Targeting Endothelial Cell Junctions with Negatively Charged Gold Nanoparticles

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Supporting Information

ABSTRACT: Endothelial leakiness or permeability directly determines the access of any bionanotechnology to the target tissue site. Currently, cancer nanotechnology relies on tumor-derived endothelial leakiness, which suffers from unreliability, inhomogeneity of leakiness, and uncontrollability. Nanomaterials by themselves are capable of inducing endothelial leakiness (NanoEL) without any tumor involvement by targeting the endothelial cell junctions; this NanoEL phenomenon not well understood. Here, we showed that the negatively charged Au nanoparticles (NPs) induce significantly higher NanoEL than positively charged nanoparticles. We hypothesized and showed that in both *in vitro* and *in silico* models that cell junction targeting arose for the negatively charged particles due to a succession of repulsive-sedimentary interactions between the negative particle and the negatively charged glycocalyx found on the cell membrane surface. On the contrary, NPs with positive charges are attracted stably by the negatively charged glycocalyx and remained in situ for long enough, which eventually are endocytosed into the cells as contrasted to localization toward the cell junction. There are implications to how nanoparticle charges could be tuned to induce (or avoid) endothelial leakiness by design.

■ INTRODUCTION

Many bionanotechnologies are introduced via the vasculature especially in cancer bionanotechnology.^{1,2} The prevailing assumption is that the nanomedicine leaves the vasculature passively due to leaky blood vessels arising from the enhanced permeability and retention (EPR) effect.^{3–6} However, that has some flaws because the EPR effect is the byproduct of a nutrient-starved tumor and the distribution of leakiness cannot be controlled extrinsically.^{7–11} Moreover, only mature tumors are starved enough to induce the EPR effect and not immature and small tumors. Herein lies the logic discordance, if cancer bionanotechnology only relies on the EPR effect, then those nanomedicine can only target mature tumors and not the immature and small tumors. From a clinical practice perspective, treatment of early cancer is always beneficial for the patients as immature cancer possesses much fewer anticancer drug resistance mutations. The sole reliance on the EPR effect will then preclude the targeting of those easier-to-kill immature tumors. We reasoned that if leakiness could be induced by nanotechnology design without an over reliance on the tumor, we will be able to

also target tumors which do not induce the EPR effect. Figuring out the design parameters of nanotechnology then becomes an important determinant of inducing this leaky outcome of endothelial cells.

Certain nanoparticles could induce endothelial leakiness in endothelial cells that form capillary beds. This nanoparticle effect is coined as “nanomaterial induced endothelial cells leakiness” (NanoEL). By virtue of their nanoscale dimensions, the nanoparticles entered into the nanosized gaps between microvascular capillary endothelial cells and disrupted the protein interactions that hold neighboring cells. This disruption together with the natural cell tension changed the nanosized gaps to result in micron sized gaps between cells which is about 3–4 orders of magnitude increase in gap distance, so large that large particles and even whole cells can pass through.¹² This has significant implications to nanomedicine and nanotoxicology as endothelial cells

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join together forming our blood vessels. Earlier, we have systematically shown that size and density are important determinants of NanoEL.^{13,14}

In our continual pursuit to understand the nanoparticle contributions to the NanoEL effect in an attempt to control it, through this study, we solved an outstanding question of why negatively charged nanoparticles can induce NanoEL much more significantly than positively charged nanoparticles. Now clearly, if this nanoactivity occurs at the cell junction, the cell junction has to be targeted in the first place. From a sedimenting nanoparticle perspective, the area of the cell–cell junction is only but a sliver of area compared to adjacent expanse of the cell membrane. Since the sedimentation of nanoparticles is a probabilistic process, why was this thin sliver targeted in the first place? We had some clues. We noticed that on the surface membrane of endothelial cells, there exists a molecule-thick layer of negatively charged polysaccharides called glycocalyx.¹⁵ Therefore, we hypothesized that the bulk of the sedimenting negatively charged particles that were approaching the cell membrane are repelled away by this negatively charged glycocalyx cell membrane, then reapproaches, and this repulsion-reapproach cycle is repeated until the particle reaches a region where there is no negative charge, which is where the location of the cell junction is. For imagery sake, we call this the “bouncing particle” hypothesis.

We set about to test this hypothesis using inert gold nanoparticles of primary size of 24 nm differing in their surface charge; namely highly negative, low negative, low positive and highly positive charge. We found that indeed the negatively charged nanoparticles caused more NanoEL compared to the positively charged nanoparticles. In addition, the negatively charged NPs tend to localize to the cell–cell junctions possibly explained by a directional diffusion along the cell membrane due to successive electrostatic repulsion-sedimentation cycles. Conversely, positive charged nanoparticles are electrostatically attracted to the negatively charged cell membrane and glycocalyx, resulting in increased residence time for subsequent endocytosis. *In silico* modeling further corroborated our *in vitro* results and provided more insights on how charge of the nanoparticles interact with the inherent electrostatic fields on cell membranes can actually influence directionality of nanoparticles diffusion on the surfaces of cells which in turn changes the final outcome of the nanoparticles.

RESULTS AND DISCUSSION

Figure 1a displayed the average zeta potential of the various Au NPs with different charges in ultrapure water, −39.1, −21.6, +14.8, and +39.5 mV (rounded off and renamed as −40, −20, +15, and +40 mV nanoparticles), respectively. In addition, hydrodynamic diameter of Au NPs with the charge of −40, −20, 15, and 40 mV samples was 62.59, 68.43, 37.66, and 40.45 nm in ultrapure water, whereas the hydrodynamic diameter in cell culture media is 50.22, 59.31, 68.36, and 68.33 nm respectively, as shown in Figure 1b. It is clear to conclude that the diameter of negatively charged Au NPs in medium is smaller than that in ultrapure water respectively, while the positively charged NPs have an opposite trend, because the negatively charged proteins in medium (−18 mV at pH 7)^{16,17} tend to form a protein corona surrounding positively charged NPs and repel the negatively charged NPs.^{18,19} In addition, Figure 1c(i–iv) shows representative transmission electron microscope (TEM) images of Au NPs with different charges. Au NPs with different charges of −40, −20, 15, and 40 mV are spherical with uniform diameter of around 24 nm (Figure S1). Moreover, Au NPs have high biocompatibility (Figure S2).

Figure 1. Characterization of the differentially charged but similar sized Au NPs. (a) Zeta potential of Au NPs with different charges. (b) Hydrodynamic diameter of Au NPs with different charges in water and cell culture medium. Particles are renamed to −40, −20, 15, and 40 mV based on zeta potential data of Figure 1a. (c) TEM images of Au NPs with different charges, (i) −40, (ii) −20, (iii) 15, and (iv) 40 mV. Scale bar: 50 nm.

Negative Charge on Au NPs Induces Significantly More NanoEL. Using this library of NPs with various charges, the capability of the NPs inducing NanoEL was examined by immunofluorescence staining assay, as shown in Figure 2a. The confluent monolayer of endothelial cells of the negative control group did not show any observable leakiness (Figure 2a). Confluent monolayers of endothelial cells were exposed to Au NPs with different charges at the same 10 μM concentration. Negatively charged Au NPs (−40 and −20 mV) were able to induce endothelial cell leakiness while the positively charged NPs could not result in noticeable gaps as shown in Figure 2a. In addition, −40 mV Au NPs group could induce larger and more gaps between endothelial cells than that by the −20 mV Au NPs group (Figure 2b); thus demonstrating that the negatively charged Au NPs were able to induce a more significant NanoEL effect as compared to positively charged Au NPs. This result is further supported by the quantitative result obtained from transwell assay (Figure 2c(i,ii)). To quantify endothelial leakiness, we plated a monolayer of endothelial cells on transwell inserts with 0.4 μm pore size. Once the monolayer reached confluence, there were treated with Au NPs of 50 μM concentration for 10 and 15 min. As a proxy for endothelial cell leakiness, FITC-dextran flux crossing the endothelial layer through the leaky sections was measured. As shown in Figure 2c(i,ii), the negatively charged Au NPs groups (−40 mV and −20 mV) again showed significantly higher NanoEL over the control and positively charged Au NPs groups (15 mV and 40 mV) over two time points. In addition, Figure S3 displays the degrees of NanoEL induced by Au NPs different concentrations (10 μM (1.97 mg/L), 20 μM (3.94 mg/L) and 100 μM (19.7 mg/L)) for 15 min, demonstrating that NanoEL is dependent on Au NPs dose. Moreover, this trend is observed quickly over a short time period which will be earlier than endocytosis influences setting in. Overall, these results imply that the negative charge on NPs appears to induce the NanoEL effect.

Successive Repulsion-Sedimentation “Bouncing” Drives Particles toward the Cell Junction. It is important to elucidate the underlying reason for the charge dependent NanoEL phenomenon. It has been reported that TiO_2 , silica and gold could disrupt the integrity of VE-cadherin pairs by the physical force generated by sedimentation and diffusion of NPs.^{12–14,20,21} However, most of the top view area of a confluent

Figure 2. Negatively charged NPs induced noticeably more NanoEL than positively charged NPs. (a) Immunofluorescence images of NanoEL induced by Au NPs with different charges (−40, −20, 15, and 40 mV) at the same concentration of 10 μM after 15 min of NPs exposure. Scale bar: 25 μm. (b) Quantification analysis of (i) gap area between endothelial cells or (ii) number of gaps induced by Au NPs with different charges. Permeability quantification of (c) experimental groups at different exposure time points, 10 and 15 min. Permeability induced by Au NPs with negative charges (50 μM) were consistently more pronounced than that of positive charges with just (i) 10 min and (ii) 15 min of treatment time. Data are means ± S.D., $n = 5$, Student's t test, $p < 0.05$, *Significant against the corresponding nontreated negative control (NC, white filled color).

cell monolayer is cell membrane rather than cell–cell junctions, thus the majority of sedimenting Au NPs would land on cell membranes and should not be targeting the cell junctions which only represent a miniscule proportion of the top view area.²² Because the resting potential of most cell membrane is negatively charged,^{23,24} we hypothesized that electrostatic repulsions of NPs with membranes may play a role in this targeting of the cell junction. After treating cells with negatively charged Au NPs, there were observable gaps formed between endothelial cells, while the positively charged NPs did not result in noticeable gaps between endothelial cells. This increased leakiness result of the negatively charged Au NPs group has been demonstrated by the immunofluorescence staining and the permeability transwell assay (Figure 2). In addition, it is interesting to observe that most of the negatively charged Au NPs are found at the boundaries of the endothelial cells especially at the gap areas despite that most of the area of the confluent monolayer of endothelial cells is cell membrane. As an internal control and considering that it is only 15 min of treatment, most particles if found on the membrane would not have been endocytosized and thus should still be on the membrane surface. Instead, the negatively charged Au NPs (−40 and −20 mV) are localized at the edges of cell membranes next to the gaps (Figure 3a and b). There were also comparatively insignificant amount of negatively charged Au nanoparticles found on the cell membrane itself (Figure 3a, b). On the contrary, most of the positively charged NPs were found on the surface of the cell membrane rather than on the cell–cell junctions (Figure 3a, b). Therefore, it can be deduced that there was a driving force to direct negatively charged NPs to the cell–cell junctions. It has been reported that on the luminal surface of the endothelial cell membrane, there is a layer of glycocalyx that is negatively charged. This glycocalyx layer consists of glycoproteins and proteoglycans.^{25,26} Syndecan-4, a glycocalyx protein, for example has long branch-like heparin chains with multiple negatively charged SO_3^- groups²⁷ that may increase the capture of positively charged particles. Endothelial cells are held together by VE-cadherin (VEC) where each endothelial cell contributes one VEC molecule of each pair; with many pairs establishing a robust cell–cell junction. Each VEC pair is held together by intermolecular bonding that requires positively charged Ca^{2+} ions.²⁸

Figure 3. Negatively charged and positively charged Au NPs tend to localize to the cell junctions and membrane surfaces, respectively. Scanning electron microscope (SEM) images of endothelial cells. (a, b) Au NPs of −40 mV charge and −20 mV charge (red arrows) were localized at the edges of the cells around the gaps with no observable localization of Au NPs on the cell membrane surface. (c, d) Positively charged Au NPs found to be on the cell surface with no observable gaps between cells. All treatments were done at 10 μM for 15 min. (e) Nontreated negative control. Red arrows show examples of Au NPs in the midst of many examples within each frame.

It is therefore possible that a charge polarization across the cell membrane surface may have arisen from the discrete distribution of different proteins at the luminal surface to the edge or cell junction. This polarization can then bring about a distinct difference in the interaction of particles at the different extracellular locations.²⁹

After being treated for 30 min with Au NPs, the endothelial cells were lysed and the lysate were centrifuged at low speed to

pellet down the Au NPs. Any proteins found to be interacting with Au NPs would also be pulled down by this centrifugation step. The proteins were then stripped off and detected with Western blotting. Consistent with the earlier study,¹³ negatively charged Au NPs interacted with cell junctional VEC but not intracellular protein, α -tubulin, showing that the interaction is there is no obvious combination between positively charged Au NPs and VEC (Figure 4a). Because the only extracellular location of VEC is at the cell junction, the negatively charged particles must have also been in the cell junction in order for it to interact with VEC in the first place (Figure 4a). Conversely, positively charged particles were not found to have interacted with VEC, which by the same logic would mean that they are not at the cell junction (Figure 4a). Combining these pull-down experimental evidence together with the SEM experimental data, it would suggest that negatively charged nanoparticles may have sedimented almost on the surface of the cell membrane but was successively repelled and sedimented and repelled again by the negatively charged glycocalyx until it reaches a region where there is no negatively charged glycocalyx; namely the cell junctions. Meanwhile, the almost opposite results of the positively charged Au NPs could be attracted to and interacted stably with the glycocalyx on the cell membrane, as illustrated in Figure 4c, d. Differences in initial sedimentation rate between the positively and negatively particles cannot explain the experimental observations as the size and effective density of the particles are almost the same.

Simulated and Computational “Bouncing Particle” Model in NanoEL. To support these experimental findings, we developed a computational model to analyze the phenomena. We are interested in the average time for one NP to diffuse within the medium and finally arrive at the cell–cell junction between two cells. This time is defined as the mean first passage time (MFPT). In our case, we also have to consider the influence of electrostatic field due to the existence of glycocalyx layer, similar as the electrophoresis. Consider the MFPT is $t_K = \frac{1}{2j \cdot nd}$, where $j = j_f + j_e$ is the flux of NPs caused by both the free diffusion and electrophoresis, $2d$ is the width of the cell–cell junction (Figure 5a), and n is the normal of the absorbing wall, our goal is to find the flux of NPs given the boundary conditions. The mathematical derivations and *in silico* modeling details are provided in the Experimental Section.

According to the simulation results, the electrostatic potential is homogeneous at the cell–cell junctions and interior of the glycocalyx layer, as shown in Figure 5b(i). But at the boundary of glycocalyx layer, there is a steep gradient of electrical potential which means NPs with positive or negative zeta potential will encounter a large force at the boundary. For NPs with negative zeta potential (positive mobility), the density is higher at the gap, as shown in Figure 5b(ii); the cell–cell junction is between point M and N. Conversely, for NPs with positive zeta potential (negative mobility), the density is lower at the cell–cell junction (as shown in Figure 5b(iii)) than that at the cell membrane covered by glycocalyx. It means the NPs flux at the gap is smaller than it at the cell membrane. However, neutral NPs (mobility equals to zero) disperse uniformly at the cell–cell junction and the cell membrane as shown in Figure 5b(iv). Furthermore, we obtain the MFPT for NPs with different zeta potentials to target the cell–cell junction (Figure 5c). We find the MFPT increases with the zeta potentials (ascending from negative to positive). In addition, the time for NP with the charge of -40 mV arriving cell–cell junctions is merely 1.26% of that for NP (40 mV) to target cell–cell junction. Because in reality, both positively and

negatively charged particles have the same effective density,^{13,30} the sedimentation rate will be the same. Both groups of particles would then arrive at the boundary layer where now repulsive (for negatively charged particles) and attractive (for positively charged particles) forces dominate over the gravitational forces. Since the theoretical time it took for the negatively charged particles to reach the cell junction is overwhelmingly shorter than that taken by positively charged particles, it becomes clear that there will be much more negatively charged particles localized at the cell junction while most of the positively charged particles remained at the cell membrane and not at the cell junction at the same short time of the experiment. For positive zeta potential ranges, the decrease in time it takes for the *in silico* particles to reach the cell junction is an exponentially correlated function of its net positive charge value (Figure 5c), which again supports our postulation that positively charged particles are attracted and bound to a negatively charged endothelial glycocalyx and subsequently remained bound such that the larger the positive charge, the stronger the interaction with the cell membrane instead of the cell junction. Now after crossing the zero zeta potential point toward the negative scale, the time function begins to diverge from the trend line and plateau off at around -40 mV (Figure 5c). Again, this corroborates well with our experimental results such that it is expected that a minimum negative charge will be sufficient to successively repel the particles along the glycocalyx until it reaches the cell membrane, albeit at a slower pace if the negative charge is weak. Overall, these theoretical results thus corroborated very well with our SEM results (Figure 3). If particles are in reality moving as free diffusion entities, movement of the particles will be random and stochastically evenly distributed and therefore it is expected that there will not be any targeting to any certain region of the cell surface. If instead there is an electrostatic field as depicted in our model, for negatively charged particles, there will be a negatively charged “ground” that electrostatically repels the negatively charged NPs approaching this “ground”. The *in silico* calculations together with the experimental data showed that diffusion is indeed directionally along the plane of the cell membrane for the negatively charged NPs and not “into the plane”. Consequently, the NP with highly negative zeta potential will be more likely to target the cell–cell junctions which are always found at the end along the plane of the membrane, thus causing NanoEL, thus supporting our “bouncing particle” model. The converse is observed in the positively charged particles such that electrostatic attraction prevails at the cell membrane, their persistent presence activates the endocytosis pathway and is endocytosized into the cell from the cell surface. This higher propensity of positively charged particles versus negatively charged particles to be more easily endocytosized has been reported in other cell types (3T3-L1 cells, human cervical carcinoma cell, etc.).^{31,32} Overall, we believe that charge of nanoparticles do play a major role in directing their movement in interesting ways previously unimaginable. Understanding nanoparticle charge movement can be applied in controlling NanoEL which can help in accessing tumors without an over reliance on the EPR effect.

CONCLUSION

In summary, these results have demonstrated that the intrinsic property of NPs like charge is able to impact NanoEL effect. In both the experimental and *in silico* models, the negatively charged Au NPs could be repelled by the negatively charged glycocalyx on plasma membrane and sedimenting and again repelled in a bouncing manner toward the cell–cell junctions.

Figure 4. Negatively charged Au NPs bind to VE-cadherin at the cell junction. (a) After Au NPs (30 min, 50 μM) treatment, centrifugation at low speed pellet down the Au-NPs. Any bound proteins would be eluted and identified with specific immunoblotting. VE-Cadherin bound to the negatively charged Au NPs but not the positively charged Au NPs. Since VE-cadherin can only be found at the cell junction, that would show that negatively charged Au NPs are found at the cell junction. (b) Electrostatic attraction between positively charged Au NPs and syndecan-4. Heparan sulfate chains of syndecan-4. (c) Schematic of the bouncing particle model resulting from a series of electrostatic repulsion and attraction respectively between cell membrane and Au NPs with (i) negative charge and (ii) positive charge.

Therefore, for one of the possible applications, the charge of NPs could be tuned to induce a leaky effect that is nanoparticle driven and not tumor dependent pathway such that we can now control

access to the tumor without relying on the tumor's intrinsic EPR induction schemes. This NanoEL effect may alleviate some of the uncontrollable and unreliable nature of over-reliance on the EPR

Figure 5. *In silico* model validates the “bouncing particles” model. (a) Diagram of the model. The cell membrane is covered by a layer of negatively charged glycocalyx, which resulted in an electrical field in solution. Due to the effect of electrical double layer (EDL), the electrical field can only influence a limited region above the layer. (b) Contour of electrical field and probability density distribution. The cell gap is between points M and N (depicted in a). (i) Distribution of electrical potential. (ii) Distribution of probability density for NPs with negative zeta potential (positive mobility) showed the high probability of the eventual location of the negatively charged particles to be between M and N. (iii) Distribution of probability density for NPs with positive zeta potential (negative mobility). There is a higher probability of localization of positively charged particles to be found outside of M and N region (cell junction). (iv) Distribution of probability density for neutral NPs (mobility equals to zero). (c) Relation between the time for one NP to arrive the cell–cell junction and the zeta potential of NPs. As zeta potential becomes more negative, the dimensionless time for that particle to reach the cell junction becomes shorter and shorter.

effect and increase overall accessibility to tumors or even tumors without initial leaky vasculature.^{9,33} Complementing NanoEL with the intrinsic vascular bursts in the tumor vasculature will further enhance the efficacy of current cancer drugs and future nanomedicine.

EXPERIMENTAL SECTION

Characterization of Au NPs. Au NPs (Nanjing Jcnano Technology Co., Ltd.) with same diameter (~24 nm) were stabilized by cetyltrimethylammonium bromide (CTAB) and then CTAB was replaced by different ligands (thiol modified polyethylene glycol with either carboxylic groups or amine groups; SH-PEG-COOH (average Mw = 5000) and SH-PEG-NH₂ (average Mw = 5000)). The different concentrations of surface modification of SH-PEG-COOH and SH-PEG-NH₂ were used to obtain Au NPs of different charges (−39.1, −21.6, +14.8, and +39.5 mV). The concentrations of Au NPs were detected with inductively coupled plasma optical emission spectroscopy (ICP-OES). The shape and primary size of the Au NPs were characterized by transmission electron microscopy (TEM; JEM 2010, JEOL, Japan) and ImageJ software. In addition, the hydrodynamic diameter

and zeta potential of Au NPs were measured by using dynamic light scattering (DLS; Malvern co., UK).

Cell Culture and Au NP Treatment. Human microvascular endothelial cells (HMVECs) (Lonza) were cultured in EndoGRO (Merk Millipore) supplemented with 5% FBS at 5% CO₂ and 37 °C. For all NanoEL induction experiments, cell seeding density was fixed at 40 000 cells/cm² and allowed to grow until they reached confluency after 2 days. Prior to Au NPs treatment, the NPs were spun down, and supernatant was removed and diluted further to avoid the influence from the stock buffer of Au NPs suspension. Thereafter, Au NPs were sonicated in cell culture medium using a probe sonicator (Micron TM XL 2000, Qsonica, USA) for 2 min on ice.

Permeability Transwell Assay. HMVECs leakiness was detected using Transwell insert (polyester membrane with 0.4 μm pore; Corning Costar, Cambridge, MA), which has been reported previously.¹² The transwells were hydrated with the EndoGRO medium for 2 h. HMVECs were plated on these hydrated transwell membranes and left to culture for 2 days to obtain a confluent monolayer. Thereafter, Au NP suspension with fluorescein isothiocyanate dextran (FITC-dextran 40,000 MW_{av}) (1 mg/mL; Sigma-Aldrich, USA) in complete culture media was added into the transwells with confluent monolayer of cells, for 10 and 15 min.

After treatment, the fluorescent intensity was measured in the 96-well plate format with microplate reader at excitation/emission wavelength of 492/520 nm. Leakiness index is defined as various FITC dextran fluxes derived for the experimental groups of interest normalized to that of the untreated control group.

Visualization of Inter cellular Gap Formation (Leakiness) with Immunofluorescence (IF) Staining. The HMVECs were grown on 8-well chamber slides for 2 days to achieve confluent cell monolayer. After being treated with Au NPs with different charges for 15 min, the endothelial cells were washed by 1×PBS solution, fixed with 4% paraformaldehyde solution for 15 min, permeabilized with 0.2% Triton X-100 for 15 min followed by blocking with 2% bovine serum albumin (BSA) for 1 h on ice. Thereafter, fixed cells were incubated with primary anti-VE-cadherin antibody at 1:200 dilution (Cell Signaling Technology, USA) in 0.2% BSA and 0.1% Triton X-100 PBS solution overnight at 4 °C. Then the cells were washed with PBS for 3 times and incubated with the secondary Alexa 488-chicken anti rabbit antibody (1:400, Invitrogen, USA) and Hoechst dye (Invitrogen) for 1 h. Finally, the labeled slides were washed and mounted using ProLong antifade reagent (Invitrogen, USA) and nuclei stained with DAPI. The immunofluorescence images were captured with Leica DMI6000 Fluorescent Microscope.

Visualization of NanoEL through Scanning Electron Microscope. Silicon wafers were treated with 0.1% polylysine solution overnight, air-dried and decontaminated by UV light for 30 min for each side of the wafers. HMVECs were seeded on the “front-side” of the silicon wafers directly. After obtaining a confluent monolayer, HMVECs were treated with Au NPs of different charges for 15 min, fixed with 4% paraformaldehyde for 30 min, and treated with 0.2% Triton X-100 for 15 min. Freeze-dry the samples and then conduct SEM characterization.

Au NP-VE-Cadherin Pull-Down Assay. HMVECs were cultured to 100% confluency and treated with Au NPs for 30 min. The cells were first washed with PBS and their protein extracted with mild detergent RIPA buffer. The lysate was centrifuged at 1000 × g, 15 min, 4 °C to isolate any Au NPs. The isolated Au NPs were then washed twice with RIPA buffer and any protein that was bound on the Au NPs were eluted out by boiling for 5 min in Laemmli’s sample buffer containing protease inhibitors. The Au NPs bound proteins and the whole cell lysate then were separated with SDS-PAGE gel and electro-transferred to PVDF membrane. The membrane was first blocked with 5% BSA and probed with anti VE-cadherin (1:1000, Cell Signaling Technology, 2500S), α -tubulin (1:5000, Sigma-Aldrich T9026) overnight at 4 °C. The membranes were then washed three times with wash buffer. The specific bands were marked with horseradish peroxidase (HRP) conjugated secondary antibodies (Santa Cruz) used at 1:10,000 dilution. The protein bands were detected with Immobilon Western Chemiluminescent HRP substrate (Merck Millipore) and the chemiluminescence image captured with Chemiluminescence Imaging System (Syngene, UK).

Cytotoxicity Studies. HMVECs were plated down on 24-well plate, cultured for 12 to 24 h, and then treated with Au NPs for 12 h. The entire medium and cells were collected into the micro centrifuge tube. The cells were then mixed with propidium iodide (50 μ g/mL) dye. Cell viability was quantified using the Tali Image Cytometer (Thermo-Fisher Scientific).

Statistical Analysis. Statistical analysis of the experimental data was performed using OriginPro 2017 software.

Theoretical Analysis. We assume that the cells grow on the substrate with equal distribution and consider two cells connected with each other by VE-Cadherin pairs, forming cell–cell junctions between points M and N in the model as shown in Figure 5a. To simplify this problem, we assume there are many replicated slices out of the plane. Selecting a particular slice (in plane) as the model, we further simplified it to a two-dimensional problem. It is supposed that there are N nanoparticles per length in medium and the total number of NPs in the field of the cell membrane (the region between $z = 0$ and $z = H$) is constant such that when one NP is internalized by the cell, another NP will appear at $z = H$. If we think of a large number of similar systems, we inferred that the probability of NPs at an arbitrary point (x, z) is stable. It means the probability density function p satisfies

$$\frac{\partial p}{\partial t} = 0. \quad (1)$$

And the flux of NP j is independent of time t . So, the time for one NP to target the junction between two cells is

$$t_K = \frac{1}{2j \cdot nd} \quad (2)$$

where $2d$ is the width of the cell–cell junction and n is the normal of absorbing wall. From a viewpoint of diffusion, we know the concentration of NPs, $c = NLp$, where N is the number of nanoparticle per length and L is the size of the cell. According to Fick’s law, we know

$$\frac{\partial c}{\partial t} = \nabla \cdot j \quad (3)$$

And the flux of NPs caused by free diffusion is

$$j_f = -D\nabla c = -2DNL\nabla p \quad (4)$$

where D is the diffusion coefficient. Then we can obtain

$$\frac{\partial p}{\partial t} = \nabla^2 p = 0 \quad (5)$$

This describes the behavior of probability density functions under free diffusion. And the probability density p must satisfy

$$\int_{\Omega} p d\Omega = 1 \quad (6)$$

Now we consider the boundary condition. Once the NP traverses across the negatively charged glycocalyx, it will be internalized by the cell. So it can be deemed the probability density of NPs at $z = 0$ is zero.

$$p(x, 0) = 0. \quad (7)$$

As mentioned above, once one NP disappears at $z = 0$, another NP will appear at $z = H$. So there is an influx at $z = H$. And we assume the chance where the NP appear is equal for every points at $z = H$. Thus, the influx is a constant.

$$\frac{\partial p}{\partial y}(x, H) = \text{constant} \quad (8)$$

For the two sides of the model ($x = \pm L$), we suppose there is no exchange between the neighbors. So there is no flux at $x = \pm L$,

$$\frac{\partial p}{\partial x}(x = \pm L, z) = 0 \quad (9)$$

Now we consider the influence of the electrostatic field. The cell membrane is covered by a negatively charged glycocalyx layer, which influences the movement of the NPs with positive or negative zeta potential, similar to electrophoresis. The velocity of NPs can be obtained by the theory of electrophoresis

$$\mathbf{v} = \mu_e \nabla \phi \quad (10)$$

where ϕ is the electrical potential, and μ_e is the electrophoretic mobility. According to Hückel’s work,³⁴ under the condition of a “thick double layer” which demands the radius of NP, a , much larger than the Debye length λ_D , μ_e can be calculated by the following equation

$$\mu_e = \frac{2\varepsilon_r \varepsilon_0 \zeta}{3\eta} \quad (11)$$

where ε_r is dielectric constant of dispersion medium, ε_0 is permittivity of free space ($C^2 N^{-1} m^{-2}$), η is dynamic viscosity of dispersion medium (Pa s), and ζ is zeta potential. Then the flux of NPs due to electrophoresis is

$$j_e = c\mathbf{v} = c\mu_e \nabla \phi = 2NLp\mu_e \nabla \phi \quad (12)$$

Combining eq 12 with eq 3, we obtain the flux of NPs caused by both the free diffusion and electrophoresis j .

$$j = j_f + j_e \quad (13)$$

Then combining eq 13 with eqs 1 and 3, we can obtain

$$\nabla(D\nabla p - \mu_p \nabla \phi) = 0 \quad (14)$$

And the electrostatic field caused by the negatively charged layer in electrolytes can be calculated by the Debye–Hückel equation³⁵

$$\nabla^2 \phi = \lambda_D^{-2} \phi - \frac{\rho_E}{\epsilon_r \epsilon_0} \quad (15)$$

where ρ_E is the charge density of the glycocalyx layer. To simplify the problem, charge is assumed to be uniformly distributed on the glycocalyx layer. Biologically, there is no glycocalyx at the cell–cell junctions

$$\rho_E = \rho_0 \text{ at } x \in (-L, -d) \cup (d, L) \quad y \in (-\infty, \infty) \text{ and } z \in (0, h)$$

And the boundary condition for eq 15 is

$$\phi(x, H) = 0, \frac{\partial \phi}{\partial x}(x = \pm L, y) = 0$$

To reflect the intrinsic relationship among the governing parameters, we select the Debye length λ_D , the diffusion coefficient D , the permittivity of the dispersion medium $\epsilon_r \epsilon_0$, the charge density ρ_0 as unit parameters to get the dimensionless parameters as follows

$$\bar{d} = \frac{d}{\lambda_D}, \bar{L} = \frac{L}{\lambda_D}, \bar{H} = \frac{H}{\lambda_D}, \bar{p} = p \lambda_D^2, \bar{t} = \frac{Dt}{\lambda_D^2}, \bar{N} = N \lambda_D, \bar{\mu}_E = \frac{2\rho_0 \lambda_D^2 \epsilon_r}{3D\eta}, \bar{\phi} = \frac{\phi \epsilon_r \epsilon_0}{\lambda_D^2 \rho_0}, \bar{\rho}_E = \frac{\rho_E}{\rho_0}$$

Equations 12 and 13 can be rewritten as follows

$$\nabla(\bar{\nabla} \bar{p} - \bar{\mu}_E \bar{\nabla} \bar{\phi}) = 0 \quad (16)$$

$$\nabla^2 \bar{\phi} = \bar{\phi} - \bar{\rho}_E \quad (17)$$

It is assumed that the length and width of cells are much larger than the width of cell–cell junction (about 20–25 nm).³⁶ So beyond the junction, we select a region with length of 320 nm to consider in the model. According to Debye's theory, the electrostatic effect can only influence a limited region around the atom with size of Debye length. Then, we select $\bar{H} = \bar{h} + 3$. And the typical parameters used in our work are listed in Table S1. Finally, finite element method is employed to solve eqs 14 and 15.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemmater.8b00840.

Characterization, cellular responses of endothelial cells, and modeling parameters used in simulation (PDF)

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Author Contributions

D.T.L. came up with the hypothesis, designed the *in vitro* study. J.W. performed all the *in vitro* experiments and analyzed the data. L.Z. and X.S. designed and performed the *in silico* study. All authors analyzed and discussed the results and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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