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Screening the toxicity of manufactured nanoparticles in cell lines

María Luisa Fernández-Cruz

Department of Environment – DETC group
National Institute for Agriculture and Food Research and Technology (INIA) – Madrid – Spain
fcruz@inia.es

COMMISSION RECOMMENDATION (2011/696/EU) of 18 October 2011 on the definition of nanomaterial

✓ Nanomaterial (NM)

A natural, incidental or manufactured material containing **particles**, in an unbound state or as an **aggregate** or as an **agglomerate** and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm.

In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %.

By derogation from the above, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials.

✓ Particle/Nanoparticle (NP)

A minute piece of matter with defined physical boundaries (one or more external dimensions is in the size range 1 nm - 100 nm).

✓ Agglomerate

A collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components.

✓ Aggregate

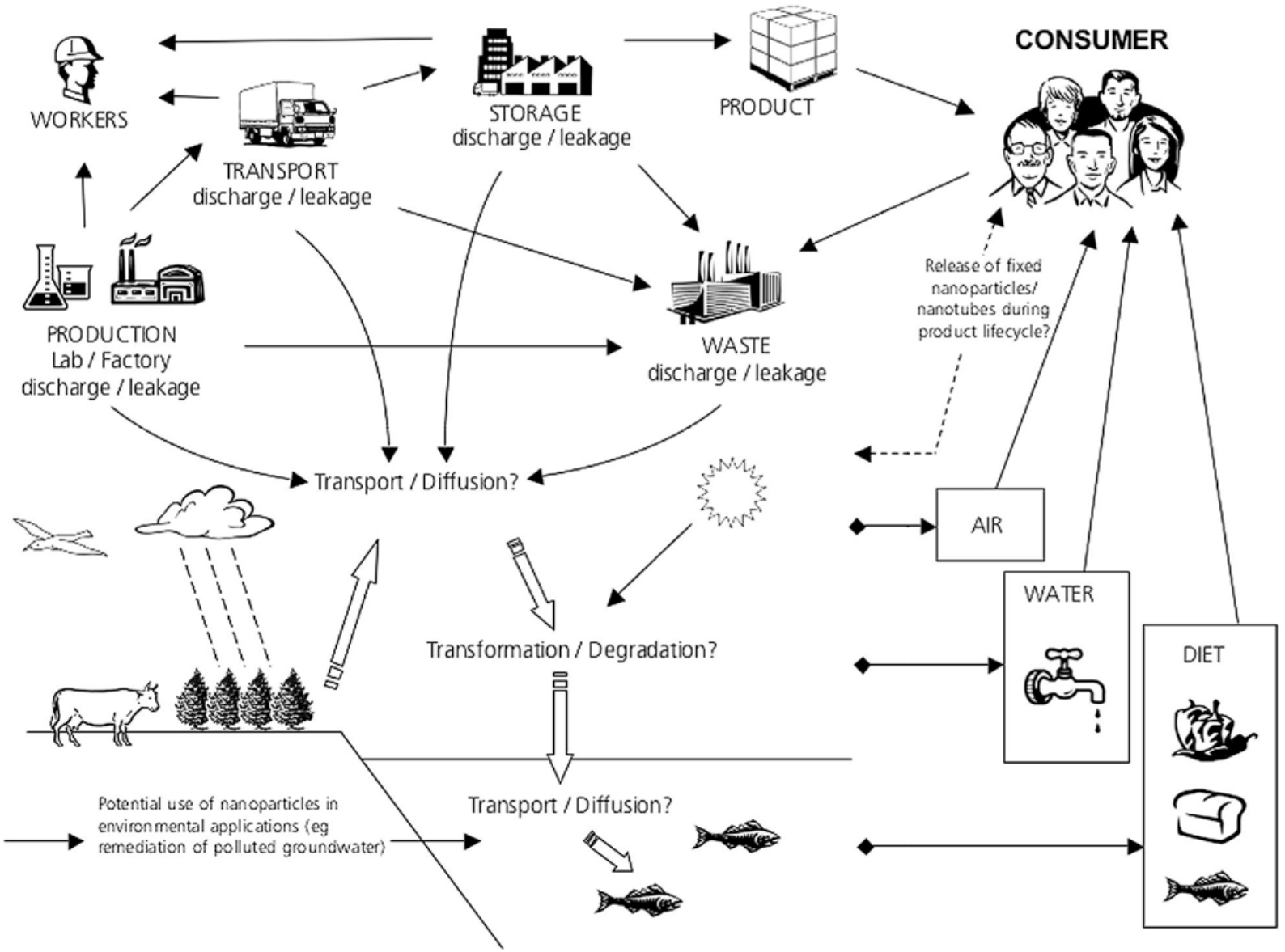
A particle comprising of strongly bound or fused particles.

Current applications

- ✓ Healthcare
  - ➢ Targeted drug delivery
  - ➢ Regenerative medicine
  - ➢ Diagnostics
- ✓ Cosmetics
- ✓ Electronics
- ✓ Textiles
- ✓ Paints and coatings
- ✓ Information technology
- ✓ Environmental protection
- ✓ Food and food packaging
- ✓ Aerospace…..

EPA,http://www.rsc.org/chemistryworld/News/2010/June/30061001.asp

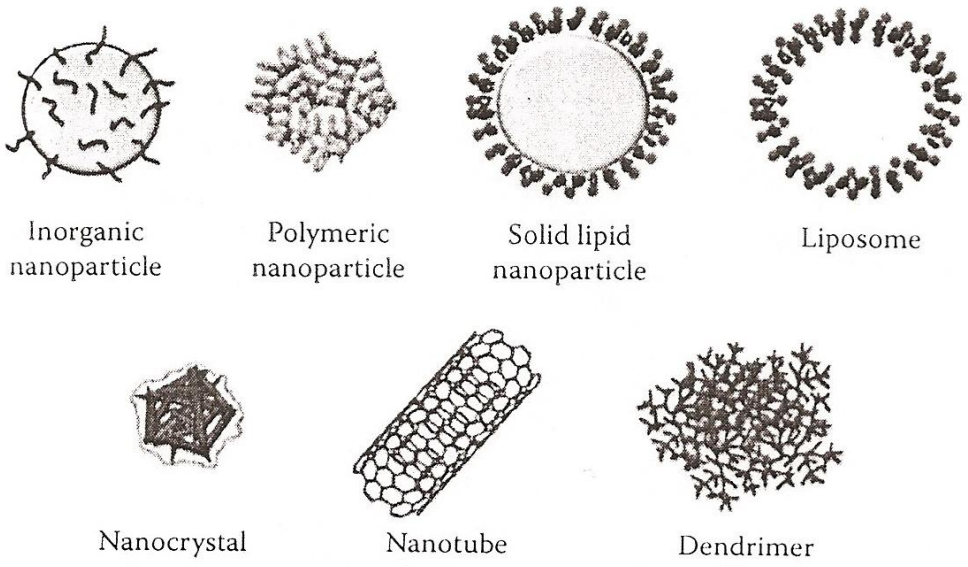
Possible exposure routes for nanomaterials



Source: Royal Society (2004). Nanoscience and nanotechnologies. Oportunities and uncertainties. London: Royal Society, p.37.

Classification of Nanomaterials

Three nano-dimensions (1-100 nm)	Two nano-dimensions (1-100 nm)
Nanoparticles Nanocapsules Fullerenes Dendrimers Quantum dots Nanostructures Nanopore	Nanofibers Nanowires Nanotubes
	One nano-dimension (1-100 nm)
	Nano thin-film



Medical Nanotechnology and Nanomedicine by Harry Tibbals, 2011

Physico-chemical properties of a NM for a same composition

Size

Surface area

Shape

Coating

Solubility

Agglomeration

Aggregation

Ion release

Critical NM Interactions

Biological transformations

Interaction with macromolecules

Physical and chemical transformations

Regulatory context

REACH: REGULATION (EC) No 1907/2006 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC

CLP: REGULATION (EC) No 1272/2008 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006

Although there are no explicit requirements for NMs under **REACH** and **CLP**, they meet the regulation's substance definition and therefore the provisions apply. The substance definition under these regulations indicates that:
"Substances, including substances at the nanoscale, manufactured or imported in volumes of 1 tonne or more per year have to be registered under **REACH**". The information required in the registration is set out in Annexes VI-X of REACH and increases with the tonnage manufactured or imported.

Other Regulations where special mention is done to NMs

Biocides, Water Framework Directive, Groundwater Directive, Drinking Water Directive, Urban Waste Water Directive,Sewage Sludge Directive , Landfill Directive, Waste Framework Directive, Air Quality Directive, Industrial Emissions Directive, Cosmetics, …

Environmental Risk Assessment (ERA) under REACH

Exposure compartments

- ✓ Surface water
- ✓ Groundwater
- ✓ Marine water
- ✓ Sewage treatment plants
- ✓ Soil
- ✓ Sediment
- ✓ Air
- ✓ Secondary poisoning

Fate

- ✓ Persistence/Degradation
- ✓ Bioavailability (adsorption/desorption)
- ✓ Distribution and bioaccumulation

Predicted environmental concentration (PEC)

Ecotoxicity

- ✓ Short-term aquatic ecotoxicity (invertebrates, growth inhibition study on algae, fish)
- ✓ Long-term aquatic ecotoxicity (invertebrates, fish)
- ✓ Bioaccumulation in fish
- ✓ Effects on terrestrial organisms (effects on microorganisms, short/long-term toxicity to invertebrates and plants, long-term toxicity to sediment organisms, reproductive toxicity to birds)

Predicted non effect concentration (PNEC)

PEC<PNEC
no environmental risk

Difficulties to apply the RA of substances to RA of substances at nanoscale

- ✓ The behaviour and effects of substances at nanoscale are dependent on several characteristics including size, concentration, surface area and overall surface reactivity and the RA have to take into account these characteristics.

- ✓ Current test guidelines may need to be modified.

- ✓ Relevant exposure scenarios and risk management measures will need further adjustments

Following slides are some examples of *in vitro* studies performed by our group as screening tools to understand the behaviour and cytotoxicity of NMs at cellular level.

Behaviour in the exposure media
- Changes in size
- Changes in concentration
- Stability with time
- Ion release from metallic NP

Internalization in the cell line

Cytotoxicity in different cell lines :
- At different intracellular levels (mitochondria, lysosome and cell membrane) to have an initial idea of the intracellular target of acute toxicity
- Dose-response curves to establish the concentration that produces a 50% of decrease in viability and to identify the concentration that doesn't produce an observed effect (NOEC)

The *in vitro* studies will be the basis to follow upon:
- NM selection from similar ones for a certain application
- Selection of the doses for *in vivo* studies.
- Decisions on the performance of the *in vivo* studies

Connolly et al. Nanoscale Research Letters 2013, 8:315
http://www.nanoscalereslett.com/content/8/1/315

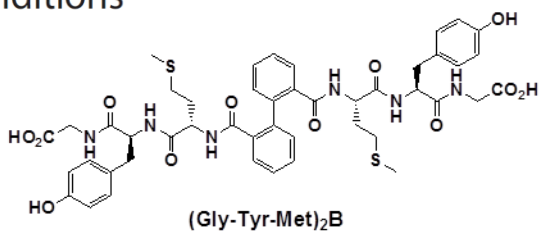
Nanoscale Research Letters
a SpringerOpen Journal

NANO EXPRESS Open Access

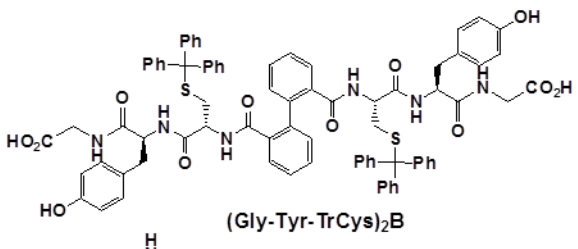
Peptide-biphenyl hybrid-capped AuNPs: stability and biocompatibility under cell culture conditions

Mona Connolly[1], Yolanda Pérez[2*], Enrique Mann[3], Bernardo Herradón[3], María L Fernández-Cruz[1*] and José M Navas[1]

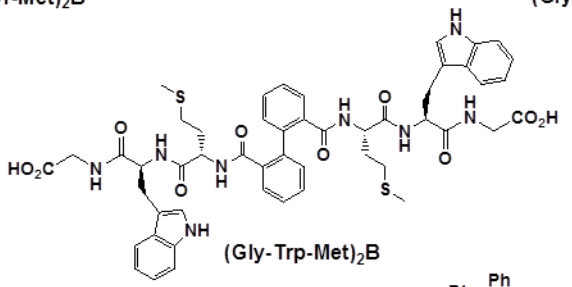
Figure 1 Peptide-biphenyl hybrid (PBH) ligands
Tr = Trityl, B = 2, 2'-(bis)carbonylbiphenyl.



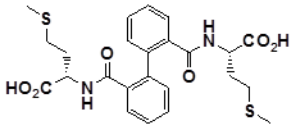
$(Gly\text{-}Tyr\text{-}Met)_2B$



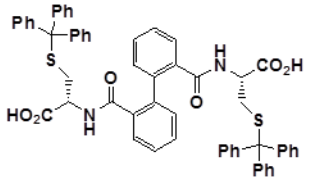
$(Gly\text{-}Tyr\text{-}TrCys)_2B$



$(Gly\text{-}Trp\text{-}Met)_2B$



$(Met)_2B$



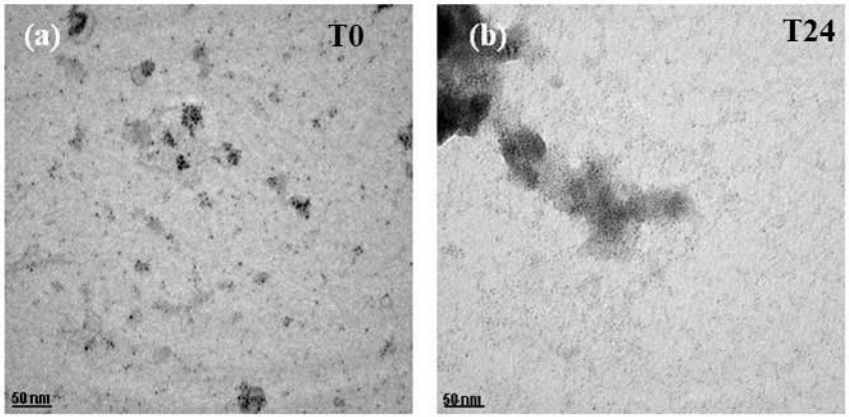
$(TrCys)_2B$

	Size (nm)	Number of Au atoms	PBH units per Au NP
$Au[(Gly\text{-}Trp\text{-}Met)_2B]$	1.6	126	8
$Au[(Gly\text{-}Tyr\text{-}TrCys)_2B]$	1.8	180	40
$Au[(Gly\text{-}Tyr\text{-}Met)_2B]$	1.5	104	7
$Au[(Met)_2B]$	2.3	375	57
$Au[(TrCys)_2B]$	2.3	375	97

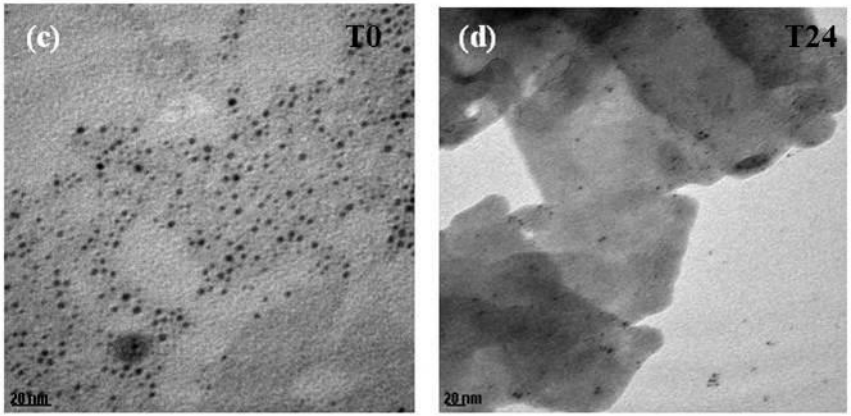
Size measurements by DLS of PBH-capped AuNps (100 µg/ml) under different conditions over time

	Milli-Q water		EMEM/S+		EMEM/S-	
	T0	**T24**	**T0**	**T24**	**T0**	**T24**
	Size nm (mean ± SD; n=3)					
Au[(Gly-Trp-Met)$_2$B]	148±2	148±1	242±4	243±6	233±15	1239±26
Au[(Gly-Tyr-TrCys)$_2$B]	143±1	143±1	261±1	261±2	251±15	195±2
Au[(Gly-Tyr-Met)$_2$B]	591±73 161±5	507±65 150±12	987±205 203±13	987±207 201±9	407±21	1230±8
Au[(Met)$_2$B]	229±23 38±6	228±10 40±3	190±13 27±9	190±4 28±3	1568±28	1368±25
Au[(TrCys)$_2$B]	205±1	205±1	261±3	260±4	271±23	908±23 97±3

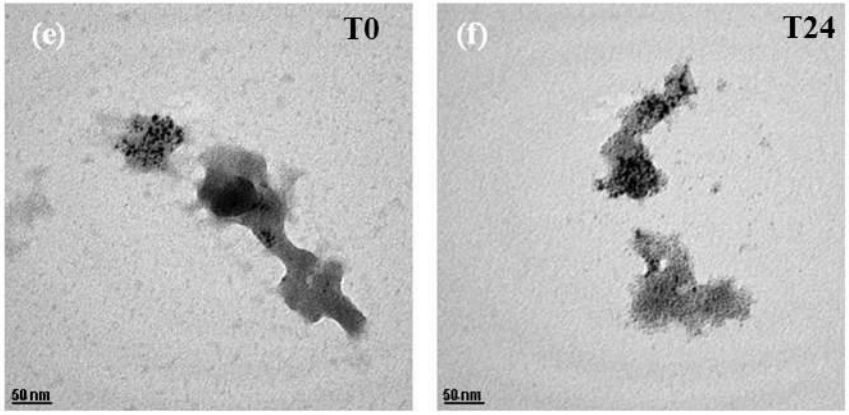
Au[(TrCys)$_2$B]



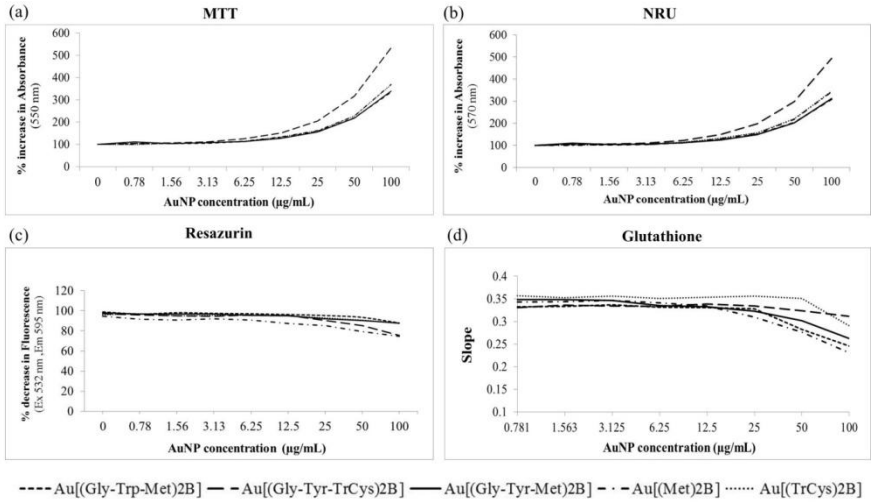
*Au[(Gly-Tyr-TrCys)$_2$B]



Au[(Gly-Tyr-Met)$_2$B]



TEM images of AuNPs in EMEM/S-

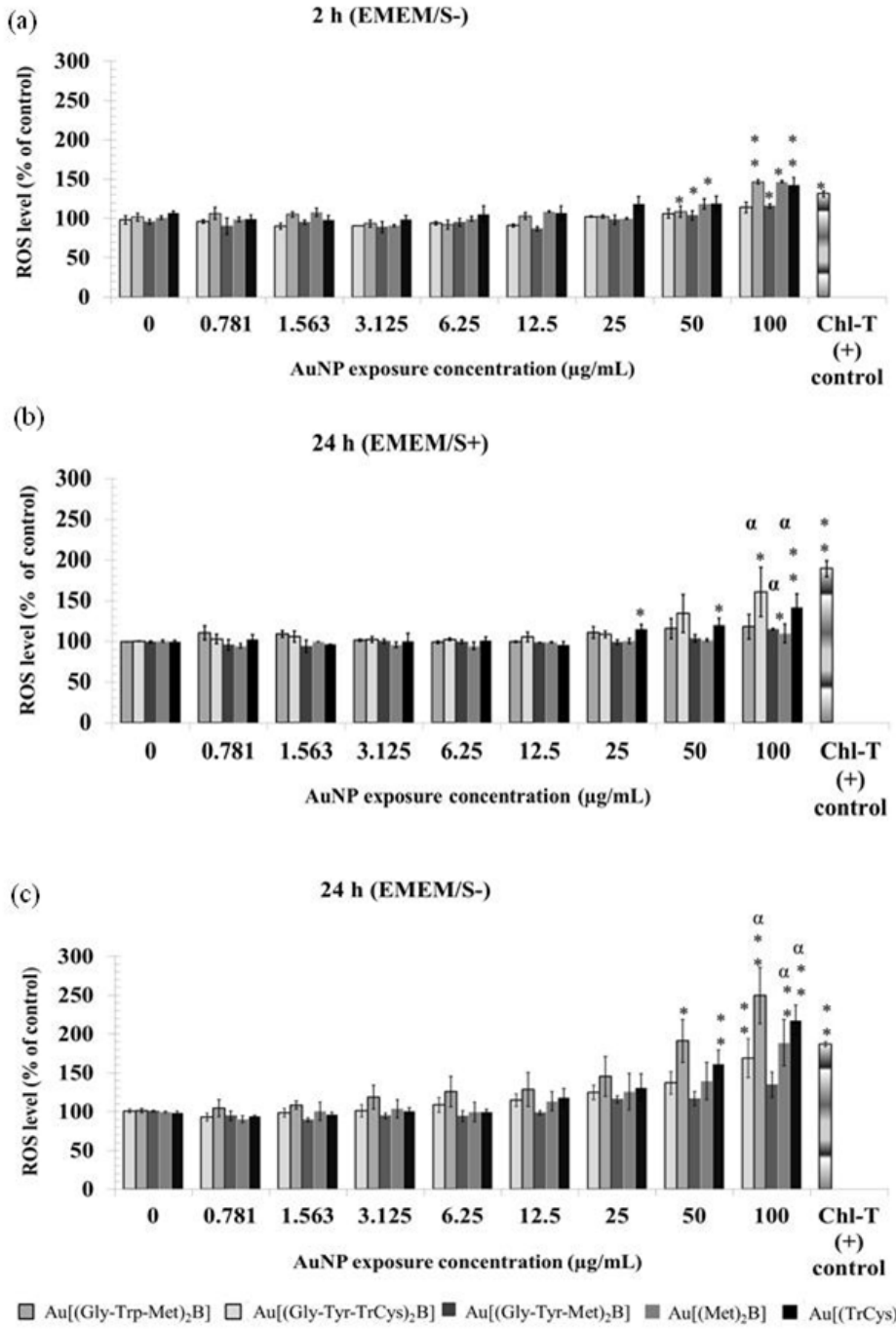


AuNP interference with the toxicity assays

Cytotoxicity of PBH-capped AuNPs following 24- and 48-h exposure (EMEM/S-), using resazurin assay

Also shown are the measured interferences in percent (%) of the control. Average values of three independent measurements are presented (mean ± SEM). Also, **α** indicates significant differences between response to 24 h and 48 h exposure. **P*<0.05 and ***P*<0.01, significant differences from control values. Bold emphasis is used to signal the most stable AuNP

		Exposure concentration (μg/ml)			
Exposure duration	**AuNP**	**12.5**	**25**	**50**	**100**
Au[(Gly-Trp-Met)$_2$B]	24 h	97±1	97±1	96±1	94±0.3**α**
Viability (%)	48 h	98±1	98±2	91±1	69±4**α**
Measured interference (%)		96±2	95±2	94±4	88±4
Au[(Gly-Tyr-TrCys)$_2$B]	24 h	98±1	96±1	93±1**	90±1**
Viability (%)	48 h	95±2	100±2	95±3	87±2*
Measured interference (%)		96±3	90±6	85±7	76±6
Au[(Gly-Tyr-Met)$_2$B]	24 h	96±1	96±1	96±1	91±2**α**
Viability (%)	48 h	94±1	91±6*	81±6**	71±5**α**
Measured interference (%)		95±2	92±2	90±4	88±4
Au[(Met)$_2$B]	24 h	97±1	96±0.4	93±0.4*	94±2* **α**
Viability (%)	48 h	97±1	91±3*	88±4**	68±4 ** **α**
Measured interference (%)		93±1	91±	91±2	89±5
Au[(TrCys)$_2$B]	24 h	98±1	97±1	92 ±2*	88±1**
Viability (%)	48 h	94±4	93±1	88±2 **	77±1**
Measured interference (%)		95±1	93±	91±3	87±4



Conclusions

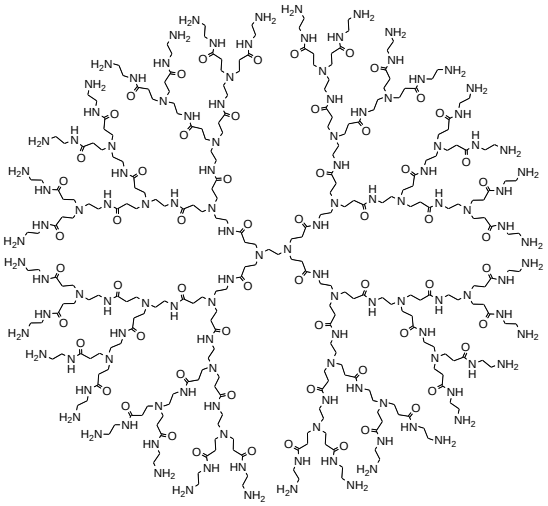
- Depending on the structure of the PBH capping ligand, the behaviour of AuNPs differed both in terms of stability and biocompatibility.
- The stability of these particles over time is dictated by both the structure of the PBH ligand and the surrounding medium.
- The most stable particle was the one capped with $(Gly\text{-}Tyr\text{-}TrCys)_2B$.
- Cytotoxicity and ROS production are only observed for concentrations higher than 25 µg/ml indicating a low toxicity of this capped AuNPs

Anal Bioanal Chem (2012) 404:2749–2763
DOI 10.1007/s00216-012-6256-4

ORIGINAL PAPER

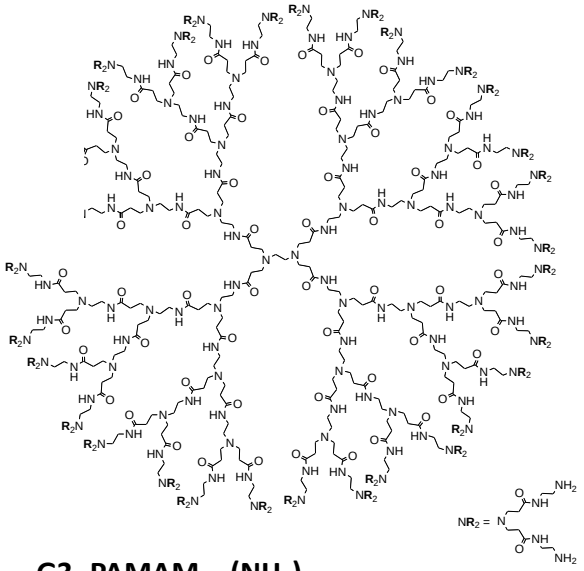
In vitro dose–response effects of poly(amidoamine) dendrimers [amino-terminated and surface-modified with *N*-(2-hydroxydodecyl) groups] and quantitative determination by a liquid chromatography–hybrid quadrupole/time-of-flight mass spectrometry based method

M. D. Hernando · P. Rosenkranz · M. M. Ulaszewska · M. L. Fernández-Cruz · A. R. Fernández-Alba · J. M. Navas



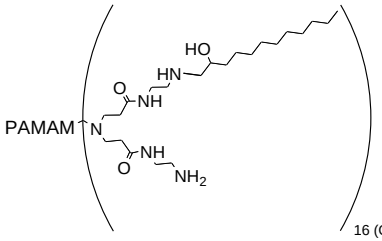
G4- PAMAM-(NH2)64

Empirical Formula (MW: 14214.36): $C_{622}H_{1248}N_{250}O_{124}$



G3- PAMAM – $(NH_2)_{32}$

Empirical Formula (MW: 6908.94): $C_{302}H_{608}N_{122}O_{60}$



G3-PAMAM-50% C_{12}

Empirical Formula (MW: 9858.09): $C_{494}H_{992}N_{122}O_{76}$

G4-PAMAM-50% C_{12}

Empirical Formula (MW: 20112.66): $C_{1006}H_{2016}N_{250}O_{156}$

Cytotoxic effects of the G3 and G4 PAMAM dendrimers

	H4IIE			RTG-2		
	NOEC (24h : 72h) (µg/ml)					
	MTT	**NRU**	**LDH**	**MTT**	**NRU**	**LDH**
G3-PAMAM	31.2: 500	500: 500	500: 31.2	500 :31.2	500: 500	500: 500
G4-PAMAM	7.8: 500	500: 500	250: 3.9	500 :31.2	500: 15.6	500: 500
G3-PAMAM mod	62.5: 125	250: 125	3.9: 7.8	500: 250	500: 500	31.2: 31.2
G4-PAMAM mod	62.5: 32.2	250: 125	62.5: 31.2	500 : 500	500: 500	31.2: 62.5

Extracellular and intracelular concentrations of the G3 and G4 PAMAM dendrimers

	H4IIE	RTG-2
	extracellular : intracellular concentration (µg/ml)	
G3-PAMAM	25.2: ND	18.7: ND
G4-PAMAM	7.2: 7.8	6.7: 6.5
G3-PAMAM mod	3.4: 16.5	4.2: 7.3
G4-PAMAM mod	5.5:15.3	6.2: 7.8

Toxicology in Vitro 26 (2012) 888–896

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Effects of cerium oxide nanoparticles to fish and mammalian cell lines: An assessment of cytotoxicity and methodology

P. Rosenkranz [a,*], M.L. Fernández-Cruz [a], E. Conde [b], M.B. Ramírez-Fernández [c], J.C. Flores [c], M. Fernández [b], J.M. Navas [a]

[a] Departamento de Medio Ambiente, Instituto Nacion
[b] División de Química, Centro de Investigaciones Ene
[c] Departamento de Química Inorgánica, Universidad

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informa
healthcare

Species-specific toxicity of copper nanoparticles among mammalian and piscine cell lines

Lan Song[1], Mona Connolly[2], Maria L. Fernández-Cruz[2], Martina G. Vijver[1], Marta Fernández[3], Estefanía Conde[3], Geert R. de Snoo[1], Willie J.G.M. Peijnenburg[1,4], & Jose M. Navas[2]

[1]Institute of Environme
Instituto Nacional de In
Spain, [3]CIEMAT. Avda. (
Bilthoven, The Netherla

Nanotoxicology, August 2013; 7(5):935–952

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Comparative cytotoxicity induced by bulk and nanoparticulated ZnO in the fish and human hepatoma cell lines PLHC-1 and Hep G2

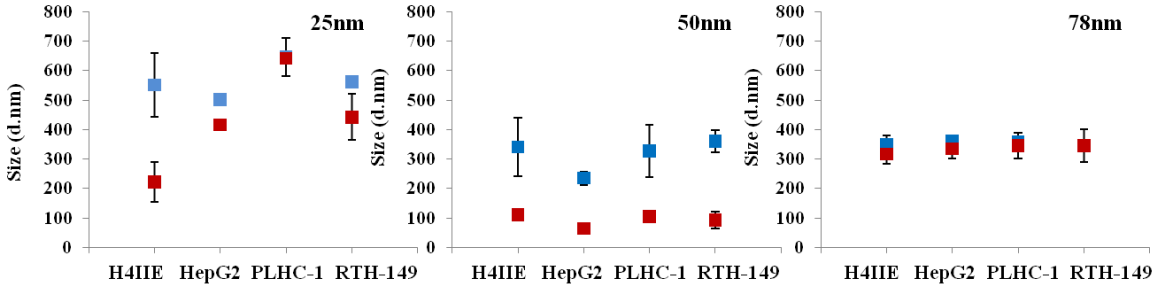
Maria Luisa Fernández-Cruz[1], Tobias Lammel[1], Mona Connolly[1], Estefania Conde[2], Ana Isabel Barrado[2], Sylvain Derick[3], Yolanda Perez[4], Marta Fernandez[2], Christophe Furger[3] & Jose Maria Navas[1]

[1]Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Departamento de Medio Ambiente, Carretera de la Coruña Km 7 Madrid, Spain, [2]CIEMAT Avda. Complutense 40, Madrid, Spain, [3]NOVALEADS, 10 avenue de l'Europe, Ramonville, France and [4]Departamento de Química Inorgánica y Analítica (E.S.C.E.T.), Universidad Rey Juan Carlos, Móstoles, Madrid, Spain

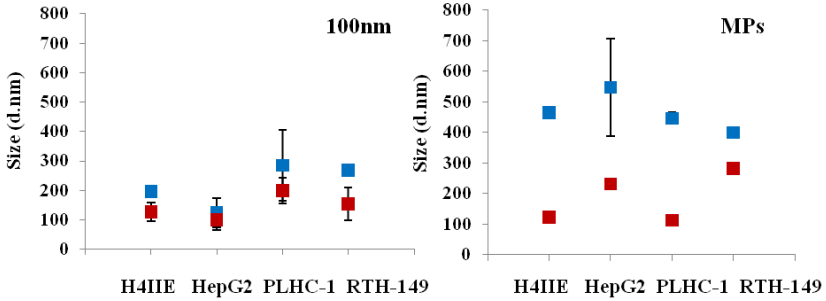
NPs	bulk/salt	cell lines
CeO_2 NM 211 (10 nm) (JRC) CeO_2 NM 212 (20-25 nm) (JRC)	Micron CeO_2 NM 213 (<5µm) (JRC)	RTG-2 cells (rainbow trout *Oncorhyncus mykiss* gonadal tissue) (ATTC) H4IIE rat hepatoma cell lines (ECACC)
ZnO NP (<100 nm) (Sigma-Aldrich) ZnO NP 6% aluminium doped (<50 nm) (Sigma-Aldrich) ZnO NP (20-30 nm) (Tecnan)	ZnO fine powder (<5 µm) (Sigma-Aldrich)	PLHC-1 fish hepatocellular carcinoma cell lines (topminnow fish (*Poeciliopsis lucida*) HEP G2 human hepatocellular carcinoma cell lines (ATTC)
Cu NP (25 nm) (IoLiTec, Inc.) Cu NP (50 nm) (IoLiTec, Inc.) Cu NP (78 nm) (NanoAmor) Cu NP (100 nm) (IoLiTec, Inc.)	Cu MP (500 nm) (NanoAmor) $Cu(NO_3)_2$ salt	PLHC-1 RTH-149 fish hepatoma (rainbow trout) H4IIE HEP G2

Characterisation of the size (nm) of 100 µg/ml suspension of NP in water and/or exposure medium by DLS

	Water	Exposure medium				
	T0	T0	T24	T48	T72	
CeO_2 NM 211 (10 nm)	304±6	330±19	312±7	290±6	301±8	EMEM+
CeO_2 NM 212 (20-25 nm)	264±18	304±16	270±7	266±6	281±7	EMEM+
Micron CeO_2 NM 213 (<5µm)	425±56	411±100	336±13	310±18	298±	EMEM+
ZnO NP (<100 nm)		1134/1123	1060/1280			EMEM-/α-MEM-
ZnO NP 6% Al doped (<50 nm)		1260/1978	2166/1091			
ZnO NP (20-30 nm)		2978/2504	2481/3753			
ZnO fine powder (<5 µm)		1421/957	1564/859			



Cu NP (25 nm)
Cu NP (50 nm)
Cu NP (78 nm)
Cu NP (100 nm)
Cu MP (500 nm)
Blue T0
Red T24



		Exposure medium		
	Nominal concentration (µg/ml)	Measured concentration (µg/ml)	Soluble fraction concentration (µg/ml)	
CeO_2 NM 211 (10 nm)	100	14.82±1.58		
CeO_2 NM 212 (20-25 nm)	100	29.72±2.24		
Micron CeO_2 NM 213 (<5µm)	100	30.81±8.78		
ZnO NP (<100 nm)	100	50.9±5.5	38.7±5.9 (α-MEM) 5.9±1.8 (EMEM)	24h incubation 10 min 1,000g/ 180,000g
ZnO NP 6% Al doped (<50 nm)	100	58.4±18.7		
ZnO NP (20-30 nm)	100	51.7±16.1		
ZnO fine powder (<5 µm)	100	40.5±0.66		
Cu NP (25 nm)	200	153.1±6.1	75%	24h incubation 20 min 13,362g
Cu NP (50 nm)	200	150.4±20.0	41%	
Cu NP (78 nm)	200	89.2±29.5	23%	
Cu NP (100 nm)	200	150.2±34.9	41%	
Cu MP (500 nm) (NanoAmor)	200	100.0±16.9	82%	

	Most sensitive test/LOEC real conc (µg/ml) (24h exposure)				Assays tested
	H4IIE	RTG-2			
CeO_2 NM 211 (10 nm)	MTT/0.68	Nt			MTT NRU LDH no interf.
CeO_2 NM 212 (20-25 nm)	MTT/0.48	Nt			
Micron CeO_2 NM 213 (<5µm)	MTT/Nt	Nt			
	HEP G2 NP	HEP G2 SUP	PLHC-1 NP	PLHC-1 SUP	
ZnO NP (<100 nm)	MTT/LDH/LUCS/18	MTT/NRU/LDH/6	MTT/9	MTT/10	MTT NRU LDH LUCS no interf.
ZnO NP 6% Al doped (<50 nm)	MTT/NRU/LUCS/16	MTT/NRU/LDH/6	MTT/3	MTT/LDH/20	
ZnO NP (20-30 nm)	MTT/LUCS/12	MTT/NRU/LDH/6	MTT/12	MTT/LDH/20	
ZnO fine powder (<5 µm)	LDH/7	MTT/NRU/LDH/6	MTT/NR/LDH/14	MTT/20	

		H4IIE	HepG2	PLHC-1	RTH-149
		IC_{50} (µg/ml)			
copper suspension	25nm	20±3	39±5	83±16	91±27
	50nm	55±15	42±2	67±20	115±12
	78nm	56±18	48±3	58±22	182±74
	100nm	76±5	57±1	59±5	104±4
	MPs	26±12	26±12	45±18	74±14
	$Cu(NO_3)_2$	54±9	43±4	109±10	120±15

We also perform studies *in vivo* in fish

- Acute toxicity studies

- Accumulation studies. We have already presented preliminary results in NANOTOX 2014.

Tissue distribution of zinc and subtle oxidative stress effects after dietary administration of a ZnO nanoparticle to rainbow trout

M Connolly[1], M Fernández[2], E Conde[2], F Torrent[3], JM Navas[1], ML Fernández-Cruz[1*]

*[1]Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Madrid, Spain, [2]CIEMAT, Madrid, Spain [3]ETS Ingenieros de Montes, Universidad Politécnica de Madrid, Spain *email: fcruz@inia.es*

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To think about

- ✓ **Which objectifs should be defined to avoid concerns about NMs related to human health and environment?**
  - To generate new basic knowledge to resolve the major uncertainties and knowledge gaps in regard to release during life cycle of the product, chemical and biological interactions and toxicological properties of NMs.
  - To stablish read accross rules in relation to toxicological properties for the different nanoforms
  - To develop safe by design strategies that avoid the release of the NPs from the NM.

- ✓ **At which stage in the timeline of an European project should we consider the study of toxic effects?**
  - Always there is a possibility of human exposure directly or via environment
  - Always there is a possibility of release to the environment

- ✓ **What are the tools to be considered? Is there lack of generic approaches?**
  - At moment REACH regulation or others, depending on the use of the NM, should be followed as well as recent advances in guideline studies and risk assessment approaches within these regulations

Aknowledgments to collaborators

Thank you for your attention