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Investigation of the uptake and cytotoxic mechanisms of amine-modified silver nanoparticles

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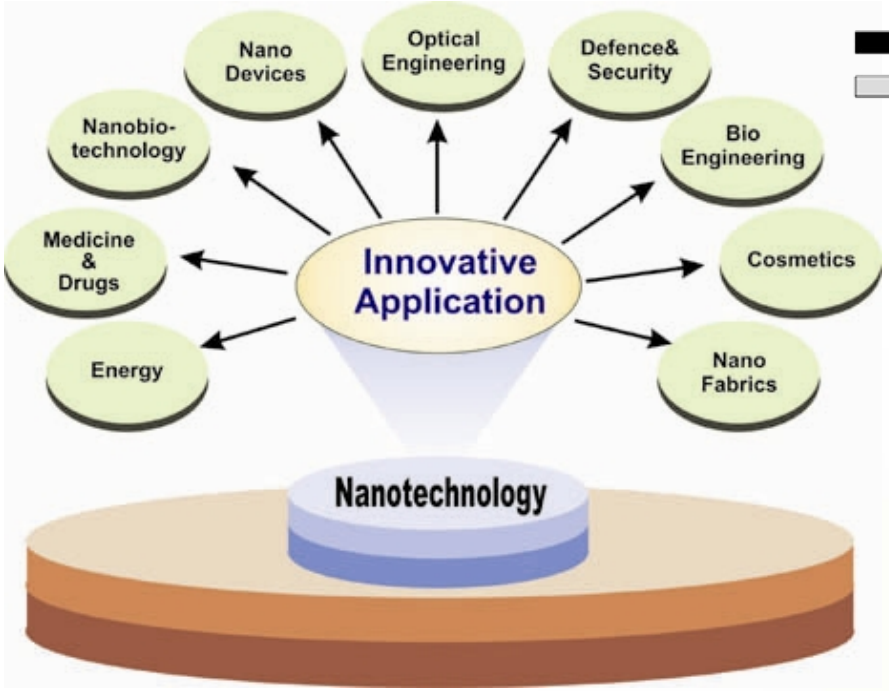
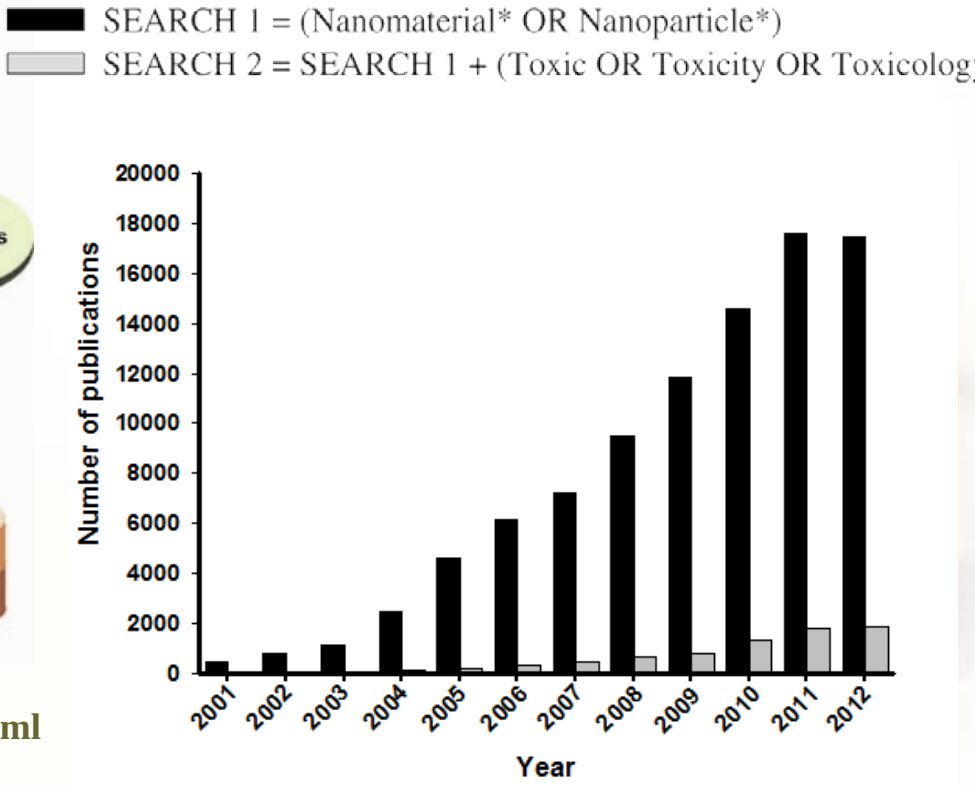
INTRODUCTION



http://www.nanorev.in/nanorev---nanotechnology.html



the impact of NP on biological system, especially their toxic effects and mechanism is still limited

Table 1. Applications of commonly used nanoparticles

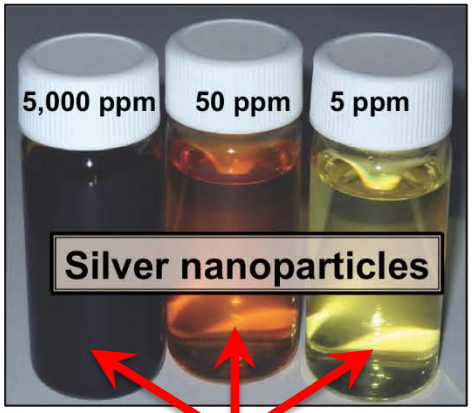
Nanoparticle Type	Abbreviation	Applications
Carbon nanotube (single or multiwalled)	CNT (SWNT, MWNT)	Cell delivery, biosensors
Silver	Ag	Antimicrobial
Quantum dot	QD	Fluorescent imaging
Aluminum	Al	Fuel additive
Iron	Fe	Magnetic imaging
Titanium dioxide	TiO_2	Paint, water treatment, food, cosmetics
Zinc oxide	ZnO	Transparent sunscreens
Manganese oxide	MnO	Catalysis, batteries

Adv Exp Med Biol. 2012;745,58-75

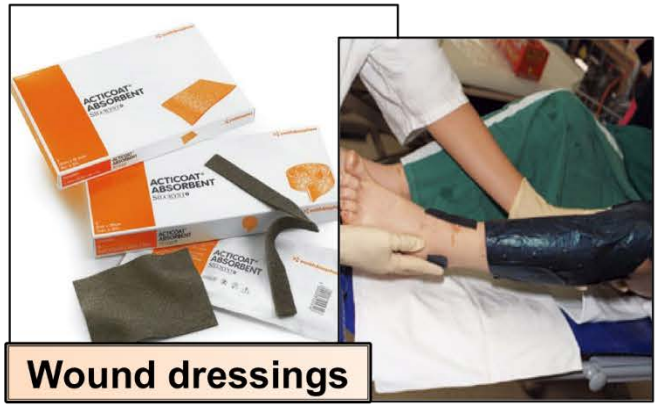
Numerous commercial applications of AgNPs lead to an increased risk of human exposure

- **Due to the effective antimicrobial activities and surface plasmon resonance properties, AgNPs have gained particular interest for various commercial applications (e.g. wound dressings and photovoltaics).**

(Zhang et al. *Nanoscale Res. Lett.*, 2016, 11:80)



Colloidal nanosuspension



Future Trends in Clinical Applications of AgNPs

Substitute for

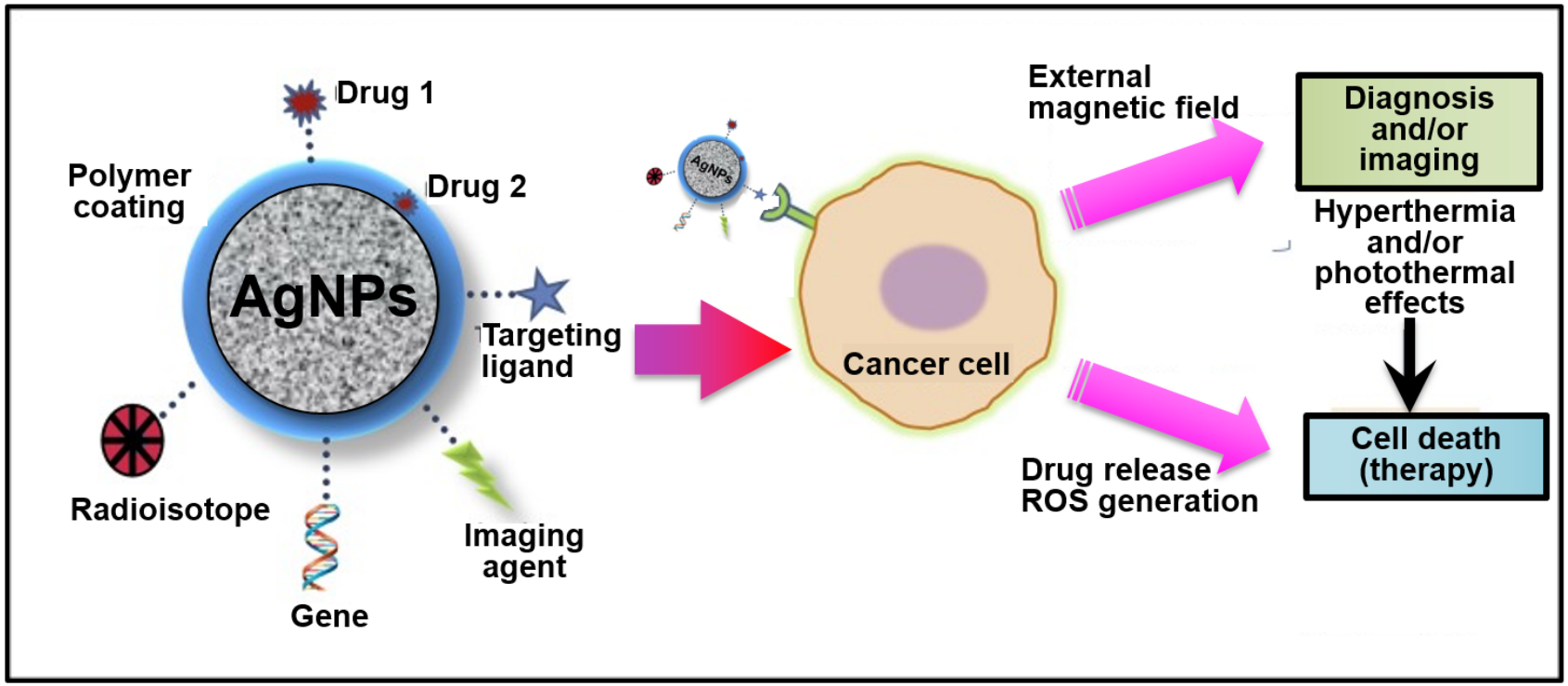
Antimicrobial Silver NPs

Being applied for

Conventional oral or injectable antibiotics

Oral hygiene (to reduce formation of dental plaque)

(Franci et al., *Molecules*, 2015, 20:8856-8874)
(Mattos et al., *Int. J. Biomater.*, 2015, 2015:485275)



(Sharma et al., *Drug Discovery Today*, 2015, 20:1143-1151)

the increase of human exposure and potential adverse health effect has led to increasing public concern

Nanotoxicology, September 2010; 4(3): 284–295

informa
healthcare

Nano-silver – feasibility and challenges for human health risk assessment based on open literature

FRANS M. CHRISTENSEN[1*], HELINOR J. JOHNSTON[2†], VICKI STONE[2], ROBERT J. AITKEN[3], STEVE HANKIN[3], SHEONA PETERS[3], & KARIN ASCHBERGER[1*]

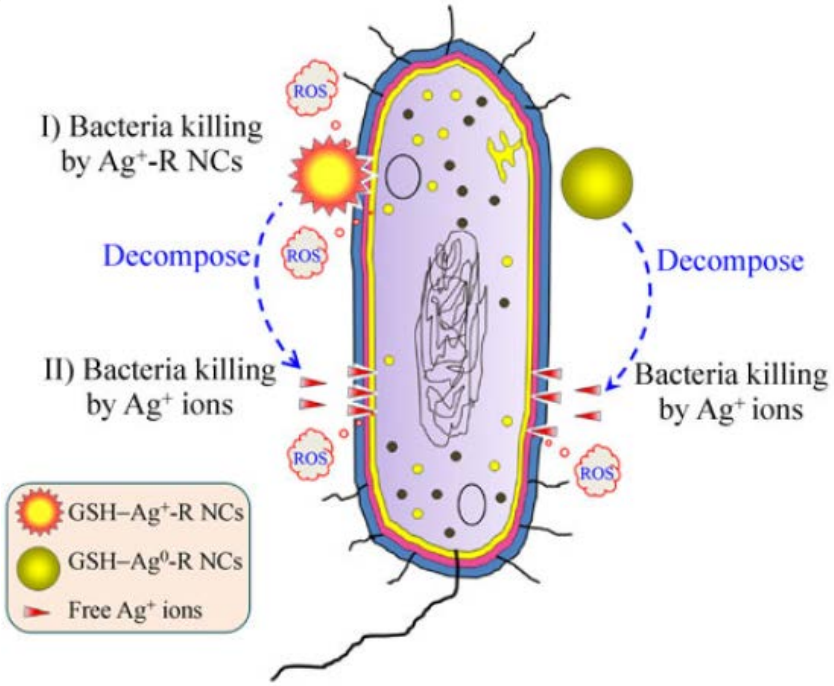
Gaps in the available data set, both in relation to exposures and hazard, do not allow reaching any definite conclusions that could be used for regulatory decision making.

Results show that repeated inhalation in the workplace and possibly consumer inhalation may cause risks. Also (uncontrolled) nano-silver drug intake and burn treatment of large parts of the body with wound dressings may cause risks.

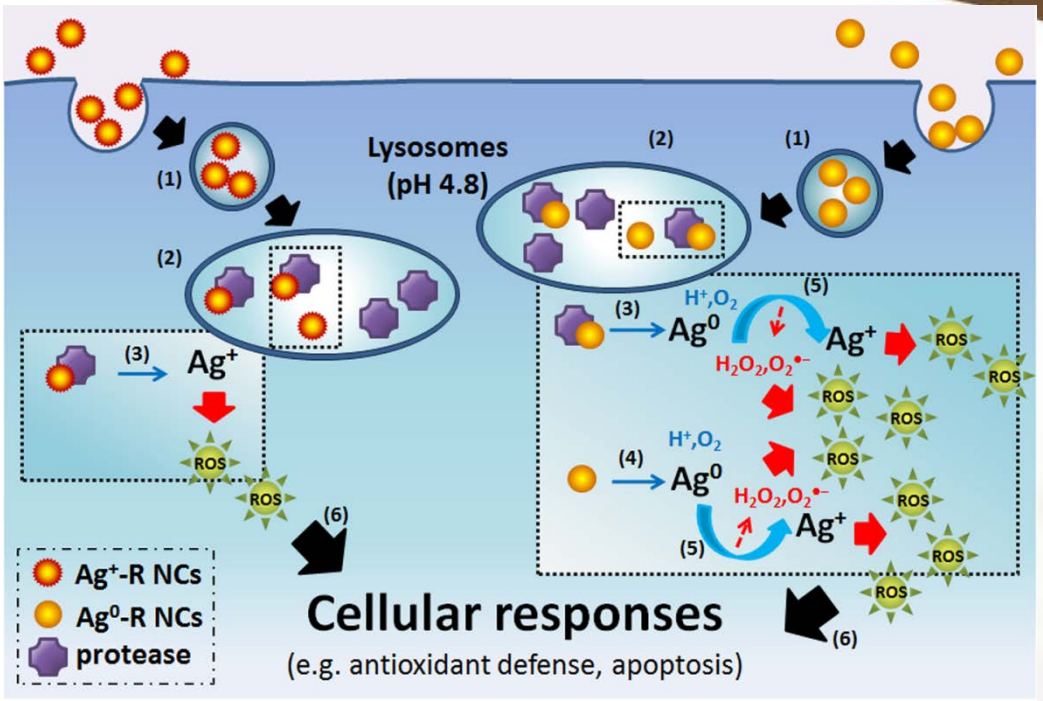
Main future work should focus on generating occupational and consumer exposure data, as well as toxicity data on absorption (are particles or only ions absorbed?),

information on genetoxicity, and further information on the toxicity following inhalation exposure to sizes and agglomeration states as uncounted in the workplace.

Silver as an antimicrobial, meanwhile may trigger cytotoxicity



Nano Research 2014; 7: 301–307

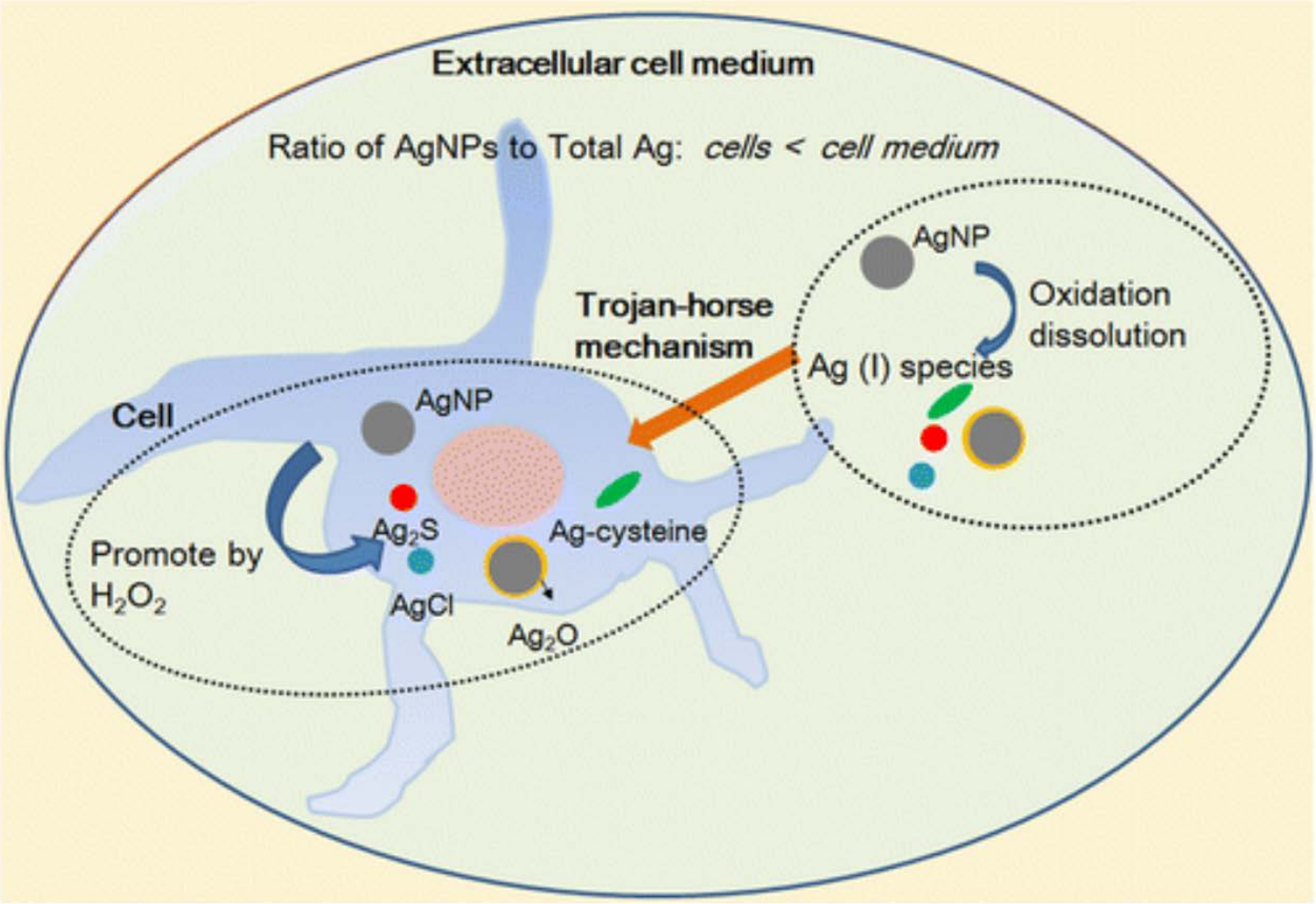


Biomaterials (2014)

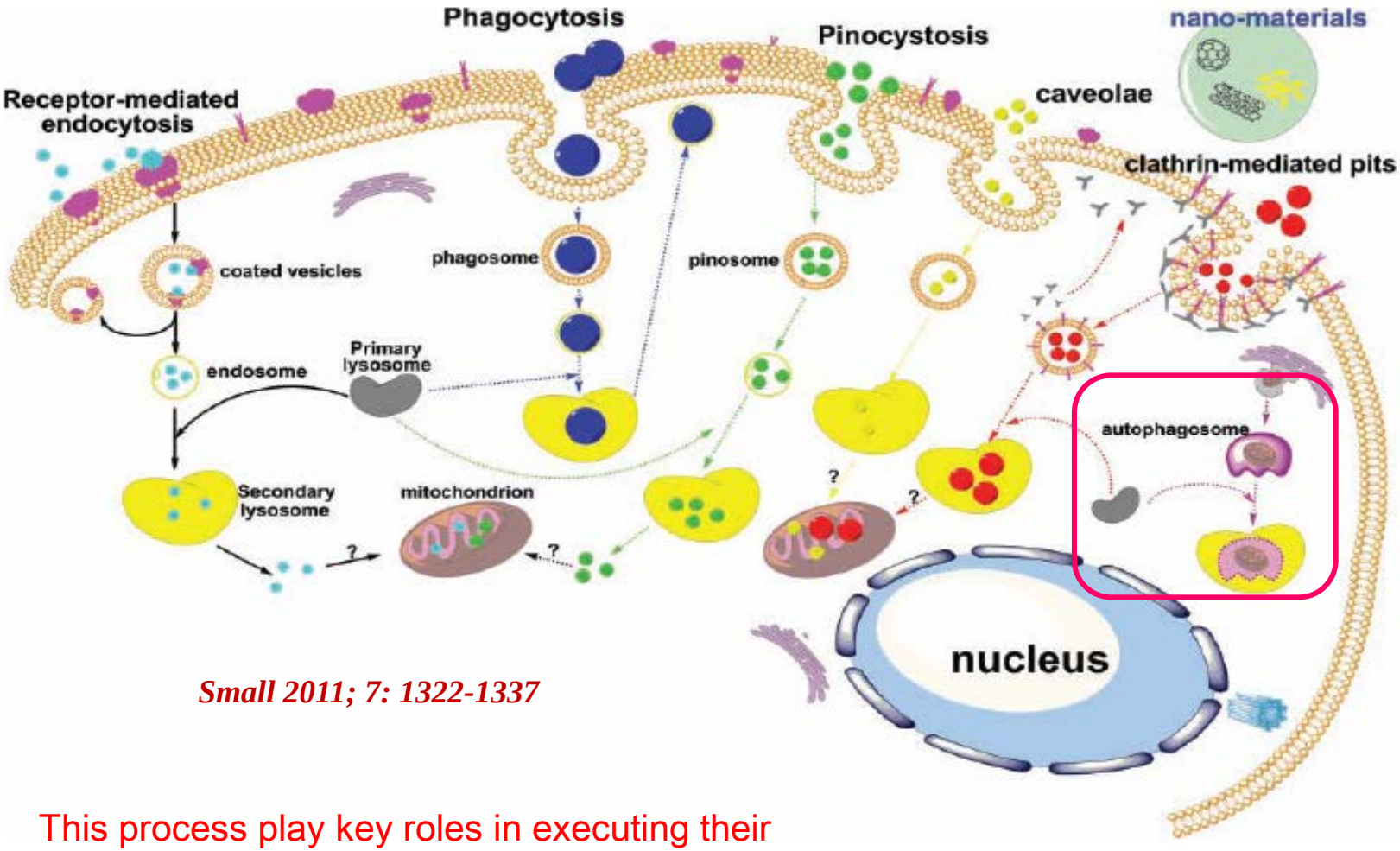
Environ Sci Technol. 2015 Mar 17;49(6):3813-21. doi: 10.1021/es504705p. Epub 2015 Feb 27.

Trojan-horse mechanism in the cellular uptake of silver nanoparticles verified by direct intra- and extracellular silver speciation analysis.

Hsiao IL[1], Hsieh YK[1], Wang CF[1], Chen IC[1], Huang YJ[1].



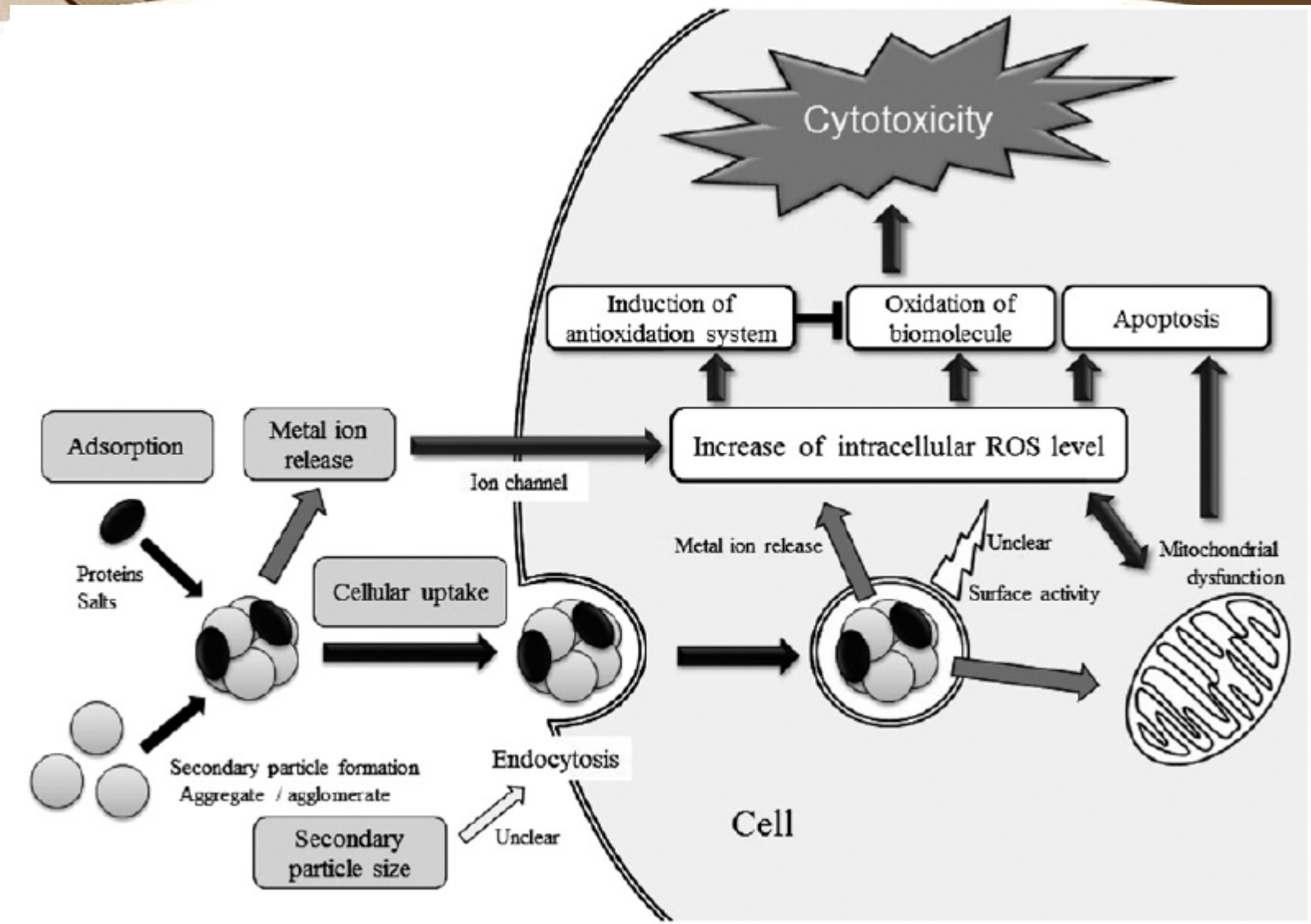
Schematic of the known pathways for intracellular uptake of nanoparticles



Small 2011; 7: 1322-1337

This process play key roles in executing their biomedical functions and in toxicity

Mechanisms of AgNP cytotoxicity in the mammalian cell



Chem. Res. Toxicol 2012; 25: 605–619

Toxicity testing methods suggested by NCL

Toxicity testing
on the cellular
response pathways

NANOTECHNOLOGY CHARACTERIZATION LABORATORY

U.S. Environmental Protection Agency (EPA) 2009

National Research Council (NRC) 2007

As the preferred toxicity testing strategy in the 21st century

Toxicity	
Oxidative Stress	
Hep G2 Hepatocyte Glutathione Assay	
Hep G2 Hepatocyte Lipid Peroxidation Assay	
Hepatocyte Primary ROS Assay	
Cytotoxicity (necrosis)	
LLC-PK1 Kidney Cytotoxicity Assay (MTT and LDH Release)	GTA-1
Hep G2 Hepatocarcinoma Cytotoxicity Assay (MTT and LDH Release)	GTA-2
Cytotoxicity (apoptosis)	
LLC-PK1 Kidney Apoptosis Assay (Caspase 3 Activation)	GTA-5
Hep G2 Hepatocarcinoma Apoptosis Assay (Caspase 3 Activation)	GTA-6
Hep G2 Hepatocarcinoma Homogeneous Apoptosis Assay (Caspase 3/7 Activation)	GTA-14
Autophagy	
Autophagic Dysfunction Assay: Qualitative Analysis of MAP LC3I to LC3-II Conversion by Western Blot	GTA-11
Autophagic Dysfunction in LLC-PK1 Cells	GTA-12

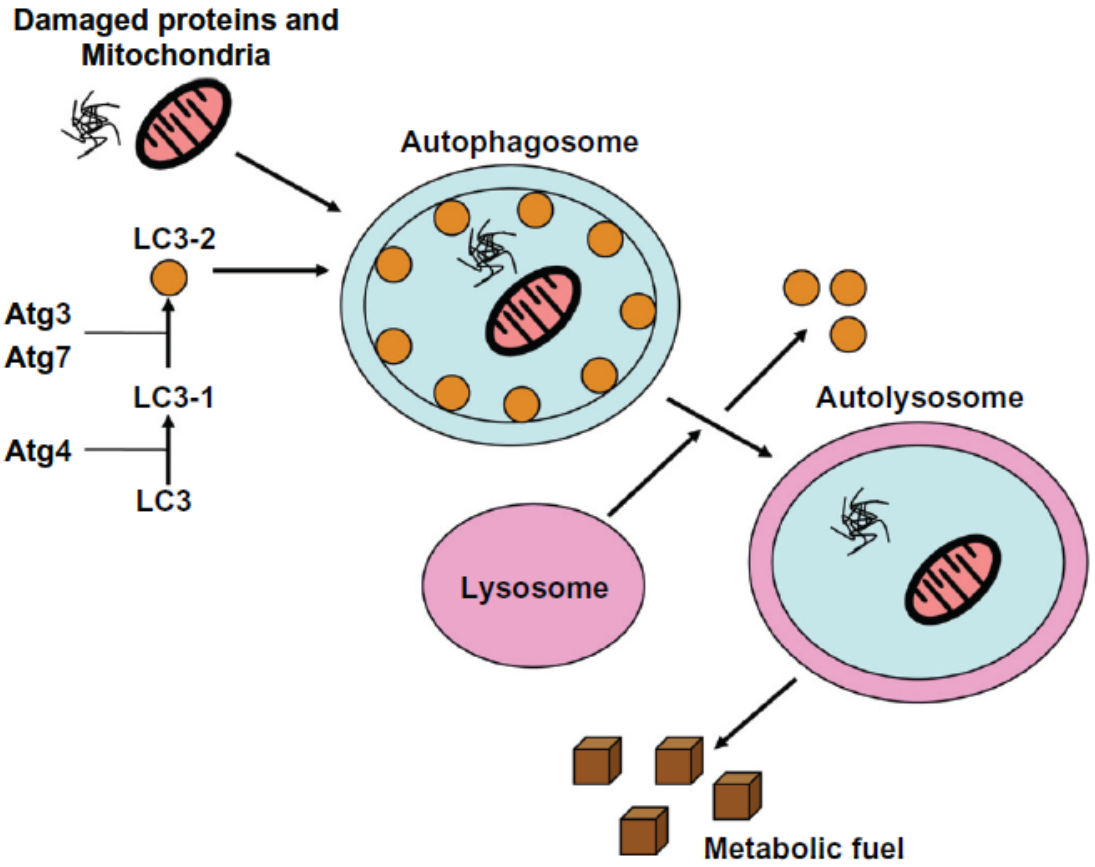


Figure 1 Autophagy produces metabolic fuel through the degradation of biomolecules.
Notes: Damaged proteins, organelles, and other biomolecules are sequestered into double-membrane vesicles called autophagosomes. LC3 is essential for autophagosome maturation. The mature autophagosomes fuse with the lysosome, and biomolecules are degraded by hydrolytic enzymes into metabolic fuel.
Abbreviation: LC3, lipidated cytosolic-associated protein light chain.

Nanomaterials **2014**, *4*, 548-582; doi:10.3390/nano4030548

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nanomaterials

ISSN 2079-4991

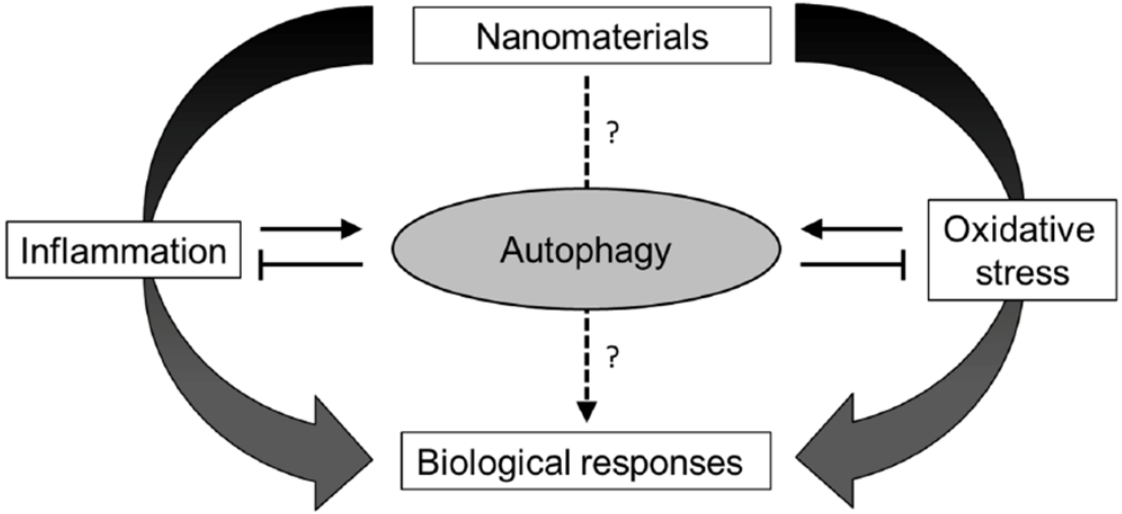
www.mdpi.com/journal/nanomaterials

Review

Autophagy as a Possible Underlying Mechanism of Nanomaterial Toxicity

Vanessa Cohignac [1,2,†], Marion Julie Landry [1,2,†], Jorge Boczkowski [1,2] and Sophie Lanone [1,2,*]

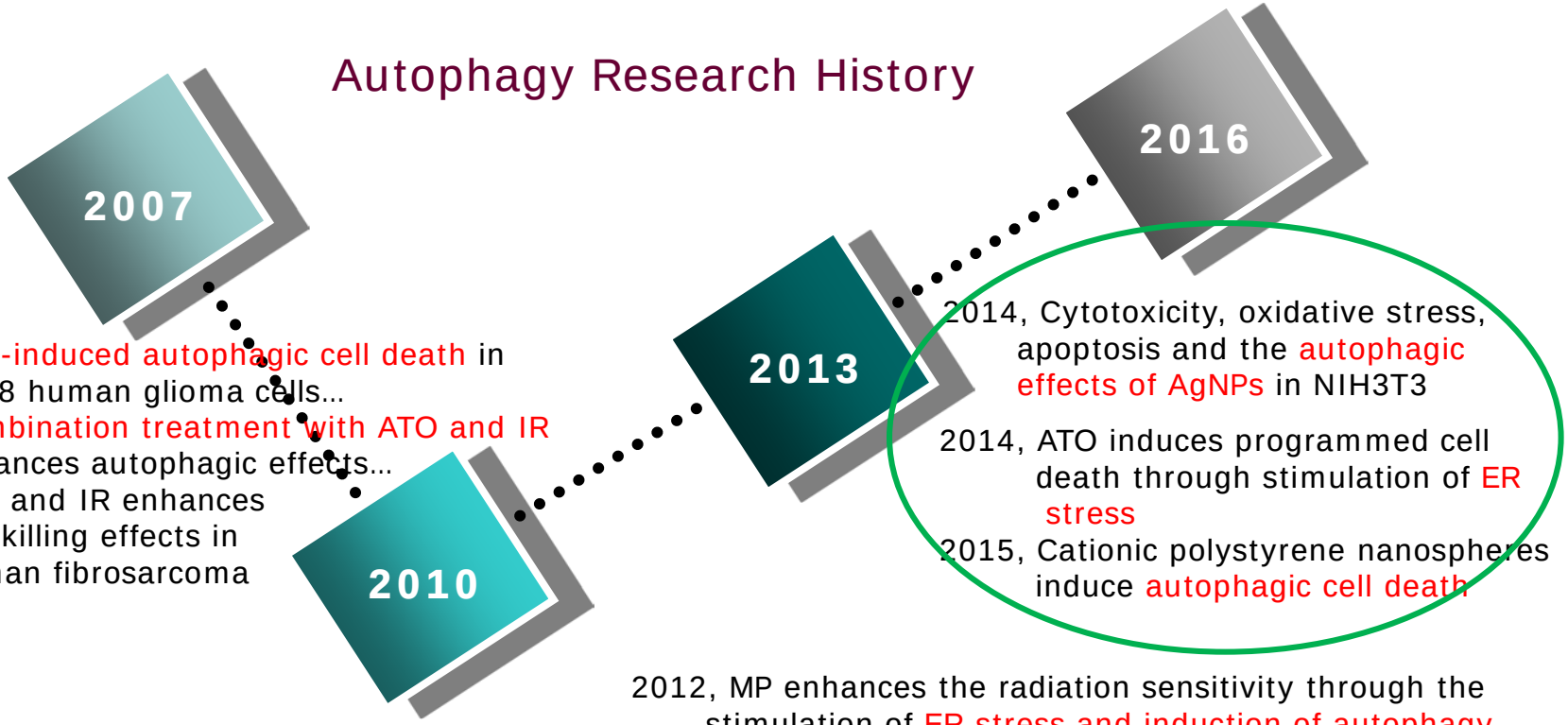
Figure 2. Hypothetic relationship between the autophagy and the biological responses to nanomaterial.



B-H Mao, J-C Tsai, C-W Chen, S-J Yan*and Y-J Wang*. Mechanisms of silver nanoparticle-induced toxicity and important role of autophagy. Nanotoxicology. In press (2016)

Y-H Lee, C-Y Fang, H-W Chiu, F-Y Cheng, J-C Tsai, C-W Chen* and Y-J Wang*. Endoplasmic reticulum stress-triggered autophagy and lysosomal dysfunction contribute to the cytotoxicity of amine-modified silver nanoparticles in NIH 3T3 cells. J. Biomed. Nanotech. Revising. (2016)

Autophagy Research History



2007, ATO-induced autophagic cell death in U118 human glioma cells…

2009, Combination treatment with ATO and IR enhances autophagic effects…

2010, ATO and IR enhances cell-killing effects in human fibrosarcoma

2010, Pterostilbene-induced Apoptosis and Autophagy in Human Bladder Cancer Cells

2011, Synergistic effects of arsenic trioxide and radiation in osteosarcoma cells through autophagy and apoptosis

2011, ATO induces autophagy and apoptosis through down-regulation of survivin

2012, MP enhances the radiation sensitivity through the stimulation of ER stress and induction of autophagy

2012, Monascuspiloin Induces Apoptosis and Autophagic Cell Death in Human Prostate Cancer Cells

2012, Chemopreventive Effects of Pterostilbene on Urethane Induced Lung Carcinogenesis via the Induction of Apoptosis and Autophagy.

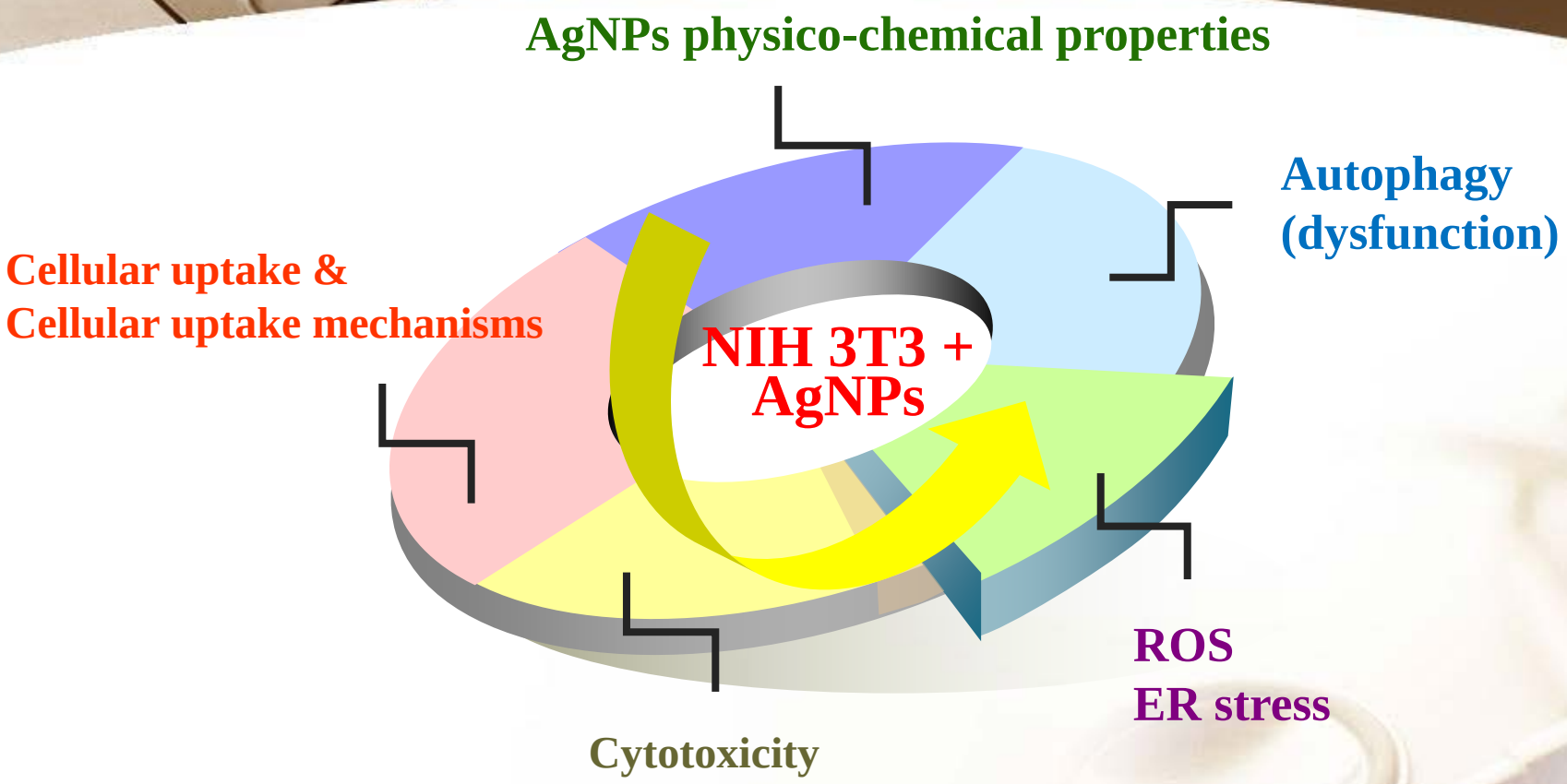
2013, SAHA enhance radiosensitivity and suppresses lung metastasis in breast cancer in vitro and in vivo

2014, Cytotoxicity, oxidative stress, apoptosis and the autophagic effects of AgNPs in NIH3T3

2014, ATO induces programmed cell death through stimulation of ER stress

2015, Cationic polystyrene nanospheres induce autophagic cell death

Experimental considerations for performing in vitro nanoparticle toxicity studies



(1)Analysis the physicochemical parameters of AgNPs.
(2)Cellular uptake and mechanisms of AgNPs.
(3)Cytotoxicity of AgNPs.
(4)The toxic mechanisms of AgNPs.

AgNPs physical-chemical characterization and dispersion in water or cell culture media

Table 1 Characterization of AgNPs

Characterization	Method	Condition	Results
Size	TEM	Dry	26.2 ± 7.6 nm
Morphology	TEM	Dry	Spherical
Composition and purity	EDX	Dry	Ag (99.6%)
Zeta potential	PALS	Water	-28.4 mV
Hydrodynamic size	DLS	Water	40.1 ± 1.7 nm
Polydispersity index (PDI)	DLS	Water	0.204
λmax	UV-Vis	Water	389 nm
Hydrodynamic size	DLS	DMEM	92.9 ± 2.1 nm
Polydispersity index (PDI)	DLS	DMEM	0.289
λmax	UV-Vis	DMEM	398 nm

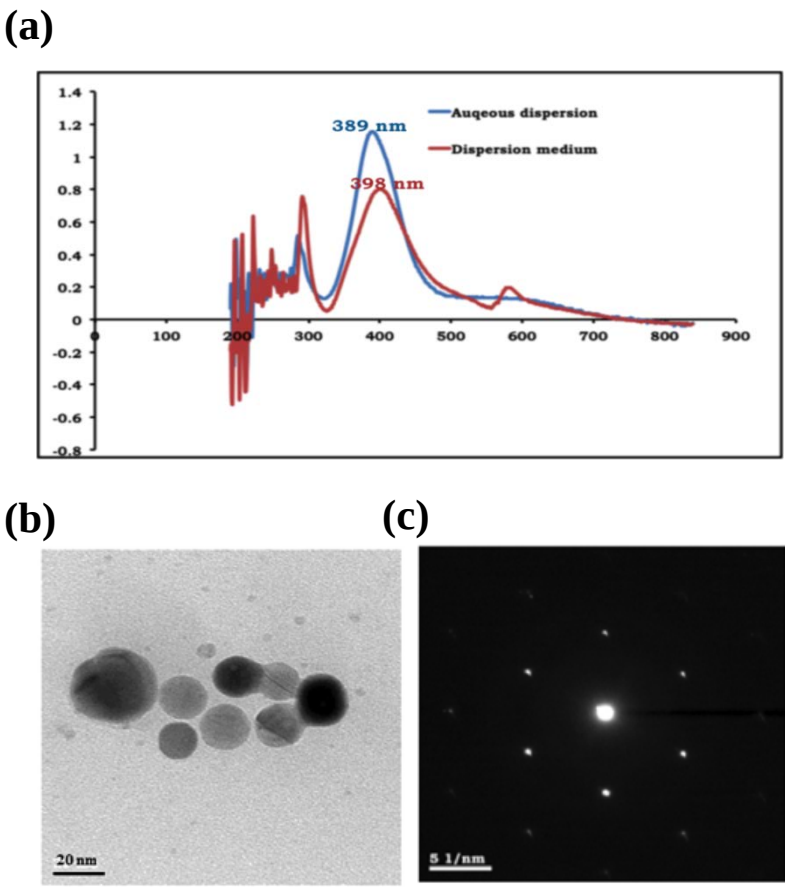


Fig. 1. Characterization of AgNPs. (a) UV-vis spectra of AgNPs in water or DMEM plus 1% FBS. (b) Transmission electron microscope (TEM) documentation of NP morphology: AgNPs were mainly spherical in shape with a mean diameter of 26 ± 7.6 nm (mean± SD) (scale bar representing 20 nm). (c) An X-ray diffraction (XRD) pattern of AgNPs.

Cellular uptake of silver nanoparticles

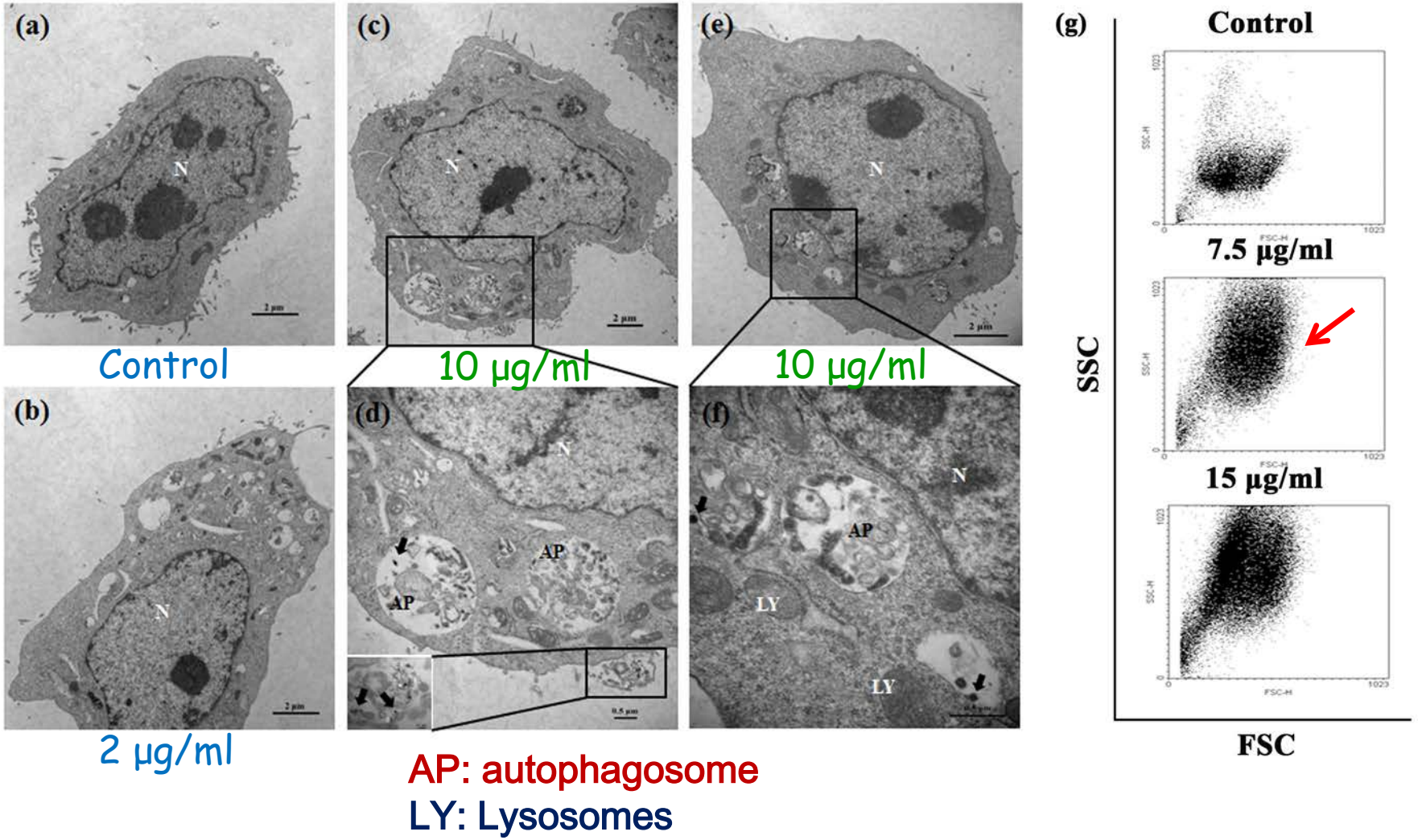


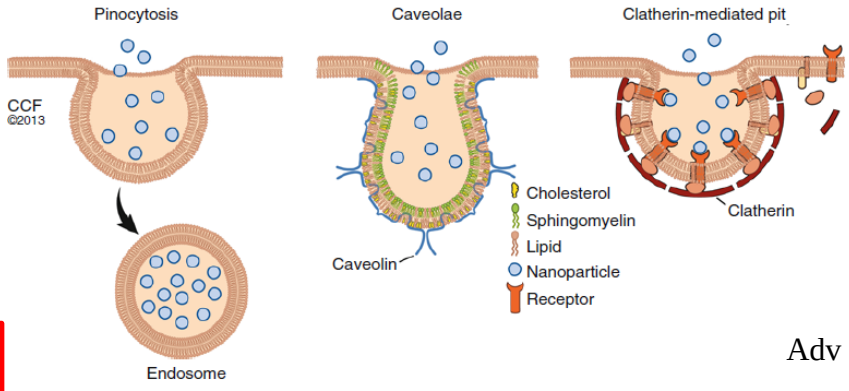
Fig. 2. Cellular uptake of AgNPs by NIH 3T3 cells. TEM images demonstrated the interaction of AgNPs with cells. Scale bars: 2 μm (a, b, c and e); 0.5 μm (d and f) The results of side-scatter(ed) light of flow cytometry demonstrated that AgNPs were apparently engulfed by NIH 3T3 cells.

Cellular uptake pathway of AgNPs

Nystatin→Cav

Chlorpromazine→Clath

Wortmannin→Pin



Adv Exp Med Biol. 2014:811:135-56

Fig. 5.1 Schematic of different cellular uptake mechanisms for NPs. The mechanisms of cellular uptake are determined by the physical characteristics of NPs. NPs with targeting ligands are generally internalized by clathrin-mediated endocytosis. Caveolae-mediated endocytosis is responsible for internalization of anionic NPs, whereas pinocytosis is the mechanism of choice for large NPs and microparticles

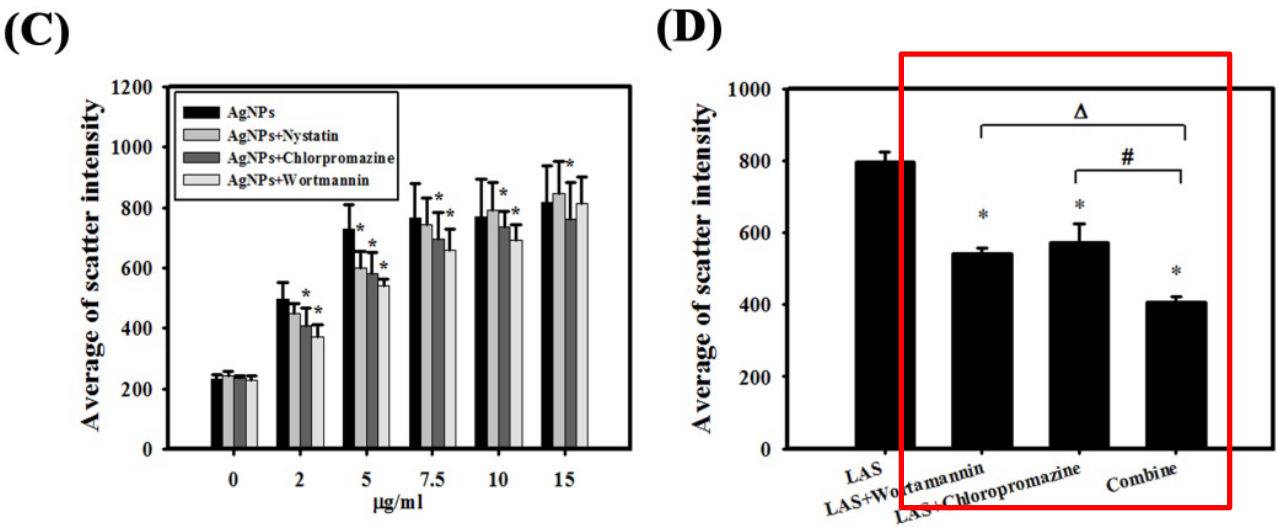
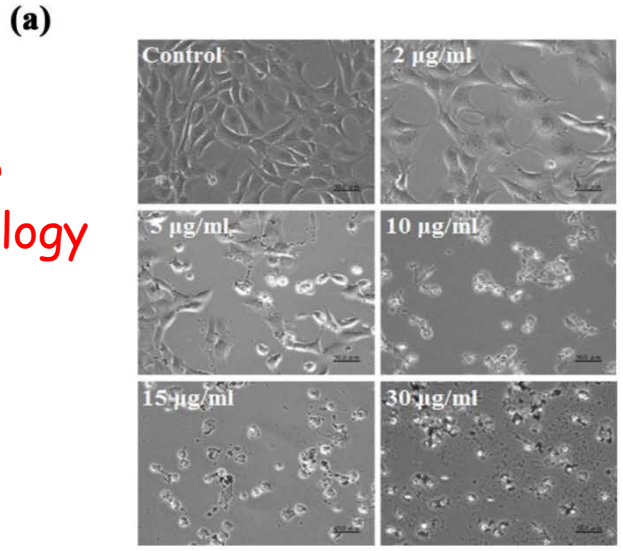
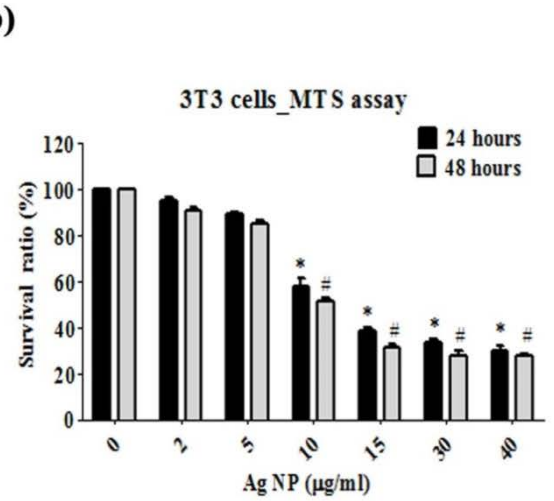


Fig. 8. (C) and (D) Cellular uptake pathway for AgNPs internalization in NIH 3T3 cells. Cells were pre-incubated with different pharmacological inhibitors for 30 min (Nystatin 7.5 μM; Chlorpromazine 10 μM; Wortmannin 200 nM) and particle uptake was analyzed by flow cytometric side scatter. Combine group means that cells were treated with AgNPs (5 μg/ml), Chlorpromazine and Wortmannin. (n=3, Δ,*, # , $p<0.05$).

Effects of AgNPs on cellular morphology and cytotoxicity

Cellular morphology

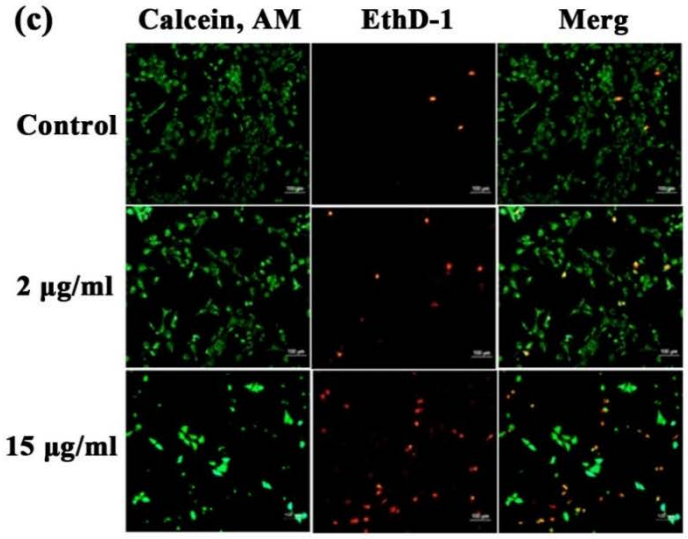




MTS assay

Live/Dead cell viability assay

100X



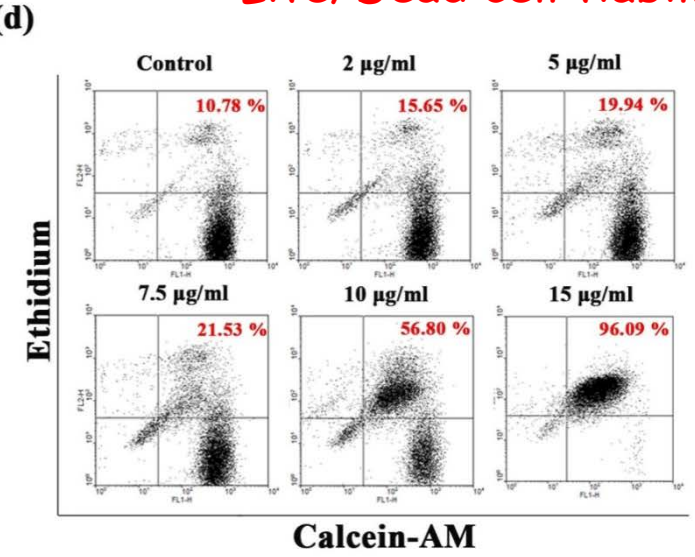


Fig. 3. Cytotoxicity of AgNP-treated NIH 3T3 cells. Cells were grown for 1 day in 6-well plates and exposed to AgNPs at the different concentrations for 24 hrs.

Effects of AgNPs on cellular reactive oxygen species (ROS), glutathione (GSH) and the expression of heme oxygenase 1 (HO-1)

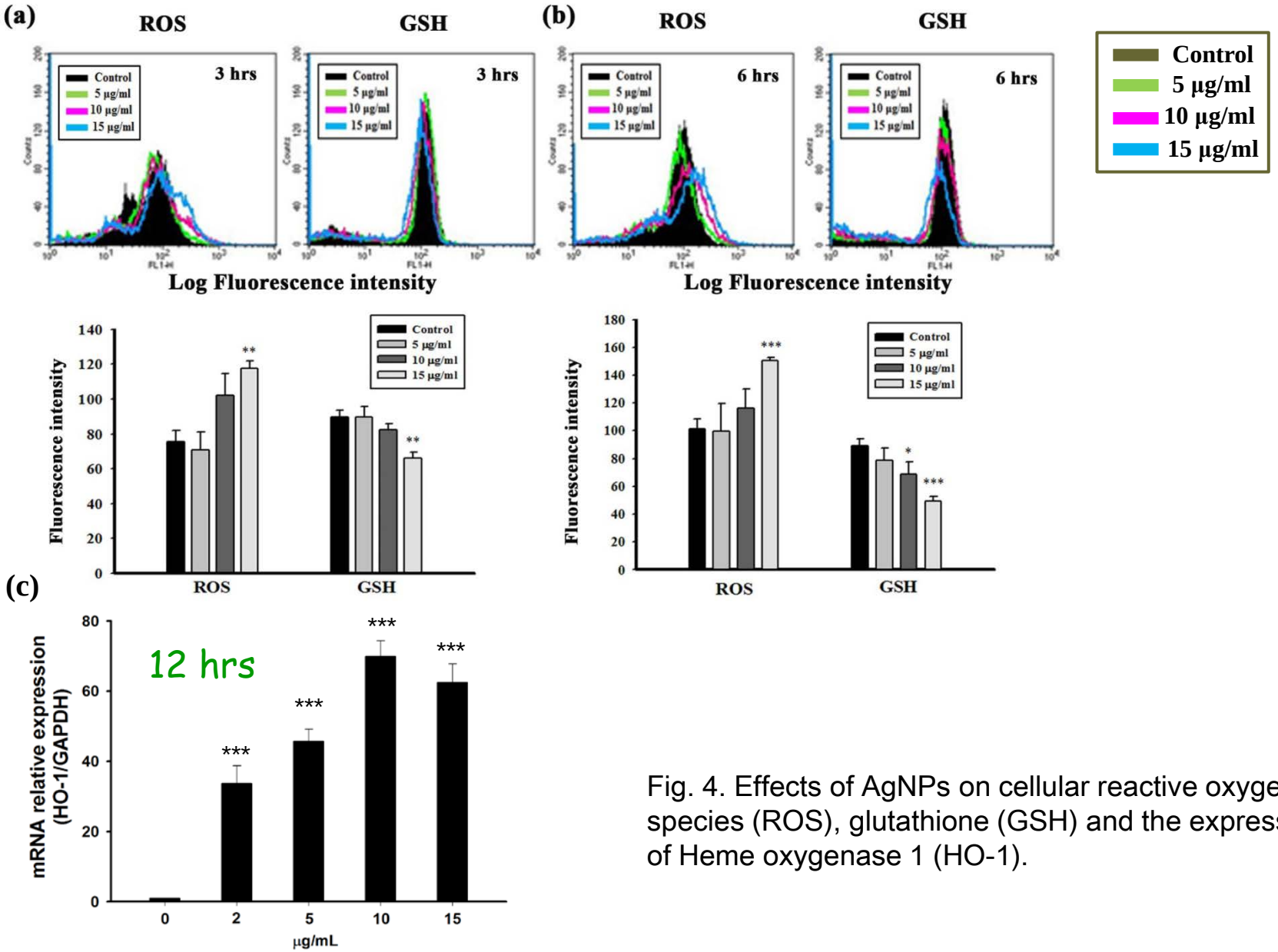
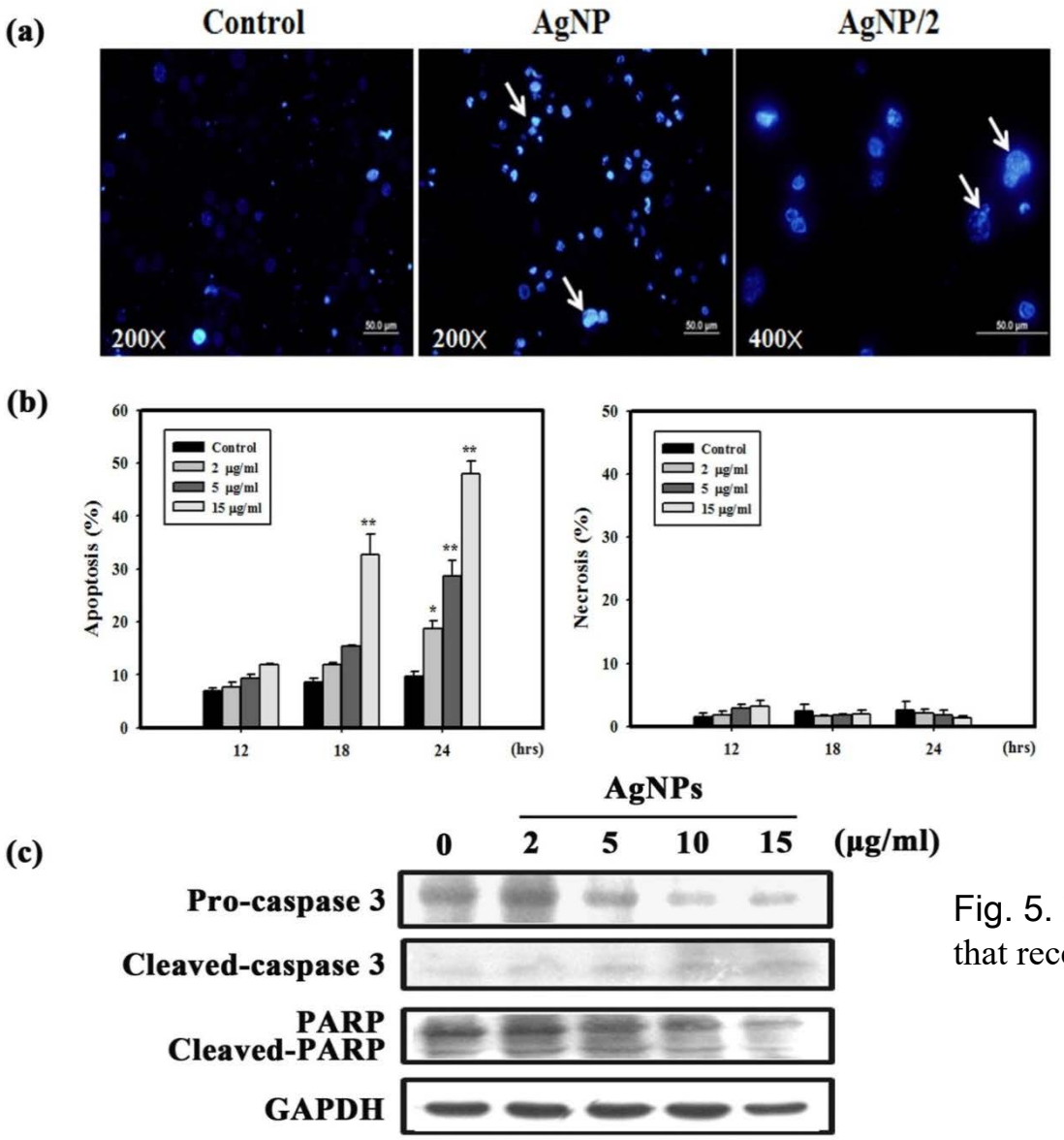


Fig. 4. Effects of AgNPs on cellular reactive oxygen species (ROS), glutathione (GSH) and the expression of Heme oxygenase 1 (HO-1).

Measurements of apoptosis and necrosis in NIH 3T3 cells treated with AgNPs



DAPI staining:
apoptotic bodies

Annexin V/PI:
Early apoptosis; Necrosis

Fig. 5. Measurement of apoptosis in NIH 3T3 cells that received various treatments. Scale bars: 50 μm.

AgNPs induced morphological and biochemical markers of autophagy in NIH 3T3 cells

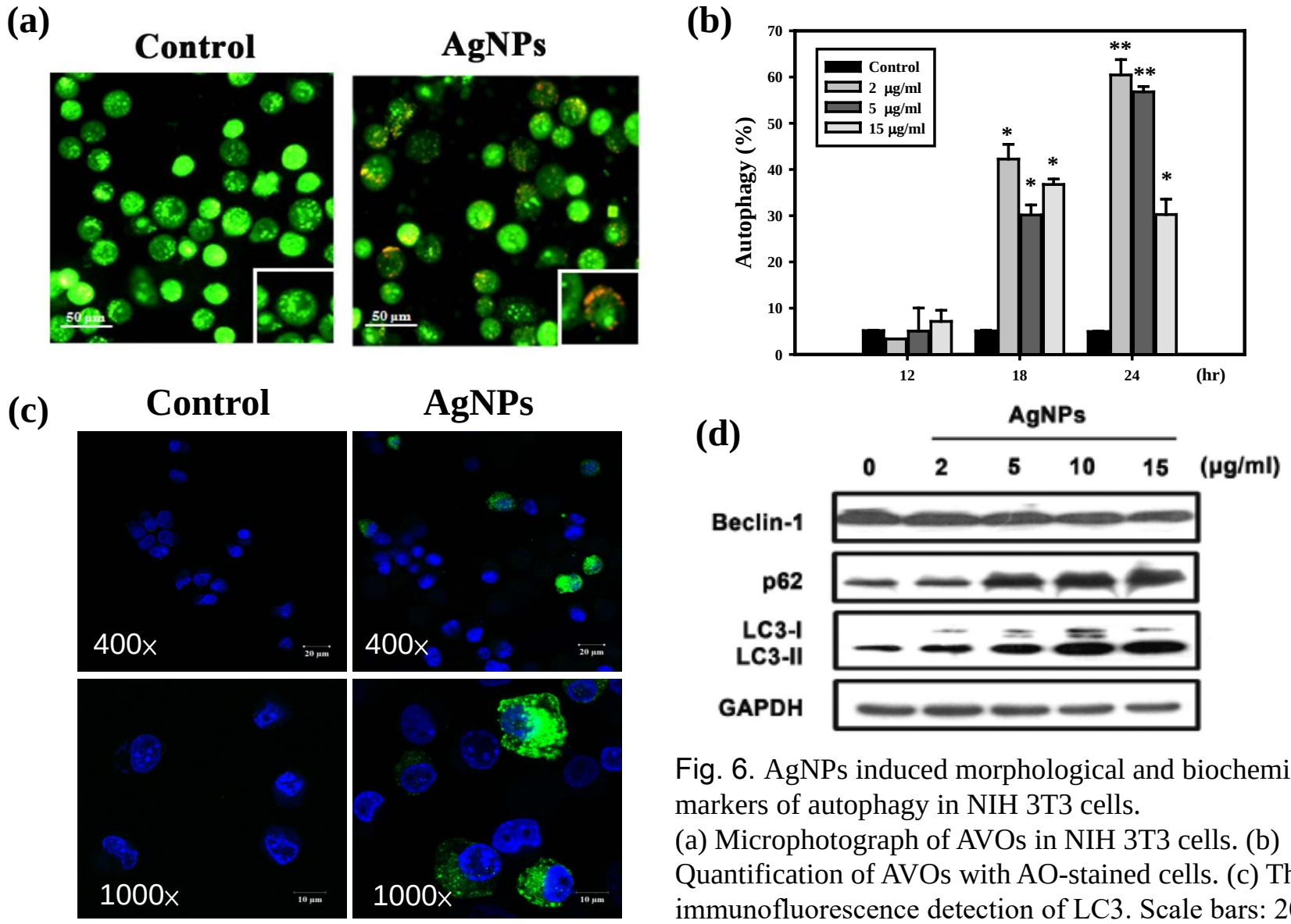
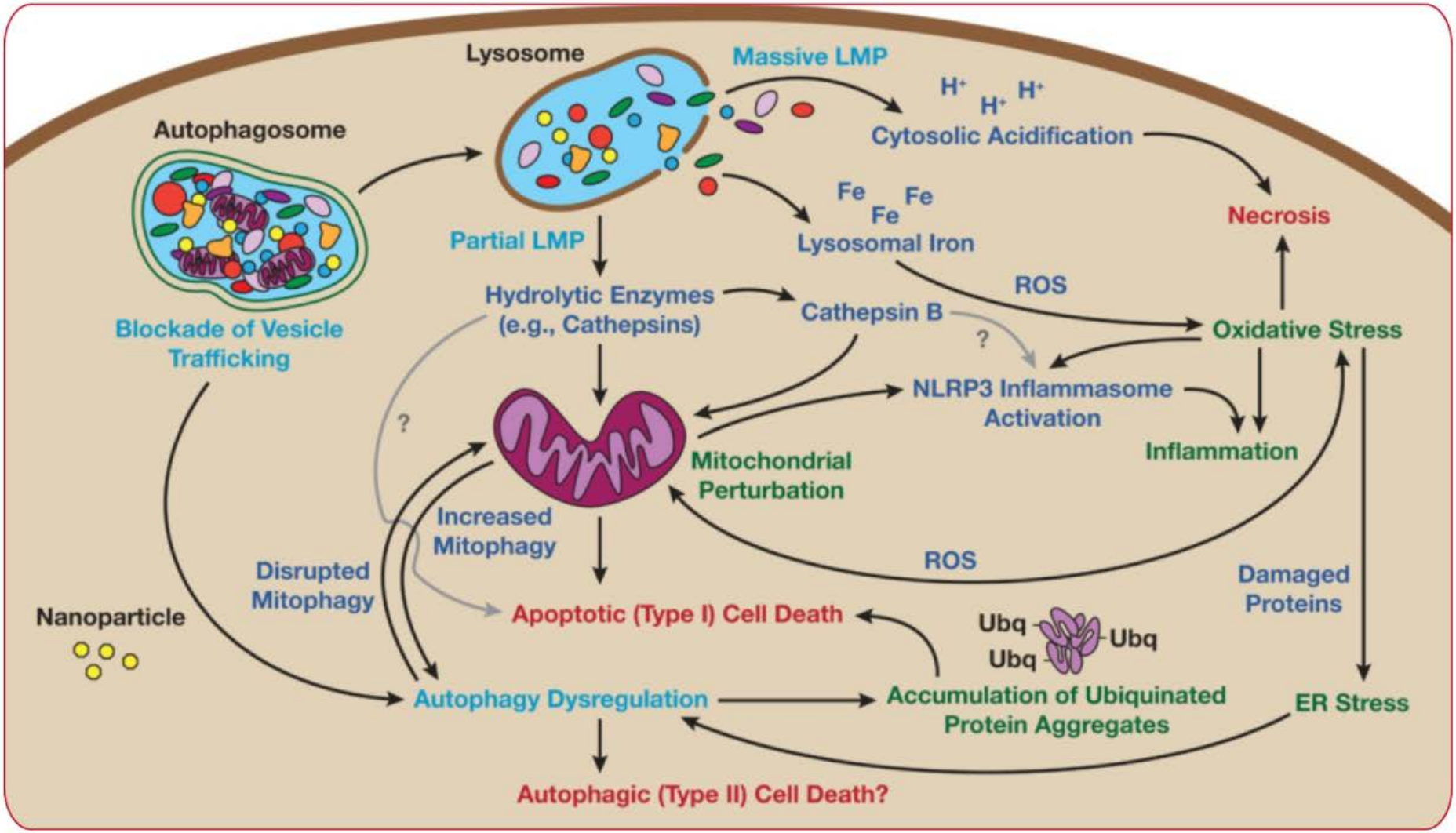


Fig. 6. AgNPs induced morphological and biochemical markers of autophagy in NIH 3T3 cells.
(a) Microphotograph of AVOs in NIH 3T3 cells. (b) Quantification of AVOs with AO-stained cells. (c) The immunofluorescence detection of LC3. Scale bars: 20 μm (up); 10 μm (down). (d) Western blotting.

Autophagy and lysosomal dysfunction as emerging mechanisms of nanomaterial toxicity



Stern *et al. Particle and Fibre Toxicology* 2012, **9**:20
http://www.particleandfibretoxicology.com/content/9/1/20 (14 June 2012)

Effect of SAS and LAS on uptake and localization of AgNPs

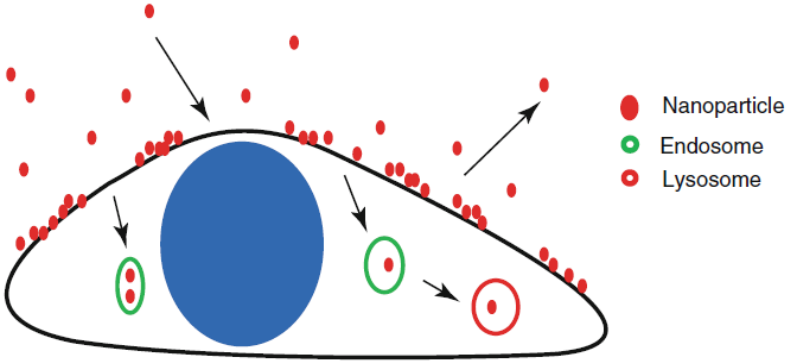


Fig. 8.1 Nanoparticle-cell membrane surface adhesion is dependent on the material and bio-molecular surface identity of the nanoparticle

Adv Exp Med Biol. 2014:811:135-56

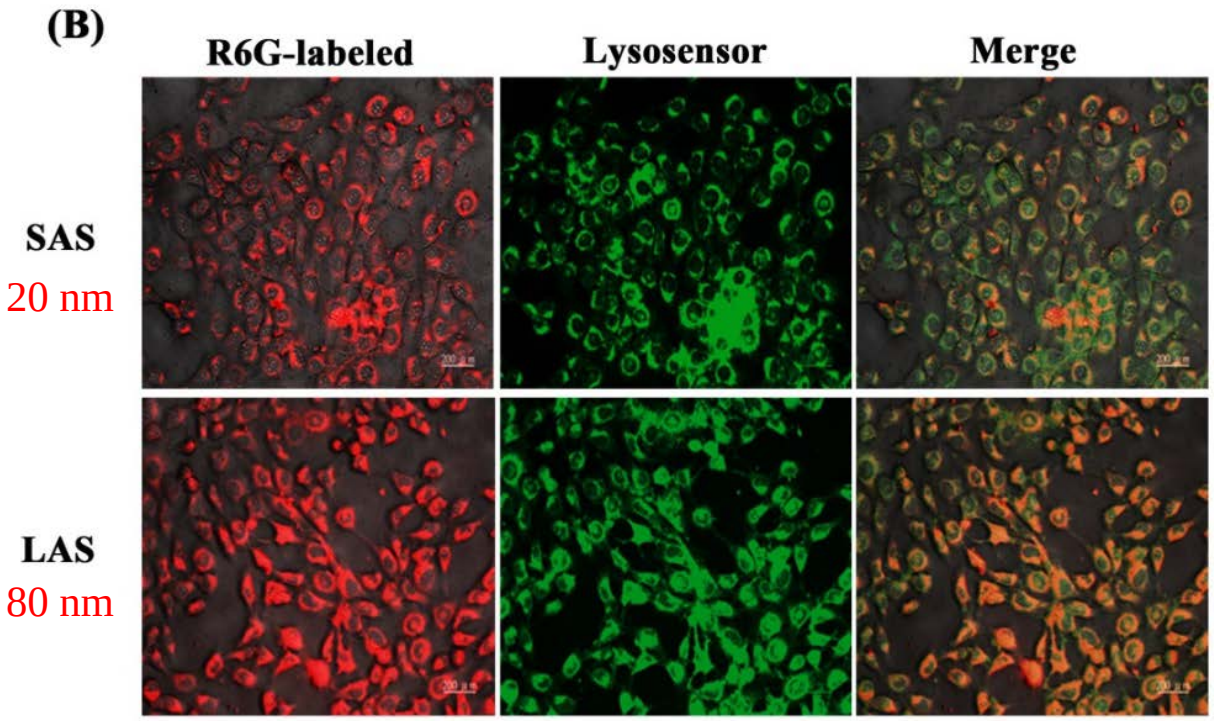
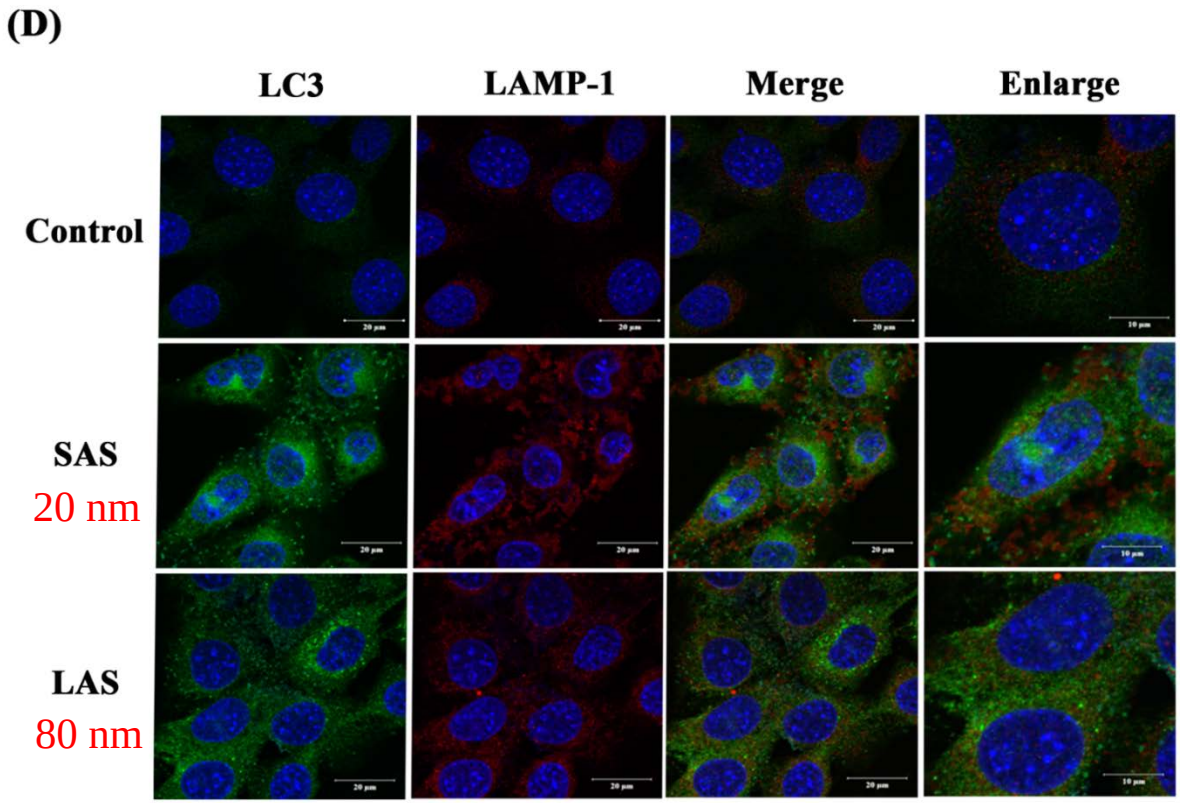
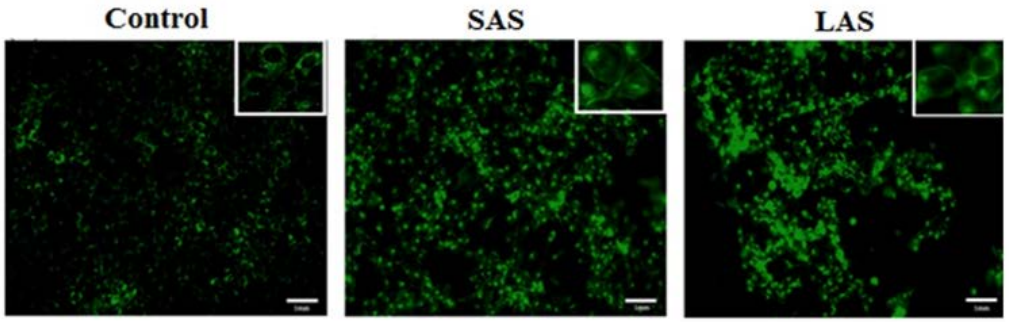


Fig. 7. Localization of AgNPs in NIH 3T3 cells. NIH 3T3 cells were treated for 3 hrs with 15 μg/ml R6G-SAS and R6G-LAS then incubated with lysosensor DND-189 for lysosome staining. Scale bars are 200 μm.

Autophagosome formation and lysosomal dilatation by AgNPs treatment



lysosome associated membrane protein-1 (LAMP-1)

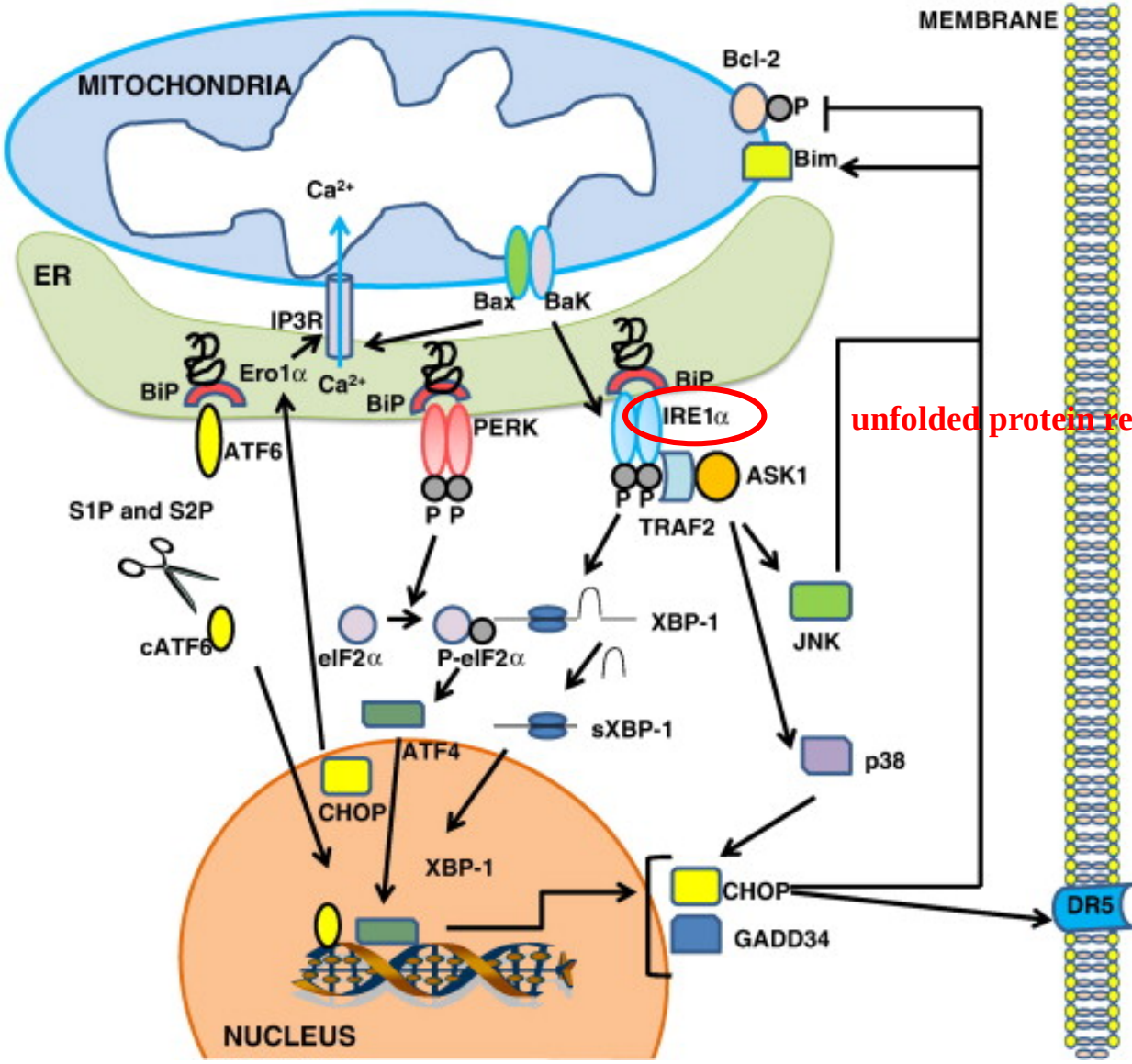


LysoSensor green DND-189

The vacuoles that formed after SAS treatment corresponded closely to dilated lysosomes

Fig. 8. Double-immunocytochemical staining of NIH 3T3 cells with anti–LC3 and anti–LAMP-1 antibodies. Cells were sham washed or treated for 12 hrs with SAS or LAS. A nearly complete separation between autophagosomes and dilated lysosomes was evident in SAS-treated cells.

ER stress signaling

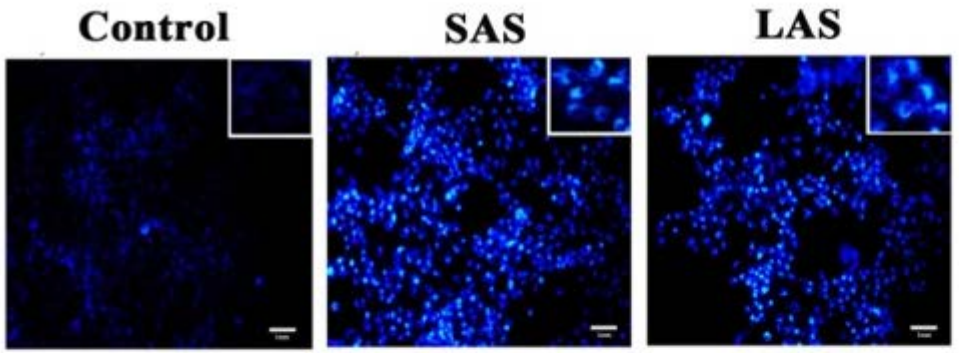


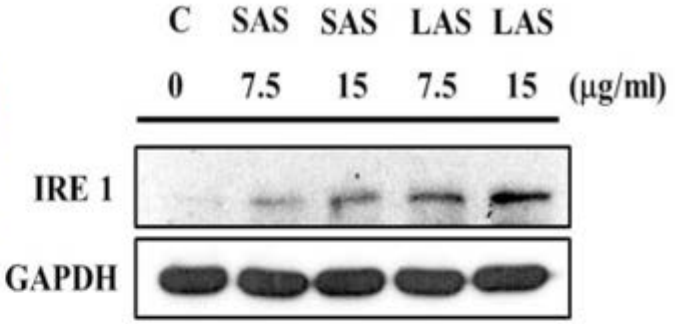
Biochim Biophys Acta. 2013 Dec;1833(12):3460-70.

◆ ER stress is an important cellular self-protection mechanism.

allowing cells to adapt to stress by initiating autophagy or inducing apoptosis

Effects of AgNPs on Endoplasmic Reticulum (ER) stress





ER-Tracker Blue-White DPX, an ER-specific dye

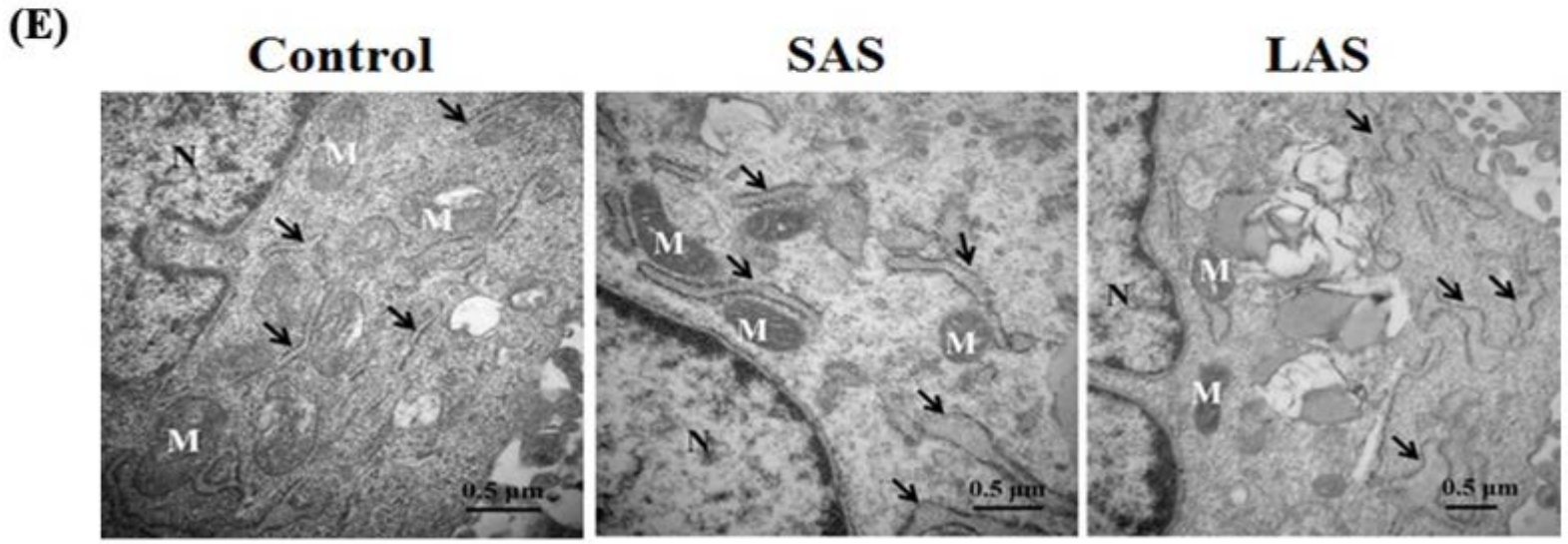


Fig. 9. Effect of AgNPs on ROS production and ER stress. Cells were treated with several doses (2, 5, 7.5, 10 and 15 μg/ml) of AgNPs for 6 hrs and 12 hrs.

AgNPs induced autophagy via ER stress

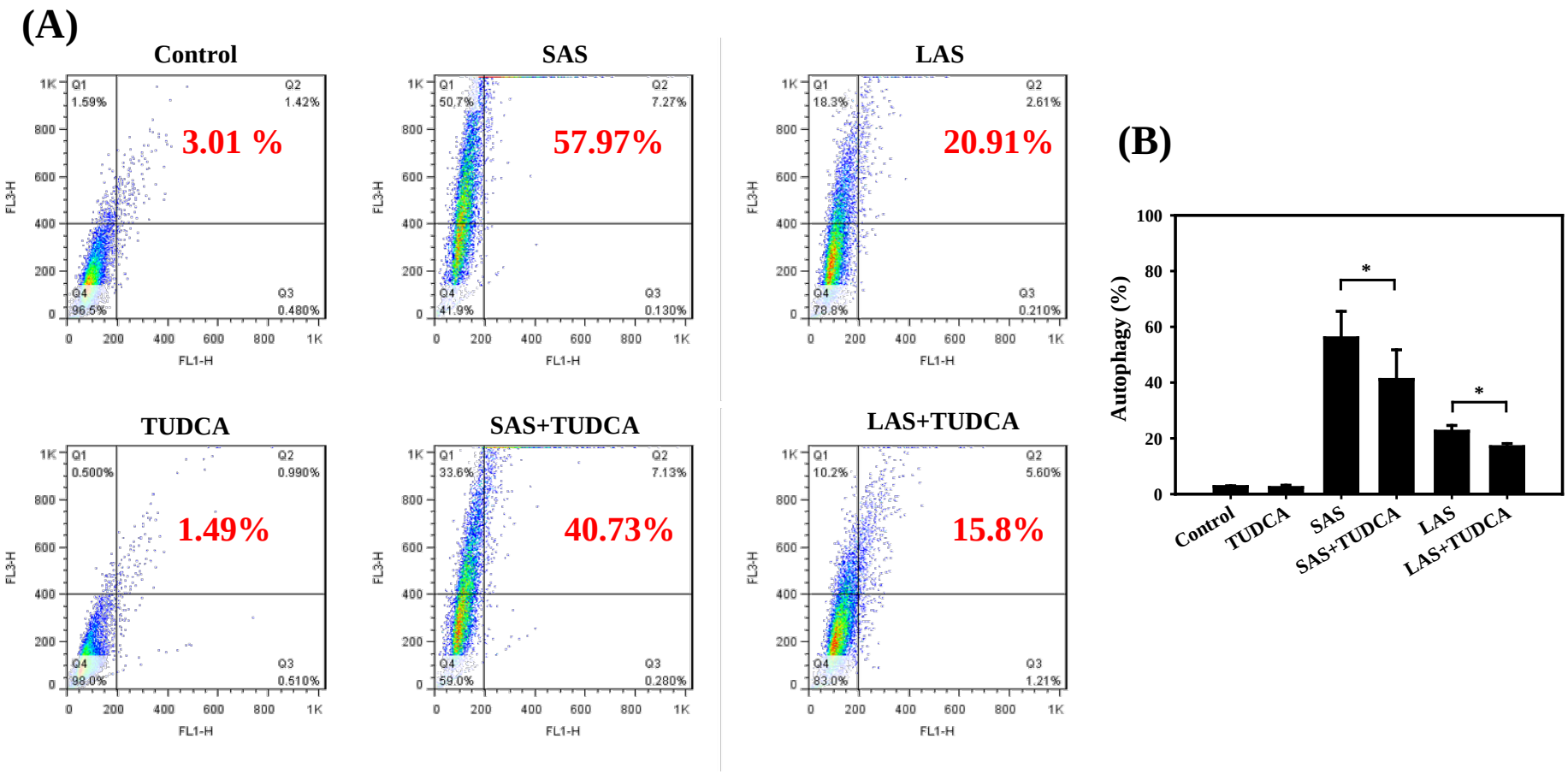
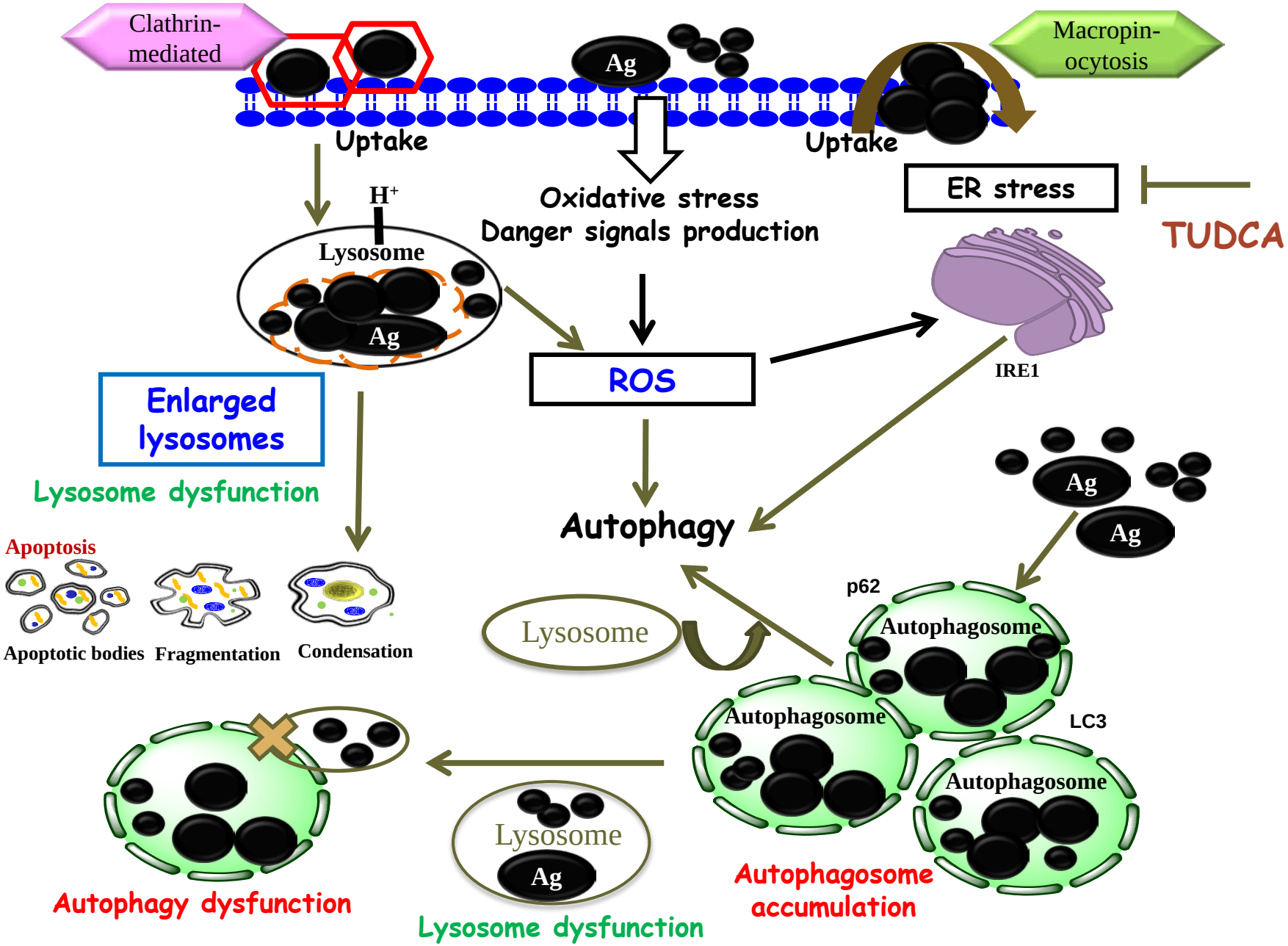


Fig. 10. AgNPs induced autophagy via ER stress in NIH 3T3 cells. Cells were treated with 15 μg/ml AgNPs with or without 500 μM TUDCA for 12 h. Detection of green and red fluorescence in AO-stained cells using flow cytometry. (A)Control; (B)SAS; (C)LAS; (D)TUDCA; (E)SAS+TUDCA; (F)LAS+TUDCA. (n=3,* $p<0.05$.)

Summary


Clathrin-mediated
Ag
Macropin-ocytosis
Uptake
Uptake
H+
Lysosome
Ag
Oxidative stress
Danger signals production
ER stress
TUDCA
IRE1
ROS
Enlarged lysosomes
Lysosome dysfunction
Autophagy
Ag
Ag
Apoptosis
Apoptotic bodies
Fragmentation
Condensation
Lysosome
p62
Autophagosome
Autophagosome
LC3
Autophagosome
Autophagosome accumulation
Lysosome
Ag
Lysosome dysfunction
Autophagy dysfunction

Conclusion

The induction of ER stress and autophagy were the critical toxic effects associated with AgNPs

The smaller-sized AgNPs resulted in more significant cytotoxicity than the larger-sized AgNPs through the induction of more extensive dysfunction of autophagy.

This study illustrates the influence of AgNPs on biological systems and may provide insights to guide the development of safe products for biomedical applications of AgNPs.

References:

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Conclusion

Table 3. Summary of cellular and molecular mechanisms underlying AgNPs toxicity

1. Induction of oxidative stress

-Lipid peroxidation (e.g. damage to the plasma membrane, disruption of the lysosomal membrane integrity)

-DNA damage, protein misfolding and aggregation, organellar injury, etc.

2. Activation of intracellular signaling pathways

- Stimulation of ER stress, apoptosis, atuophagy, and inflammasome pathways

3. Interference with mitochondrial electron transport and aerobic respiration

- Mitochondrial impairment

4. Defective autophagy

-#Blockade of autophagosome-lysosome fusion

5. Release of toxic Ag cations

-*Interference with copper homeostasis

unique for AgNPs and other like metallic NPs; * AgNPs-specific

Mechanisms of silver nanoparticle-induced toxicity and important role of autophagy. Nanotoxicology. In press (2016)






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