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SCHOOL Nicolás Cabrera  
"Fluorescent Nanoparticles in  
Bio-medicine"  
16th-20th July, 2012  
Madrid, Spain



Universidad  
Zaragoza



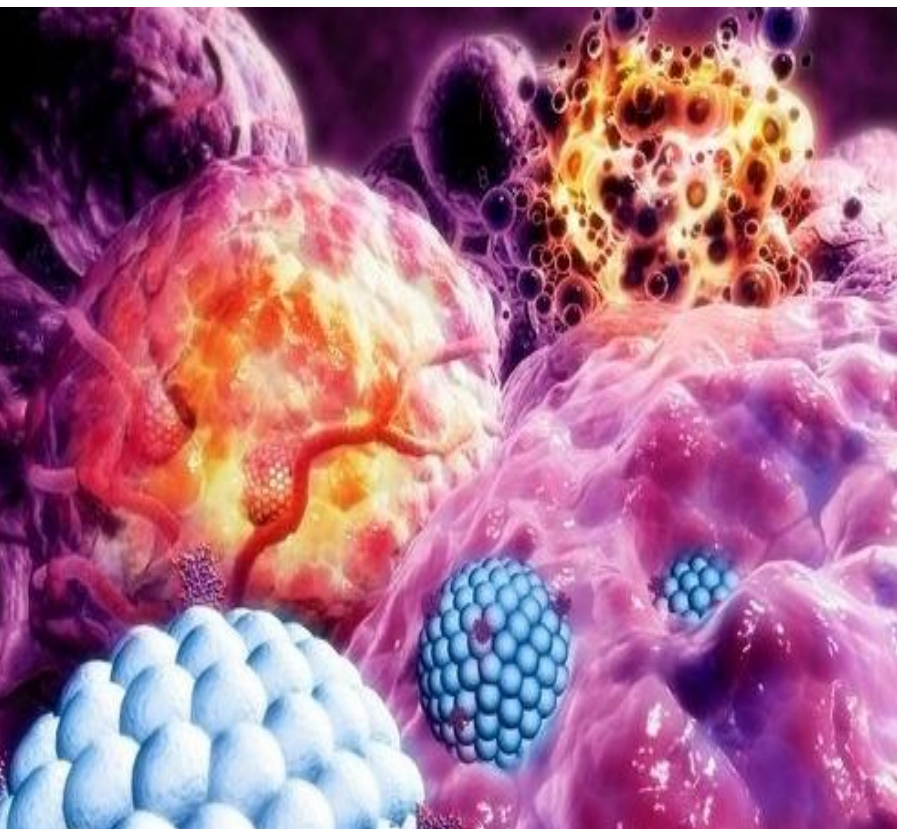
# *In vitro toxicity studies of polymer coated superparamagnetic iron oxide nanoparticles*

BY

Lamiaa M.A. Ali<sup>1,2</sup>, Victor sorribas<sup>2\*</sup>, Rafael Piñol<sup>1</sup>, Lierni Gabilondo<sup>1</sup>, Angel Millan<sup>1</sup>, Fernando Palacio<sup>1\*</sup>

<sup>1</sup> Institute of Material Science of Aragón. CSIC – Zaragoza University, and Condensed Matter Physics department. Faculty of Science, Zaragoza, Spain.

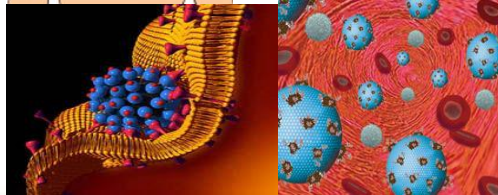
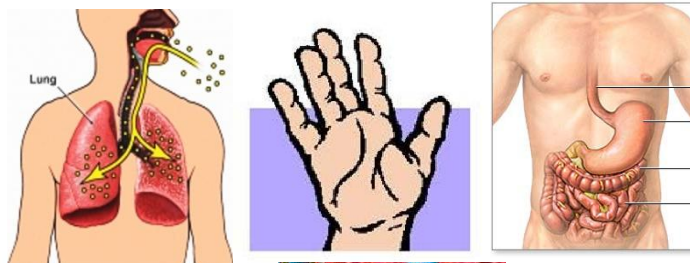
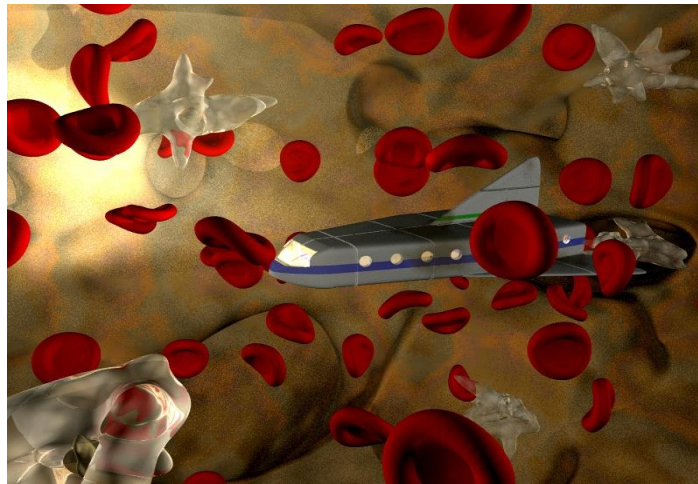
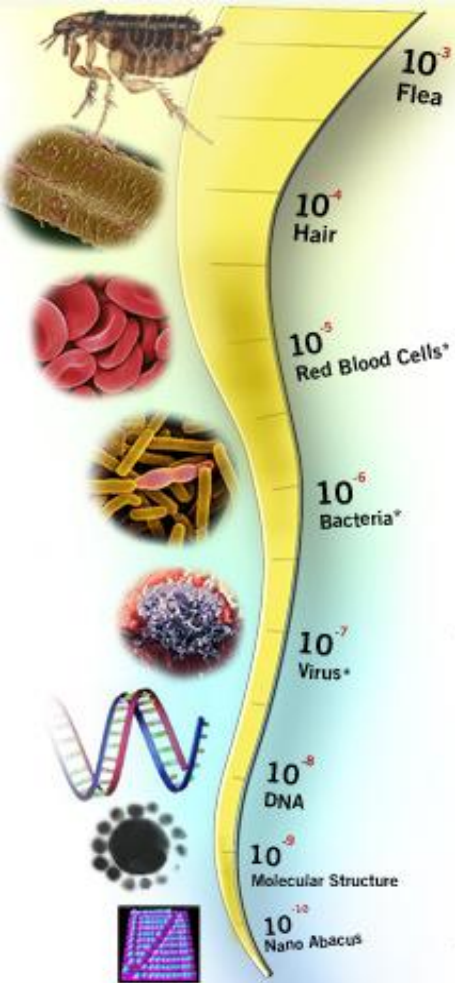
<sup>2</sup> Laboratory of Molecular Toxicology, Faculty of Veterinary, Zaragoza University, Zaragoza, Spain.



Nanotechnology

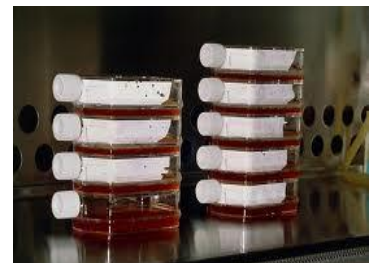
Nanomedicine

Nanotoxicology



*In Vitro*

*In Vivo*



# Types of nanomaterials

Nanomaterial	Trade Name	Application	Target	Adverse Effects	Manufacturer	Current Status
<b>Metallic</b>						
Iron oxide	Feridex	MRI contrast	Liver	Back pain, vaso-dilatation	Bayer Schering	FDA approved
	Resovist	MRI contrast	Liver	None	Bayer Schering	FDA approved
	Combidex	MRI contrast	Lymph nodes	None	Advanced Magnetics	In phase 3 clinical trials
	NanoTherm	Cancer therapy	Various forms	Acute urinary retention	MagForce	In phase 3 clinical trials
Gold	Verigene	In vitro diagnostics	Genetic	Not applicable	Nanosphere	FDA approved
	Aurimmune	Cancer therapy	Various forms	Fever	CytImmune Sciences	In phase 2 clinical trials
Nanoshells	Auroshell	Cancer therapy	Head and neck	Under investigation	Nanospectra Biosciences	In phase 1 clinical trials
<b>Semiconductor</b>						
Quantum dot	Qdots, EviTags, semiconductor nanocrystals	Fluorescent contrast, in vitro diagnostics	Tumors, cells, tissues, and molecular sensing structures	Not applicable	Life Technologies, eBioscience, Nanoco, CrystalPlex, Cytodiagnostics	Research use only
<b>Organic</b>						
Protein	Abraxane	Cancer therapy	Breast	Cytopenia	Abraxis Bioscience	FDA approved
Liposome	Doxil/Caelyx	Cancer therapy	Various forms	Hand-foot syndrome, stomatitis	Ortho Biotech	FDA approved
Polymer	Oncaspar	Cancer therapy	Acute lymphoblastic leukemia	Urticaria, rash	Rhône-Poulenc Rorer	FDA approved
	CALAA-01	Cancer therapy	Various forms	Mild renal toxicity	Calando	In phase 2 clinical trials
Dendrimer	VivaGel	Microbicide	Cervicovaginal	Abdominal pain, dysuria	Starpharma	In phase 2 clinical trials
Micelle	Genexol-PM	Cancer therapy	Various forms	Peripheral sensory neuropathy, neutropenia	Samyang	For phase 4 clinical trials

## Theranostic tool

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graph LR; A[Theranostic tool] --- B[Diagnosis]; A --- C[Therapy];
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### Diagnosis

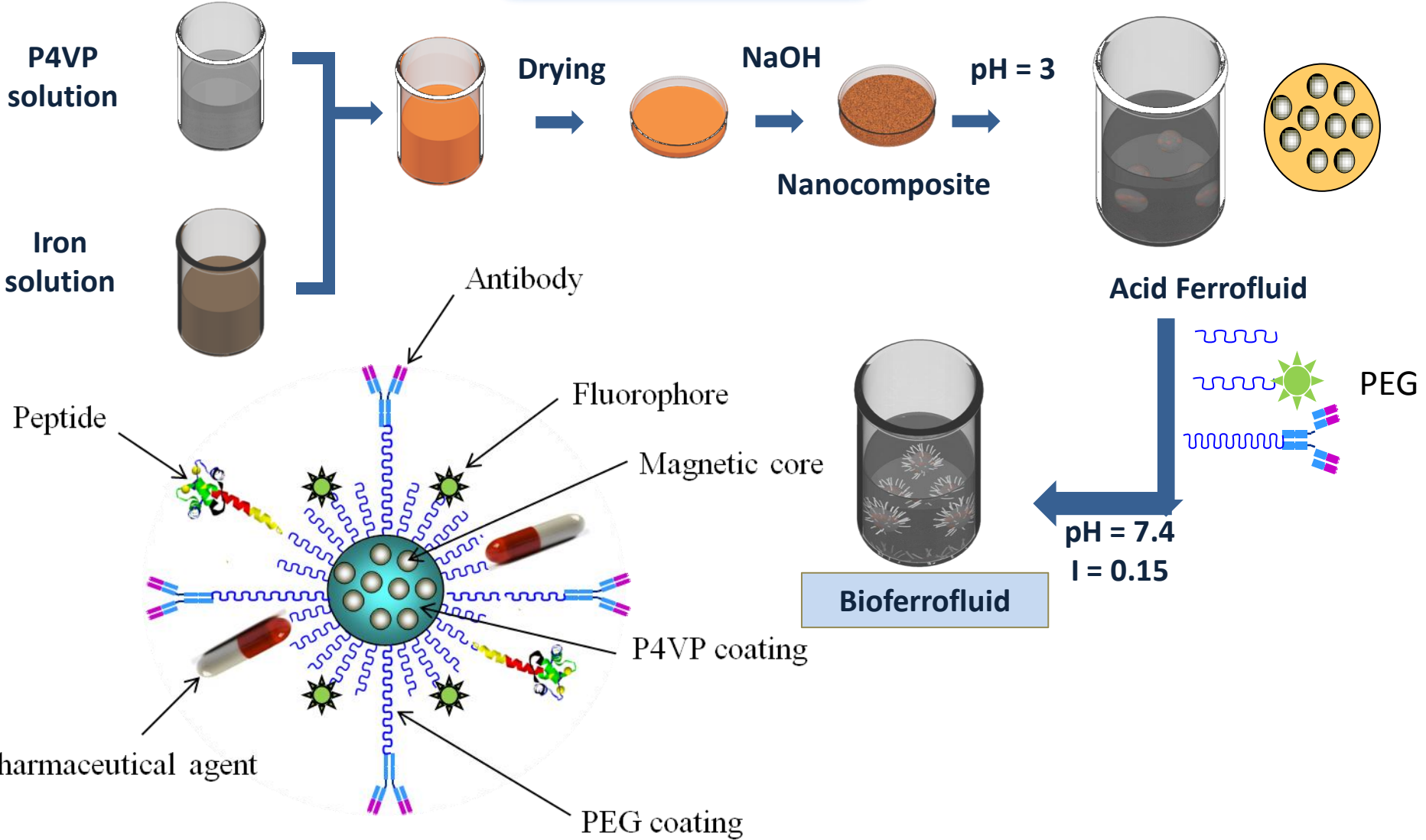
- MRI contrast agent:
  - \* Cancer imaging
  - \* Stem cells tracking  
(regenerative medicine)
  - \* Monitoring the transplanted tissues.
- Bio detection of pathogens
- Detection of protein

### Therapy

- Hyperthermia.
- Drug and gene delivery
- Detoxification.
- Immunization.
- Cell separation  
(cellular therapy) .

# SPIONs preparation

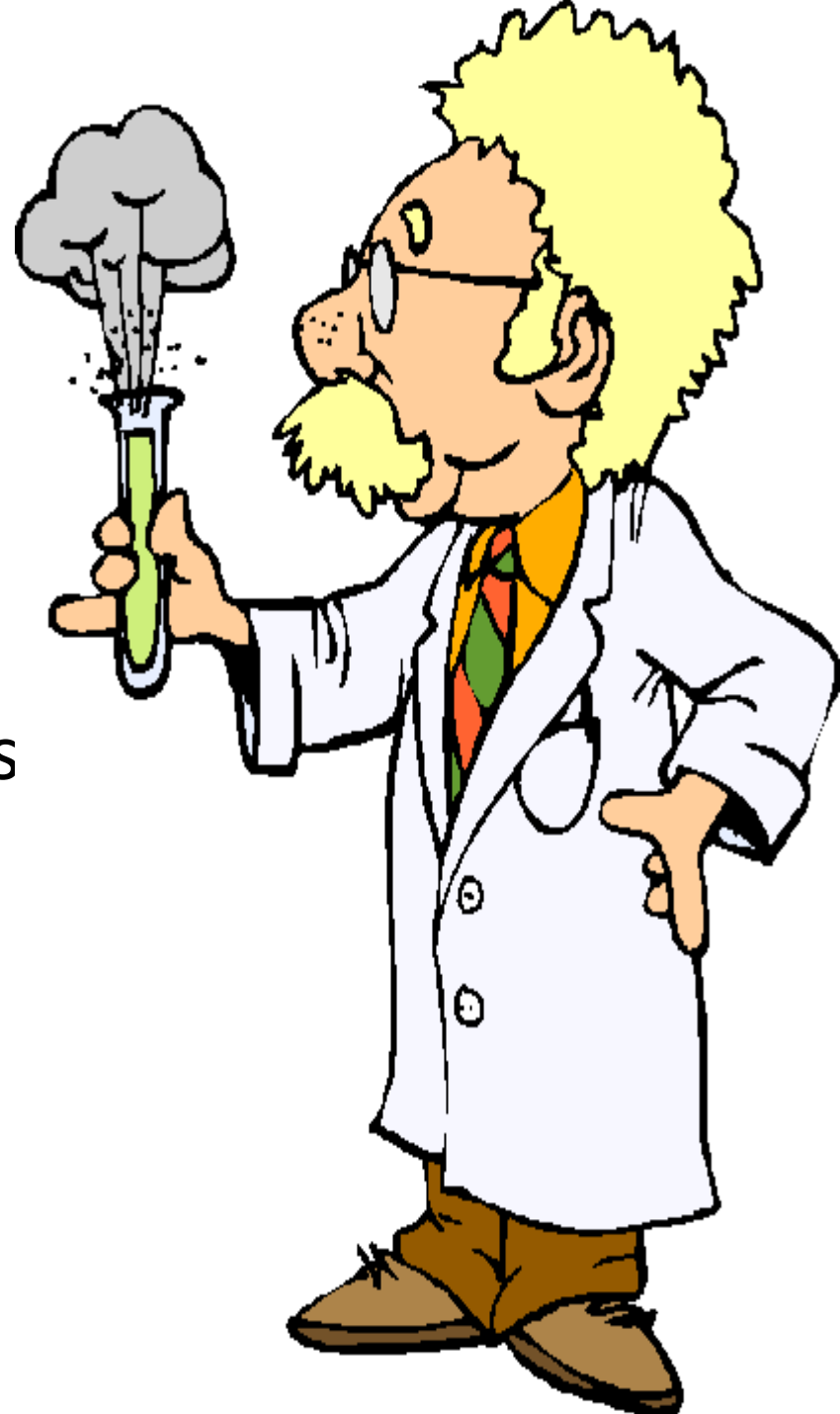
## POLYMERIC ROUTE



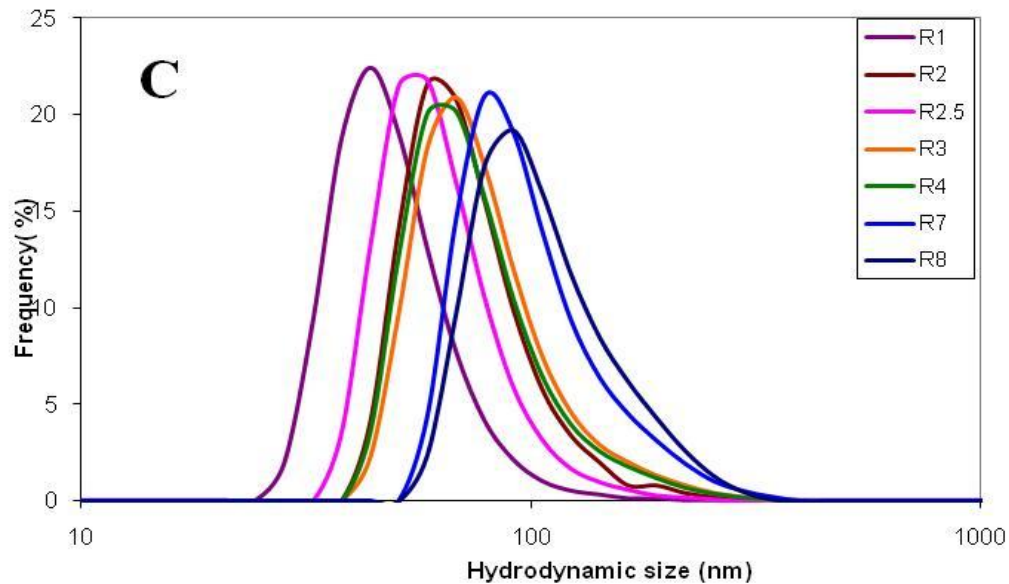
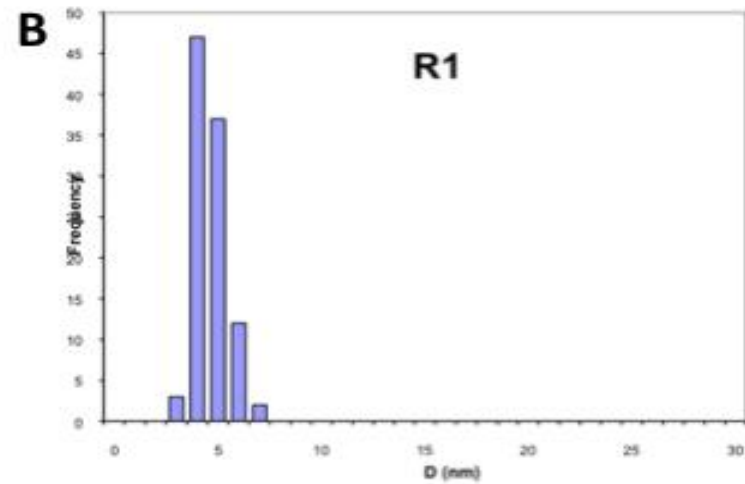
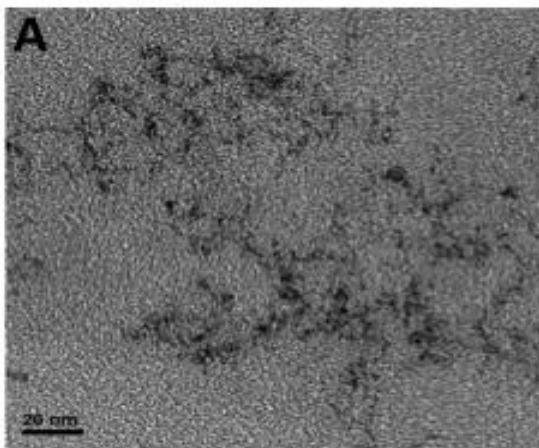
•Millan A, Palacio F, Falqui A, Snoeck E, Serin V, Bhattacharjee A, Ksenofontov V, Gütlich P, Gilbert I. Maghemite polymer nanocomposites with modulated magnetic properties. *Acta Mater* 2007; 55: 2201.  
•Millan A, Palacio F, Ibarz G, Natividad E. Systems containing magnetic nanoparticles and polymers, like nanocomposites and ferrofluids, and their applications. Patent ES2308901B1



- **Controlling polydispersity** nanomaterial is crucial, otherwise different batches of the same nanomaterial might display different behaviours inside the cell.



	Core diameter (nm)	Hydrodynamic diameter (nm)
R1	4.1	50.5
R2	7.4	64.9
R2.5	8.8	75.5
R3	9.4	79.4
R4	12.1	82.1
R7	13.3	104.0
R8	14.4	112.0

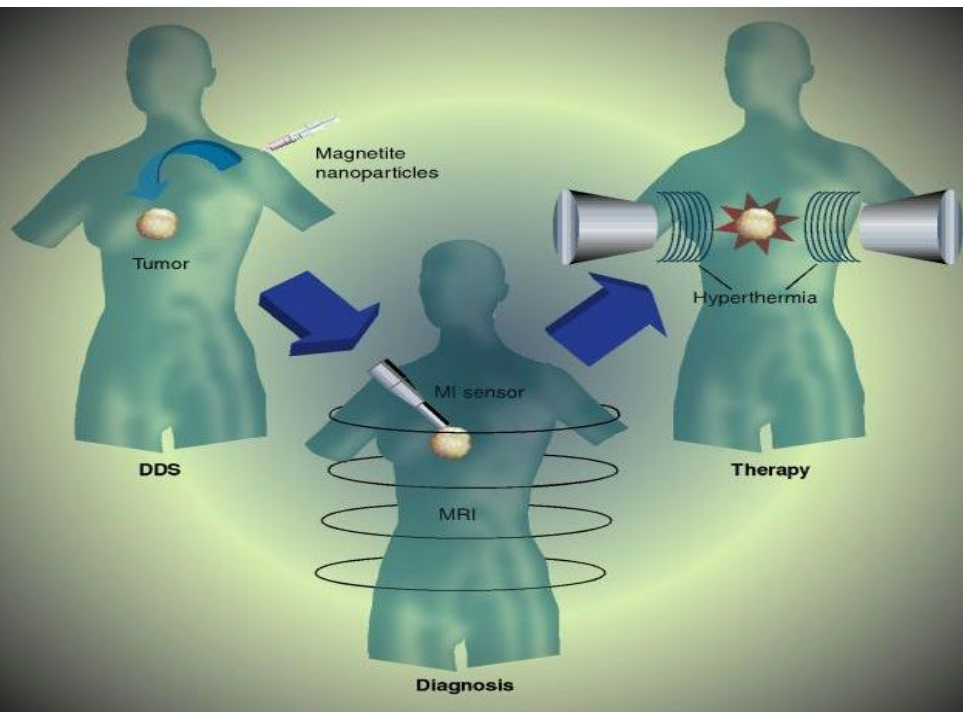


**Fig 1.** Analysis of ferrofluids. A. TEM images of iron oxide nanoparticles in the ferrofluid (R1) . B. Size distribution of spherical iron oxide nanoparticles. C. The hydrodynamic diameter of all R series.



## ▶ Rationale

To study the toxicological behaviour of our ferrofluids *in vitro* and *in vivo*, in order to introduce them in the market as a therapeutic tool (Hyperthermia) or as a diagnostic tool (MRI contrast agents).



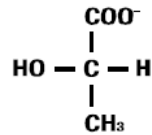
# Cytotoxicity



# LDH ASSAY

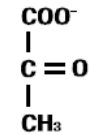
- Colorimetric assay.

1. Step:



lactate

LDH

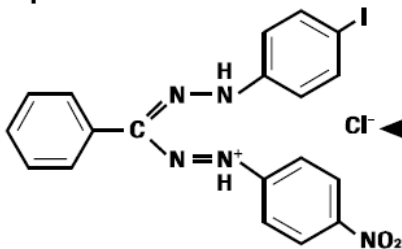


pyruvate

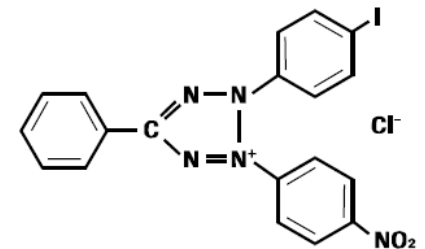
NAD<sup>+</sup>

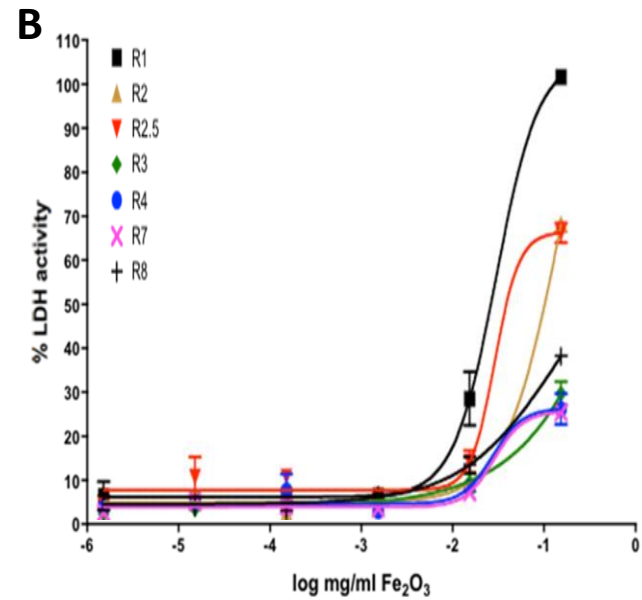
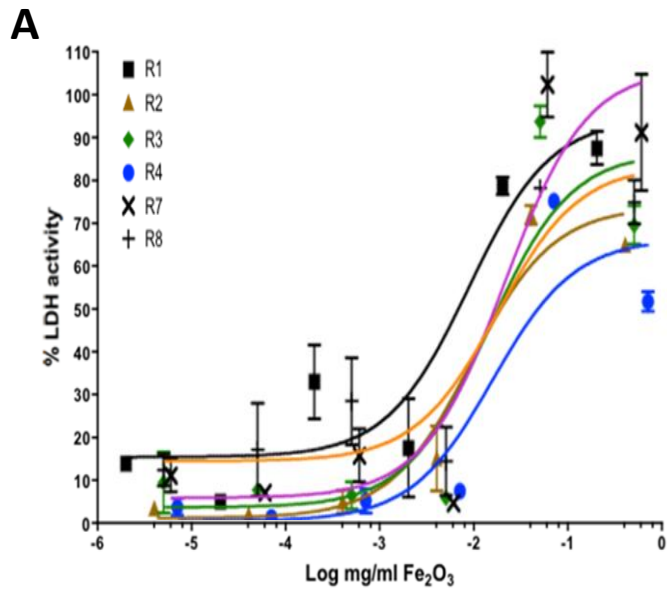
NADH + H<sup>+</sup>

2. Step:



Catalyst





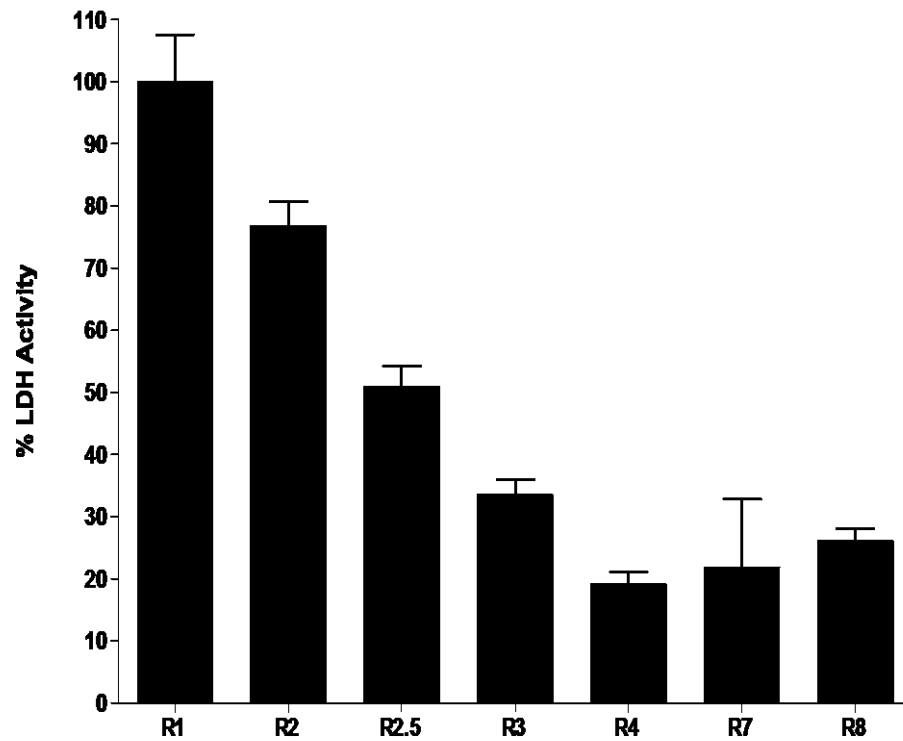
**Fig 2.** LDH activity measurement for 2 cell lines, VSMC (A) and OK cells (B) after 24h of incubation with different conc. of ferrofluids.

LC50 for VCMC.

	Day 1	Day 2	Day 3	Day 4	Day 5
R1	0,0093	0,0136	0,0089	0,0087	0,0095
R2	0,0099	0,0092	0,0084	0,0082	0,0076
R2.5	0,0025	0,0037	0,0096	0,0020	0,0036
R3	0,01581	0,0140	0,0146	0,0135	0,0224
R4	0,01915	0,0156	0,0112	0,0100	0,0073
R7	0,02686	0,0208	0,0198	0,0254	0,0220
R8	0,02363	0,0231	0,0174	0,0185	0,0166

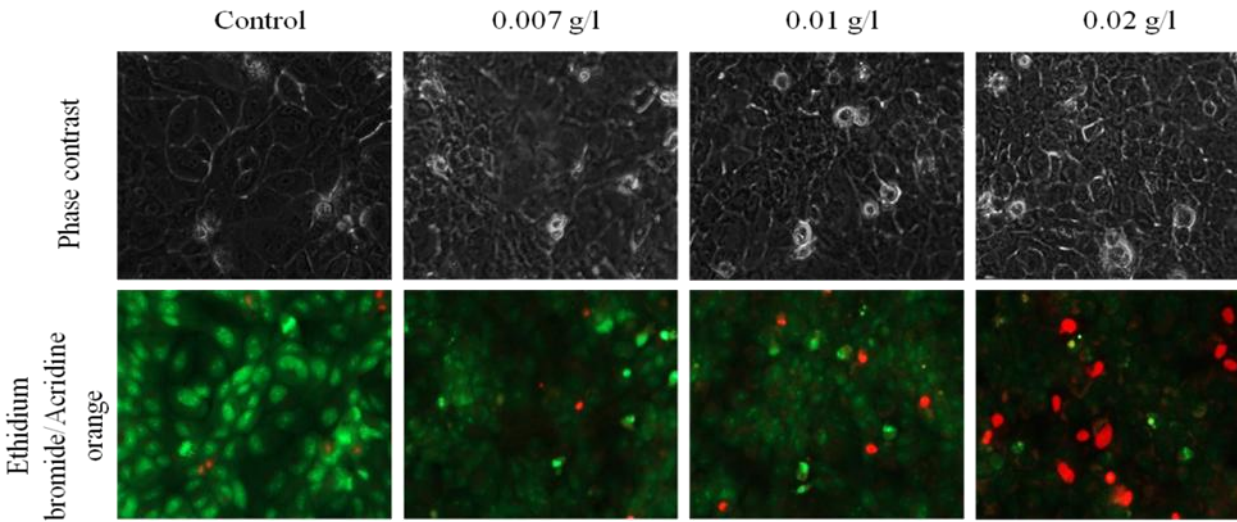
LC50 for OK cells.

	R1
Day 1	0.0896
Day 2	0.0407
Day 3	0.0312
Day 4	0.0292
Day 5	0.0279



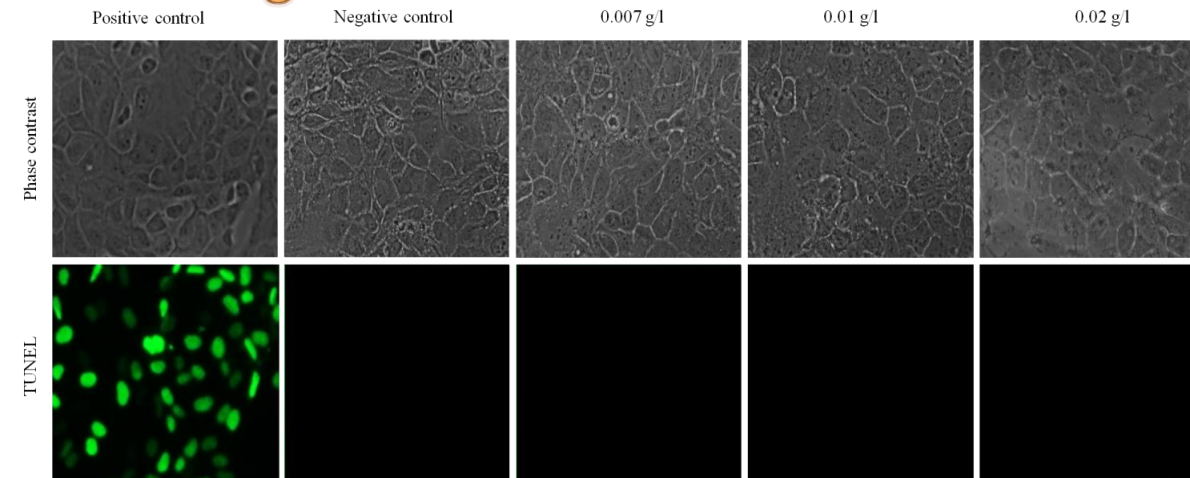
**Fig 3.** Effect of the nanoparticle diameter on the OK cell death after 7 days of incubation with 0.01 g/l  $\text{Fe}_2\text{O}_3$

## Acridine Orange/Ethidium bromide



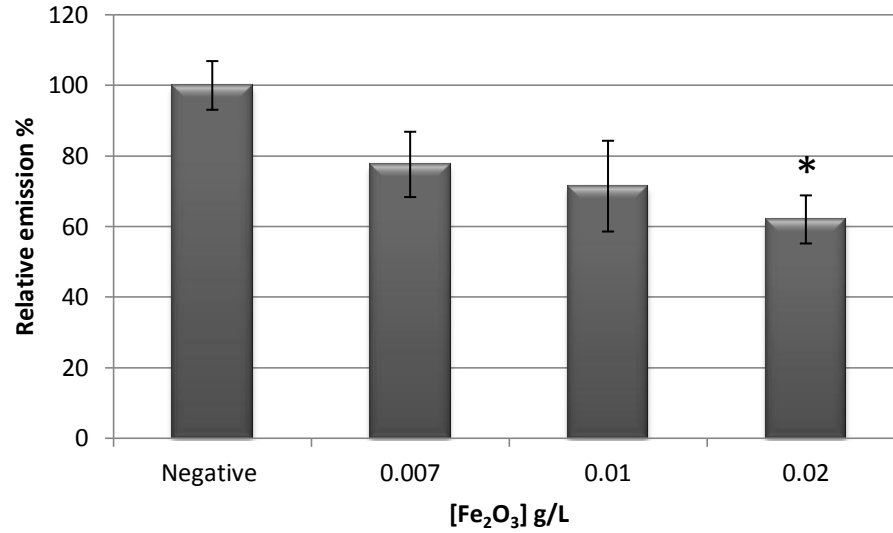
**Fig 4.** Fluorescence micrographs of AO/EB stained OK cells treated with R1 nanoparticles using the indicated concentration of nanoparticles for 24h

## DNA Fragmentation



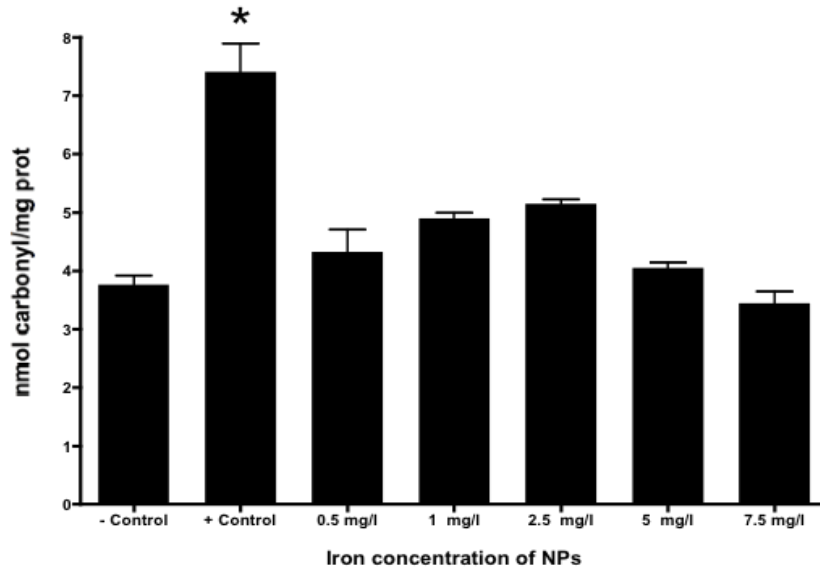
**Fig 5.** Fluorescent micrographs (magnification $\times 40$ ) of DNA fragmentation stained OK cells treated with different conc. of R1 for 24h.

# Caspase 3



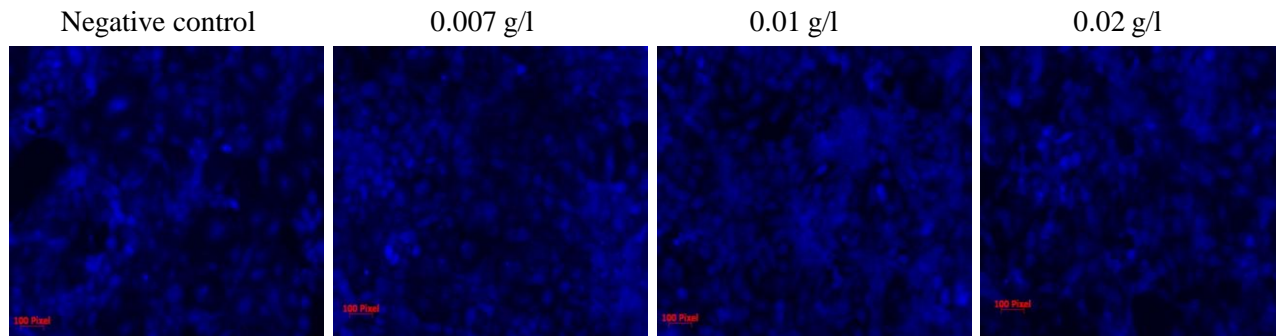
**Fig 6.** Caspase 3 relative emission measurement in OK cells after 24h of incubation with the indicated conc. of R1.

# Oxidative stress



**Fig 7.** Colorimetric determination of carbonyl protein content using different concentrations of R1 nanoparticles for 24 hours.

# Glutathion (GSH) level



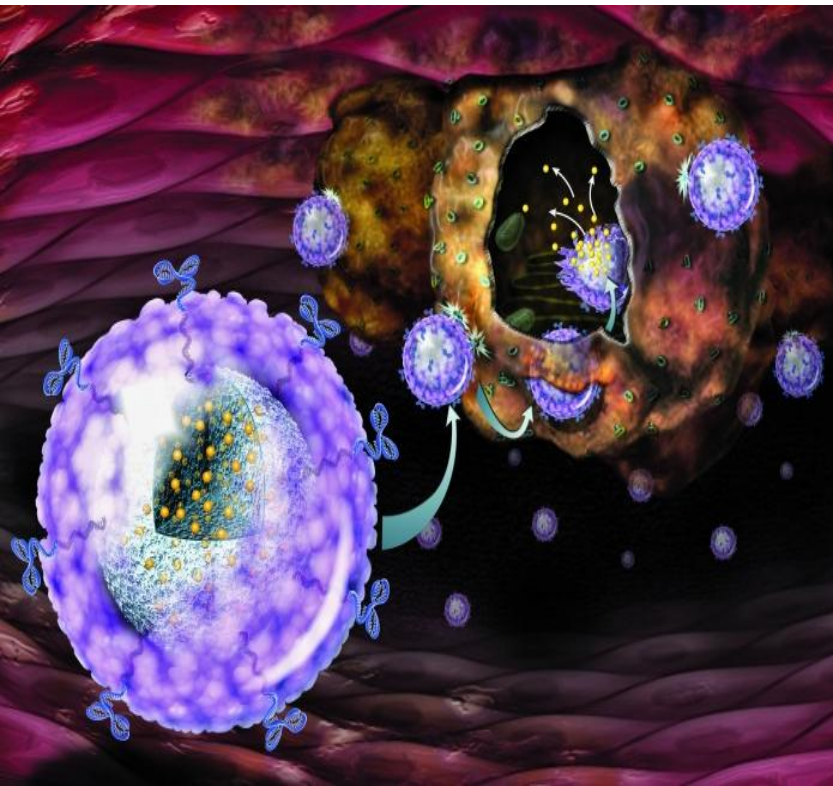
**Fig 8.** Fluorescence micrographs of Thiotracker stained OK cells treated with R1 nanoparticles using the indicated concentration of nanoparticles for 24h



# CONCLUSIONS

- The nanoparticles toxicity is higher in the VSMC in compared to OK cells.
- R1 is the more toxic than R8 (size dependent toxicity).
- Our nanoparticles causing cell death by necrosis .
- No oxidative stress action was detected after 24h of nanoparticles incubation with OK cells.

# Studies at the nano-biointerface level (Uptake, uptake kinetics, and the mechanism of endocytosis)



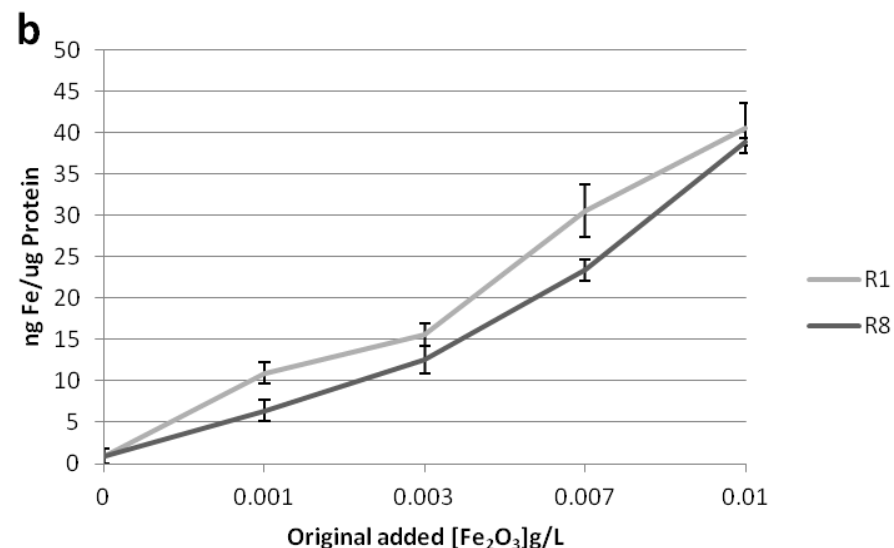
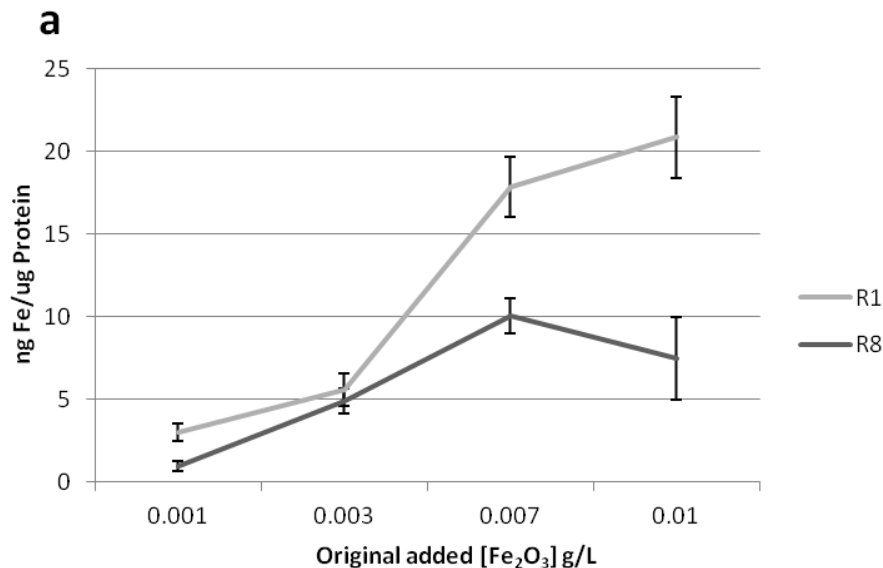
Good understanding of the nano-bio interface interactions helps to :

1. Disclose valuable information related to some nanomaterial implications as toxicity.
2. Determine the intracellular fate of the nanomaterial.
3. Design an efficient drug delivery system capable of escaping from unfavorable cellular conditions (e.g. lysosomes).
4. Improve the subcellular targeting research area in the near future.

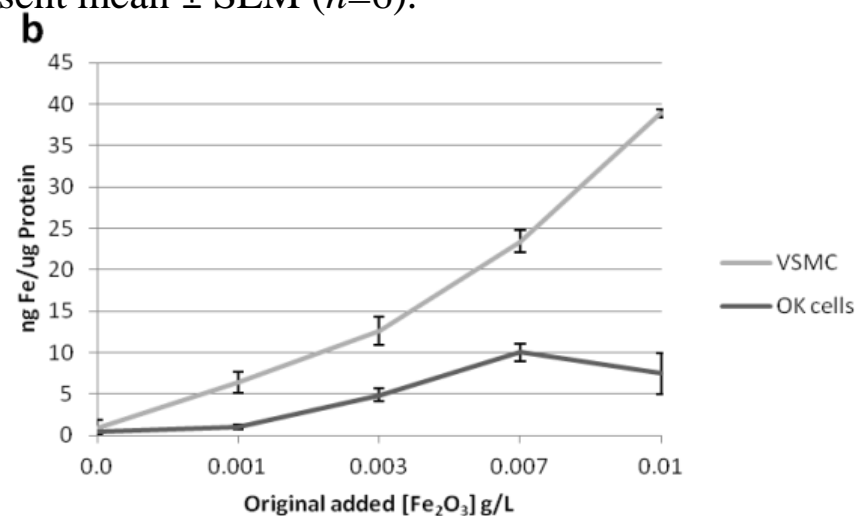
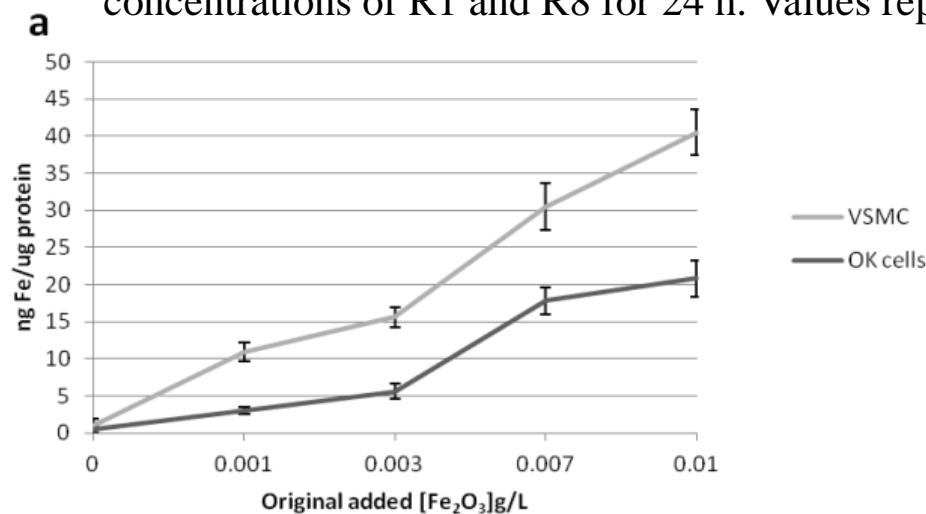
Understanding the interactions of these materials with cells is crucial for improving their behaviour in-vivo and in-vitro. Especially uptake into cells and degradation in intracellular compartments is of high importance. ]

# Factors affect on the nanomaterials uptake

1. Nanomaterial physicochemical properties (shape, size, surface charge, surface charge density, and surface charge arrangement)
2. Suspending medium ( pH, Ionic strength, temperature, and presence of large organic molecules as proteins)
3. Cell type, and cell density.

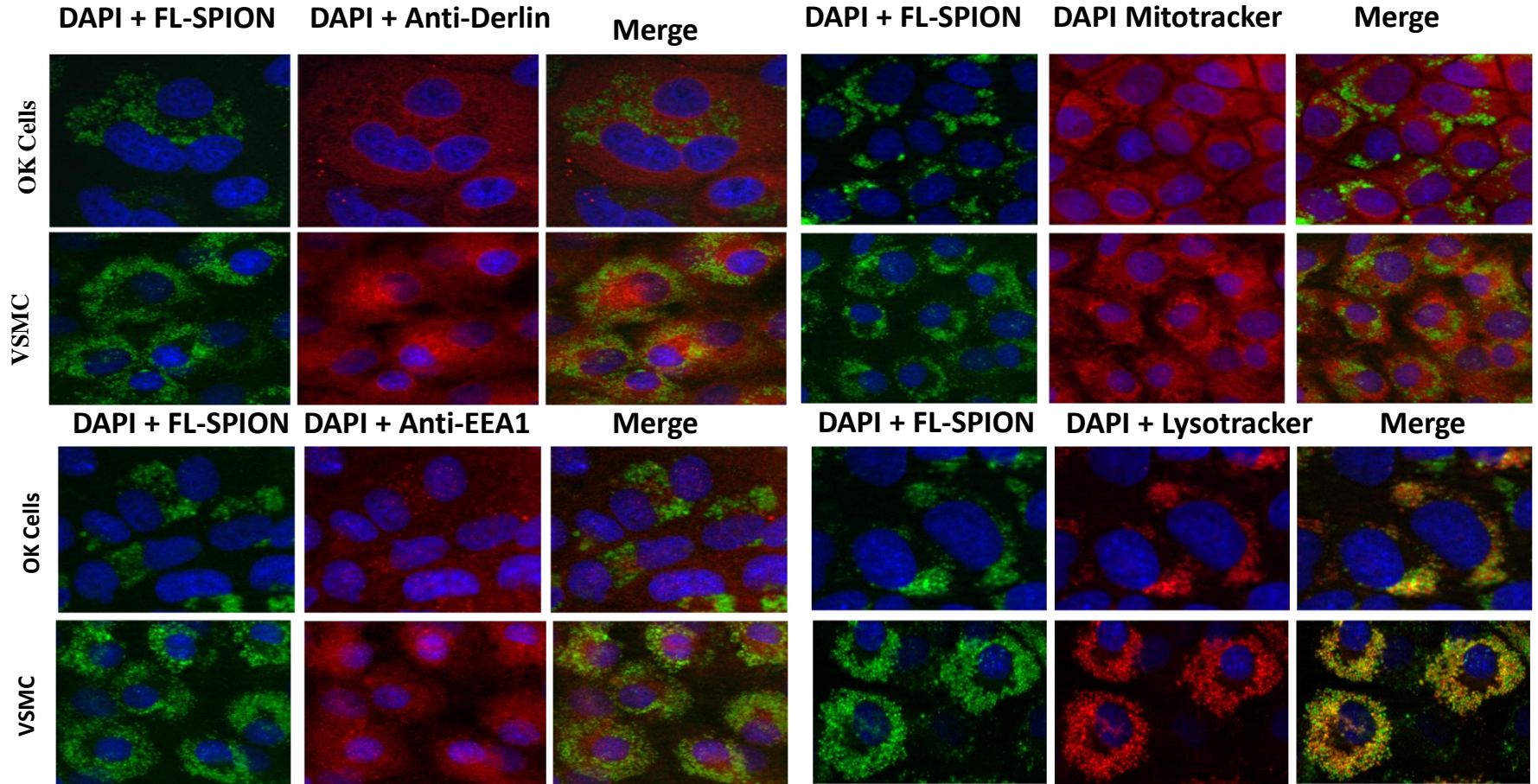


**Fig 9.** Quantitative iron detection for OK cells (a) and VSMC (b), after incubation with different concentrations of R1 and R8 for 24 h. Values represent mean  $\pm$  SEM ( $n=6$ ).



**Fig 10.** Effect of cell type on the nanoparticles uptake. Both cell lines (OK and VSMC) were incubated with different concentrations of R1 (a) and R8 (b) for 24 h. Values represent mean  $\pm$  SEM ( $n=6$ ).

# Subcellular localization



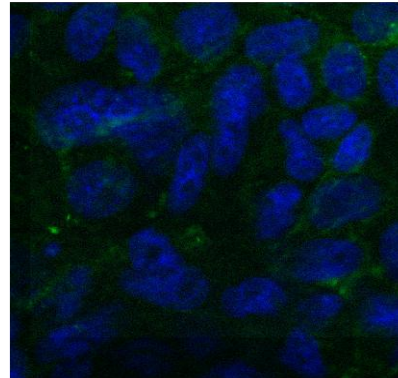
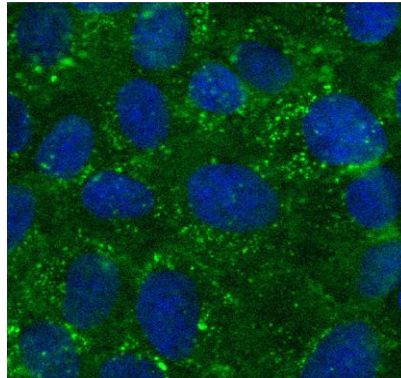
**Fig 11.** Subcellular localization of nanoparticles using markers of the endoplasmic reticulum (Anti-Derlin Ab), mitochondria (Mitotracker), early endosomes ( Anti-EEA1), endolysosomal compartments (lysotracker) by fluorescence microscopy. Cells were treated with fluorescent nanoparticles (R8) at 0.007 g/l  $\text{Fe}_2\text{O}_3$  for 24 h.

# Endocytosis mechanism

Control

K<sup>+</sup>-depletion

Potassium depletion

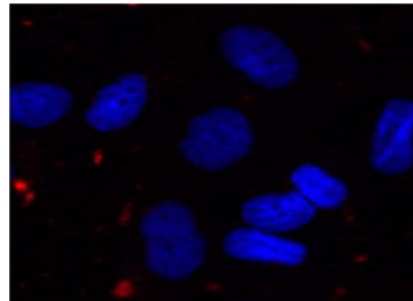
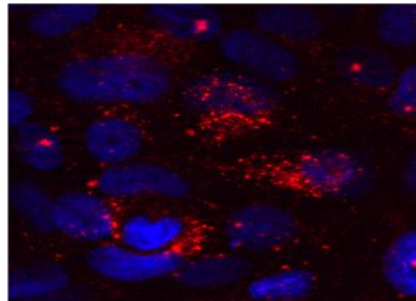


Control

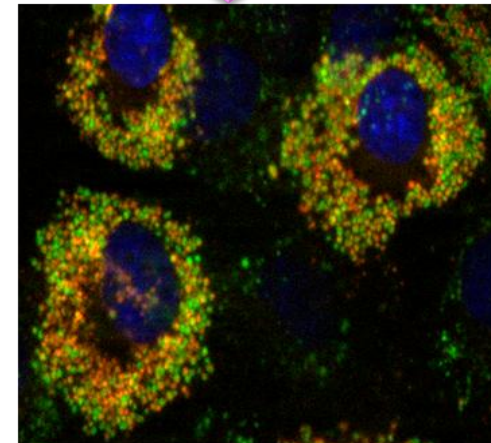
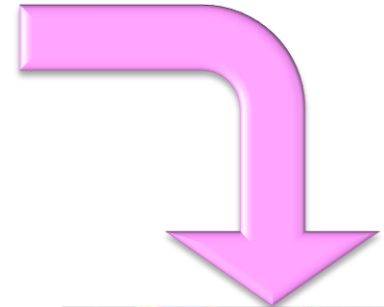
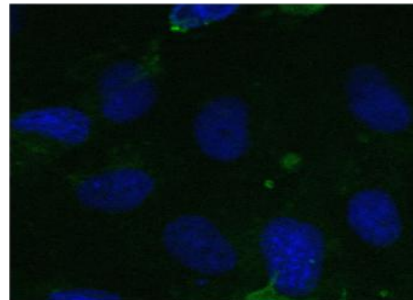
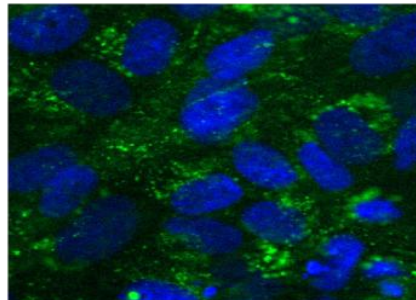
Chlorpromazin

Clathrin coated pit inhibitor

Transferrin



Nanoparticles



# CONCLUSIONS

- The size dependent uptake was conspicuous in each cell line (OK or VSMC), since R1 internalized more than R8 into the cells. This could explain the toxic effect associated with R1 compared to R8 in both cell lines.
- A cell type dependent nanoparticle (R1, R8) uptake was indicated, since VSMC taken up more nanoparticles in compared to OK cells.
- Fluorescent nanoparticles were not found in the endoplasmic reticulum, early endosomes, mitochondria or the nucleus. They are, however, observed inside the endo-lysosomal compartments (lysosomes and late endosomes) after 24 hours of incubation in both cell lines (OK and VSMC).
- The existence of fluorescent R8 in the endo-lysosomal compartments suggests the mechanism that is responsible for the nanoparticle internalization which is Clathrin dependent endocytosis that was confirmed with the help of pharmacological inhibitors.



# Special thanks



Prof. Victor Sorribas



Prof. Fernando Palacio



Prof. Angel Millan



Rafa Piñol



Lierni Gabilondo

"Everything is poison, there is  
poison in everything. Only  
the dose makes a thing not a  
poison."

**Thanks for your attention**

Even I



Paracelsus

"The father of toxicology"



## Gloomy Forecast for States, Even if Economy Rebounds

Task Force Finds Problems Deep and Often Masked

By MARY WILLIAMS WALSH and MICHAEL COOPER

WASHINGTON — The fiscal crisis for states will persist long after the economy rebounds as they confront rising health care costs, underfunded pensions, ignored infrastructure needs, eroding revenues and expected federal budget cuts, according to a report issued here Tuesday by a task force of respected budget experts.

The problems facing states are often masked by lax budget laws and opaque accounting practices, according to the report, an independent analysis of six large states released by the State Budget Crisis Task Force.

It said that the financial collapse of 2008, which caused the most serious fiscal crisis for states since the Great Depression, exposed deep-set financial challenges that will worsen if no

action is taken.

"The ability of the states to meet their obligations to public employees, to creditors and most critically to the education and well-being of their citizens is threatened," warned the chairmen of the task force, Richard Ravitch, a former lieutenant governor of New York, and Paul A. Volcker, a former chairman of the Federal Reserve.

The report added a strong dose of fiscal pessimism just as many states have seen their immediate budget pressures begin to ease. And it called into question how states will restore the services they have cut during the downturn, saying that the loss of jobs in prisons, hospitals, courts and agencies have been more severe than in any of the past nine recessions.

"This is a fundamental shift in the way governments have responded to recessions and appears to signal a willingness to 'unbuild' state government in a way that has not been done before," it said, noting that court systems had cut their hours in many states, delaying actions including divorce settlements and criminal trials.

The report arrived at a delicate political moment. States are deciding whether to expand their Medicaid programs to cover the uninsured poor as part of the new health care law, with the federal government pledging to pay the full cost at first. Public-sector unions feel besieged, as states and cities from Wisconsin to San Jose, Calif., have moved to save money on pensions. And Washington's focus on deficit reduction — with big budget cuts scheduled for after the fall election — has made cuts to state aid inevitable.

Continued on Page A3

## Lamiaa M.A. Ali is searching for a post-doc position



Lamiaa has started her searching journey, Email: [miss\\_limo@yahoo.com](mailto:miss_limo@yahoo.com) or [limo@unizar.es](mailto:limo@unizar.es)

## At Fiscal Cliff, Anti-Tax Vow Gets New Look

By JONATHAN WEISMAN

WASHINGTON — Senate Democrats — holding firm against extending tax cuts for the rich — are proposing a novel way to circumvent the Republican pledge not to vote for any tax increase: Allow all the tax cuts to expire Jan. 1, then vote on a tax cut for the middle class shortly thereafter.

The proposal illustrates the lengths lawmakers are going to in an effort to include new federal revenues in a fix for the "fiscal cliff," the reckoning in January that would come when all Bush-era tax cuts expire, and automatic

## Christie's Brashness Blunts Hopes to Join Ticket

By KATE ZERNIKE

WASHINGTON — Chris Christie asked for an audience at the Brookings Institution last week, and being one of the nation's most popular and provocative governors, got one.

Before a capacity crowd, he contrasted his commanding executive style with an "alooof" president. With his usual blend of bombast, self-deprecation and choreographed frankness, he po-

LOOKING OUT FOR NO. 2

Aides to Mitt Romney try to leave no document unturned in vetting possible running mates. Page A16.

sitioned himself as a master of compromise who could straight-talk Washington out of its partisan torpor.

The appearance was a reminder why some prominent conservatives and Republican donors have been promoting Mr. Christie as the perfect running

mate for Mitt Romney. He has a record of fiscal conservatism in a moderate state. His aggressive approach to political combat has excited the Tea Party contingent, but he also has influential allies on Wall Street and in other power centers of the Republican Party. His rumpled, real-guy persona would complement Mr. Romney's wealthy, pressed-jeans image.

Democrats in New Jersey, his home state, mocked his address at Brookings as the "Tampa Try-Continued on Page A24

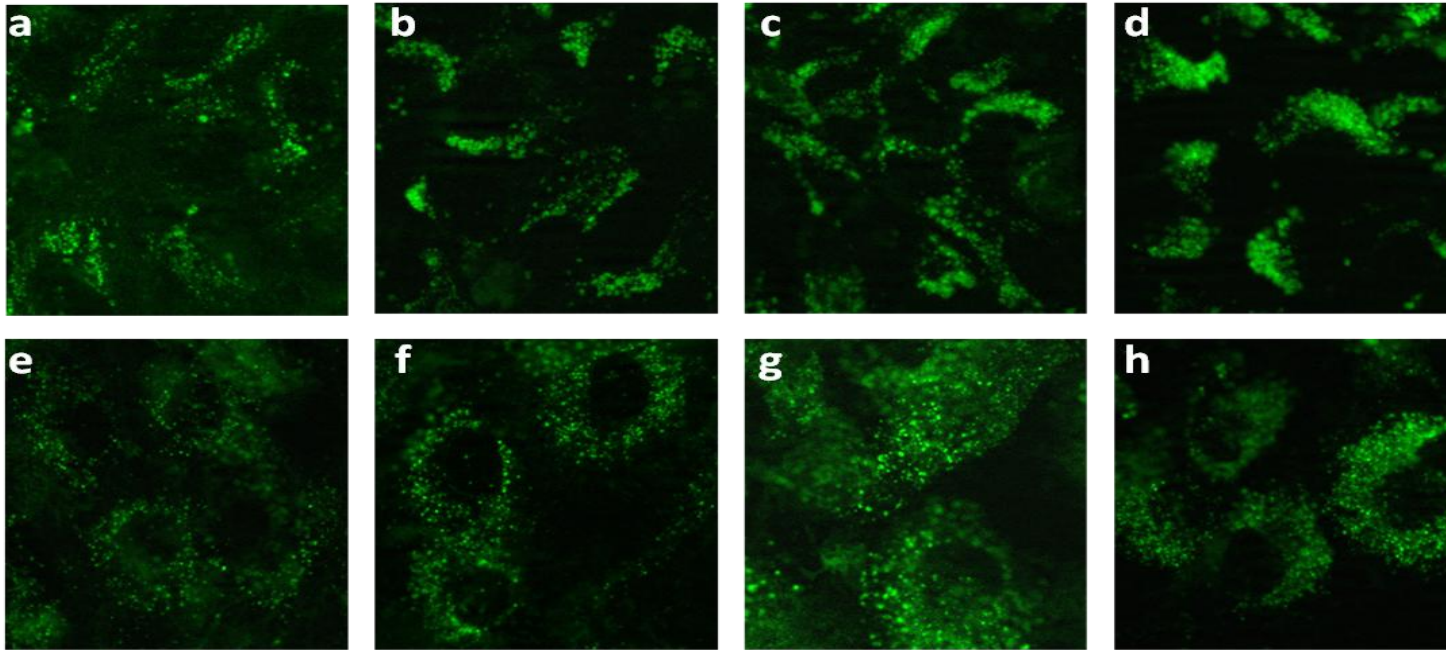
## Refugees Escape Hard-Liners From North

By ADAM NOSSITER

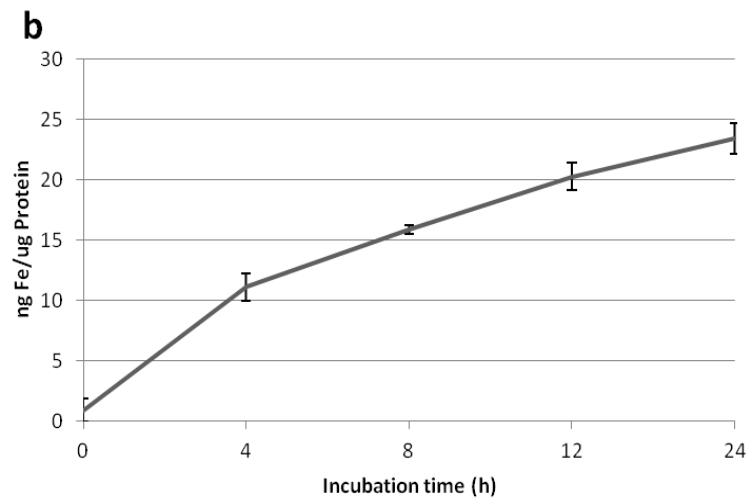
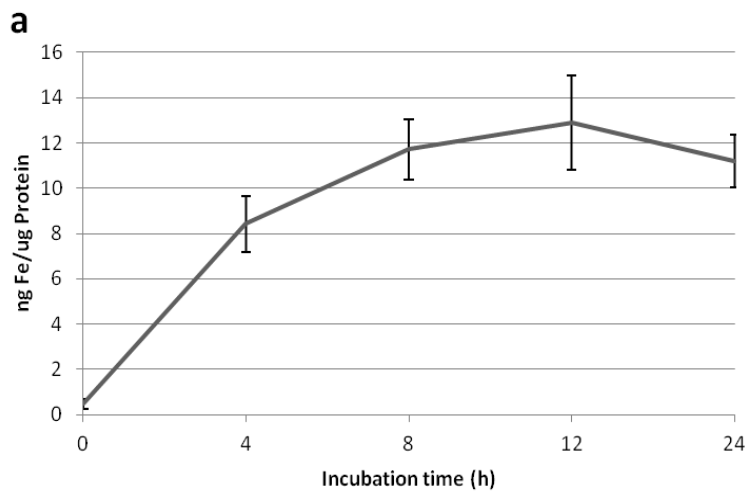
MBERA, Mauritania — The vast desert expanse of northern Mali has become a magnet for Islamic extremists who have tightened their grip on Timbuktu and other far-flung towns, imposing a strict form of justice that is prompting tens of thousands of people to flee what some are likening to an African Afghanistan.

Rattled recent arrivals at a

# Uptake kinetics

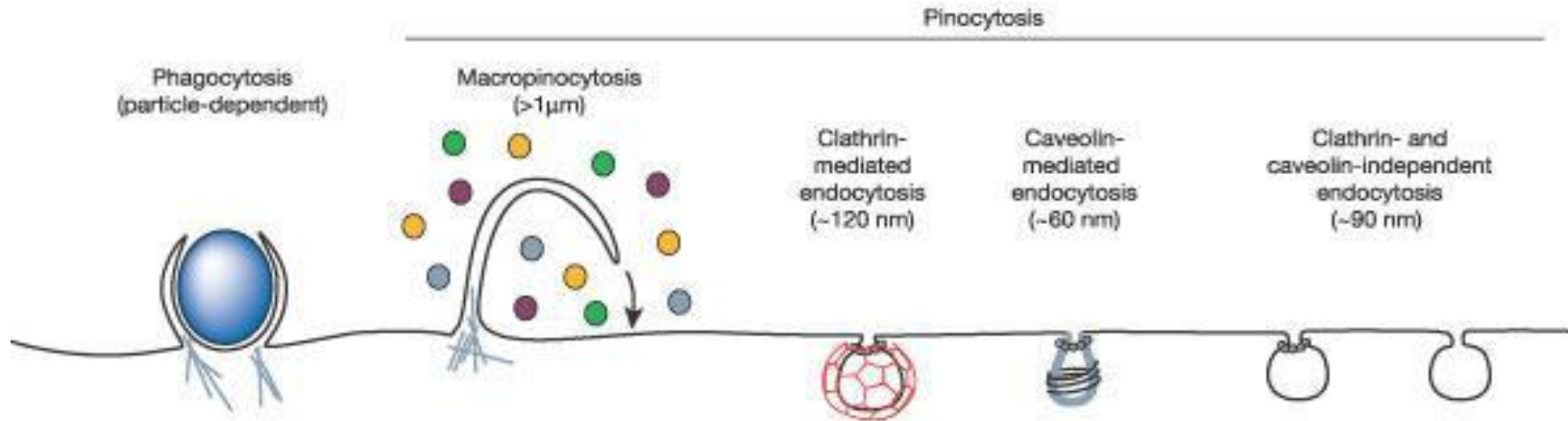


Internalization of fluorescently labeled R8 (112nm) in OK and VSMC at different time points of incubation. Upper panel is OK cells after 4h (a), 8h (b), 12h(c), and 24h (d) of incubation, lower panel is VSMC after 4h (e), 8h (f), 12h (g), and 24h (h) of incubation.



Time dependent uptake of R8 in OK cells (a) and VSMC (b). Values represent mean  $\pm$  SEM ( $n=6$ ).

# Endocytosis



Different categories of endocytosis mechanisms