Electronic Supplementary Material (ESI) for Environmental Science: Nano.

Supplementary Section

Polyacrylic acid coated nanoparticles elicit endothelial cell apoptosis and diminish vascular relaxation in ex vivo perfused iliac arteries of the cane toad (*Rhinella marina*).

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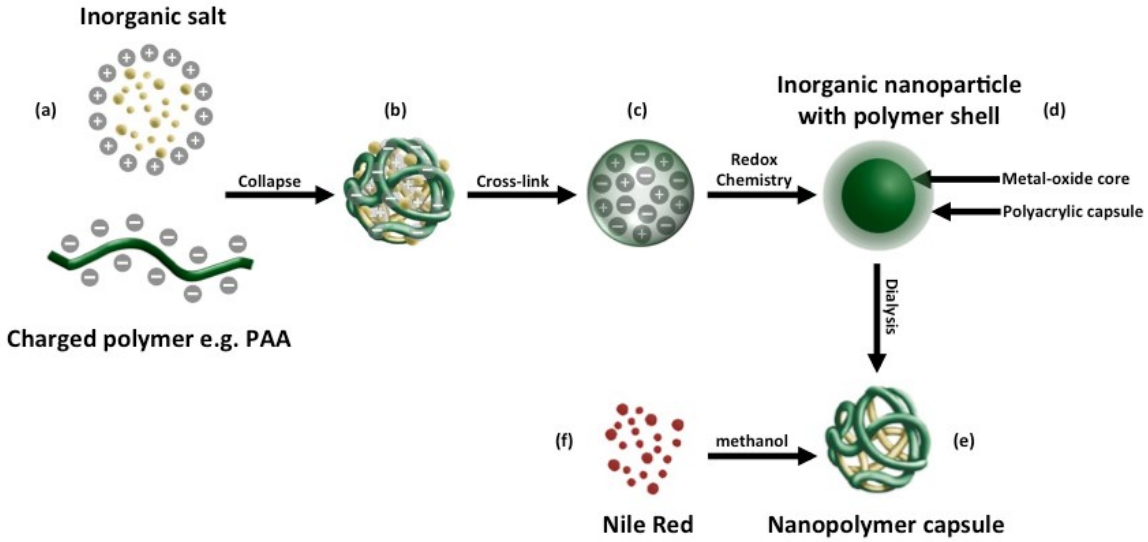
PAA-NP Synthesis Protocol

The functionalized derivatives used in this study consisted of polyacrylic acid (PAA)-encapsulated metal oxide TiO_2 (rutile) and a Nile Red dye that were manufactured by Vive Crop Protection Inc. (Toronto, ON, Canada) and were kindly donated. The NP template was synthesized via interaction between oppositely charged PAA (120 kDa) polymer chains and inorganic counterions (i.e. inorganic Ti) (**Figure S1a**), resulting in condensed orb-like structures of negative charge that are less than 10 nm in size (**Figure S1b**). These structures were then stabilized by cross-linking polymer chains either chemically or through ionizing radiation in order to maintain their integrity in suspension (**Figure S1c**). Finally, redox and precipitation reactions were used to convert the counterions encapsulated within the cross-linked coating to inorganic-oxide NPs, which are filtered through a membrane as a final purification step before being lyophilized for long-term storage (**Figure S1d**).[1-2] The PAA-NPs that contained the fluorescent dye, Nile Red, were also produced for this study and used as a photoluminescent NP to track uptake and translocation across tissues and cells. PAA-Nile

Red NPs were produced in a similar manner as described above, where 0.2 g Nile red (excitation/emission wavelengths [Ex/Em] = 552/636 nm) were dispersed in methanol, causing an association between a dialysis-hollowed polymer NP capsule (**Figure S1e)** and the fluorescent dye, and then lyophilized (**Figure S1f**). The interaction of metal-oxides or dyes with the PAA polymer is defined by non-covalent interactions such as charge-charge interactions, hydrophobic interactions, polymer-chain interactions, van der Waals forces or ionic interactions.[1] Working suspensions were made from lyophilized stocks and resuspended in HEPES-buffered saline. Lyophilized stocks were stored at 4°C and protected from light.



Supplemental Figure S1. Schematic diagram of polymer-coated NP synthesis, provided by Vive Crop Protection Inc. (http://vivecrop.com/). Interaction between oppositely charged polymer chains and counterions (a) results in condensed orb-like structures (b). These structures are then stabilized by cross-linking polymer chains either chemically or through ionizing radiation (c). Redox and precipitation reactions are used to convert the counterions encapsulated within the cross-linked coating to inorganic NPs (d). Dispersing Nile Red dye in methanol causes an association between the polymer NPs and the fluorescent dye (e) to make the Nile Red-loaded nanocapsules (f).

PAA-NP Characterization

Manufacturer measurements for PAA-NP size, pH, metal purity and percent metal composition are summarized in **Supplemental Table S1**. To determine trace metal content, lyophilized PAA-NPs were weighed, acidified with HNO_3 then measured using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, Varian Vista-Pro CCD Simultaneous) equipped with an autosampler (Varian SPS 3). ICP-OES was coupled with Total Organic Carbon (TOC-VCPH Shimadzu analyzer) oxidative combustion-infrared analysis equipped with an autosampler (ASI-V) and solid sample module (SSM-5000A) (**Supplemental Table S2**). Results from Supplemental Tables S1 and S2 showed that sizes ranged between 3-9 nm and metal purity is above 98%. The pH of suspended PAA-NP were neutral.[3]

In addition to manufacturer measurements, we have conducted extensive physico-chemical characterization of stock PAA-NPs. NP shape and primary particle size was confirmed with Transmission Electron Microscopy (TEM), using a JEOL-2010 (LaB6 filament) electron microscope with an accelerating voltage of 200 kV. Nanoparticles (10 μg/mL) in ultrapure water were drop-coated onto a carbon coated copper grids (200 m) and air-dried at room temperature to remove any residual solvent prior to analysis. TEM imaging of PAA-NPs confirmed information from the manufacturer that the PAA-NP metal cores were 3-9 nm in size.[4-5]

Supplemental Table S1. Product lot number, particle size (nm), pH, total metal (%) and purity (%) of PAA-TiO_2. Properties not measured for PAA-Nile Red due to the absence of a metal core.[3]

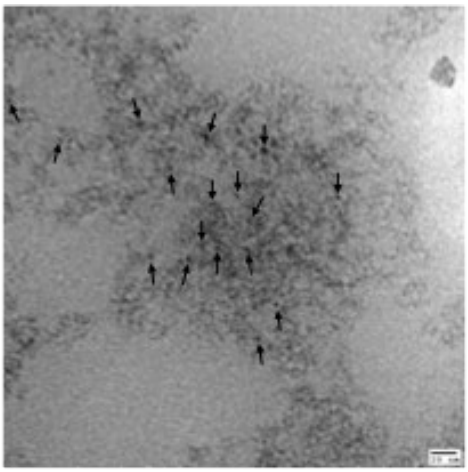
Nanoparticle	Lot Number	Size (nm)[a]	pH	Total Metal (%)[b]	Purity (%)[c]
PAA-TiO_2	PB 42	3-9	7.0	46	98

1. [a]Exclusive of PAA coating.
2. [b]Total metal, Ti.
3. [c]Purity is exclusive of Na^+ stabilizer and PAA coating.

Supplemental Table S2. Trace metal (>0.1%), excluding Na^+, of PAA-TiO_2 as analyzed by the manufacturer, Vive Crop Protection. Dashes indicate trace metal not present or below the ICP-MS detection limit.[3]

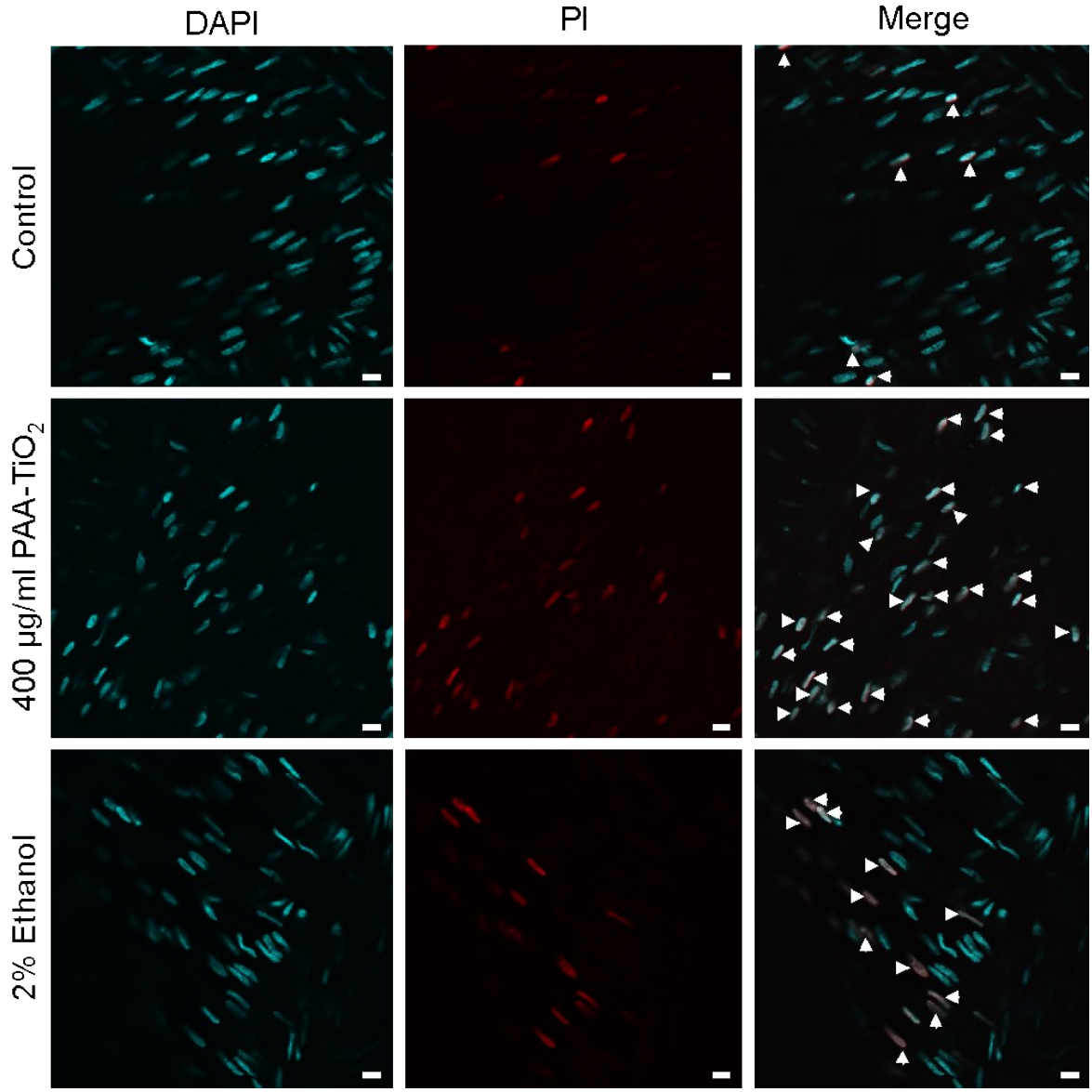
Trace metal (%)	PAA-TiO_2
Ag	0.5
Al	0.3
B	0.1
Ca	
Co	0.1
Cr	-
Ga	-
Gd	-
Ho	-
Ir	-
K	-
Li	-
Mg	-
P	-
Pr	-
Rb	-
Si	-

Zn -

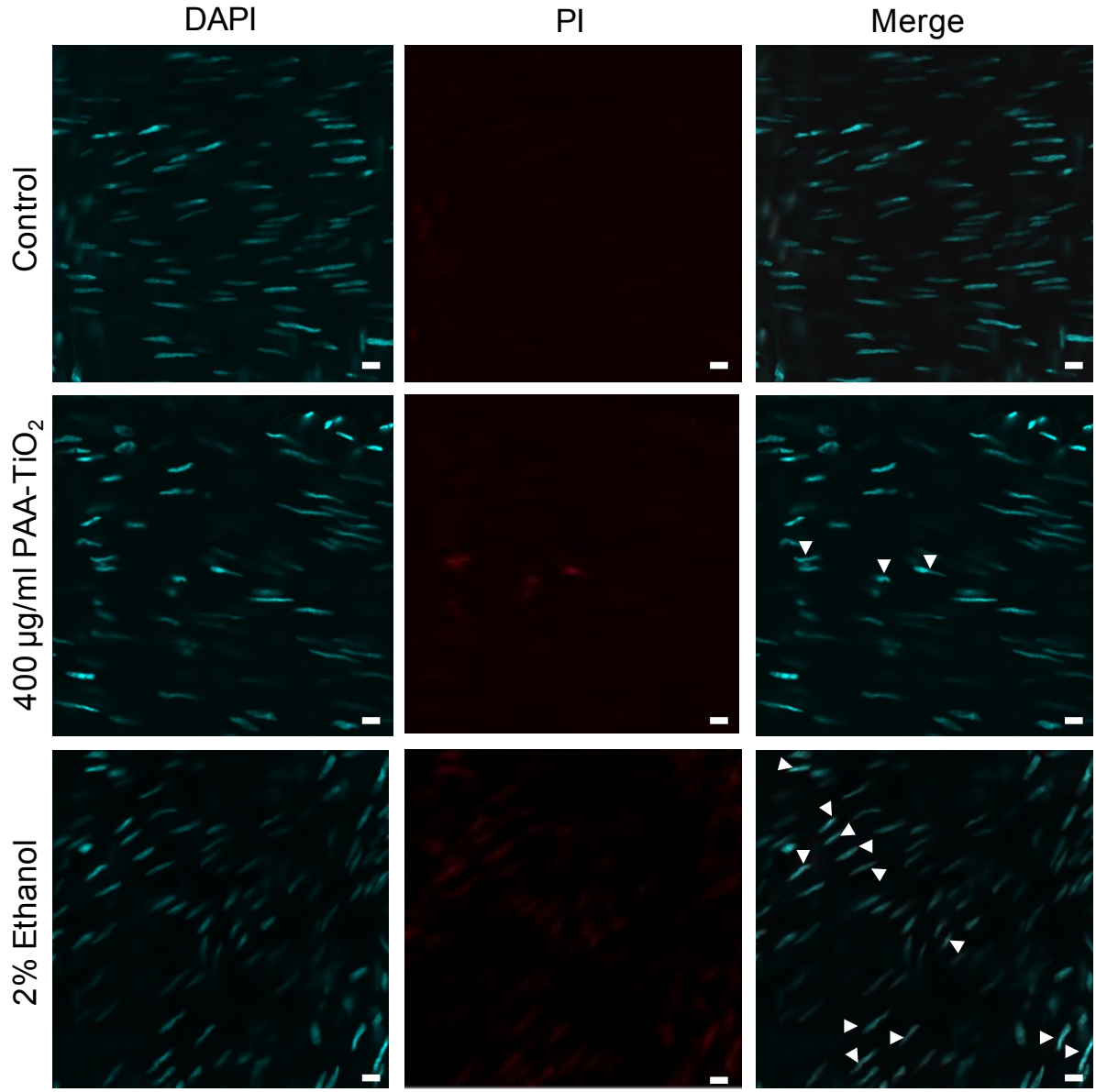


Supplemental Figure S2. Transmission electron micrographs (JEOL-2010 (LaB6 filament) electron microscope with an accelerating voltage of 200 kV) of Vive Crop Protection PAA-TiO_2 showing primary NP sizes ranging between approximately 3 to 9 nm (arrows). Scale bars are 20 nm. Image adapted from.[5]

Endothelial and smooth muscle cell death

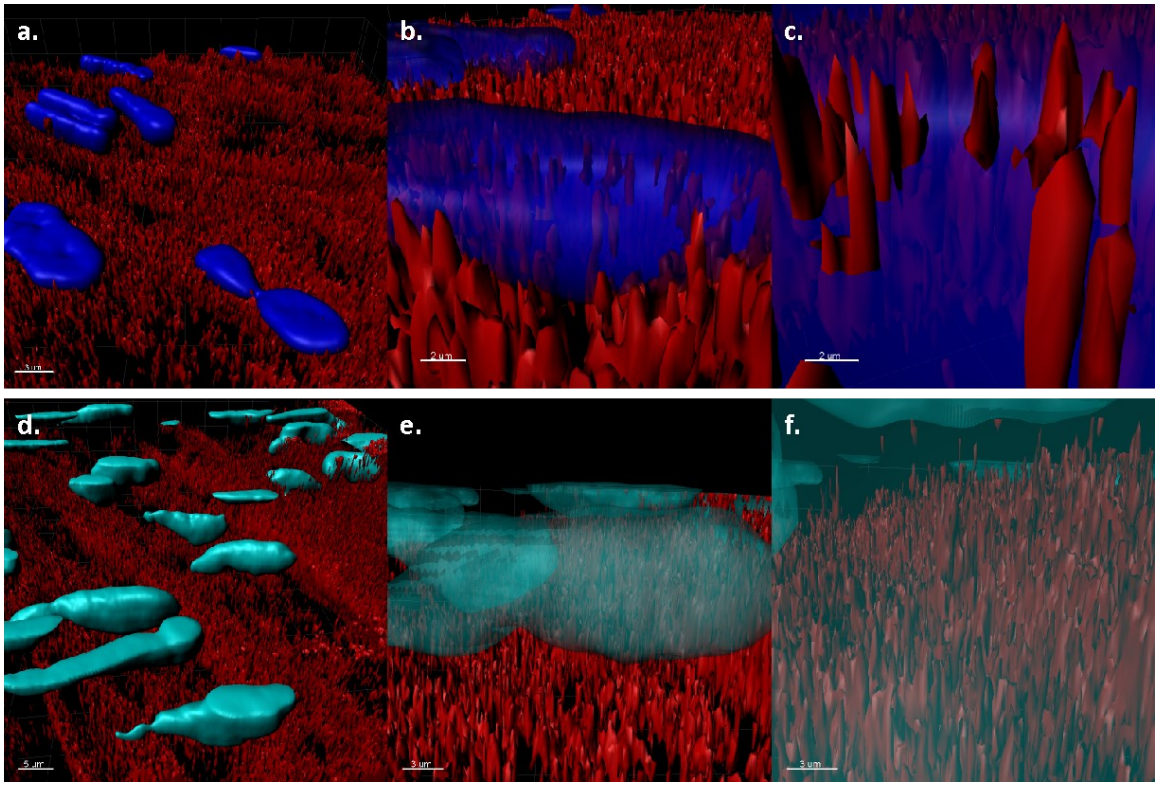


Supplementary Figure S3. Endothelial cells from cane toad iliac arteries perfused for 2 h with HEPES-buffered saline (control; *n* = 3), 400 µg/ml PAA-TiO_2 NPs (*n* = 3), or 2% ethanol (positive control; *n* = 3). The cells were stained with PI (red) and DAPI (blue; nucleus). In the merged images, white arrows indicate dead or dying cells. Scale bar = 10 µm.



Supplementary Figure S4. Smooth muscle cells from cane toad iliac arteries perfused for 2 h with HEPES-buffered saline (control; *n = 3*), 400 µg/ml PAA-TiO_2 NPs (*n* = 3), or 2% ethanol (positive control; *n* = 3). The cells were stained with PI (red) and DAPI (blue; nucleus). In the merged images, white arrows indicate dead or dying cells. Scale bar = 10 µm.

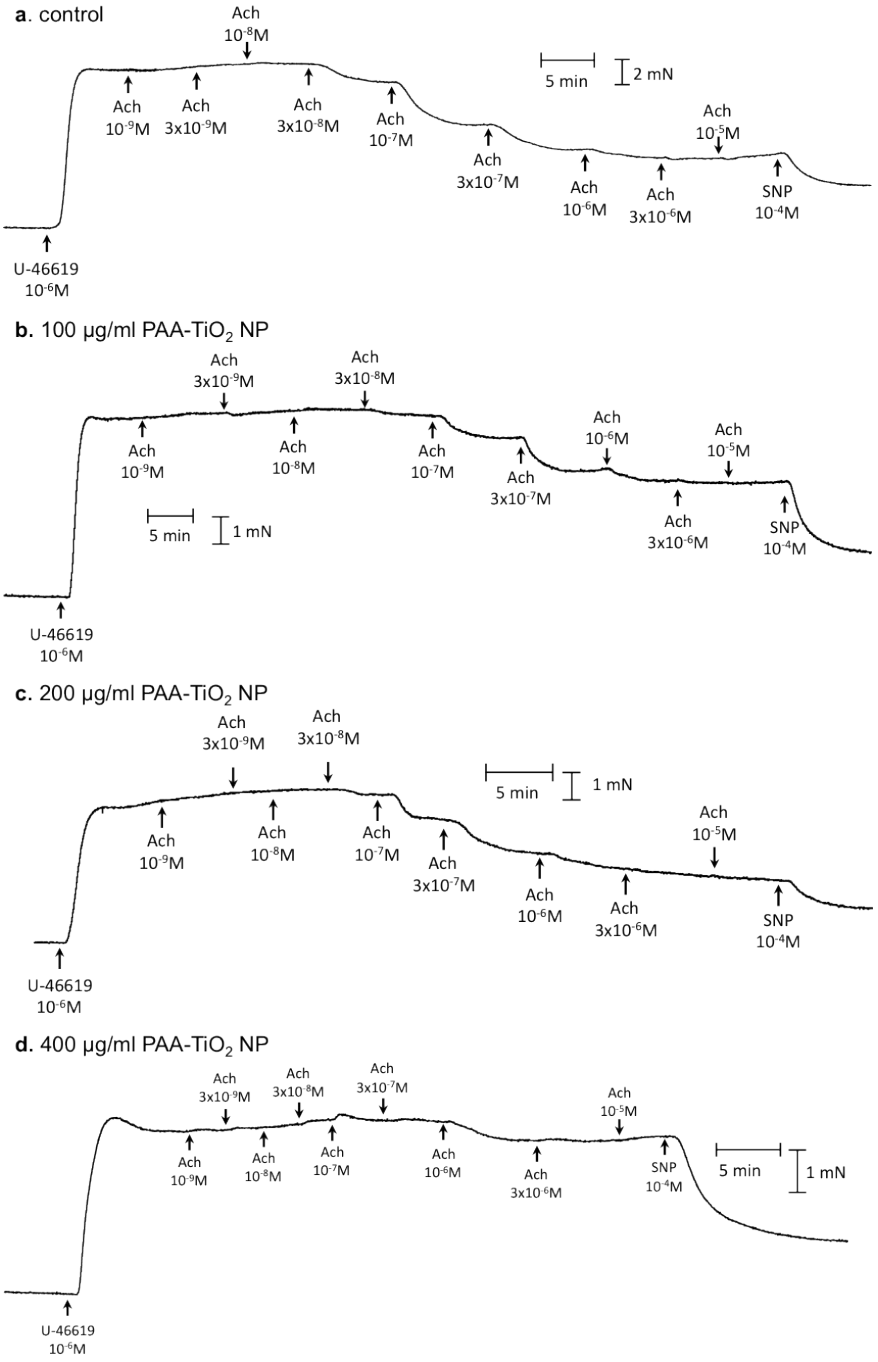
PAA-NR NP uptake in cells



Supplementary Figure S5. Representative laser scanning confocal microscopy (60x objective lens x 3.14 zoom) three-dimensional images of whole-mounted cane toad iliac artery showing endothelial (blue) and vascular smooth muscle cell (cyan) nuclei at distance (a. and d.), close-up to nuclei (b. and e,) and inside the nuclei (c and f). DAPI was used as a fluorescent probe for cell nuclei, with surface transparency reduced to 65% for three-dimensional reconstruction in panels b,c,e and f to show interior of nucleus. Results demonstrate penetration of PAA-Nile Red NPs (red) inside endothelial cell nuclei but not smooth muscle cell nuclei. Scale bars for three-dimensional images are between 2 - 5 µm.

Myograph tension recordings

Representative tension recordings demonstrate concentration-dependent ACh-mediated relaxation in iliac arteries after pre-perfusion of vessels for 2 h with HEPES-buffered saline (control; Figure S6a), HEPES-buffered saline containing 100 µg/mL PAA-TiO_2 NP (Figure S6b), 200 µg/mL PAA-TiO_2 NP (Figure S6c) or 400 µg/mL PAA-TiO_2 NP (Figure S6d). SNP (10^{-4} M) was added at the completion of ACh-mediated vasodilation to confirm maximum relaxation capacity of the vessels.



Supplementary Figure S6. Representative tension recordings showing the concentration-dependent vasodilatory effects of ACh (10^{-9} M – 10^{-5} M) on cane toad iliac arteries perfused for 2 h with HEPES-buffered saline (a; control) or perfused for 2 h with 100 µg/mL PAA-TiO_2 NPs (b), 200 µg/mL PAA-TiO_2 NPs (c), and 400 µg/mL PAA-TiO_2 NPs (d). SNP (10^{-4} M) was added at the completion of the experiment to confirm the maximum relaxation capacity of the arteries.

References

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4. Ortega, V. A.; Ede, J. D.; Boyle, D.; Stafford, J. L.; Goss, G. G., Polymer-Coated Metal-Oxide Nanoparticles Inhibit IgE Receptor Binding, Cellular Signaling, and Degranulation in a Mast Cell-like Cell Line. *Advanced Science* **2015,** *2* (11), 1500104.
5. Ortega, V. A.; Katzenback, B. A.; Stafford, J. L.; Belosevic, M.; Goss, G. G., Effects of polymer-coated metal oxide nanoparticles on goldfish (Carassius auratus L.) neutrophil viability and function. *Nanotoxicology* **2015,** *9* (1), 23-33.