.; Cui, P. F.; Xing, L.; Lee, J.; Kim, D.; Jiang, H. L.; Oh, Y. K. Applications of pi-pi stacking interactions in the design of drug-delivery systems. *J. Controlled Release* **2019**, *294*, 311–326.
- (60) Lee, K.-S.; Kim, H.-J.; Li, Q.-L.; Chi, X.-Z.; Ueta, C.; Komori, T.; Wozney, J. M.; Kim, E.-G.; Choi, J.-Y.; Ryoo, H.-M. Runx2 is a common target of transforming growth factor β 1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. *Mol. Cell. Biol.* **2000**, *20*, 8783–8792.
- (61) Li, N.; Zhou, L.; Xie, W.; Zeng, D.; Cai, D.; Wang, H.; Zhou, C.; Wang, J.; Li, L. Alkaline phosphatase enzyme-induced biomineralization of chitosan scaffolds with enhanced osteogenesis for bone tissue engineering. *Chem. Eng. J.* **2019**, *371*, 618–630.
- (62) Du, H.; Chandaroy, P.; Hui, S. W. Grafted poly-(ethylene glycol) on lipid surfaces inhibits protein adsorption and cell adhesion. *Biochim. Biophys. Acta* **1997**, *1326*, 236–248.