Anticancer effects of nanometallic oxides and their ligands with photosensitizers in osteosarcoma cells

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We studied the cytotoxic effects in Osteosarcoma (U2OS) cells to different nanosized metallic oxides e.g. zinc oxide nanowires (ZnO-NRs), manganese di-oxide nanowires (MnO_2 NWs), ferric oxide nanoparticles (Fe_2O_3 NPs) individually and their complexed forms with photosensitizers photofrin®, 5-Aminolevulinic acid (5-ALA), and protoporphyrin IX (Pp IX). Cellular toxicity was assayed by cellular morphology, reactive oxygen species (ROS) detection, MTT assay under ultraviolet (UV), visible light and laser exposed conditions. Prominent cell death with above cited nanomaterials in their complexed forms with Photosensitizer was observed in labeled U2OS cells. This cell death might be due to their synergetic effect via the release of singlet oxygen species in Osteosarcoma cells showing their anticancer-cell effects.

(Received September 7, 2015;

Keywords: Osteosarcoma cell, reactive oxygen species (ROS), MTT assay, photodynamic therapy (PDT)

1. Introduction

Osteosarcoma being an aggressive heterogenic primary cancerous tumor of bone is most commonly found in adolescents and young adults, involving osteocytic cells [1]. It is malignant tumor of long bones usually in the region of metaphases involving osteoid-producing neoplastic cells and less commonly in the axial skeleton and short bones [2, 3]. Treatment of bone cancer prior to the use of chemotherapy was sole surgery were not found successful/appealing as were expected but currently replaced by treatment modalities of photodynamic therapy (PDT) [4-6]. At the nanoscale level nanomedicine and nanotechnology, have introduced numerous NPs with different chemical and physical properties for cancer diagnostics and treatment [7]. While, nanotechnology analysis and develops tools measuring in size from almost 1-1000 nm (mainly 1-200 nm), the purpose of which is to explain biological systems [8]. Due to altered properties of nanomaterials (NMs) and matchable size of very complex biological structures, can allocate them to readily interrelate with said complex structures on both cell surface, with in the cell and qualitative affect the tissues activity in self-motivated and selective manners. Physical and chemical properties of nanomaterials disagree from their bulk material counter parts because of an increase in their relative surface area and quantum effects [9-11].

MnO_2 nanowires induce cytotoxicity in U2OS cells involve the liberation of reactive oxygen species (ROS), nitrogen reactive species (NWs), and formation of oxidative stress, DNA oxidative damage culminating in cell necrosis [7]. Nanomaterial structures bring more unique and novel properties to MnO_2, which may induce unpredictable impacts on safety and human health. Nanotoxicities of various metal oxides e.g. MnO_2 NWRs, Fe_2O_3 NPs and ZnO NWs have attracted much attention of researchers [7].

Moreover, in order to shrink the size of bulk materials to nanoscale realm, nano-size-dependent properties of nanomaterials e.g. MnO_2, Fe_2O_3 and ZnO are manifested. ZnO nanostructures are the very precious known among all the materials so far, the growth of which is facilitated by self organized growth properties of this material and can emit intrinsic (white light) which is a basic need for PDT [12]. Enhanced intracellular and nuclear delivery of nanoparticles (NPs) mediated ligands result in significant and rapid induction of apoptosis and necrosis [13]. NPs prefer to intratumor accumulation because of its specific architecture and used as drug delivery vectors resulting in large intratumor localization, reducing the harmful nonspecific side effects of chemotherapeutics [14]. Nanowires (MnO_2 and ZnO) and nanoparticles (Fe_2O_3) contain several properties to play a dominant role in their enhanced optical, biomedical and structural effects on normal as well as precancerous human cells [15]. MnO_2 exerts cellular toxicity via direct or indirect formation of reactive oxygen species (ROS) the direct oxidation of biological molecules with disruption of cellular calcium and iron homeostasis [16, 17]. Manganese dioxide in a neutral medium have pH 7.4, has moderate oxidizing

activity while, highest in acidic but least in alkaline medium [18]. It has been reported that Fe2O3 NPs can induce significant toxic effects due to having large surface area. The toxicity of these nanoparticles is influenced in dose dependent manner [19]. The nanomaterials may induce ROS, inflammatory response, liberation of apoptotic entities and reduction of mitochondrial functions to the biological cells results in cellular toxicity [20]. Nanoparticles such as ZnO have been proved to be more toxic in conjugated form (i.e. complexed with porphyrin) under the exposure of UV light. However, bare porphyrin showed insignificant cytotoxicity [21]. These nanomaterials (i.e. ZnO) behave lightly in the absence of light. Conversely, ZnO NMs can penetrate through abnormal cells (Cancerous cells) liberating cell apoptosis by RNS or ROS [22-24].

The motives of this study were to observe the cytotoxic effects of ZnO NRs, MnO2 NWs, and Fe2O3 NPs individually and their complexed forms with photosensitizer in Osteosarcoma cells, synergetic effect of nanomaterials with PS along with intracellular release of ROS.

2. Experimental Detail

Hydrothermal processes were used for the synthesis of zinc oxide nanorods (ZnO NRs), manganese dioxide nanowires (MnO_2 NWs) [25-27], Fe_2O_3 nanoparticles (Fe_2O_3 NPs) [28] and were tested for their cytotoxicity in Osteosarcoma cells. The final synthesized nanomaterials were characterized by different techniques (i.e. X-ray diffraction and Scanning Electron Microscopy). Powder X-ray diffraction (XRD) of the NRs, NWs and NPs was evaluated using a Rigaku Geiger flux diffractometer having Cu Kα radiation source where (λ=1.5406 Å).

Cell Culturing

In cell culturing process, Osteosarcoma (U2OS) cell line was seeded in plastic tissue-culture flasks (Nunc Wiesbaden Germany) in Dulbecco's Modified Eagle Medium (DMEM) with Hanks salts, also supplemented with 10% fetal bovine serum (FBS) and 2mM L-glutamine and with some non-essential amino acids. Furthermore, for proper attachment to the substratum, the cells were incubated at 37 ºC for 24 hours. Same protocol was adopted in previous published data by Fakhar *et al*. [29]. After that, the cells were harvested via 0.25% trypsin once the confluence was reached to 65-75% [4-5, 30-31].

Preparation of stock solution

Phosphate buffer saline (PBS) was used to make the stock solution of all the nanometallic oxides and the drugs. However, PpIX was dissolved in dimethyl sulfoxide. The pH and the concentration of the solutions were kept at 7.4 and 20 mg/ml respectively. Furthermore, the solutions were stored in dark after sonication of 45 min. In the next step, the stock solutions were diluted with DMEM and the working drug solutions were prepared (concentration ranging from 0.05µg/ml to 200µg/ml.

Labeling of cells with nanomaterials and their ligands for Reactive Oxygen Species (ROS) Measurement

The cells were washed many times with PBS in order to remove the cell culturing medium and cells were exposed to the various concentrations (0.05-200 µg/ml) of working drugs. The cells exposed to the nanometallic oxides (ZnO NRs, MnO_2 NWs and Fe_2O_3 NPs) and their ligands (photofrin®, 5-ALA and PpIX) were incubated for 15 hours at 37 ºC in humidified air containing CO_2 (5%). The whole process was done in 96-well plates whose last three columns were used as control (without labeling of working drugs). The medium containing drugs and metal oxide was removed by washing twice in DMEM and ROS measurements were taken by using the 2', 7'-Dichlorofluorescein diacetate once incubated in dark at 37 ºC for half an hour. A significant production of ROS can liberate possible changes/injury to cell membrane, mitochondria or nucleus [32-33].

Subsequently, the cells were irradiated via UV light for 2 minutes at a light dose of 10 J/cm^2. Again the ROS production was assessed by a multifunctional micro plate reader (POLAR star Galaxy). Furthermore, the cells were examined via Nikon Eclipse 400 epifluorescence microscope and images were taken with a CCD camera and DU 897E.

Laser Irradiation

Optimal concentration of the photosensitizers (Photofrin®, 5-ALA and PpIX) was assessed in osteosarcoma cells by applying different concentrations ranging from 0.05µg/ml to 200µg/ml. After 24 hours incubation with photosensitizer in 96 wells plates, treated cell line was then irradiated through light via Photocure AKTILITE CL 16 for 7 minutes. This is LED based narrow band (630-635 nm) red light technology device and it illuminates up to 40x50 mm with delivery of different doses (0-160 J/cm^2) of 635 nm. First six wells (containing U2OS) having different concentrations of the working drugs were exposed with a light of dose 30 J/cm^2 while the other 2 wells were treated as control. The experiment was repeated three times for accuracy of the measurements. Furthermore, the cells were washed using ice-cold PBS to remove the old media. Then DMEM were added into the cells and plates were kept in the incubator for 24 hours for MTT Assay as discussed in previous published data [34-35].

MTT Assay

For nanometallic oxides cytotoxicity tests, 96-wells plates (flat bottomed and microtiter) were seeded out such that 1×10^5 cells/well (U2OS cells) and were incubated with varying concentrations (0.05-200 µg/ml) of MnO_2

NWs, ZnO NWs and Fe_2O_3 NPs for 15 hours. Thereafter DMEM was used to wash and prepare the cells for MTT test. The MTT assay was performed via the addition of MTT (0.25 mg/mL in PBS) to the cell culture dishes for 3 h at 37 ˚C. The dishes were then dried, and the formazan product dissolved in DMSO. Thereafter absorption was measured using micro-well plate reader at a wavelength of 540 nm [31, 34-40].

3. Results and discussion

Fig. 1a shows typical XRD data for MnO_2 nanowires. The XRD pattern exhibited a pure tetragonal phase of α-MnO_2. The results are verified with JCPDS card no. 44-0144 (a = 9.956 Å, b = 2.860 Å). Figure 1b shows the XRD peaks of the Fe_2O_3 nanoparticles. The patterns are verified with JCPDS No. 84-0306 and are consistent with hematite phase of Fe_2O_3. The average crystallite size was calculated by Scherer Formula which is 34 nm as reflected by two strong peaks at 110 and 104 [35]. Figure 1c shows XRD pattern of the as grown ZnO nanorods. 1c. The typical peaks for ZnO NRs were indexed to the hexagonal phase of ZnO (wurtzite).

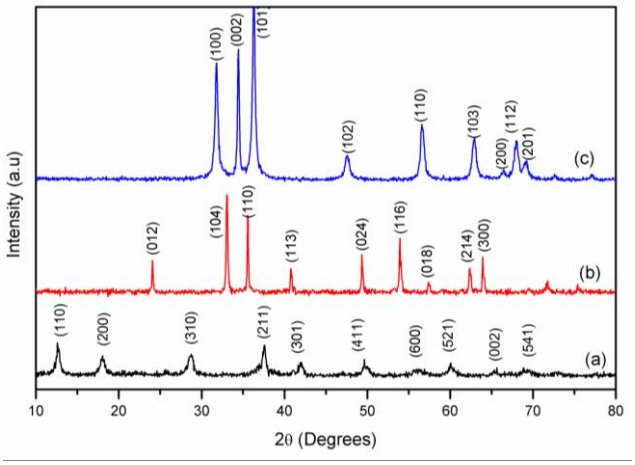


Fig. 1: XRD Pattern of (a) MnO_2 NWs, (b) Fe_2O_3 NPs and (c) ZnO NRs

Scanning electron microscope (SEM) was used to examine the size and morphology of the multiple samples of nanomaterials.eg ZnO NRs, MnO_2 NWs and Fe_2O_3 NPs. SEM images of the nanomaterials, ZnO NRs, MnO_2 NWs and Fe_2O_3 NPs are shown in Fig. 2a, 2b and 2c respectively. The diameter of the ZnO nanorods is in the range of 100-105 nm while MnO_2 NWs with diameter in the range of 85-100 nm are shown in figure 1a and 1b respectively and α-Fe_2O_3 NP is in the range of 100-150 nm is shown in Fig 1.c.

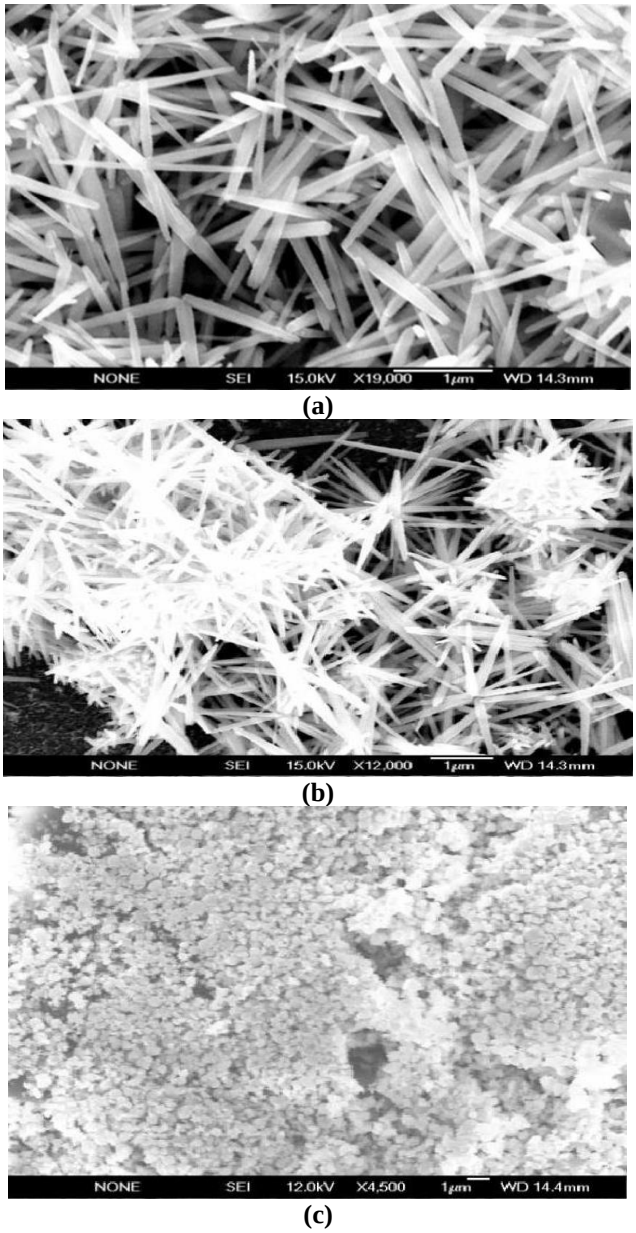
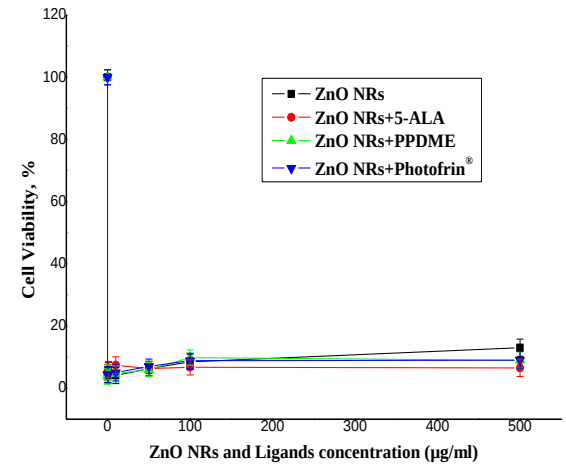
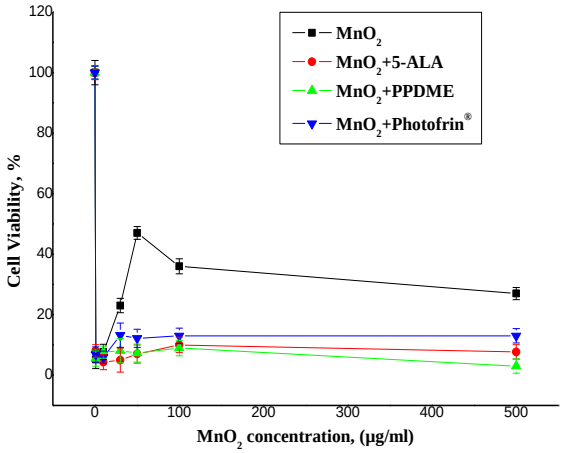


Fig. 2: SEM Image of (a) ZnO NRs, (b) MnO_2 NWs and (c) Fe_2O_3 NPs

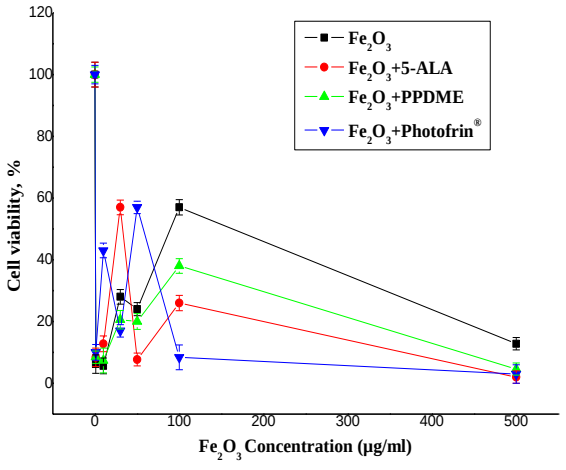
In the present study, U2OS cell line was used for purpose to see the possible effects of nanomaterial based PDT for the treatment of bone cancer. The criteria to evaluate the activity of nanoparticles/nanowires conjugated with photosensitizer in said cells with specific concentration was, cell morphology, cell viability loss, ROS detection in dark as well as under UV exposure, intracellular spectroscopy. It is worth mentioning that no such data of study with different ligands of metallic oxides with photosensitizer under UV and in dark was available. However, many researchers quoted different PDT parameters e.g. time of incubation of photosensitizers, drug concentration, and optimum dose of UV-Visible light, complexed form of metallic oxides with photosensitizers, cell line type [14-16], instead of cytotoxicity of multiple ligands of nanomaterials with photosensitizer.



(A)



(B)

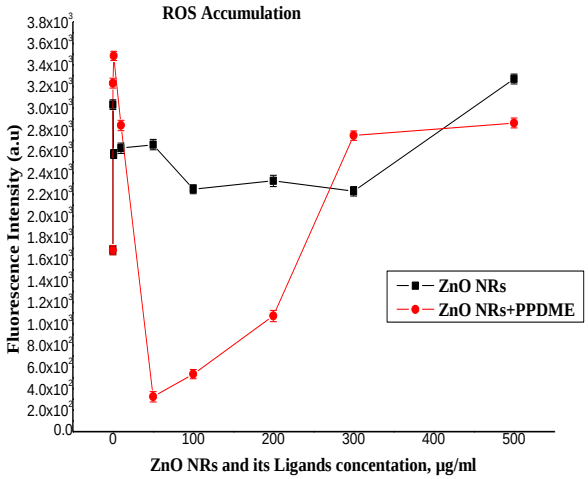


(C)

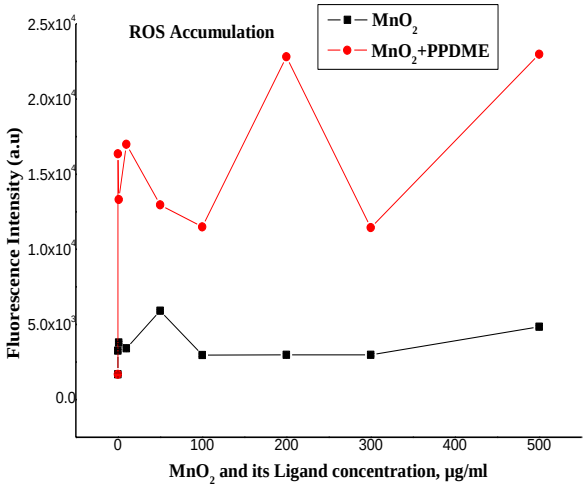
Fig. 3: (A) Cell viability of Osteosarcoma cells treated with different concentration of ZnO NRs and their ligands with Photosensitizers (B). Cell viability of Osteosarcoma cells treated with different concentration of MnO_2 NWRs and their ligands with Photosensitizers (C). Cell viability of Osteosarcoma cells treated with different concentration of Fe_2O_3 NPs NWRs and their ligands with Photosensitizers

Fig. 3 (A) shows, bare ZnO NWs and their conjugated forms with 5-ALA, PpIX and Photofrin®, same pattern, almost superimposed template of cytotoxicity (loss in cell viability 95% with conjugated forms), deducting that ZnO NWs synergize the effectiveness of photosensitizers. This data shows that ZnO NWs have convincing killing effect for osteosarcoma cells. In Figure 3 (B), we have noted that MnO_2 NWRs initially with 1 µg/ml, loss in cell viability was maximum (78% loss) and then decreasing trend was seen up till 50 µg/ml, finally becoming stable at 200 µg/ml which was almost 82 %. While in case of ligands of MnO_2 NWRs with photosensitizers (5-ALA, PpIX and Photofrin®) are almost similar pattern of loss in cell viability was seen which almost 93% was, showing their candidature as anticancer ligands for U2OS cell. Figure 3 (C) shows the cytotoxicity is dose dependent manner of bare Fe_2O_3 NPs and their complexes with photosensitizers (5-ALA, PpIX and Photofrin®) for osteosarcoma cells [19]. Results are conclusive that ZnO NWs, Fe_2O_3 NPs and ligands of MnO_2 NWRs with photosensitizers might be convincing and appealing candidates for treatment of osteosarcomatous cells. Aminolaevulinic acid, itself is not a photosensitizer, a precursor in the haem synthesis, PpIX accumulate in the cells causing photosensitization [4-6]. In Figure 3 author focused/examined the cytotoxicity of individual and complex of photosensitizers (Photofrin®, 5-ALA and PPDME) with ZnO NRs, MNO_2 and Fe_2O_3 in bone marrow carcinoma. For treatment purpose, Photofrin® gave good response as compare to 5-ALA and PPDME. When ZnO NRs labeled with osteocarcinoma, about 90 % cell viability loss were assessed by microplate reader. The same nature of work were done in previous reported data in the presence and absence of UV light by considering foreskin fibroblast as experimental biological model. About 5% cell viability were recorded when suggested cells were labeled with MnO_2 nanowires conjugated with PPDME as shown in fig. (c). In addition, Fe_2O_3 with Photofrin® show very nice compatibility for treatment of Osteosarcoma cellular model, about3-4 % cells viable were found in this sunerio. In Figure 4(A) with bare ZnO NWs, there is increasing trend in accumulation of ROS at 0.5 µg/ml up till 5 µg/ml which is in agreement with ZnO NPs [40] while their ligand with Pp IX shows synergetic trend of ROS accumulation and these results are in consistence with our previous work (submitted for publication) with melanoma and fibroblast cells. Manganese dioxide displays small increasing trend in ROS accumulation up till 10µg/ml in Fig. 4 (B), while its conjugated forms with PpIX shows enhanced increasing trend of ROS at 0.5 µg/ml and both of these values correspond their viability in U2OS cells shown in figure 3(B). Ferric oxide nanoparticles in its individual form displays increasing trend in ROS accumulation at 0.5 µg/ml is seen Fig. 4(C), and its complexed form with PpIX shows no synergetic effects as with above nanowires. Researchers have quoted the importance of ROS for cancer killing effect in their series of experiments [41-44]. In Figure 5, we assessed % cell viability under laser exposure (30 J/cm^2, 6.5 minutes time of irradiation) of labeled cells with different concentrations of photosensitizers (1-200 µg/ml). Loss in cell viability with 200 µg/ml of 5-ALA and Photofrin® was 80% and 70% respectively, while, 84% with 10 µg/ml of PpIX,

suggesting their effectiveness as anticancer photosensitizers.



(A)



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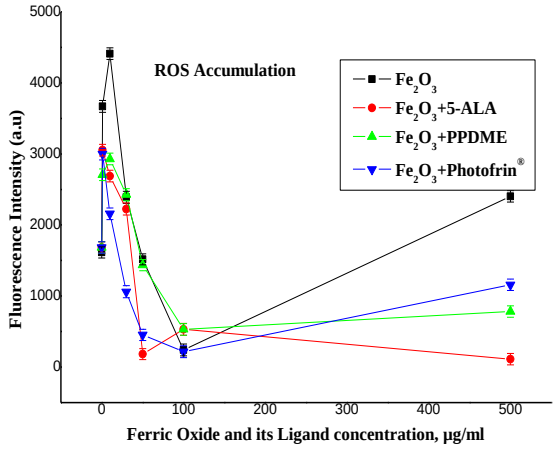
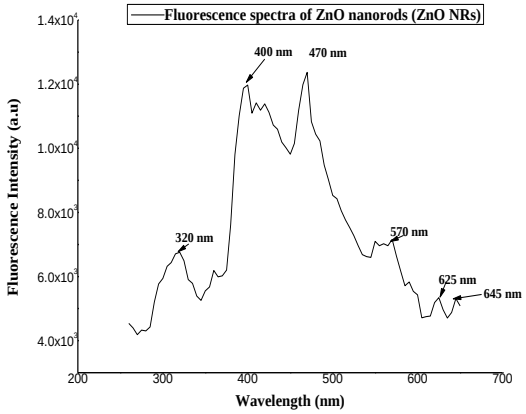
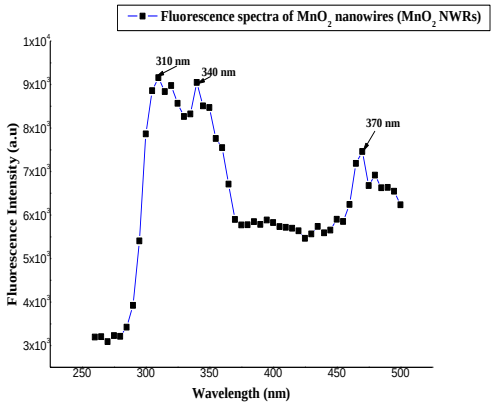


(C)

Fig. 4: (A) Reactive oxygen species (ROS) accumulation in Osteosarcoma cells treated with ZnO NRs (B) Reactive oxygen species (ROS) accumulation in Osteosarcoma cells treated with MnO_2 NWRs (C). Reactive oxygen species (ROS) accumulation in Osteosarcoma cells treated with Fe_2O_3 NPs



(A)



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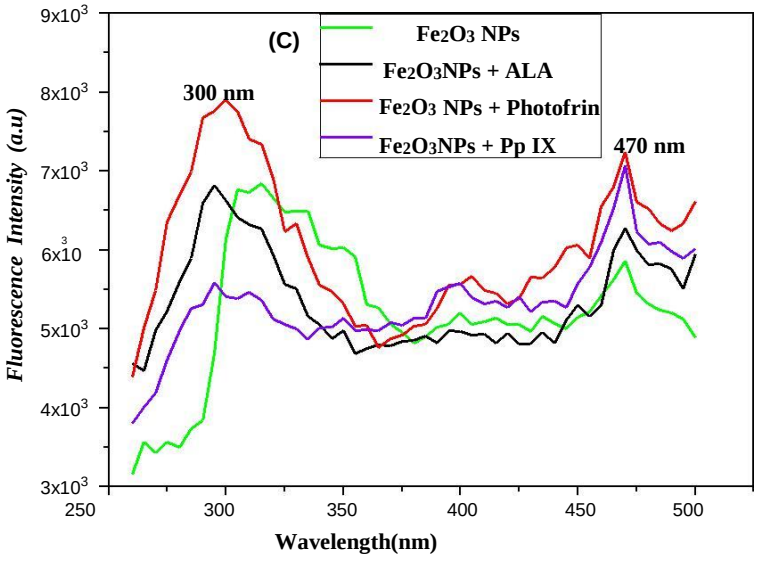

(C)

Fig. 6: (A) Fluorescence Emission Spectrum of ZnO NRs (B). Fluorescence Emission Spectrum of MnO_2 NWRs (C). Fluorescence Emission Spectrum of Fe_2O_3 NPs

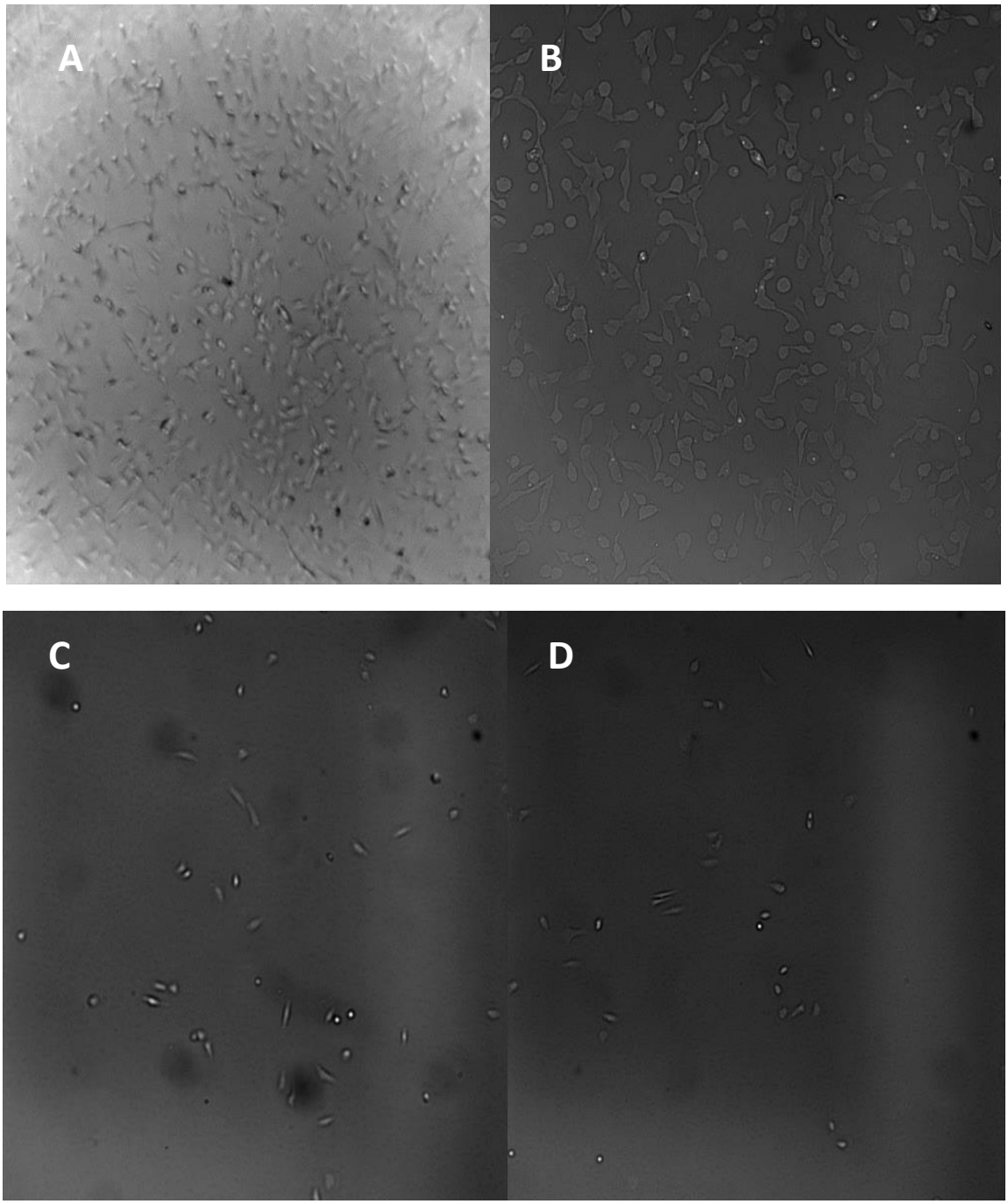

Fig. 7: (A-D) Cell (Osteosarcoma) Morphology

Fluorescence spectra of bare and conjugated forms metal oxides were evaluated to identify the emission nature light which is responsible to excite the photosensitizer. In Fig. 6 (A), we can see six fluorescence spectra peaks from ZnO NWs with their ligands described in our published data [12]. In case of MnO_2 NWs and their ligands, three well defined emission peaks, 280 nm, 310 nm, and 370 nm of fluorescence spectrum in case of MnO_2 NWRs are seen in Fig.6(B) that are consistent to previously published results [45]. Two prominent peaks in Fe_2O_3 NPs and their ligands are seen in Fig. 6(C). Osteosarcoma cells with their cell boundaries and nucleus can be seen in Figure 7(A-B) as control. In Figure 7 (C), few cells with fluorescence are observed; look to be bulging from their background medium. Dead cells clusters are seen in Figure (D).

4. Conclusion

Cytotoxic effects of nanometallic oxides e.g. zinc oxide nanowires (ZnO NWs), Iron oxide nanoparticles (Fe_2O_3 NPs), manganese di-oxide nanowires (MnO_2 NWRs) and their ligands with photosensitizers are

explored having multiple concentrations (0.05-200 µg/ml) in Osteosarcoma cells. Results are interpreted by cellular morphology, intracellular reactive oxygen species (ROS) detection, spectrofluorimetry and MTT assay under UV as well as visible laser exposed conditions. Data is suggestive that ZnO NWs, MnO_2 NWs and Fe_2O_3 NPs when complexed with photosensitizers synergize each other's effectiveness. Such ligands are having significant and convincing cytotoxic effects due to the liberation of ROS in Osteosarcoma (U2OS) cells and can be assigned as toxic agents for Osteosarcoma cells.

Acknowledgements

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project No. RGP -VPP-293.

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