Published in final edited form as:

ACS Nano. 2011 October 25; 5(10): . doi:10.1021/nn201904t.

Single Molecule Detection of H₂O₂ Mediating Angiogenic Redox Signaling on Fluorescent Single-Walled Carbon Nanotube Array

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Abstract

Reactive oxygen species, specifically hydrogen peroxide (H₂O₂), activate signal transduction pathways during angiogenesis, and therefore play an important role in physiological development as well as various pathophysiologicals. Herein, we utilize a near infrared fluorescent single-walled carbon nanotube (SWNT) sensor array to measure the single molecule efflux of H₂O₂ from human umbilical vein endothelial cells (HUVEC) in response to angiogenic stimulation. Two angiogenic agents were investigated: the pro-angiogenic cytokine, vascular endothelial growth factor A (VEGF-A) and the recently identified inorganic pro-angiogenic factor, europium (III) hydroxide in nanorod form. The nanosensor array consists of SWNT embedded within a collagen matrix that exhibits high selectivity and sensitivity to single molecules of H₂O₂. A calibration from 5 to 400 nM quantifies the production of H₂O₂ at nanomolar concentration in HUVEC with 1 s temporal and 300 nm spatial resolution. We find that the production of H₂O₂ following VEGF stimulation is elevated outside of HUVEC, but not for stimulation via nanorods while increased generation is observed in the cytoplasm for both cases, suggesting two distinct signaling pathways.

Keywords

single-walled carbon nanotube; near infrared fluorescence; single molecule detection; hydrogen peroxide; redox signaling; angiogenesis; europium (III) hydroxide nanorods

Angiogenesis is a process of new blood vessel creation from existing vasculature that plays an important role in physiological and pathophysiological processes such as wound healing, ischemic heart and limb disease, tumor growth and metastasis, rheumatoid arthritis and atherosclerosis.¹⁻³ Vascular endothelial growth factor (VEGF) is an angiogenic cytokine promoting proliferation, migration and capillary formation of endothelial cells (EC).^{4,5} It has been reported that VEGF signaling in EC induces the increased production of reactive oxygen species (ROS) intracellularly^{6,7}. It is known that ROS serve an anti-microbial function⁸ in addition to intracellular signaling.⁹ Hydrogen peroxide (H₂O₂) is generated primarily from dismutation of superoxide produced by NADPH oxidase (Nox) in the EC membrane,¹⁰ and mediates cellular signal transduction via oxidation of protein tyrosine phosphatases (PTP) and the activation of kinases and transcription factors during angiogenesis.¹¹⁻¹³ Among ROS, H₂O₂ is a dominant oxidant in cellular redox signaling

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processes due to its much longer lifetime (half-life, 1 ms) and higher concentration (steady-state level, 100 nM) than other ROS such as superoxide and hydroxyl radical.⁹

Recently, lanthanides have attracted interest as therapeutic tools to manipulate angiogenic signaling.¹⁴ Specifically, europium (III) hydroxide nanorods show unique pro-angiogenic properties involving EC proliferation and vascular sprouting like other pro-angiogenic cytokines such as VEGF and basic fibroblast growth factor.¹⁵ However, the mechanism of angiogenesis induction in EC for this inorganic nanomaterial remains unknown. In particular, a central question is whether H₂O₂ participates as an intercellular in addition to intracellular signaling molecule during angiogenesis in EC. To this extent, a comparison of the redox signaling pathways between nanorods and VEGF may elucidate the angiogenic mechanism of the former. This mechanism may also inform the development of new therapeutic strategies for diseases in which angiogenesis plays an important role such as cardiovascular diseases and cancer.

Several approaches have been reported to detect cellular H₂O₂. Organic dye-based probes typically depend on chemical reaction to fluorescent products.^{16–19} They often lack reversibility prohibiting observation of signaling over long times. Reversible probes for H₂O₂ detection remain an active area of research, with examples including genetically-encoded fluorescent indicators²⁰ and europium-doped nanoparticles²¹. However, quantitative detection remains difficult²⁰ and selectivity toward H₂O₂ is often compromised²¹. These approaches also cannot quantify H₂O₂ efflux from cells, particularly at the single molecule level necessary to resolve physiological signaling⁹ in living cells. Therefore, there is still a longstanding need to develop probes capable of providing selective, spatiotemporal and quantitative information of H₂O₂ production in living organisms in order to better understand its roles as a redox signaling molecule.

There are several promising applications of single-walled carbon nanotubes (SWNT) to biological and medical research areas.^{22–27} In particular, semiconducting SWNT appear to be a suitable nanomaterial for fluorescent optical sensors^{28–36} in biological and medical research due to their stable photoluminescence (PL)³⁷ in near infrared (nIR) region with no-photobleaching threshold^{38,39}, which allows long exposures and integration times compared to other probes. SWNT PL is also sensitive to environmental changes including charge transfer^{33,40,41} and local dielectric change^{29,42,43}, which causes intensity alteration and photoemission shift. Recently, it has been demonstrated that nIR fluorescent SWNT can detect small molecules even at the single molecule level by monitoring the stepwise fluorescence quenching of single SWNT sensor.^{44–47} In addition, we have shown that SWNT embedded in collagen are able to selectively and reversibly detect H₂O₂ against other ROS.^{45,46} However, it is a remaining challenge to quantitatively detect small molecules like H₂O₂ at nanomolar concentrations produced from living cells, particularly as a part of diverse signaling pathways.

Herein, we apply our SWNT/collagen sensor to the problem of single molecule detection of H₂O₂ efflux during angiogenic signaling triggered by VEGF or the artificial pro-angiogenic factor, europium (III) hydroxide nanorods, in EC. The SWNT/collagen sensor is calibrated for H₂O₂ at nanomolar concentration to quantify its production in living EC stimulated by VEGF or nanorods. The production of H₂O₂ outside EC is quantitatively and spatiotemporally measured for all stimulations inducing angiogenesis on the SWNT/collagen sensor, which enables us to understand the mechanism of its generation for angiogenic redox signaling in living EC. We find that H₂O₂ is mainly produced near the EC membrane after VEGF stimulation while it is generated in the EC cytoplasm for nanorods stimulation during angiogenesis, indicating distinct signaling pathways.

Results and Discussion

In order to quantitatively detect H_2O_2 generated from human umbilical vein endothelial cells (HUVEC) for angiogenic signaling (Figure 1a), we first calibrated the SWNT/collagen sensor for H_2O_2 at nanomolar concentrations in PBS. The SWNT/collagen sensor array was prepared by slowly drying the diluted solution of collagen-suspended SWNT (final concentration of SWNT, $0.19 \mu\text{g/mL}$) at room temperature on a glass-bottomed petri dish as previously described.⁴⁵ As shown in Figure 1b, an area of $580 \times 580 \text{ nm}^2$ (inset: 2×2 pixels in a diffraction-limited image) corresponds to single SWNT, which is distinctly distributed on the glass surface of a petri dish. The sensor array shows very bright nIR fluorescence when it was observed by the nIR fluorescence microscope with a $100\times$ TIRF objective (excitation with 658 nm laser). In addition, the nIR fluorescence of the SWNT/collagen sensor is very stable without fluctuation in the absence of H_2O_2 during measurement for 20 min as shown in the fluorescence time-trace (Figure 1c, without H_2O_2). However, the stepwise fluorescence quenching is clearly observed upon addition of H_2O_2 ($50 \mu\text{M}$) into the solution (Figure 1c, H_2O_2 addition), indicating single molecule adsorption of H_2O_2 on the sidewall of a single SWNT. These fluorescence time-traces demonstrate that the sensor only responds to H_2O_2 . Next, we added serially-diluted solutions of H_2O_2 at nanomolar concentrations from 12.5 to 400 nM into the SWNT/collagen sensor in PBS in order to obtain the calibration curve for H_2O_2 . After the fluorescence image was monitored in real-time for 20 min upon addition of H_2O_2 , 100 diffraction-limited spots (2×2 pixels/spot, 100 SWNT sensors) in the fluorescent image movie (Figure 1b) were selected in the order of the highest intensity to the lowest intensity to be analyzed by the analysis algorithm similar to that reported before.⁴⁵ Then, we calculated the number of transitions caused by H_2O_2 on single SWNT sensor and plotted the total number of transitions for 100 SWNT sensors as a function of H_2O_2 concentration. To calculate the number of transitions corresponding to H_2O_2 adsorption on each selected SWNT, each time-trace was subjected to an error-minimizing step-finding algorithm⁴⁸ where the intrinsic steps in intensity are easily identified within the noise. Best-fit traces were obtained in a manner analogous to linear regression, where the final regression minimized the error between the fitted curve and the experimental data. Based on the best-fit traces, the number of transitions was calculated. As shown in the representative traces (Figure 2a–d), there was some variation in the number of states observed in 1200 s frames from 3 to 10 for single molecule adsorption of H_2O_2 , indicating that its ideal sensitivity could go down to single molecule level. We find that the total number of transitions for 100 SWNT sensors increases with increasing the concentration of H_2O_2 (Figure 2e). However, the best correlation was not obtained at the current stage and the error bar was large because the traces not having real transitions were also counted for total transitions. Therefore, we selected the traces of SWNT sensors having sharp real-transitions by looking at them one by one, and then sent the selected traces to the algorithm to obtain the total number of transitions. As depicted in Figure 2f, the total number of transitions shows better correlation with the concentration of H_2O_2 at the range of 12.5 – 400 nM after selection of good traces from 100 SWNT sensors, which gives a reliable calibration curve. Since the steady-state concentration of H_2O_2 for signaling in living cells is approximately 100 nM within the cell,⁹ the calibration of the SWNT/collagen sensor at nanomolar concentration can be effectively applied to quantification of its production related to angiogenic signaling in HUVEC.

We then utilized this sensor to quantitatively detect H_2O_2 generated from angiogenic redox signaling in living HUVEC to investigate the mechanism of its production stimulated by either VEGF or europium (III) hydroxide nanorods as pro-angiogenic factors. In EC, VEGF stimulates EC proliferation by producing H_2O_2 ,^{11–13} as a critical step in angiogenesis. It is observed that nanorod stimulation at $10 \mu\text{g/mL}$ results in an increase in EC proliferation compared to unstimulated control, confirmed by [^3H]-thymidine incorporation assay³

(Supplementary Figure 1a). Interestingly, $\text{Eu}(\text{OH})_3$ nanorod stimulation of HUVEC increases phosphorylation of mitogen-activated protein kinase (MAPK) similar to the case of VEGF stimulation (Western blot analysis, Supplementary Figure 1b) and part of the known signaling pathway in angiogenesis.^{49–52} These results clearly verify that these nanorods have pro-angiogenic properties in EC, similar to other cytokines such as VEGF.

In order to detect H_2O_2 generated from HUVEC stimulated by VEGF or $\text{Eu}(\text{OH})_3$ nanorods, the cells were re-plated onto the SWNT/collagen sensor array in a petri dish filled with a complete EBM medium. After cell adhesion on the sensor array, the complete EBM medium was replaced with a serum-starving one (0.2% fetal bovine serum). The cells on the sensor array in the serum-starving medium were incubated further for 12 h at 37°C, and then stimulated with VEGF (10 ng/mL) or the nanorods (10 $\mu\text{g/mL}$). The nIR fluorescence response of the sensors underneath a single cell was then monitored in real-time for 20 min to quantitatively detect H_2O_2 produced from HUVEC and analyzed by the algorithm to calculate flux. Figure 3a shows the white-light image of a single HUVEC stimulated with VEGF on the top of the SWNT/collagen array, showing normal morphology. As shown in Supplementary Figure 2, the morphology of the cells stimulated with the $\text{Eu}(\text{OH})_3$ nanorods is also normal and similar to the VEGF-stimulated case. Hence, we observe no apparent cytotoxicity from nanorod exposure, as reported previously¹⁵. Figure 3b shows the image of 100 brightest SWNT sensors underneath a single cell to locate individual SWNT (2×2 pixels). As shown in the intensity time-traces (Figure 3c, red), the stepwise fluorescence quenching of SWNT/collagen sensors occurs underneath a cell stimulated by VEGF or $\text{Eu}(\text{OH})_3$ nanorods, demonstrating that the sensor recognizes H_2O_2 outside of HUVEC after stimulations. Based on this stepwise quenching, we can calculate the number of transitions on each single sensor and generate the spatial map of H_2O_2 flux around the HUVEC (Figure 3d). Sensors at different locations have different numbers of transitions. This array of nanosensors is unique in that it is capable of providing spatial information of H_2O_2 production at the single cell level.

Next, we quantitatively compared the total number of transitions for a stimulated cell to one for an unstimulated cell. As shown in Figure 3e, the number of transitions (71 ± 9) for the unstimulated cell constitutes a background signal. This background was observed for the case of A431 cells in a recent study of EGFR signaling.⁴⁶ Unstimulated cells can produce H_2O_2 as redox signaling molecules for other physiological responses, and also as a part of cell respiration. In this case, Nox has been implicated in the basal production of H_2O_2 in the HUVEC membrane as suggested before,^{46,53,54} as a major source of ROS during angiogenesis^{6,11,12}. For VEGF stimulation, the total number of transitions (127 ± 17) observed for the same period of time (20 min, 100 SWNTs) is statistically greater than that of the unstimulated case (Figure 3e). We conclude that VEGF induces the elevated efflux of H_2O_2 near the cell membrane for angiogenic signaling to promote the cell proliferation and the phosphorylation of MAPK in HUVEC as shown in Supplementary Figure 1. The increased production of H_2O_2 after VEGF stimulation in HUVEC is attributed to the Nox-dependent signaling pathway activated by the growth factor, VEGF, mainly taking place in the cell membrane as demonstrated before^{6,12,55}. In contrast, the SWNT/collagen array underneath HUVEC stimulated by $\text{Eu}(\text{OH})_3$ nanorods shows no increase in the total number of transitions (67 ± 8) compared to unstimulated control. Additionally, we added manganese (III) tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP, 20 $\mu\text{g/mL}$)^{56,57} to the $\text{Eu}(\text{OH})_3$ nanorods-stimulated cell as a mimic of cell-permeable superoxide dismutase (SOD)^{58,59} that is an enzyme to catalyze the dismutation of superoxide into H_2O_2 in living cells. As shown in Figure 3e, no increase in the total number of transitions (53 ± 21) over control is observed, even after additional MnTBAP stimulation. We conclude that H_2O_2 production in HUVEC near the cell membrane does not happen in the case of nanorods stimulation. Next, we quantitatively estimated the concentration of H_2O_2 production induced by VEGF or

nanorods stimulation using the calibration curve (Figure 2e) based on the total number of transitions. As shown in Figure 3f, it was found that 20 nM of H_2O_2 is generated outside of the cells after VEGF stimulation, which is 10 times higher than the background concentration (2 nM) without stimulation. However, for the nanorods stimulation, 1.5 nM of H_2O_2 appears to be generated outside of the cell membrane, which is similar to the unstimulated case. Therefore, we can expect that the production mechanism of H_2O_2 triggered by $\text{Eu}(\text{OH})_3$ nanorods for angiogenic redox signaling might be different from one stimulated by VEGF in HUVEC.

In order to investigate intracellular production of H_2O_2 following nanorod stimulation, we utilized the organic fluorescent probe, carboxy- H_2DCFDA ⁶⁰, to detect H_2O_2 in the cytoplasm of HUVEC. After the cells were stimulated with VEGF, nanorods or nanorods/MnTBAP, carboxy- H_2DCFDA (25 μM) was incubated for 30 min at 37°C. As shown in Figure 4, the VEGF stimulation leads to fluorescence increase in the cytoplasm of HUVEC compared to unstimulated control, indicating that H_2O_2 is increased by VEGF-induced signaling inside HUVEC as well as outside near the cell membrane, as detected by the SWNT/collagen sensor. Additionally, the fluorescence increase is also observed in the cytoplasm after $\text{Eu}(\text{OH})_3$ nanorod or nanorod/MnTBAP stimulation compared to one in the absence of stimulation. This clearly demonstrates that the production of H_2O_2 is elevated inside HUVEC upon nanorods stimulation although it is not observed outside near the cell membrane.

A notable difference between VEGF and nanorods stimulation is the location of H_2O_2 production in HUVEC according to the experimental results. The VEGF stimulation leads to Nox activation in the cell membrane to produce superoxide that is quickly converted into H_2O_2 by extracellular SOD^{53,54,61}. This proximity production of H_2O_2 near VEGFR in the cell membrane is effectively detected by the SWNT/collagen sensor underneath the cells, which eventually promotes VEGF-induced angiogenic signaling via oxidation of PTP colocalized in caveolae/lipid rafts⁵³. However, we can suggest that the mechanism of H_2O_2 production induced by $\text{Eu}(\text{OH})_3$ nanorods in HUVEC might not be the same case as VEGF. When we investigated the fate of the nanorods inside of the cells after stimulation, they appear to be amorphous (Supplementary Figure 3, Strano: we have to get the TEM image from the Mayo collaborator).¹⁵ The trivalent nanorods (Eu^{3+}) can be easily reduced to divalent Eu^{2+} in the cytoplasm as reported before,²¹ which can result in the change in the nanorod crystal structure from hexagonal to amorphous phase after incubation inside cells for the certain period of time. Therefore, we can speculate that the nanorods could play a role as an oxidant to directly inactivate PTP or phosphatase and tensin homolog (PTEN) in the cytoplasm via oxidation of cysteine, which leads to the increase of mitochondrial H_2O_2 production⁶². The diffusion of H_2O_2 generated in the cytoplasm across the HUVEC membrane is so limited that it could not be effectively detected by SWNT/collagen sensor outside cells.⁶³ This proposed mechanism for H_2O_2 production stimulated by $\text{Eu}(\text{OH})_3$ nanorods can be supported by its increased production in the cytoplasm, but not outside near the cell membrane.

In summary, we show that the SWNT/collagen sensor is able to quantitatively detect single molecule level of H_2O_2 produced in living HUVEC under stimulation by the growth factor, VEGF and the artificial pro-angiogenic factor, $\text{Eu}(\text{OH})_3$ nanorods for angiogenic signaling. Indeed, the spatial and temporal production of H_2O_2 is also reported by the single molecule detection approach using fluorescent SWNT. The quantitative detection of H_2O_2 is used to assess the mechanism of its production for two different stimulations, suggesting that it is induced near the membrane for the VEGF stimulation, but in the cytoplasm for the nanorods case. This is the first demonstration to quantitatively detect H_2O_2 at nanomolar

concentration generated from the living organisms to decipher complex redox signaling pathways in relation to angiogenesis.

Methods

Preparation of SWNT/collagen sensor array

An 1 mg portion of SWNT was added in 1 mL of collagen solution (3.41 mg/mL) containing 0.02 M of 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 was decanted. A 20 μ L portion of collagen-suspended SWNT solution was added to a 40 μ L of collagen solution (3.41 mg/mL), and the resulting solution was diluted with an acetic acid solution (0.02 M, 4.14 mL). A 400 μ L of final SWNT solution (0.19 μ g/mL SWNT) was added in each petri dish, and gently dried at room temperature for 20 h. Before being used for cellular experiments, it was extensively washed with water and PBS (pH 8).

Calibration of SWNT/collagen sensor for H₂O₂ and data analysis

Before experiments, the sensor was washed several times with PBS (pH 7.4). A 2 mL portion of PBS was added to the sensor in a petri dish, which was placed on the top of the nIR fluorescence microscope (Carl Zeiss, Axiovert 200) attached with a 2D InGaAs array (Princeton Instruments OMA 2D) with a 100x TIRF objective (658 nm laser excitation). After focusing on the sensor, the fluorescence response was imaged and monitored over time at 1 sec/frame over the course of 1200 s upon addition of 88 μ L of a 9.11 μ M-H₂O₂ solution (final concentration, 400 nM). A solution of H₂O₂ was serially diluted from 400 to 12.5 nM in order to obtain the calibration curve. The calculation for the number of transitions from a movie monitoring the entire SWNT film fluorescence over time was determined using an algorithm. In the first step, a MATLAB routine selected 100 diffraction-limited spots (2 $\times$ 2 pixels) in the nIR images, in the order of the highest intensity to the lowest intensity. Each obtained time-trace was then subjected to an error-minimizing step-finding algorithm where the intrinsic steps in intensity can be identified within noisy data. To detail this further, 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 location 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 location prior to the transition. A similar analysis was performed to 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 was calculated. In order to obtain the calibration curve for H₂O₂, the traces having sharp real-transitions were selected by going through all traces of 100 SWNT sensors with eyes, and then the selected trances were sent to the algorithm to calculate the total number of transitions.

Single molecule detection of H₂O₂ generated from living HUVEC

After primary HUVEC confluent was over 90%, a complete EBM medium was removed from a flask. After washing cells with PBS twice, a 1.5 mL portion of trypsin/EDTA solution was added into the flask and incubated for 5 min at 37°C. After addition of 1.5 mL of trypsin neutralizing solution to the cells, they were centrifuged down in a microcentrifuge tube for 5 min at 1500 rpm. The supernatant was removed from the tube, and 1 mL of complete EBM medium was added. After gently mixing the cells, 50 µL of the cell solution was added into the SWNT/collagen sensor array in a petri dish filled with 2 mL of a complete EBM medium. The cells were incubated to adhere on the top of the sensor array for 12 h at 37°C. The complete EBM medium was then replaced to a serum-starving EBM medium (0.2% FBS), and incubated further for 12 h. Finally, the cells were treated with each stimulus such as VEGF-A (10 ng/mL), Eu(OH)₃ nanorods (10 µg/mL) and Eu(OH)₃/MnTBAP (20 µg/mL) in L-15 medium not containing FBS. The fluorescence response to H₂O₂ generated from the cells was imaged, monitored and analyzed as same as described above for calibration of the sensor.

Acknowledgments

This work was supported by a Beckman Young Investigator Award to M.S.S. and the National Science Foundation. A seed grant from the Center for Environmental Health and Science at MIT is also appreciated. J.H. Kim is grateful for the postdoctoral fellowship from the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2007-357-D00086).

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Figure 1.

Single molecule detection of H_2O_2 mediating angiogenic redox signaling. a) Schematic diagram of H_2O_2 production in angiogenesis and sensing platform. b) nIR fluorescence image of a SWNT/collagen sensor array showing emission from single isolated SWNT sensors. *Inset*: diffraction-limited spot (2x2 pixels) corresponding to a single SWNT. c) Representative fluorescence time-traces (red) in PBS with and without H_2O_2 (50 μM), showing clear stepwise fluorescence quenching.

Figure 2.

Calibration of SWNT/collagen sensor for H_2O_2 . a) Representative fluorescence time-trace (red) monitored for 20 min showing three transition states, b) four transition states, c) eight transition states, d) nine transition states upon addition of H_2O_2 (400 nM) in PBS. e) Correlation of the total number of transitions with H_2O_2 concentration for all 100-SWNT sensors. f) Calibration curve for H_2O_2 at nanomolar concentration from 12.5 to 400 nM after selecting the traces having sharp real-transitions, showing that the total number of transitions increases with increasing H_2O_2 concentration.

Figure 3.

Single molecule detection of H_2O_2 produced from living HUVEC for angiogenic redox signaling. a) White-light picture of single cell stimulated by VEGF-A on the top of SWNT/collagen sensor array. b) 100 Diffraction-limited spots of SWNT underneath a cell, which are selected for analysis of fluorescence response to H_2O_2 . c) Representative fluorescence time-traces (red) selectively responding to H_2O_2 produced under VEGF-A and $\text{Eu}(\text{OH})_3$ nanorods stimulation in HUVEC, showing stepwise quenching. d) Spatial distribution plot of the number of transitions after VEGF-A stimulation on HUVEC. e) The total number of transitions calculated from 100-selected SWNTs over the course of 20 min upon treatment of stimuli such as VEGF-A, $\text{Eu}(\text{OH})_3$ nanorods and $\text{Eu}(\text{OH})_3/\text{MnTBAP}$ to HUVEC. f) Estimated concentration of H_2O_2 produced from HUVEC stimulated by VEGF-A, $\text{Eu}(\text{OH})_3$ nanorods and $\text{Eu}(\text{OH})_3/\text{MnTBAP}$ using the calibration curve (Fig. 2e).

Figure 4. Intracellular detection of H_2O_2 in HUVEC after stimulation. a) White-light picture of cells and fluorescence image for unstimulated one, b) for VEGF-A, c) for $\text{Eu}(\text{OH})_3$ nanorods, d) for $\text{Eu}(\text{OH})_3/\text{MnTBAP}$. The results show that H_2O_2 production increases in the cytoplasm after stimulation. Carboxy- H_2DCFDA ($25 \mu\text{M}$) was used for detection of H_2O_2 inside of cells.