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Efficient delivery of biological cargos into primary cells by electrodeposited nanoneedles via cell cycle-dependent endocytosis

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ABSTRACT

Nanoneedles are a useful tool for delivering exogenous biomolecules to cells. Although therapeutic applications have been explored, the mechanism regarding how cells interact with nanoneedles remains poorly studied. Here, we present a new approach for the generation of nanoneedles, validated their usefulness in cargo delivery, and studied the underlying genetic modulators during delivery. We fabricated arrays of nanoneedles based on electrodeposition and quantified its efficacy of delivery using fluorescently labeled proteins and siRNAs. Notably, we revealed that our nanoneedles caused the disruption of cell membranes, enhanced the expression of cell-cell junction proteins, and downregulated the expression of transcriptional factors of NFkB pathways. This perturbation trapped most of the cells in G2 phase, in which the cells have the highest endocytosis activities. Taken together, this system provides a new model for the study of interactions between cells and high-aspect-ratio materials.

KEYWORDS

Electrodeposition, nanostructure, intracellular delivery, mechanobiology, cell cycle

The delivery of exogenous biomolecules into cells is the foundation of genome editing and cell therapy.¹ Existing approaches often involve the use of viruses, electric fields, or chemicals that are either dangerous, toxic, or inefficient.² Hence, there is an unmet need of developing efficient and cost-effective methods for intracellular delivery with minimal cytotoxicity and cellular stress.

Microneedles with a feature size of 100 – 500 μm have demonstrated functionality for intracellular delivery. However, the delivery efficacy via microneedles is usually low (8%^{3,4} – 20%⁵) through passive diffusion. In addition, some efforts have been paid to actively deliver plasmids into the cells through microneedle-mediated electroporation.⁶ Although the efficacy was boosted to 50%, the setup requires a high voltage (> 50V) to operate.

In contrast, high-aspect-ratio nanomaterials, especially vertical nanoneedles with a feature size of 100 – 1000 nm, have demonstrated a broad utility, efficiency (up to 95%), and minimal cytotoxic effect in intracellular delivery *in vitro*,⁷ including nucleic acids,⁸ nanoparticles,⁹ therapeutic drugs,¹⁰ proteins,¹¹ and metabolites.¹² In addition, nanoneedles have been introduced for *in situ* gene/immune therapy^{13,14} in animal models and achieved encouraging results *in vivo*. Nanoneedles may prove to be a safe and effective tool for intracellular delivery.

Despite the success at the laboratory level, the translation of nanoneedles from bench to clinic is very limited. Several key challenges remain unsolved. Indeed, the fabrication of nanoneedles commonly involves the use of reactive-ion etching (RIE),^{2,8–10,12,13,15} which is expensive, non-scalable, labor-intensive, and requires access to the centralized cleanroom facility. The use of RIE significantly hampers the large-scale manufacturing of nanoneedles. Moreover, prior studies have utilized established cancer cell lines to measure/demonstrate the delivery activities, such as leukemic lymphocytes,^{16,17} and adherent carcinoma.^{8,9,13,16,18} These cancer cell lines are generally easy to transfect and therefore are not a good representation of the primary or stem cells used as therapeutics.¹⁹

Moreover, the principle of nanoneedle-mediated intracellular delivery is not systematically understood. It has been proposed in some studies that nanoneedles

simply penetrate the cell membrane through membrane disruption^{2,12,20,21} while others suggest that the penetration model oversimplifies the cell-nanoneedle interface.^{11,22,23} The presence of nanoneedles may promote the uptake of biomolecules through a dynamic biochemical and biomechanical regulation of cell membranes.^{11,24–27} A recent study also highlights that nanoneedles can even regulate the expression of nuclear genes via mechanotransduction.²⁸ Taken together, these findings indicate the need for in-depth investigations of the cell-nanoneedle interface.

We recently introduced electrodeposited electrodes for studying the interaction between cells and nanostructures.^{29,30} Our electrodeposition method is highly tunable to the fabrication of microarchitectures with different degrees of nanostructuring in a template-mediated^{31–33} or template-free manner.³⁴ Notably, nanoneedles with highly tunable geometries and densities can be deposited onto the bare gold surface in a few minutes with a simple setup.³⁴ This motivated us to adapt electrodeposition as a means for rapid, RIE-free fabrication of nanoneedles.

Here, we report the first use of electrodeposition to fabricate gold nanoneedles for intracellular delivery in hard-to-transfect primary and stem cells. Our electrodeposition method is rapid, RIE-free, scalable, and relatively low-cost. We investigated the relationship between nanoneedle morphology, delivery efficiency, and cytotoxicity. We found the gold nanoneedles with different morphology had minimal effects on the viability and apoptosis of cells based on the staining of Annexin V. Highly spiky nanoneedles deposited under the voltage of 0 mV and -300 mV resulted in better delivery efficiency. Various exogenous biomolecules (dextran, albumin, transferrin, quantum dot, siRNA) were successfully delivered with the help of electrodeposited nanoneedles into primary and stem cells, including pluripotent stem cells, mesenchymal stem cells, endothelial cells, and primary fibroblasts. We also studied the mechanism underlying the improved efficiency. It was found that the electrodeposited nanoneedles promote the endocytosis of biomolecules via arresting cells on the G2 cell cycle. Further microarrayed and mass spectrometric analysis reveals that such cell cycle arrest

originated from the limited adhesion on the cell membrane, transduced to the nucleus via the NF κ B pathway, and achieved through regulating E2F and CDK proteins in the nucleus. Taken together, our findings prove the utility of electrodeposited gold nanoneedles for the generic delivery of biomolecules and provide biological insights into the interaction between complex nanostructures and sensitive primary cells beyond the current membrane disruption model. We believe this novel perspective is useful for the design of next-generation multifunctional biointerfaces.

Our nanoneedles are generated on a mask-free gold-coated glass substrate (Fig. 1A). The fabrication procedure only involves one-step electrodeposition running at direct current potential amperometry (DCPA). This one-step procedure can be performed at any benchtop potentiostat, offering great simplicity compared to other fabrication methods that typically require access to a dedicated cleanroom facility for photolithography and RIE.^{2,28,35,36} Another advantage of electrodeposition is its ability to fabricate a large surface area at a time – we have validated the DCPA can be performed properly on 5-inch gold-coated glass wafers. The scalability is potentially useful when scaling up the application of nanoneedles for the large-scale manufacturing of therapeutic cells.

During DCPA, the morphogenesis of nanoneedles can be precisely controlled via tuning the potential on cathodes. Increasingly positive potential limits the nucleation of gold ion (AuCl₄⁻) in HAuCl₄ solution to metallic gold.³⁷ As a result, the nanoneedles deposited under a higher positive potential have smaller size and spikiness of needles,³⁴ as revealed by the scanning electron microscopy (SEM, Fig. 1B). Hence, to favor the formation of nanoneedles, it is desired to apply zero or negative potentials to speeds up the nucleation – this yields in the creation of nanostructured microarchitectures (NMA) consisting nanoneedles on its deposition front (Fig. 1B). The diameter of deposited nanoneedles is tunable through the potential - potentials of 0 mV and -300 mV generate nanoneedles with diameters of 100 and 500 nm, respectively. It is worth noting that the electrodeposition is not able to control the orientation of NMAs and associated

nanoneedles compared to RIE-based approaches.^{2,28,35,36} However, considering its high degree of nanostructuring, the NMAs still contain sufficient amounts of nanoneedles oriented upright for direct contact with the cell membranes.

Characterization of NMA biocompatibility is a prerequisite for delivery. We therefore evaluated the cytotoxicity of NMAs using an Annexin V/Dead cell staining assay by flow cytometry, where Annexin V stains apoptotic cells and NucGreen Dead dye stains necrotic cells. We assessed the viability of HES2, a human embryonic stem cell line (hESC) after 2 days' culture on NMAs with different spikiness (Fig. 1C) and observed minor changes in terms of viability and apoptosis (Fig. 1D). This demonstrates the short-term biocompatibility of NMAs and enables its use as a means for intracellular delivery. To have a better idea of the impact of NMAs on hESCs, we recultured the Day 2 cells released from the NMAs. A cocktail of SOX2/Oct4/SSEA5 was utilized to quantify the phenotypes of hESCs using flow cytometry (Fig. 1E and S1A) and fluorescent microscopy (Fig. S1B). We did not observe noticeable alternation in cell morphology and expression of pluripotent markers. This finding indicates that the NMAs are not only biocompatible but also have little impact on phenotypic variation in the short term (2 days).

We performed a series of experiments to deliver various biomolecules into hESCs. We tested delivery with Alexa Fluor 647-labeled transferrin, a glycoprotein protein that mediates the transport of iron through blood plasma. The uptake of transferrin is strictly regulated through clathrin-mediated endocytosis³⁸ and therefore is a good indicator of endocytotic activities. We found that the cells on NMAs experienced much higher uptake of transferrin compared to ones on flat vessels (81.9% vs 42.2%, Fig. 2A). A similar trend was also observed with quantum dots, a nanomaterial that is largely taken by clathrin-mediated endocytosis.³⁹ Interestingly, the delivery efficiency of sharp nanoneedles (0 mV and -300 mV) is considerably better than mild nanopits (Fig. 2B). Taken together, this suggests that the presence of NMAs, especially high aspect-ratio nanoneedles, promotes the clathrin-mediated endocytosis. Indeed, this observation is in high agreement with

a previous study reporting that the nanoneedles help the formation of clathrin-coated pits for vesicle intake.¹¹

Endocytosis is a major pathway for the uptake of many common biomolecules, including dextran.⁴⁰ We next used dextran with different molecular weights to benchmark the generality of upregulated endocytosis (Fig. 2C). A global increase of uptake was observed from the molecular weight of 3kDa to 500kDa, although the uptake of 500k dextran on NMAs was still relatively low (< 30%) after 24 hours. Hence, we concluded that the presence of NMAs benefits the endocytotic intake of common biomolecules and could effectively help the delivery of biomolecules up to 70kDa. In addition, we observed little (< 20%) uptake of albumin on flat and NMA conditions, although the albumin is internalized mostly through endocytosis.⁴¹ The mechanism behind this observation is unclear. The added albumin may interact with the soluble fibroblast growth factors (FGF) present in the hESC medium^{42,43} before the cellular uptake.

We also seeded multiple different primary cells, including adipose-derived stem cells (ADSC), human umbilical vein endothelial cells (HUVEC), primary fibroblast cells (18-CO, isolated from human colon tissues), and BYS-0113 human induced pluripotent stem cells (hiPSC, Fig 2D). Upregulated intake of dextran and albumin were consistently observed, indicating that NMA-mediated upregulation of endocytosis is applicable to multiple cell types.

Common transfection reagents, such as lipofectamine, utilize endocytosis to deliver specific DNA and RNA into cells.⁴⁴ Hence, the NMA-mediated upregulation of endocytosis may also benefit the delivery of DNA and RNA. To test this, we delivered a variety of small interfering RNA (siRNA) into hESCs, hiPSCs, and ADSCs. The siRNA was fluorescently labeled by Cy5 or FAM. The transfection efficiency was quantified by flow cytometry (Fig. 3A – 3C). We observed an upregulated intake of siRNA at the RNA level. To verify the integrity and functionality of delivered siRNA, we examined the knockdown (KD) of siRNA-targeted protein at the protein level (Fig. 3D and 3F). Similar to the results at the RNA level, cells cultured on NMAs had lower expression of the target protein,

indicating a higher KD efficiency. We achieved a 75% KD of GAPDH on hESCs and 90% KD of Vimentin on ADSCs, which is comparable to the well-optimized KD protocol for stem cells.⁴⁵ It is worth noting that we achieved such efficiency with only one round of transfection in 24 hrs while the previous protocol requires multiple rounds of transfection in 72 hrs to 6 days.⁴⁵ Hence, NMA-mediated upregulation of endocytosis holds great potential for rapid and efficient DNA/RNA delivery.

Next, we sought to investigate the mechanism of the increased endocytosis. A comprehensive proteome analysis was performed based on mass spectrometry. Differential protein expression was carried out by comparing the total peptide number from each sample. Proteins were considered differentially upregulated if fold change (NMA/control) is greater than 4 (equivalent to $\log_2(\text{FC}) > 2$). Lists of upregulated proteins were submitted to Metascape⁴⁶ for over-representation analysis (Fig. 4A). Not surprisingly, membrane trafficking, cytoskeleton-dependent intracellular transportation, and organelle organization occur in the top-10 enriched pathways. This matches well with our observation of upregulated endocytotic activities. Interestingly, cell cycle and cell phase-related pathways also show up multiple times in the top 10 enriched pathways, revealing a possible alternation in cell cycle. With this in mind, we performed cell cycle analysis using flow cytometry and noticed a significant modulation (Fig. 4B and 4C). Compared to the cells seeded on a flat substrate, the one on the NMAs had a noteworthy increase in the percentage of G2/M phase. Quantitative analysis revealed a negative correlation between the potential of deposition (equivalently to the spikiness of NMAs) and the percentage of cells in the G2/M phase. Highly spiky NMAs (0 mV and -300 mV) can arrest around 60% of cells on the G2/M phase. To accurately identify the phase of arrest, we stained the samples with histone H3 pS28, a M phase-specific phosphorylated protein and confirmed less than 2% of the cells on 0 mV NMA substrate were on M phase (Fig. 4D). With this in mind, we concluded that the cells on spiky NMAs underwent significant G2 arrest.

The uptake of exogenous biomolecules is a cell-cycle-dependent process. Numerous studies have demonstrated the uptake of nanoparticles,⁴⁷ lipoplex,⁴⁸ polyplex,⁴⁸ metal compound,⁴⁹ as well as membrane permeability,^[38] are strongly correlated to the cell cycle. Cells in G2 cycles have much higher delivery/transfection efficiency compared to the ones in G0/G1 cycle. For some cell lines, such difference can be up to 30 – 500 fold higher as quantified by the activity of transfected luciferase.⁴⁸ These studies support our observations that the highly spiky NMAs induced G2 arrest and such arrest benefited the delivery of exogenous biomolecules.

To identify the key regulators in this microenvironment-induced cell cycle arrest, we subsequently performed mRNA-based microarray analysis. We surveyed 48 and 96 essential genes in cell cycle regulation and cell adhesion, respectively (Fig. 4D and S2). For the cell cycle, we found the E2F family was significantly upregulated when seeding cells on NMAs. Within, expression of *E2F4* has been reported to regulate a stable G2 arrest in prostate carcinoma.⁵⁰ Most of the members in E2F family, including *E2F2* and *E2F4*, were recently identified to highly expressed in non-G0/G1 cells, indicating their roles in maintaining cells in S-G2-M phases.⁵¹ In addition, the top downregulated gene in the cell cycle is *CDKN1B*, a promotor for G1 arrest.⁵² Hence, we concluded that the NMA causes G2 arrest by upregulating E2F family and downregulating *CDKN1B* in the nucleus. For cell adhesion, we noticed that over 90% of the genes were upregulated on NMAs and the top upregulated genes are corresponding to cell-cell junction (*ICAM1*, *HAS1*). This finding is consistent with our previous observation that cells underwent limited adhesion and membrane disruption on NMA and therefore promote cell-cell adhesion to avoid anoikis.³⁴

To search for the possible pathways that transduce the mechanical signaling from the membrane to the nucleus for cell cycle arrest, we performed TRRUST analysis (Transcriptional Regulatory Relationships Unraveled by Sentence-based Text mining)⁵³ using the list consisting of regulated genes/proteins. There are three shared genes (*SP1*, *RELA*, *NFKB1*) in the transcriptional regulators of the hits from

cell cycle and adhesion (Fig. 4F and Fig. S3) at the RNA level. *SP1* is also a shared gene when overlapping transcriptome and proteome. Indeed, *SP1* interacts with most of the top hits discussed above, such as E2F families,^{54,55} *COL11A1*,⁵⁶ *ICAM1*,⁵⁷ and *HAS1*.⁵⁸ This suggests the central role of *SP1* of converting mechanical alternation into cell cycle arrest. Interestingly, *SP1*, *RELA* and *NFKB1* are all involved in the NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway where *RELA* and *NFKB1* encoded two members of the NFκB protein family.⁵⁹ With this in mind, we believed NFκB pathway is very likely to mediate the NMA-induced cell cycle arrest.

We performed a dot blot of 41 proteins involved in NFκB pathway to deconvolute the mechanotransduction from membrane to nucleus. We observed a noticeable decrease (> 20% compared to flat control) of *NFKB1*, *STAT1*, *STAT2*, *IKKγ*, *IL-17 RA*, *RELA*, and *MYD88* (Fig. 4G and Fig. S4). Within, the presence of *NFKB1* and *RELA* at the protein level confirms the validity of the transcriptional analysis. Indeed, the expression and translocation of *NFKB1* and *RELA* are directly regulated by *IKKγ* in NFκB pathway.⁶⁰ *IKKγ*, also known as NEMO, can be modulated by various stimuli from ECM, including compressive force,^{61,62} and fluid shear.^{63,64} For example, dynamic tensile strain has shown a negative impact on *IKKγ* expression in human chondrocytes.⁶¹

With the data from transcriptome and proteome in mind, we proposed a schematic of the mechanism of NMA-mediated cell cycle arrest and upregulated endocytotic activities (Fig. 4H). The inadequate surface on the sharp needle of NMA forces seeded cells to undergo limited adhesion, upregulated cell-cell adhesion, and introduced mechanical strain stress. Such stress was sensed by *IKKγ* and transduced via *RelA* and NFκB to the nucleus. *RelA* and NFκB react with *SP1* in the nucleus, promoted the expression of E2F families, downregulated the expression of *CDKN1B*. Such alteration pushed cells to depart from the G1 phase and be arrested at the G2 phase, which favors the endocytosis of exogenous biomolecules.

In summary, we developed a rapid, RIE-free protocol to fabricate nanostructured microarchitectures (NMA) with tunable nanostructuring via electrodeposition. NMAs were found to significantly promote the endocytosis of proteins and siRNA in primary cells, which improves the efficiency of gene knockdown. We performed a comprehensive analysis of transcriptome and proteome to reveal the underlying mechanism. We concluded that endocytosis is a result of cell cycle arrest transduced from NMAs to nucleus through the NF- κ B pathway. Taken together, our results demonstrate that the presence of high-aspect-ratio nanostructures in biointerfaces can induce more complex biological responses than membrane deformation. The interaction with nanostructures will alter multiple intracellular processes, such as signaling transduction and cell cycle. Importantly, the nanostructure-induced cell cycle arrest can be utilized to improve the cellular endocytic process and yield efficient intracellular delivery. It will be useful to consider the features characterized when designing the future multifunctional biointerfaces to further heighten the efficiency of drug delivery strategies.

EXPERIMENTAL SECTIONS

The experimental sections are included in the supporting information.

SUPPORTING INFORMATION

Supporting Information (SI) is available from free of charge on the website. The SI contains experimental sections, details of antibodies, and additional data characterizing the cellular behavior on nanostructured microarchitectures.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Z. W and S.O.K conceived and designed the experiments. Z. W, H. W, S. L, M. L performed the experiments and analyzed the data. All authors discussed the results and contributed to the preparation and editing of the manuscript.

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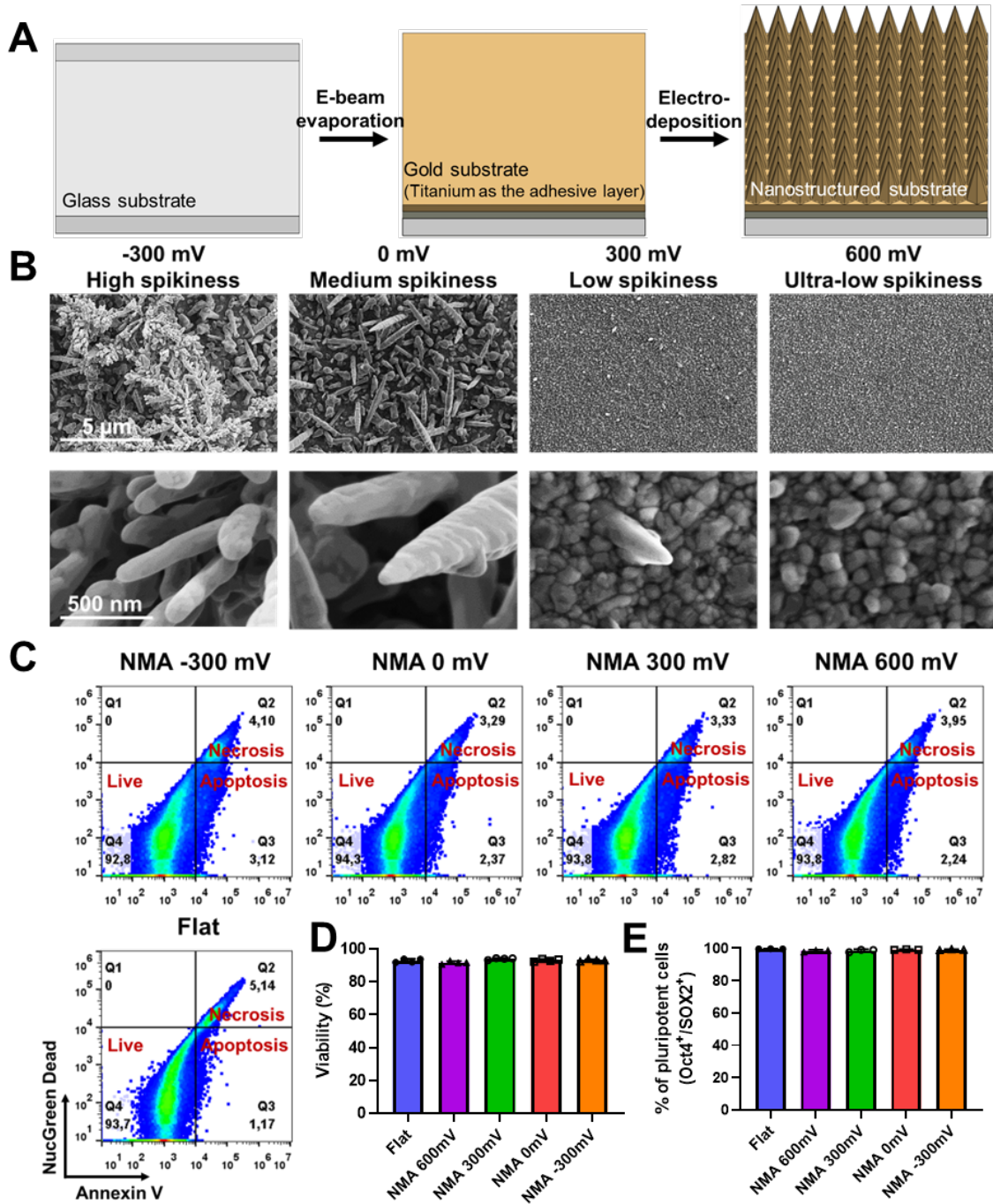


Figure 1. Electrodeposition of biocompatible nanostructured microarchitectures (NMAs) with tunable morphology and density of nanoneedles. (A) One-step fabrication of NMAs via electrodeposition. (B) Morphology of NMAs deposited under different potentials. (C) Representative flow cytometric profile showing the percentage of live, apoptotic and necrotic cells recovered from NMAs. (D) Quantitation of cell viability. (E) Quantitation of pluripotency.

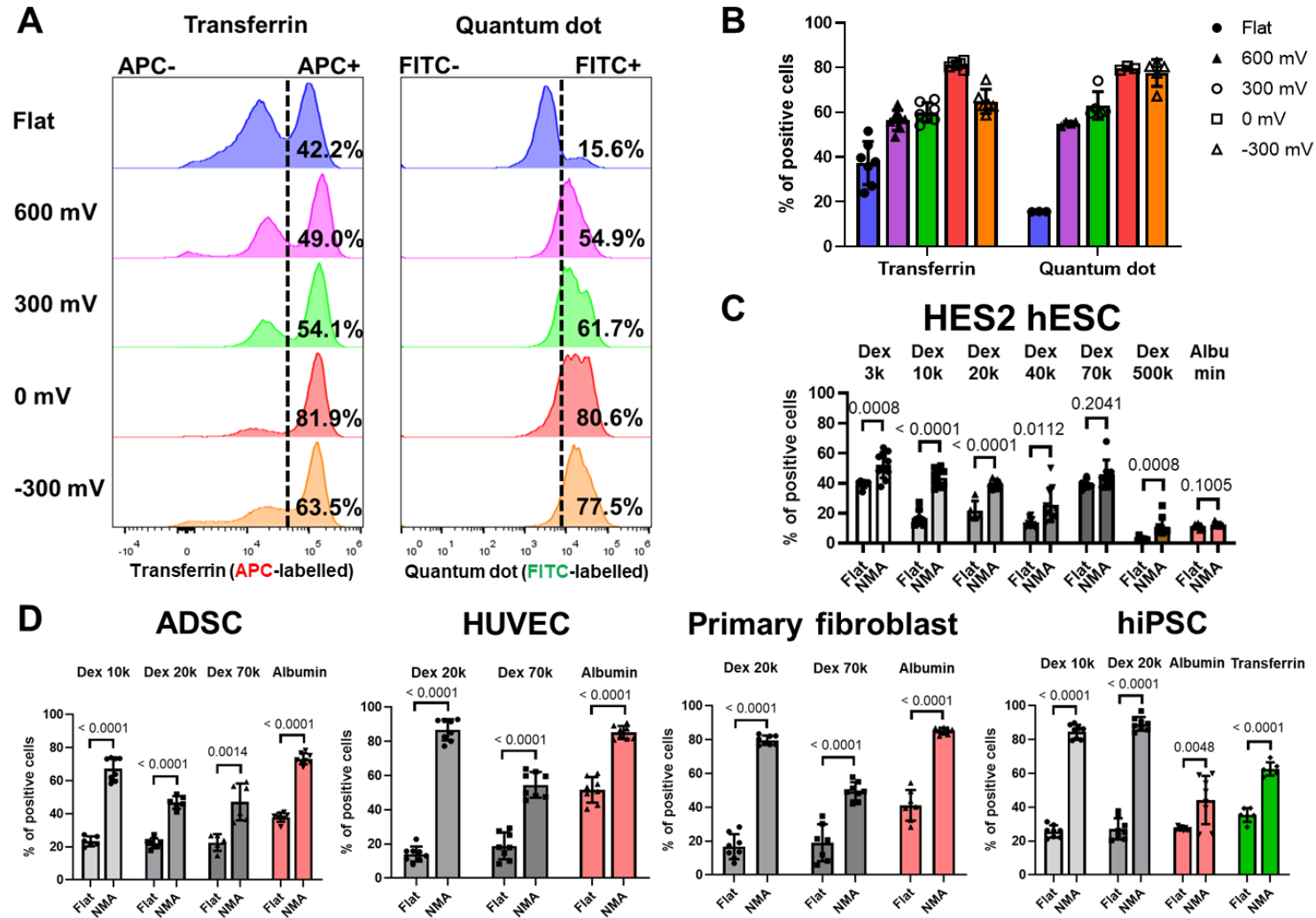


Figure 2. NMAs containing high-density nanoneedles promote the delivery of various biological molecules across multiple hard-to-transfect primary cells. (A) Representative flow cytometric profile of HES2 embryonic stem cells treated with fluorescently labeled transferrin for 6 hrs and quantum dots (QD) for 24 hrs. (B) Quantitation of deliver efficiency on different substrates. (C) Quantitation of deliver efficiency of dextran cargoes on HES2 cells after 6 hrs. (D) Quantitation of deliver efficiency of NMA 0mV substrates for multiple primary cells after 24 hrs. Each dot represents a technical replicate. At least 3 biological replicates for each group.

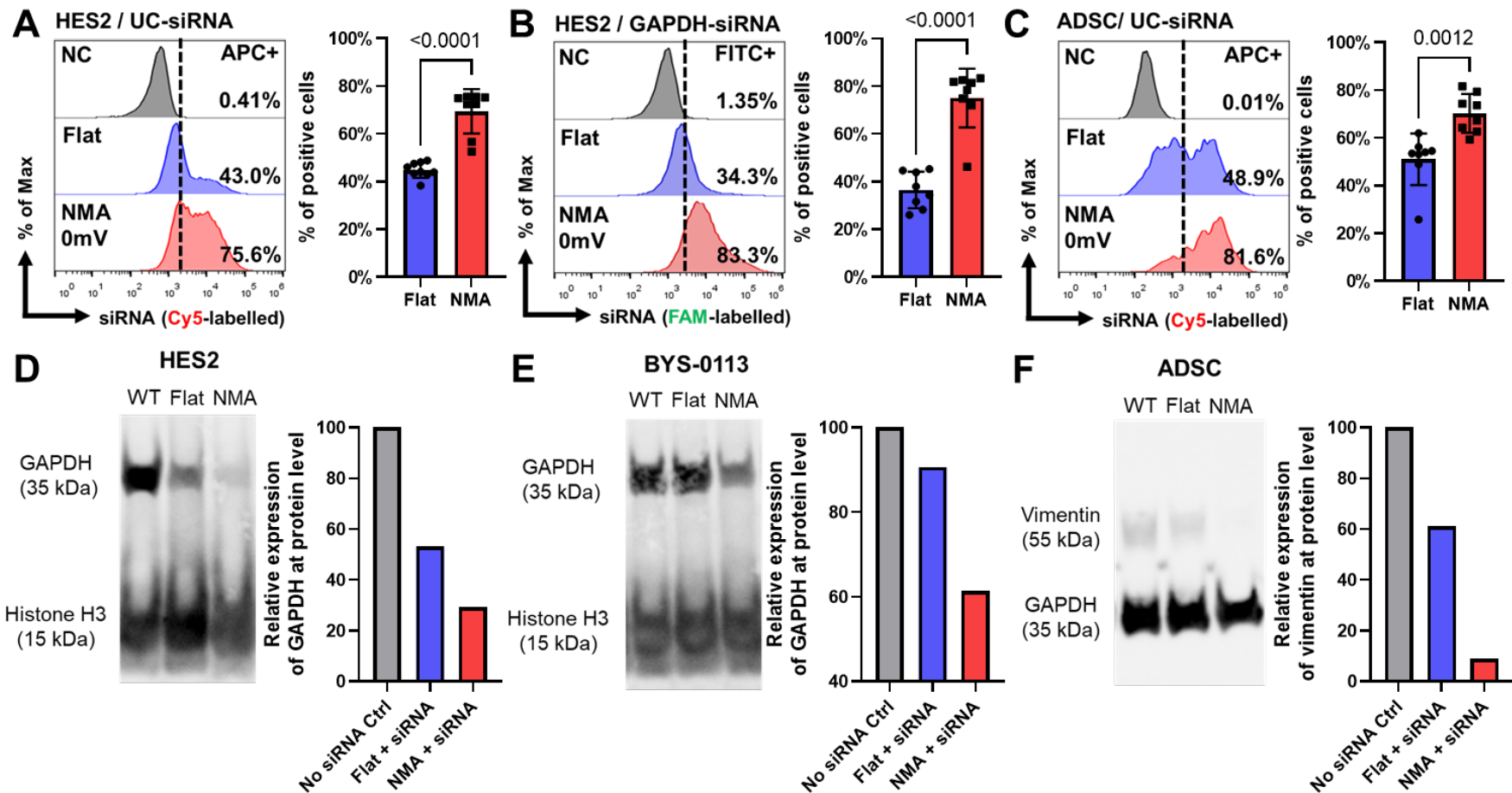


Figure 3. NMAs containing high-density nanoneedles promote the lipofection of siRNA and yield significant knockdown at the protein level. (A-C) Representative flow cytometric profile of HES2 embryonic stem cells (A-B) and adipose-derived stem cells (ADSC) treated with fluorescently labeled siRNA for 6 hrs. (D-E) Quantitation of the GAPDH expression on HES2 and BYS-0113 cells 60 hours post-lipofection. (F) Quantitation of the Vimentin expression on ADSC cells 60 hours post-lipofection.

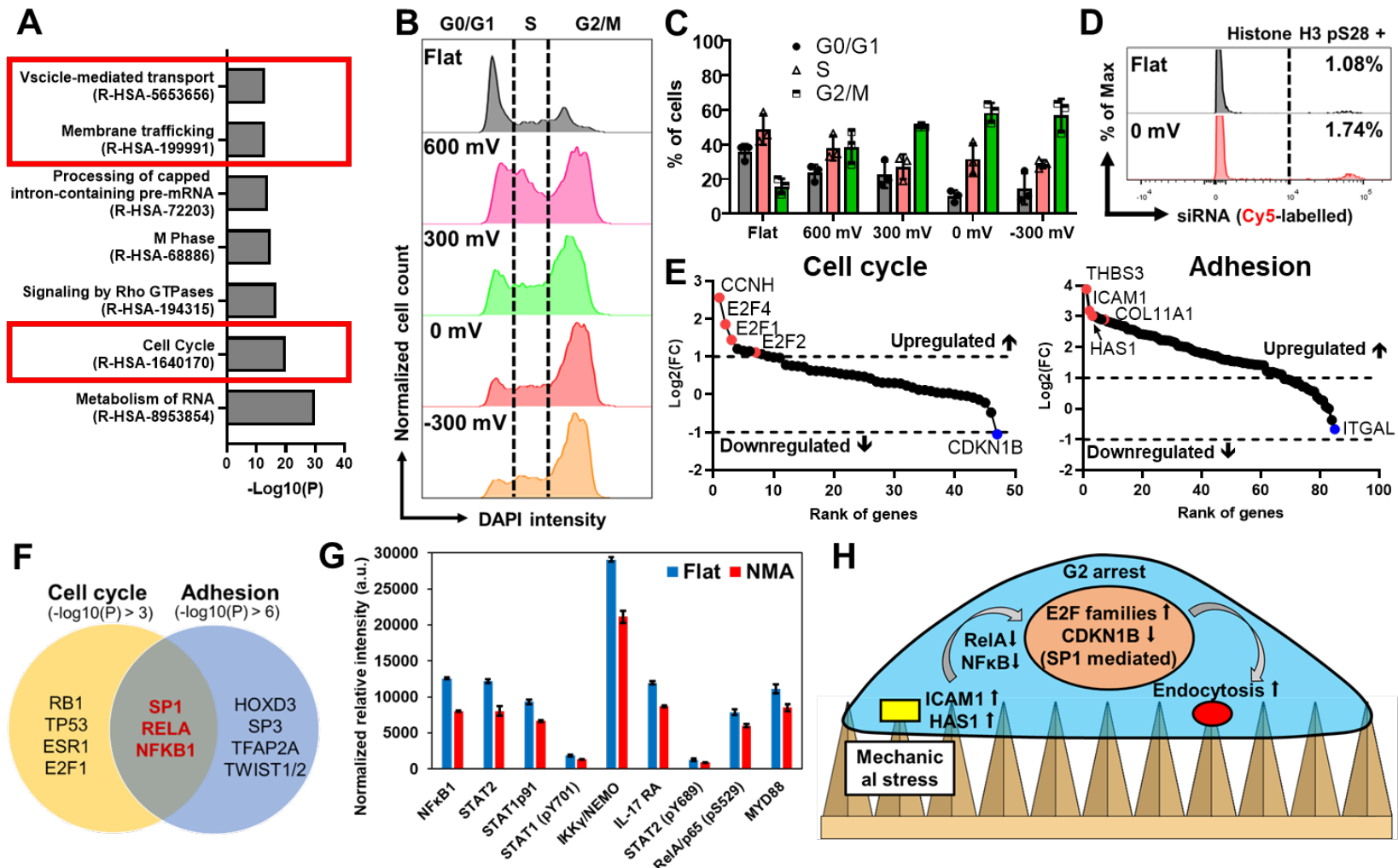
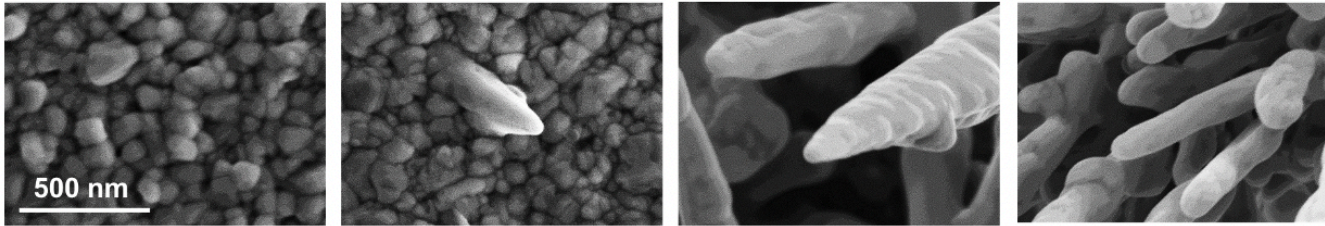
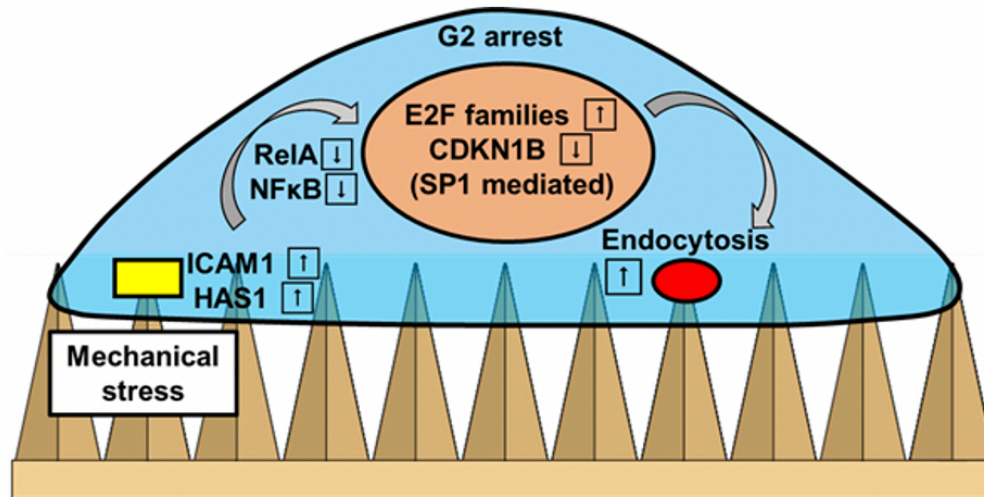


Figure 4. NMA promotes membrane traffic via cell cycle arrest. (A) Top enriched pathways from label-free mass spectrometry analysis. (B) Flow cytometric analysis of the cell cycle of cells seeded on the NMA. (C) Quantitation of the percentage of cells in each cell cycle. (D) Flow cytometric analysis of the expression of histone H3 pS28, a mitosis-specific biomarker. (E) Differentially expressed genes in the process of cell cycle and cellular adhesion revealed by Taqman qPCR array. (F) Results of TRRUST analysis revealing the NFKB-related TFs play a key role in the signal transduction. (G) Dot plot results regarding the major proteins involved in the NFKB pathways. The error bar indicates standard deviation from duplicates. (H) Proposed mechanism of signaling transduction for NMA-mediated cell cycle arrest and endocytosis promotion.



Increased transfection



TOC Graphic