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Inorganic nanoflowers make new blood vessels

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EXPERIMENTAL SECTION

Materials.

Zinc nitrate hexa hydrate [$Zn(NO_3)_2.6H_2O$], aqueous ammonium hydroxide[aq.NH_4OH, 28-30%], fluorescent dye 2′7′-dichlorofluorescein diacetate (*DCFA*), were purchased from Aldrich, USA and were used without further purifications for the synthesis of ZnO nanoflowers. The Human umbilical vein endothelial cells (HUVEC) and its individual components for making EBM complete media were obtained from Lonza, USA. EA.hy926 endothelial cells were purchased from American Type Culture Collection (ATCC) [^{3}H]Thymidine was purchased from Amersham Biosciences, Piscataway, NJ. Tris-EDTA (TE buffer: YBP24771) was purchased from Fisher Scientific.

Synthesis of ZnO nanoflowers by microwave method

ZnO nanoflowers were synthesized in domestic microwave oven (DMO)[1, 2] by the interaction of aqueous solution zinc(II)nitrate and aq.NH_4OH at atmospheric pressure in an open reflux system. In a typical synthesis, 1 ml of aqueous NH_4OH were added to 39 ml of aqueous solution of Zn(II)nitrate (297 mg) [OH/Zn = 4, molar ratio] in a 100 ml round-bottomed flask. The reaction mixture was irradiated for 5 to 60 min [designated as Zn5: 5min, Zn10: 10 min, Zn20: 20 min, Zn40: 40 min and Zn60: 60 min] with 60% of the instrument's power (on/off irradiation cycles ratio of 3/2) in order to control the reaction and reduce the risk of superheating the solvent. The microwave refluxing apparatus is a modified domestic microwave oven (GOLD STAR 1000W with a 2.45 GHz). In the post-reaction treatment, the resulting products were collected, centrifuged at 10,000 rpm, washed 3 times with Millipore water followed by ethanol and Millipore water again, and then dried overnight under vacuum at room temperature. The yield of the as-prepared products was more than 90%.

Preparation of ZnO nanoflowers in trsi-EDTA

ZnO nanoflowers were suspended in sterile TE (1X tris- EDTA, pH = 7.4) buffer and make stock solution of 10 mg/mL. The ZnO nanoflowers solution was used for all cell culture experiments and *in vivo* experiments after 15 minutes of irradiation under UV light inside the tissue culture hood. Freshly prepared suspension of ZnO nanoflowers in TE buffer was prepared each time before all *in vitro* and *in vivo* experiments.

Cell culture experiments

HUVECs were cultured at 100 mm tissue culture plates for ~24 h at 37°C and 5% CO_2 in EBM complete media. After 70% confluence, the cells were plated in to corresponding plate for thymidine incorporation assay, cell cycle assay, wound healing scratching assay, cellular uptake and detection of reactive oxygen species (ROS) etc.

Cell proliferation assay

The [^{3}H]-thymidine incorporation assay was carried out as reported [1, 2]. Briefly, (2 x10^4) of HUVECs were seeded in 24-well plates, cultured for 1 day in EBM, serum-starved (0.2% serum) for 24 hours, and then treated with different concentrations (0, 5, 10 and 20 μg/mL) of ZnO nanoflowers. After 24 hours of incubation with ZnO, 1 μCi [^{3}H]-thymidine was added into 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[3].

Cellular uptake

HUVECS cells (10^6 cells / 10ml) were cultured in 100 mm plate in EBM complete media and after 70% of confluence the complete media was replaced by EBM starved media containing 0.2% of FBS. After 24 hour of starvation, the cells treated with ZnO

nanoflowers and the cells were extensively washed with PBS, trypsinized, and neutralized. The cells were then collected by centrifugation, re-suspended in trumps solution, and submitted for TEM for observation of cellular uptake of nanoparticles in the cytoplasmic compartment.

Determination of ROS:

HUVECS cells (10^5 cells / 2ml) were cultured in six well plates and treated with ZnO nanoflowers in EBM complete media using cover slips. After 24 h of incubation of HUVECs, EBM complete media was replaced by EBM starved (0.2%FBS) media and next day the HUVECs were incubated for another 24 h with ZnO nanoflowers at different concentrations (5-20µg/mL), TBHP (10 µg/mL) and VEGF (10 ng/mL). After 24 h of incubation, cells were incubated with 20µm of DCFDA (Sigma Aldrich: D6883) for 30 min and the cells were washed eight times with DPBS and finally the fluorescence images of treated and untreated HUVEC cells in EBM media were examined with Olympus IX71, Olympus U-CMAD3, T2 Tokyo Japan. The green fluorescence emission (λ_{Em}= 525 nm) indicating the presence of ROS was collected with a 10X microscope objective after excitation at λ_{Ex} = 488 nm (blue).

Egg yolk angiogenesis assay

Incubated chicken eggs at fourth day were purchased from the Government Poultry Station, Potheri, Chennai. The eggs were broken and gently placed in sterile Petri dishes under aseptic condition. Filter paper discs were soaked in ZnO nanorad solution (1ug, 10ug, 20ug), 10ng VEGF and TE buffer were then placed on the egg yolks, which were incubated for another 4 h. Images were taken at 0, 2 and 4 h of incubation using Olympus camera (10 megapixel) adapted to a stereomicroscope. Images were analysed using Angioquant software (Niemistö *et al.*, 2005).

Wound healing assay:

EA.hy926 (1 × 106) cells were seeded on 24-well plates to confluence, and the monolayers of EC were scratched with sterile pipette tip (20-µl). The cells were washed with PBS and incubated with ZnO nanorad solution (1ug, 10ug, 20ug), 10ng VEGF and TE buffer for 4 h as described elsewhere (Staton et al., 2004). Bright field images were taken with 4x magnification under an inverted microscope and analyzed using Image J image analysis soft-ware (Release @ 4.0 3.2). The wound healing effect was calculated as the percentage of remaining cell-free area (at 8 hours) compared with the initial wound area.

CHARACTERIZATION TECHNIQUES

ZnO nanoflowers synthesized at different reaction time in a domestic microwave oven were thoroughly characterized by several physico-chemical techniques, which are described below.

X-ray diffraction (XRD):

The structure and phase purity of the as–synthesized ZnO nano flowers were determined by X-ray diffraction (XRD) analysis using a Bruker AXS D8 Advance Powder X-ray diffractometer (using $CuK_{\alpha}\lambda$ =1.5418 Å radiation).

Thermo-gravimetric (TG) and Differential Scanning Calorimetric (DSC) Analysis

TGA of the as-synthesized samples was carried out under a stream of nitrogen at a heating rate of 10°C/min from 30°C to 700°C using a METTLER TOLEDO TGA/STDA 851. DSC analysis of the as-synthesized samples was carried out on a METTLER TOLEDO TC15, using a stream of nitrogen (20 ml/min) at a heating rate of 4°C/min in a crimped aluminum crucible from 30°C to 600°C.

Transmission electron microscopy (TEM) study

The morphology of as-synthesized ZnO nanomaterials synthesized at different reaction time (designated as Zn5: 5min, Zn10: 10 min, Zn20: 20 min, Zn40: 40 min and Zn60: 60 min) was determined by TEM on a FEI Technai 12 operating at 80KV. In order to observe the cellular uptake of the ZnO nanoparticles inside the cytoplasmic compartment of the cells using TEM, the experimental procedures were carried out according to published literature. [1]

Scanning electron microscopy (SEM) study

Scanning electron microscope (SEM) is an indispensable and powerful tool and it is used to study the surface morphology of materials. Here, Hitachi S-3000 N, Japan (SEM) has been used to investigate morphology of ZnO nanomaterials. SEM images of ZnO products indicate the flower like structures.

RESULTS AND DISCUSSION:

Zinc oxide (ZnO) nanoflowers were synthesized by the reaction between aq.NH_4OH soln. and zinc nitrate (OH/Zn =4) in a domestic microwave oven (DMO). The characterization of ZnO nanflowers was carried out by several physico-chemical techniques such as XRD, TGA-DSC, TEM, SEM etc. The crystal structures of the as-synthesized product, obtained after MW heating at different times, were identified using X-ray diffraction (XRD) analysis, which indicated the crystalline nature of all the products (designated as Zn5: 5min, Zn10: 10 min, Zn20: 20 min, Zn40: 40 min and Zn60: 60 min) (**Fig. 1A**). All reflections were distinctly indexed to a pure wurtzite crystallinity phase of ZnO nanofowers.

A representative TGA-DSC profile for the as-synthesized ZnO nanoflowers obtained after 60 min irradiation of microwave heating (sample Zn60) is shown in SI-Fig.1. The TGA pattern of the as-synthesized product (SI-Fig.1.a) shows the occurrence of two distinct weight losses in two steps with an overall weight loss of 7.3% between 30°C and 600°C. The DSC pattern also shows two distinct endothermic peaks at two steps in the same temperature range (SI-Fig.1.b). The first one, a broad endothermic peak in the temperature range of 30°C to 125°C (SI-Fig.1. a) is associated with the release of 0.24 wt% of residual water and carbon dioxide which are physically adsorbed onto the surface of the as-synthesized material [4, 5]. The second step weight loss (7 wt%) in the TGA begins around 170°C and ends at 260°C, and a corresponding well-defined endothermic peak is detected with a sharp peak at 242°C (Fig.). This second weight loss could be attributed due to the decomposition of the residual ammoniacal complexes-$[Zn(NH_3)_4]^{2+}$ on surfaces of ZnO particles, which formed in the synthesis process of ZnO in aqueous solution [6]. The second weight loss does not correlate with the weight loss of ~18% corresponds to the conversion of $Zn(OH)_2$ to ZnO. The combination of results from XRD, TGA, DSC and TEM indicate that the as-synthesized material is ZnO nanoflowers.

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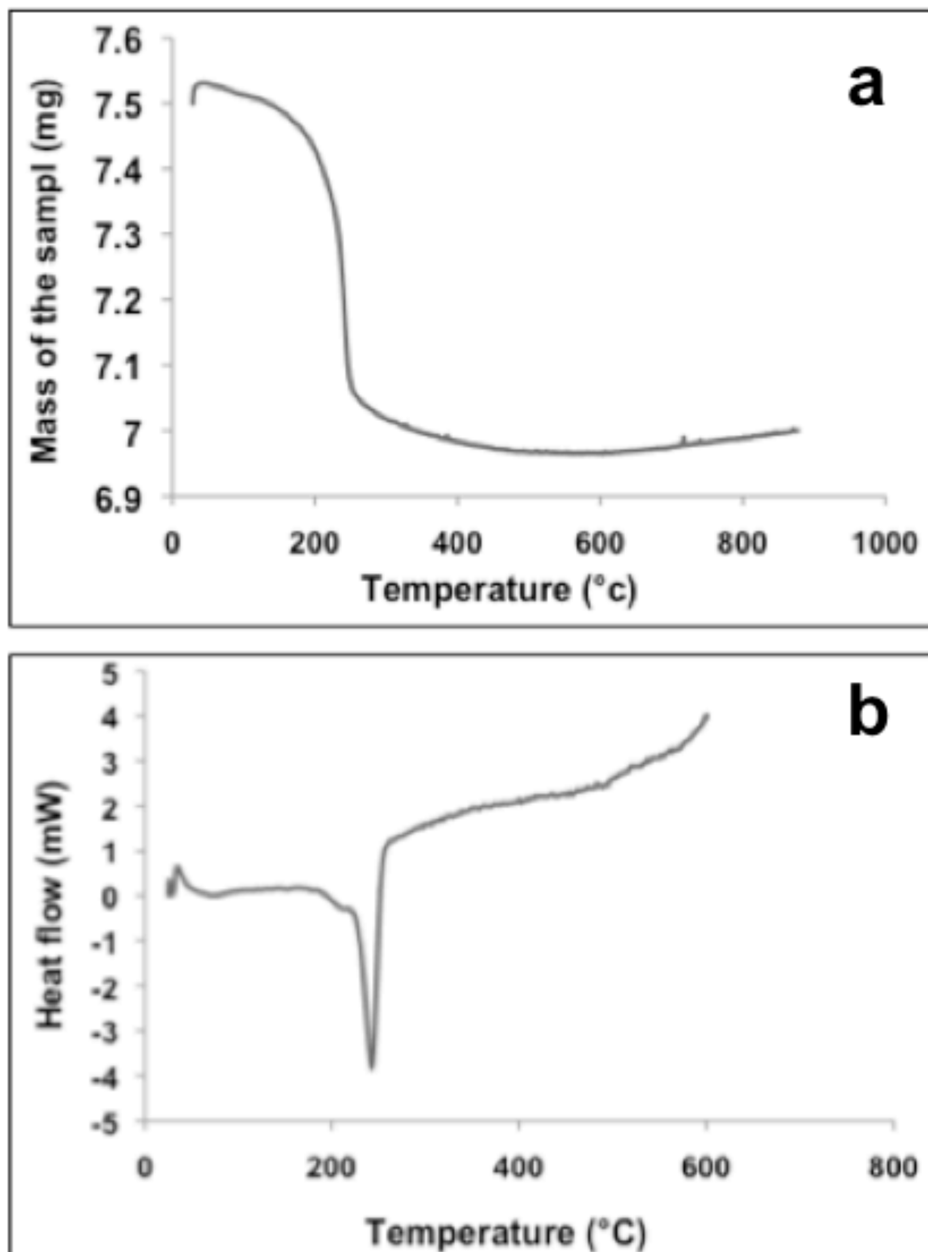
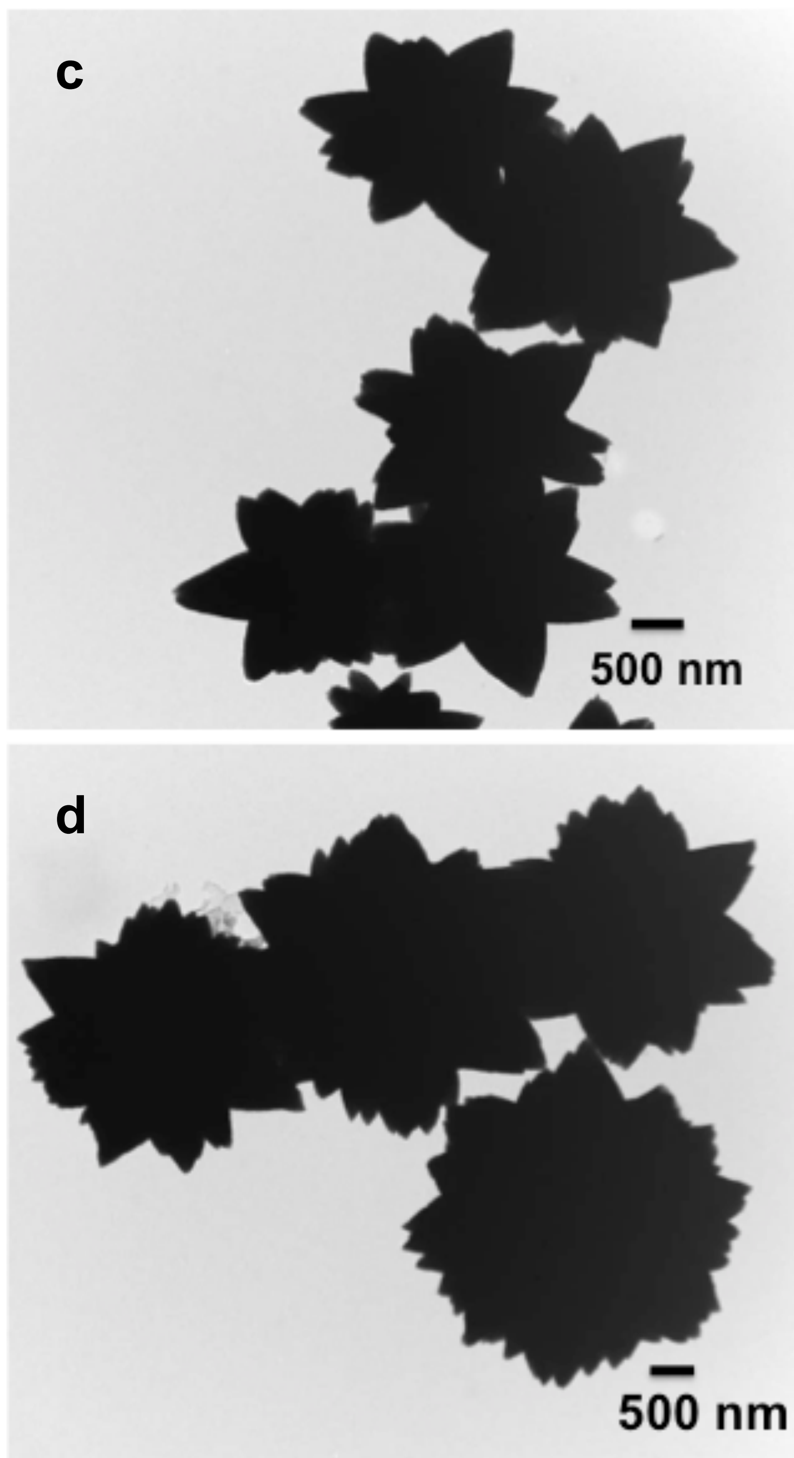
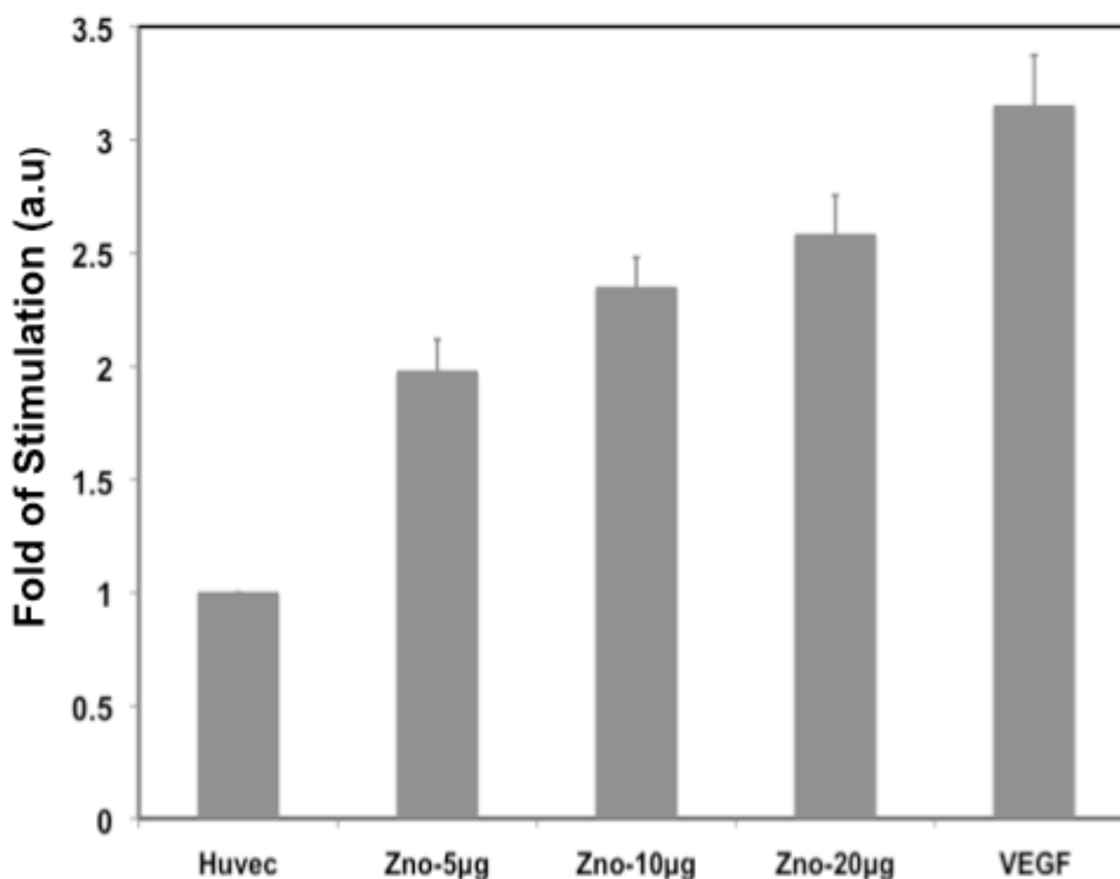


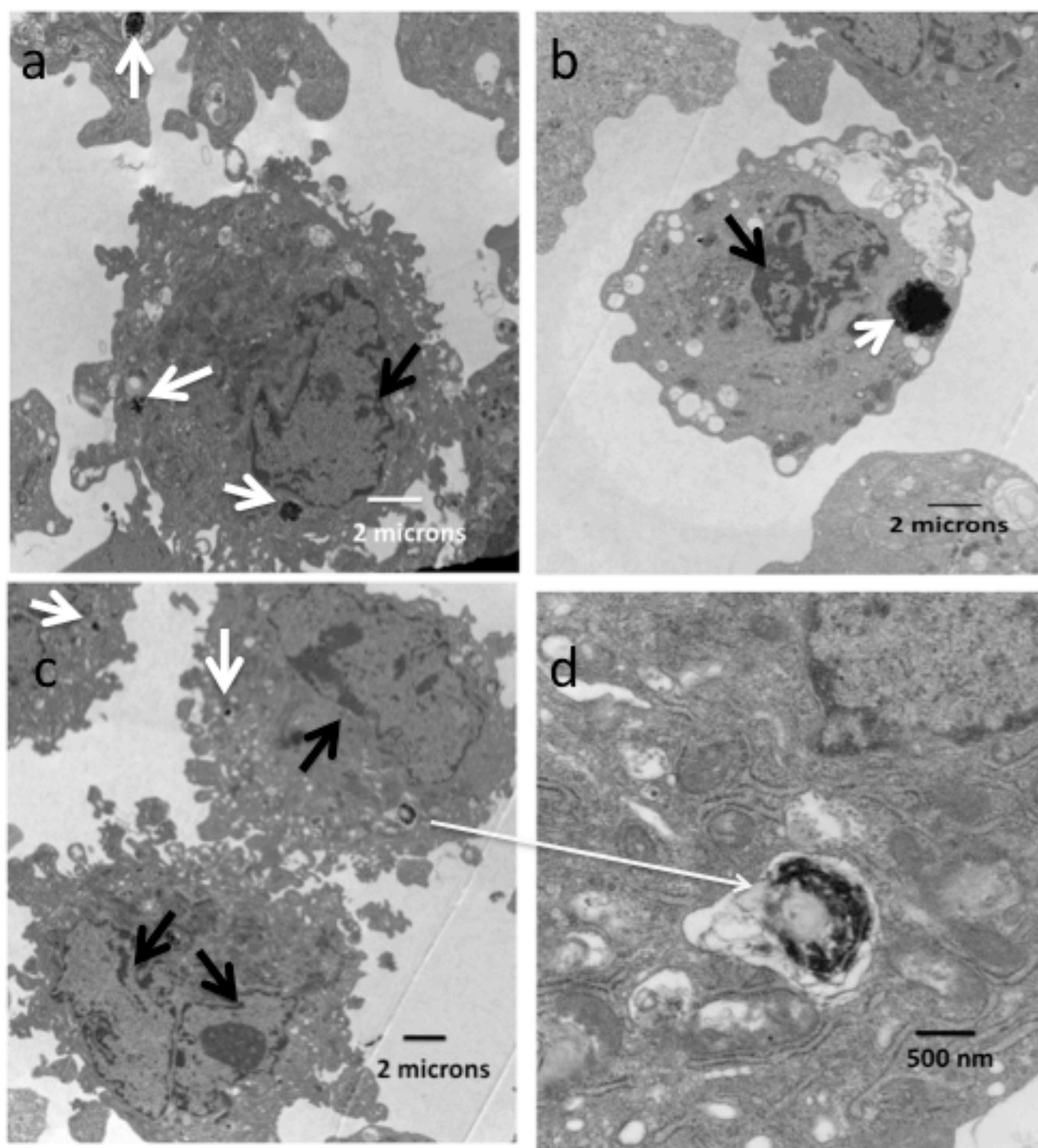
SI-Fig.1.(a-b): TGA & DSC anaalysis of ZnO nanoflowers (Zn60). (A) TGA and DSC of as-synthesized ZnO nanoflowers obtained after 60 min of microwave irradiation. The results shows that as-synthesized nanomaterials is ZnO nanoflowers.



SI-Fig.1 (c-d): TEM images of ZnO nanoflowers. (c-d) High magnificationTEM images of microwave-assisted as-synthesized ZnO nanomaterials obtained after (c) -10 min (Zn10) and –(d) 20 min (Zn20) of microwave heating. Both TEM images clearly show that the as-synthesized material consists entirely of nanoflowers.



SI-Fig.2: Cell proliferation assay using radioactive [^{3}H]-thymidine assay of HUVEC in presence of ZnO nanoflowers (Zn05). (**a**) The effect of ZnO nanoflowers obtained after 5 min of microwave heating (Zn05) on HUVEC proliferation at different concentrations (Zn-5µg, Zn-10µg, and Zn-20µg) indicates the dose dependent cell proliferation. VEGF (VF) was used as a positive control. The data are statistically significant where $p \leq 0.05$ [(mean ± one standard deviation) of three separate experiments performed in triplicates].



SI-Fig.3: Internalization of ZnO nanoflowers (20µg/mL) in HUVEC was observed by TEM images. Black arrows marked the presence of nucleus and white arrows marked the nanoparticles. The results indicate the uptake of nanoflowers inside the cytoplasmic compartment of HUVEC.

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