LUMINESCENT NANOMATERIALS FOR BIOLOGICAL LABELLING

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INTRODUCTION

"Biomedical imaging research has leveraged the benefits of significant advances in electronics, information technology and, more recently, nanotechnology. Substantial progress in the ability to fabricate nanoparticles and the discovery of their novel size dependent physical and chemical features has drawn the attention of researchers in this area. The development of targeted contrast agents such as fluorescent probes has made it possible to selectively view specific biological events and processes in both living and nonviable systems with improved detection limits, imaging modalities and engineered biomarker functionality. The fabrication of luminescent-engineered nanoparticles is expected to be integral to the development of next generation therapeutic, diagnosis and imaging technologies." [1]

Aim of the work

- Preparation of novel luminescent systems based on gold nanoparticles and small sized europium oxide nanoparticles.
- Photoluminescent properties studies of the systems.
- Applcation of microscopy techniques (Confocal, Fluorescence microscopy and TEM) for the *In vitro* studies of cellular uptake.

CELLS

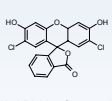
Macrophages [2]: white blood cells that crawl around in the extracellular fluids and gobble up microbes and other foreign material by phagocytosis. Macrophages are key players in the immune response to foreign invaders such as infectious microorganisms. Tumour-associated macrophages (TAM) are involved in tumour angiogenesis and anti-tumour immune response. In certain malignant diseases including the lung, breast, cervix, bladder, as well as squamous and renal cell carcinomas, accumulation of macrophages within the tumour mass is associated with a poor prognostic outcome. Infiltration of skin tumours by macrophages is an important step in tumour progression, although the mechanisms of macrophage recruitment to the tumour mass and the subsequent effects on tumour growth are still poorly understood.

Human Neuroblastoma IMR-32 [3]: A common neoplasm of early childhood arising from neural crest cells in the sympathetic nervous system, and characterized by diverse clinical behaviour, ranging from spontaneous remission to rapid metastatic progression and death. Neuroblastoma is the most common solid tumor outside the brain in infants and children.
The human neuroblastoma cell line IMR-32 exhibits both cholinergic and adrenergic properties. We have used IMR-32 cells to study the uptake of luminescent nanoparticles.

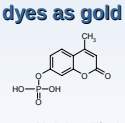
PREPARATION AND CHARACTERISATION OF NANOPARTICLES

Gold nanoparticles stabilised by luminescent ligands were prepared in aqueous solution, by $NaBH_4$ reduction of $NaAuCl_4$ in the presence of Luminescent ligand acting as stabiliser. The reaction mixture was heated at 50-60°C for 25-50 h obtaining cherry red sols. The particles were purified by centrifugation (3500 r.p.m.;30min).GNPs were characterized by UV-vis, PL, HRMAS 1H NMR spectroscopies and TEM.

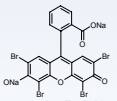
Luminescent dyes as gold stabilisers



2',7'-Dichlorofluorescein

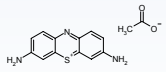


4-Methylumelliferyl phosphate



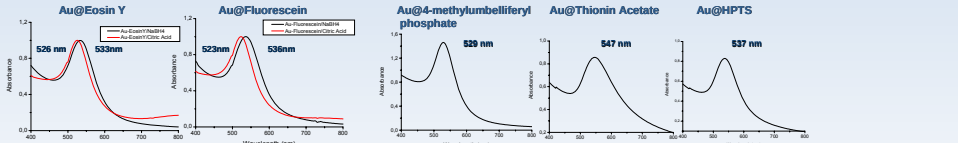
Eosin Y disodium salt

HPTS
8-Hydroxy-1,3,6-pyrenetrisulfonic acid

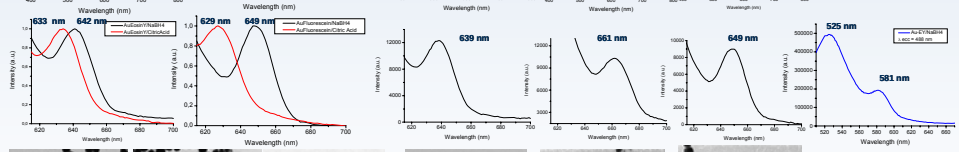


Thionin acetate
3,7-Diaminophenothiazin-5-ium acetate

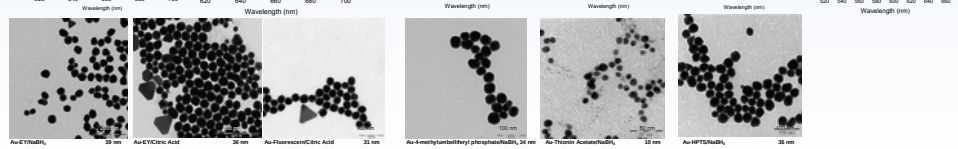
UV-vis spectra



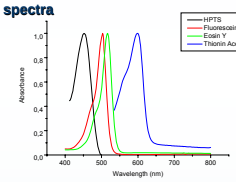
PL spectra



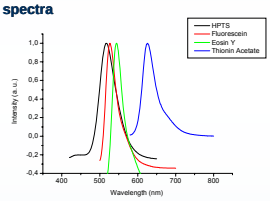
TEM



UV-vis spectra



PL spectra



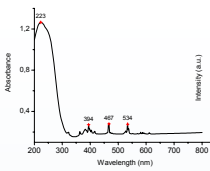
PREPARATION OF 7 nm Eu_2O_3 NPs FROM W/O MICROEMULSIONS [5]

For the preparation of Eu_2O_3 NPs, two microemulsions having composition: water*/CTAB/n-octane/1-butanol were used as microreactors for the precipitation of the precursor $Eu_2(CO_3)_4(OH)_2$.
By calcination at 900°C for 6h, 7 nm sized Eu_2O_3 NPs were obtained.

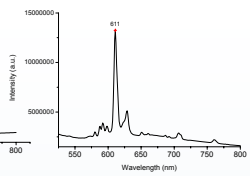
The particles were characterized by Diffuse Reflectance UV-vis, ATR FTIR, XRD, Fluorescence spectroscopy and TEM.

* (Water = $Eu(NO_3)_3$ or $(NH_4)_2CO_3$ aqueous solution)

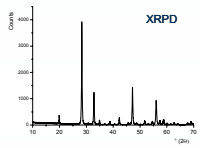
DRIFT UV-vis Excitation spectra



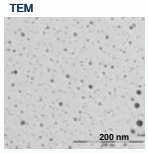
Photoluminescence Emission spectra (λ_{Ex}= 464 nm)



XRPD

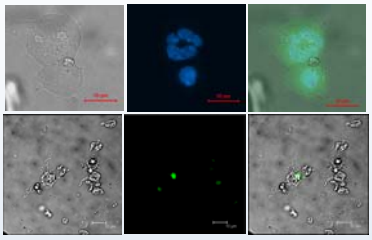


TEM



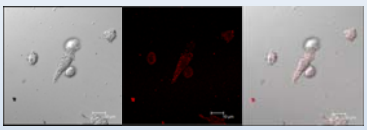
10 and 35 nm Au@AVA NPs in Macrophages

AVA= 4-Aminovaleric acid [4]

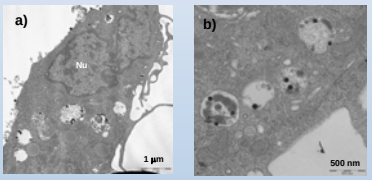


Fluorescence Microscopy Au@AVA/BH4 (10 nm)

Confocal Microscopy Au@AVA/BH4 (10 nm)

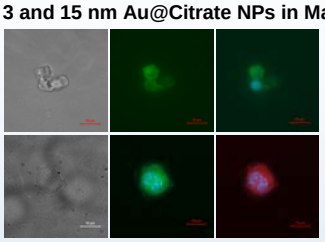


Confocal Microscopy Au@AVA/Citric Acid (35nm)



TEM
a) Au@AVA/BH4 (10 nm)
b) Au@AVA/Citric Acid (35nm)

3 and 15 nm Au@Citrate NPs in Macrophages

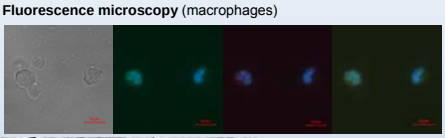


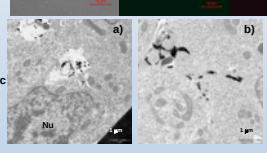
Fluorescence Microscopy Au@Citrate (3nm)

Fluorescence Microscopy Au@Citrate (15nm)

$Au@EosinY/NaBH_4$

Fluorescence microscopy (macrophages)





TEM (IMR-32)

Cellular uptake suggested mechanisms:
a) **endocitosys**
NPs present in endosomes
b) **diffusion**
NPs present in cytoplasm

SAMPLE PREPARATION FOR TEM

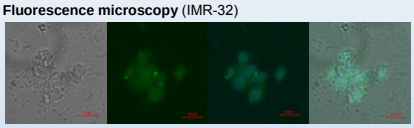
Incubation: 1h, 37°C, CO_2 flow 5%
Fixation: 4% PFA, 2.5% Glutaraldehyde
Rinse: PBS
Post fixation: 1% OsO_4
Staining: 0.5% Uranyl acetate
Dehydration: EtOH
Embedding in epoxy resin
Polymerization 60°C, 48h
Ultra thin cutting (Microtrome)
Mounting on TEM grids
Staining of thin sections: 5% Uranyl acetate Reynolds Lead Citrate

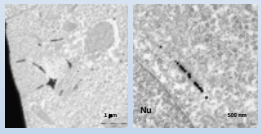
SAMPLE PREPARATION FOR FLUORESCENCE and CONFOCAL MICROSCOPY

Incubation: 1h, 37°C, CO_2 flow 5%
Pellet centrifugation: 1300 r.p.m.; 3min
Fixation: Cryro-spray
Rinse: PBS
Mounting on Microscopy glass slides
With addittion of Gel Mount with DAPI

$Au@4\text{-}Methylumelliferyl\ phosphate/NaBH_4$

Fluorescence microscopy (IMR-32)



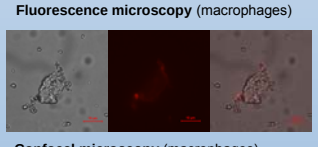


TEM (IMR-32)

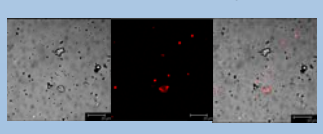
Cellular uptake suggested mechanism: **diffusion**
NPs present in cytoplasm.
NPs associated to the ATP dependent cytoskeleton filaments (microtubules)

$7\ nm\ Eu_2O_3$ NPs

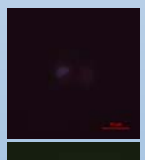
Fluorescence microscopy (macrophages)



Confocal microscopy (macrophages)



$Au@Thionin\ Acetate/NaBH_4$



Fluorescence microscopy (Macrophages)

35 nm Au NPs = red emission

DAPI = blue emission (Nucleus)

35 nm Au NPs = red emission

< 15 nm Au NPs = green emission

DAPI = blue emission (Nucleus)

Au NPs in Nucleus

Gold colloids constituted by nanoparticles, that are stabilised by chromophores, have been prepared by a particular preparation method, designed in order to obtain 30-50 nm diameter particles. For the preparation, two different reducing agents ($NaBH_4$ and Citric acid) and stabilisers having photoluminescence (PL) signals in different Vis spectrum region, have been applied. Absorption and emission spectra showed no peaks due to free ligands, and were substancially different. PL spectra of GNPs, obtained by excitation at the absorption wavelength value (523-547 nm range), showed values in a 596-661 nm range, depending on the ligand kind and particles size. We have also found that the type of reducing agent influences the GNPs PL emission. Moreover, when excited at 488 nm, gold sols showed two PL peaks, centered at 525 and 581 nm, in accordance with Fluorescence and Confocal Microscopy observations. Indeed, when FITC fluorescence filter is used (λ_{ex} = 488 nm) the particles internalized in cells, showed green fluorescence signals, while Texas Red fluorescence filter is used (λ_{ex} = 568 nm) the particles showed red fluorescence signals. Luminescent GNPs were tested as labelling agents in two different cellular systems (macrophages and neuroblastoma cells). The cellular uptake of these particles was studied using Confocal and Fluorescence microscopy and the particles internalization was finally confirmed by TEM.
We have found an almost general correlation between the fluorescence signal colour and the particles size. In the case of NPs smaller than 10 nm in size (eg. 3 nm Au@Citrate, 10 nm Au@4-aminovaleric acid) only green fluorescence signals are found applying FITC filter. On the contrary, if the particle diameters are 15 nm or larger, red fluorescence signals are found when Texas Red fluorescence filter is applied. However, in some cases, for particles with broader size distribution, green and red fluorescence signals were both observed. This allowed us to distinguish types of particles capable of internalization into different cell compartments (eg.Thionin Acetate stabilised GNPs; larger particles were found in macrophage nucleus and the smaller ones in the cell's cytoplasm, i.e. endosomes). To confirm the efficient cellular uptake, and suggest GNPs internalization mechanism, we have studied our GNP-cell systems by TEM. For GNPs found in cell endosomes, endocitosys as cellular uptake mechanism is suggested [6], and for those found in cell cytoplasm, uptake via diffusion mechanism is suggested.
All these findings propose these particles as labelling agents in wide range of cell systems, and could be eventually applied in experiments *in vivo*.

References

1. P. Sharma et al., *Adv. Colloid Interface Sci. 123-126*, **2006,** 471-485.
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