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Functionalized up conversion rare earth nanoparticles for bio imaging of cancer cells

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Abstract*—In recent yearsupconversion nanoparticles (UCNPs)have being investigated due to their potential applications in biomedicine such as fluorescent biolabels, among many others. The luminescence of this kind of NP's are effectively activatedby near infrared radiation (NIR) and upconvertto luminescence in the visible region. Besides, their luminescence is not faded as compared with organic dyes and fluorescent proteins. In this study, UCNPs made of Y_2O_3:Yb^{3+}, Er^{3+} (1%, 10% mol) and Gd_2O_3:Yb^{3+}, Er^{3+} (1%, 10% mol) were functionalized with aminosilanes and folic acid (UCNP-NH_2-FA) and characterized with transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FTIR) and luminescence measurements. Moreover, cytotoxicity was analyzed via colorimetric assays MTT (methy-134 thiazolyltetrazolium) in two cancer cell lines: cervical adenocarcinoma cells (HeLa) and breast cancer cells MB-MDA-231. It is found that the functionalized UCNPs were non-cytotoxic in all cancer cell lines. Confocal images revealed that UCNP-NH_2-FA conjugates as a target to attract cells with overexpressed folate receptor (FR). The UCNPs offer a great potential to be used as bio labels because their fluorescence was clearly localized into cell cytoplasm.*

***Keywords*— luminescence, nanophosphors, lanthanide, biomedical**

I. INTRODUCTION

Nanotechnology has arisen with different types of nanoparticles and nanomaterials for several applications in biomedicine. Our special interest is in bioimaging applications, using upconversion nanoparticles(UCNPs) to identify certain types of cancer cells [1,2]. The nanoparticles can upconvert the near infrared (NIR) energy (λ= 980 nm) into visible light[3]. In this context, the UCNPs of Y_2O_3:Yb^{3+}, Er^{3+} (1%, 10% mol) and Gd_2O_3:Yb^{3+}, Er^{3+} (1%, 10% mol) were synthesized by sol-gel method[4], the hosts were the oxides Y_2O_3 and Gd_2O_3 and they were combined with two rare earth ions $Er^{3+}$and Yb^{3+}. The ion Yb^{3+} receives the energy from the NIR laser at 980 nm wavelength and it shows high quantum yield. The excited Yb^{3+} ion transfers the energy to the Er^{3+} ions, which results in their excitation to a higher level $^4I_{11/2}$. The ions have a non-radiative decay to $^4I_{13/2}$ level and further radiative decay to theground state with 661 nm of wavelength emission (red) this event is called energy transfer upconversion (ETU)[5]. The color of the emission varies according with the percentage of dopants elements [6].One of the challenges of the biolabels, is the chemical durability of the UCNPs, in this study theyhave a silica core shell that is useful because we prevent possible cytotoxicity into the cells[7]. Also, the functionalization of the UCNPs with specific ligands is necessary to label specific cancer cells.There are different types of biological ligands; in this case, folic acid has been use for several authors with successful results with cervical adenocarcinoma cells (HeLa) and breast cancer cells MB-MDA-231[8]. After the UCNPs were coated with a thin silica shell by Stöber method[9], they were functionalized with amine group (APTES/TEOS) to enable folic acid (FA) conjugation[10]. The FAare able to bind to the folate receptor (FR) available on cancer cells surface.Further this bindingprovoke the internalization of the NPs into the cytoplasm via endocytosis[11]. The FR has three isoforms (α, β and γ), the studied cells have FR-α overexpressed[12].The cytotoxicity of the UCNPs was tested by a cell viability assay based on the reduction of the MTT reagent (methy-134

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thiazolyltetrazolium) for HeLa and trypan blue assay for MB-MDA-231. They were characterized before and after functionalization. The UCNPs presented an average size of 50 nm for the Gd_2O_3:Yb^{3+}, Er^{3+} and 70 nm for the Y_2O_3:Yb^{3+}, Er^{3+}, showed strong luminescence emission and they were non-cytotoxic.

II. MATERIALS AND METHODS

2.1 Sol-gel synthesis and ultrasonication

The precursors used were: $Y(NO_3)_3$ (Alfa Aesar 99.9965%) or$Gd(NO_3)_3$ (Alfa Aesar 99.99%) with$Yb(NO_3)_3$ (Alfa Aesar 99.9%) and$Er(NO_3)_3$ (Alfa Aesar 99.9%). Theywere prepared by sol-gel synthesis and the annealing temperatures were 1200°C for Y_2O_3 and 900°C for Gd_2O_3[4, 6]. The UCNPs were ultra-sonicated with high intensity ultrasonic processor (Sonics & Materials, Inc.) at 70% of the amplitudefor about 30 min with 20 ml of isopropanol/ethanol [6].

2.2 Silica and Aminosilane functionalization

The biolabels must accomplish specific characteristics in order to target onto a specific tumour; the size has to be less than a 100 nm and the surface need to have the proper ligands that bind to the surface of the cells.The UCNPs were functionalized with aminopropyltrimethoxysilane (APTES)[8], the amino groups (NH_2) formed on the surface of the UCNPs are easily able to bind with folic acid (FA). TheFAcan be linked with the folic acid receptors on the cell surface (FR).It was found that when FA was chemically linked with amine or c-carboxyl groups, its FR binding affinity were hardly affected[13].The UCNPs were coated with a thin silica shell; we used the Stöber synthesis[9]. The UCNPswere coated with the TEOS technique (Tetraethyl orthosilicate, Sigma Aldrich 98%) [14]. They were ultrasonicated after the coating and then the aminosilane functionalization (UCNP-NH_2) was done mixing the coated UCNPswith ethanol, 0.02 ml of 3-Aminopropyl-trimethoxysilane (APTES, 98% Sigma Aldrich), 0.14 ml of TEOS and 0.2 ml of ammonium hydroxide, for 4 hours.Theywere dried and collected [8, 14].

2.3 Folic acid conjugation

Folic acid is also known as folate and it has a high affinity binding reagent that could recognize folate receptor (FR). The cancer cells studied have a tumor marker over-expressed on the surface[8]. The UCNPs were functionalized with folic acid ligands; the reaction was prepared on a Schlenk system. The solution was agitatedinto the darkness. The resulted UCNP-NH_2-FA were centrifuged (6000 rpm for 15 min), washed with DMSO (3 x 45 ml) and ethanol (5 x 45ml). Finally they were vacuum dried overnight at 30°C. The UCNP-NH_2-FA turned into a yellow color [15].

2.4 Characterization

To study the crystallinity of the UCNPs we did the X-Ray diffraction (XRD) analysiswith the Phillips X'Pert-MPD, equipped with Cu Kα radiation (λ= 0.15406 nm). The results obtained were compared with the database PCPDFWIN [6]. To study the morphology and the nanoparticle size we used the transmission electron microscopy (TEM) brand JEOL JEM-2100-F. We used the fluorescence spectrometer (PL, Hitachi® FL-4500) with 980 nm for excitation to analyze the photoluminescence of the UCNPs. FTIR analysis was done to confirm the aminosilane-folic acid functionalizationin the range of 400–4000 cm^{-1}(Thermonicolet 1700).

2.5 Cell culture

We obtained from American Type Culture Collection (ATCC) the human cervix carcinoma HeLa cells (ATCC CCL-2) and breast cancer cells (MDA-MB-231). Theywere cultivated in RPMI-1640 media supplemented with 10% Fetal Bovine Serum (FBS, BenchMark, Gemini Bio Products), 1% Penicillin streptomycin (Sigma-Aldrich), 1% L-glutamine and 1.5 g/l sodium bicarbonate. Cells were propagated in growth medium and maintained at 37°C and 5% CO_2.

MCF7 cells were cultured in Dubelcco's Modified Eagle Media (DMEM, Sigma Aldrich, MO, USA) supplemented with 10% SFB, 1% antibiotic/antimicotic, 1% L-glutamine, 1.5. g/l sodium bicarbonate and 0.01 mg/ml of human recombinant insulin. Cells were propagated in growth medium and maintained at 37°C with 5% CO_2.

2.6 Cytotoxicity assay

The evaluation of the cell membrane integrity is one of the most common ways to measure cell viability and cytotoxic effects. The assay based on the reduction of the MTT reagent (methy-134 thiazolyltetrazolium) measures the reducing potential of the cell using a colorimetric reaction. The viable cells reduce the MTT reagent to a colored formazan product. The bare UCNPs and functionalized UCNP-NH_2-FA were tested using the TOX1 in vitro toxicology assay kit (Sigma-Aldrich). The test was done in a 96-well plate containing 10,000 cells per well. The UCNPs were ultrasonicated and diluted at 0.001 µg/mL to 1 µg/mL in RPMI-1640 media. Hela cells were incubated for 24 hr at 37°C and 5% CO_2. The positive control was the incubation of cells in complete RPMI-1640 media without UCNPs, simulating cell behavior under ideal conditions. DMSO (Dimethyl sulfoxide) was used to induce cell death for negative control. After incubation time, cells were

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washed with phosphate buffer solution pH 7.4 (PBS 1x) and MTT reagent was added to the plate. Cytotoxicity was evaluated by absorbance measurements with an ELISA plate reader (Thermo Scientific, USA) at 570 and 690 nm. All data obtained from incubated UCNPs were normalized to data from three positive control wells with no UCNPs in three independent experiments[16].

2.7 Trypan blue assay

Vital dyes, such as trypan blue, are normally excluded from the inside of healthy cells; but if the cell membrane has been compromised, they cross the membrane and stain intracellular components. This staining method is described as a dye exclusion method, because cells are very selective in the compounds that pass through the membrane, the trypan blue is not absorbed by viable cells however, it traverses the membrane in a dead cell.Thetrypan blue assay was used to measure the cytotoxicity of the UCNPs (bare and functionalized) with breast cancer cells (MB-MDA-231).The cell viability was calculated as the number of viable cells divided by the total number of cells within the grids on the hemacytometer. The cells are considered non-viable if they take up trypan blue. The solution of trypan blue was prepared with 0.4% solution of in buffered isotonic salt solution pH 7.4 (PBS 1x) and 0.1 mL of trypan blue stock solution to 1 mL of cells. The cells were loaded on the hemacytometer and examined immediately under a microscope at low magnification. The quantities of blue staining cells were counted and the number of total cells was also verified [17]. All experiments were done in triplicate and the results were expressed as mean ± standard deviation of three independent experiments. The data were evaluated by analysis of variance (ANOVA), followed by Tukey's Multiple Comparison Test, using Graph Pad Prism 6.0 software. The results were considered statistically significant when $p < 0.05$.

2.9 Confocal microscopy cell imaging

In this study, the nucleus of the fixed cells was strained withthe fluorescentstainDAPI (4', 6-diamidino-2-phenylindole). This dye binds strongly to A-T (adenine-thymine) rich regions in DNA and it is used extensively in fluorescence microscopy.We seeded 300,000 cells in RPMI-1640 media and incubated overnight at 37 °C and 5 % CO_2 into a culture Petri dishes coated with Poly-d-lysine (Mat-Tek P35GC1.5-10C). The HeLa and breast cancer cells (MB-MDA-231) wereincubated with 1 µg/mL of UCNP-NH_2-FA for 24h at 37 °C and 5 % CO_2. After incubation, the cells were rinsed with PBS 1x and fixedwith 4 % formaldehyde-PBS solution at 4 °C for 15 min.Then they were permeabilized with 0.5 % Triton X/PBS 1x for 15 min at 4 °C. The nuclear staining was achieved by incubated the cells with DAPI at 0.5 ng/µL in darkness for 10 min at RT, followed by five washes with PBS. Nuclear staining with DAPI was also visualized with an inverted laser-scanning microscope OlympusFluoView FV1000 (Japan) using an argon ion laser for excitationat 405 nm wavelength and filters for emission of DAPI. UCNPsfluorescent was detected using the NIR laser (980 nm) and RFP (red fluorescent protein) filter channel (excitation at 487 nm and emission at 610 nm). Cells were visualized with a 63 × (DIC), 1.4 N.A. planapochromaticoil immersion objectives. The imaging parameters used produced no detectable background signal from any source other than from UCNPs and DAPI. The MetaMorph software for Olympus was used to capture the images [18].

III. RESULTS

3.1 TEM images of UCNPs

The compositions obtained for the UCNPs synthesized by the sol-gel method wereand $(Y_{0.89}Yb_{0.1}Er_{0.01})_2O_3$ and $(Gd_{0.89}Yb_{0.1}Er_{0.01})_2O_3$ respectively.In figure 1 are shown the TEM images of bare and functionalized UCNPs of Y_2O_3:Er^{3+}/Yb^{3+} (1%, 10% mol) and Gd_2O_3:Er^{3+}/Yb^{3+} (1%, 10% mol). The form of the UCNPs is mostly spherical. The average size of UCNPs Y_2O_3:Er^{3+}/Yb^{3+} were 70 nm (+/- 10 nm) and 50 nm for Gd_2O_3:Er^{3+}/Yb^{3+}. We observed agglomerates in all the UCNPs. The silica shell is approximately of 5 nm thick.

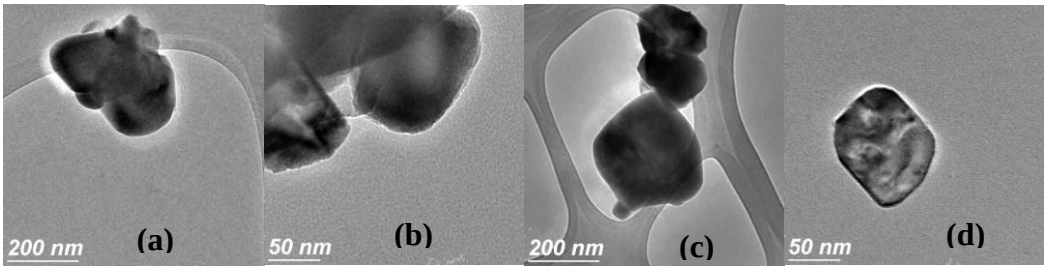

Fig.1: *TEM images of UCNPs Y_2O_3: Er^{3+}/Yb^{3+} (1%, 10% mol) (a) bare and (b) functionalizedUCNP-NH_2-FA.TEM images of Gd_2O_3: Er^{3+}/Yb^{3+} (1%, 10% mol) (c) bare and (d) functionalized UCNP-NH_2-FA.*

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3.2 Upconversion luminescence

In figure 2 are shown the upconversion luminescence spectra of bare and functionalized UCNP-NH_2-FA with 980 nm laser excitation. The red emission of the nanoparticles Y_2O_3:Er^{3+}, Yb^{3+}(1%, 10% mol) was at 661 nm of wavelength(figure 2-a) and also the same fluorescence applying for the Gd_2O_3:Er^{3+}, Yb^{3+}UCNPs (figure 2-b), with the electronic transitionof the Er^{3+} ion in the level$^4F_{9/2}\rightarrow{}^4I_{15/2}$ (660 nm). This is because the UCNPs have the same dopants in different hosts, so the luminescence effects are the same due to the energy transfer process from Yb^{3+} ion to the Er^{3+} ion. The difference between both UCNPs is the size; the Y_2O_3 UCNPs are bigger due to the size of the lattice.

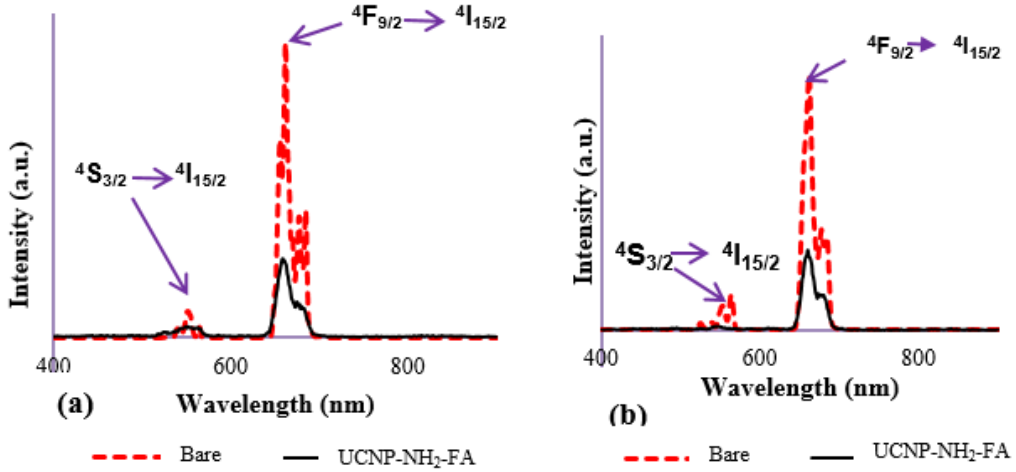
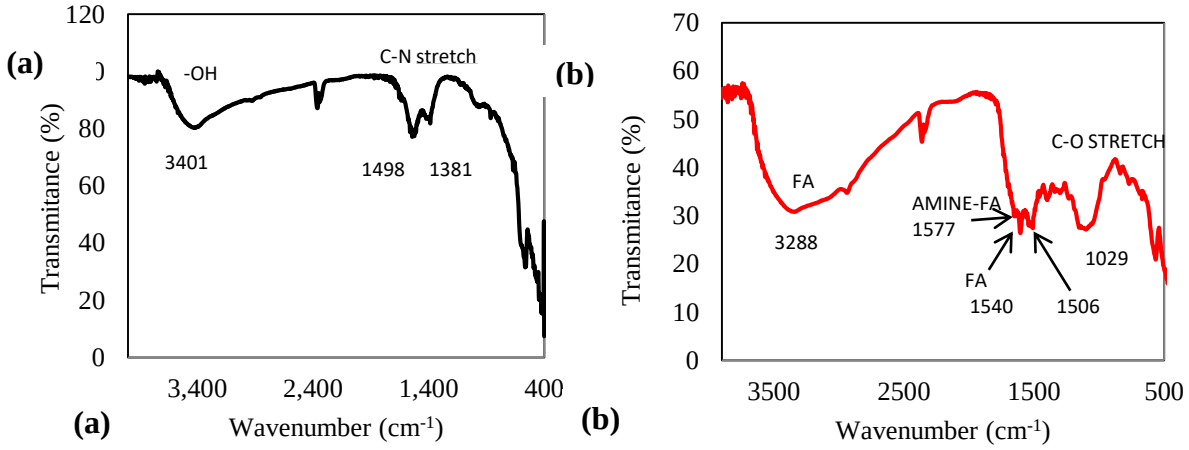


Fig.2: The upconversion emission spectra with 980 nm excitation for bare and functionalized UCNPs of (a) Y_2O_3:Er^{3+}/Yb^{3+} (1%, 10% mol) and (b) Gd_2O_3:Er^{3+}/Yb^{3+} (1%, 10% mol)

3.3FTIR analysis

The Fourier transform infrared spectroscopy analysis (FTIR) was used to verify the functionalization UCNP-NH_2-FA. With this technique, we verify thepresence of the amine groups and folic acid on the surface of the nanoparticles with an infraredspectrum of absorption or emission.The figures4-aand 4-c show the FTIR spectra of bare UCNPs. Figures 4-b and 4-dshow the aminosilane-folic acid functionalization (UCNP-NH_2-FA).



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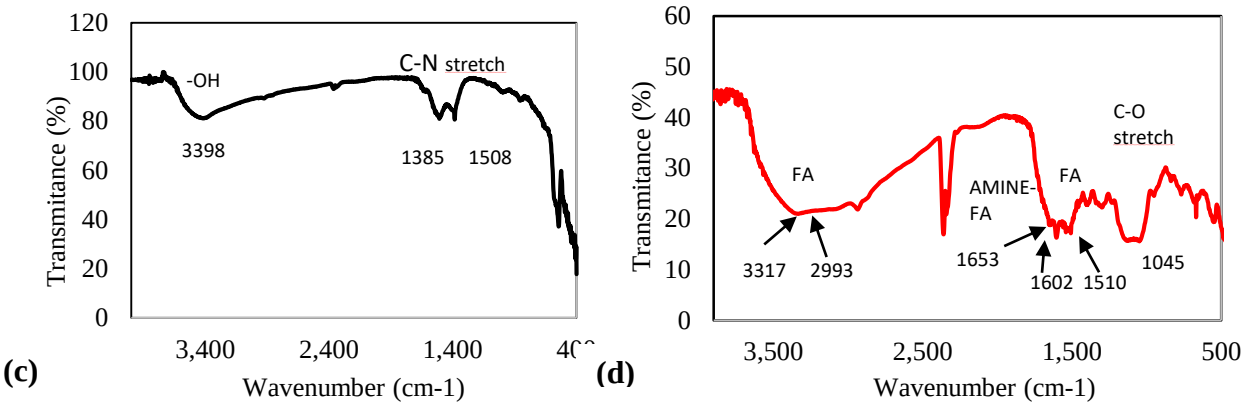

Fig.4: FTIR spectra of (a) bare UCNPs $Y_2O_3:Er^{3+}/Yb^{3+}$(1%,10% mol), (b) UCNPs with aminosilane functionalization, (c) bare UCNPs $Gd_2O_3:Er^{3+}/Yb^{3+}$(1%,10% mol), (d) functionalized UCNP-NH_2-FA.

3.4 Cytotoxicity assay and cellular imaging.

The cytotoxicity results with the MTT assay for bare and functionalizedUCNP-NH_2-FAincubated on HeLa are shown in figure 5.The UCNPs tend to agglomerate and precipitate after the addition to the well of the 96-well plate, even after been ultrasonicated, this reason causes interference with the absorbance reading of the plate.The results for $Y_2O_3:Er^{3+}$, Yb^{3+} are on figure 5-afor HeLa cellsand $Gd_2O_3:Er^{3+}$, Yb^{3+} are in figure 5-b. On both assays the functionalized UCNPs showed no cytotoxicity on both cancer cell lines.

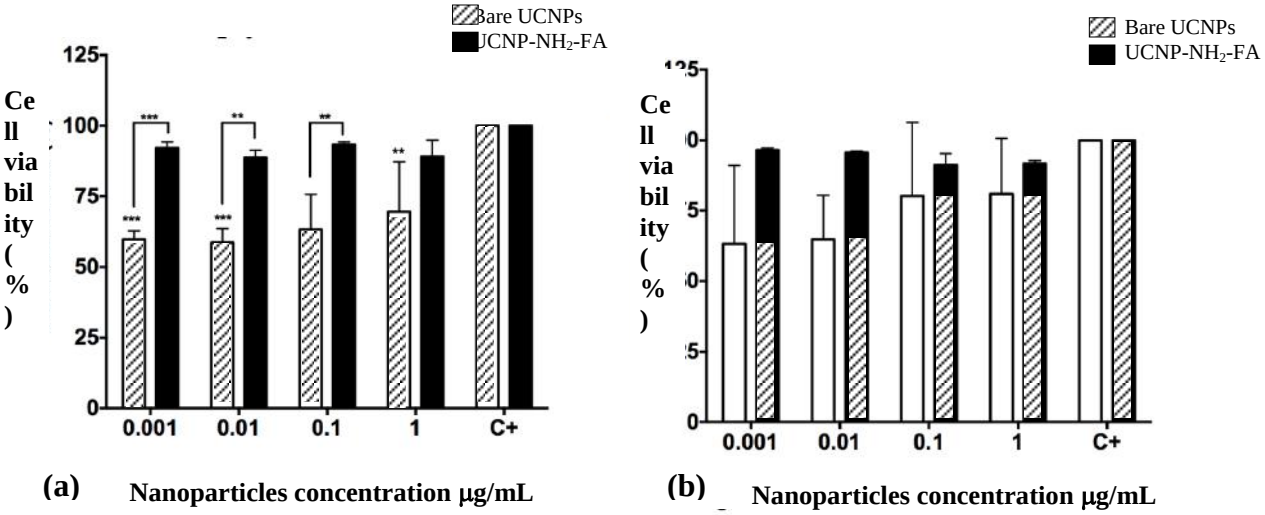

Fig.5: Cell viability assay of (A) bare UCNP $Y_2O_3:Er^{3+}/Yb^{3+}$ (1%,10%) (lined bars) and functionalized UCNP-FR (black bars) with HeLa cells, (B) bare UCNP $Gd_2O_3:Er^{3+}/Yb^{3+}$ (1%,10%) (lined bars) and functionalized UCNP-FR (black bars) with HeLa cells.

The cell viability for breast cancer cell MB-MDA-231 is shown in figure 6 shows with trypan blue assay with 0.1 and 1 µg/mL of UCNPs concentration. The results indicated in figure 6-a for UCNPs $Y_2O_3:Er^{3+}$, Yb^{3+} that there are no cytotoxicity of functionalized UCNPs with 65.71 % and 80.56% of cells viability. For the UCNPs $Gd_2O_3:Er^{3+}$, Yb^{3+}, the cell viability is more than 83% on both concentrations, as shown in figure 6-b.

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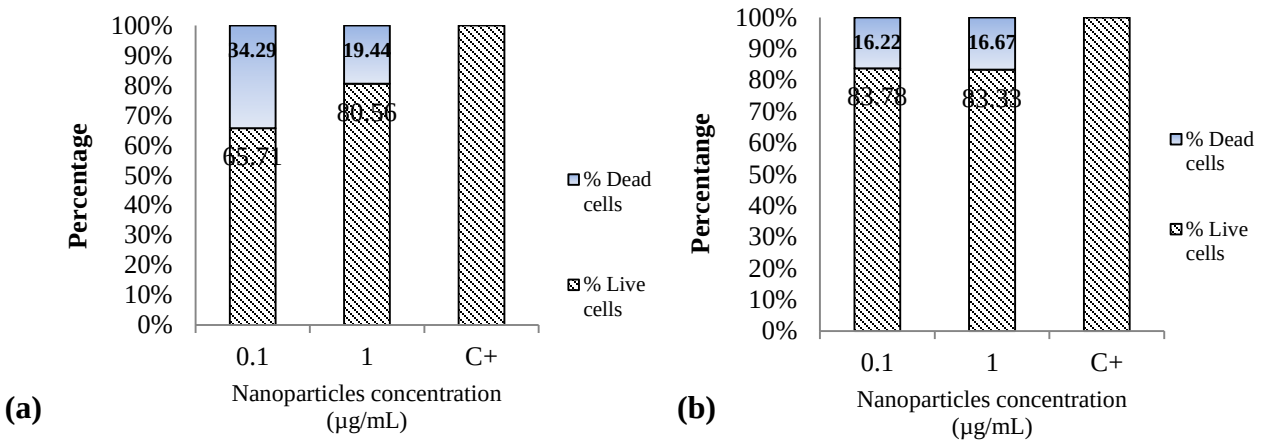

Fig.7: Results of trypan blue assay for breast cancer cells (MB-MDA-231) with the incubations of UCNP-NH_2-FA of (a) $Y_2O_3:Er^{3+}/Yb^{3+}$ and (b)$Gd_2O_3:Er^{3+}/Yb^{3+}$ with two different concentrations.

3.5 Cancer cell imaging with UCNPs

The UCNP-NH_2-FAwere excited with a 980 nm laser in cell culture medium with the characteristic green upconversion emissions; they were incubated in both cancer cell lines with a concentration of 1 µg/mL for 24 hours into the cells, then they were properly fixed and prepared in order to obtain imaging with the confocal microscope. The nucleus (N)stained with DAPI, was excited with 405 nm to obtain 461 nm of emission (blue) of the DAPI dye, shown in figures 8-a, 8-d, 9-a and 8-d. Figure 8 shows the UCNPsinto HeLa cells and figure 9 shows UCNPs into breast cancer cells (MB-MDA-231). In figures 8-b, 8-e, 9-b and 9-e it is seen the localization of functionalized UCNPs only excited with NIR 980 nm +RFP (red fluorescent protein) 8-c, 8-f, 9-c and 9-f shows the merge of the images. The UCNPs were clearlylocalized in the cytoplasmic region as expected, since folic acid ligands were used for UCNPinternalization.The confocal images of the UCNPs into the cells were observed in the laser-scanning microscope Olympus FluoView FV1000 (Japan).

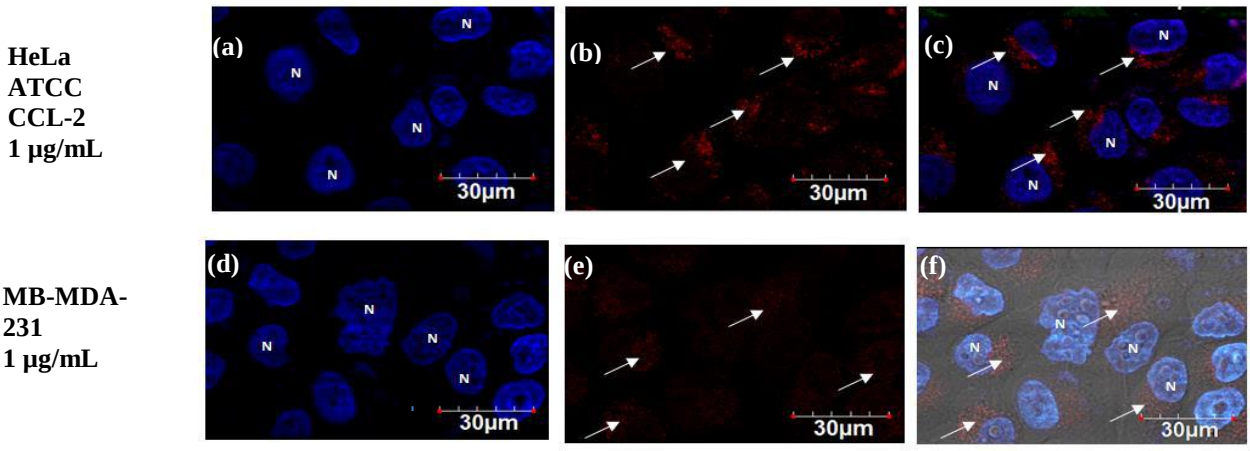

Fig.8: Fluorescence redemission by $Y_2O_3:Er^{3+}/Yb^{3+}$ (1%,10% mol) incubated with HeLa cells (concentration 1 µg/mL) for 24 h. (a) Staining with DAPI show nuclei of HeLa, MB-MDA-231 and MCF-7 cells labeled as "N" (b) Laser NIR 980 nm + RFP shows the functionalized UCNPs into the cell. (c) Overlay DAPI, Laser 980 nm +RFP localize the UCNPs activity in the cytoplasm of the cells (white arrows).

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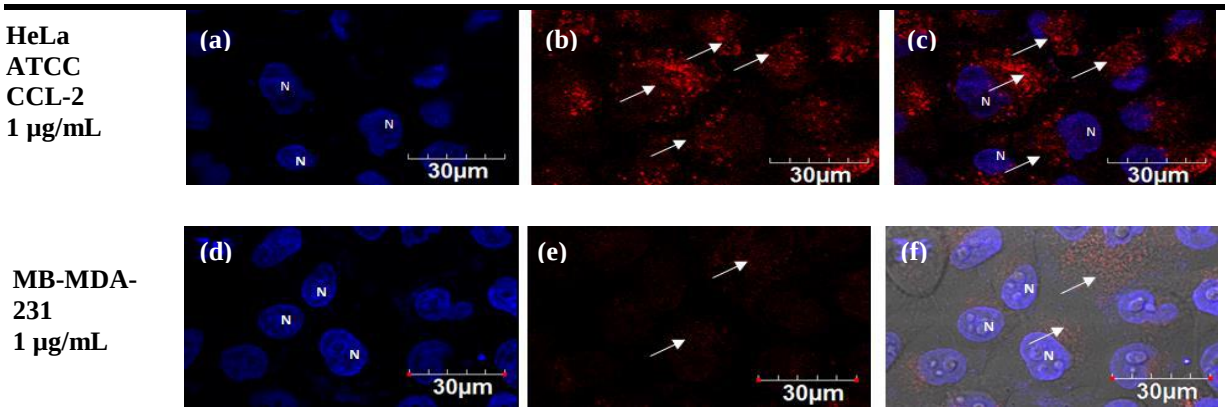

Fig.9: *Fluorescence red emission by $Gd_2O_3:Er^{3+}/Yb^{3+}$ (1%,10% mol) incubated with HeLa cells (concentration 1 µg/mL) for 24 h. (a) Staining with DAPI show nuclei of HeLa, MB-MDA-231 and MCF-7 cells labeled as "N" (b) Laser NIR 980 nm + RFP shows the functionalized UCNPs into the cell. (c) Overlay DAPI, Laser 980 nm +RFP localize the UCNPs activity in the cytoplasm of the cells (white arrows).*

IV. DISCUSSION AND CONCLUSIONS

The UCNPs synthesized by the sol-gel methodwith the hosts Y_2O_3 and Gd_2O_3 co-doped with Er^{3+} and Yb^{3+}had a shape mostly spheroidal with an average diameters of 70 nm and 50 nm (±10 nm) respectively.The photoluminescence analysis showed outstanding optical properties for bare and functionalized UCNPs, they have a stable luminescence, photostability and long lifetimes of excited states, so they overcome the problems of the currently used organic dyes. The UCNPs showed red emission spectra (660 nm). The FTIR spectra showed the prescence of the amine-folic acid bonds (NH2-FA) on the surface of the UCNPs (1612 to 1512 cm-1), the FA ligands (3331 cm-1) and the wagging –NH2 (669-793 cm-1).We evaluated the viability of the cells with bare and functionalized UCNPs with the MTT assay. The ability of the cells to reduce MTT is an indicator ofmitochondrial integrity and its functional activity is interpreted as measure of cell viability after exposure to the UCNPs evaluated.The HeLa cells incubation with different bare $Y_2O_3:Er^{3+}/Yb^{3+}$ and $G_2O_3:Er^{3+}/Yb^{3+}$at 0.001 and 0.01 µg/mLconcentrations caused a 40% of cell death. But,with UCNP-NH_2-FA, all the cells had more than 80% of viability, thus the cell death and the cytotoxic effect was reduced for all of the tested concentrations. For breast cancer cells MB-MDA-231, trypan blue assay showed more than 80% cell viability for the tested concentrations, confirming that the UCNPs are non-cytotoxic.The studies with the confocal microscope showed the UCNPs localized into the cell cytoplasm with no cytotoxicity effects to other possible cells, which confirms that they can be used as biolabels for the cancer cells studied. Further studies with *in vivo* murine models with breast and cervix cancer need to be tested to be considered as an important tool to diagnostic cancer.

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