Materials and Methods

Mechanism of Ca^{2+} change

To understand the origin of Ca^{2+}

Ethylene glycol tetraacetic acid (10 mM, EGTA, Sigma-Aldrich) was used as a chelator in EGM-2 to reduce the Ca^{2+} present. Changes in intracellular calcium present when calcium was removed from media indicated that the change in intracellular calcium originated with the regulation of an ion channel, not release from intracellular stores. Fluo-3, AM was used to measure the level of Ca^{2+} generated with a 24 h exposure to SS of 6 dynes/cm^2 and a 2 h exposure to concentrations (0, 10, 20, and 100 µg/mL) of 5, 20 nm and 50 nm Au NPs. The samples were imaged using fluorescence microscopy and quantified using ImageJ as mentioned above.

Activation of Ca2+ ion channel

To test which specific Ca^{2+} ion channel was activated due to exposure to SS and Au NPs, GSK 2193874 (1 µM, Tocris Bioscience, Bristol, UK), GsMTx4 (3 µM, Tocris Bioscience), and YM-254890 (1 µM, Fujifilm, Richmond, VA, USA) were used as channel inhibitors to TRPV4, Piezo1, and G(q)PCR Ca^{2+} ion channels, respectively. Channel antagonists were used individually to discriminate between channel activation in the presence of Au NPs, as quantified by changes in intracellular calcium measured by Fluo-3, AM (Invitrogen). Ca^{2+} present after a 24 h exposure to 6 dynes/cm2 SS and a 2 h exposure to 5, 20 nm or 50 nm Au NPs (0, 10, 20, and 100 µg/mL) were imaged by fluorescence microscopy and quantified using ImageJ as mentioned above.

Actin Alignment

Actin alignment was measured to investigate whether exposure to 5, 20 and 50 nm Au NPs caused cytoskeletal structural alteration. HUVEC were fixed and stained with Alexa Fluor 594 phalloidin with or without a 24 h exposure to 6 dynes/cm2 SS and a 2 h exposure to Au NPs. The samples were imaged using fluorescence microscopy and compared to samples without NPs. The images were further analyzed with ImageJ and MATLAB for actin alignment using a method previously used by Liu et al. to quantify the directionality of actin fibers.[44,110] To filter out low-intensity, high-frequency noise, the fluorescent images were translated to a frequency domain using a fast Fourier transform (FFT) with a bandpass filter. For better distribution analysis, the high amplitude, low-frequency waveforms were separated into 18 angle bands ranging from 0 to 180 degrees and each angle band's total pixel intensities were computed. Further, the coefficient of variation of the intensity distribution among all 18 angle bands was computed. The coefficient of variation was turned into an alignment index for easier interpretation. An index of 1 corresponded to a distribution in which all pixel intensities were concentrated in one angle band (complete alignment), whereas an index of 0 corresponded to an equitable distribution of pixel intensities across all angle bands (no alignment).[84]

Results and Discussions

Mechanisms of Ca^{2+} Change

Intracellular Calcium

To test the hypothesis that the Ca^{2+} concentration in a cell is regulated by either releasing intracellular stores or influx through membrane channels, the chelator EGTA was used to deplete calcium from cell media, thereby removing extracellular sources of the ion. The level of Ca^{2+} generated was measured using Fluo-3, AM with or without a 24 h flow and a 2 h exposure to Au NPs. The intracellular level of Ca^{2+} reduced from 151.08 ± 6.44% (no EGTA) to 17.84 ± 2.1% (EGTA), with the percentage indicating change compared to untreated HUVEC (Figure 3.5c). A similar but less sizeable trend was shown by cells treated with 5 and 50 nm Au NPs; the intracellular level of Ca^{2+} reduced from 66.47 ± 3.18 to 10.50 ± 1.07 and 53.80 ± 3.62 to 6.77 ± 1.12, respectively. The significant decrease in intracellular calcium when calcium was depleted from media indicated that the source of intracellular calcium influx upon nanoparticle dosing was tied to regulation of an ion channel, not release from intracellular stores.

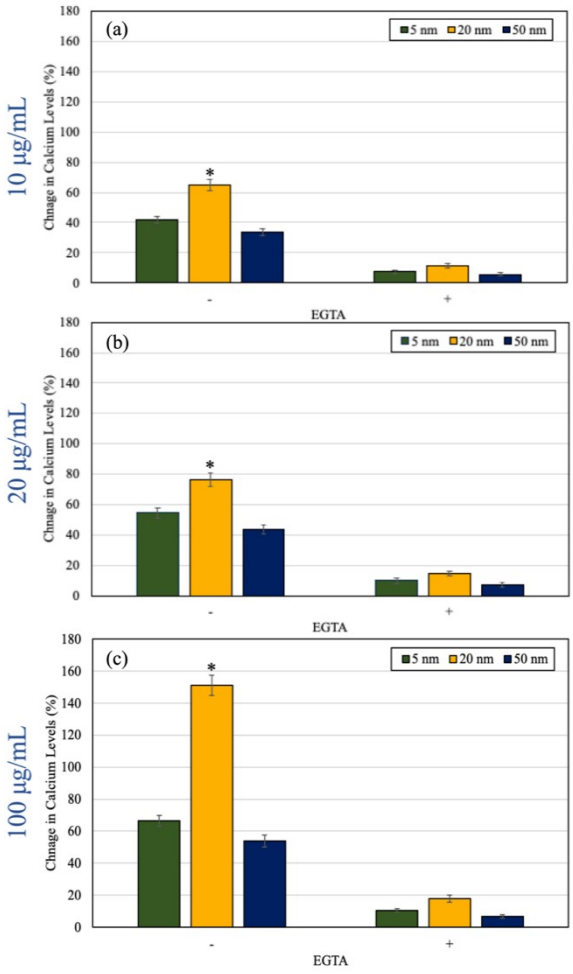

Figure S1. Percentage change of intracellular Ca^{2+} produced as compared to the positive control (cells only) after HUVEC were exposed to 5, 20, and 50 nm Au nanoparticles with (+) or without (-) EGTA in Flow at (a) 10 µg/mL (b) 20 µg/mL (c) 100 µg/mL. Data shown are mean +/- standard deviation. Data with '*' represent a statistically significant difference between the cells treated with and without EGTA, n = 4, $p < 0.05$.

Activation of Ca^{2+} ion channel

The above results indicated that intracellular Ca^{2+} change was tied to regulation of an ion channel; therefore, TRPV4 and Piezo1 were checked for activation as they are the ion channels that control influx of extracellular Ca^{2+} in ECs.[55-63] GqPCRs, which when activated lead to release of Ca^{2+} from intracellular stores in the endoplasmic reticulum were also checked for activation.[65,67,68] Channel specific inhibitors were used in EGM-2, individually, to test which specific Ca^{2+} ion channel was activated due to exposure to SS and Au NPs. GSK 2193874, GsMTx4, and YM-254890 were used as channel inhibitors to TRPV4, Piezo1, and G(q)PCR Ca^{2+} ion channels, respectively.[111-113] Fluo-3, AM (Invitrogen) was used to measure the level of Ca^{2+} generated with a 24 h exposure to flow and a 2 h exposure to 5, 20 nm, or 50 nm Au NPs. On addition of TRPV4 antagonist to HUVEC exposed to flow and 100 µg/mL 20 nm Au nanoparticles, the intracellular level of Ca^{2+} dropped drastically from 151.08 ± 6.44% (no antagonists/untreated controls) to 16.84 ± 1.12%. On the other hand, the samples treated with Piezo1 and GqPCR antagonists statistically matched untreated controls (Figure 3.6c). The above results indicated the activation of the TRPV4 channel, which further activates signaling cascades triggered by Ca^{2+}. These signaling cascades can cause changes in vesicular transport, transcription, and cytoskeletal remodeling. The detailed activation mechanism of the TRPV4 channel at the molecular level is unknown, but its response to shear, stiffening, surface expansion, stretch, and osmotic swelling & shrinking has been studied previously.[57-63,114,115] Au NPs have previously been shown to play a role in TRPV1 activation, but no link has been established in TRPV4 activation prior to these findings.[116] This new result coupled with our previous work shows 20 nm Au NPs exposure induced nanoEL by activation of the TRPV4 channel in HUVEC.

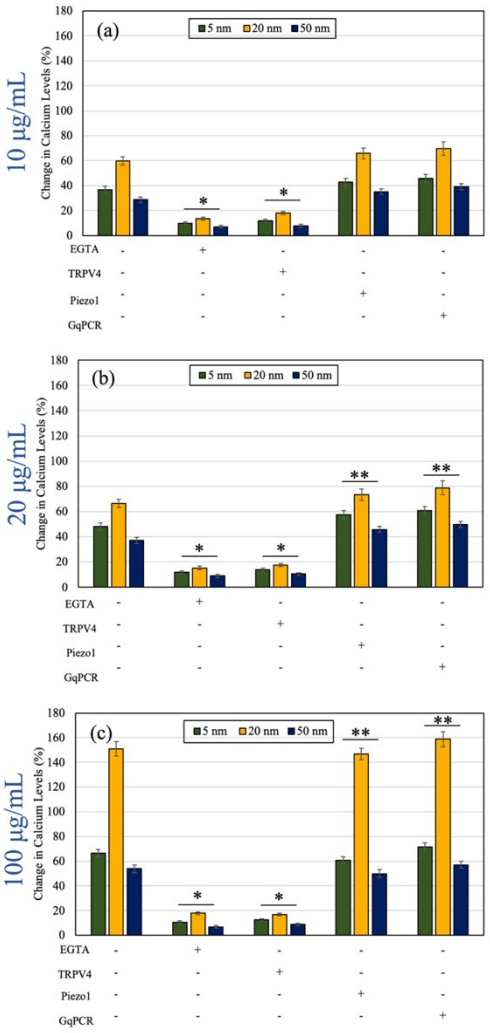

Figure S2. Percentage change of intracellular Ca^{2+} produced as compared to the positive control (cells only) after HUVEC were exposed to 5, 20, and 50 nm Au nanoparticles (100 µg/mL) with (+)/without (-) EGTA and Ca^{2+} ion channel inhibitors TRP4, Piezo1, and GqPCR in Flow at (a) 10 µg/mL (b) 20 µg/mL (c) 100 µg/mL. Data shown are mean +/- standard deviation. Data with '*' represent a statistically significant difference between the cells treated with and without EGTA & the channel antagonists, n = 4, $p < 0.05$, data with '**' represent a statistically significant difference between the cells treated with EGTA and the channel antagonists, n = 4, $p < 0.05$.

Actin rearrangement on exposure to Au NPs

Actin alignment was measured to investigate whether exposure to 5, 20 and 50 nm Au NPs caused cytoskeletal structural alteration. As we showed previously, this cytoskeletal structural alteration leads to cellular contraction, which further leads to widening of the paracellular route as cells pull away from each other.[117-120] Actin alignment was quantified using ImageJ and MATLAB after HUVEC were fixed and stained with Alexa Fluor 594 phalloidin with or without a 24 h exposure to 6 dynes/cm2 SS and a 2 h exposure to particles. An alignment index of 1 corresponded to a distribution in which all pixel intensities were concentrated in one angle band (complete alignment), whereas an index of 0 corresponded to an equitable distribution of pixel intensities across all angle bands (no alignment).[44,84] Twenty nm Au NPs showed the highest alignment index, as the alignment index increased to 464.12 ± 33.06% on exposure to 100 µg/mL 20 nm Au nanoparticles in flow from 125.67 ± 8.03% in static as compared to the untreated HUVEC (Figure 3.7c). A similar but less sizeable trend was shown by cells treated with 5 and 50 nm Au NPs, where the alignment index of 5 nm Au NP was slightly higher than 50 nm Au NP. These results were comparable to the level of Ca^{2+} seen in the previous studies, indicating that the actin rearrangement was triggered by the Ca^{2+} influx.[44,51,84,121,122] Ca^{2+} triggered actin rearrangement has been shown to be regulated via the Rho family GTPase pathway by Michalick et al.[63] The results indicated that the actin microfilament rearrangement observed in HUVEC treated with 20 nm Au NPs induced nanoEL. This type of

leakiness is also known as indirect (type II) nanoEL, as it requires secondary events of NP-EC interactions.[11] In our case, the NPs activate the TRPV4 ion channel, which leads to actin rearrangement and likely further results in movement of the 20 nm NPs through the EC barrier.

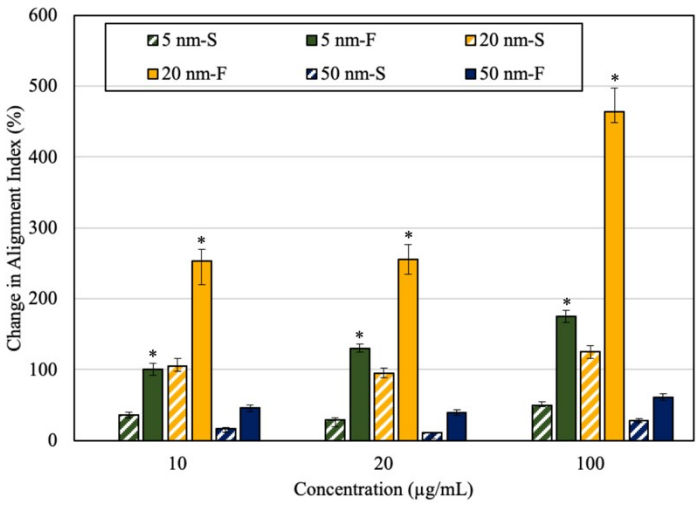

Figure S3. Change in actin alignment index as compared to positive control (cells only) on exposure to 5, 20, and 100 nm Au nanoparticle at 10 µg/mL, 20 µg/mL, and 100 µg/mL in static and flow conditions. Data shown are mean +/ - standard deviation. Data were analyzed using ANOVA and post hoc t-test; data with '*' represent a statistically significant difference between the static and flow, $n = 4$, $p < 0.05$.