Reactive Oxygen Species Driven Angiogenesis by Inorganic Nanorods

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Materials and methods:

Europium(III) nitrate hydrate [$Eu(NO_3)_3.xH_2O$, 99.99%], aqueous ammonium hydroxide [aq.NH_4OH, 28–30%], europium(III) oxide nanopowder, and *N*-acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (USA) and were used without further purification. HUVECs were purchased from American Type Culture Collection (ATCC, USA). EBM medium and the individual components for making EBM complete medium were obtained from Cambrex Bio Science Walkersville, Inc. (MD, USA). [^{3}H]Thymidine was purchased from Amersham Biosciences (Piscataway, NJ). Mn(III)tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP) was purchased from BIOMOL (USA). Catalase (C-100) was obtained from Sigma-Aldrich. Dihydroethidium (hydroethidine) was purchased from Molecular Probes. Single-walled carbon nanotubes were purchased from Nano-C, Inc. Type I collagen was purchased from BD Biosciences. Glass-bottom Petri dishes were purchased from MatTek Corp. L-15 medium was purchased from ATCC. Acetic acid was purchased from Sigma-Aldrich.

Microwave-assisted Synthesis of Europium Hydroxide [$Eu^{III}(OH)_3$] Nanorods

The synthesis of $Eu^{III}(OH)_3$ nanorods was carried out according to our previous publications with some modifications[1]. Briefly, 1 ml of aqueous NH_4OH was added to 39 ml of an aqueous solution of 676 mg Eu(III) nitrate (NH_4OH/Eu^{3+} molar ratio of 4; pH 5.5) in a 100-ml round-bottom flask. The resulting solution was irradiated for 60 min at 60% of maximum power (on/off irradiation cycles in the ratio of 3/2) in a microwave oven (GOLD STAR 1000W with 2.45 GHz frequency). In the post-reaction treatment, the microwaved products were collected, centrifuged at 10,000 rpm, washed several times

while alternating between Millipore H_2O and ethanol, washed again with Millipore H_2O, and finally dried overnight under vacuum at room temperature.

Nanorod Solution Preparation

Solid crystalline $Eu^{III}(OH)_3$ nanorods (100 mg) were added to 10 ml of sterile TE (Tris-EDTA) buffer and incubated for 5 sec in a sonicator bath to make a homogeneous suspension of 10 mg/ml. This freshly prepared stock solution was used for all cell culture experiments and *in vivo* toxicity experiments.

Detection of Endotoxin

Millipore H_2O, used for all experiments in our research, was tested for endotoxin using the Gel-clot method according to the manufacturer's instructions (Pyrotell® Multi-test; Cat # GS250; Associates of Cape Cod,MA). The formation of a gel clot indicates the presence of endotoxin in a sample. However, we did not observe any gel clots, confirming the absence of endotoxin in the water. Similarly, prior to incubation with HUVEC endothelial cells for cell proliferation studies, we tested the nanorod suspension in TE buffer for possible endotoxin contamination.

Cell Culture and Cell Proliferation Assay

HUVEC (passage 3; P3) were cultured in 100-mm plates for ~24 h at 37°C and 5% CO_2 in complete EBM medium. The [^{3}H]-thymidine incorporation assay was carried out as previously reported [1a]. Briefly, HUVEC (2 x10^4) were seeded in 24-well plates, cultured for one day in EBM, serum-starved (0.1% serum) for 24 hours, and then treated with different concentrations of $Eu^{III}(OH)_3$ nanorods with and without N-acetyl-L-cysteine, MnTBAP, and catalase. After 24 hours, 1 μCi [^{3}H]-thymidine was added to each well.

Four hours later, cells were washed with cold PBS, fixed with 100% cold methanol, and collected for the measurement of trichloroacetic acid-precipitable radioactivity. Experiments were performed in triplicate.

Zebrafish Studies

Zebrafish were grown and maintained at 28.5 °C in a 14 h day and 10 h night cycle. Mating was routinely carried out at 28.5°C, and all embryos were staged according to established protocols [2]. All zebrafish studies were performed under the MCW institutional guidelines (Animal Protocol Number 312-06-2). Adult Tg(fli1a:EGFP) line was purchased from the zebrafish information network (ZFIN). The SIV (subintestinal vessels) were analyzed at 72 hpf, and photomicrographs were taken using a Leica MZ 16FA steromicroscope, (Leica Microsystems) with 1x planapochromatic objective and 10x eyepiece in 100x zoom. The image acquisition software used was Image-Pro AMS 6.0 (Media Cybernetics, Bethesda, MD).

Superoxide anion (O_2•−) determination by an HPLC method

HPLC is a standard method for detection and quantification of intracellular superoxide anion (O_2•−) in HUVEC [3]. The product of the HE and (O_2•−) reaction has been previously characterized [3a]. A stable fluorescent product, 2-hydroxyethidium, is formed from the reaction between dihydroethidium and superoxide anion. Briefly, HUVEC were grown in EBM media with serum, which was replaced by serum-free EBM medium one day before the study. On the day of the experiment, the cells were rinsed three times with chilled Krebs-Hepes buffer and preincubated with MnTBAP for 15 min at 37°C in a CO_2 incubator before the addition of $Eu^{III}(OH)_3$ nanorods (24 hours). On the day of measurement for superoxide anion (O_2•−), the cells were again washed with fresh buffer,

a final concentration of 25 μM dihydroethidium was added, and the cells incubated for 20 minutes. The cells were washed again and incubated in fresh Krebs-HEPES buffer for an additional hour. The cells were then harvested by scraping and homogenized in chilled 100% methanol. Cell lysates were centrifuged at 12,000 rpm for 10 minutes at 4°C, and the supernatant was used for measurement of superoxide levels using a Beckman Coulter System RP-HPLC equipped with a Jasco FP1520 fluorescence detector. The excitation and emission wavelengths were 480 nm and 580 nm, respectively. Data were collected and analyzed using chromatography software (32 Karat™ Beckman Coulter).

Preparation of the SWNT/collagen sensor

SWNT/collagen sensor was prepared according to our previous literature [4]. A 1 mg portion of SWNT was added to 1 mL of collagen solution (3.41 mg/mL) containing 0.02 M acetic acid, and the resulting mixture was sonicated for 10 min in an ice bath using a probe-tip sonicator (40% amplitude, 10 W). The suspension was then centrifuged for 3 h at 16,300*g*, and the supernatant decanted. A 20-mL portion of collagen-suspended SWNT solution as added to 40 mL of collagen solution (3.41 mg/mL), and the resulting solution was diluted with 4.14 mL of a 0.02 M acetic acid solution. The final SWNT solution (400 mL of 0.19 mg/mL SWNT) was added to each Petri dish and gently dried at room temperature for 20 h. Before being used for cellular experiments, the SWNT/collagen sensor was extensively washed with water and PBS (pH 8).

Detection of H2O2 in HUVEC by Semiconducting SWNT:

Semiconducting SWNT emits stable photoluminescence (PL) [5] in the near infrared (nIR) region with no photobleaching threshold [6], which allows long exposures and integration times compared to conventional organic fluorescent dyes. Recently, nIR fluorescent

SWNT has been shown to detect small molecules, even at the single-molecule level, by monitoring the step-wise fluorescence quenching of a single SWNT sensor [4, 7]. In particular, we have shown that the SWNT/collagen sensor is capable of detecting H_2O_2 at the single-molecule level with high selectivity against other ROS. For this purpose, the SWNT/collagen sensor was prepared by slowly drying the diluted solution of collagen-suspended SWNT (final concentration of SWNT, 0.19 mg/mL) at room temperature on a glass-bottom petri dish, as previously described [4]. The SWNT/collagen sensor was clearly homogeneously distributed on glass without aggregation, such that a 2x2 pixel area (580 x 580 nm^2) corresponded to a single SWNT sensor (SI- Fig. 5b). In addition, this sensor shows very bright and stable nIR fluorescence over long exposure times.

In order to investigate H_2O_2 efflux from HUVEC stimulated by VEGF or $Eu(OH)_3$ nanorods, the cells were replated on the SWNT/collagen sensor in a petri dish filled with complete EBM medium. The cells on the SWNT sensor in the serum-starving medium (0.2% fetal bovine serum) were incubated for a further 12 h at 37^oC, and then stimulated with VEGF (10 ng/mL) or nanorods (10 mg/mL). The nIR fluorescence response of the sensors underneath a single cell was then monitored in real time for 20 min to detect H_2O_2 produced from HUVEC and analyzed using an algorithm to calculate the number of stochastic transitions in nIR fluorescence intensity caused by generated H_2O_2, as described in the supplementary information.

CHARACTERIZATION TECHNIQUES

Characterization techniques for $Eu^{III}(OH)_3$ nanorods are described briefly as follows.

X-ray diffraction (XRD)

The structure and phase purity of the as-synthesized samples were determined by X-ray diffraction (XRD) analysis using a Bruker AXS D8 Advance Powder X-ray diffractometer (using CuK_{α} λ = 1.5418 Å radiation).

Transmission electron microscopy (TEM)

Particle morphology (microstructures of the samples) was studied with TEM on an FEI Technai 12 instrument operating at 80 KV. Internalization of nanoparticles inside the cells was visualized by TEM according to our published description [8].

nIR fluorescence microscopy

Fluorescence images were taken using a fluorescence microscope (Carl Zeiss, Axiovert 200), with a CCD camera (Carl Zeiss, ZxioCam MRm) and 2D InGaAs array (Princeton Instruments OMA 2D). Videos were acquired using the WinSpec data acquisition program (Princeton Instruments). Samples were excited by a 658-nm laser (LDM-OPT-A6-13, Newport Corp) at 35 mW.

Cellular data analysis

Cellular data were analyzed as described previously [9]. In the first step, a MATLAB routine selected 100 diffraction-limited spots (2x2 pixels) in the nIR images in the order of highest to lowest intensity. Each time trace obtained was then subjected to an error-minimizing, step-finding algorithm in which the intrinsic steps in intensity can be identified within noisy data. The best-fit traces were obtained in a manner analogous to linear regression, where idealized traces exhibiting minimized error deviation from the experimental traces were selected. Specifically, the experimental trace was initially fit to a flat trace with a value equal to the mean intensity value of the experimental trace. Next, the algorithm assumes the existence of a single step where the value prior to the step is

the mean intensity of the trace before the transition, and the value after the step is the mean intensity of the trace after the transition. The location of this step is iteratively fit at each time point within the trace, and the trace resulting in the best fit is selected. Once the location of the first transition is determined, the locations of the second and third steps are determined similarly by analyzing the bisections separately. In the region prior to this first transition, the algorithm once more assumes the existence of a step and determines its location by iteratively fitting the step to each time point prior to the transition. A similar analysis was performed at the second bisection or the region after the first transition, where the algorithm once more assumes the existence of a step and determines its location. The algorithm continues fitting steps to the bisections until the best fit is obtained. Finally, the number of transitions is calculated.

Statistics

Mean and standard deviations were calculated. We calculated the P values using the Student T-Test and reported significant results ($P < 0.05$).

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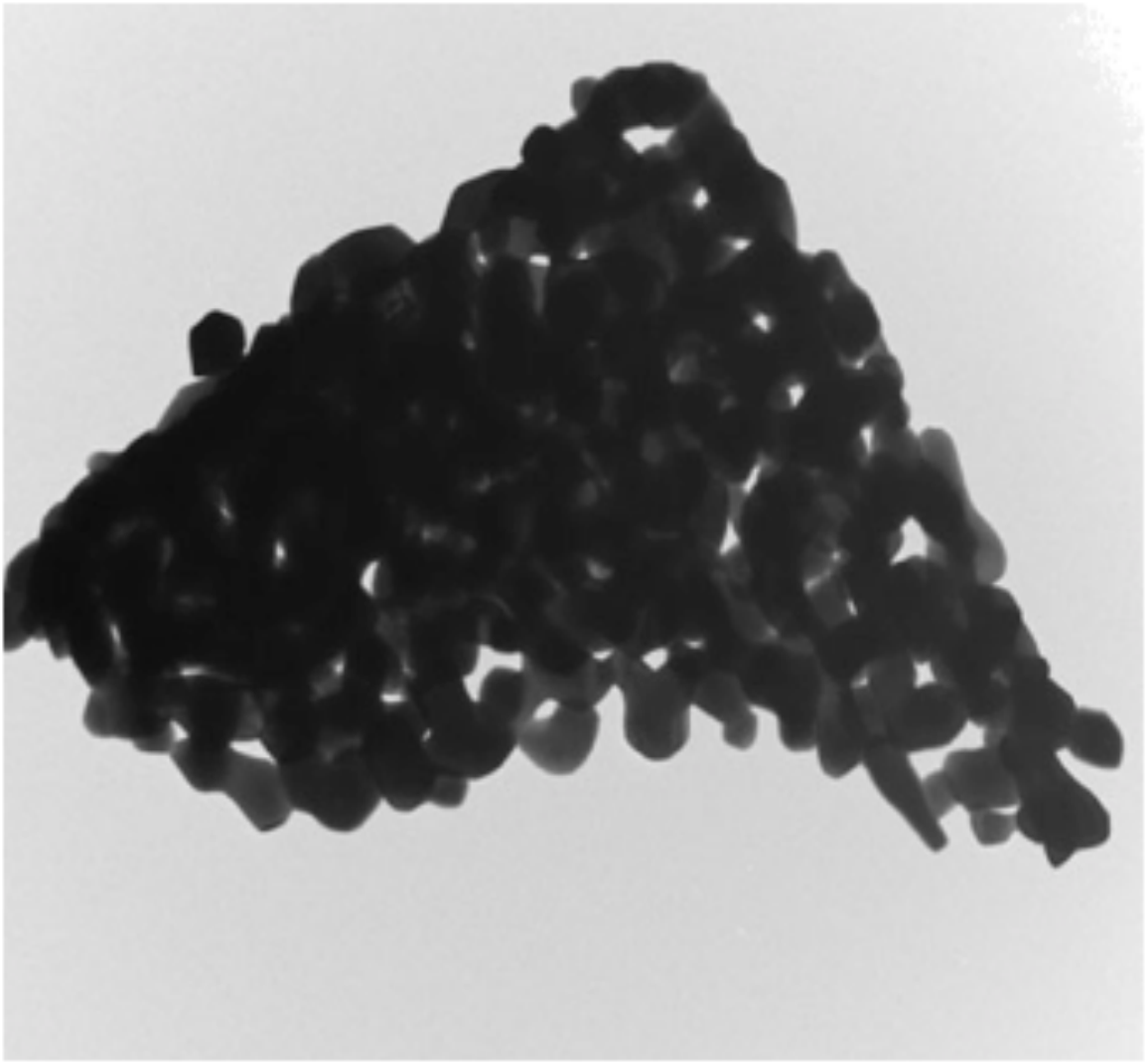
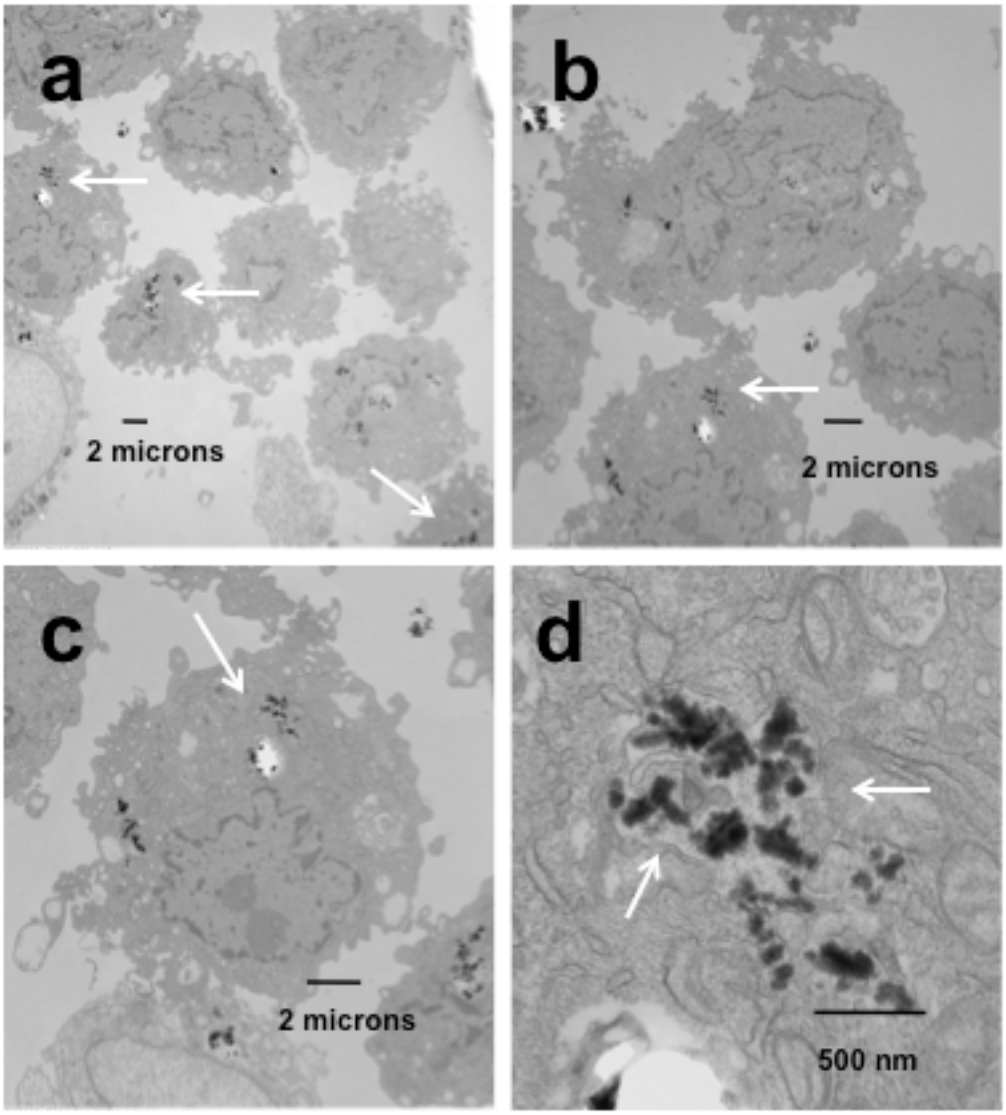
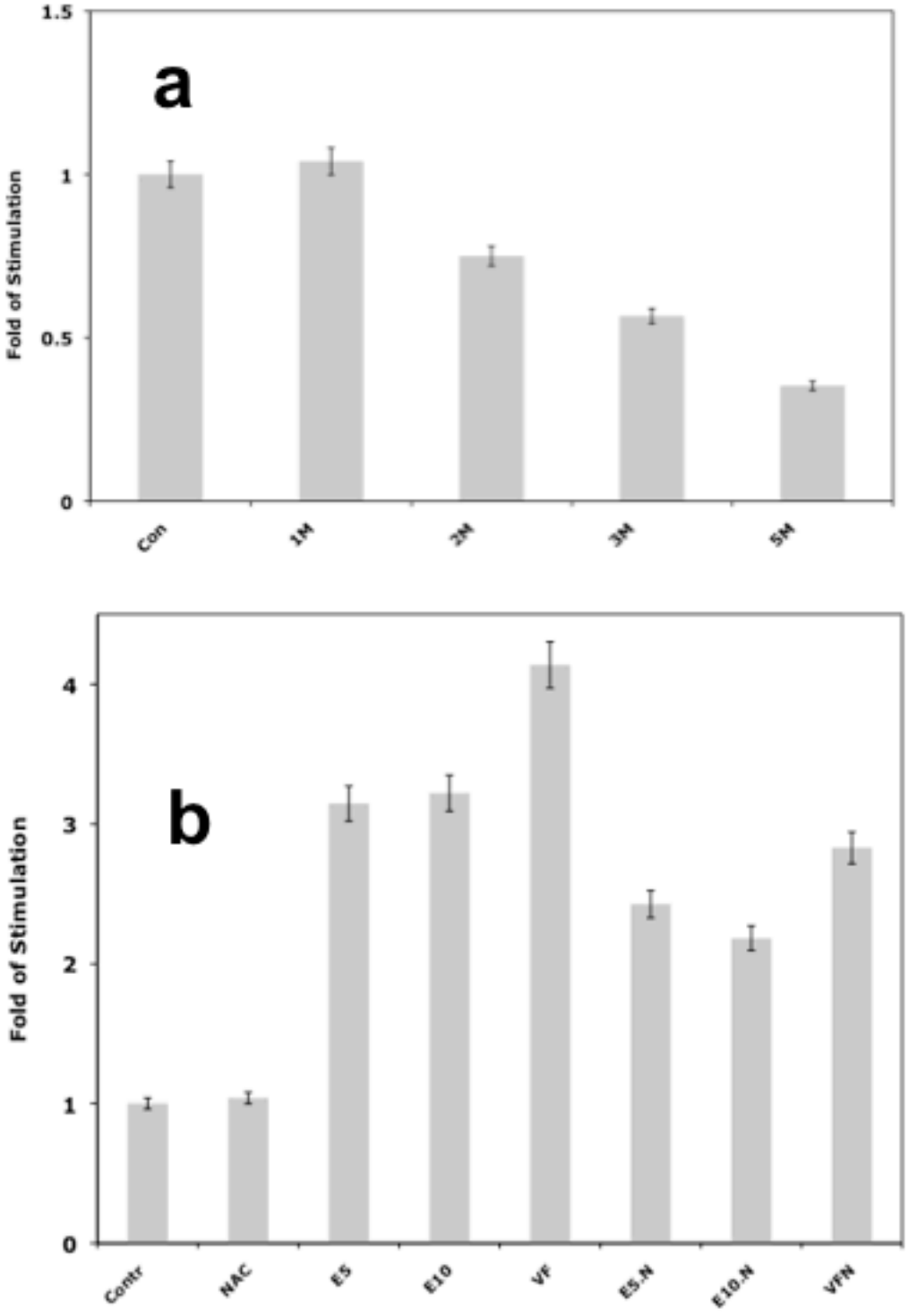


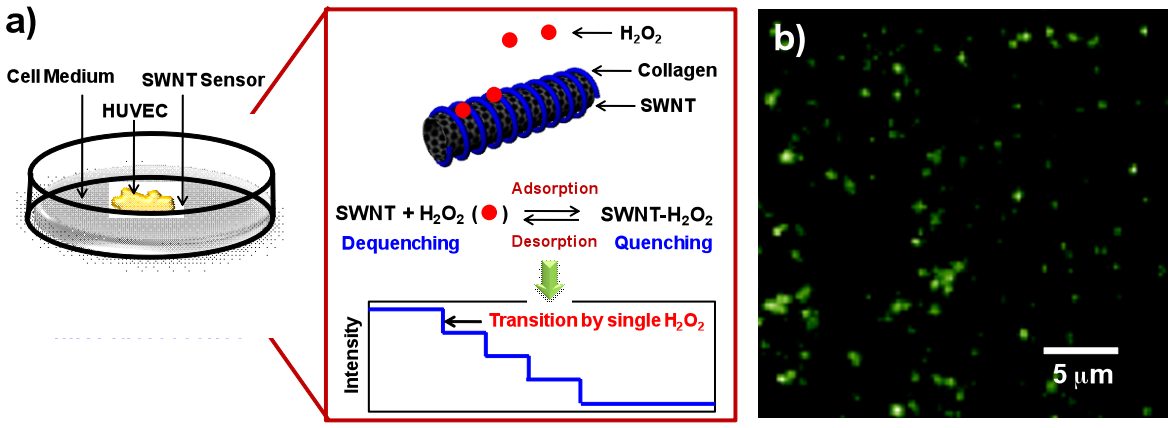
SI-Fig.1: Physical characterizations of Eu_2O_3 nanopowder. TEM picture of Eu_2O_3 nanopowder obtained from Sigma-Aldrich, **USA shows** ~200 nm particle size**.**



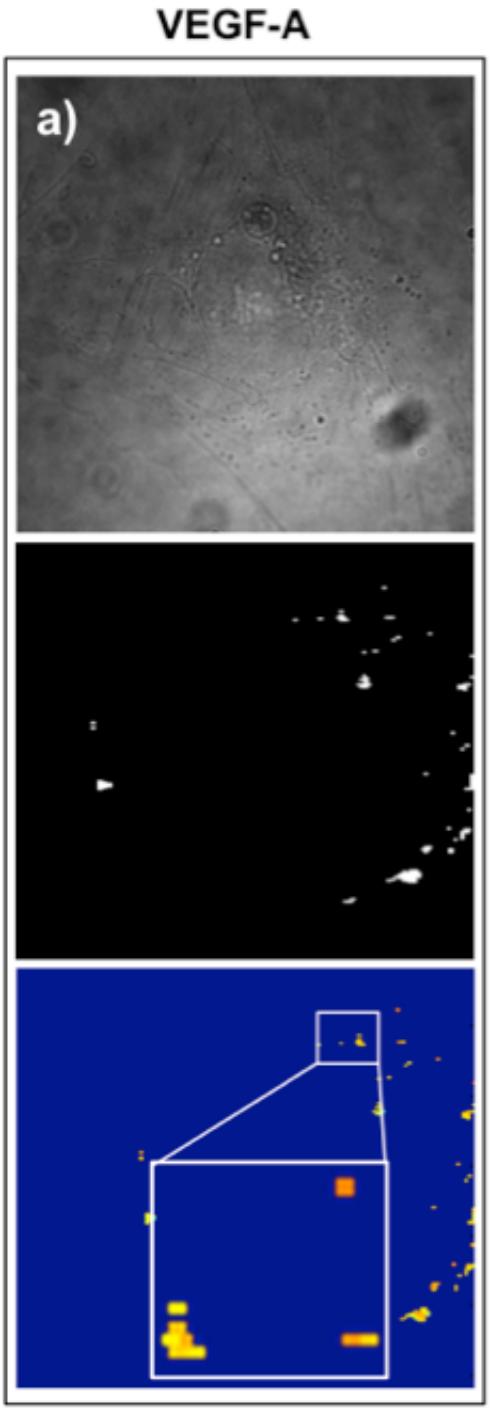
SI-Fig.-2 (a-d): Cellular uptake and internalization of [$Eu^{III}(OH)_3$] nanorods in HUVECs has been visualized using transmission electron microscopy (TEM). Cells were observed by TEM after HUVECs were exposed to [$Eu^{III}(OH)_3$] nanorods for 24 h incubation at 10 μg/mL concentrations of nanorods in EBM serum free media. Nanoparticles are clearly visible in endocytic compartments within the cell (indicated by white arrows from low magnification to high magnification in a-d).



SI-Figure-3 (a-b): Cell proliferation assay of HUVECs using radioactive [^{3}H]-thymidine. **a.** Cell proliferation assay was carried out at different concentration (1M to 5M) of *N*-acetyl-L-cysteine (NAC) and it was observed that up to 1M the HUVECs were non-toxic. However, beyond the 1(M) concentration of NAC the cells are toxic. **b.** Cell proliferation assay was carried out using nanorods and VEGF in presence of *N*-acetyl-L-cysteine as ROS scavenger. E5.N and E10.N indicate the concentration of 5 and 10 μg of nanorods at 1M NAC. The data are statistically significant where $p \leq 0.05$. [(mean±SD of 3 separate experiments performed in triplicates]. Endothelial cell proliferation, represented as fold stimulation is increased with increasing concentration of [$Eu^{III}(OH)_3$] nanorods from 1-to 10μg/ml.



SI-Fig.4. (a-b). Single molecule detection of H_2O_2 efflux produced from HUVEC on SWNT/ collagen sensor. **a**) Schematic illustration of SWNT/collagen sensor for detection of H_2O_2 through stochastic nIR fluorescence quenching. **b**) nIR fluorescence image of SWNT/collagen sensor underneath HUVEC.



SI-Figure 5: Single-molecule detection of H_2O_2 efflux from HUVEC treated with VEGF with the SWNT/collagen sensor.