Nanobiotechnology
nanomedicine/nanotoxicology

Prof Emanuele Papini

?????

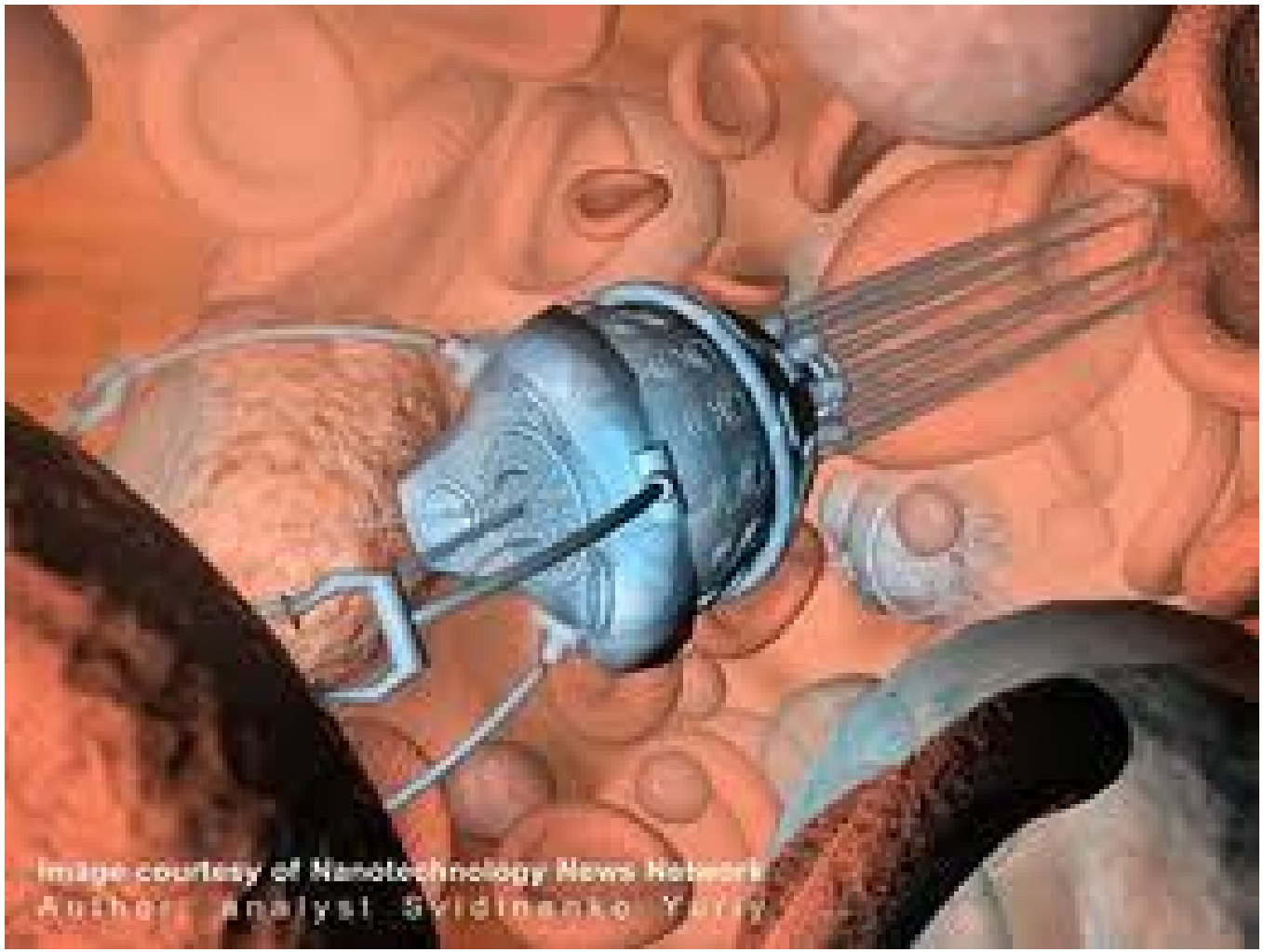


Image courtesy of Nanotechnology News Network
Author: analyst Svidinenko Yuriy

there is an increasing optimism that nanobiotechnology will be applied in medicine leading to a revolution in therapies and diagnosis, with significant advances in the fight of cancer, cure of other chronic degenerative diseases and in early and very effective/sensitive diagnosis..

among medicinal NPs, the so called ***theragnostics*** have been foresighted: a Nanosystem able to diagnose and to once treat an ill cell (e-g a cancer cells)

Future applications anticipated are in 1. drug delivery 2. diagnosis 3. production of biocompatible material (nanostrustured materials forming micro or macroscopic objects like implants or intrabody devices and apparatuses)

- The first nano-biotechnological application is the use of liposomes in the ‘70s

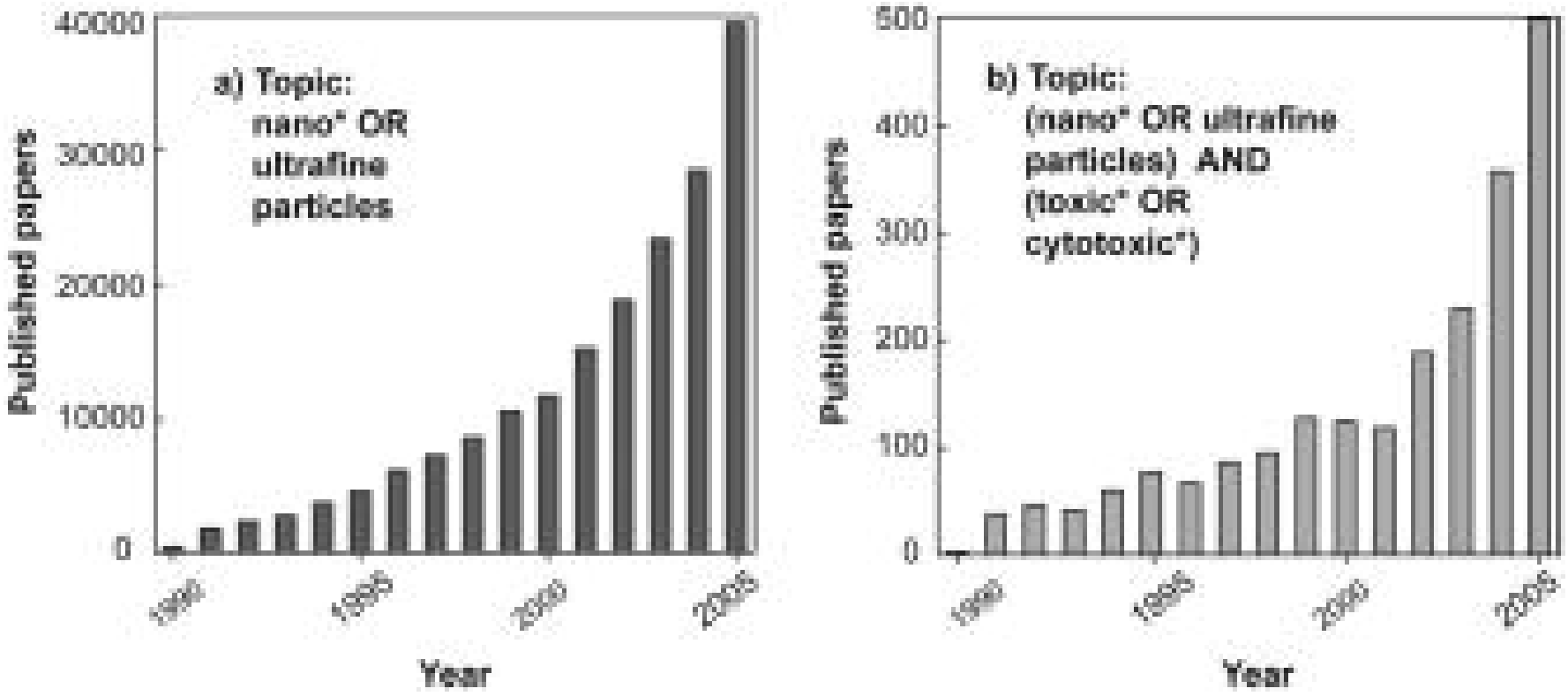
- From that initial start other nano-systems or nanoparticles (NPs) were then developed as i) drug delivery carriers ii) Imaging tools

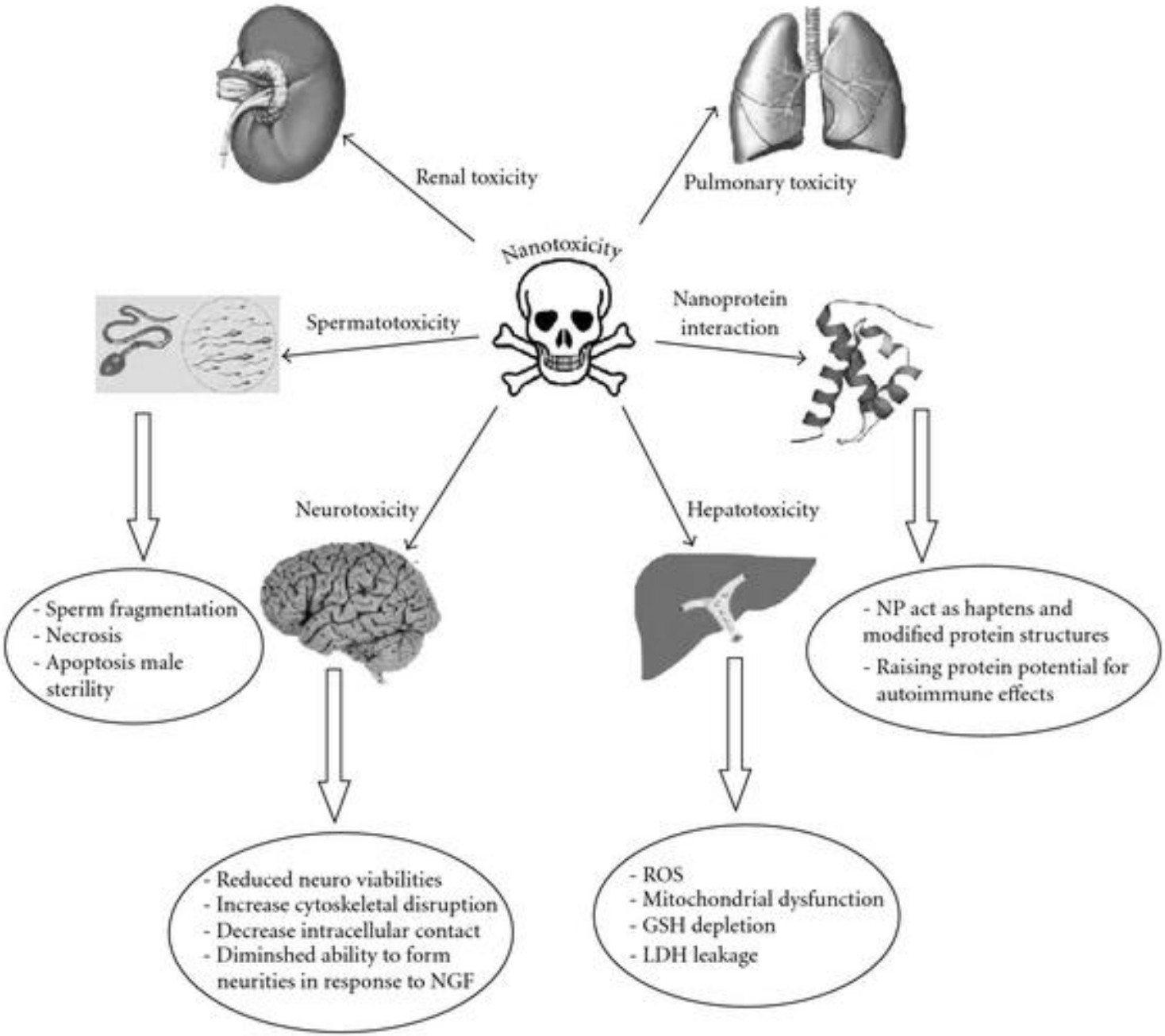
- However the toxicological issue emerged: Nanotoxicology

However NPs toxicity appears more the rule than the exception, and, as said, investigations in nanotoxicology are growing at the same rate than nanomedical studies

Indeed the attention on nanostructures generate from the discovery of the so called ultrafine particulates present in pollution (fuel derivatives fro example) and pathogenic studies are more advanced in the field of environmental nanoparticles toxicology

the toxicological issue emerged: Nanotoxicology




Renal toxicity
Pulmonary toxicity
Nanotoxicity
Spermatotoxicity
Nanoprotein interaction
Neurotoxicity
Hepatotoxicity
- Sperm fragmentation
- Necrosis
- Apoptosis male sterility
- NP act as haptens and modified protein structures
- Raising protein potential for autoimmune effects
- Reduced neuro viabilities
- Increase cytoskeletal disruption
- Decrease intracellular contact
- Diminshed ability to form neurities in response to NGF
- ROS
- Mitochondrial dysfunction
- GSH depletion
- LDH leakage

- Combustion derived nanoparticles (CDNPs) are generally environmental NPs.

They are known to be inflammogenic and to induce effects on people already suffering of respiratory or cardiovascular diseases

- Oxidative stress appears to mediate the detrimental action of such “pollution” NPs, but also lung ad systemic inflammation are induced..

What is the pathogenetic mechanism of CDNPs?

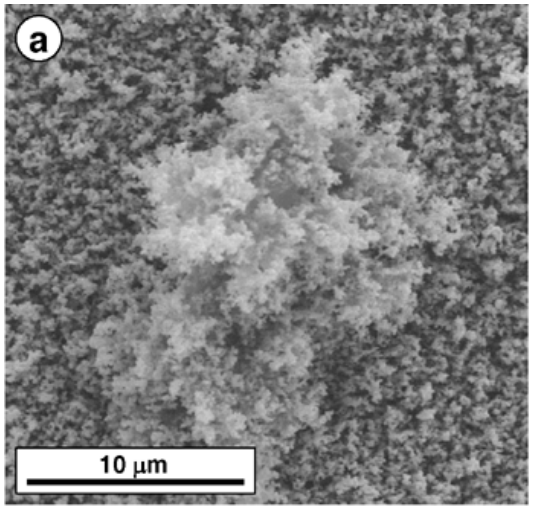
In this course I have therefore decided to start analysing the progress made in the analysis of the properies and bio-effects of environmental industrial NPs: the so called CDNPs

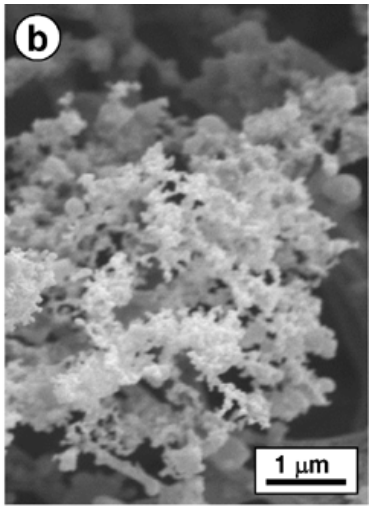
CDNPs stands for Combustion Derived Nano Particles

CDNPs are a fundamental model for what we must avoid when developing NPs for medical applications

Field emission scanning electron micrographs of combustion-derived nanoparticles

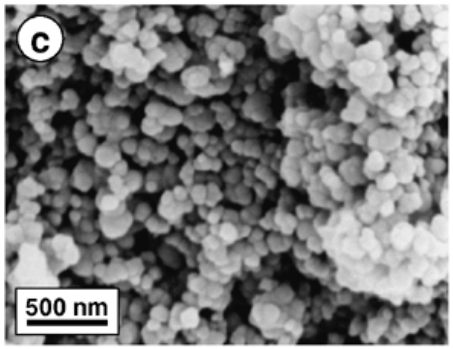
large soot nanostructured particle, lying on a dense bed of soot nanoparticles

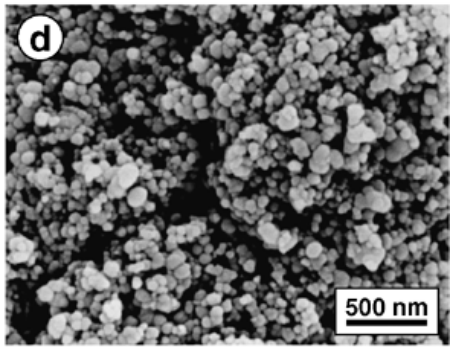




detail of the flocculated ('bunch-of-grapes') structure of soot;

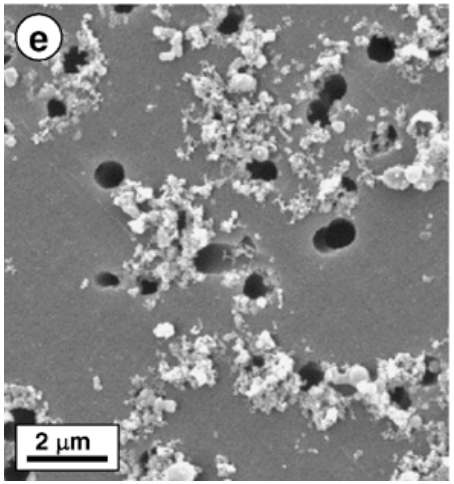
carbon black sample mixed population of fine and nanosized particles

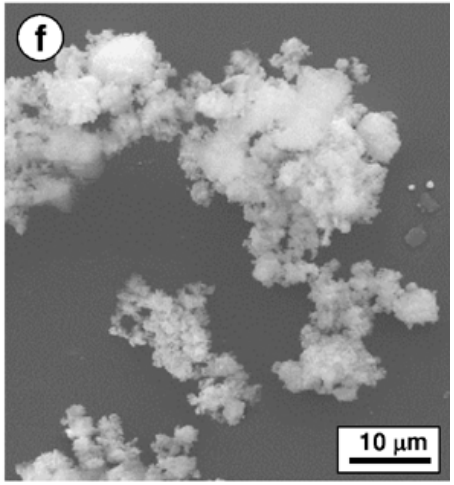




carbon black sample from a population of strictly nanoparticles

urban nanoparticles





residual oil fly ash (ROFA) particles

Airborne Perticulate Matter (PM) derives from three main human activities: 1. Industrial 2. combustion 3. automobile

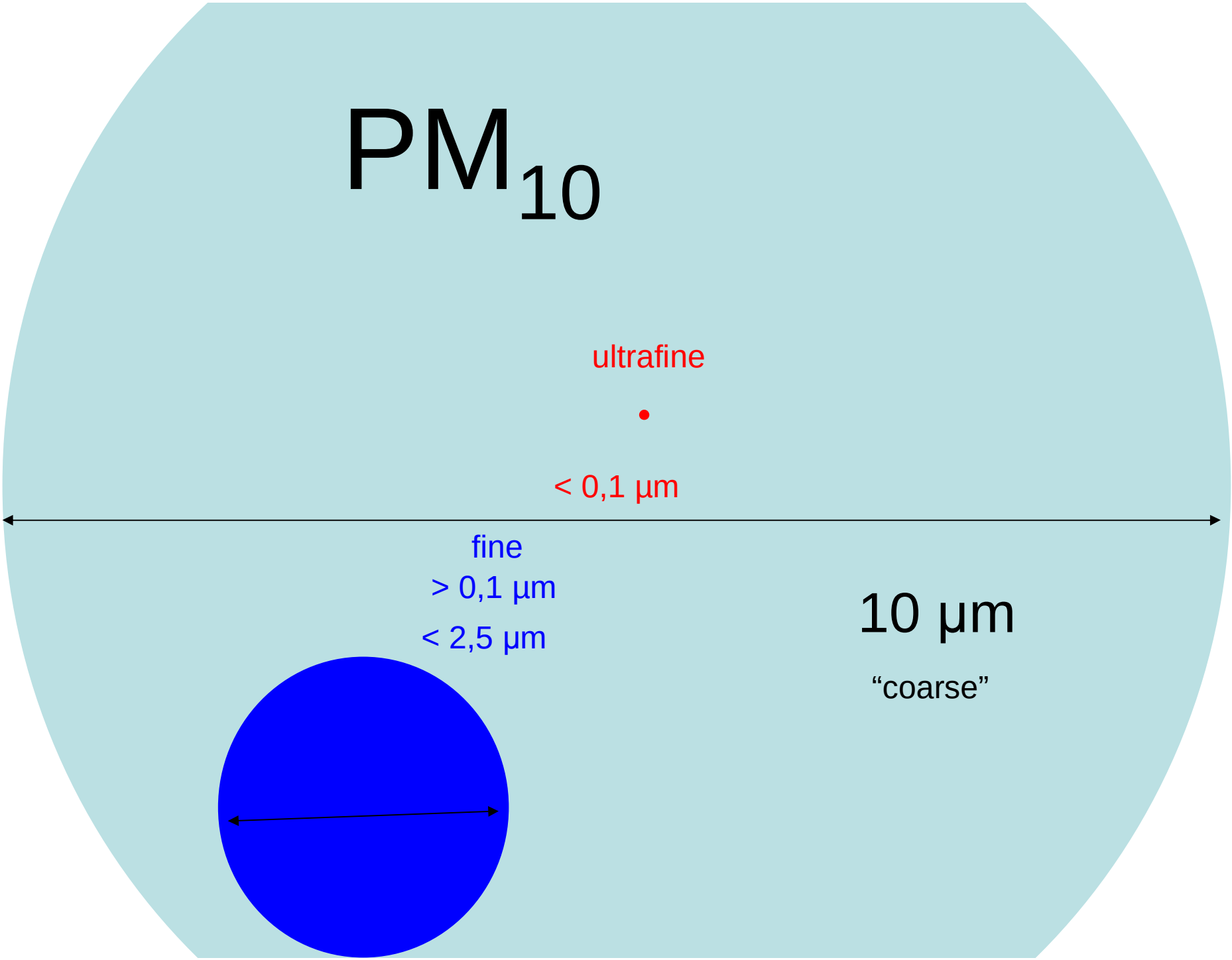
Airborne Particulate Matter is also called PM_{10} because it contains particles with a diameter lower than 10 μm

PM_{10} contains particles of three size categories:

“coarse” with a diameter comprised between 2,5 and 10 μm

“fine” particles, with a diameter comprised between 0,1 μm and 2,5 μm

“**ultrafine”** (UF) particles with an aerodynamic diameter lower than 0,1 mm (or 100 nm): these are the real NPs present in the PM10


PM10
ultrafine
< 0,1 µm
fine
> 0,1 µm
< 2,5 µm
10 µm
“coarse”

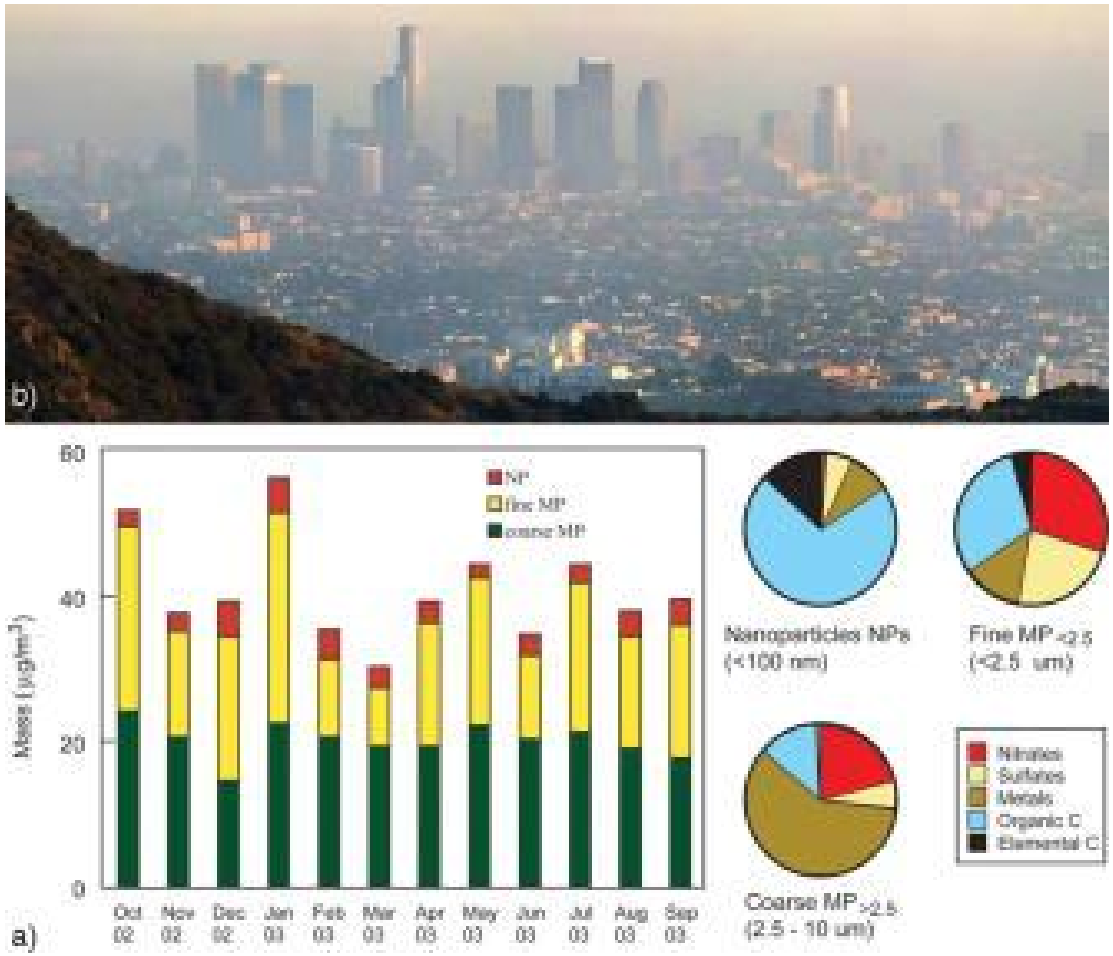
It is evident that PM10 is highly heterogeneous in mature and it is very difficult to define in terms other than size distribution

In industrial areas PM10 are generated by road transport, industrial and construction activities

While in rural areas it is mostly formed by pollen grains, fungal spores, plant materials

From the toxicological point of view the traffic exhausted derived NPs are responsible for up to 80% of human exposure!

Inhalation into the respiratory system is the major portal entry of PM10 in humans


b)
a)
Mass (μg/m³)
60
40
20
0
NP
fine MP
coarse MP
Oct 02
Nov 02
Dec 02
Jan 03
Feb 03
Mar 03
Apr 03
May 03
Jun 03
Jul 03
Aug 03
Sep 03
Nanoparticles NPs (<100 nm)
Fine MP<2.5 (<2.5 um)
Coarse MP>2.5 (2.5 - 10 um)
Nitrates
Sulfates
Metals
Organic C
Elemental C

One important aspect is the following:
Inhaled NPs can cross the epithelia and the endothelia, and possibly reach the systemic circulation wherefrom they can target various sensitive organs

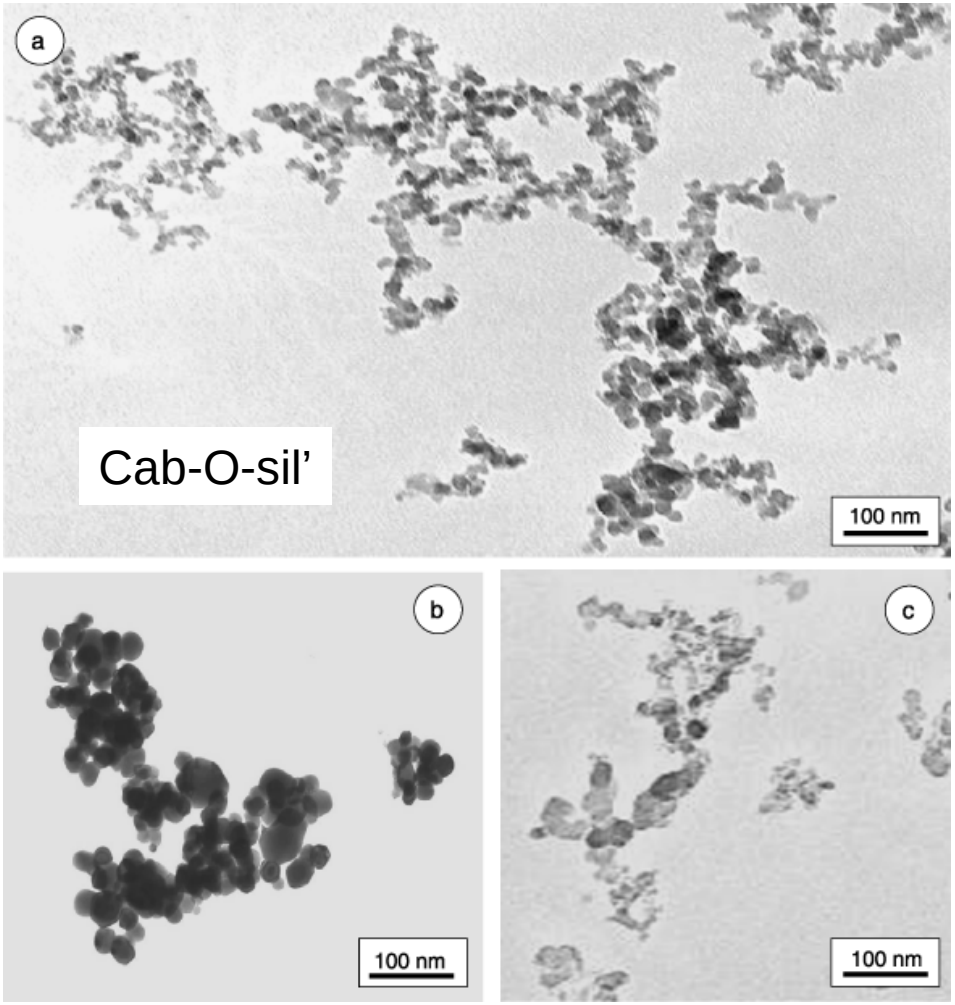
ROS generation is considered a central effect induced by toxic NPs, ROS generation is linked to inflammatory lung disease

In addition invasion of the systemic circulation has been implicated in the observed cardiac dysfunctions, observed in experimental animals and humans subjected to environmental industrial inhalation

- **The Particle Physico-chemistry**

- Nanoparticles are defined as NPs with diameter or at least one dimension lower than 100 nm
- However: many manufactured, combustion derived or natural (*volcanic activity, earth erosion, sands storms*) nanoparticles are prone to rapid agglomeration
- These agglomerates of NPs have a size in the micron range but are nonetheless “nanostructured particles” or NSPs. An example is provided by TiO2 (titanium dioxide) NPs, a manufactured material.

TEM of manufactured and combustion-derived nanoparticles (anthropogenic):



TiO**2** (Sigma, Poole, UK);

diesel exhaust particles

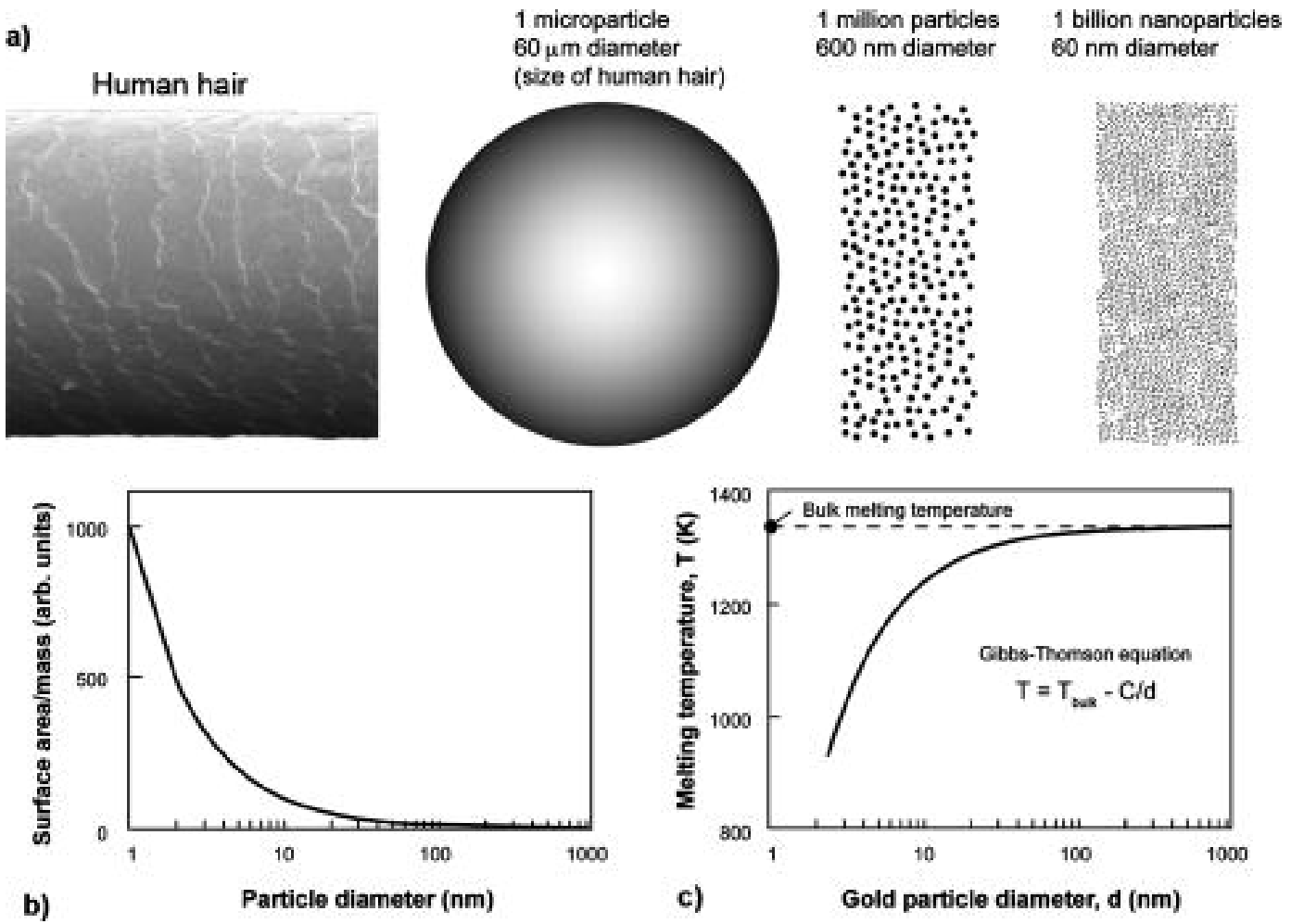
- It is important to note that TiO_2 in the nanostructured form has a much higher bio-activity compared with the same amount of substance a single crystal. This appears a general observation in nanotoxicology.
- It is suggested that the lung toxicity in humans is related to the physicochemical propensity to form NSPs but olso to the chemical nature of the NPs and the presence of other releasable constituents

CDNPs

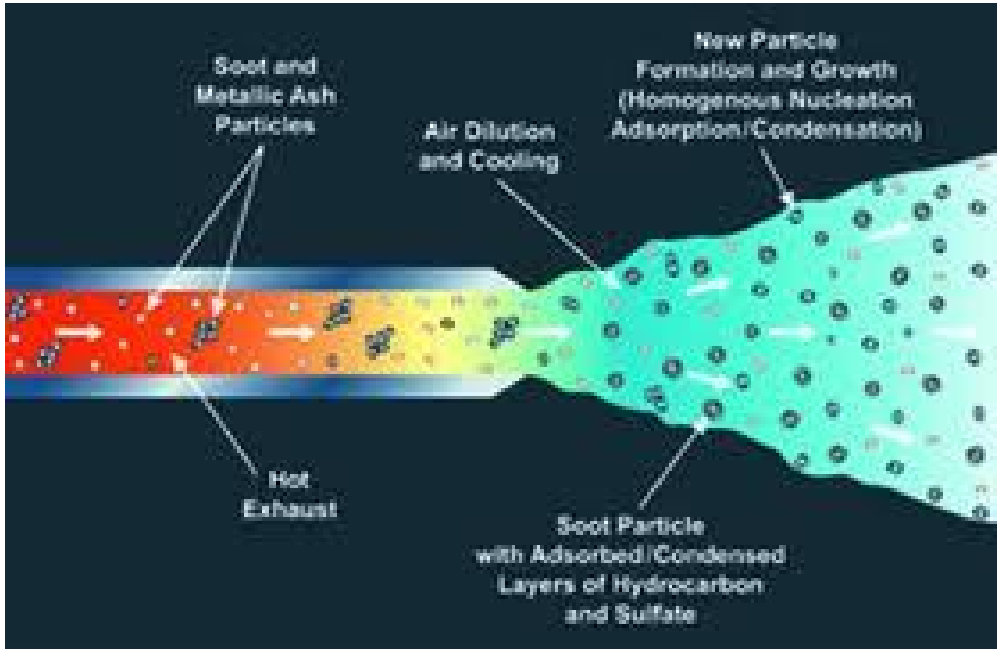
They are classified into three groups:

- Diesel Exhausted Particles (DEPs)
- Carbon Black (CB)
- Fly Ash (FA, comprising Residual Oils Fly Ash or ROFA)

- All these particles, although different in chemical nature, are poorly soluble and their high Surface Area (SA) is considered crucial for bio-effects, and the principal cause of lung chronic inflammation


a)
Human hair
1 microparticle
60 μm diameter
(size of human hair)
1 million particles
600 nm diameter
1 billion nanoparticles
60 nm diameter
Surface area/mass (arb. units)
1000
500
0
1
10
100
1000
Particle diameter (nm)
b)
Melting temperature, T (K)
1400
1200
1000
800
Bulk melting temperature
Gibbs-Thomson equation
T = T_bulk - C/d
1
10
100
1000
Gold particle diameter, d (nm)
c)

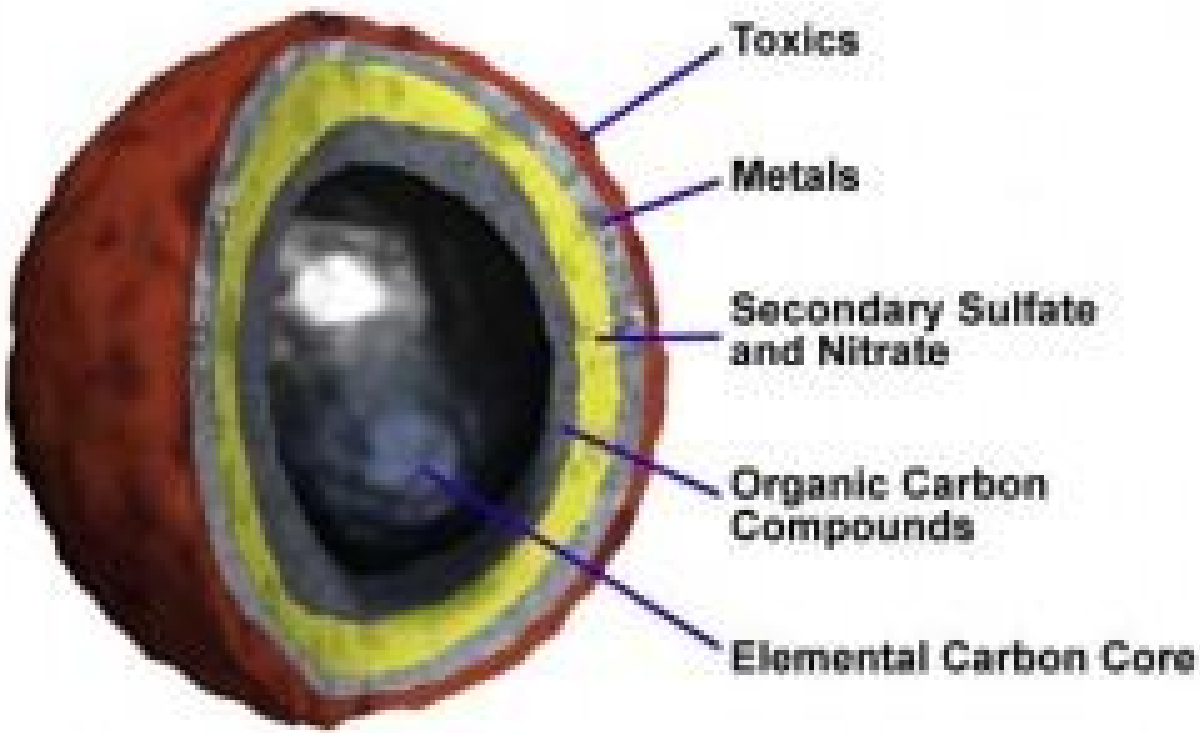
DEPs


Soot and
Metallic Ash
Particles
Air Dilution
and Cooling
New Particle
Formation and Growth
(Homogenous Nucleation
Adsorption/Condensation)
Hot
Exhaust
Soot Particle
with Adsorbed/Condensed
Layers of Hydrocarbon
and Sulfate

DEPs

- They are formed by the residue of diesel engine combustion.
- In urban areas DEPs accounts for up to 80% of the mass of PM10
- DEPs contain toxic organic molecules and toxic metals that contribute to their observed toxicity with the already mentioned high SA. Indeed transition metals, organics and SA all synergise in the production of Reactive Oxygen Species (ROS)
- We need to understand why SA is so critic: it allows the rapid dissolution of soluble species (higher surface to volume ratio compared to micro or macro materials with the *same* chemical composition..); it provides a large substrate area on which catalytic chemistry can occur in the proper conditions

DEPs



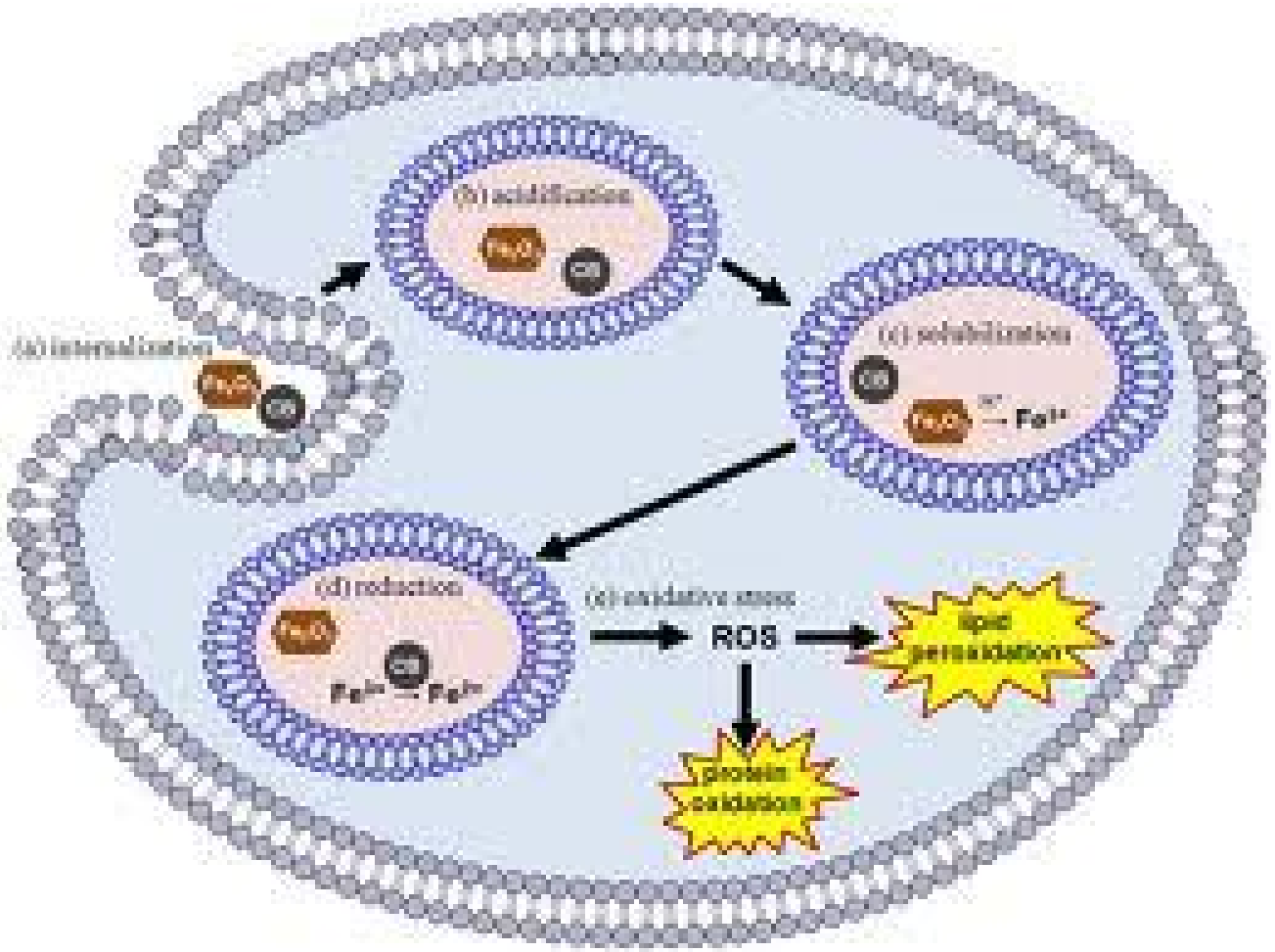
•DEPs contain toxic organic molecules and toxic metals that contribute to their observed toxicity with the already mentioned high SA. Indeed transition metals, organics and SA all synergise in the production of Reactive Oxygen Species (ROS)

CB particles

- Incomplete thermal decomposition of hydrocarbons leads to low-solubility particles in many industrial process.

- CB production can be controlled to do NPs with a range of industrial applications: e.g. the common photocopier toner
- Long term occupational exposure to CB particles can lead to chronic bronchitis with a slight reduction of lung function
- Only under excess exposure it has been observed iin early studies that CB can induce lung fibrosis
- In conclusion epidemiological studies established a relatively low risk for human health due to CB exposure

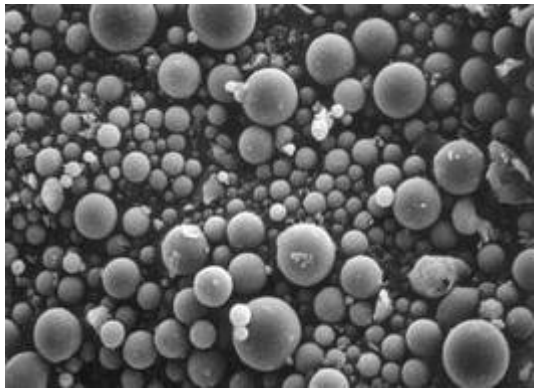
CB particles and ROS



FA

- Fly ash is a generic term for particulate matter derived from contaminants (organic and mineral) of organic fuels. There can be FA from combustion of oils (ROFAs) or from combustion of solid fuels like coal or waste

FA particles

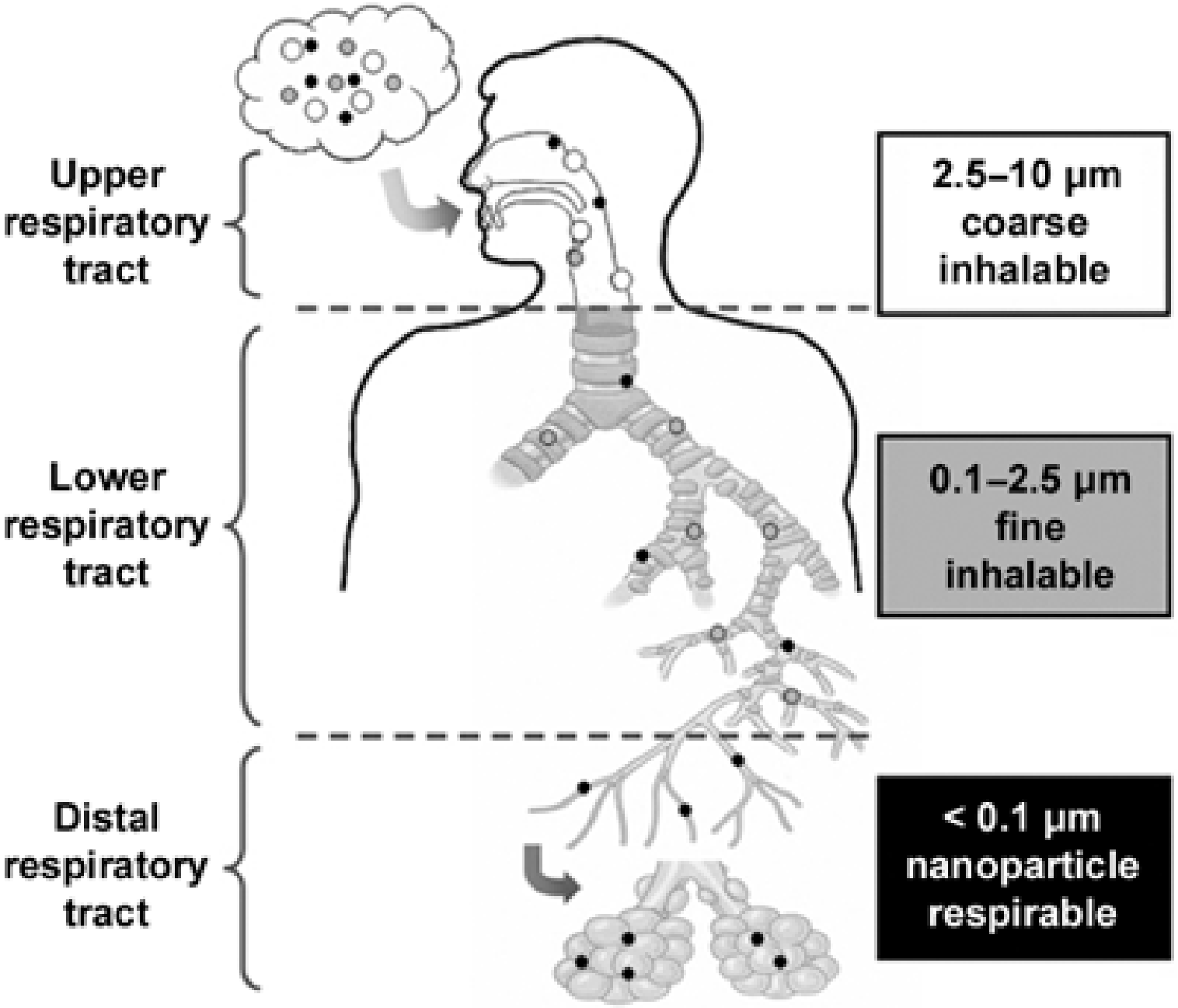


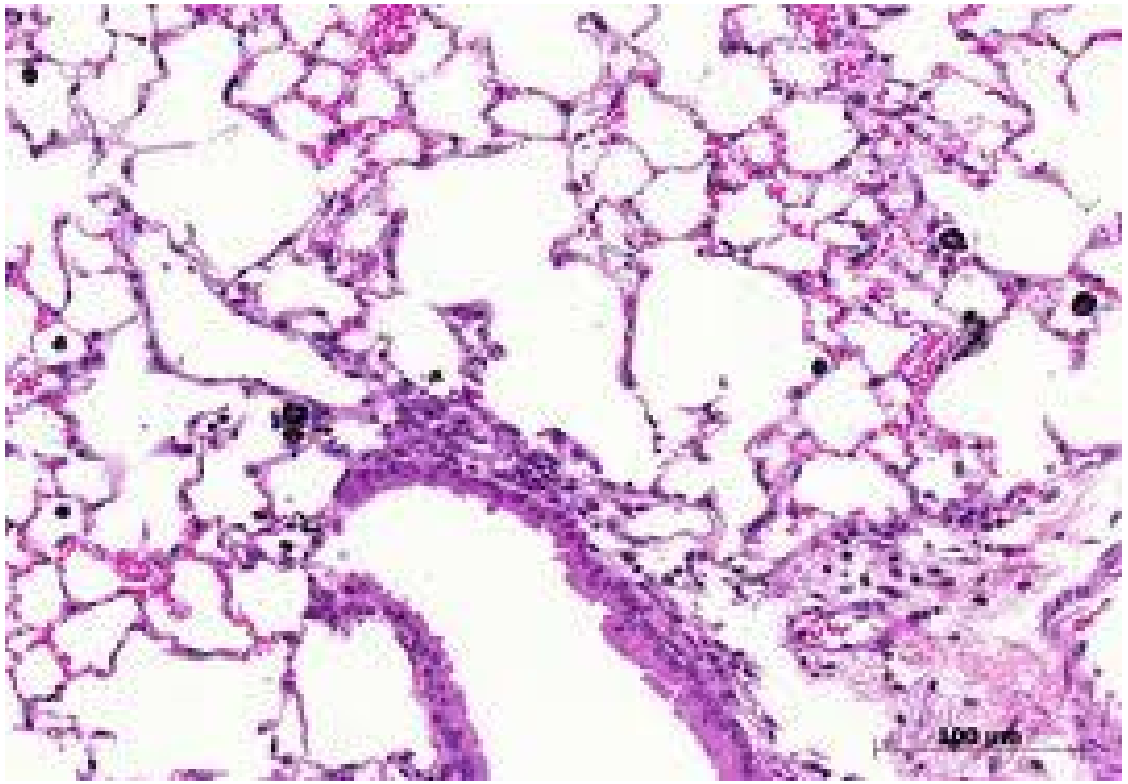
- ROFA contain transition metals, sulphates and acids incorporated in a carbonaceous particulate core. The solubility and bio-availability of these load, and in particular of transition metals is considered critic and of special interest in the evaluation of ROFA toxic effects
- FA from coal or waste combustion also derive from minerals, sulphur or other contaminants present in the fuel, and ROS generation is dependant on the bio-availability of transition metals

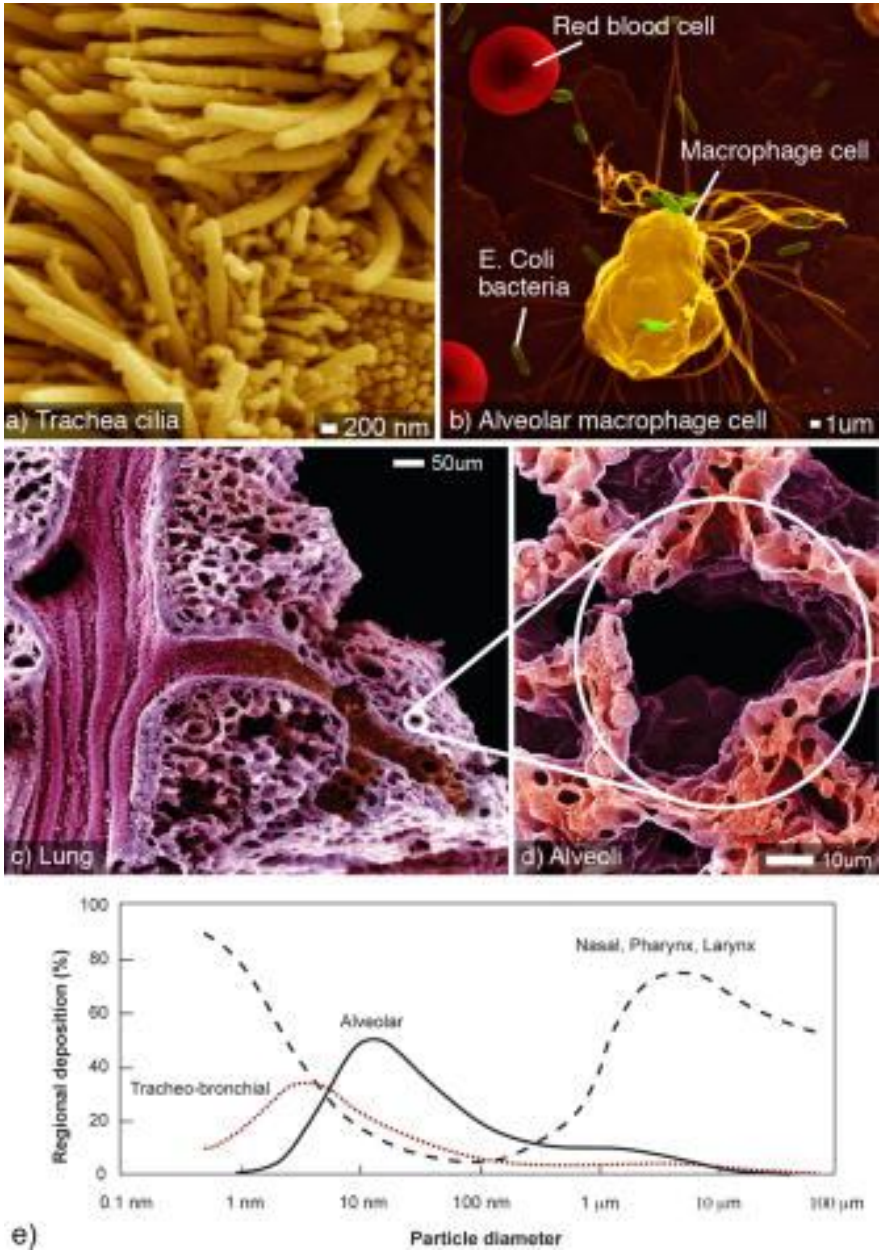
FA particles

- Although FA also contains particulate in the “coarse” fraction (2,5-10 μm) the most pathogenic part is the NSPs fraction: it contains carbonaceous aggregates of 20-50 nm and surface-bound particles of Fe, Al, Ti (~10 nm)
- However, the metal content of FA is lower compared to DEP. It is believed that the principal factor responsible for ROS production induced by FA is SA

Distribution of PM10 particles in the human respiratory system.




100 µm

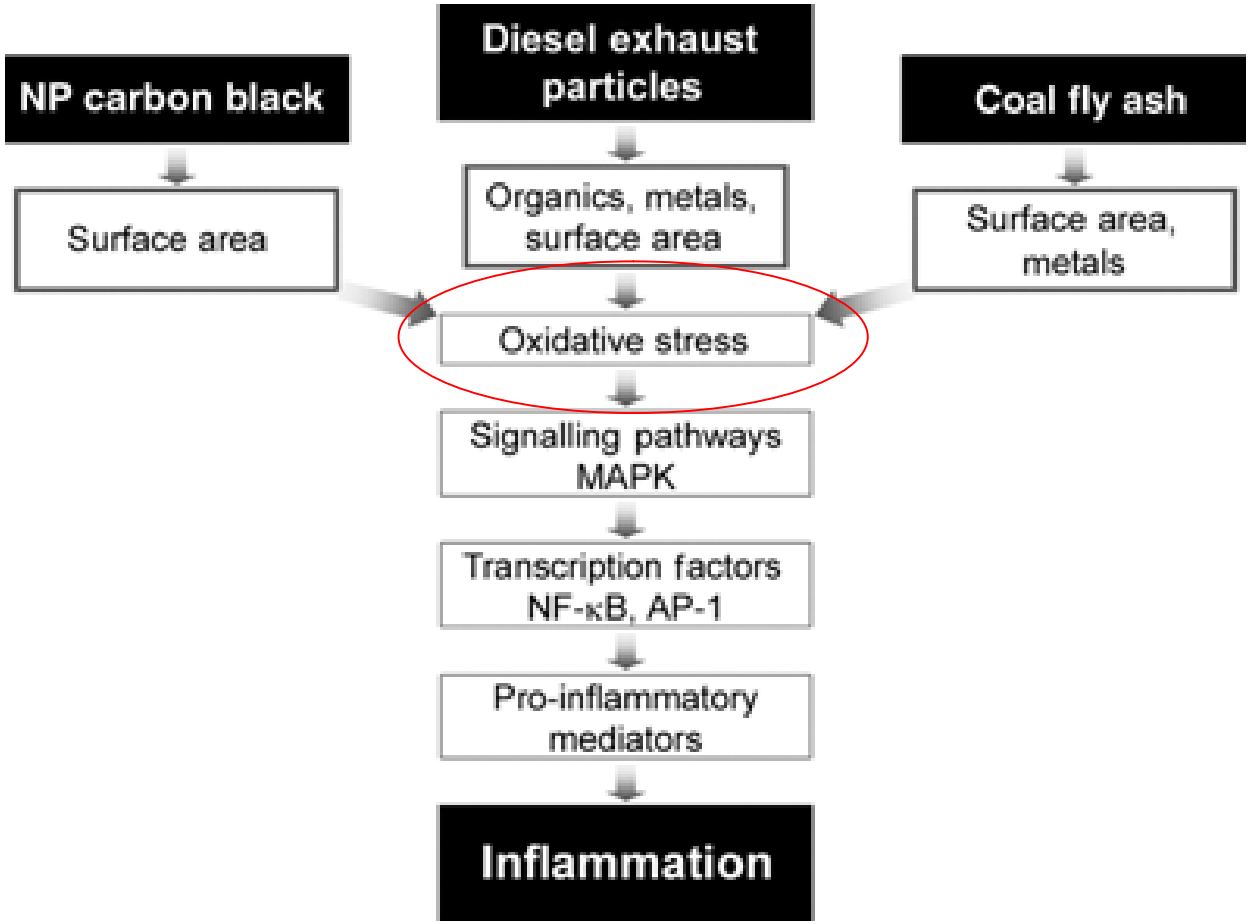

a) Trachea cilia
200 nm
Red blood cell
Macrophage cell
E. Coli bacteria
b) Alveolar macrophage cell
1um
50um
c) Lung
d) Alveoli
10um
Regional deposition (%)
100
80
60
40
20
0
Nasal, Pharynx, Larynx
Alveolar
Tracheo-bronchial
0.1 nm
1 nm
10 nm
100 nm
1 µm
10 µm
100 µm
Particle diameter
e)

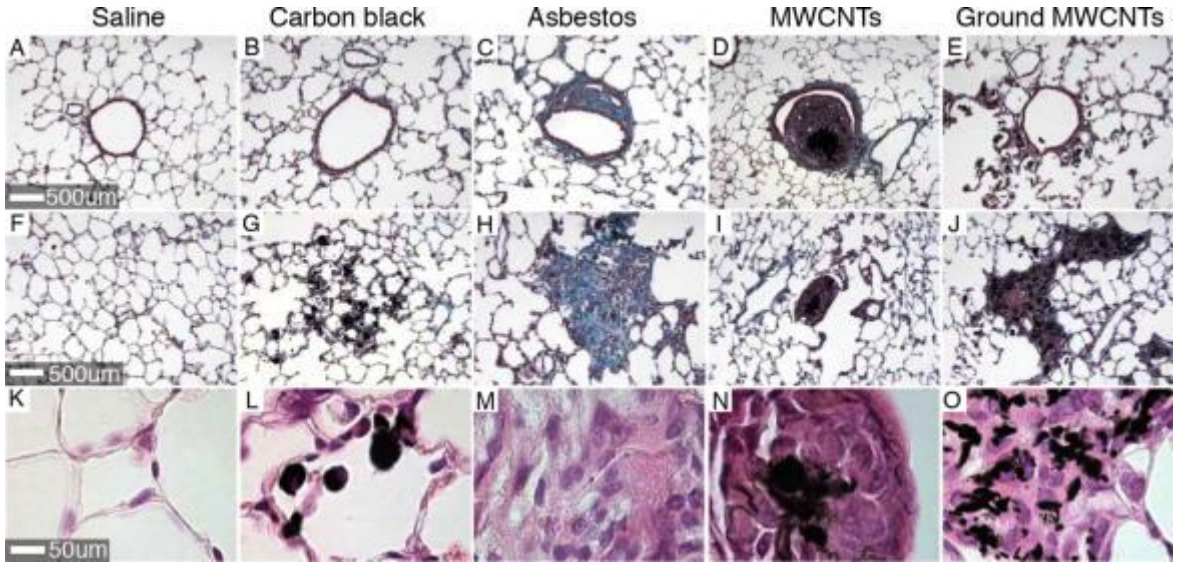
Nanoparticles toxicology

Inflammation is a common response to inhalation of CDNPs

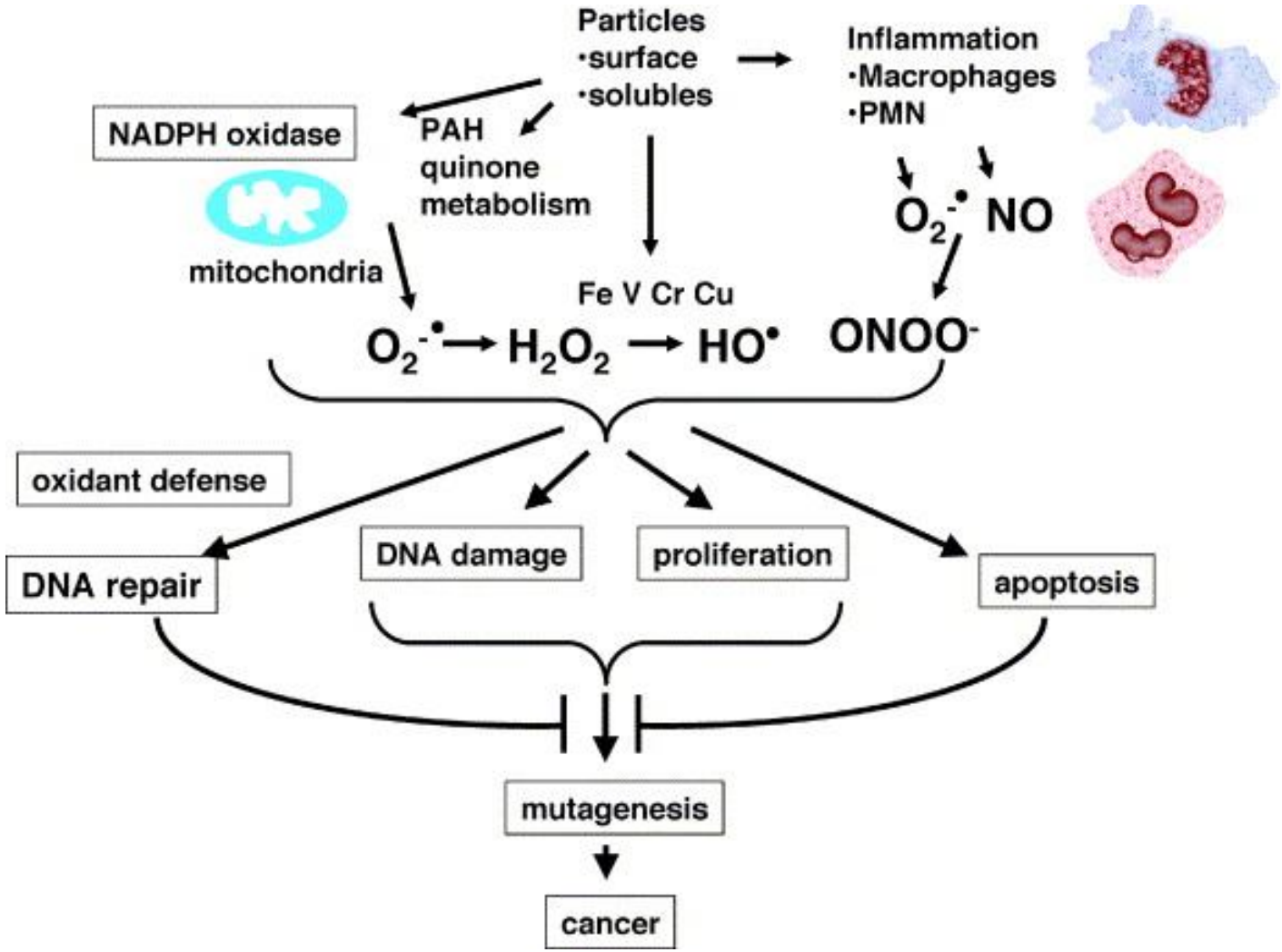
- There is a unifying hypothesis for their toxicity: ROS determine the activation of redox-sensitive transcription factors like nfk-b. This leads to fibrosis, chronic inflammation and possibly cancer

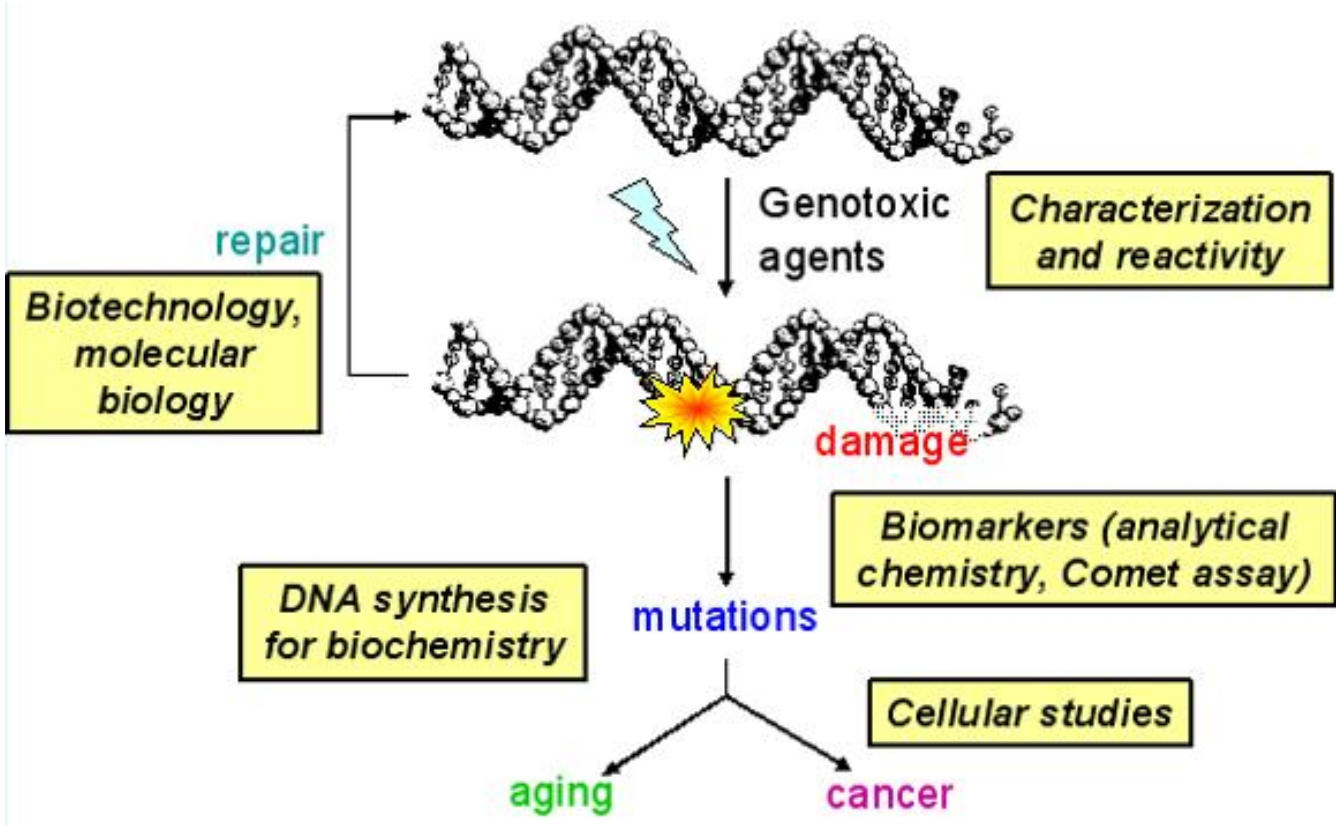
Pro-inflammatory effects




Saline
Carbon black
Asbestos
MWCNTs
Ground MWCNTs
A
B
C
D
E
500um
F
G
H
I
J
500um
K
L
M
N
O
50um

- DEP release a soluble component containing transition metals and organic, FA a lower amount of transition metals and CB no soluble fraction
- Transition metals and Polycyclic Aromatic Hydrocarbons (PAHs) interact with fluids lining the lung and undergo cycling redox reactions that produce ROS (superoxide anion = $O_2^{\cdot -}$ and hydroxyl radical = $OH^{\cdot}$)

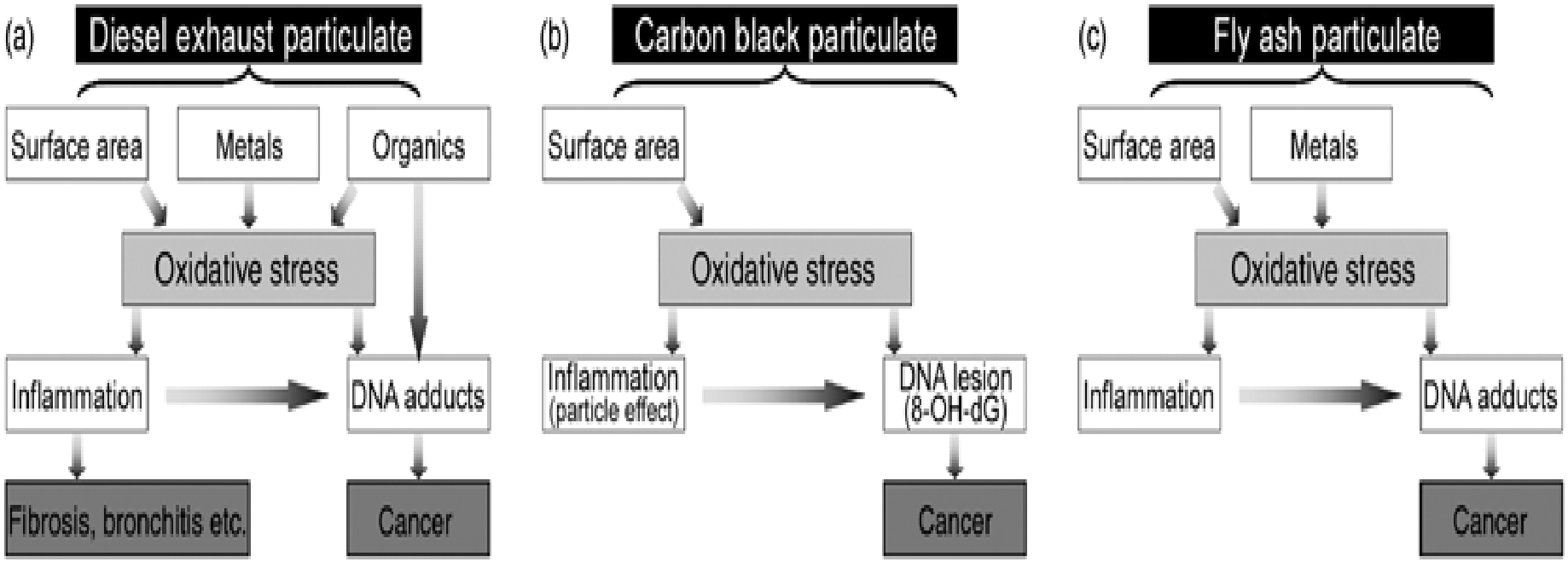

Particles
·surface
·solubles
Inflammation
·Macrophages
·PMN
NADPH oxidase
PAH
quinone
metabolism
mitochondria
$O_2^{-\bullet}$ NO
Fe V Cr Cu
$O_2^{-\bullet} \rightarrow H_2O_2 \rightarrow HO^\bullet$
$ONOO^-$
oxidant defense
DNA repair
DNA damage
proliferation
apoptosis
mutagenesis
cancer


repair
Biotechnology, molecular biology
Genotoxic agents
Characterization and reactivity
damage
Biomarkers (analytical chemistry, Comet assay)
DNA synthesis for biochemistry
mutations
Cellular studies
aging
cancer

Genotoxicity

- the mechanisms are poorly understood
- DEPs: it is known that PAHs (one of the released components of DEPs) can cause DNA adducts. In addition transition metals can also produce ROS which iin turn can result in DNA strand breakage
- CB: they are normally devoid of PAHs and transition metals and the observed genotoxicity is ascribed only to particle overload. Some authors have found that CB can induce 8-hydroxy-deoxyguanine (8-OH-dG)
- FA: the genotoxic mechanism are due to SA and transition metals release

genotoxicity



Mechanistic Hypothesis of CDNPs derived disease

- the cardiac effects are due to pulmonary inflammation which is proposed to interfere with coagulation and stability of the atheromatous plaques (*in what way?)*
- Particle inhalation triggers pulmonary nervous reflexes that disrupt the cardiac rhytm
- Lung toxicity correlates with the number of NPs

CDNPs in the lung

- It is suggested that pulmonary inflammation is propagated to endothelia and platelets systemically leading to clotting reaction and to plaque progression and rupture. The effect is indirect.
- NOTE that the *coaugulation balance* is involved in NPs pathogentic mechanisms!
- *What is the relation between inflammation and coagulation?*

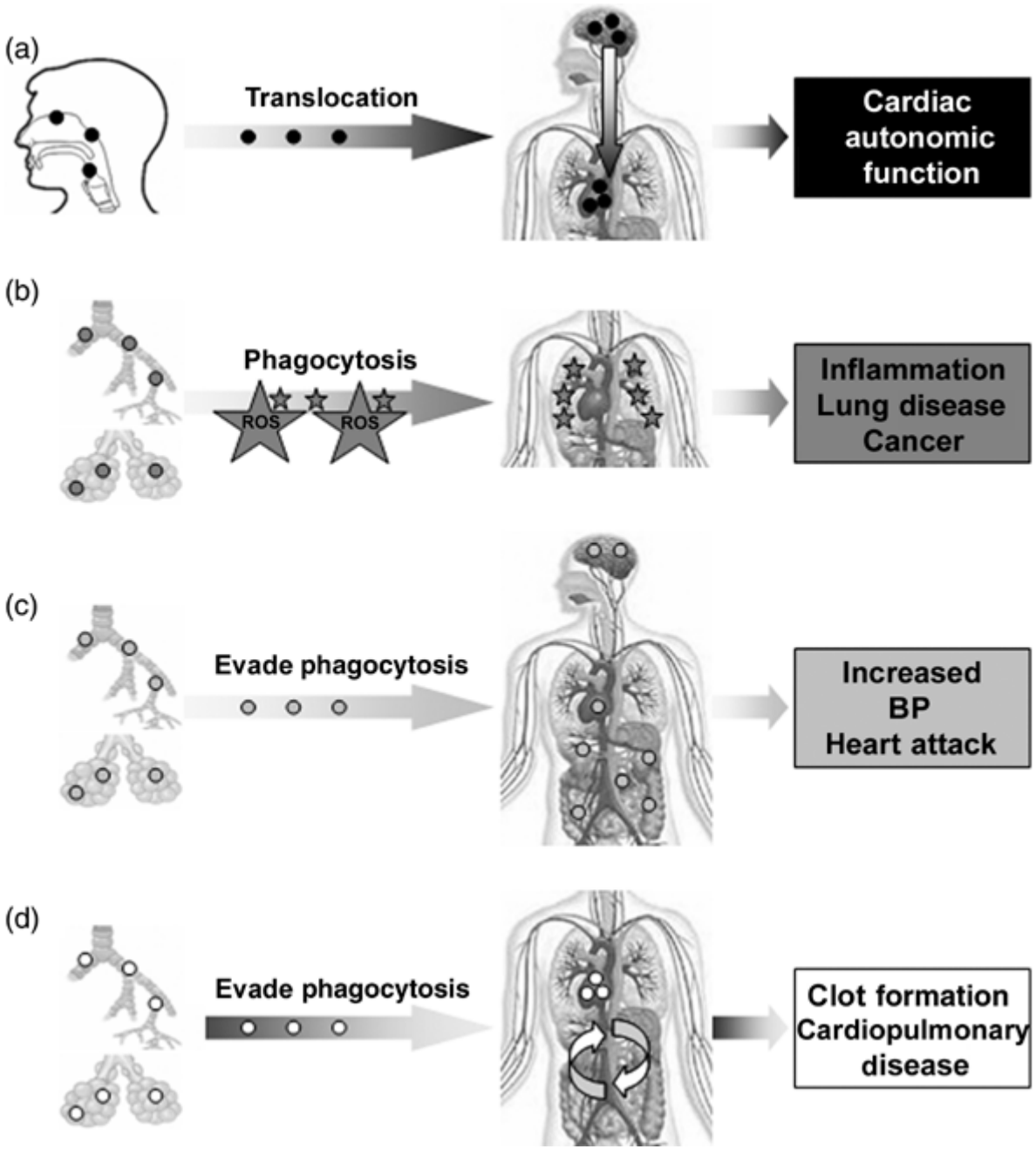
CDNPs in the lung (2)

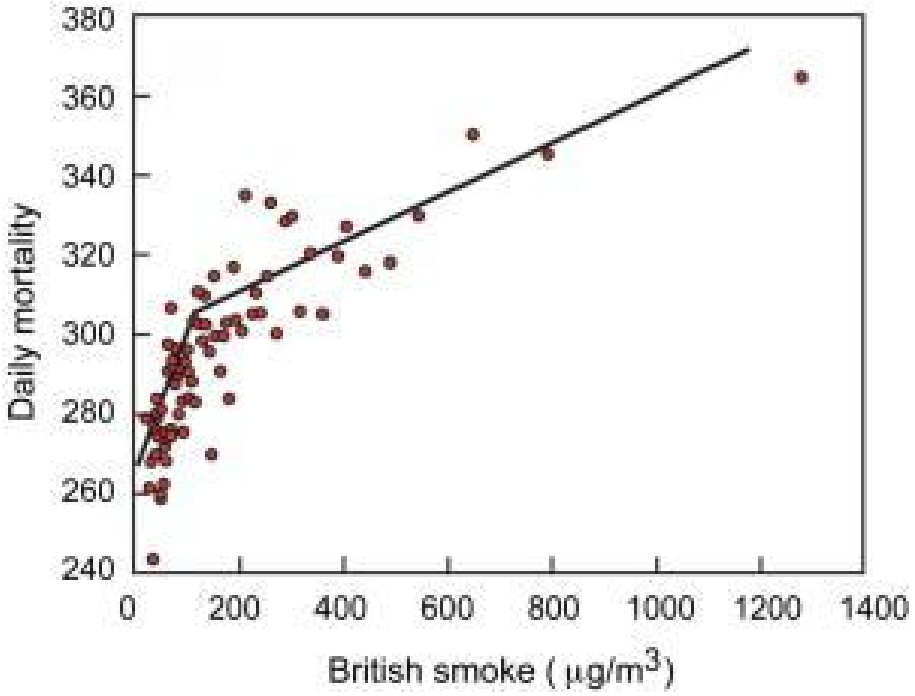
- Autonomous nerve endings in the airways walls may be affected directly by inhaled NPs or as a secondary effect of chronic inflammation. Activation of such nerve endings would induce changes in the autonomic control of the Heart Rhytm leading to Fatal Arrythmias.

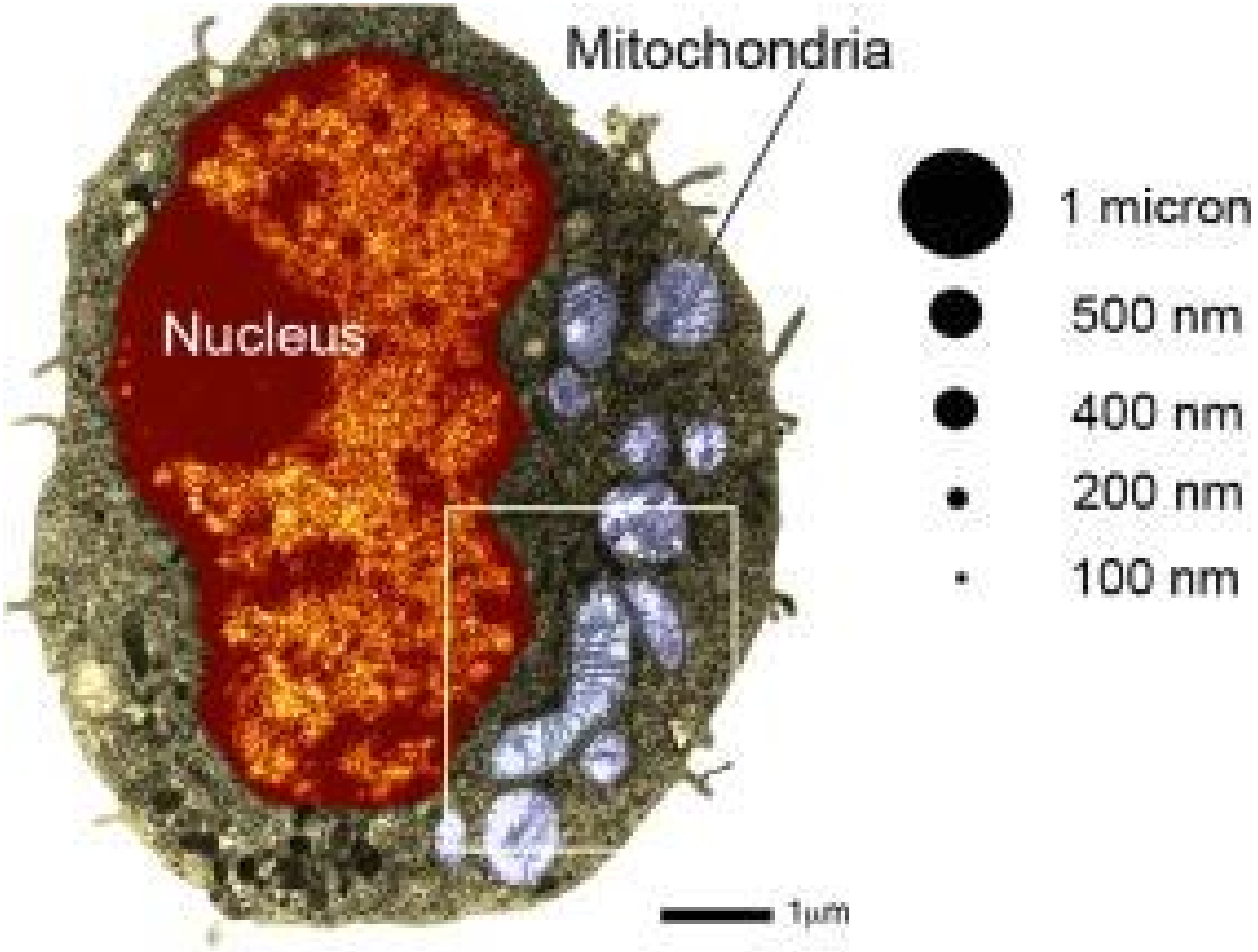
CDNPs in the BLOOD ?

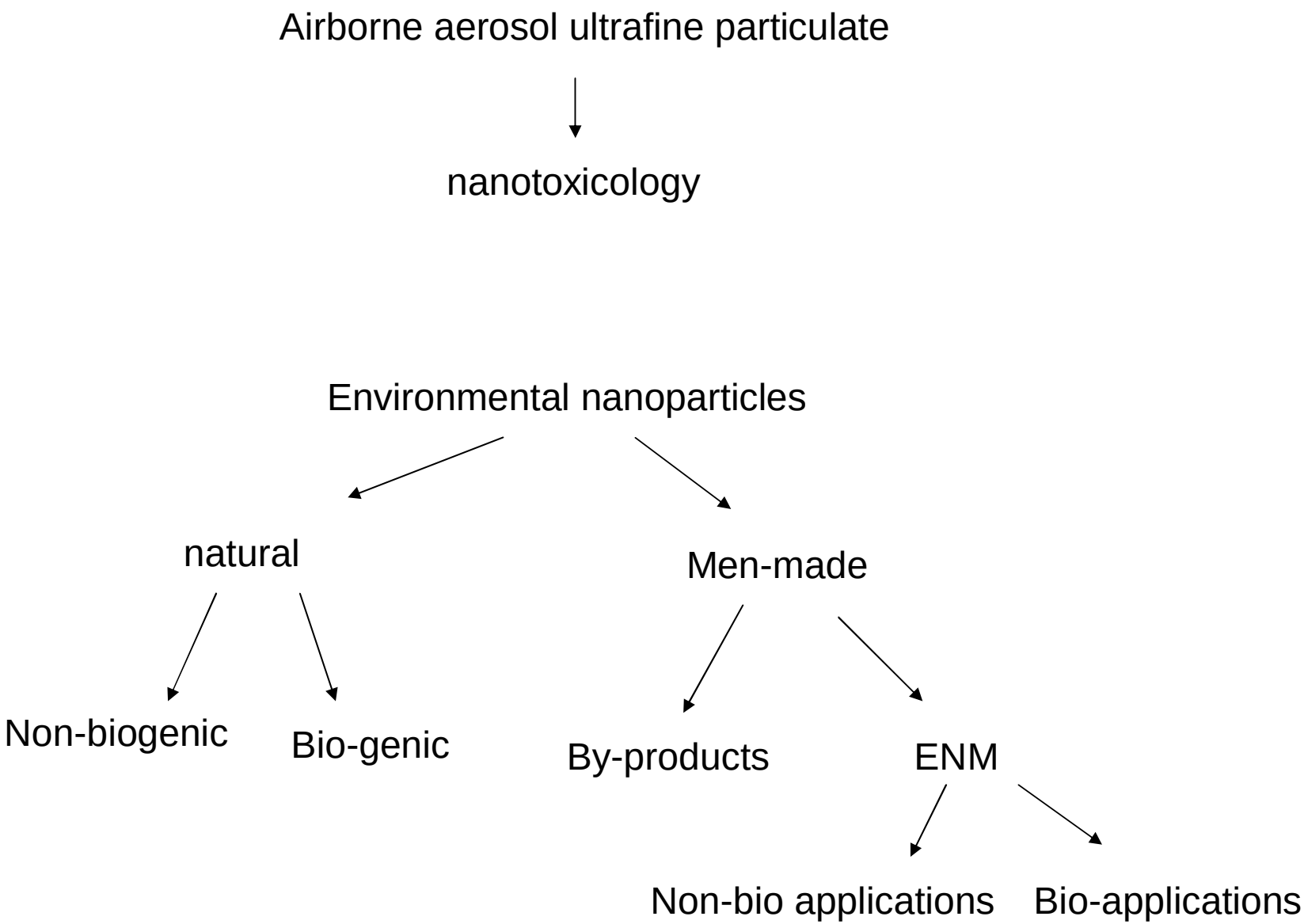
- One important aspect concerns the possibility that indeed NPs gain access to the bloodstream. It is then possible that the NPs intercat with the endothelium and the atherosclerotic lesions, induce ROS and provoke plaque destabilization eventually leading to and Acute Coronary Disease.
- It is also possible that NPs interact with circulating coagulation factors to promote thrombogenesis

The fate of nanoparticles in the human body




380
360
340
320
300
280
260
240
Daily mortality
0
200
400
600
800
1000
1200
1400
British smoke (μg/m3)


Mitochondria
Nucleus
1 micron
500 nm
400 nm
200 nm
100 nm
1µm

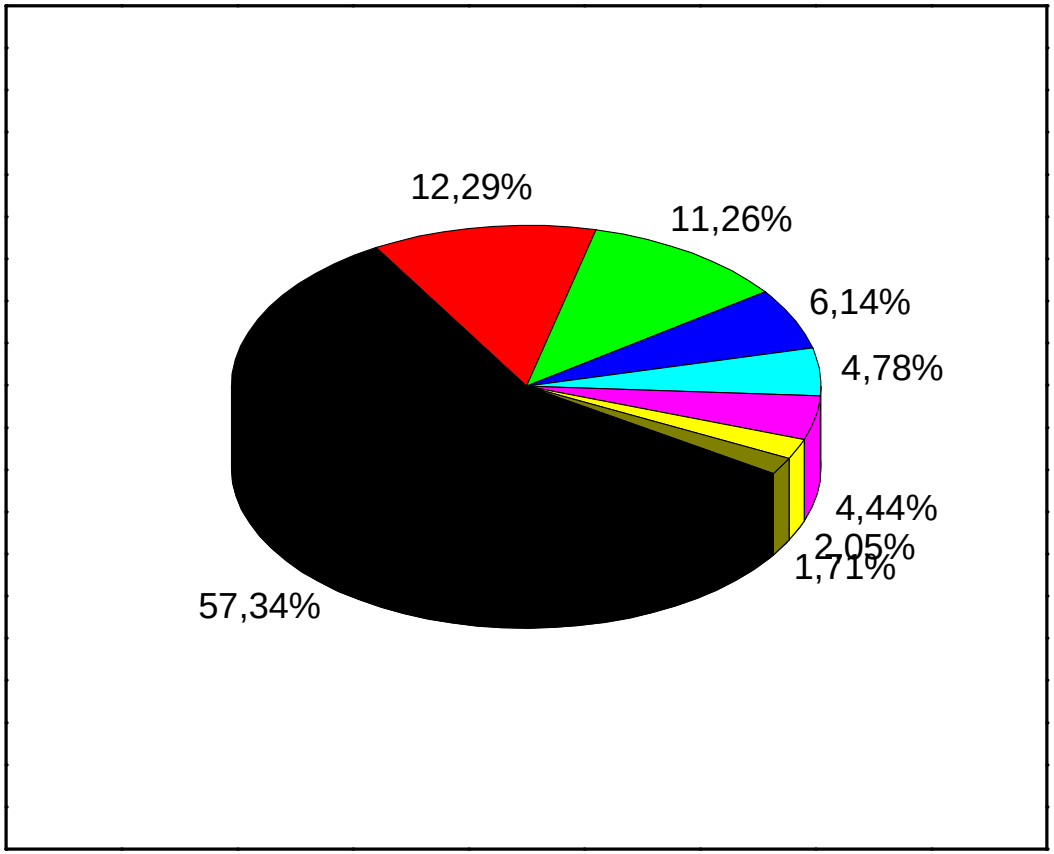

Airborne aerosol ultrafine particulate
nanotoxicology
Environmental nanoparticles
natural
Men-made
Non-biogenic
Bio-genic
By-products
ENM
Non-bio applications
Bio-applications

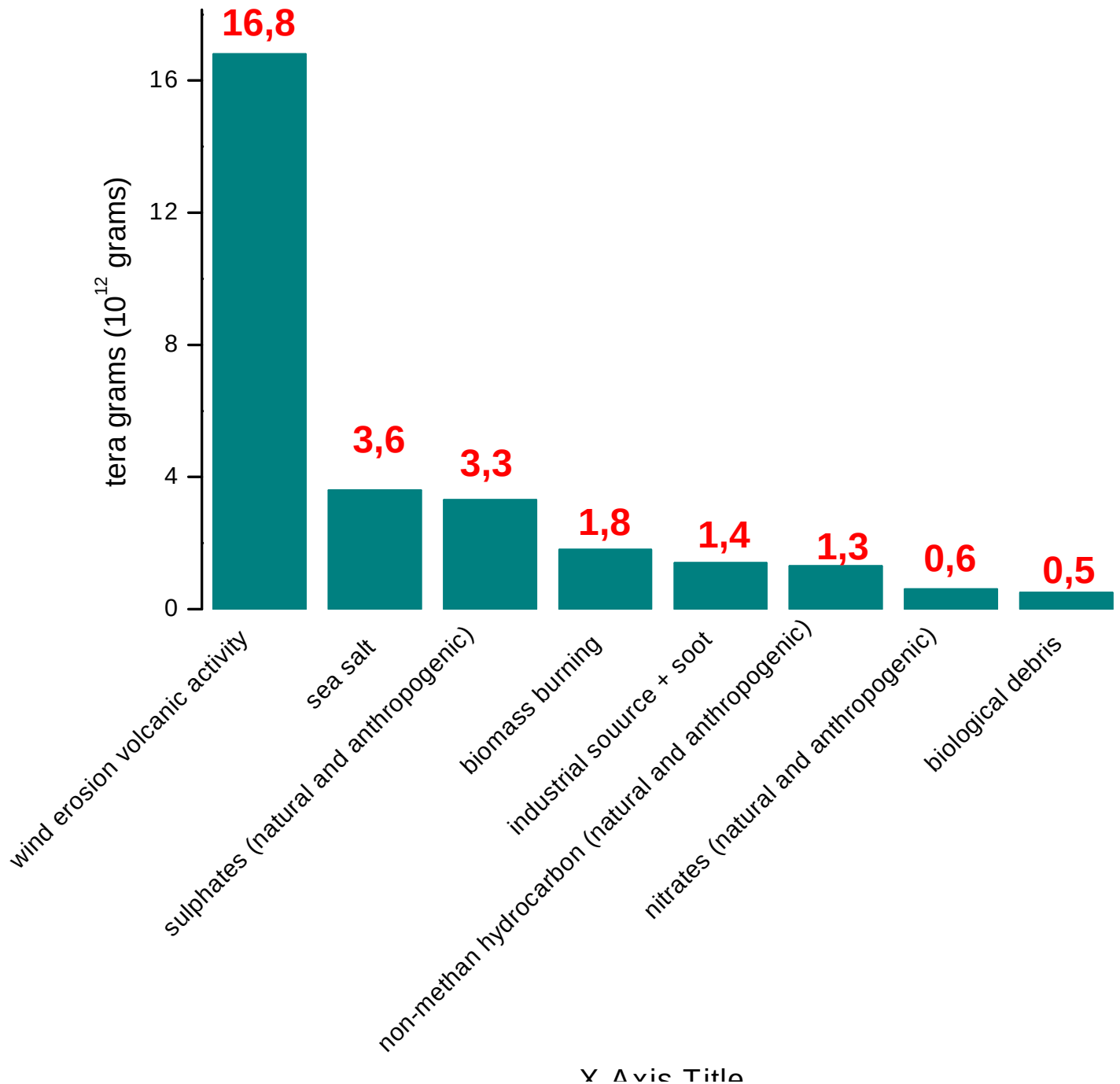
Source of nanoparticles

- NPs are abundant in nature; they are produced by several natural process
- Air pollution is not only associated with human activities (cars, industry, coal burning) but also to natural events: dust storm, volcanic eruptions, forest fires..
- All these natural situations can produce a massive amount of NPs that can affect the quality of air
- Total atmospheric aerosol: ~10 % due to human activity while ~90% to natural events

Total global aerosol

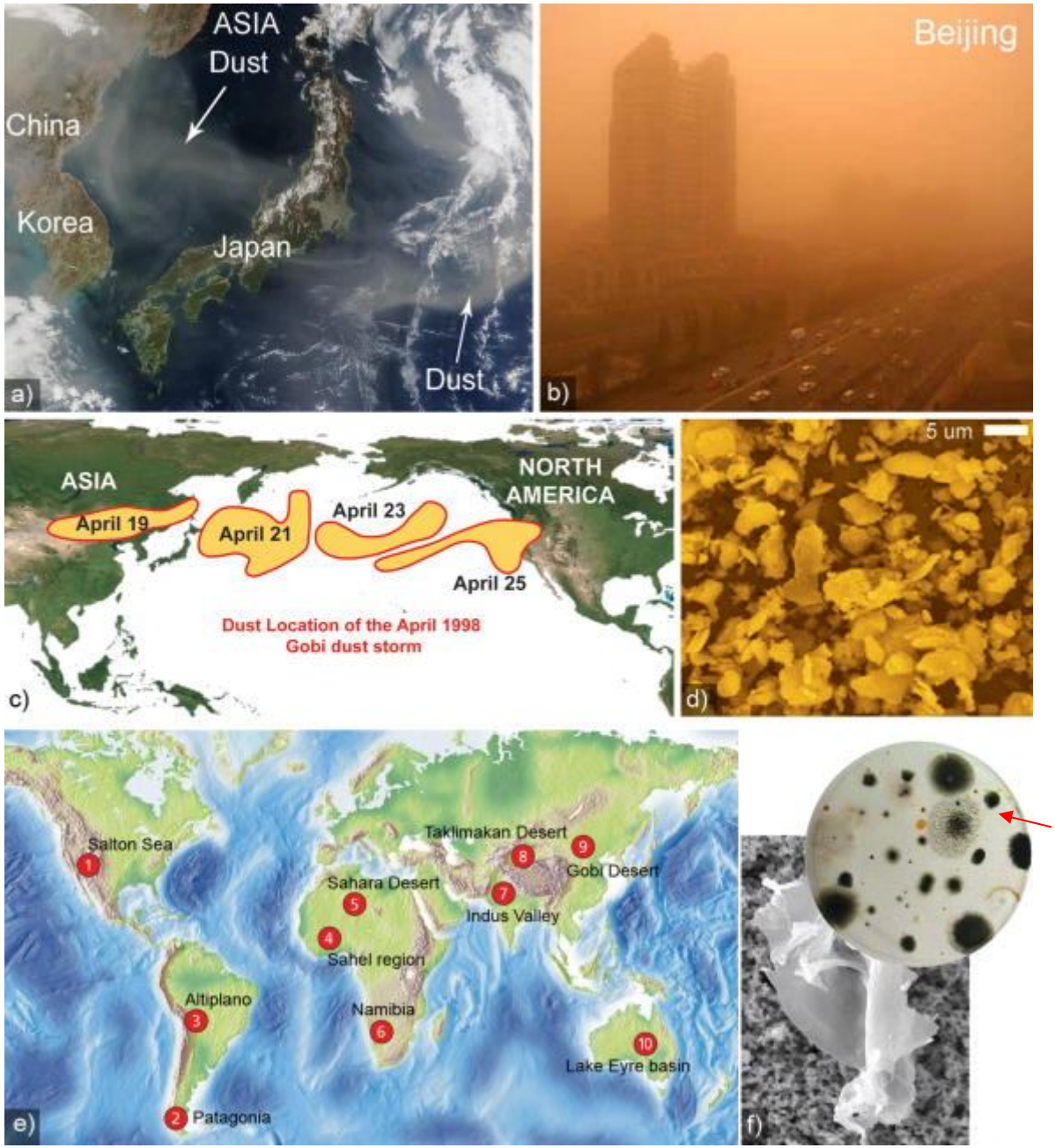
- wind erosion volcanic activity
- sea salt
- sulphates (natural and anthropogenic)
- biomass burning
- industrial souurce + soot
- non-methan hydrocarbon (natural and anthropogenic)
- nitrates (natural and anthropogenic)
- biological debris




16,8
3,6
3,3
1,8
1,4
1,3
0,6
0,5
tera grams (10^12 grams)
0
4
8
12
16
wind erosion volcanic activity
sea salt
sulphates (natural and anthropogenic)
biomass burning
industrial souurce + soot
non-methan hydrocarbon (natural and anthropogenic)
nitrates (natural and anthropogenic)
biological debris
X Axis Title

1. DUST STORM

- It represents the main single source of environmental NPs
- ~50% of troposphere and atmospheric aerosol is due to minerals from deserts
- 30-50% of particles from desert storm are < 2,5 μm in diameter
- NPs 100-200 nm can reach the concentration of 1500/cm^3
- Dust-storm NPs generated in one part of the world can affect regions thousands of kms away



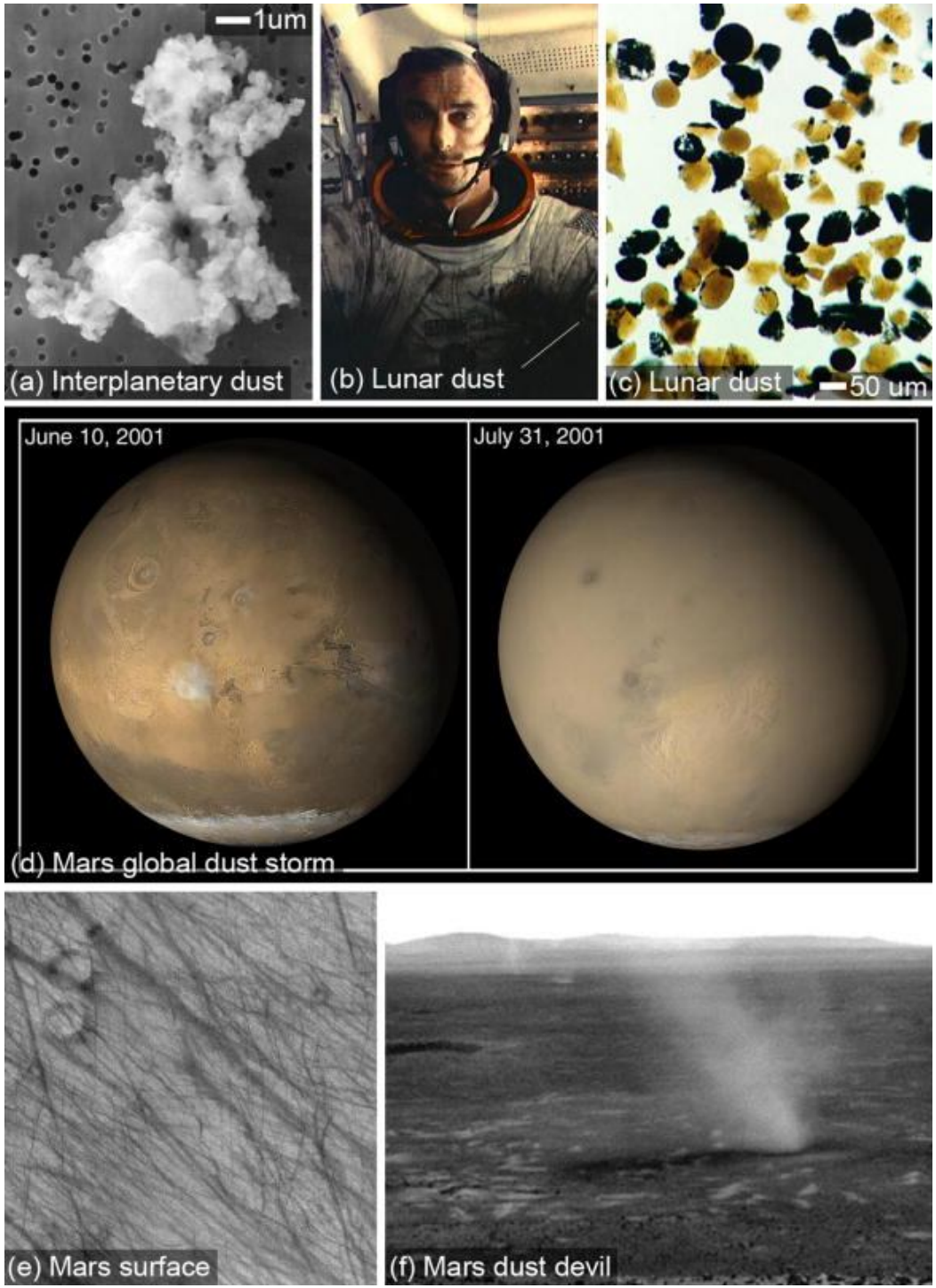
Bacteria in spread dust

Health problems posed by terrestrial airborne dust

- In subjects already affected by *asthma* and *emphysema* worsening of sympthoms
- The chemical composition of the dust is important: e.g. iron or other metals induce ROS and inflamamtion and lung scaring (fibrosis); dust may be contaminated by virus, fungi, bacteria, toxic chemical.
- ~200 types of bacteria and fungi survive UV light exposure during the iintercontinental jurney from Africa to America

2. Extraterrestrial NPs

- NPs are widely distributed outside our planet
- Lunar dust is very fine grained, in mars frequent dust devils spread fine dust in the atmosphere
- Nanostructurated dust is observed in the interplanetary space


1um
(a) Interplanetary dust
(b) Lunar dust
(c) Lunar dust
50 um
June 10, 2001
July 31, 2001
(d) Mars global dust storm
(e) Mars surface
(f) Mars dust devil

Health problems posed by extraterrestrial dust

- Lunar dust (adsorbed on space suites) contaminated the capsule and produced eye irritations in the austronauts
- Intratracheal inhalation of small amount of moon fine dust resulted in *pneumoconiosis* and *fibrosis* in animal models
- Prolonged missions to the moon or mars may increase the risk of respiratory disease in austronauts

4. volcanic activities

- Volcanic eruptions ash and gases containing MPs and NPs are propelled into the atmosphere
- Enormous mass: a single volcan can eject ~30 x 10^{6} tons of ash (18000 m altitude)
- Ash particulate can diffuse worldwide with the primery effect of decreasing the sun radiation


40 km plume
STRATOSPHERE
TROPOSPHERE
a)
St. Helen, 1980
Rabaul Volcano, 1994
b)
c)
500 m

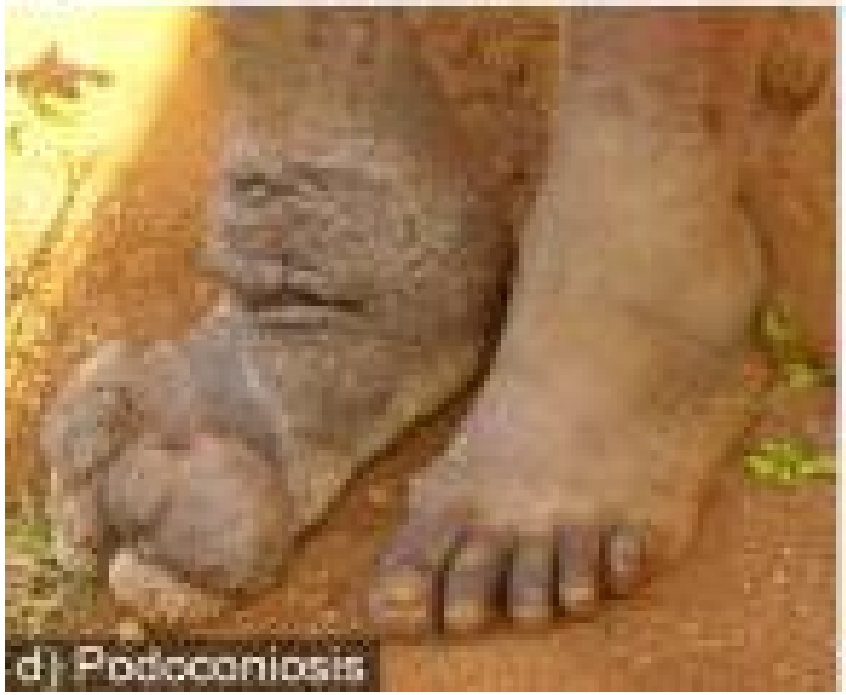
Adverse health effects of ash NPs

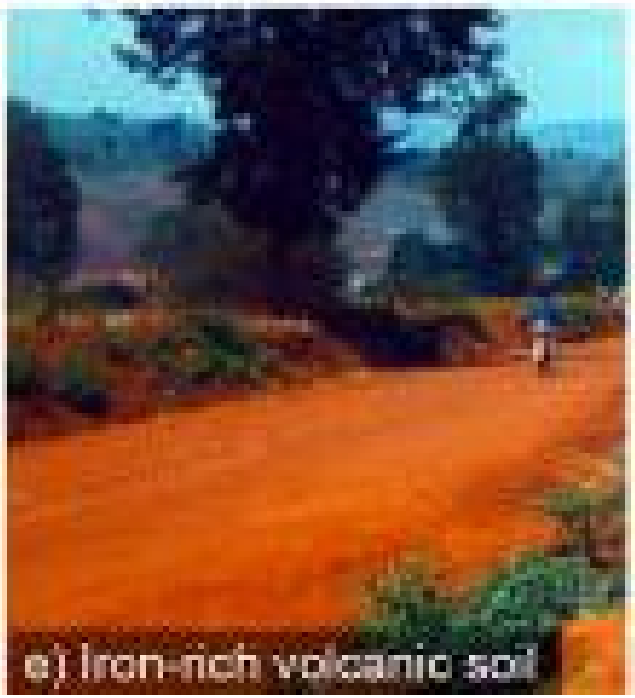
- Particles from volcanoes dust (black lava and red clay soils) contain heavy metals, well known to be toxic to humans (e.g. iron dyoxide)
- Short term effects: nose and throat irritation, bronchitidis
- Long term effects: podoconiosi, Kaposi sarcoma

Bare-foot farmer in volcanic soils (e.g. African Rift Valley) have solid particles (0,4-25 µm) in foot dermis, macrophages and local lymphnodes.

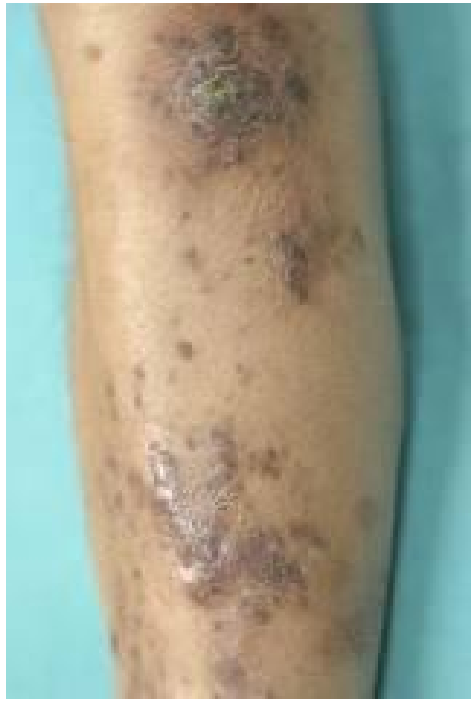
This leads to lymph drainage block (overwhealming of lymph flux) with aedema
Then turning into “hard aedema” and elephant-like foot (*elephanthiasis*)

- ~10% of peolple living in volcanic tropics suffer of podoconiosis



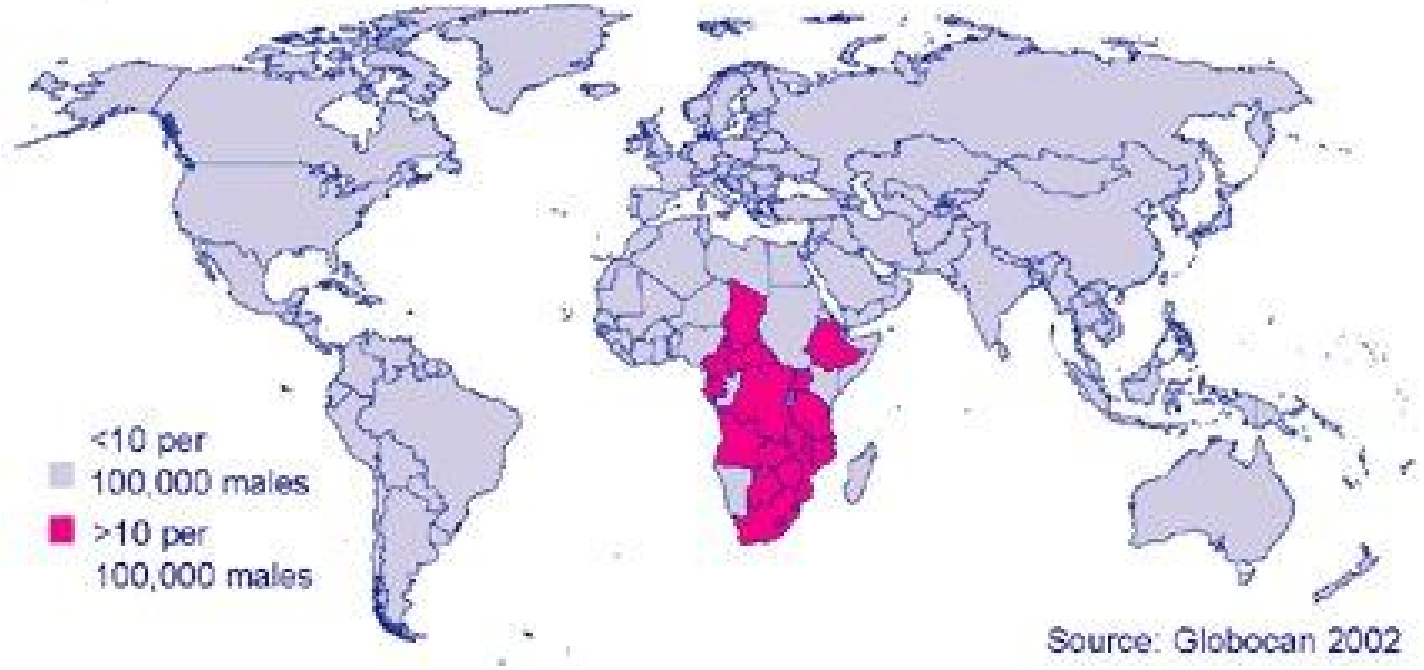


Kaposi sarcoma

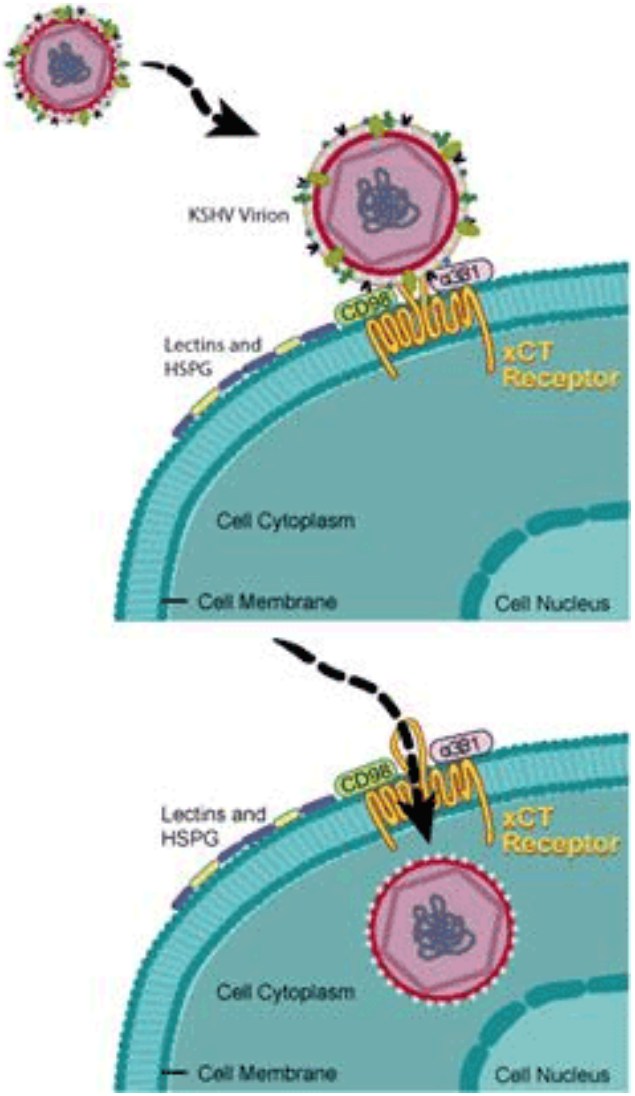


- Malignant cancer form affecting blood and lymph vessels

Figure 5.2: Kaposi sarcoma age-standardised incidence per 100,000 males



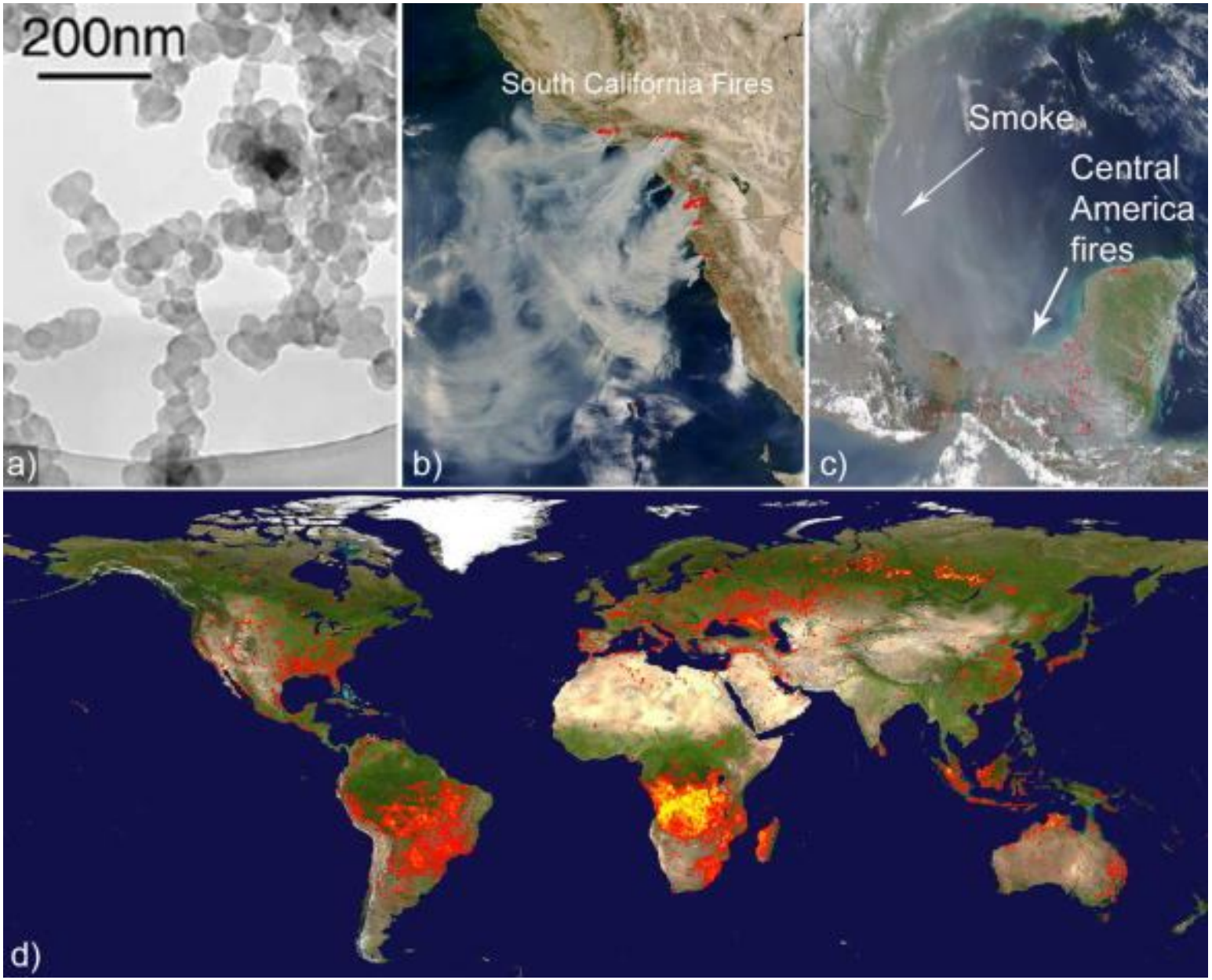
- Endemic Kaposi sarcoma in part of the world rich in volcanic soils
- Iron particles may act as a cofactor in the etiology of this carcinoma



- In 1994 Chang and Moore identified a new virus, Kaposi's sarcoma-associated herpesvirus (KSHV) as the cause of these tumours.
- It is believed that ferromagnetic particles effects on the lymphatic draining increase the risk of KSHV infection

3. Forest fires

- Forest and grass fires are due to lighting strikes and human activity: they generate and spread ash and smoke over thousands of square-miles and can significantly increase air particluate matter (included nanoparticles)

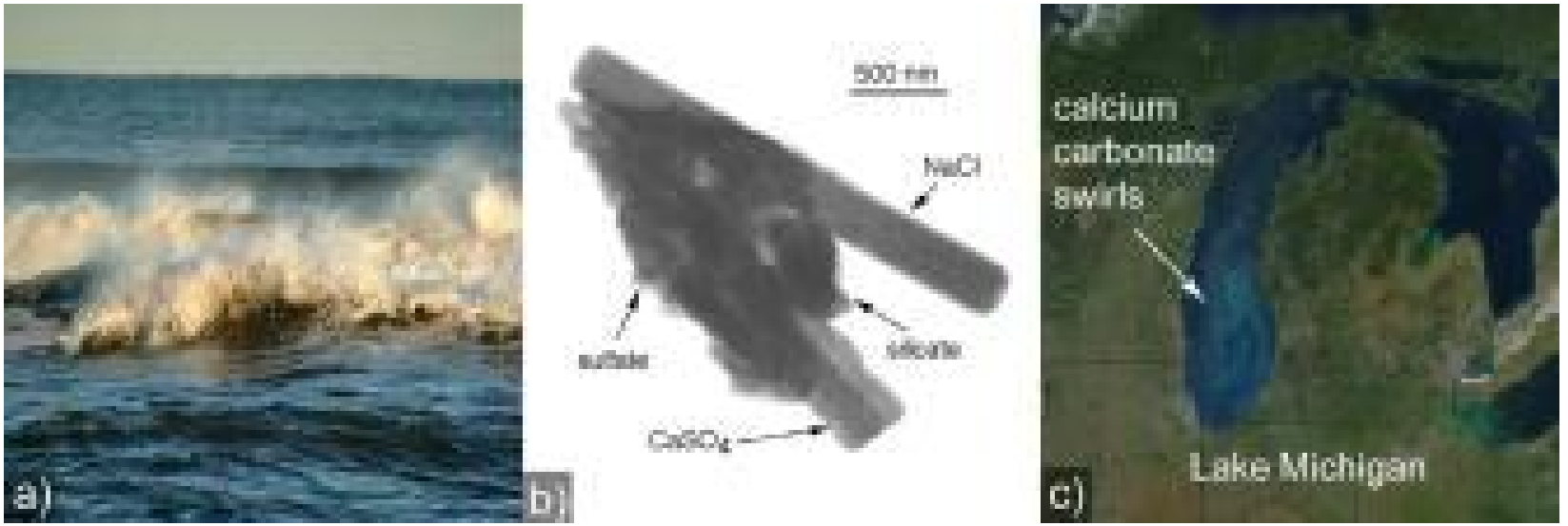

200nm
South California Fires
Smoke
Central America fires
a)
b)
c)
d)

Adverse health effects of forest fires

- Epidemiological studies showed that during forest fires medical visits increase.
- Also worsening of sympthoms of people already affected by cardio-pulmunary dysfunctions and diseases

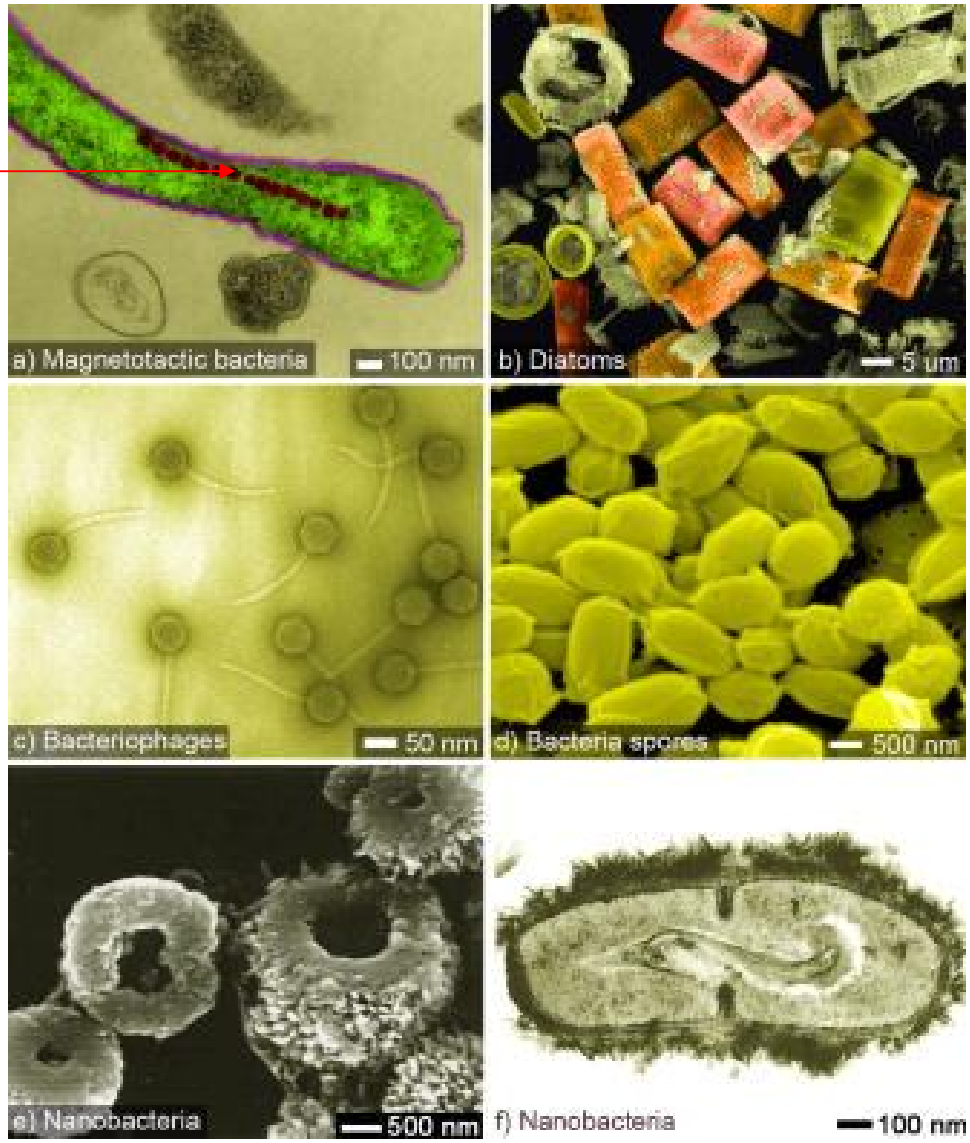
Ocean and water evaporation

- Sea/ocean spray from waves generate aerosols of sea-salt (mainly); 100 nm-several microns
- Salt aerosol has no risk and is on the contrary considered benefical to restore the mucociliary clearance in patients with respiratory diseases


a)
500 nm
NaCl
sulfate
silicate
$CaSO_4$
b)
calcium
carbonate
swirls
Lake Michigan
c)

Organism derived (biogenic NPs)

Magnetite nanoparticles



Diatomaceous Earth

virus

Bacterial spores

Bacterial mineral nanoshells

- Diatoms have cell wall formed by amorphous silica (SiO_2)
- Magnetotactic bacteria, present in fresh water and marine environments, synthesise magnetites nanoparticles
- S-layer bacteria are *nanobacteria* with a porous shell of calcium phosphate (20-100 nm Ø)

Health problems from biogenic NPs

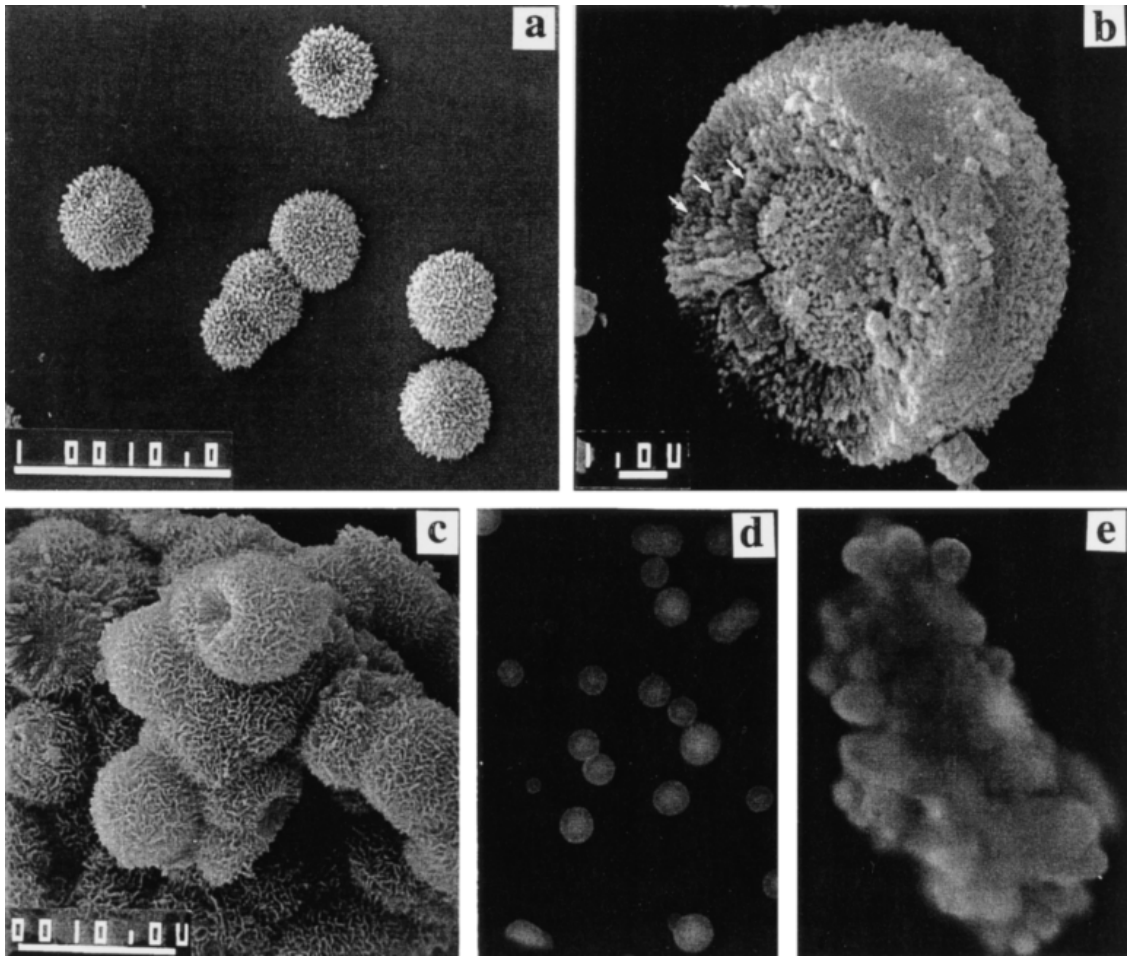
- Workers of diatomaceous earth?
- Biogenic magnetite has been associated with neurodegenerative diseases
- Nanobacteria shells have been found in blood and organs of animals and humans: suspected to contribute to ciseases involving calcifications

Diseases involving calcification processes…

- Artery plaque (atheroma)
- Aortic aneurysm
- Heart valves calcification
- Renal stones formation
- Chronic prostatitis
- Ovarian and breast cancer

- *Can nanobacteria colonies function as nuclation center for atheromas and stones formation?*

Nanobacteria and kidney stones

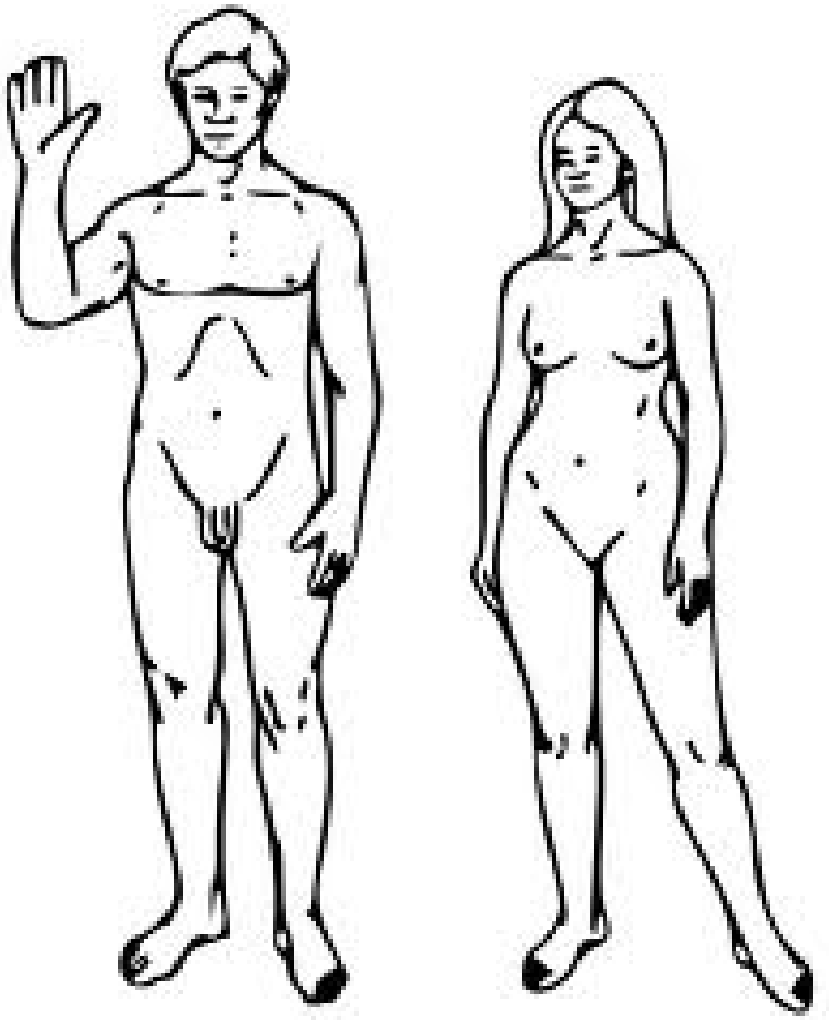


Strange aspect..

- The replication rate of nanobacteria quadruplicates in the absence of gravity

- So the calcium phosphate nano-shells are considered to be the cause of increased kidney stone formation in astronauts

Anthropogenic nano-materials



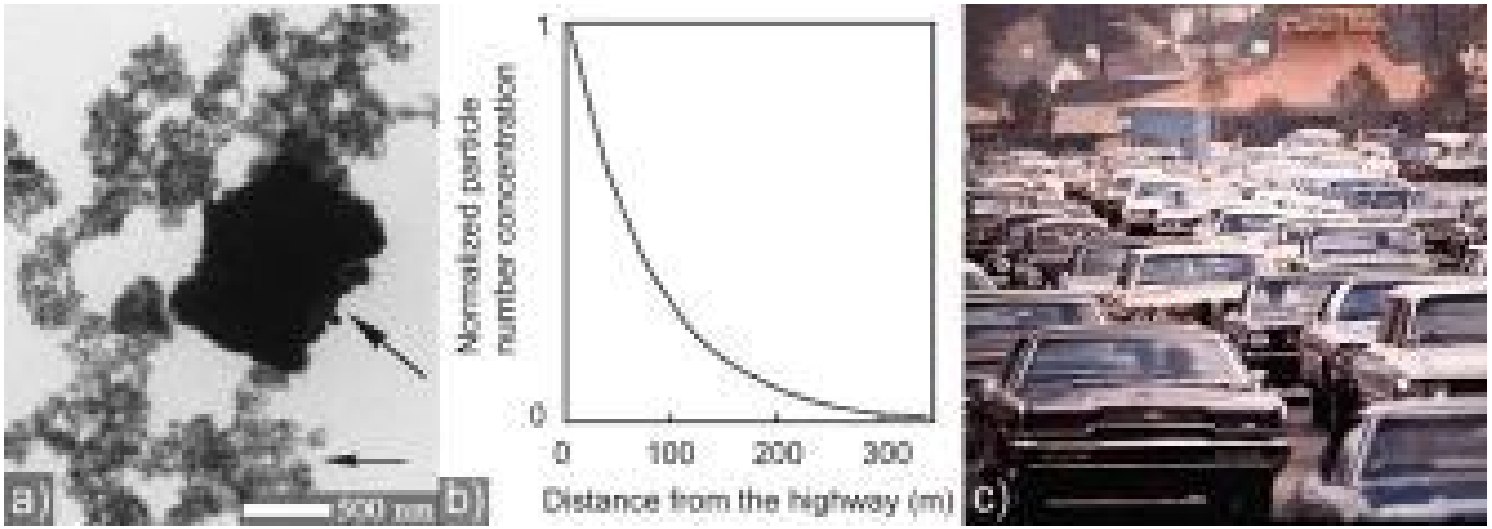
Humans have created nano-materials as by-products of:

- Simple combustion
- Chemical manufacturing
- Combustion in vehicles and airplanes engines
- Combustion of fluel oils for power generation
- Welding (it: saldatura)
- Fusion and refining of gold

In addition purpose-made nanomaterials for:

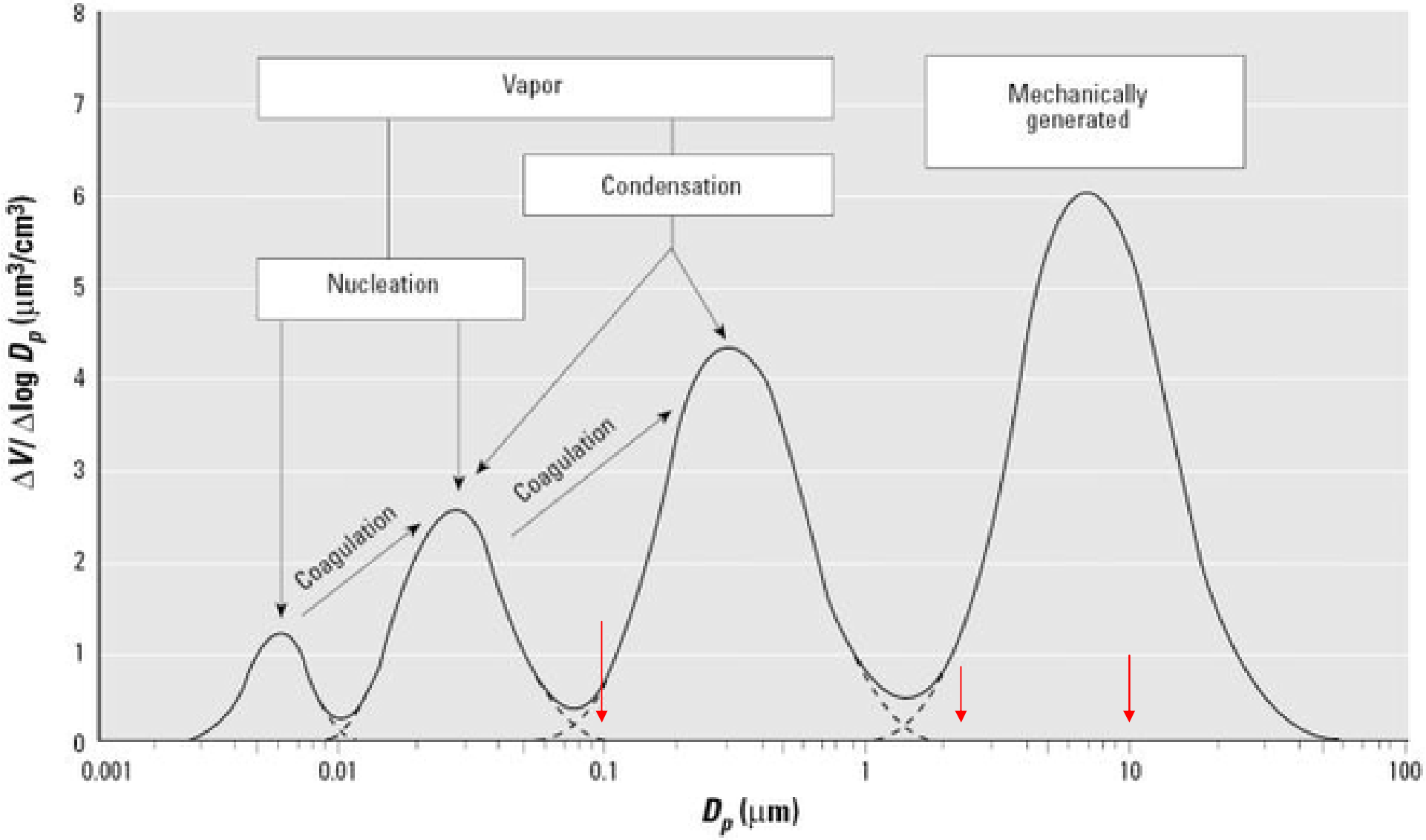
- Cosmetics
- Sporting goods
- Tyres
- stain-resistant clothings
- Sunscreens
- Tooth paste
- Food additive
- Etc etc

As we already discussed, also diesel engine exhausted NPs



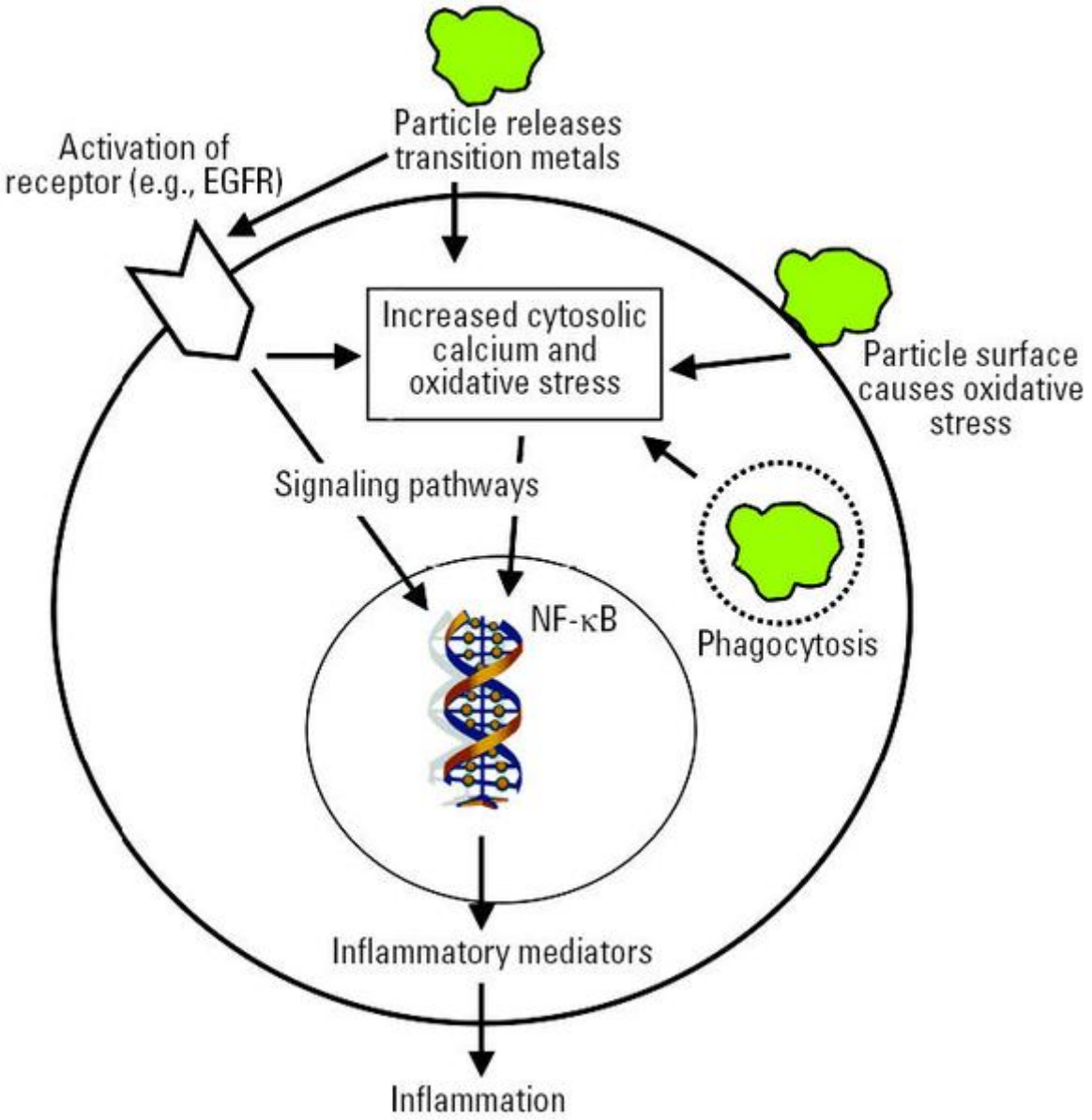
Near highways pollution is the principal source of particulate contamination

Size distribution of traffic-related particulate matter



Diesel and gasoline derived NPs

- Primary source of atmospheric micro- and nano-particles in urban areas
- Typically spherical, numerically the most abundant are NPs (10% mass BUT 90 % in number)
- Diesel NPs: 20-130 nm Ø
- Gasoline NPs: 20-60 nm Ø
- Carbon nanotubes and fibers found in smaller quantities may be very toxic and carcinogenic in the lung (like or more than asbestos fibers)

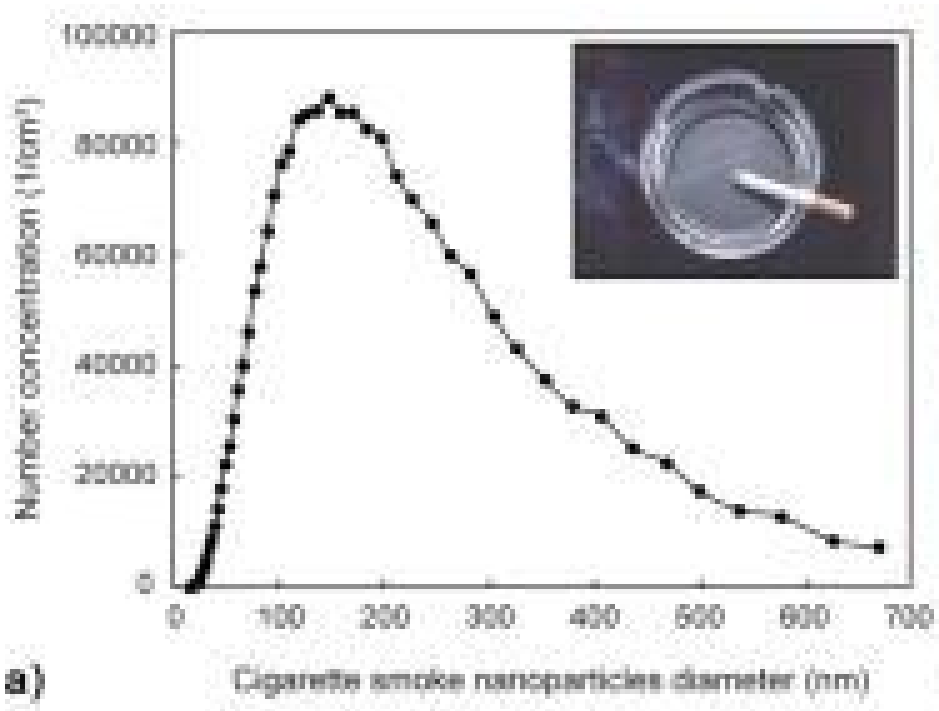

Particle releases transition metals
Activation of receptor (e.g., EGFR)
Increased cytosolic calcium and oxidative stress
Particle surface causes oxidative stress
Signaling pathways
NF-κB
Phagocytosis
Inflammatory mediators
Inflammation

Helth effects of diesel and gasoline NPs

- Respiratory problems
- Cardiovascular diseases
- Cancer (lung)
- Higher risk for people living close to high-ways and for drivers
- Drivers (diesel vehicles) show an elevate drisk of myocardial infarction

Indoor pollution

- Cooking
- Smoking (cigarette included)
- Cleaning
- Combustion (candle, fireplaces)

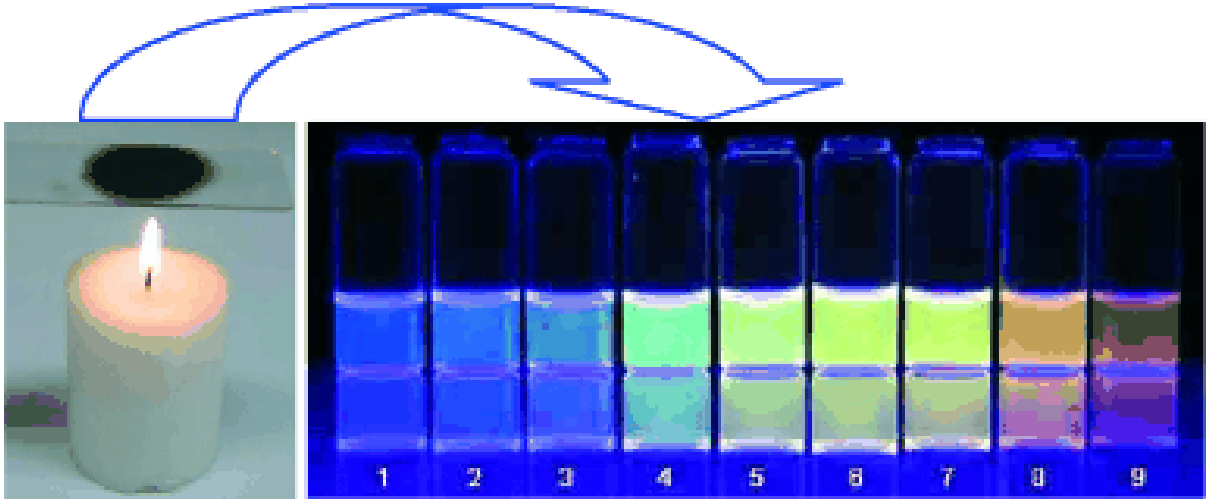


Soot (*it: fuliggine*)

Water-soluble, multicolor fluorescent carbon nanoparticles are prepared by refluxing candle soot with nitric acid

crude candle soot

purified fluorescent CNPs

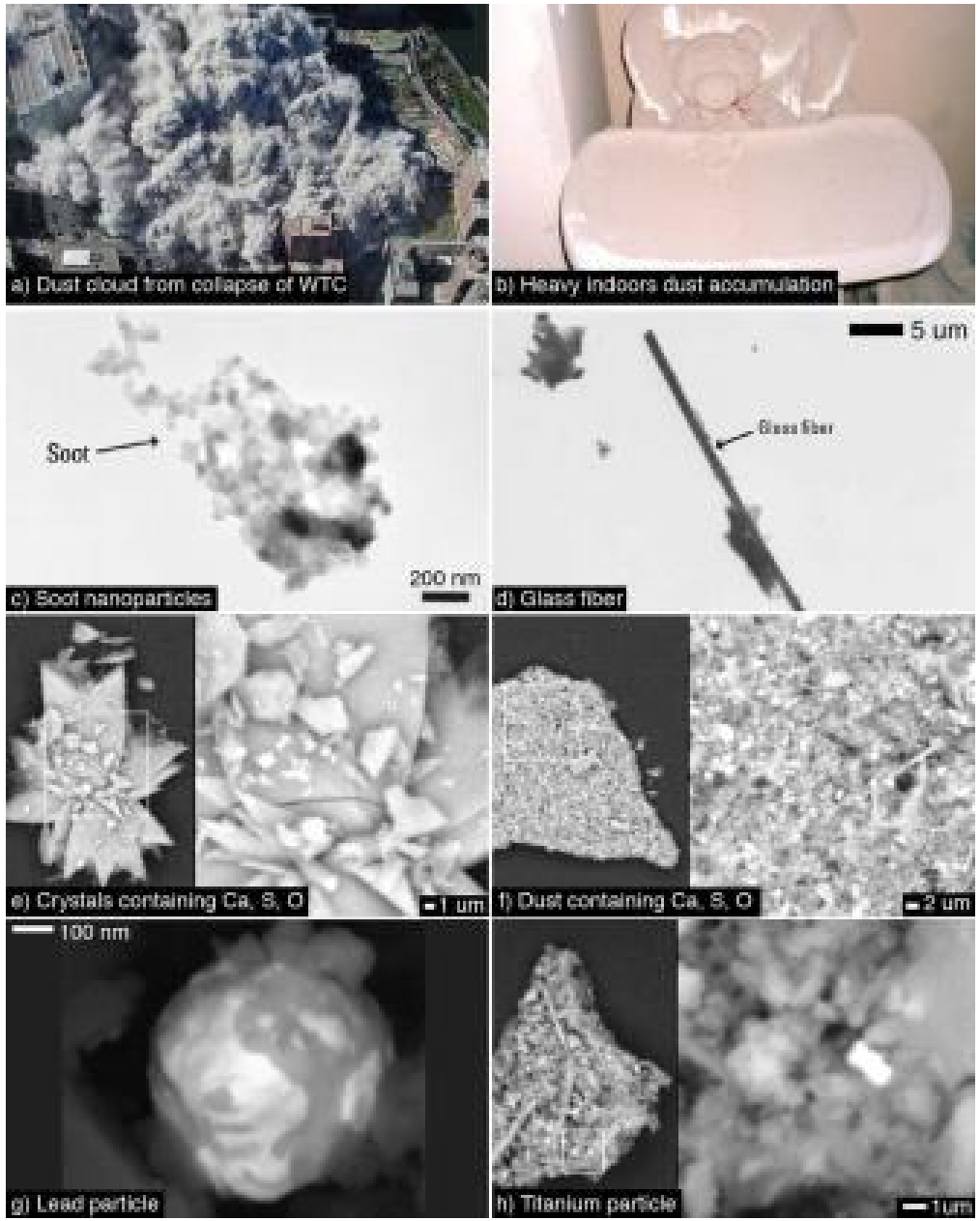


Other indoor sorce of NPs

- Textile fibers
- Skin particles
- Chemicals
- Droppings of dust mites

Building demolition

- Asbestos fibers (old buildings)
- Lead particles
- Glass fibers particulates
- Wood particles
- Paper particles fibers
- Other toxic particulates



- Firefighters in twin tower attack site showed increased bronchial hypereactivity. COUGH

- LONG TERM EFFECT UNKOWN

Table 1. UFPs/NPs (< 100 nm), natural and anthropogenic sources.

Natural	Anthropogenic	
	Unintentional	Intentional (NPs)
Gas-to-particle conversions	Internal combustion engines	Controlled size and shape, designed for functionality
Forest fires	Power plants	
Volcanoes (hot lava)	Incinerators	Metals, semiconductors, metal oxides, carbon, polymers
Viruses	Jet engines	
Biogenic magnetite: magnetotactic bacteria protoctists, mollusks, arthropods, fish, birds human brain, meteorite (?)	Metal fumes (smelting, welding, etc.)	Nanospheres, -wires, -needles, -tubes, -shells, -rings, -platelets
	Polymer fumes	
	Other fumes	Untreated, coated (nanotechnology applied to many products: cosmetics, medical, fabrics, electronics, optics, displays, etc.)
Ferritin (12.5 nm)	Heated surfaces	
Microparticles (< 100 nm; activated cells)	Frying, broiling, grilling	
	Electric motors	

cosmetics

- Delivery agents for nutrients
- E.g. fullerenes derivatives in skin creams, claimed to be antioxidant

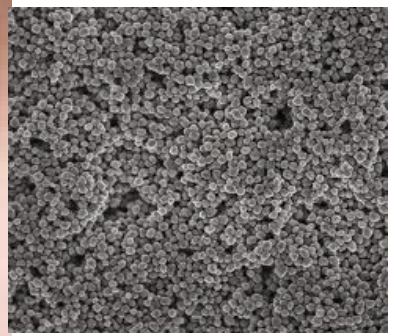
- Health effects? Not known

Other consumer products

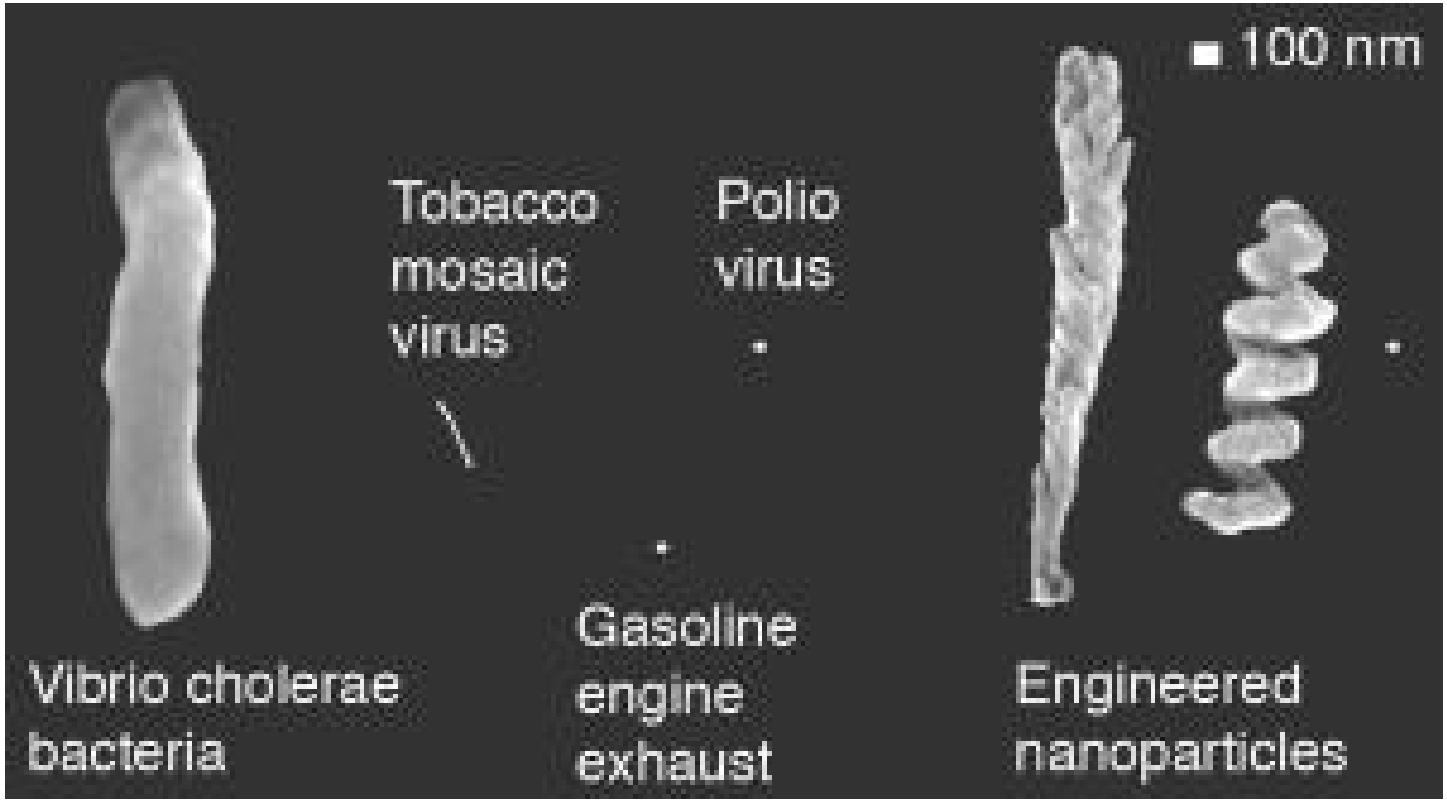
- TiO_2 (NPs Ø > 100 nm) are considered biologically inert
- White pigments
- Food colorant
- Sunscreens
- Skin creames anti-wrinkle (rughe)

- Recent observation suggest that TiO2 NPs are toxic and inflammogenic..

- Ag (silver) Nps are used as antibacterial/antifungal agents in many products e.g. tooth paste, air sanitizing sprays, socks, pillows, shampoo…

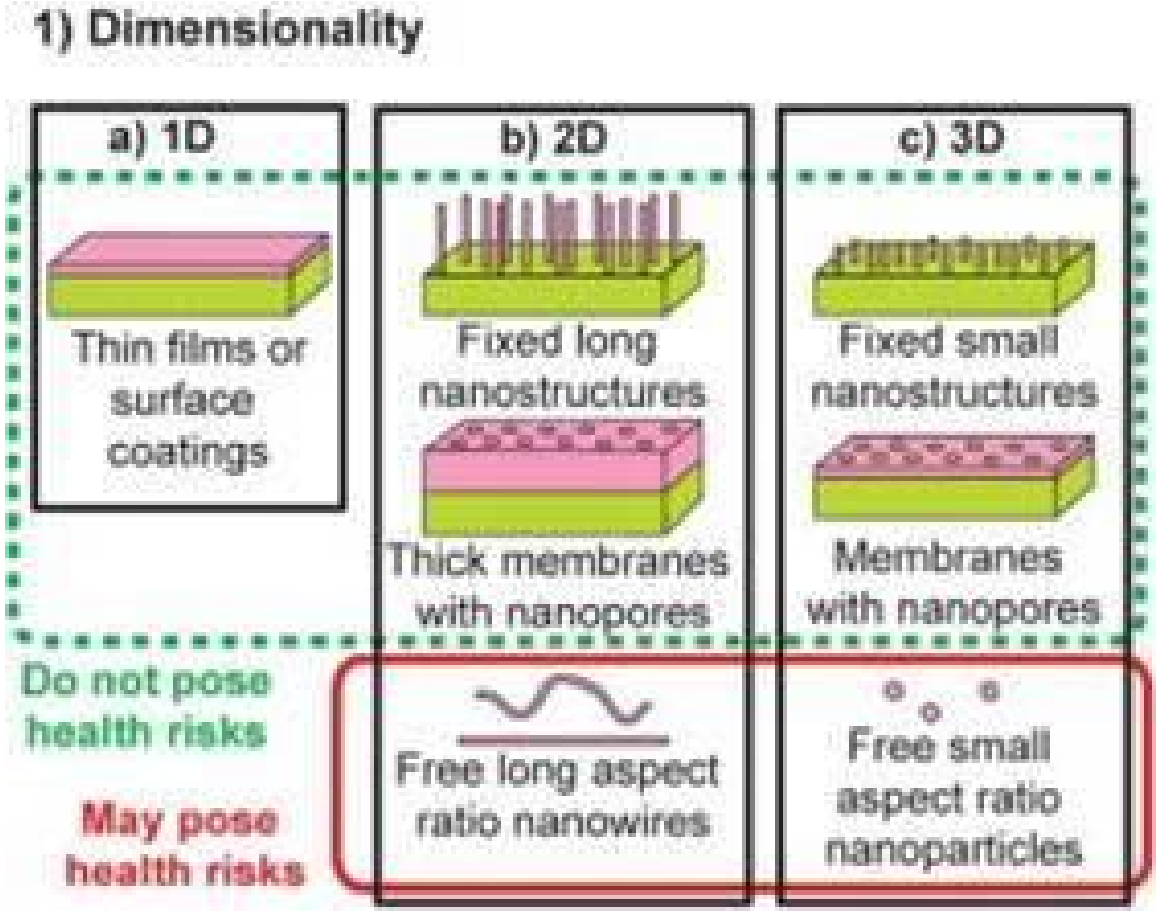


Some engineered nanoparticles compared with selected micro-(nano-)organisms and a CDNP



Engineered Nanostructures classification

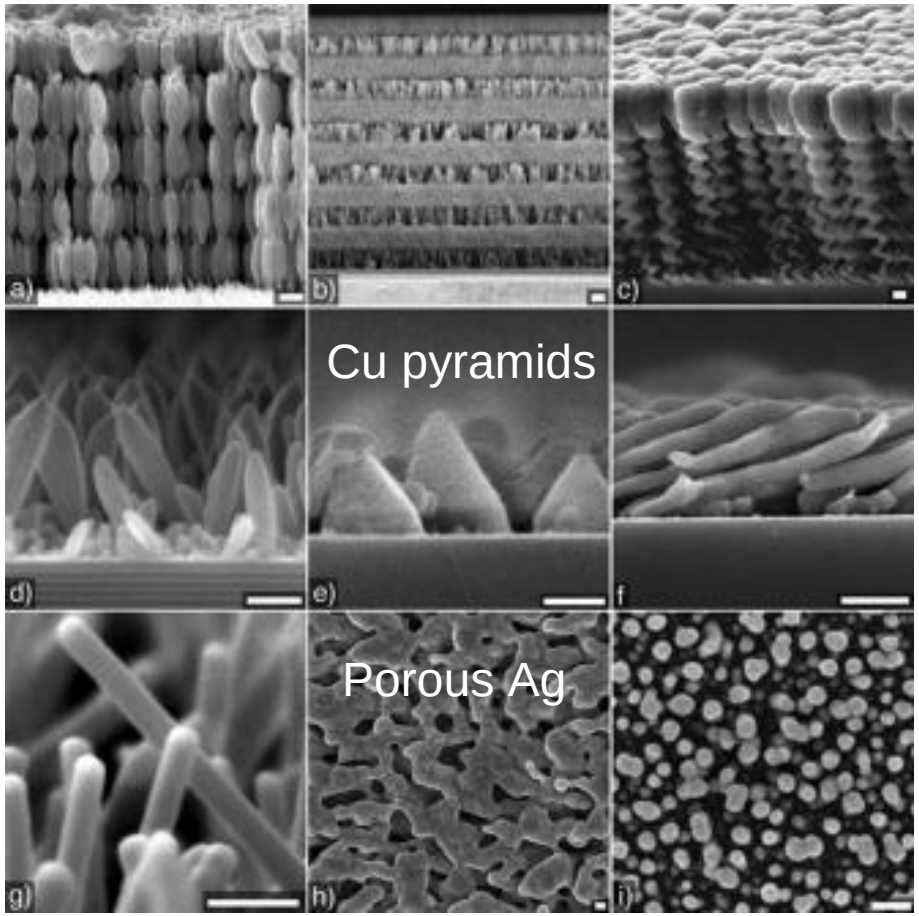
- Dimensionality
- Morphology
- Composition
- Uniformity
- Agglomeration


1) Dimensionality
a) 1D
b) 2D
c) 3D
Thin films or surface coatings
Fixed long nanostructures
Fixed small nanostructures
Thick membranes with nanopores
Membranes with nanopores
Do not pose health risks
Free long aspect ratio nanowires
Free small aspect ratio nanoparticles
May pose health risks

Bar: 100 nm

Si 12-layer structure

Si rugate filter



MgF_2 capping layer
helical film

Ti pillar

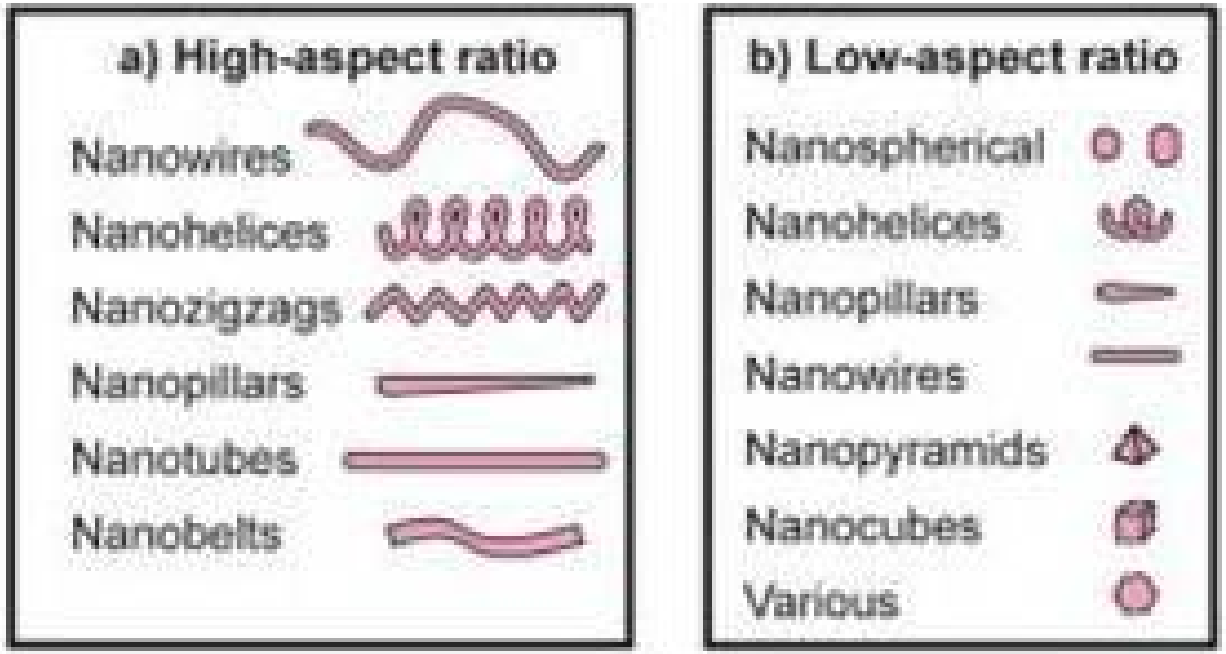
Cu oblique
columns

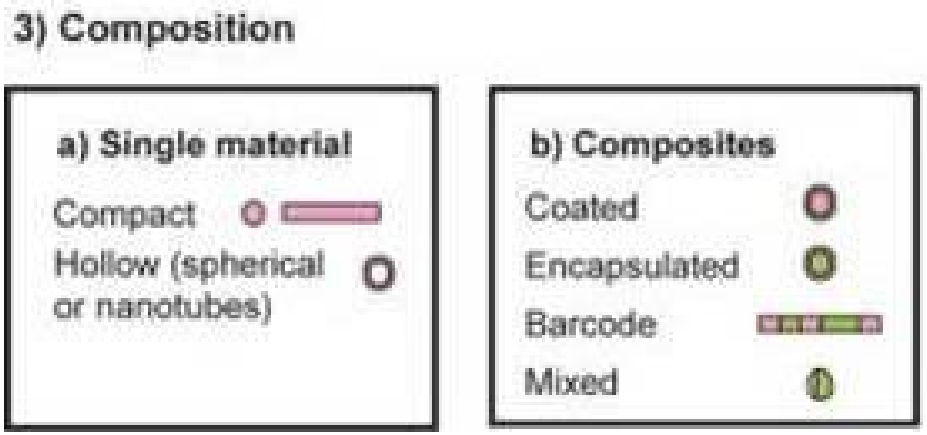
ZnO nanowires

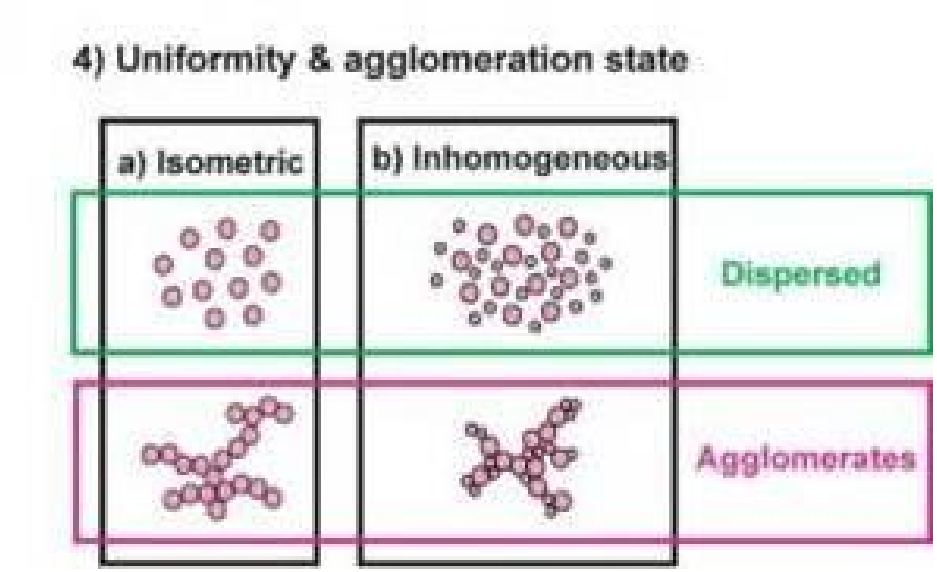
Porous Si

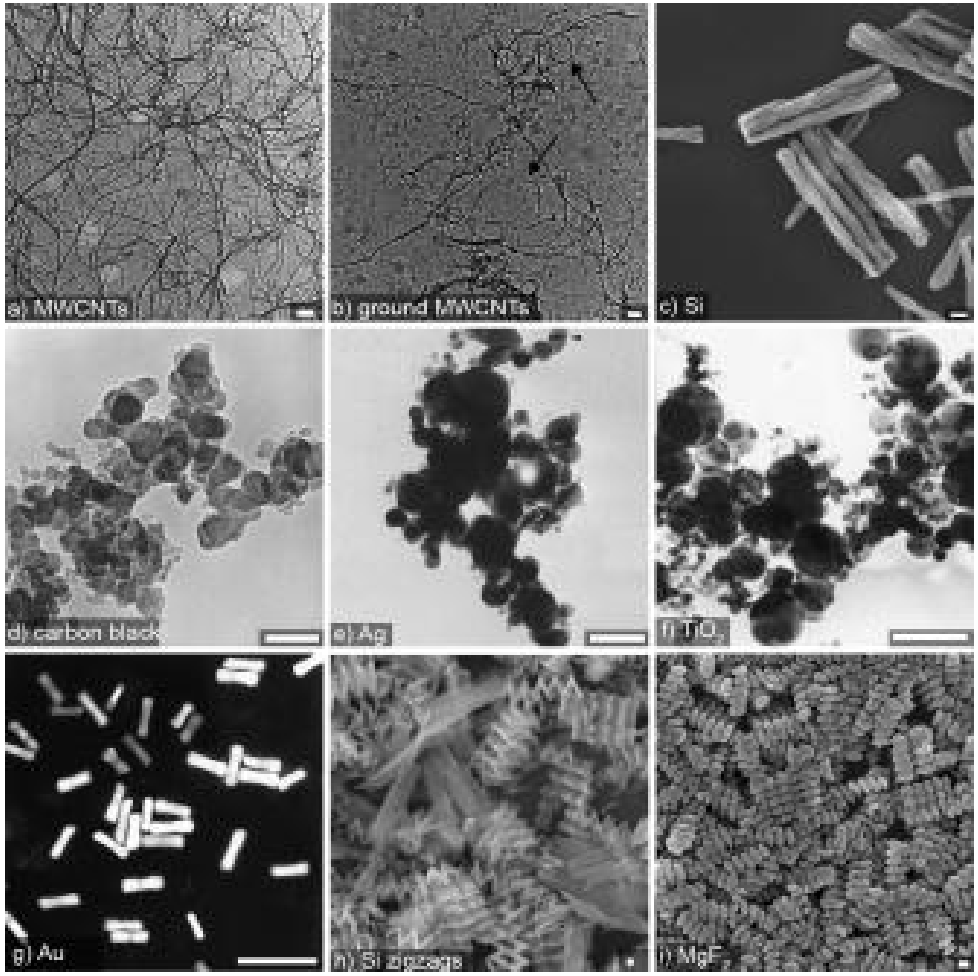
Examples of nanomaterials in thin film form

2) Morphology




3) Composition
a) Single material
Compact
Hollow (spherical or nanotubes)
b) Composites
Coated
Encapsulated
Barcode
Mixed


4) Uniformity & agglomeration state
a) Isometric
b) Inhomogeneous
Dispersed
Agglomerates

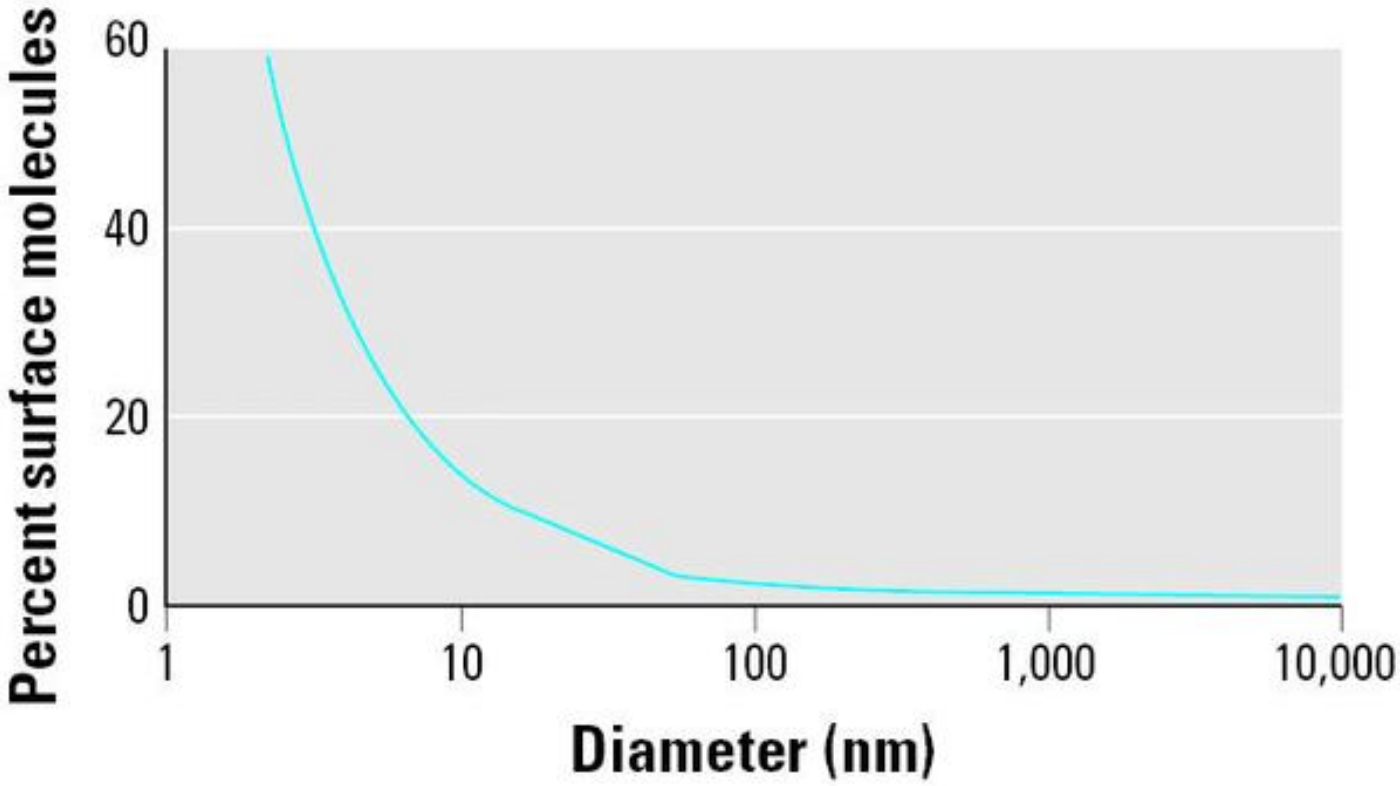

a) MWCNTs
b) ground MWCNTs
c) Si
d) carbon black
e) Ag
f) TiO2
g) Au
h) Si zigzags
i) MgF2

Main differences between nano- and bulk materials

- Surface effects

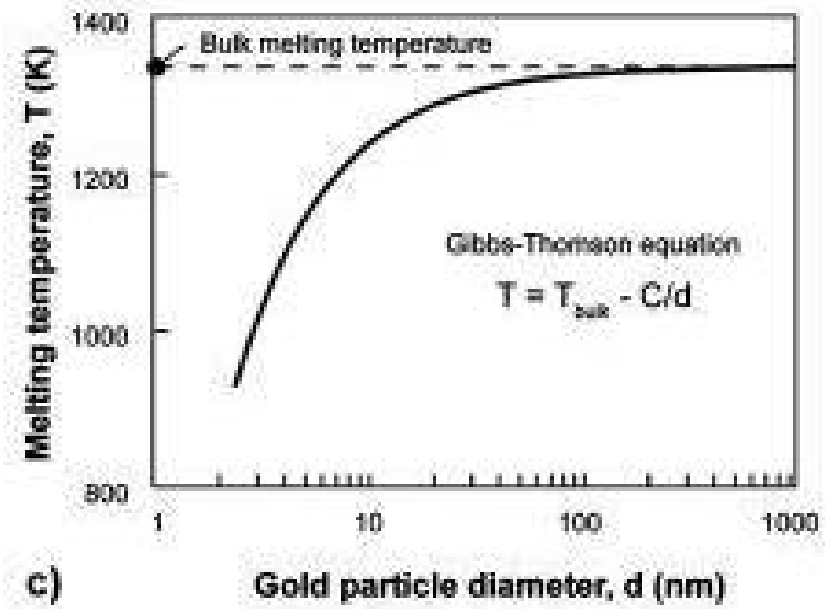
- Quantum effects

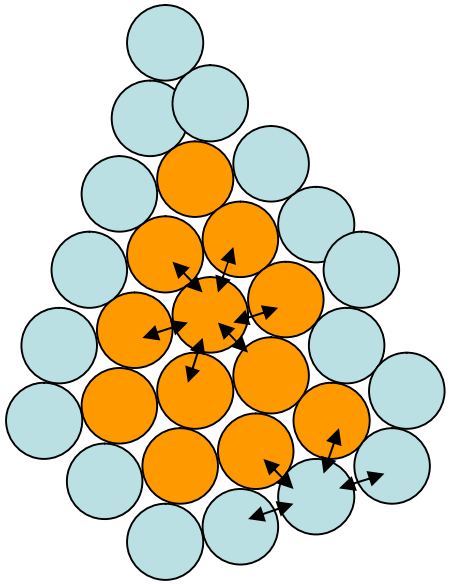
- Surface effects: it is simply due to the increase of the fraction of atoms present on the surface of the material



Temperatura di fusione

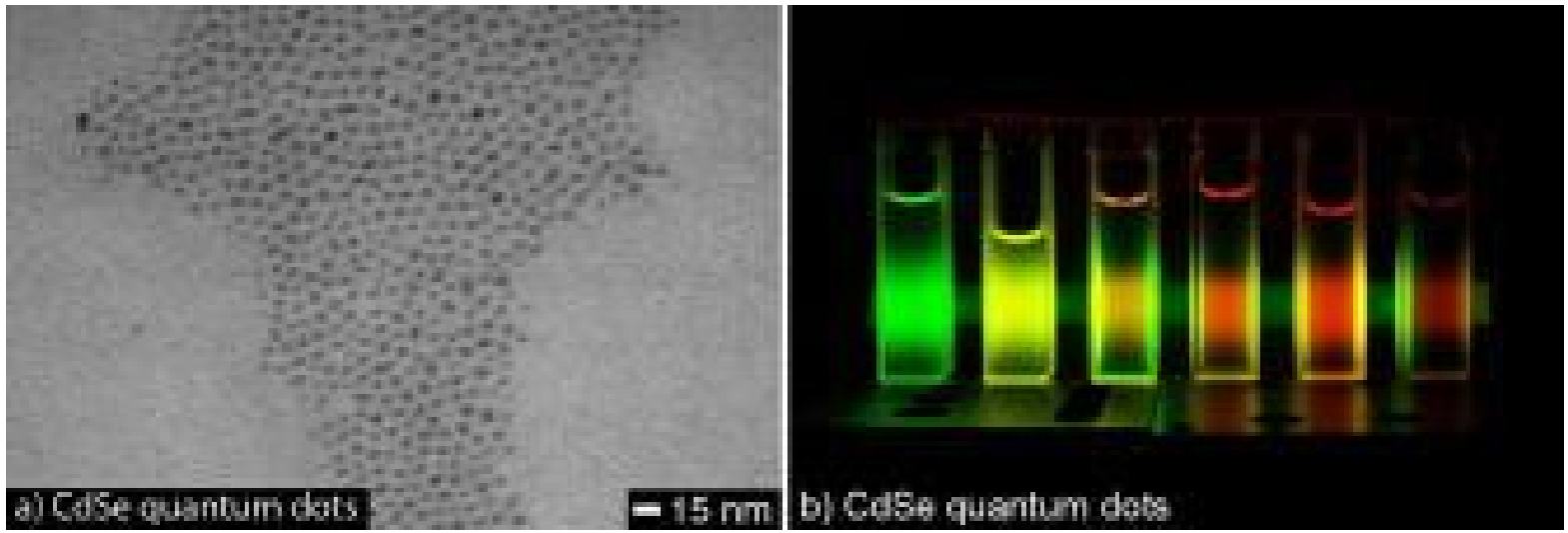
- Atoms on the **surface** have less binding than the ones **inside** the material: the melting temperature *decreases* with particle size





Quantum effects

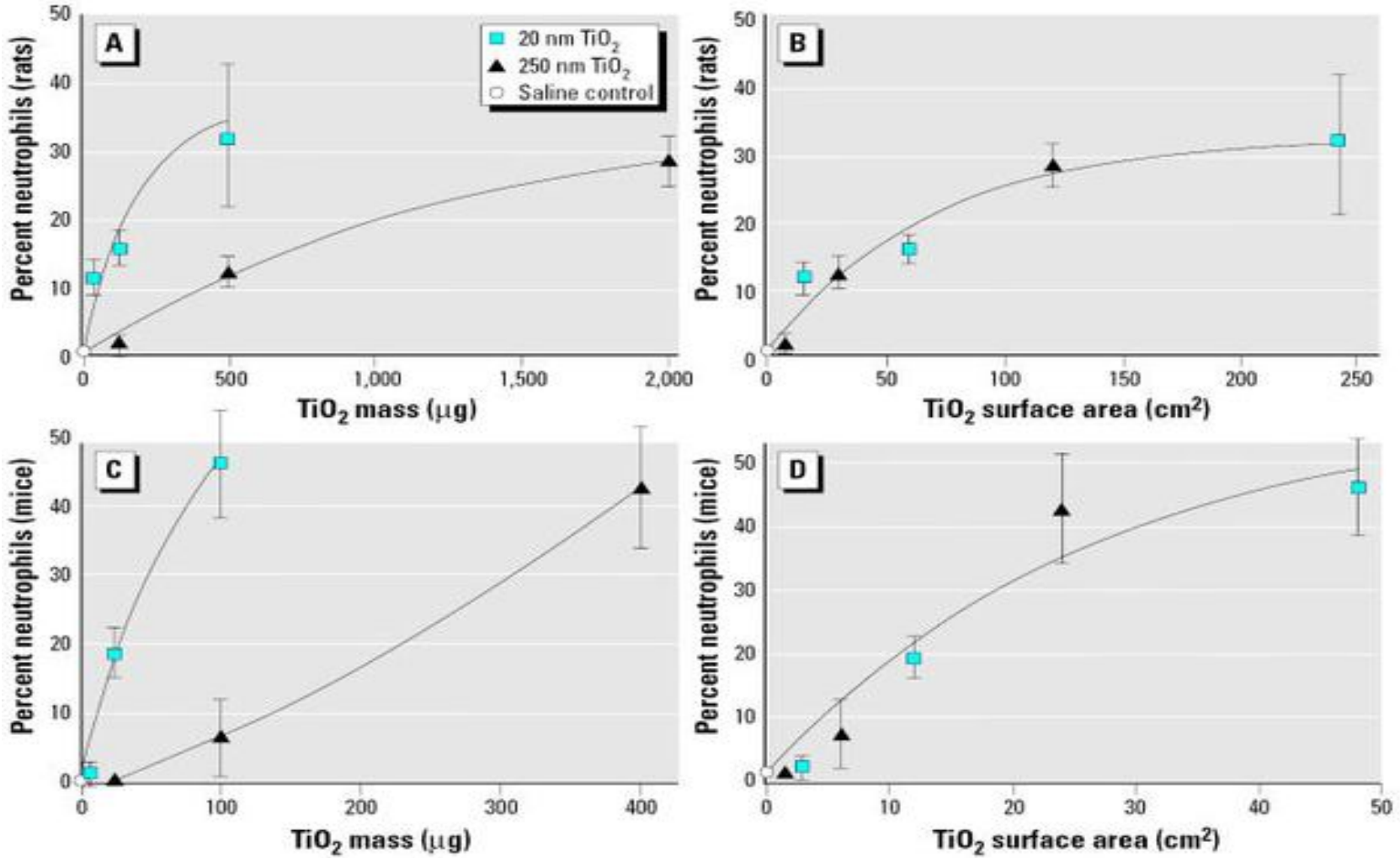
- The electronic behaviour of a Quantum Dot (QD e.g. CdSe) is similar to that of an atom: this results in a quantized energy specrum



Tipical nanotoxicological feature: Surface Area

- TiO_2 NPs
- PTFE NPs
- Amorphous SiO_2 NPs

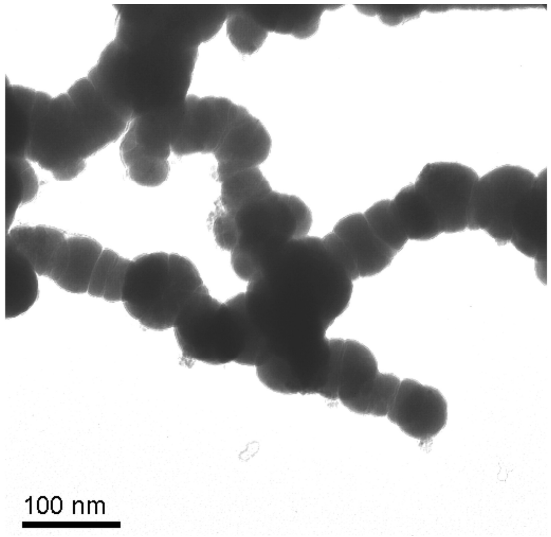
TiO_2 (titanium dioxyde; anatase) instilled in animal trachea, analysis of PMNs in the lung lavages (measure of inflammation)



- PolyTetraFluoroEthylene (PTFE) fume (heating to ~ 480 °C) contains NPs (~18 nm Ø) which are very toxic

If accidentally overheated, nonstick cookware can emit fumes that may be harmful!!

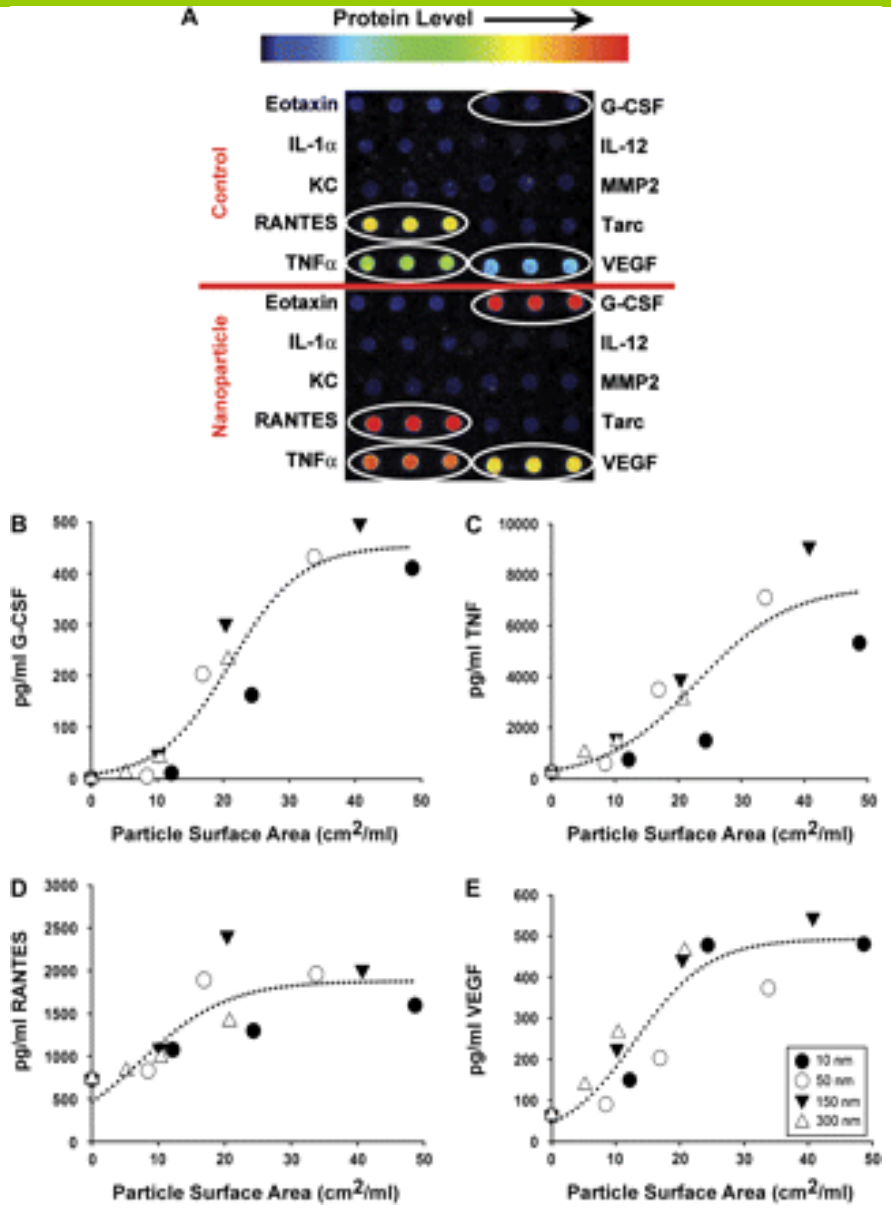
- PTFE NPs induce lung acute injury and high mortality rate *in rats*
- PTFE NPs aging results in size increase (> 100 nm Ø due to coalescence) and loss of toxicity

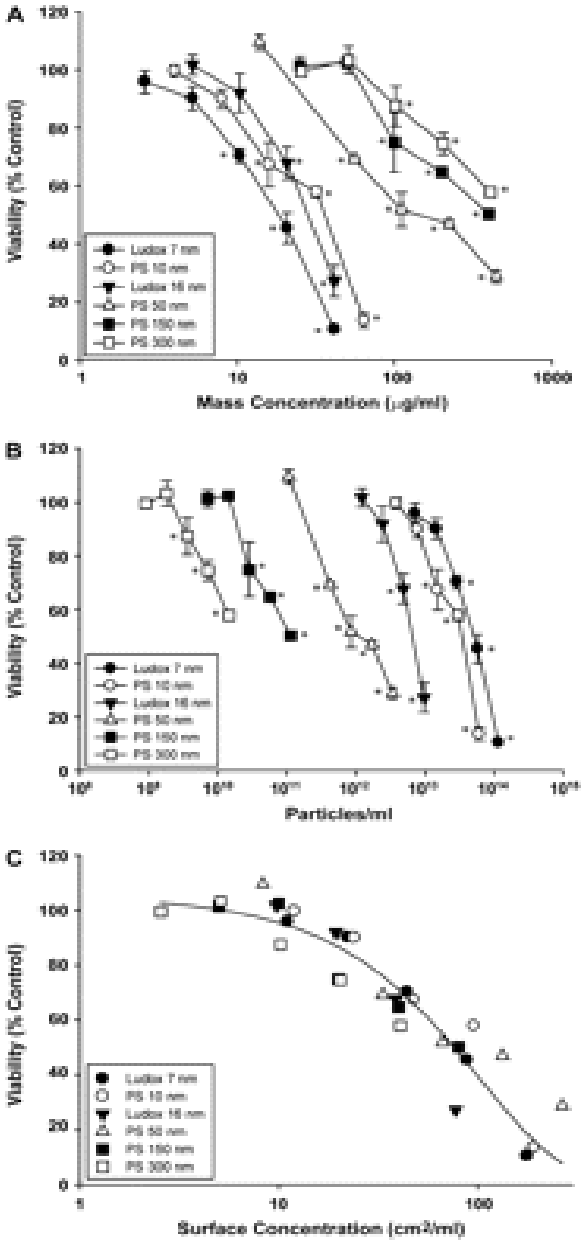


Teflon fume derived NPs

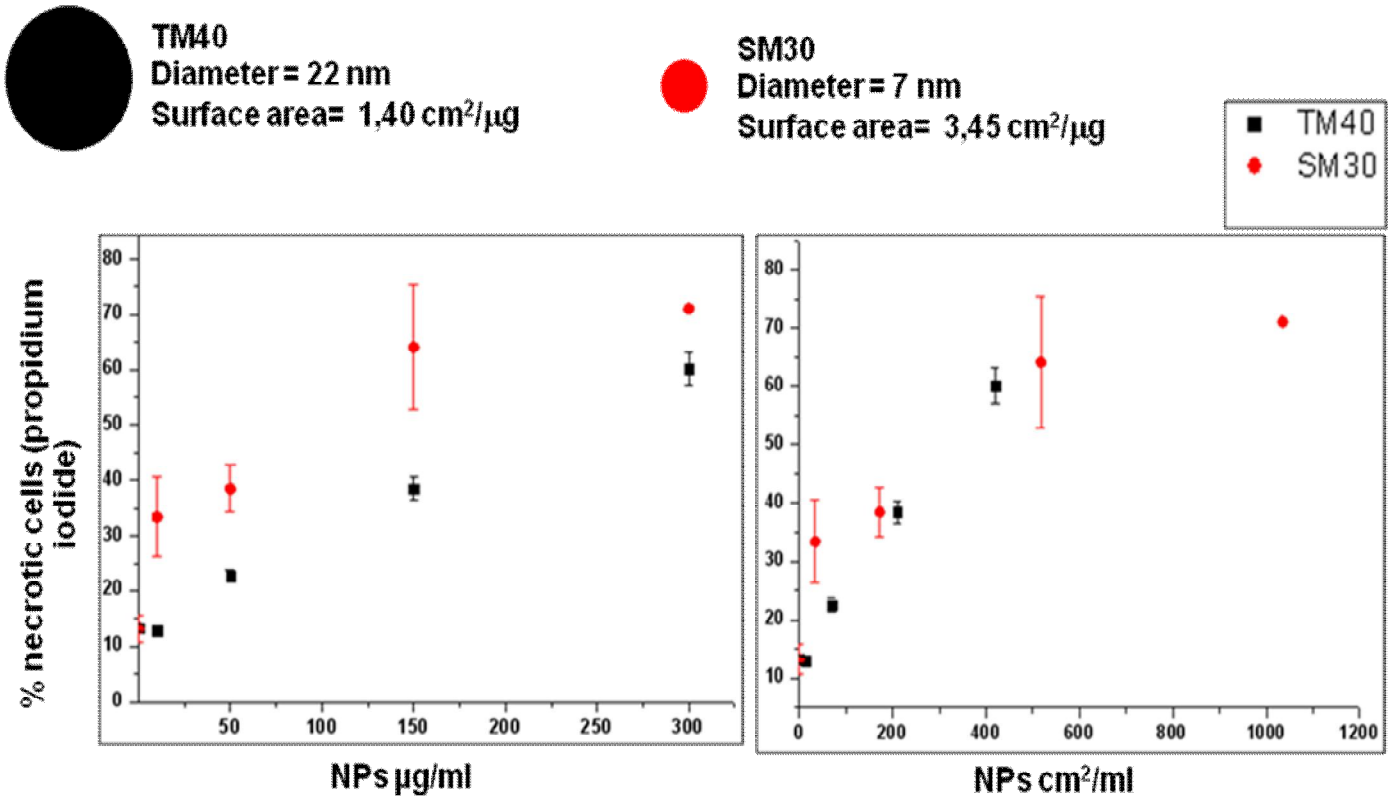
- *Caveat:* possibly also chemical modifications are responsible for such loss of toxicity, not only size increase..

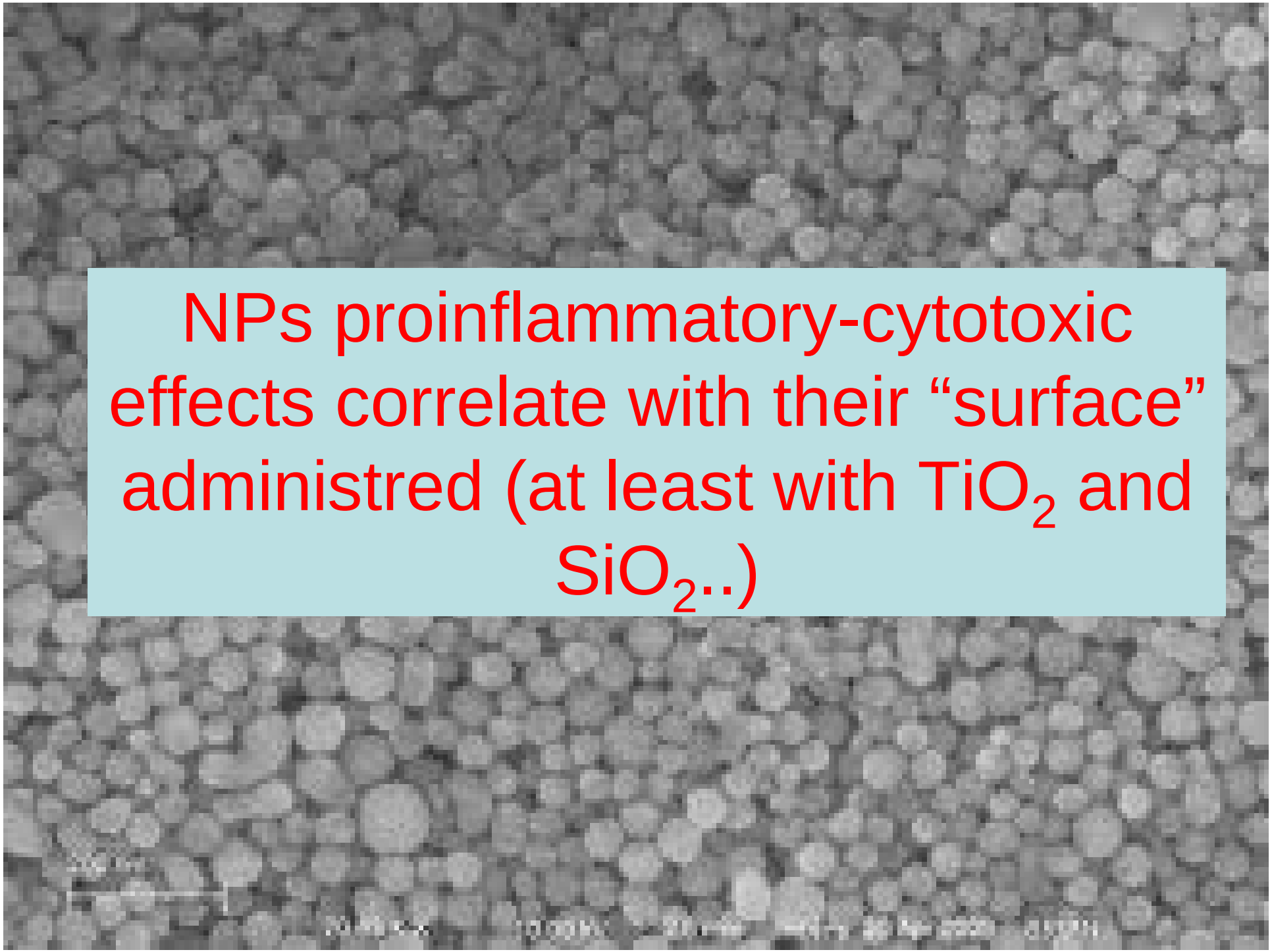
macrophages cytokines response to SiO_2 NPs





macrophages cell death due to SiO_2 NPs


TM40
Diameter = 22 nm
Surface area= 1,40 cm²/µg
SM30
Diameter = 7 nm
Surface area= 3,45 cm²/µg
TM40
SM30
% necrotic cells (propidium iodide)
NPs µg/ml
NPs cm²/ml

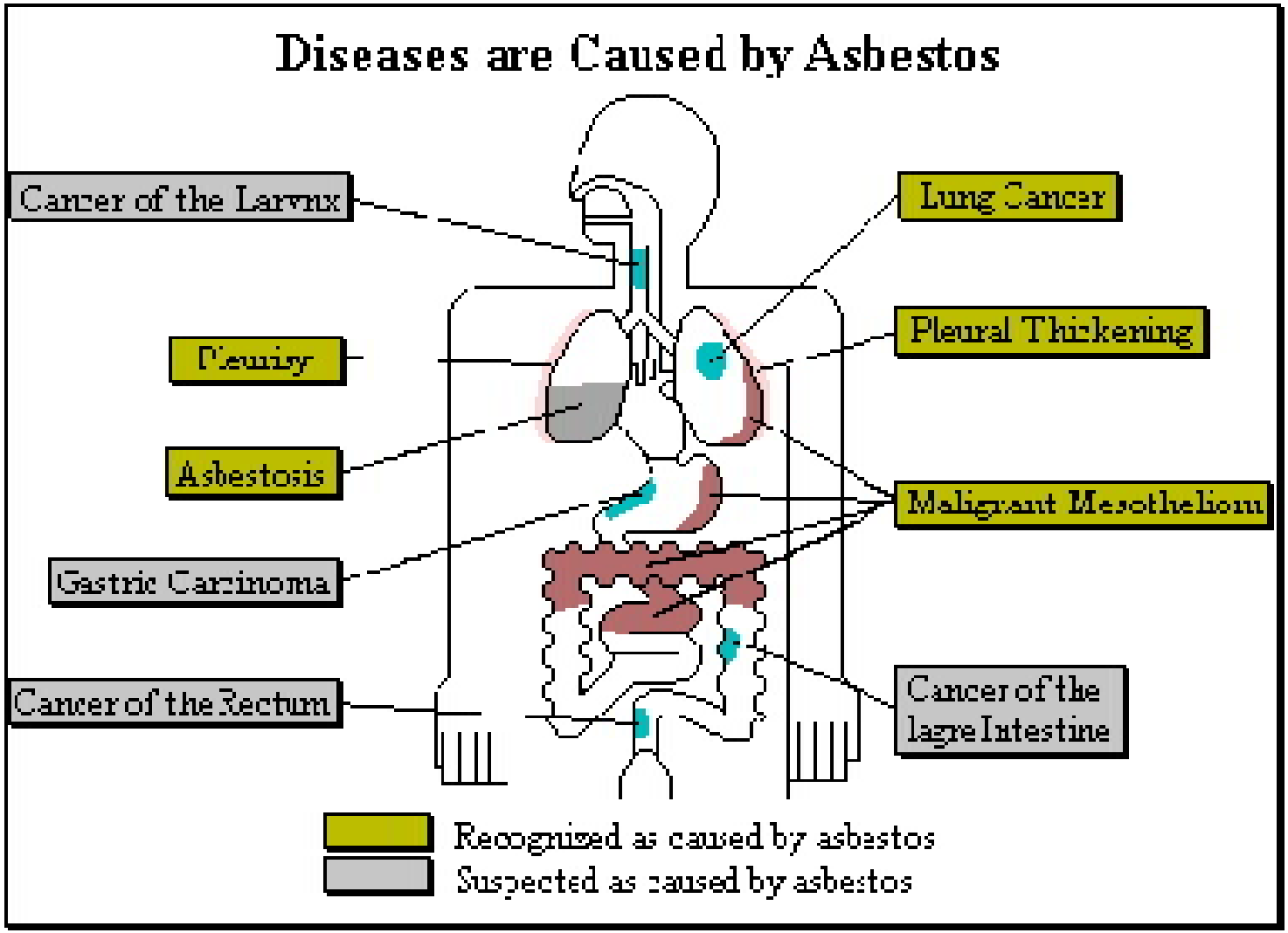

NPs proinflammatory-cytotoxic effects correlate with their “surface” administred (at least with TiO_2 and SiO_2..)

FIBERS: defined as structures with a high length/diameter ratio (aspect ratio > 3:1)

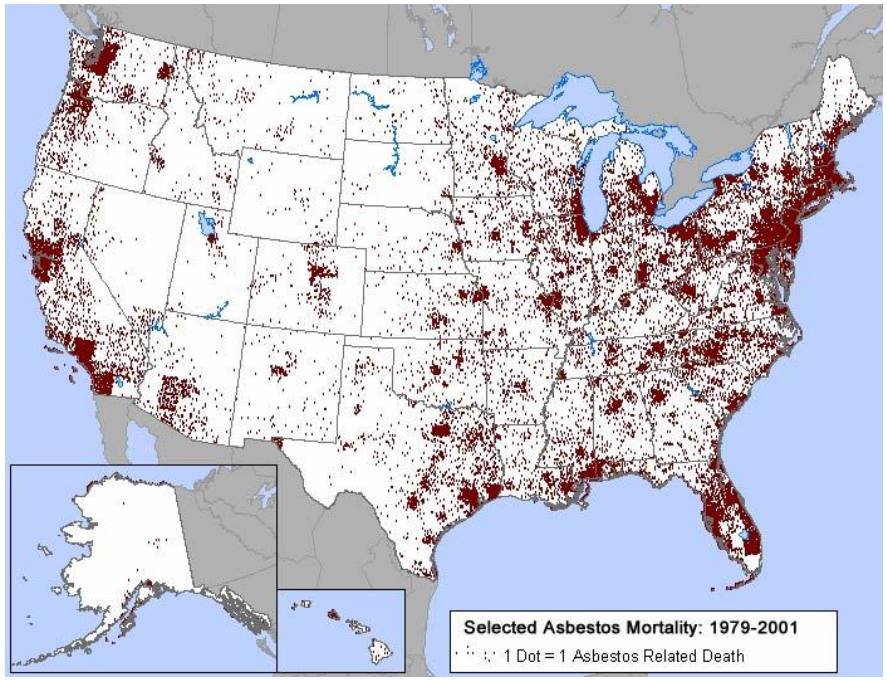
- Biopersistent vitreous fibers
- Asbestos fibers

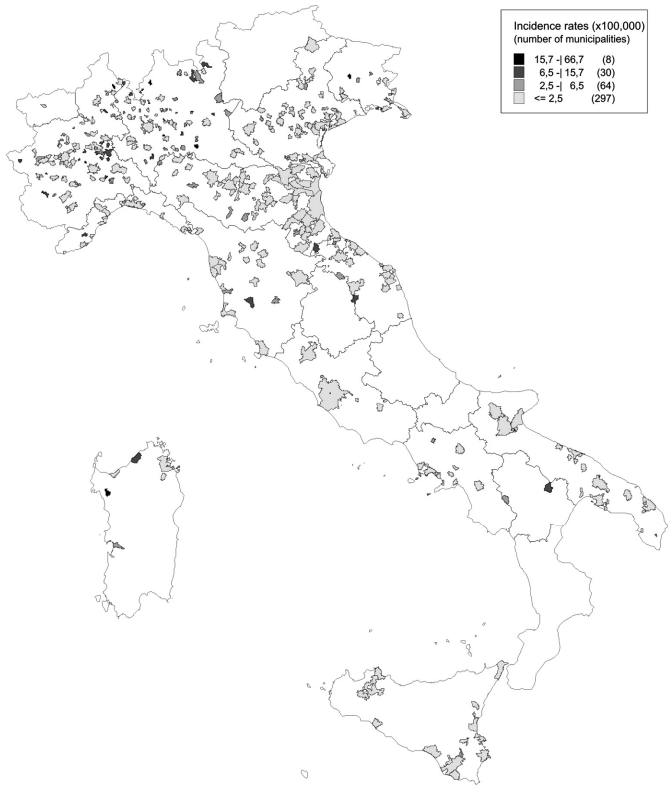
- Fibers are dangerous and associated with increased risk of *fibrosis* and *cancer* (lung carcinoma and mesothelioma)
- Three Ds are important: dose, dimension and durability

Asbestos is very toxic



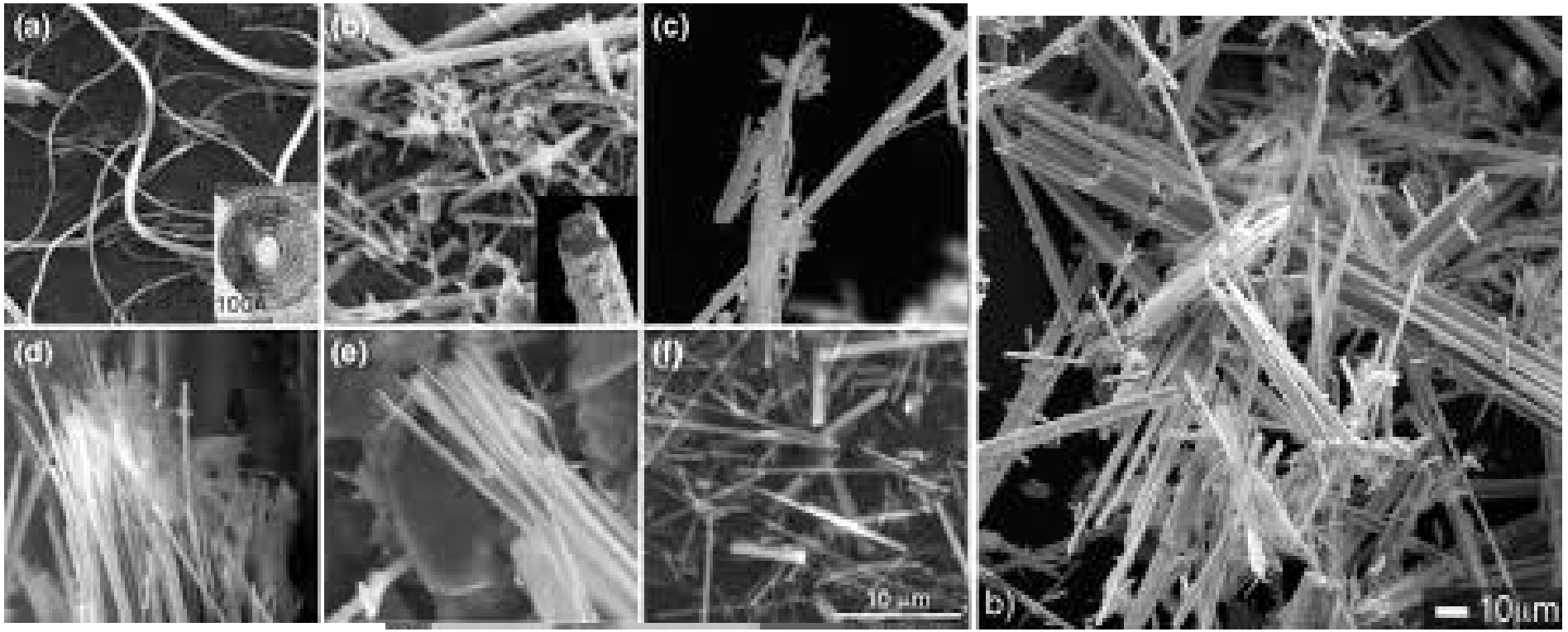
Mortality due to asbestos in the US





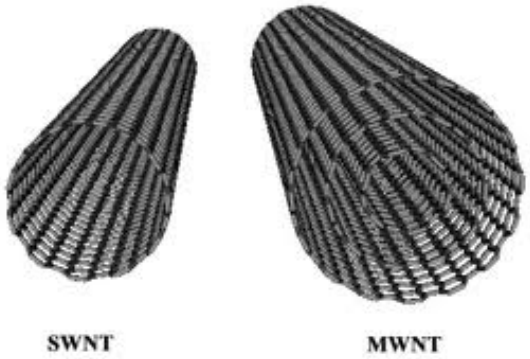
.. and Italy (mesothelioma)

Asbestos fibers

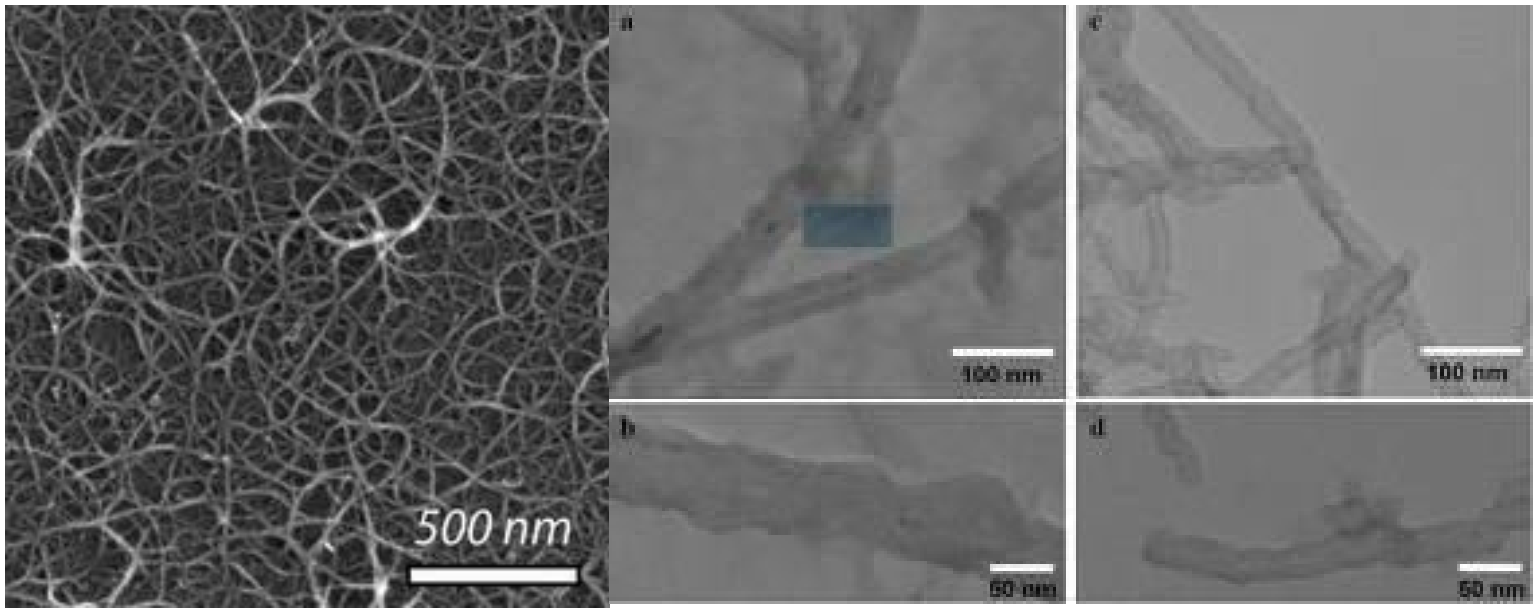


Carbon nanotubes are fibers

Single Wall Nano Tubes

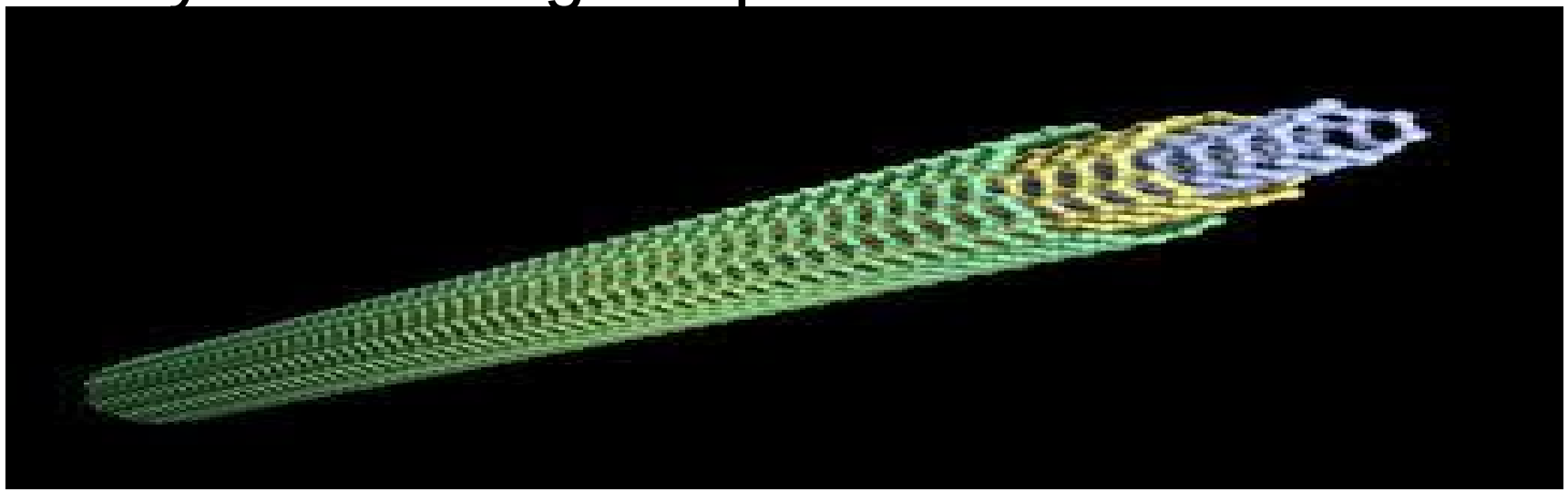


Multi Wall Nano Tubes



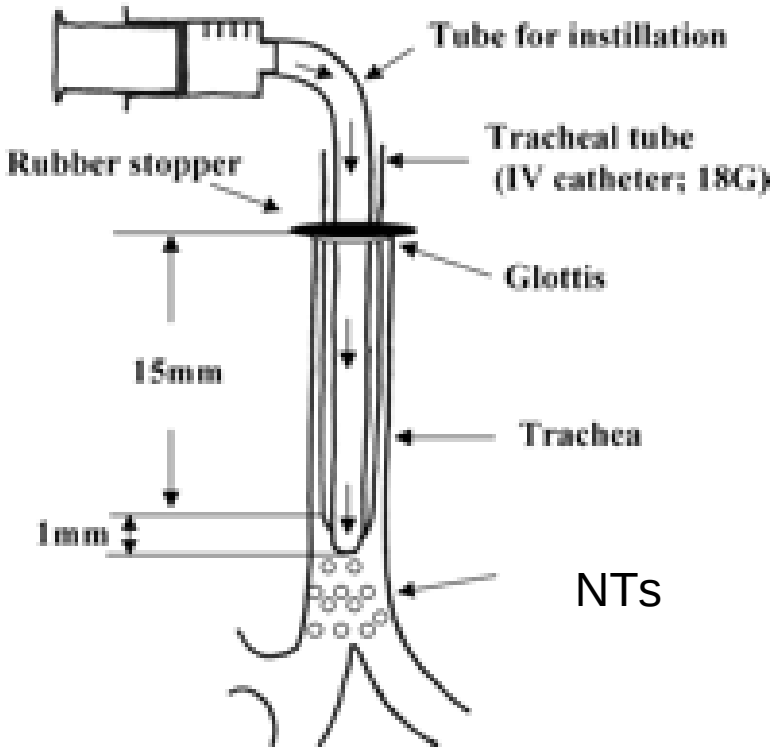
NTs

- They have a high aspect ratio > 100



- 5 µm long and 0,7-1,5 nm Ø (SWNTs) or 2-50 nm Ø (MWNTs)

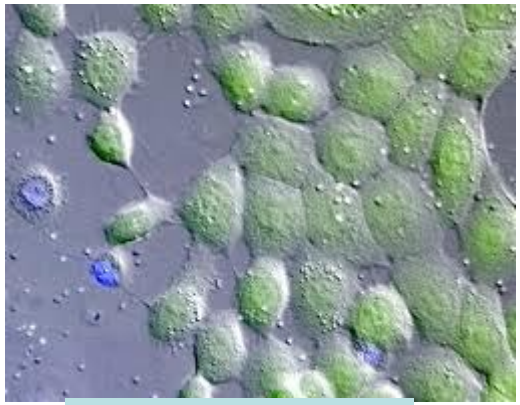
NTs Intratracheal administration in rats



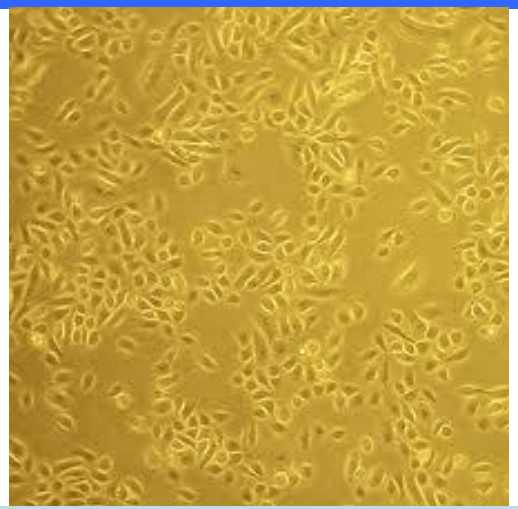
- Significant acute inflammation of the lung at high doses
- Granuloma formation

But.. Metal contaminants responsible for toxic effects?

In vitro cellular studies



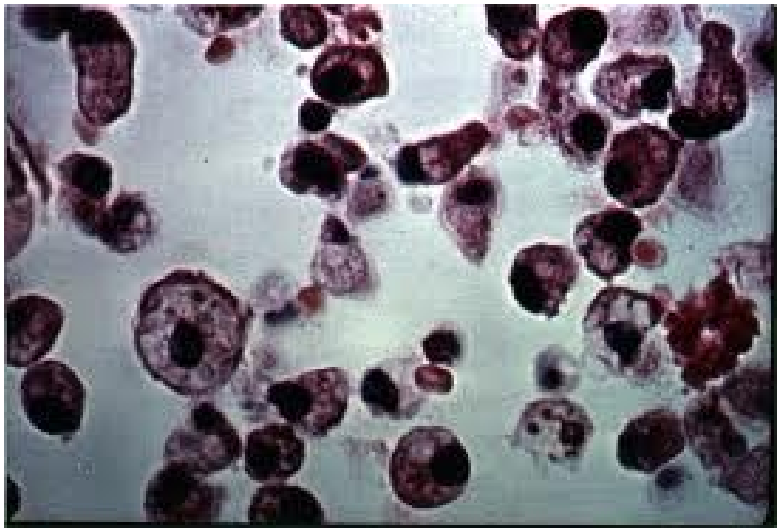
keratinocytes



Bronchial epithelial cells

SWNTs induce ROS, free radicals and depletion of antioxidants (Glutathion)
OXIDATIVE STRESS

NTs toxicity in macrophages



Mitocondria strongly affected

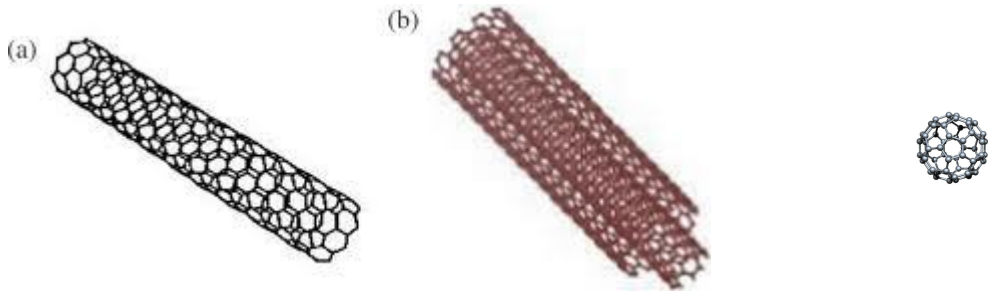
Phagocytitc activity inhibited

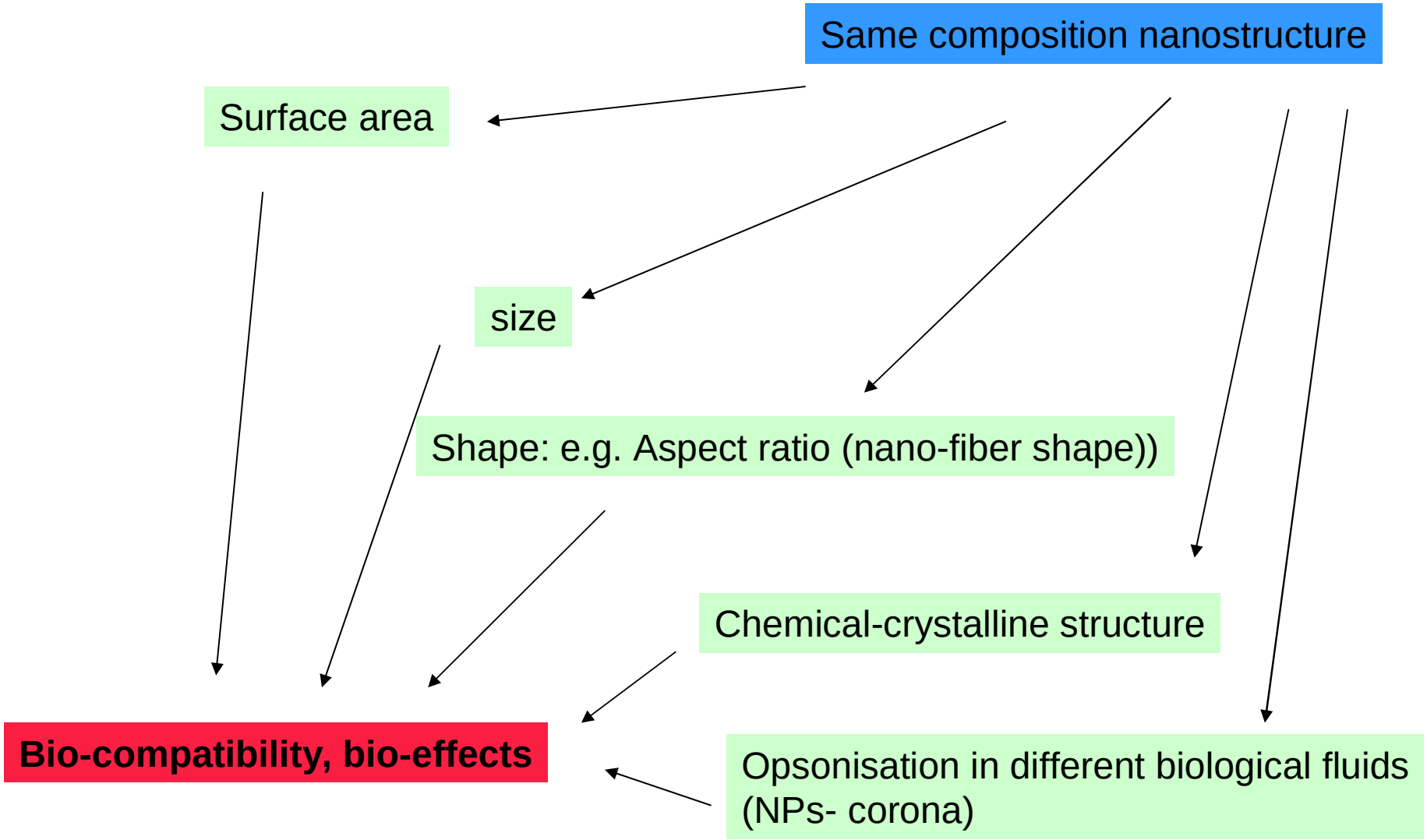
Low doses < 1 µg

Relation with aspect ratio (*fiber-osity?*)

- Order of toxicity:

- SWNTs>MWNTs> fullerenes

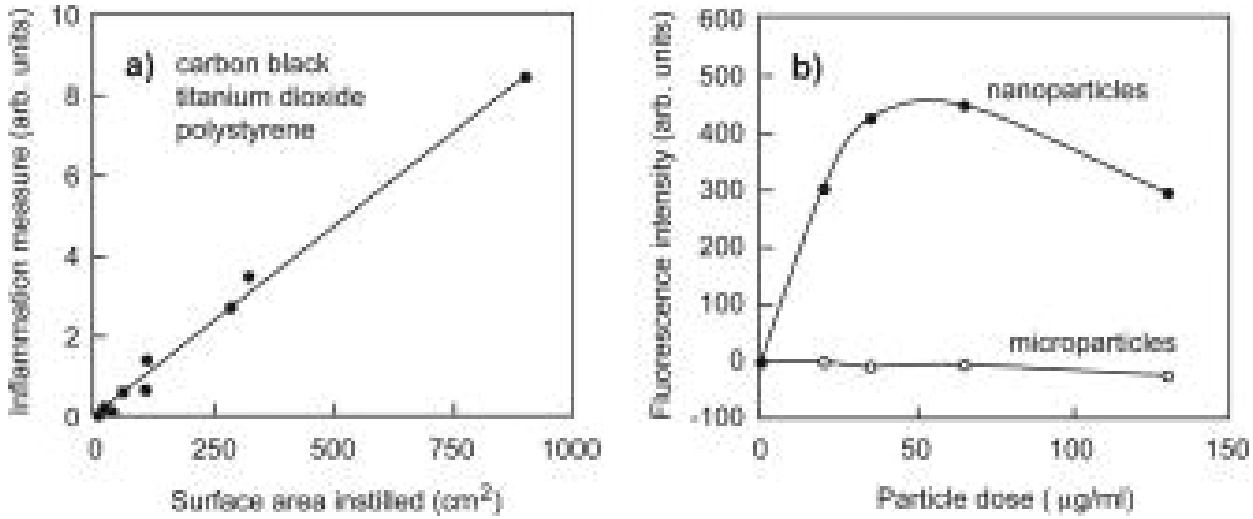



Same composition nanostructure
Surface area
size
Shape: e.g. Aspect ratio (nano-fiber shape))
Chemical-crystalline structure
Bio-compatibility, bio-effects
Opsonisation in different biological fluids (NPs- corona)

Again on the relevance of NPs size

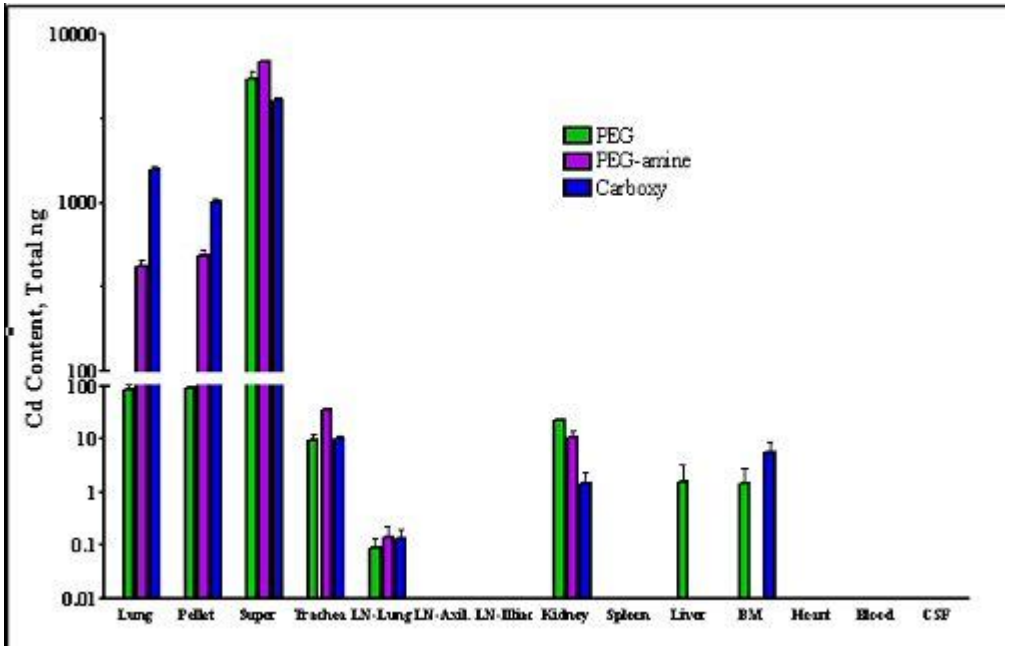
- As discussed, in the case of TiO_2 NPs, the smaller the size the higher are the acute effects (rats and mice afte inhalation or tracheal instillation), likely due to SA exponential increase..

Same surface= same effect..
This principle appears correct also comparing
mild toxic NPs like CB, titanium dioxide and polystirene



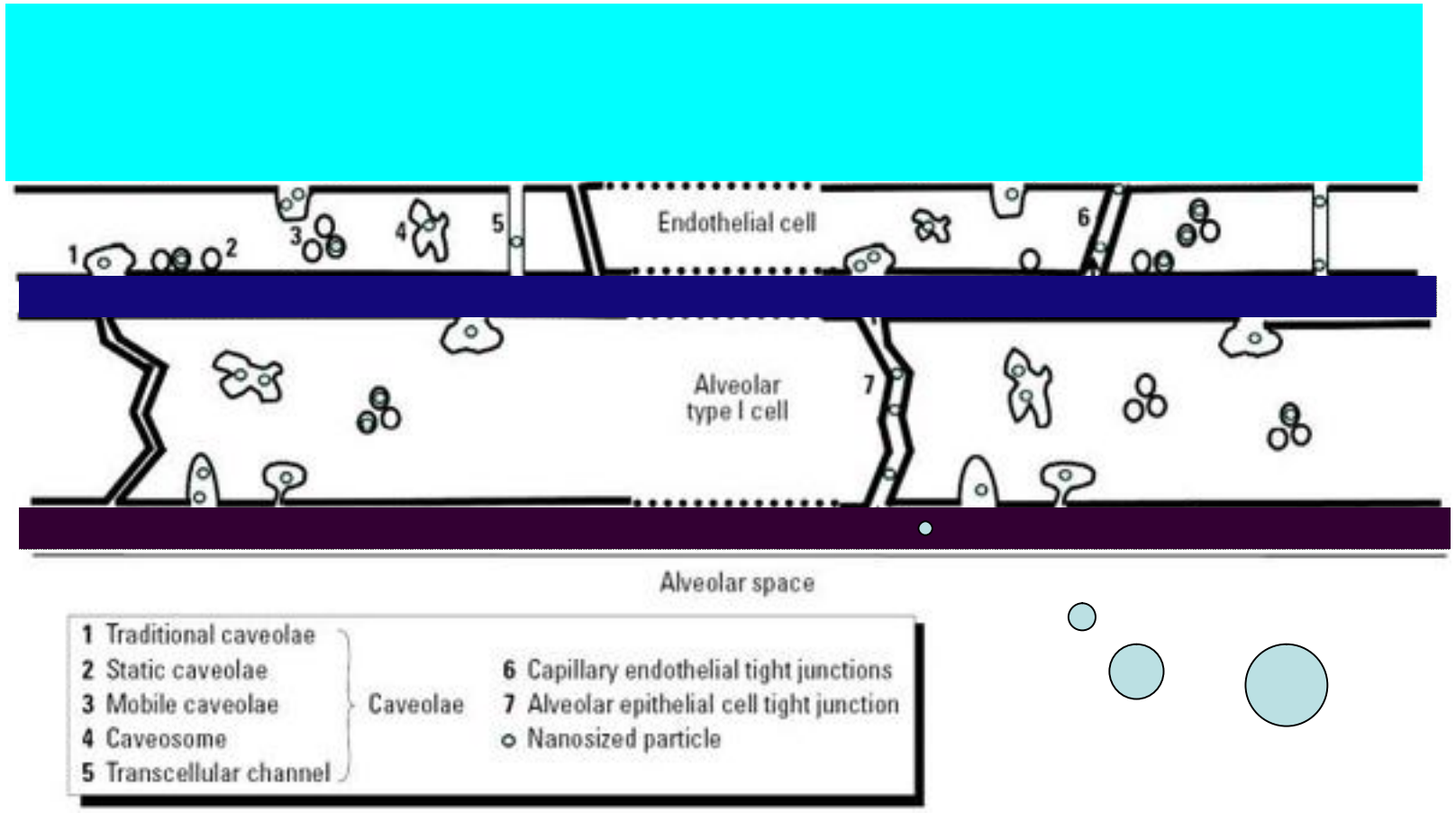
In addition, there are also long term effects which are increased by decreasing NPs size

Prolonged retention in the lung

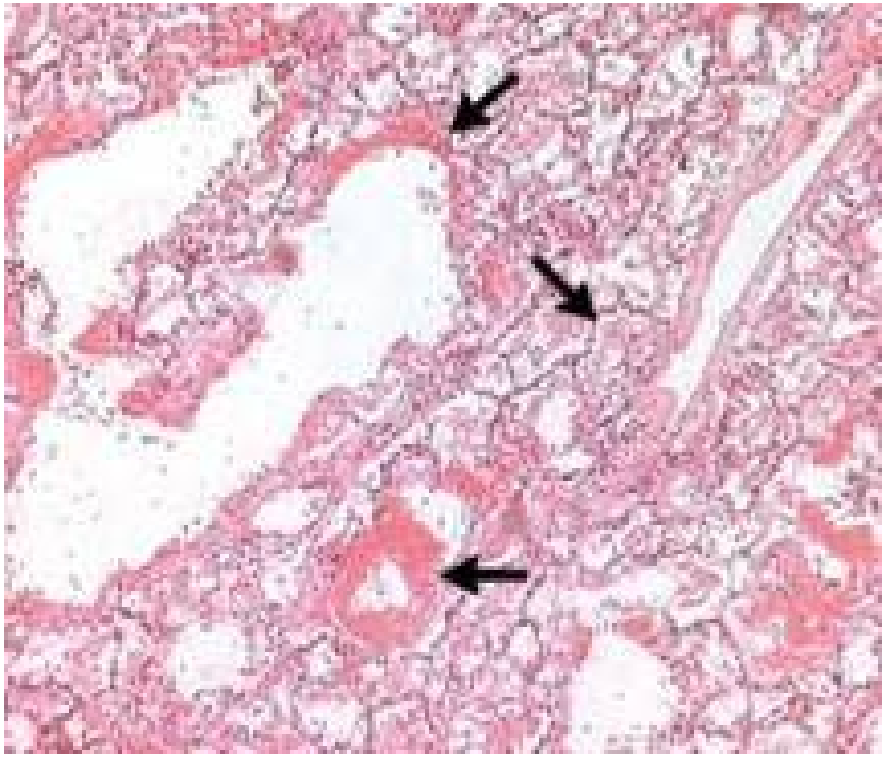


Tissue distribution of Cd in tissues immediately following intratracheal microspray exposure of rats to QDs (10 µg as Cd).

Increased translocation to the pulmunary interstitium and persistance in this comparment



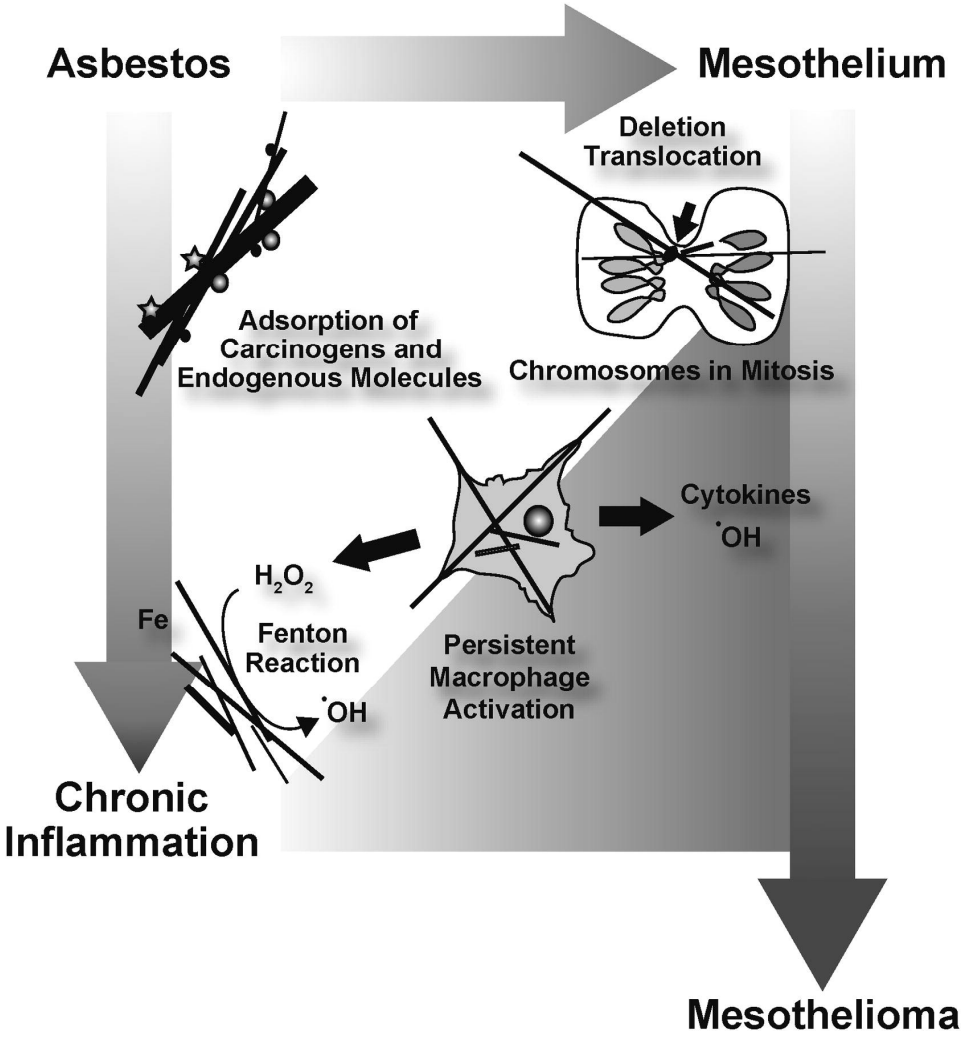
Impairment of alveolar functions



Greater epithelial effects

- Proliferation of type II alveolar epithelial cells (3% of all epithelial cells, target in lung cancer!)

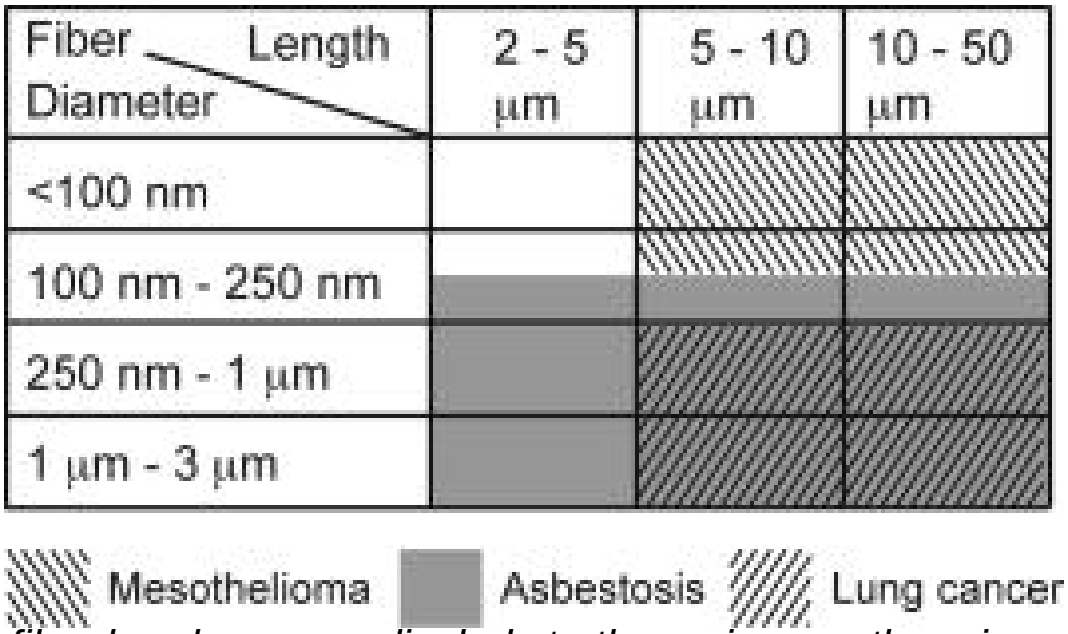
Again on fiber toxicity



- *Note: If a long fiber breaks perpendicularly to the main axys there is a*

decrease in bio-persistance and toxicity; however asbestos fibers break longitudinally!!

Longer asbestos fibers are more toxic



- *Note: If a long fiber breaks perpendicularly to the main axys there is a decrease in bio-persistance and toxicity; however asbestos fibers break longitudinally!!*

CNT toxicity

- It is controversial because of:
- Great variability of their structures: single walled, multi-walled, diameter, length, closed capped section or open ends
- In vitro CNTs are indeed very toxic to cells because they are hydrophobic and thend to aggregate: therefore they can be modifiyed by the additon of hydroxyl and carboxyl groups to make them more water soluble
- CNTs are eliminated from the lung very slowly: 81% is still present in the rat lung 60 days after exposure

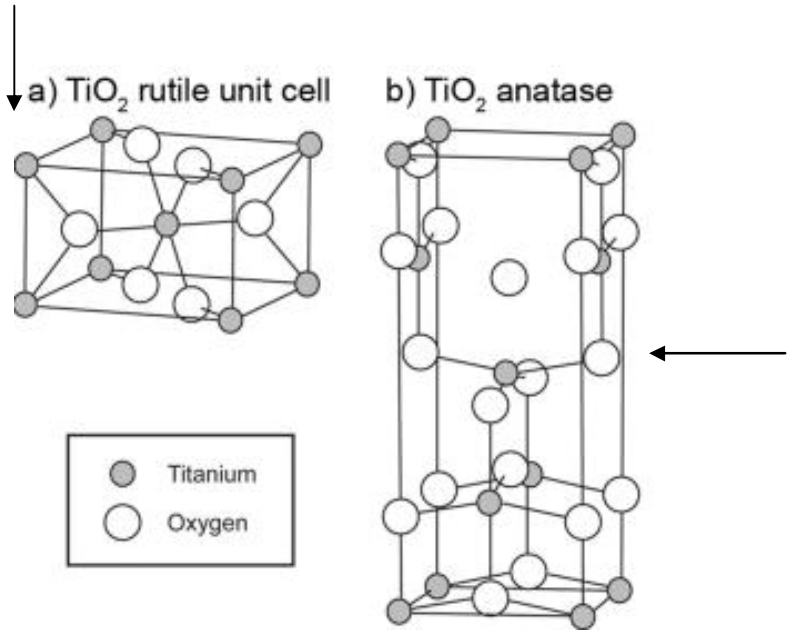
NPs chemistry and crystalline structure

- Chemistry is very critic in influencing different aspects the NPs bio-activity: cellular uptake, subcellular localization, ability to catalyse the production of ROS (*very important in toxicity!*)
- *It is important to distinguish between:*

composition *and* *chemistry*

The same composition may correspond to different chemical or crystalline structures

RUTILE: oxidative stress dependent DNA damage!



ANATASE: mildly pro-inflammatory but no DNA damage as in the case of RUTILE form

TiO2 NPs may be in two different crystalline structures

- In some cases NPs crystalline structure can change after interaction with water

- E.g. ZnS NPs (~ 700 atoms, ~ 3 nm Ø) become more ordered when in the presence of water

Oxidative stress

- Many in vivo and in vitro studies have shown that Nps of variuos chemical composition induce ROS:

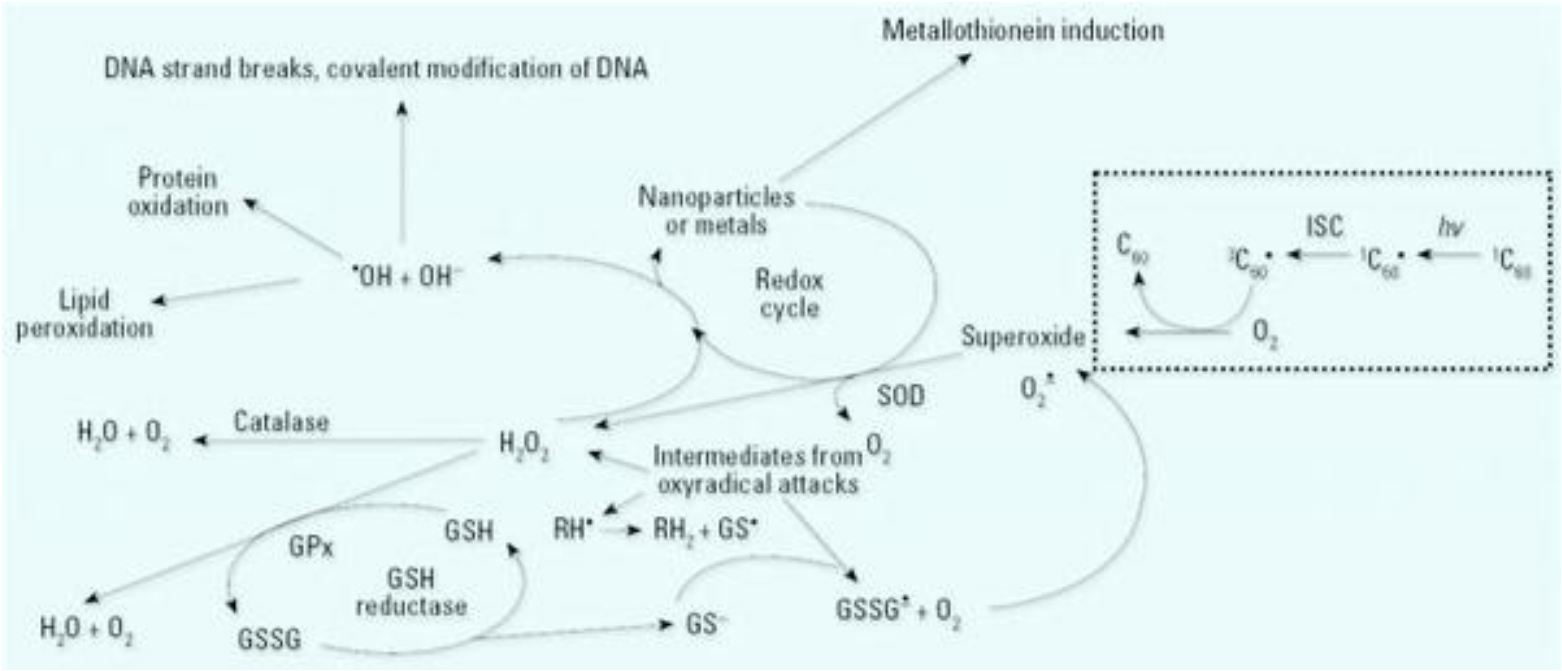
- Auto-mobile exhaust
- QDs
- CNTs
- Fullerenes

Mechanisms of ROS induction

- 1. free radicals or oxidants can be present on the surface of NPs (in particular those present in the air) e.g. NO_2 and O_3
- 2. transition metals (iron, copper, chromium, vanadium, yttrium) NPs or as components or contaminants of NPs-nanofibers (DEPs, CNTs ?) may act as catalyst in Fenton-type reactions

- 3. Alteration of mitochondria: several studies have shown that NPs can enter these organelles

- 4. activation of inflammatory cells like macrophages and PMNs

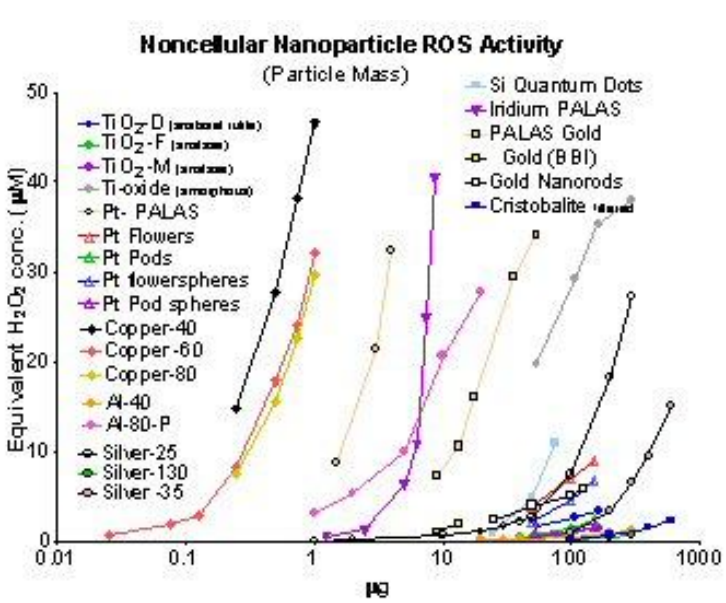

Metallothionein induction
DNA strand breaks, covalent modification of DNA
Protein oxidation
Nanoparticles or metals
ISC
hν
Lipid peroxidation
•OH + OH⁻
Redox cycle
Superoxide
SOD
Catalase
H2O + O2
H2O2
Intermediates from oxyradical attacks
GPx
GSH
RH• → RH2 + GS•
GSH reductase
H2O + O2
GSSG
GS•
GSSG• + O2

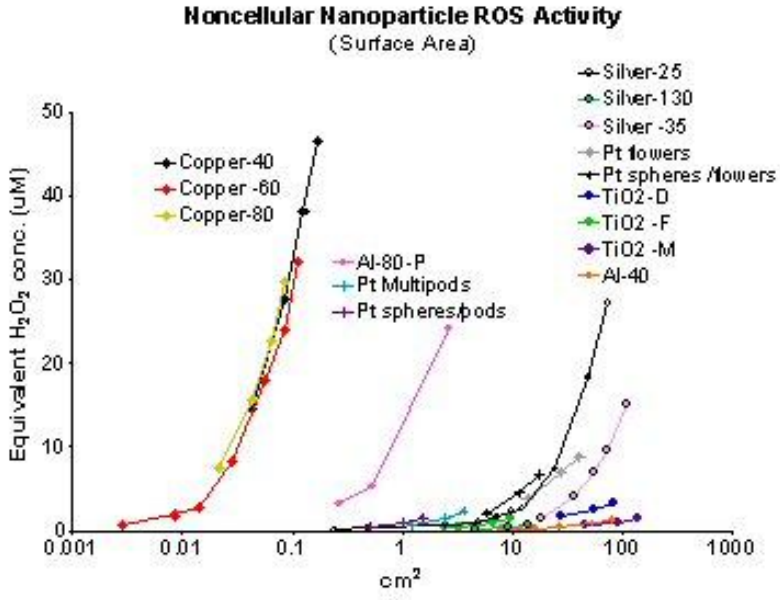
Fenton reaction

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^- + HO^\bullet \qquad (R1)$$

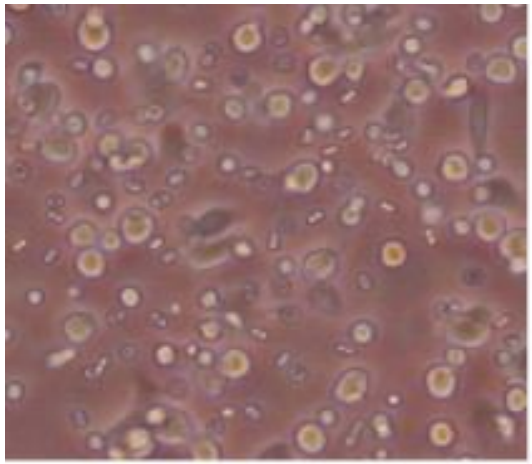
$$Fe^{3+} + H_2O \xrightarrow{h\nu} Fe^{2+} + H^+ + HO^\bullet \qquad (R2)$$

$$HO^\bullet + RH \longrightarrow H_2O + R^\bullet \qquad (R3)$$

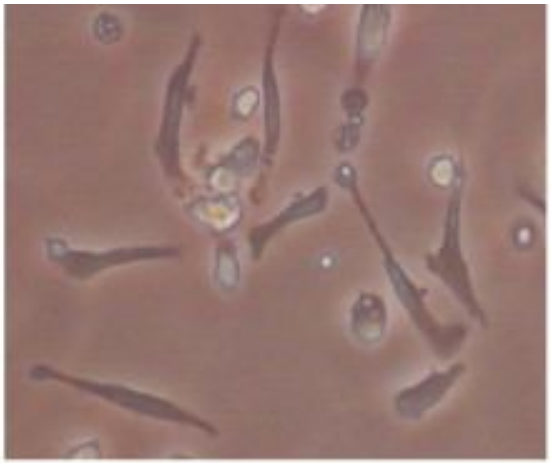

Noncellular Nanoparticle ROS Activity
(Particle Mass)
Equivalent H_2O_2 conc. (μM)
μg
Si Quantum Dots
Iridium PALAS
PALAS Gold
Gold (BBI)
Gold Nanorods
Cristobalite
Pt- PALAS
Pt Flowers
Pt Pods
Pt flowerspheres
Pt Pod spheres
Copper-40
Copper-60
Copper-80
Al-40
Al-80-P
Silver-25
Silver-130
Silver -35


Noncellular Nanoparticle ROS Activity
(Surface Area)
Equivalent H_2O_2 conc. (uM)
cm^2
Copper-40
Copper -60
Copper-80
Al-80-P
Pt Multipods
Pt spheres/pods
Silver-25
Silver-130
Silver -35
Pt flowers
Pt spheres /flowers
TiO2-D
TiO2 -F
TiO2 -M
Al-40

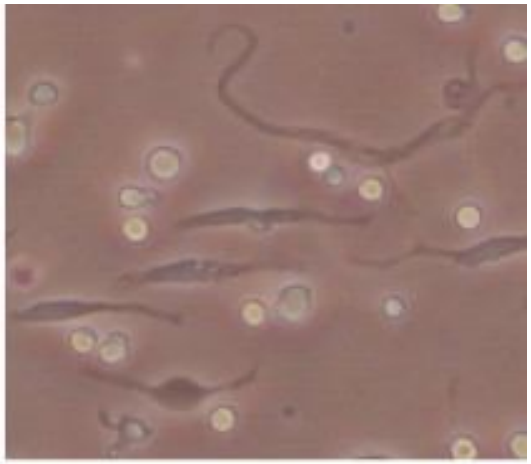
Macrophages activation by amorphous SiO_2 NPs



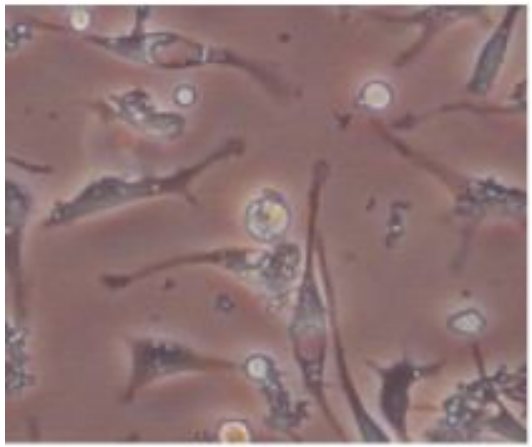
Control



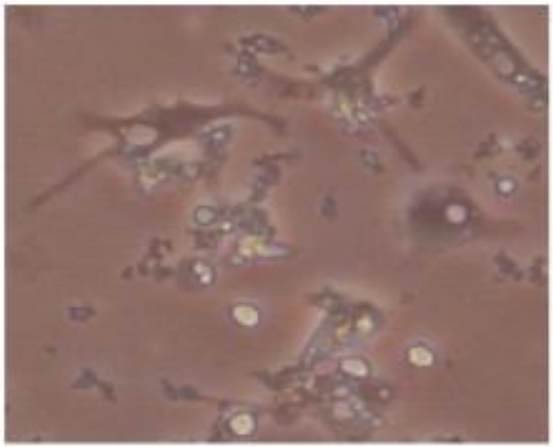
12 h



24 h



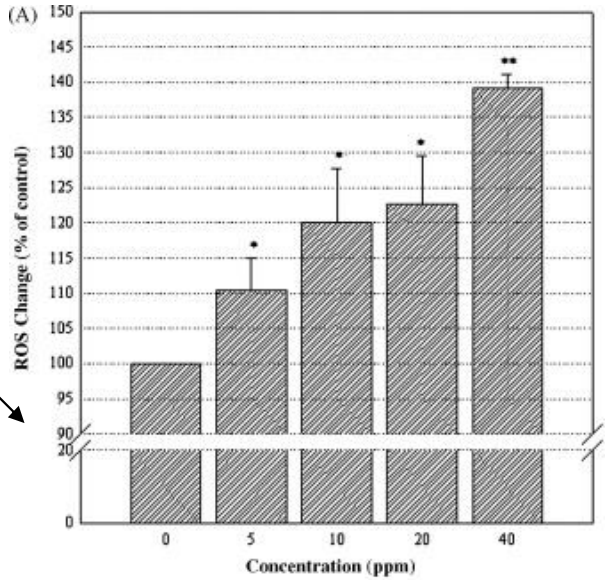
48 h

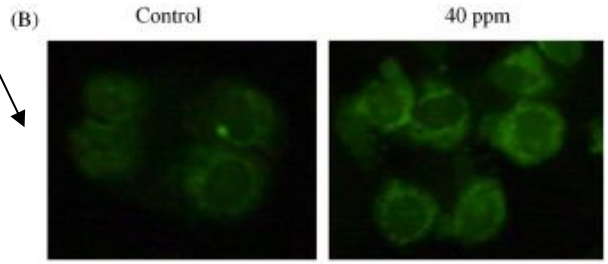


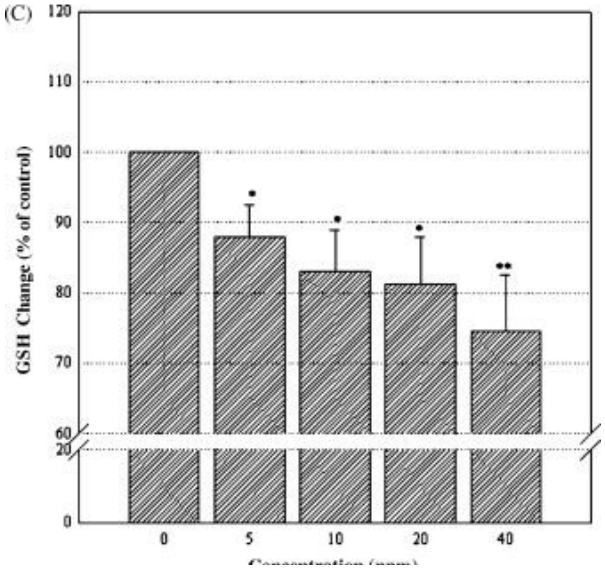
72 h

Activation of peritoneal macrophages by silica nanoparticles.
Mice were intraperitoneally injected with amorphous silica nanoparticle 50 mg/kg and were sacrificed

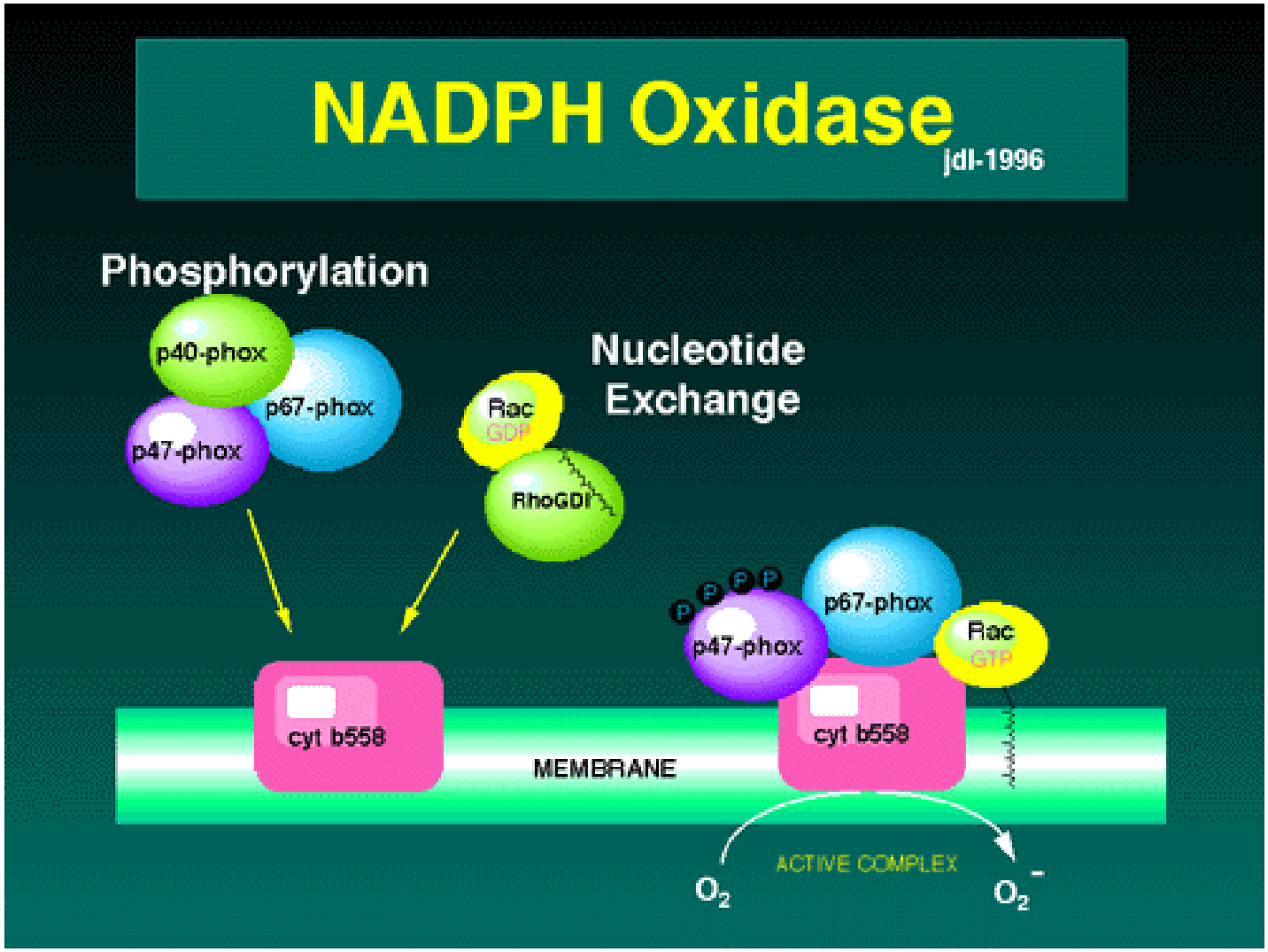
Effects of silica nanoparticles on the level of ROS and GSH in RAW264.7 cells.

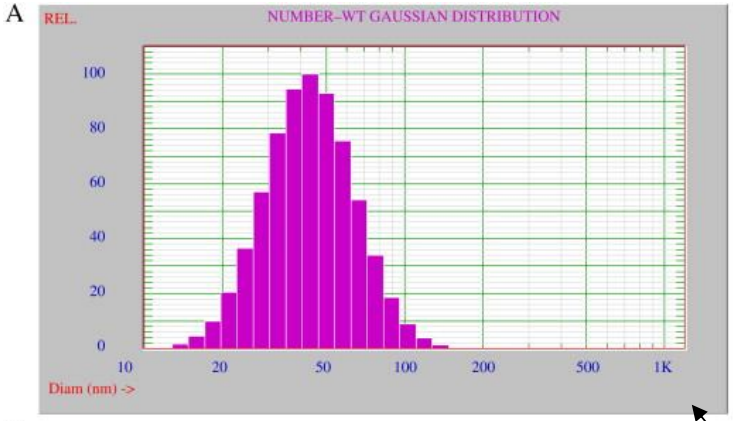


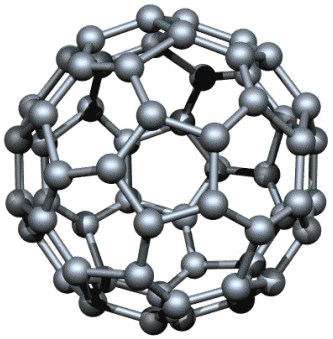




Cells were treated with silica nanoparticles for 24 hours


NADPH Oxidase
jdl-1996
Phosphorylation
p40-phox
p67-phox
p47-phox
Nucleotide
Exchange
Rac
GDP
RhoGDI
P
P
P
P
p67-phox
p47-phox
Rac
GTP
cyt b558
MEMBRANE
cyt b558
ACTIVE COMPLEX
O_2
O_2^-





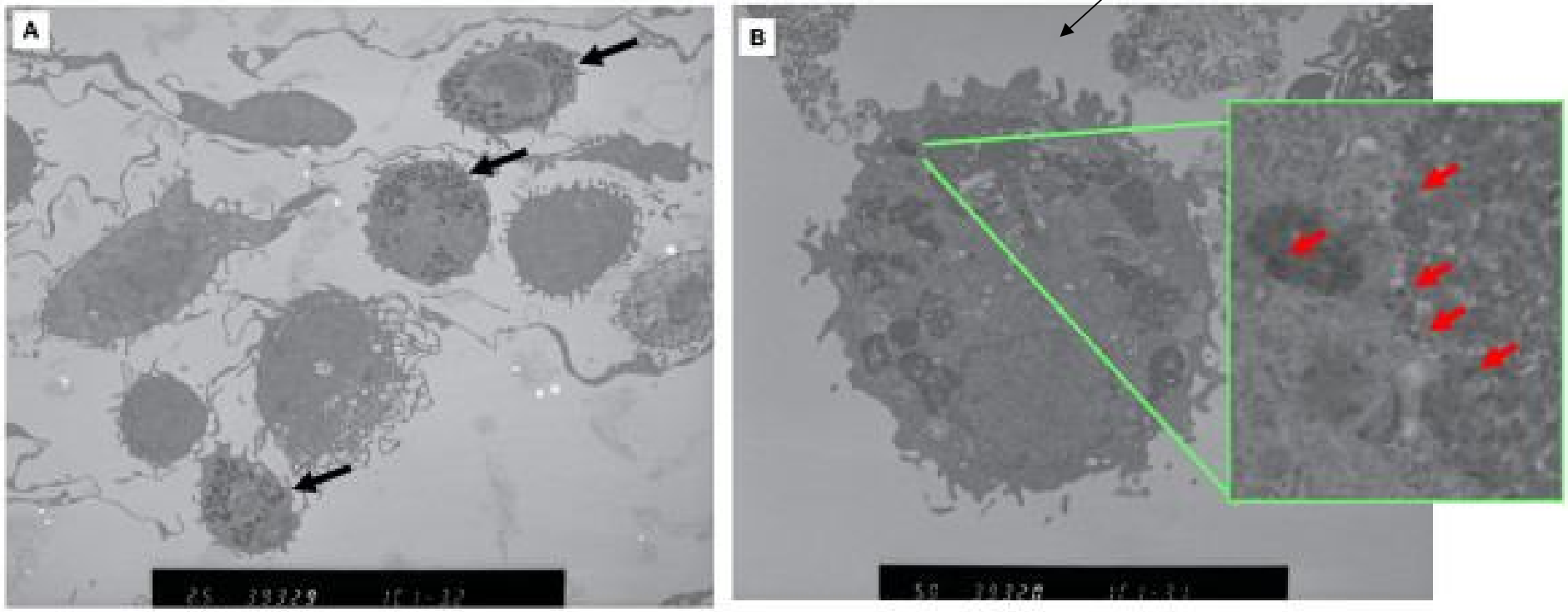
- The physico-chemical properties of fullerene C60s.

The size distribution

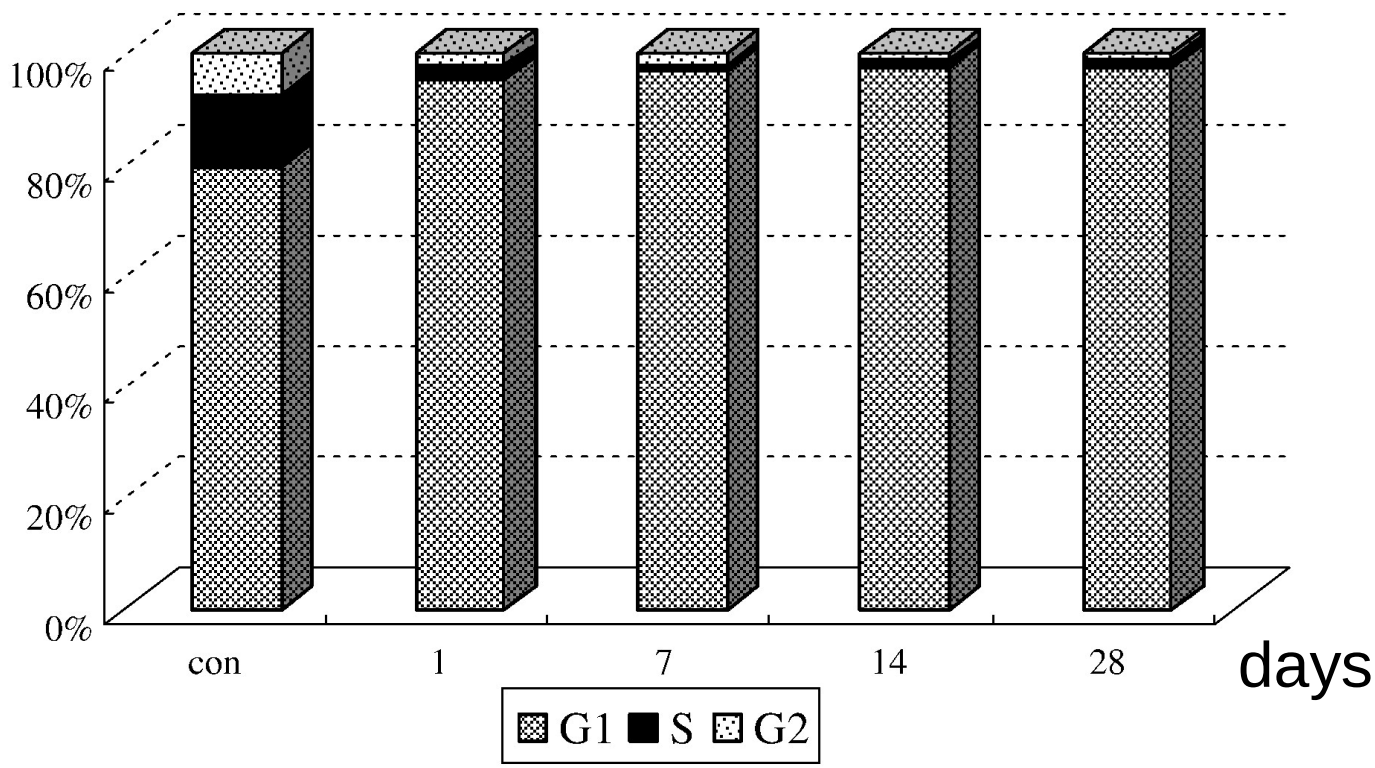
surface charge

TEM image.

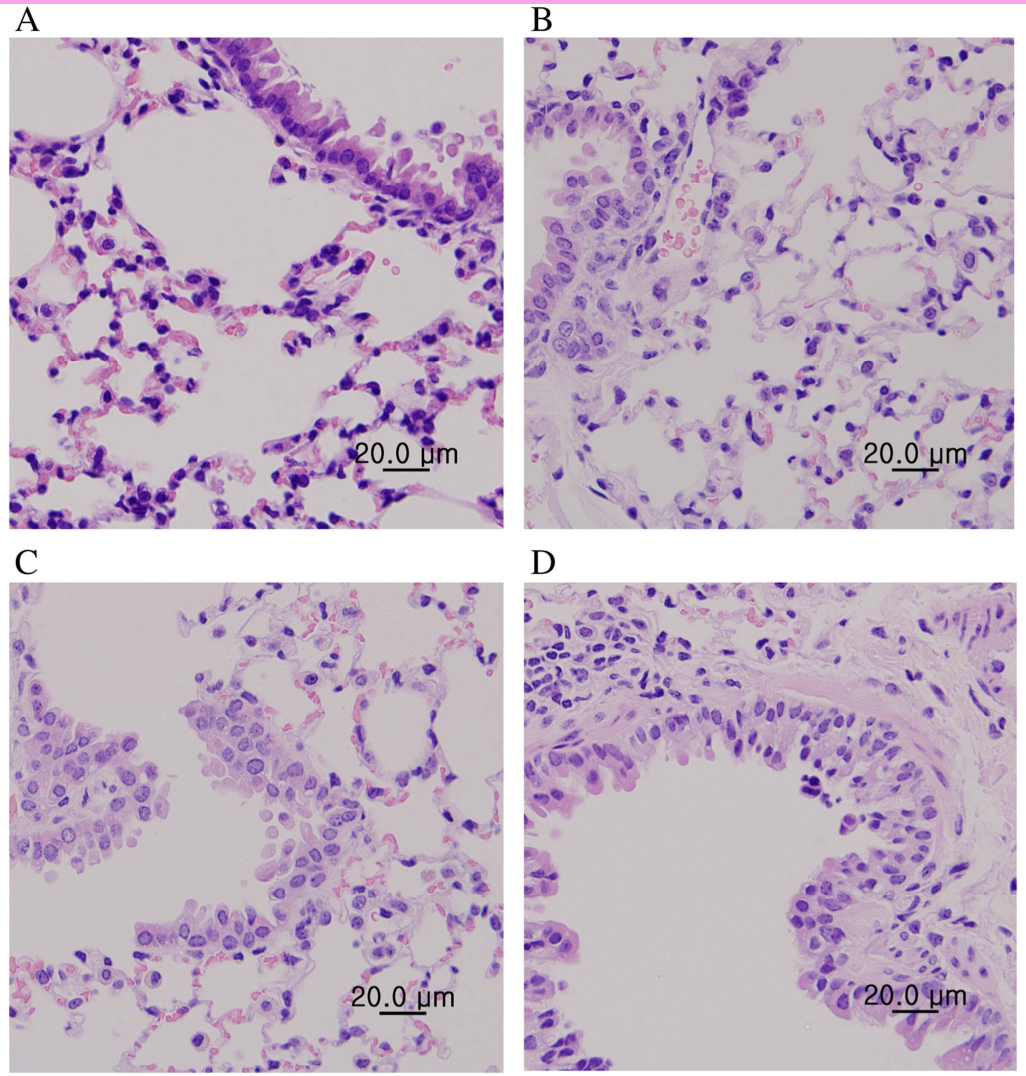
Fullerene NPs endocytosed by alveolar macrophages and PMNs

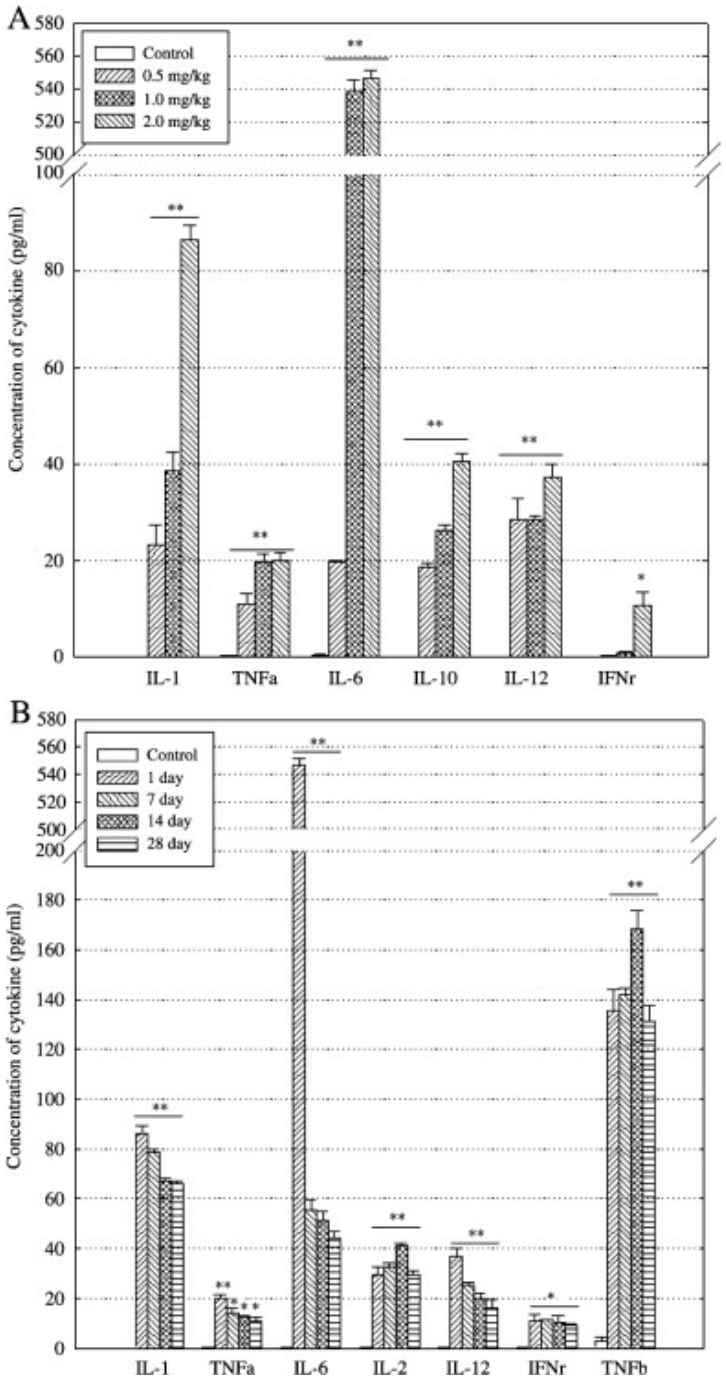


Change of cell growth following instillation of 2 mg/kg C60s



Histopathological change of tissues after a single intratracheal instillation of C60s (2 mg/kg)



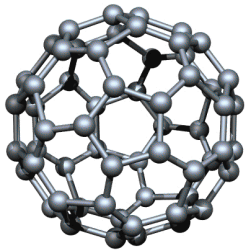


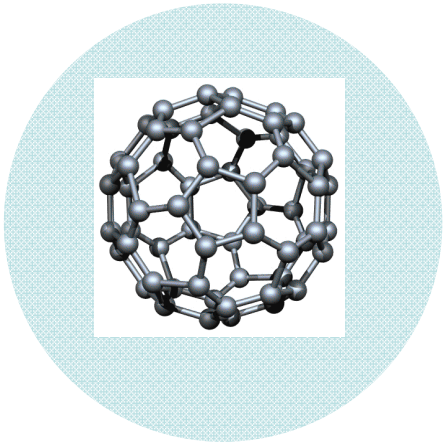
Dose dependance

Changes in cytokine levels in Bronchio-Alveolar Lavages fluid after a single instillation of fullerene C60s

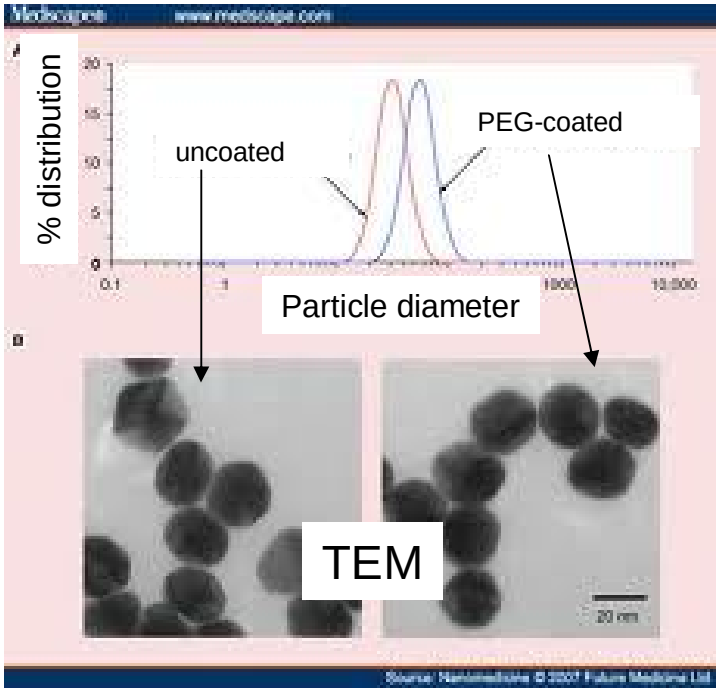
Time dependance

“stealth” Fullerene NPs to avoid negative effects ?

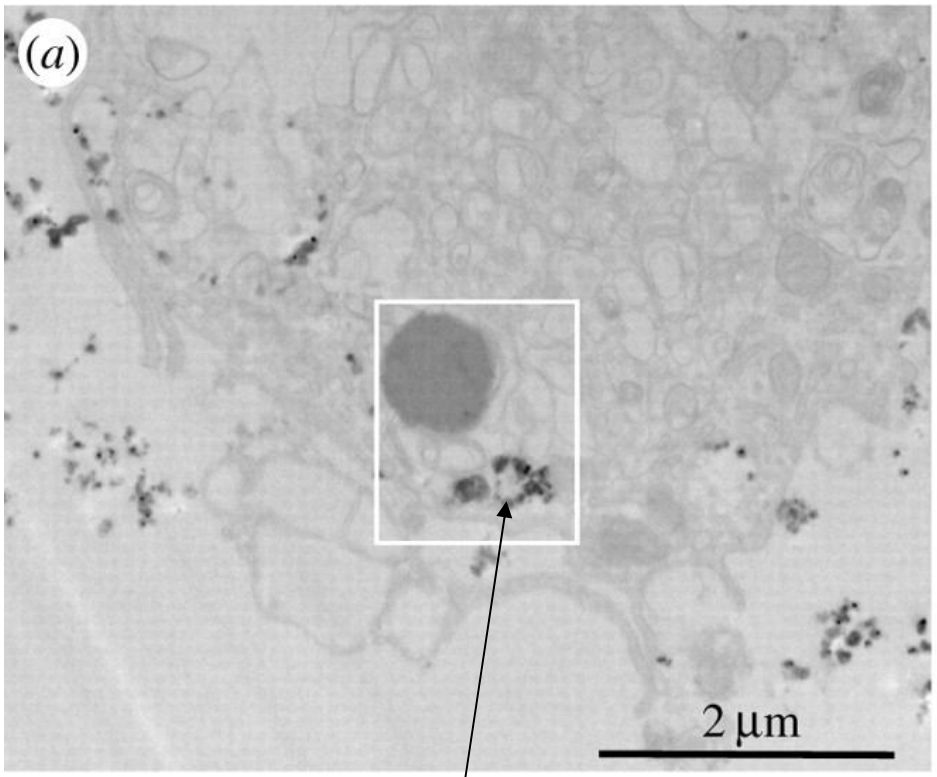


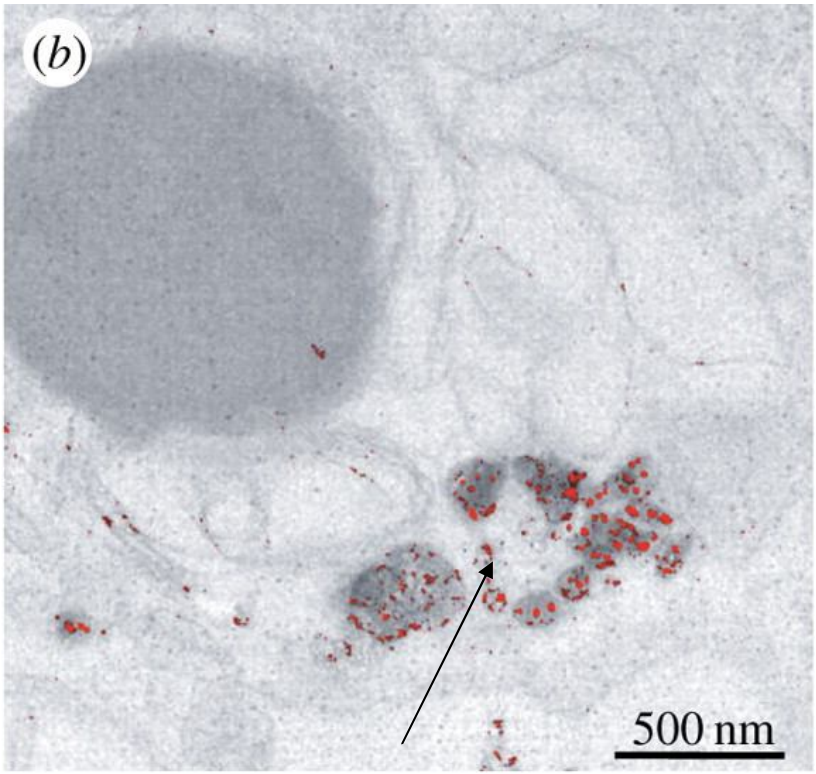


PEG-coat



energy-filtered TEM (EFTEM) analysis of yttrium as a constituent of the CNT pellet catalyst in an A 459 epithelial cell

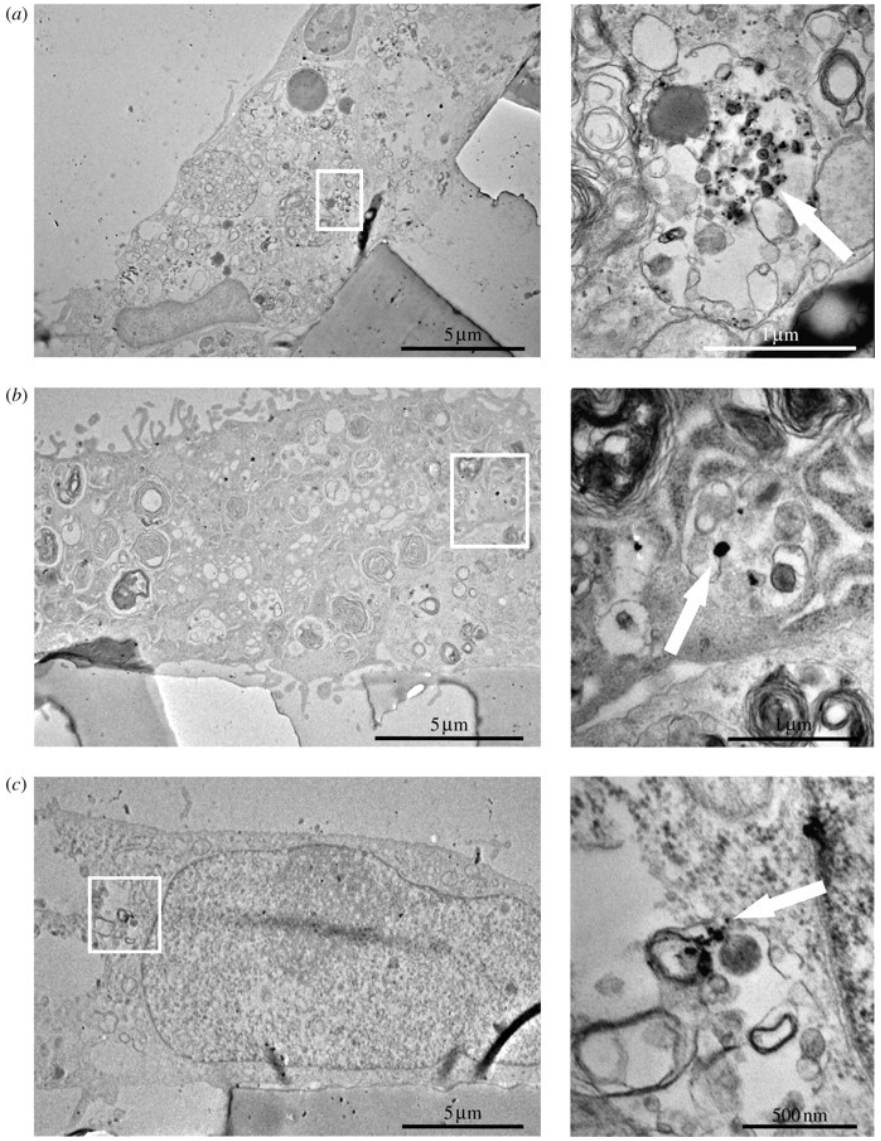




CNTs

Yttrium staining

TEM pictures of intracellular CNT pellet.

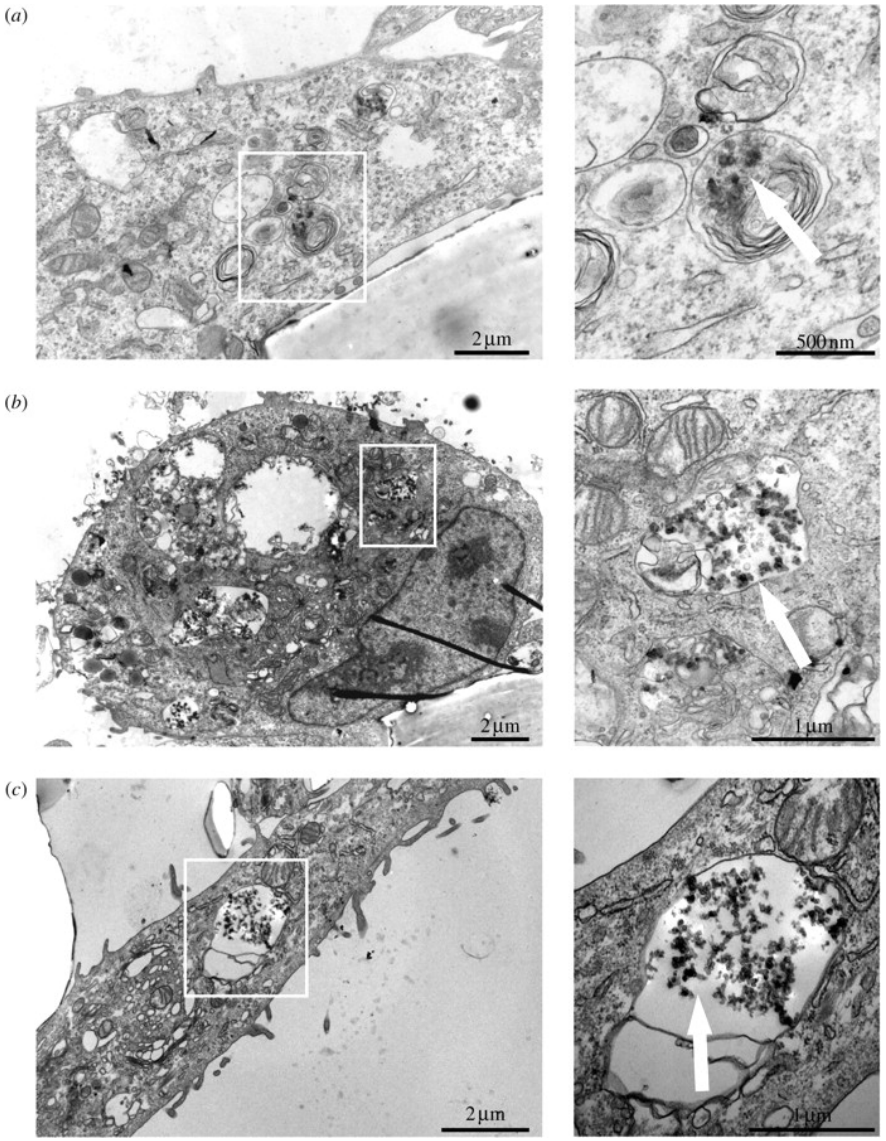


A549 Epithelial cells

macrophages

Dendritic cells

TEM pictures of intracellular DEPs.

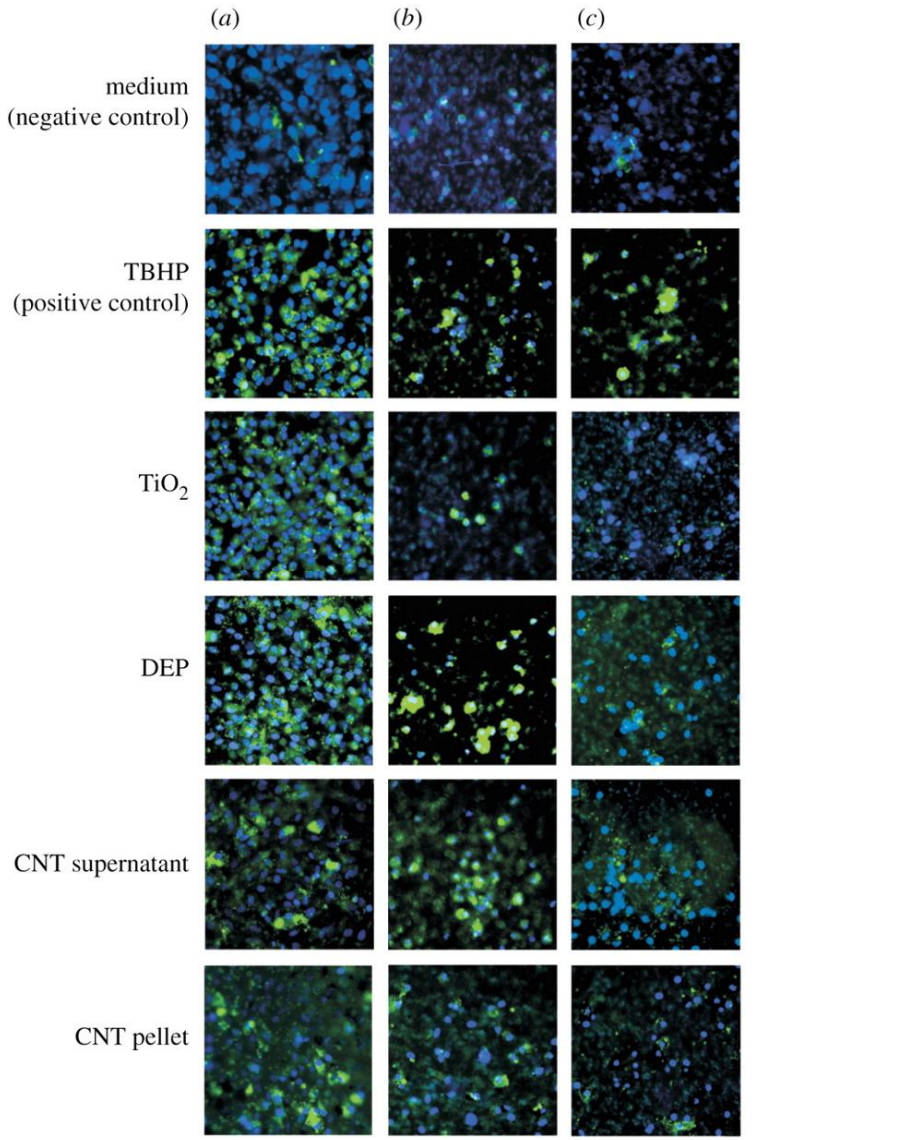


A549 Epithelial cells

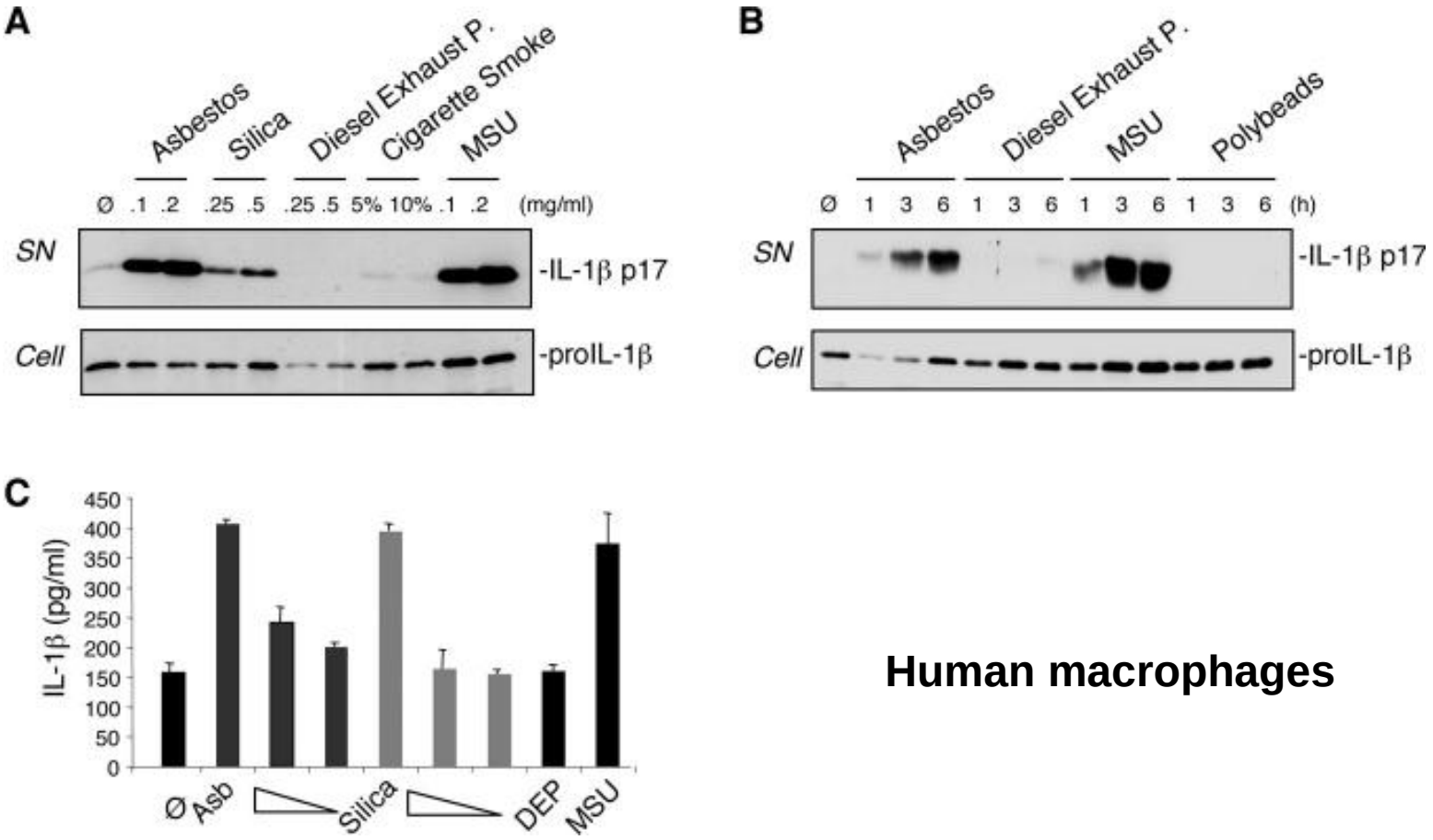
macrophages

Dendritic cells

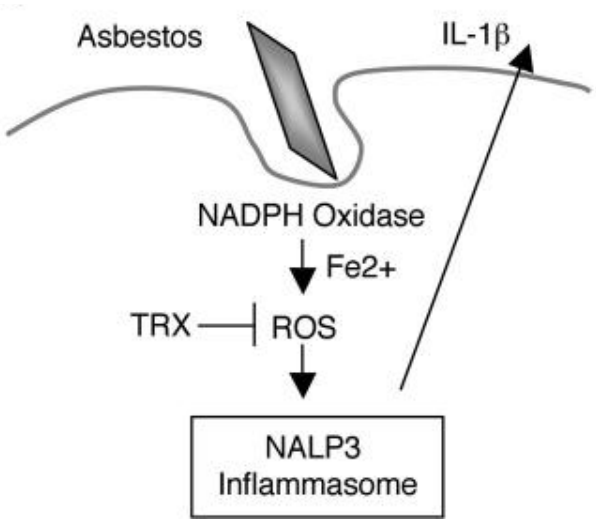
ROS production in cell cultures exposed to different nanosized particles for 24 h.



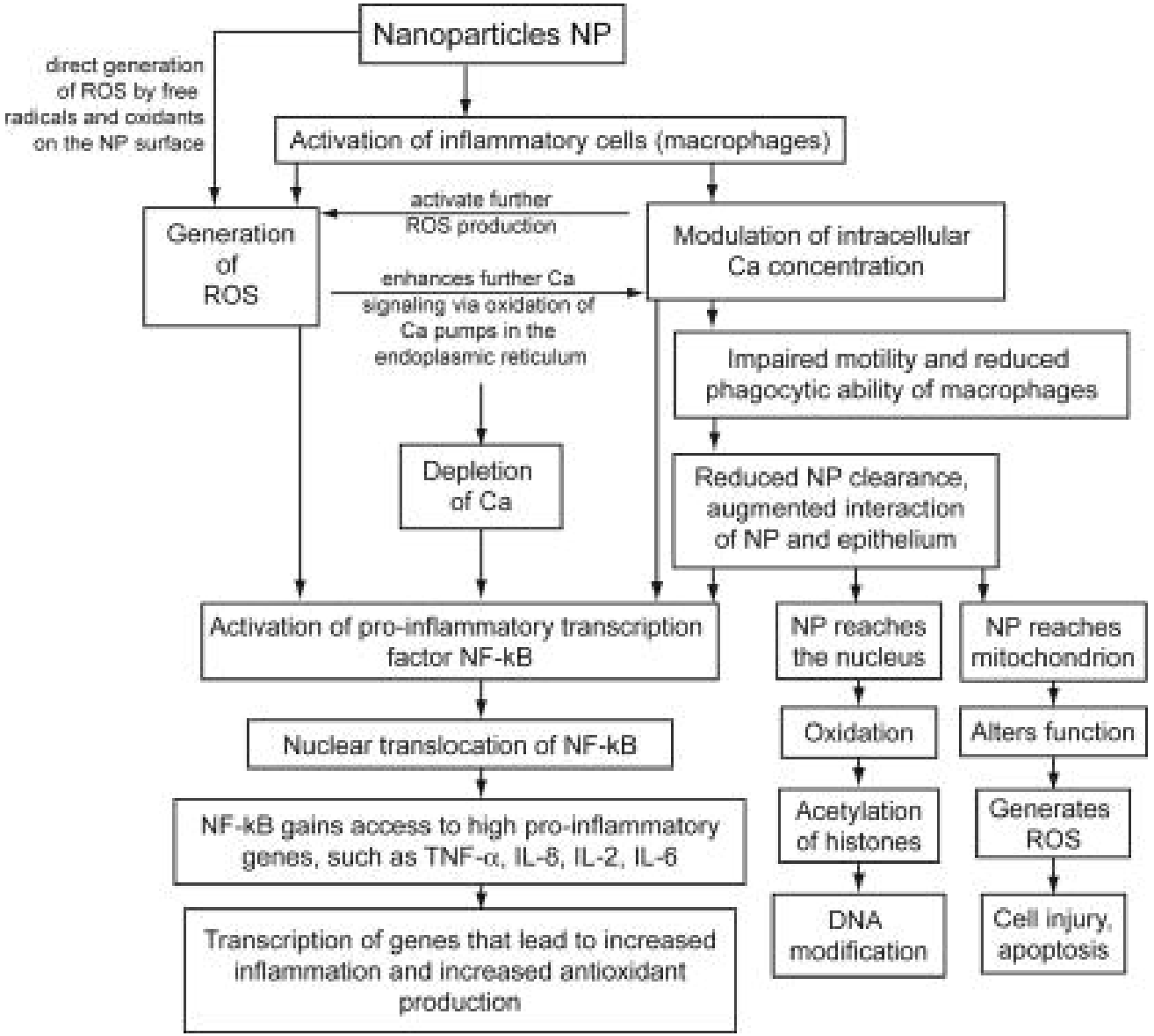
Production of IL-1b induced by different particles: amorphous SiO2 NPs, DEPs, NPs from cigarette smoke, asbestos fibers, monosodium urate crystals (MSU)



Human macrophages



The activation of the NADPH oxidase System is necessary for the activation of The IL-1b production Inflammosome


Nanoparticles NP
direct generation of ROS by free radicals and oxidants on the NP surface
Activation of inflammatory cells (macrophages)
activate further ROS production
Generation of ROS
enhances further Ca signaling via oxidation of Ca pumps in the endoplasmic reticulum
Modulation of intracellular Ca concentration
Impaired motility and reduced phagocytic ability of macrophages
Depletion of Ca
Reduced NP clearance, augmented interaction of NP and epithelium
Activation of pro-inflammatory transcription factor NF-kB
NP reaches the nucleus
NP reaches mitochondrion
Nuclear translocation of NF-kB
Oxidation
Alters function
NF-kB gains access to high pro-inflammatory genes, such as TNF-α, IL-8, IL-2, IL-6
Acetylation of histones
Generates ROS
Transcription of genes that lead to increased inflammation and increased antioxidant production
DNA modification
Cell injury, apoptosis

NPs portal entry

Respiratory tract

GASTROINTESTINAL TRACT

SKIN

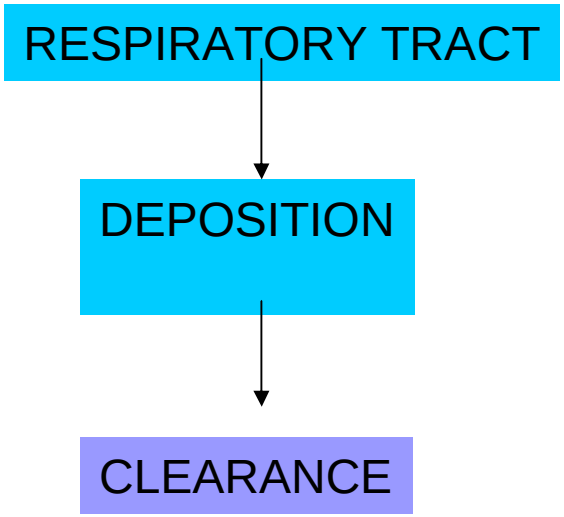
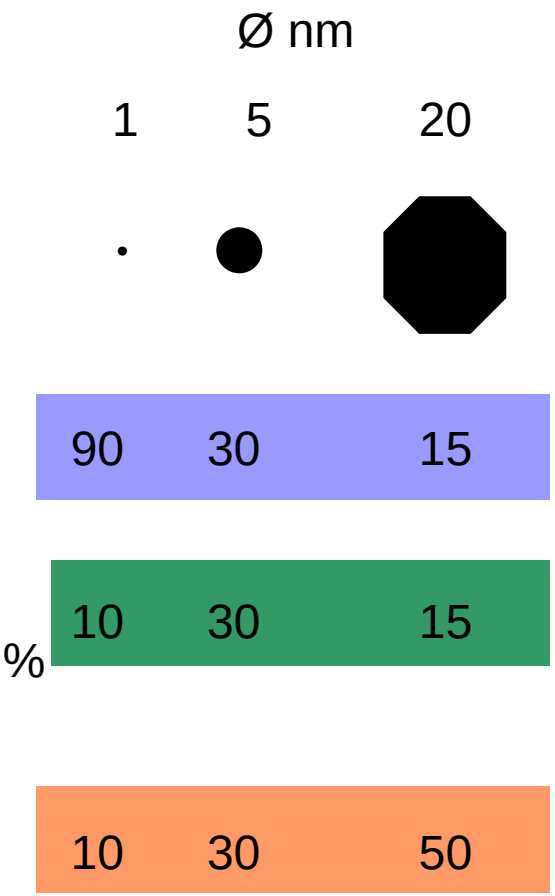
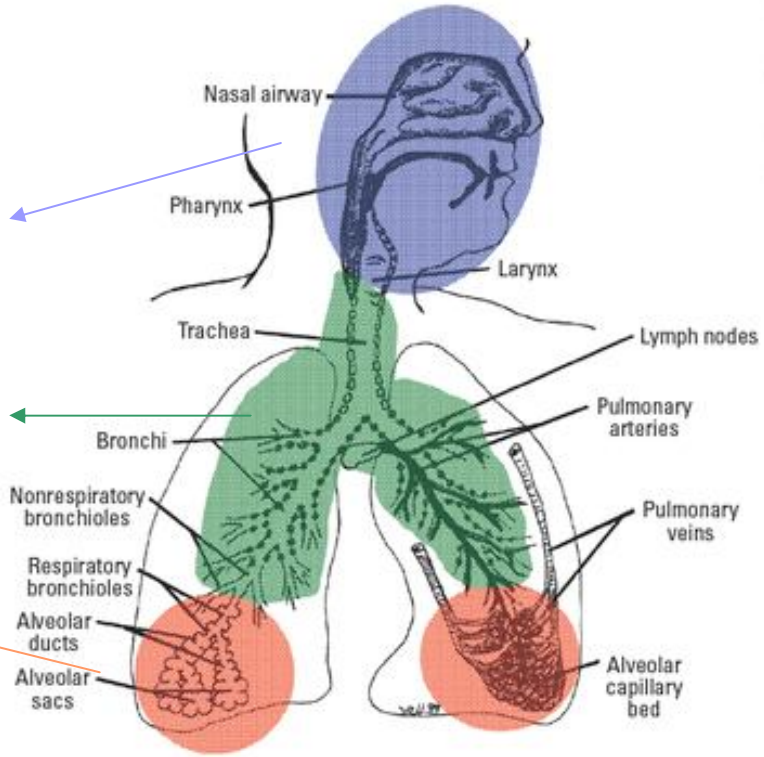


Table 3. Clearance mechanisms for inhaled solid particles in the respiratory tract.

Physical clearance processes (translocation)
Mucociliary movement (nasal, tracheobronchial)
Macrophage phagocytosis (tracheobronchial, alveolar)
Epithelial endocytosis (nasal, tracheobronchial, alveolar)
Interstitial translocation (tracheobronchial, alveolar)
Lymphatic drainage (tracheobronchial)
Blood circulation (tracheobronchial, alveolar)
Sensory neurons (nasal, tracheobronchial)
Chemical clearance processes[a]
Dissolution
Leaching
Protein binding

[a]Nasal, tracheobronchial, and alveolar regions.





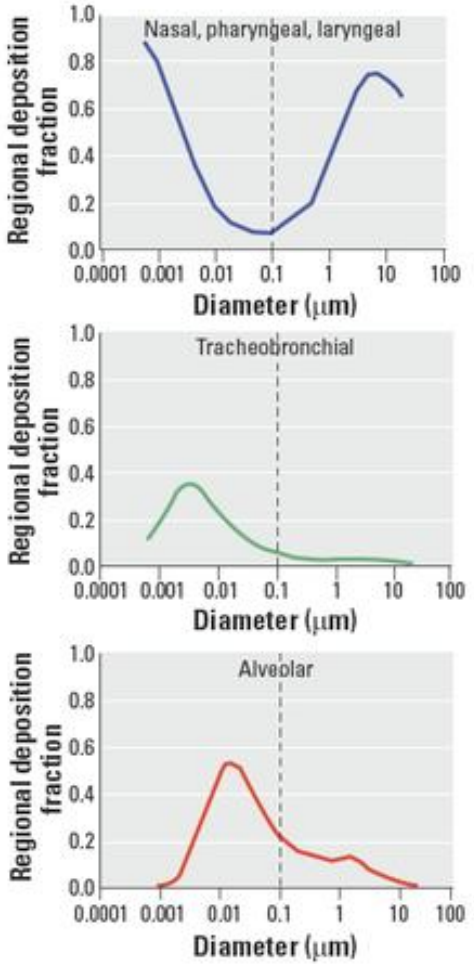
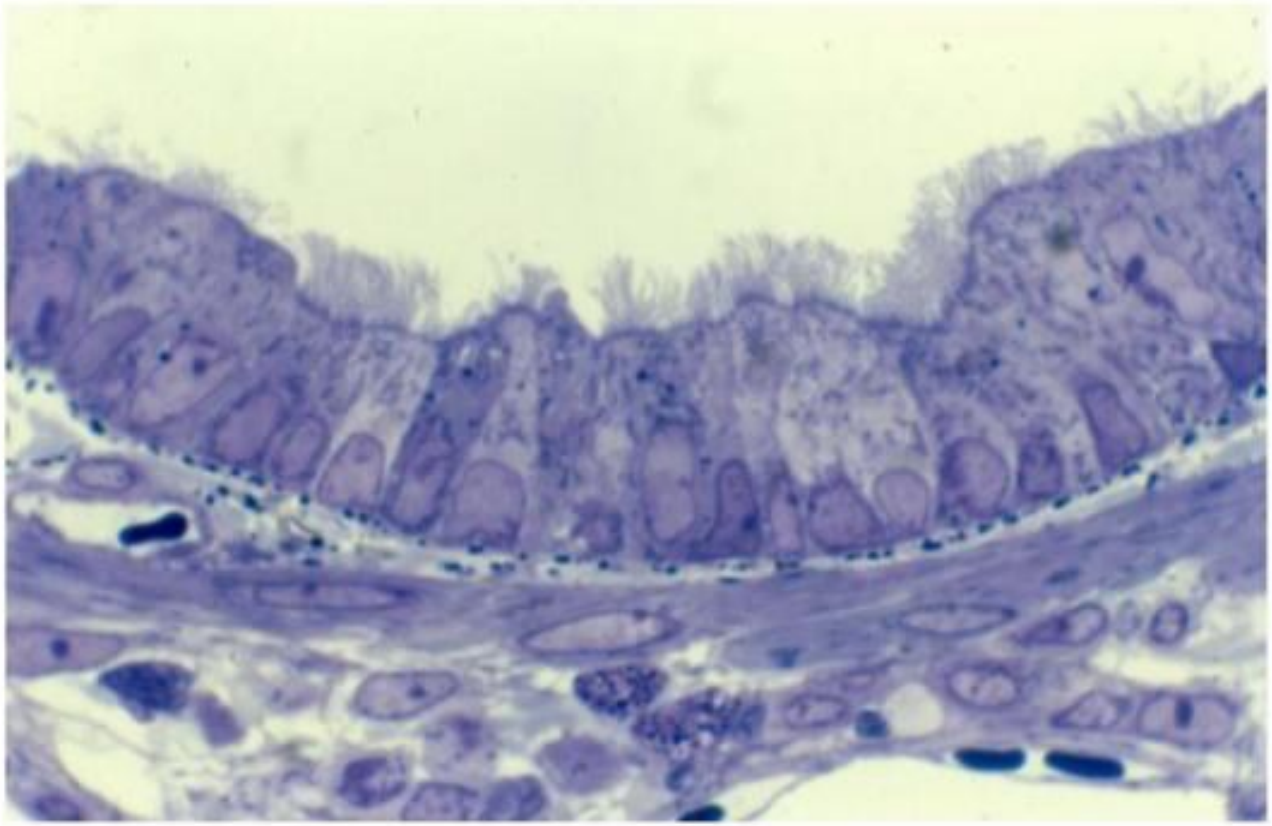


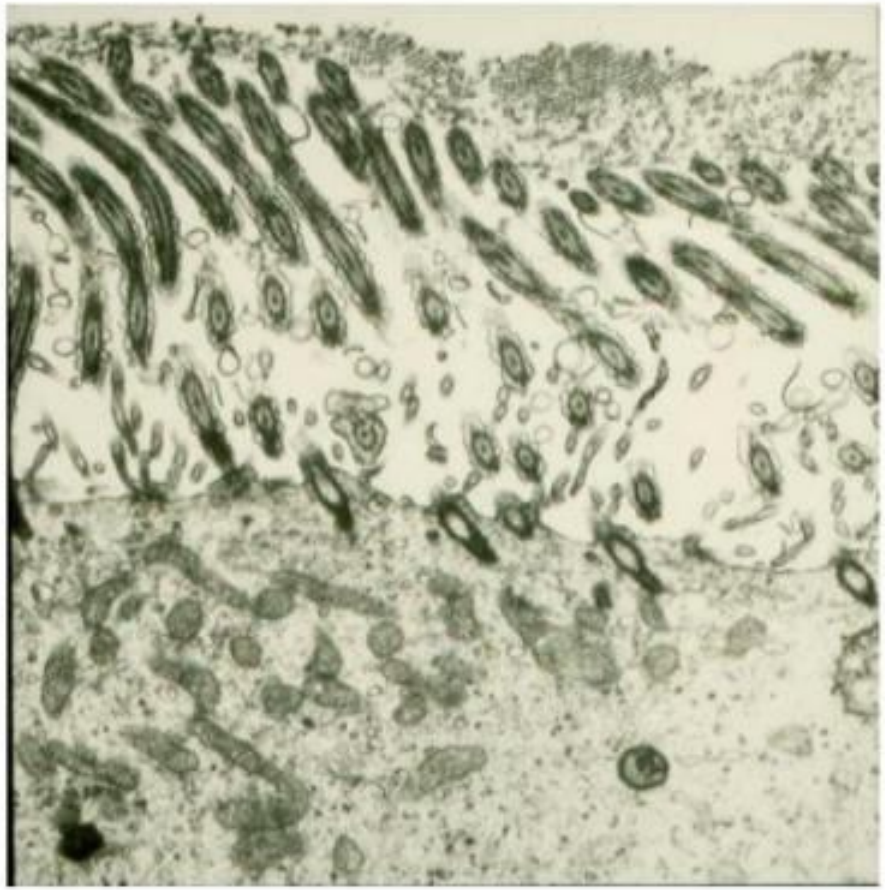
Figure 8. Predicted fractional deposition of inhaled particles in the nasopharyngeal, tracheobronchial, and alveolar region of the human respiratory tract during nose breathing. Based on data from the International Commission on Radiological Protection (1994). Drawing courtesy of J. Harkema.

- The lung internal surface area: 75-140 m^2
- Number of alveoli: ~ 300 x 10^6
- thickness of the alveolar lining fluid: ~ 200 nm
- amount of alveolar lining fluid can be ~20 ml

Normal Respiratory Epithelium



Mucociliary Escalator



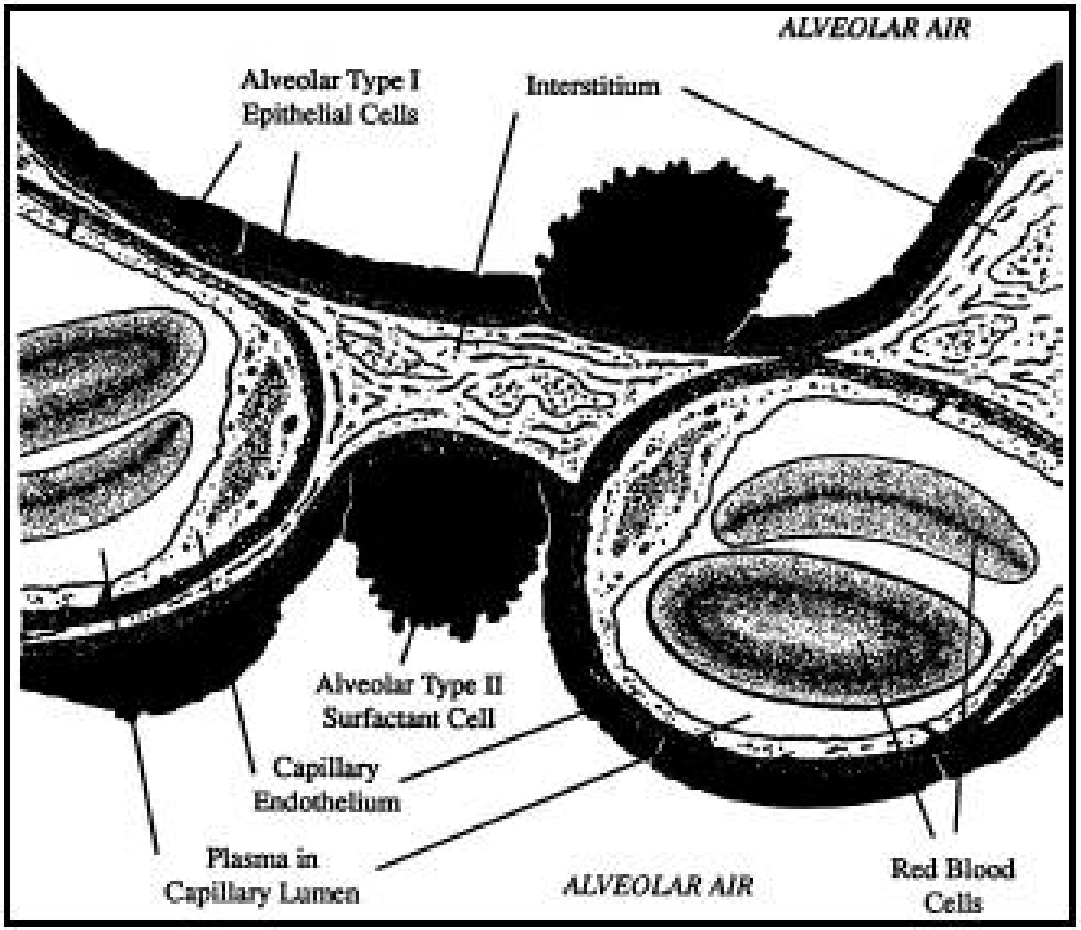
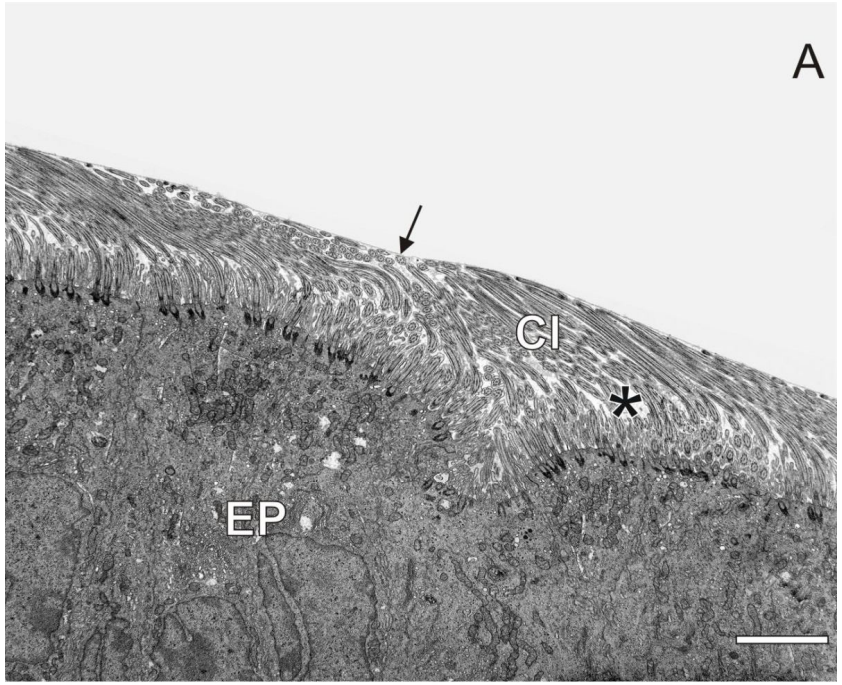
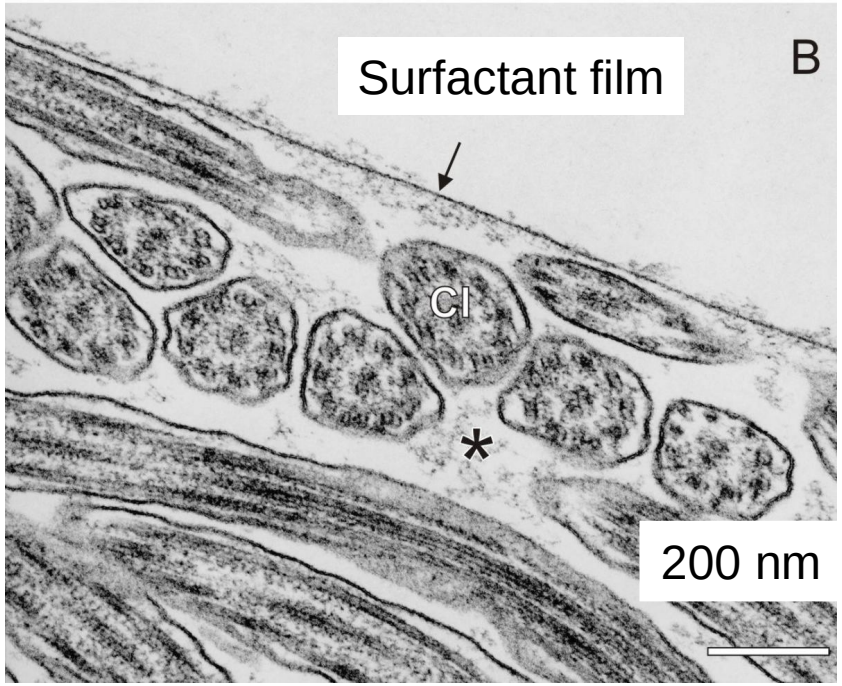
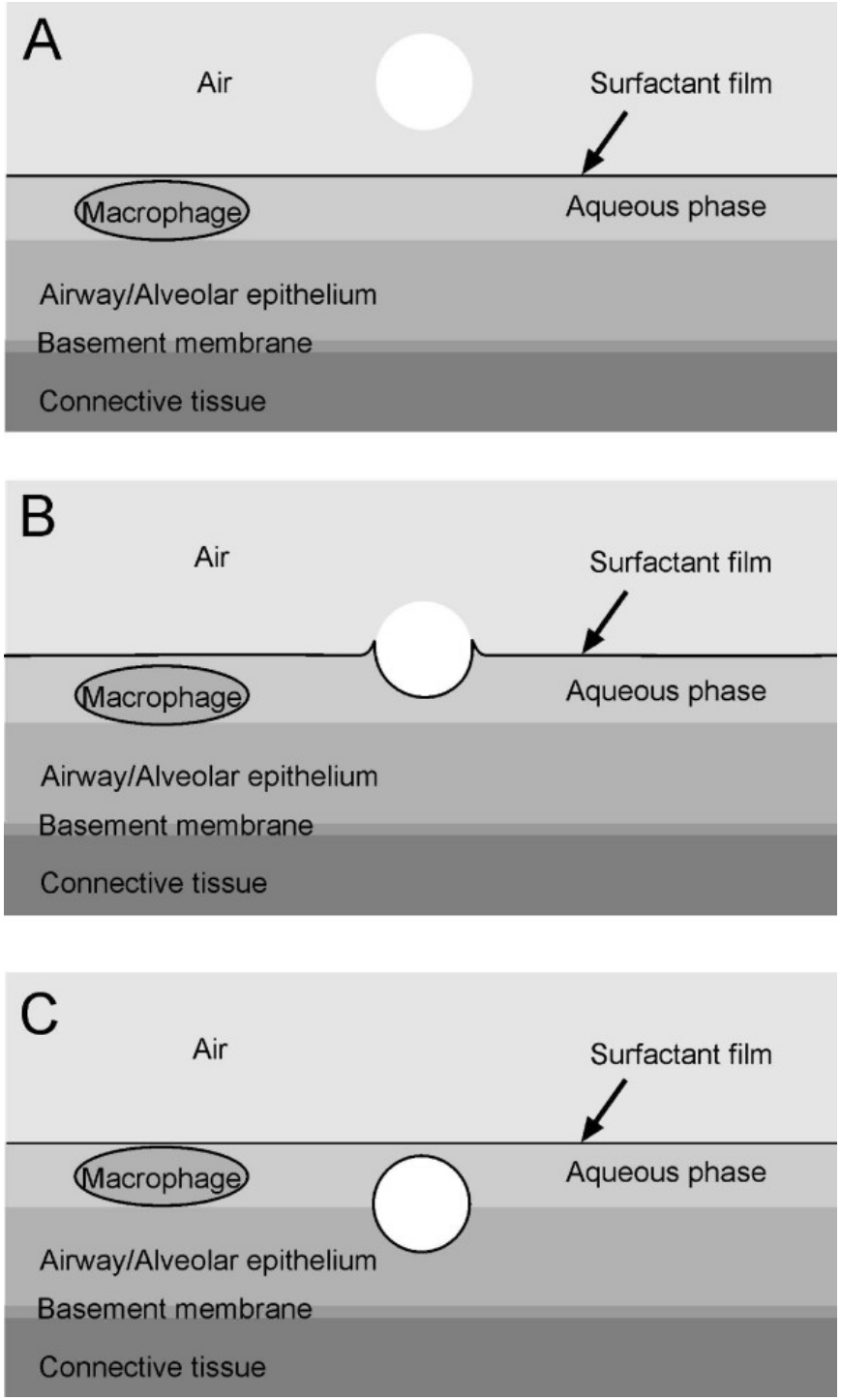


Fig. 8.15. Expanded view of the alveolar wall (redrawn from Vander, Sherman, and Luciano[866]).

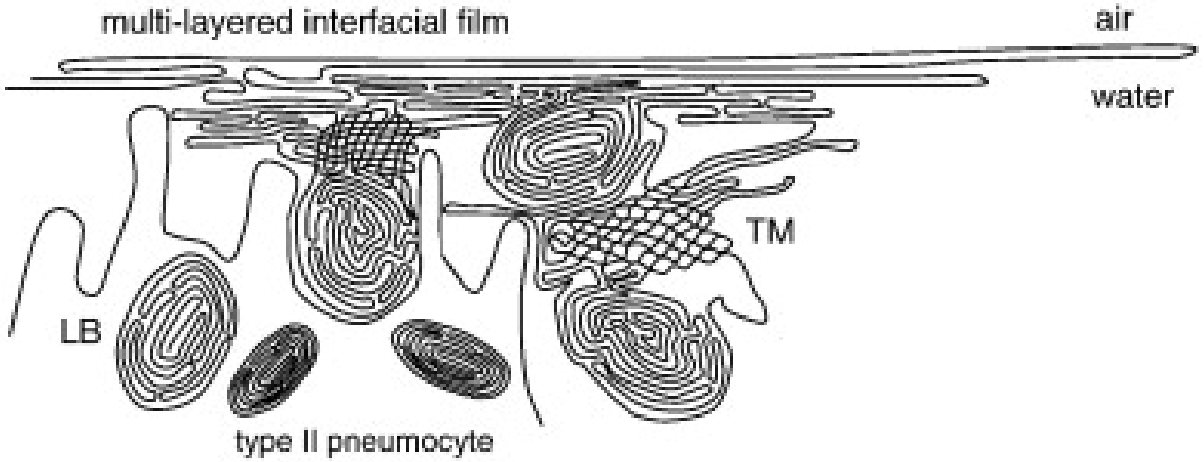
Micrographs of horse trachea

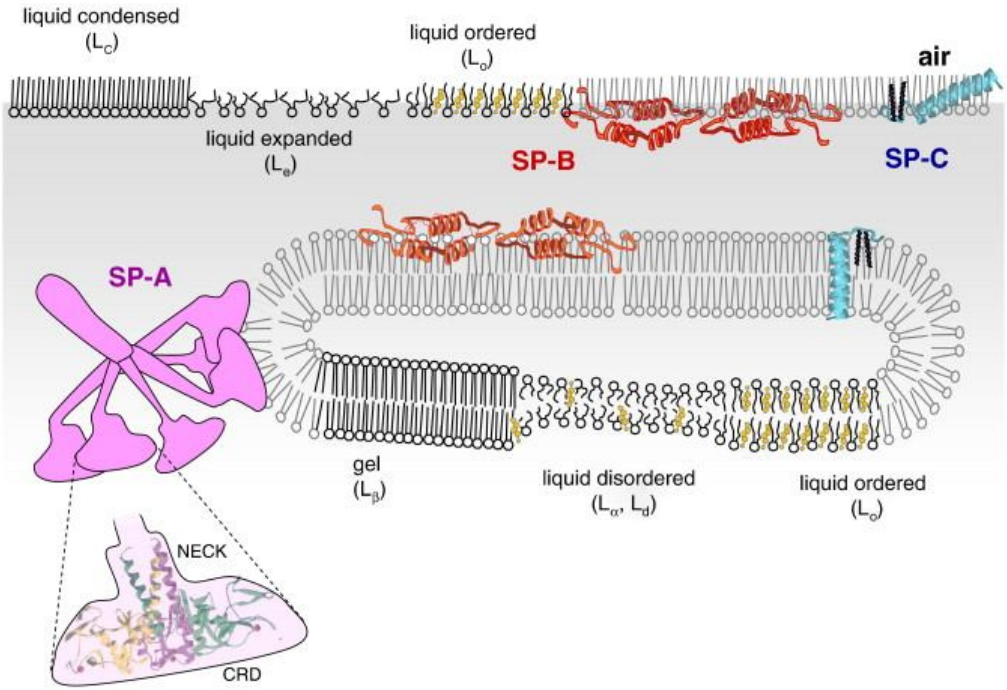




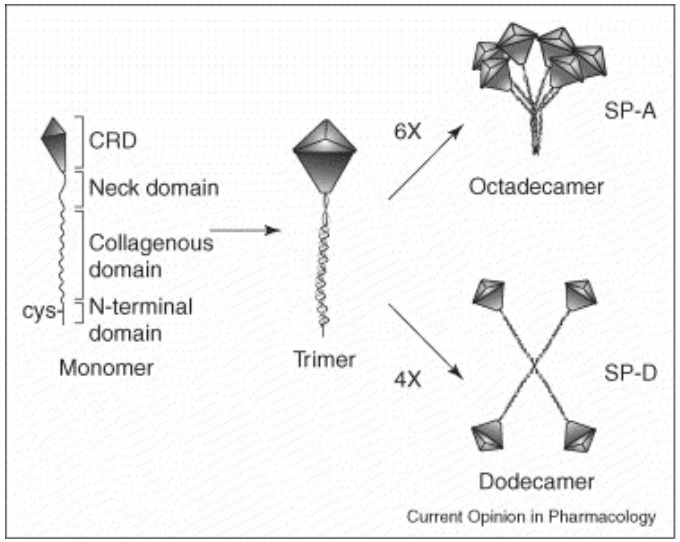

A
Air
Surfactant film
Macrophage
Aqueous phase
Airway/Alveolar epithelium
Basement membrane
Connective tissue
B
Air
Surfactant film
Macrophage
Aqueous phase
Airway/Alveolar epithelium
Basement membrane
Connective tissue
C
Air
Surfactant film
Macrophage
Aqueous phase
Airway/Alveolar epithelium
Basement membrane
Connective tissue

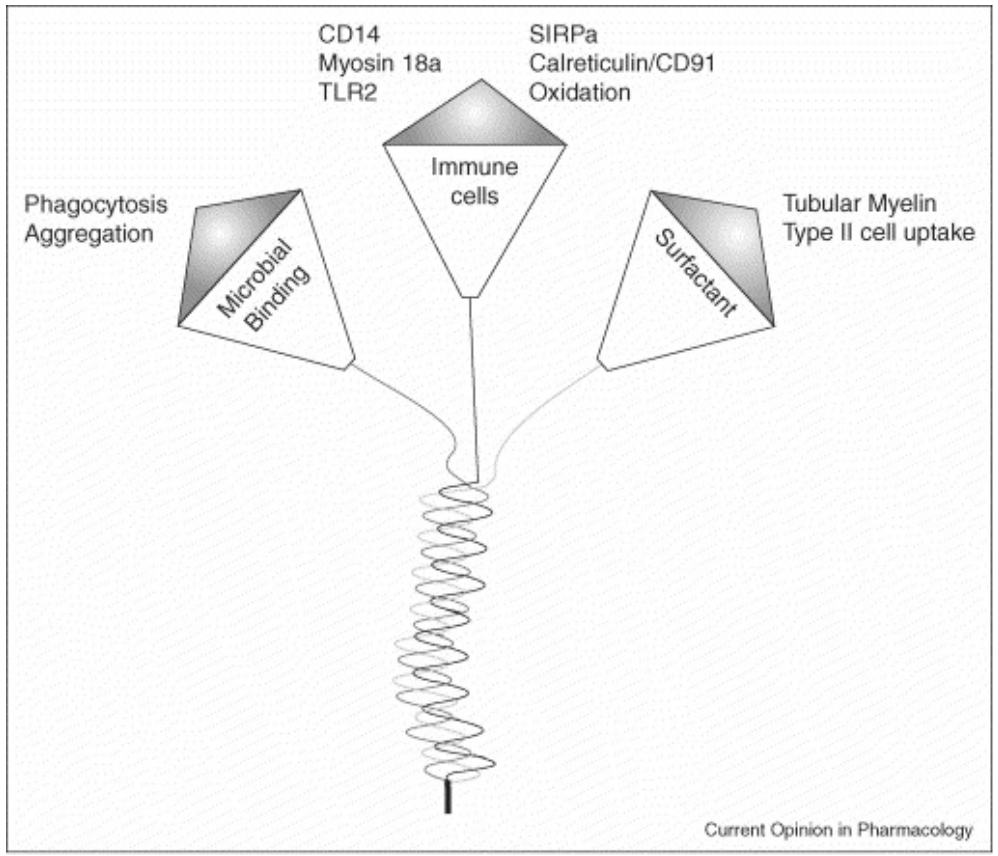
Lung surfactants



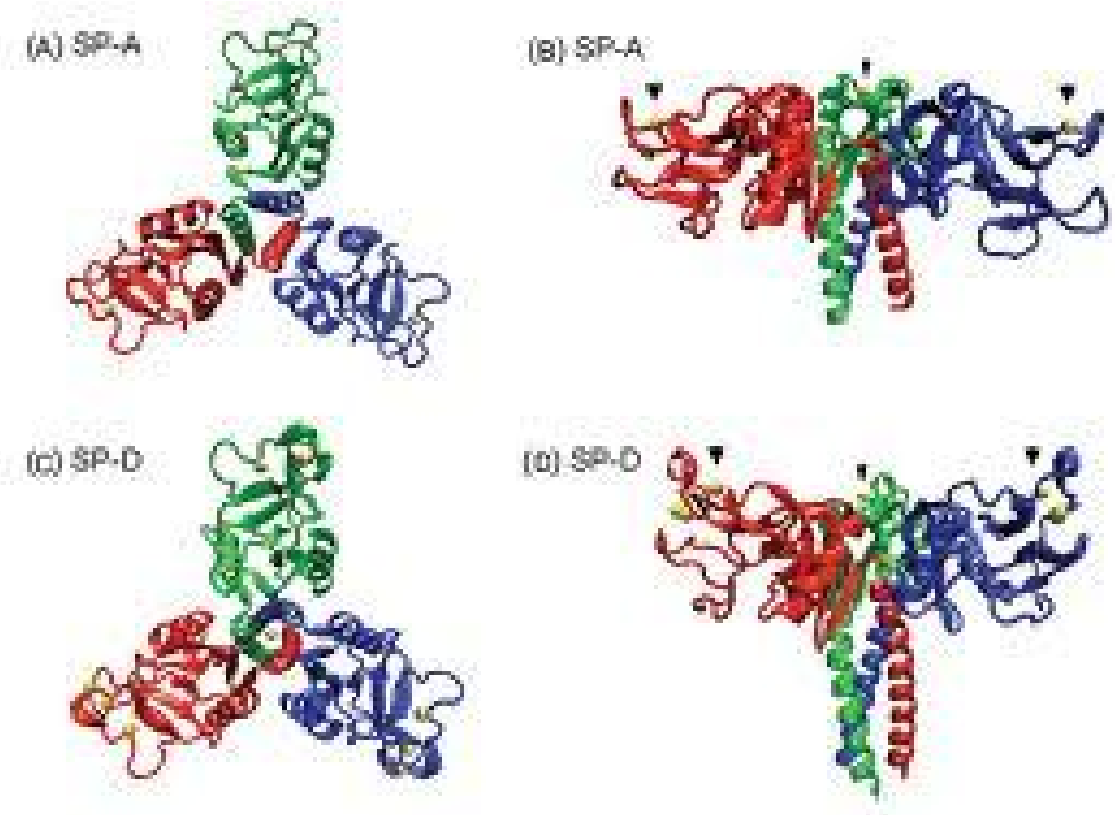


Surfactant Protein -A and -B are also named *collectins* : they bind microrganisms and mediate their phagocytosis by AM and PMNs

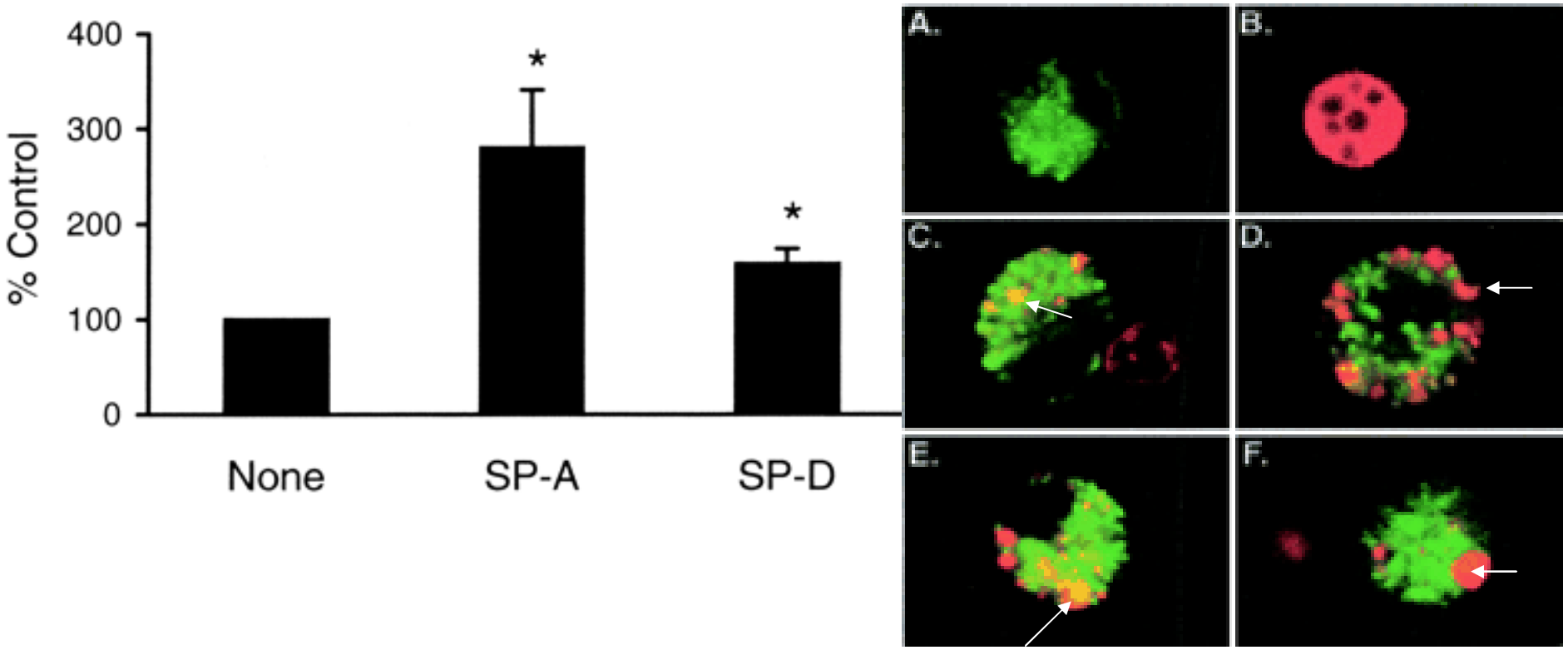




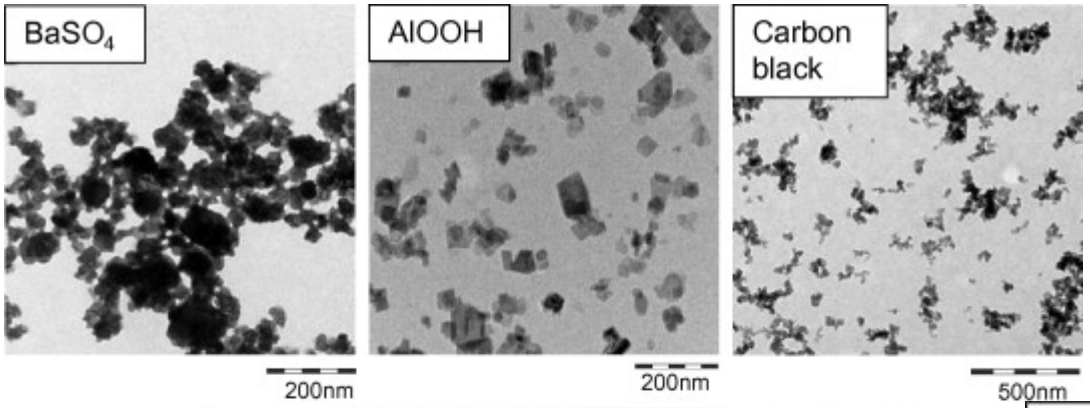
Structure of SP-A and SP-B bindi Carbohydrate Binding Domains (CDR)

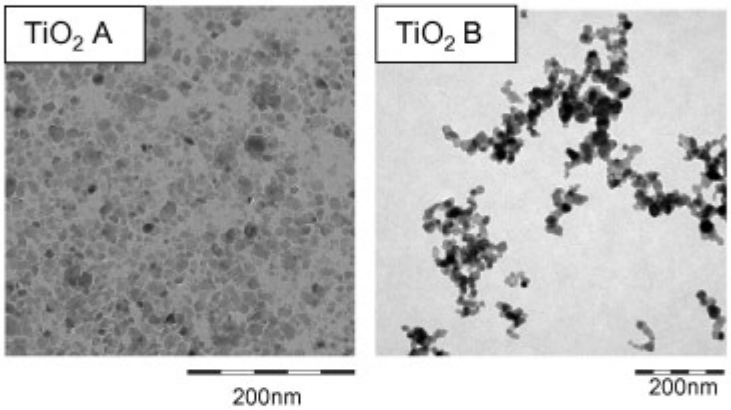


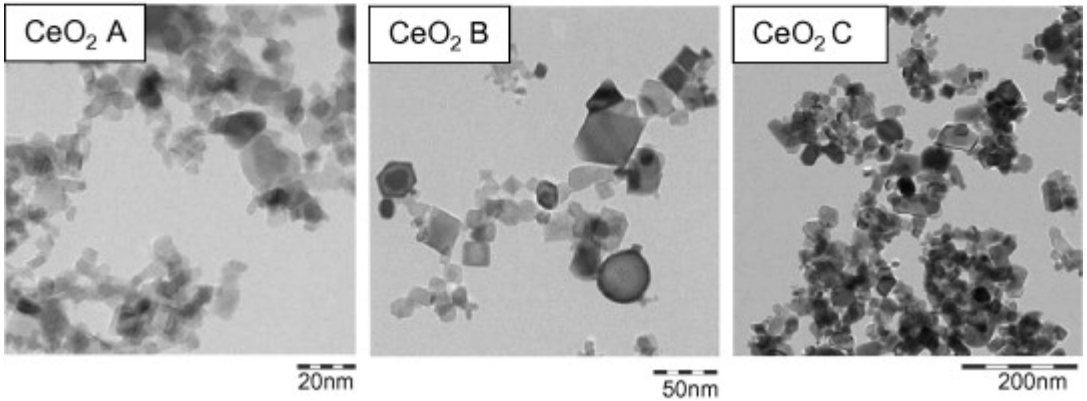
SP-A and SP-D favour AM phagocytosis of apoptotic PMNs

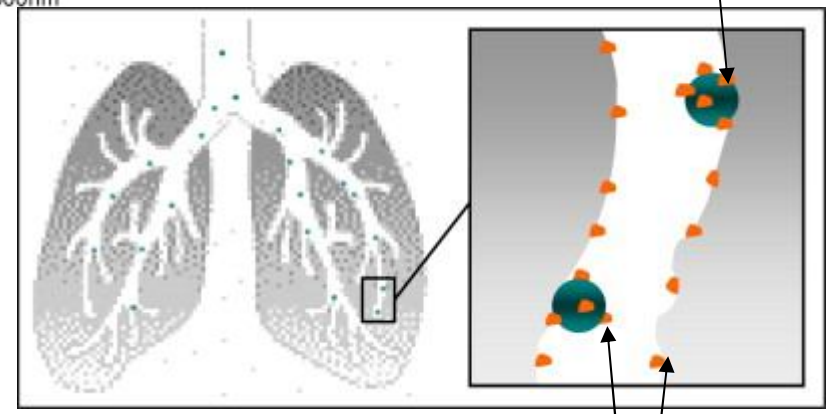


Do NPs bind to the surfactant proteins?





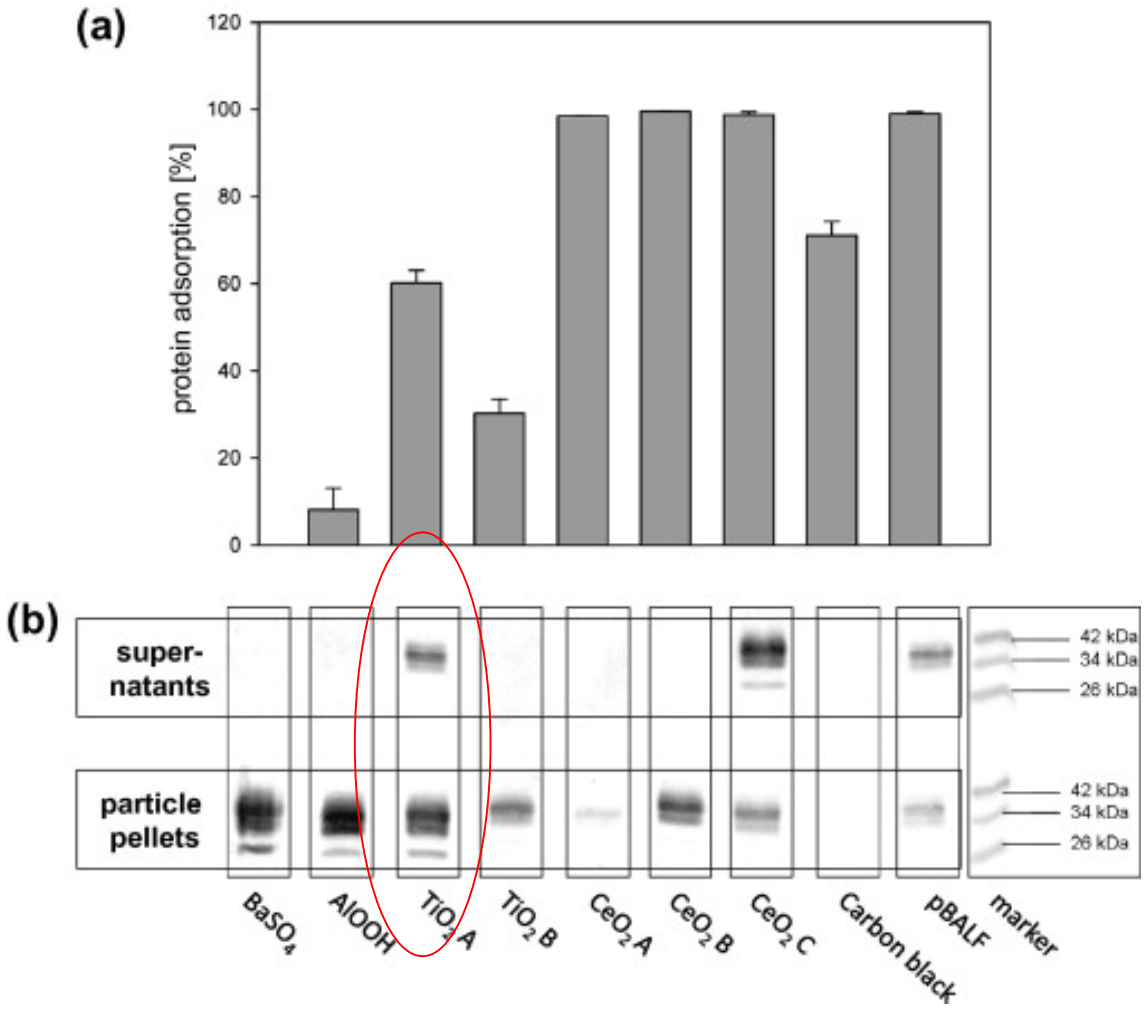




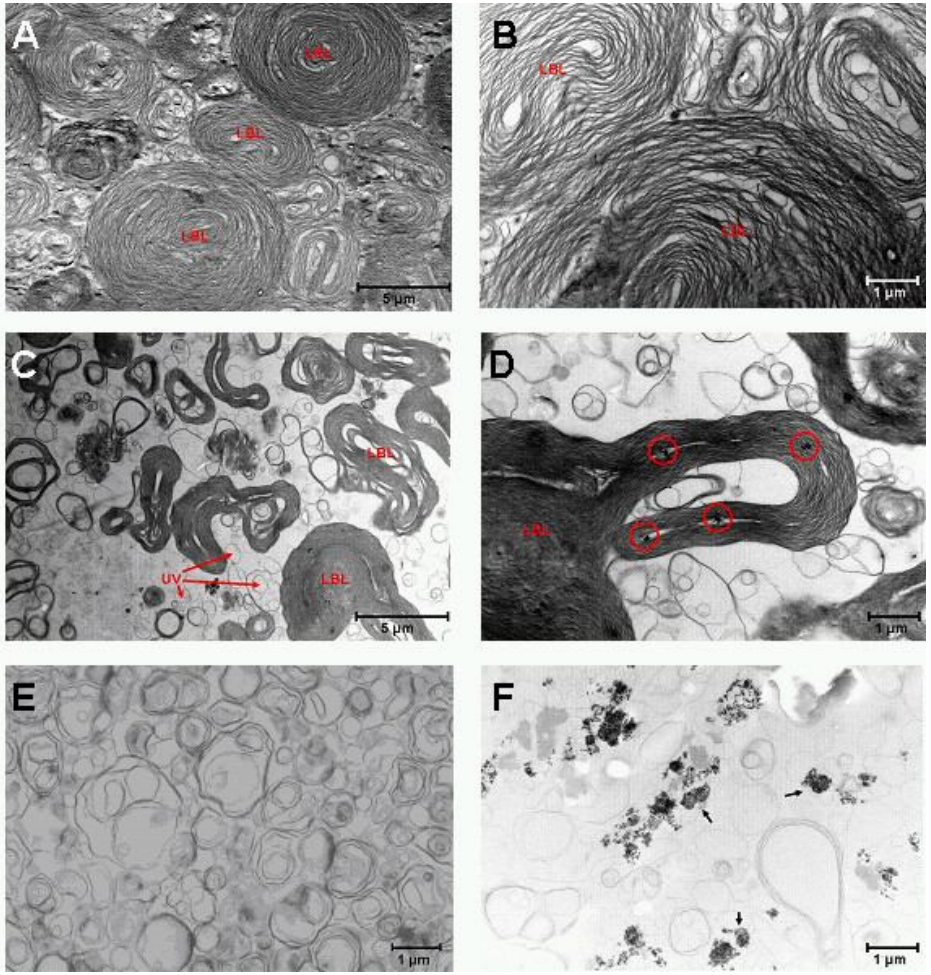
NPs

surfactant

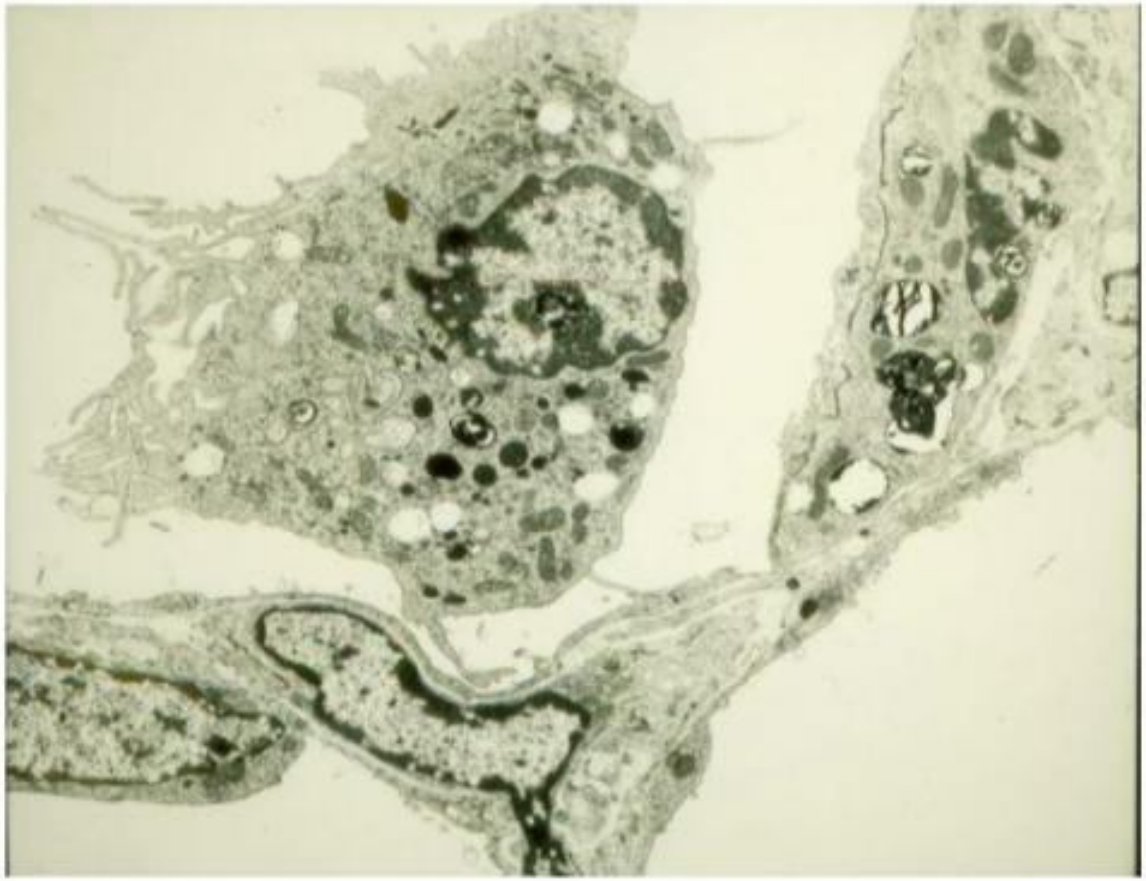
Surfactant Protein A (SP-A) binding to different NPs



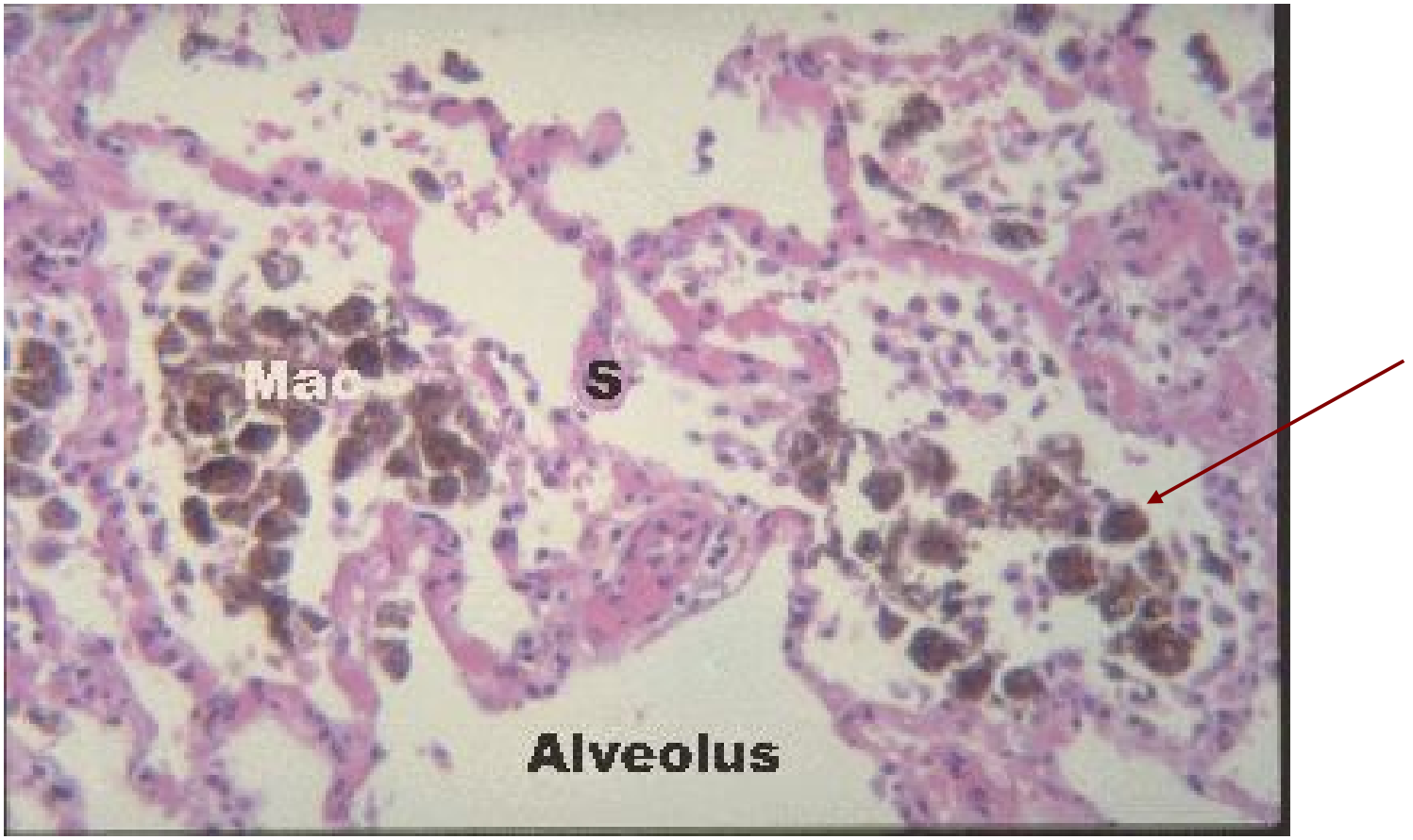
TiO_2 NPs disrupt the structure of surfactant film



Alveolar Macrophage **(AM)**



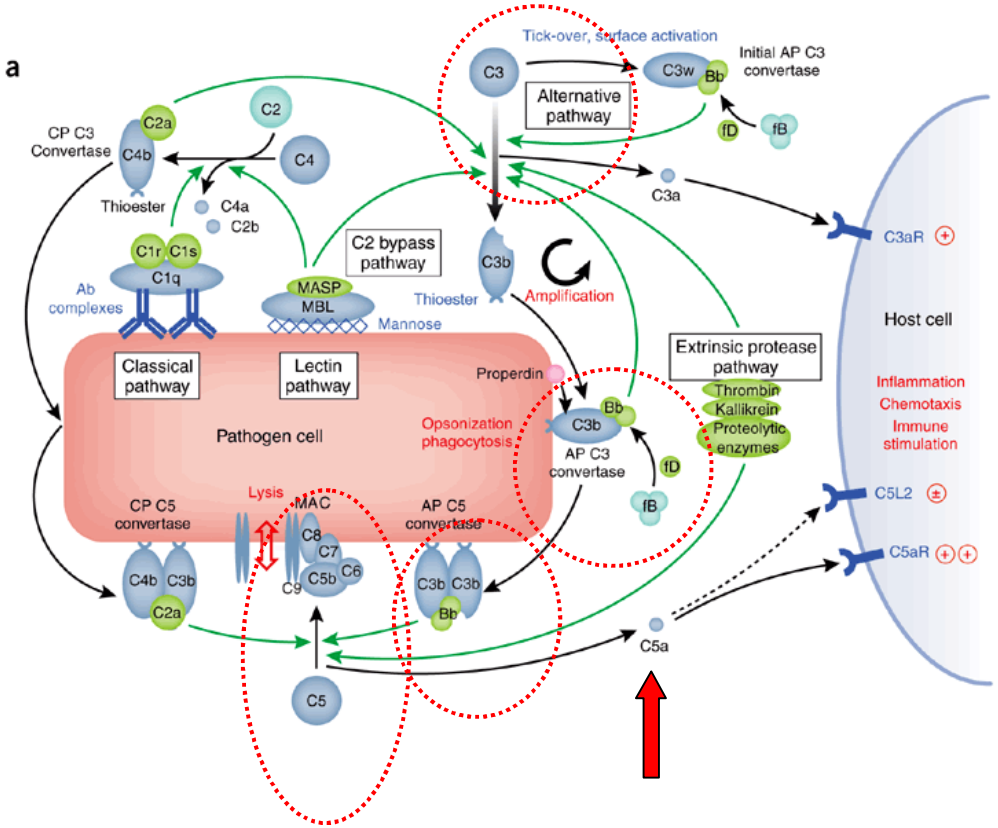
Migration of AM into the alveolar space



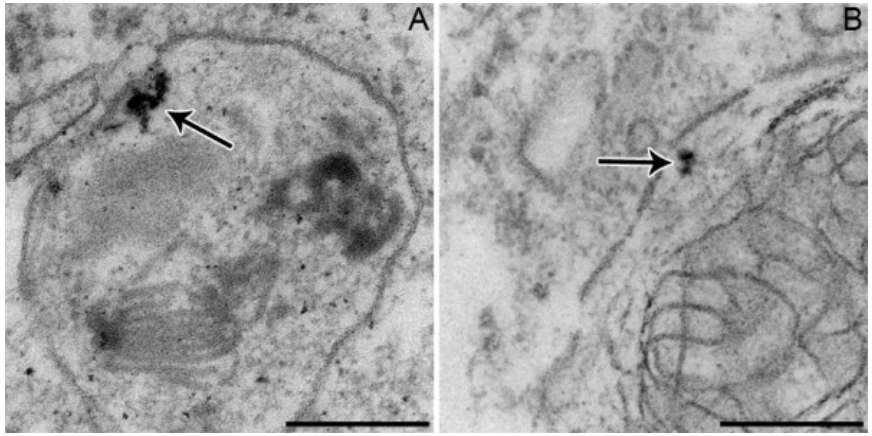
Alveolar macrophages in action

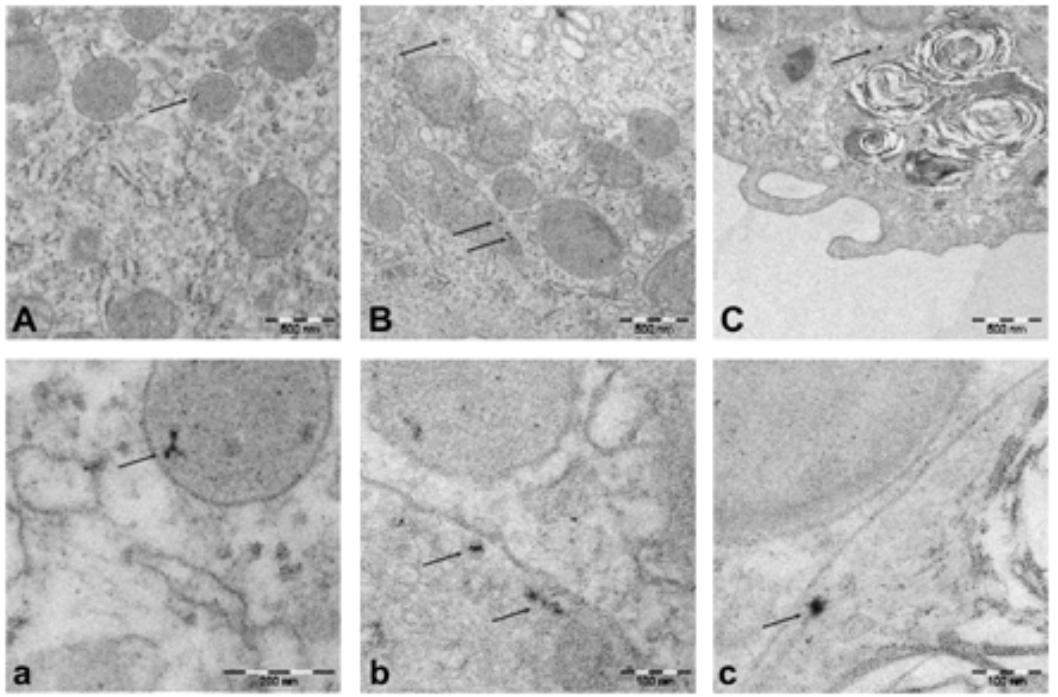
- *Are attracted by chemotactic factor like C5a of the complement cascade*
- *After NPs (and MPs) phagocytosis AM slowly move the mucociliar escalator*
- *Capture of NPs (MPs) by macrophages is completed within 6-12 hours from deposition*
- *The retention half life is 700 days in the humans (70 days in the rats!)*

Complement activation



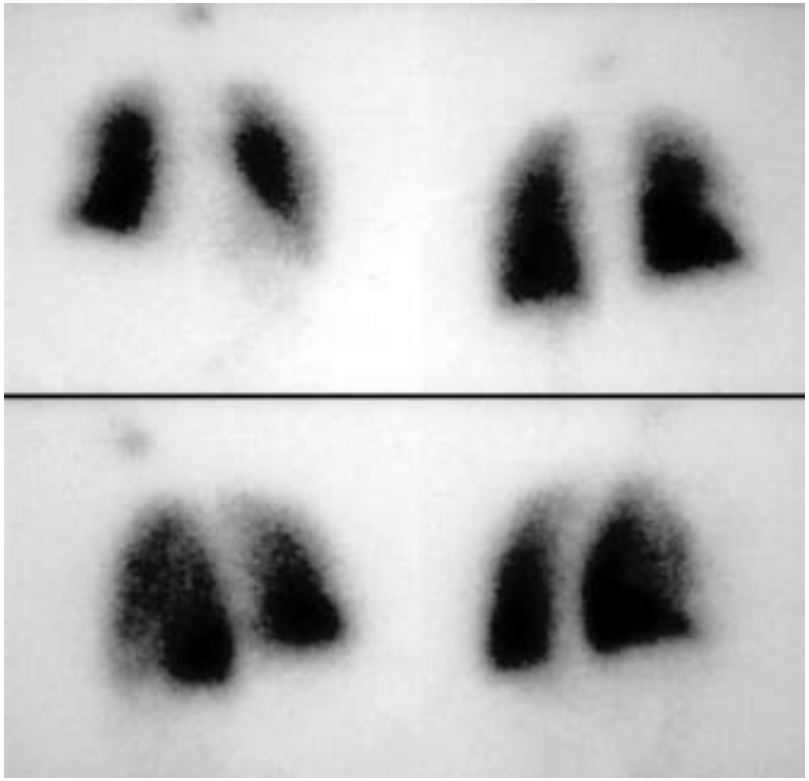
TiO_2 NPs in the phagolysosome of an alveolar macrophage



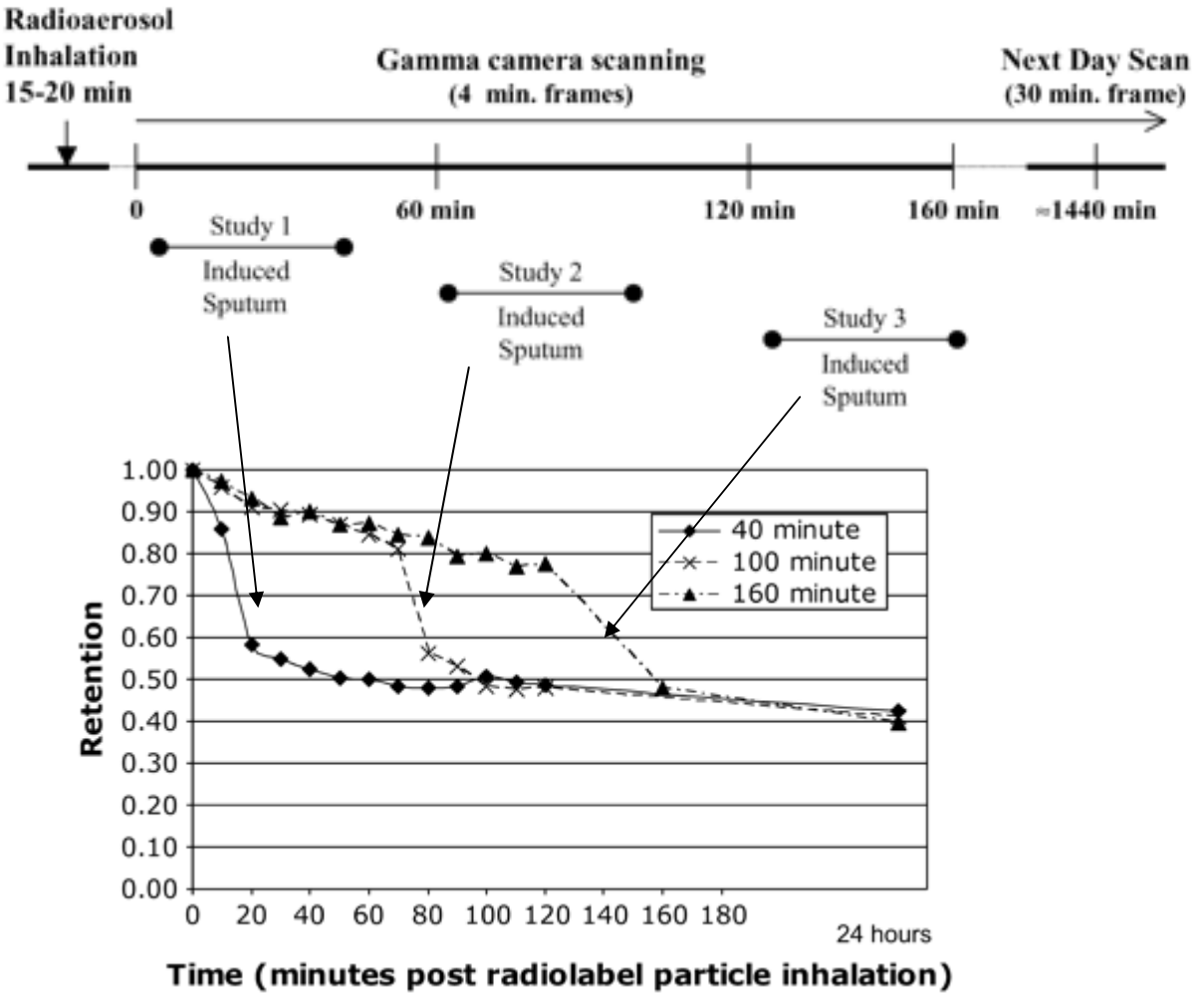


Scintigraphy Lungs inhaled with Tc99 labeled colloidal sulfur

Colloidal sulfur

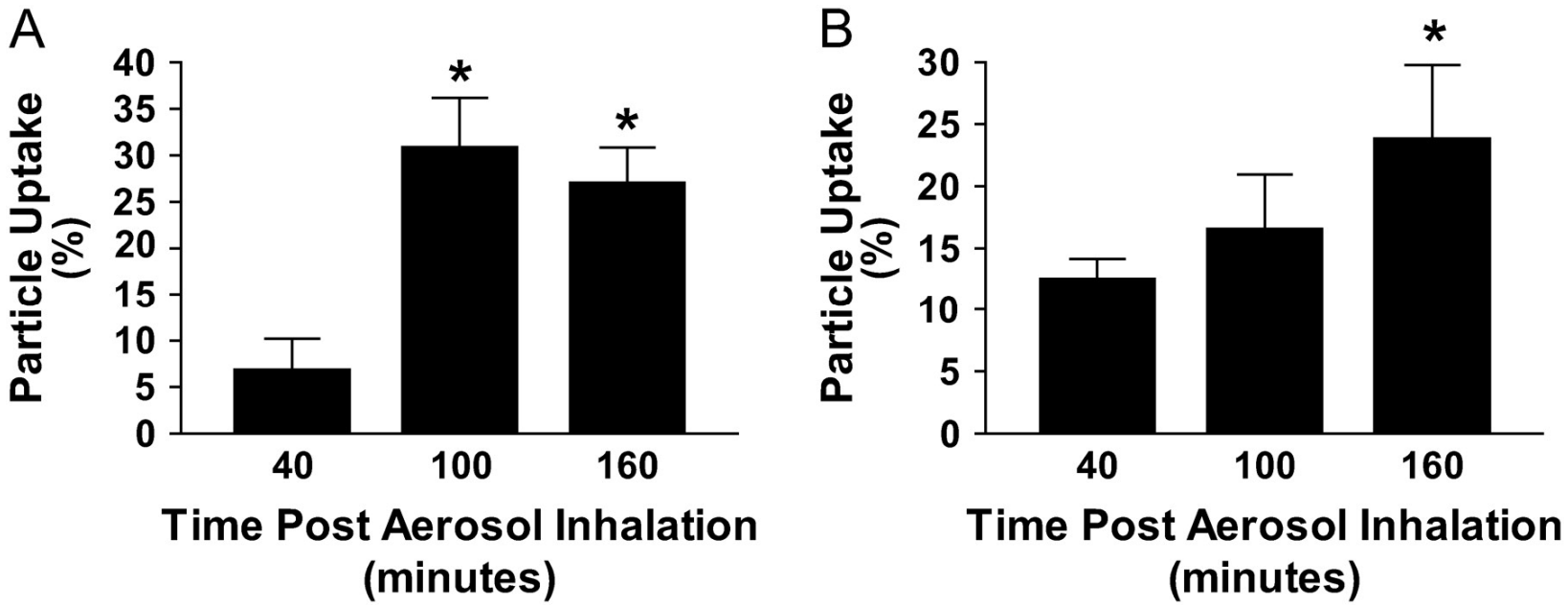


Mucociliary clearance of Radiolabeled (^{99}Tc m) sulfur colloid 220 nm Ø in human volunteers

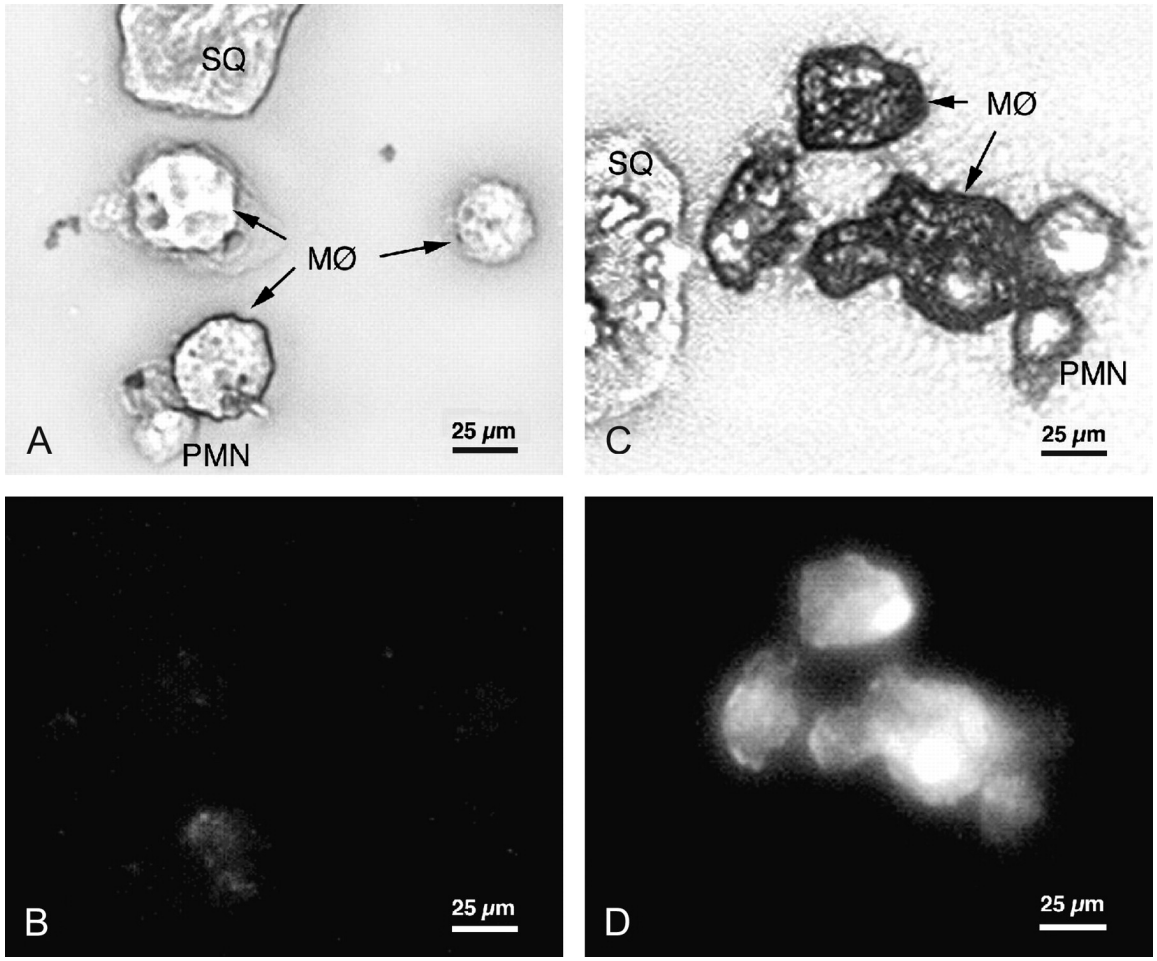


% of sulfur NPs in AM from sputum

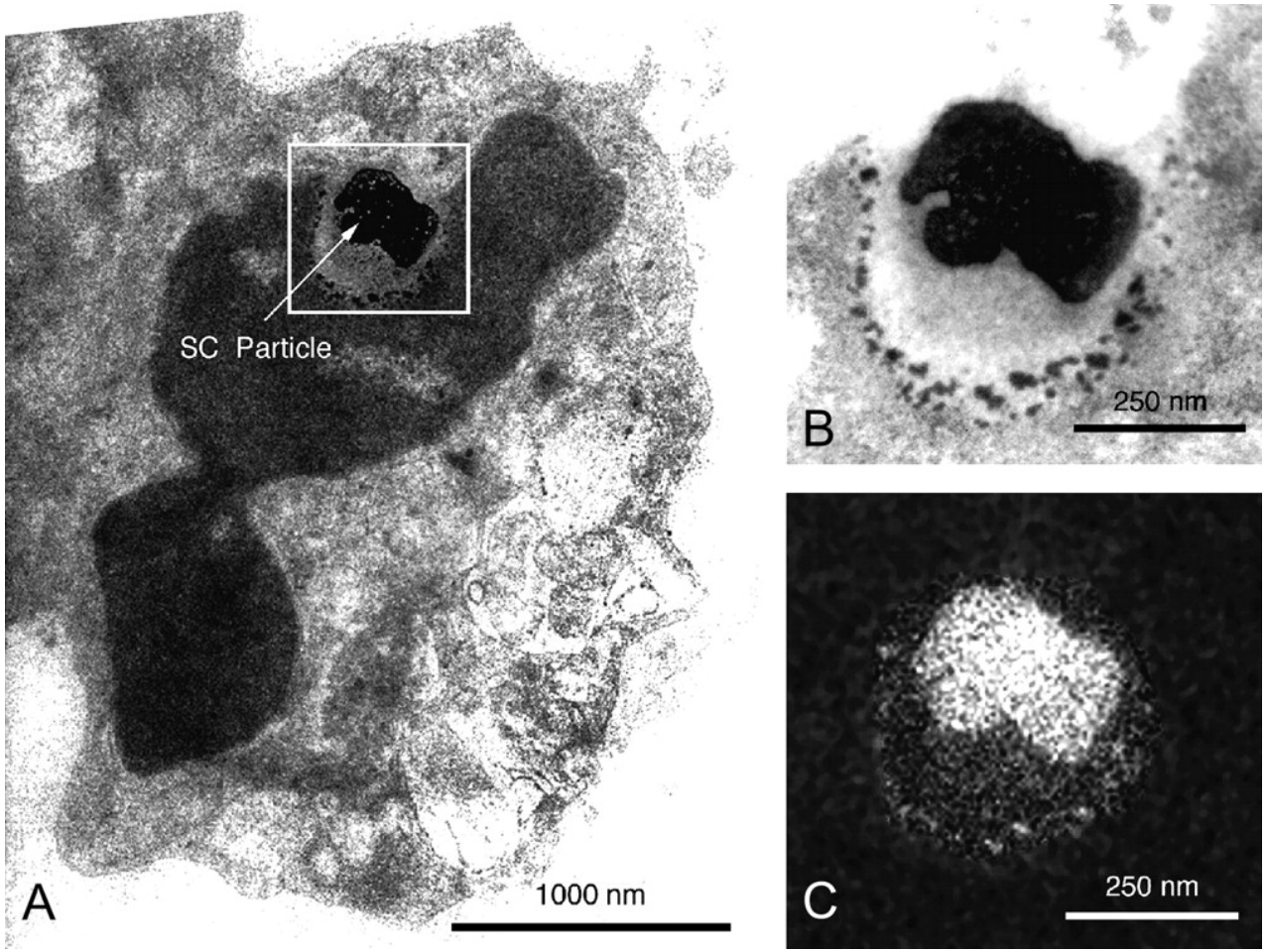
- In vivo in vitro



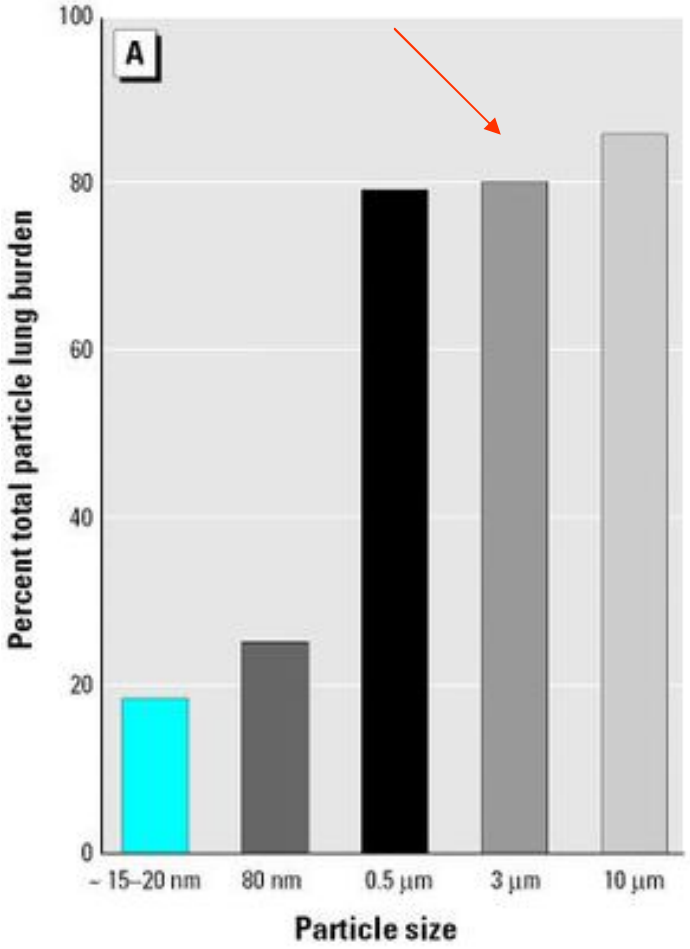
Fluorescent Sulfur NPs in AM and in PMNs



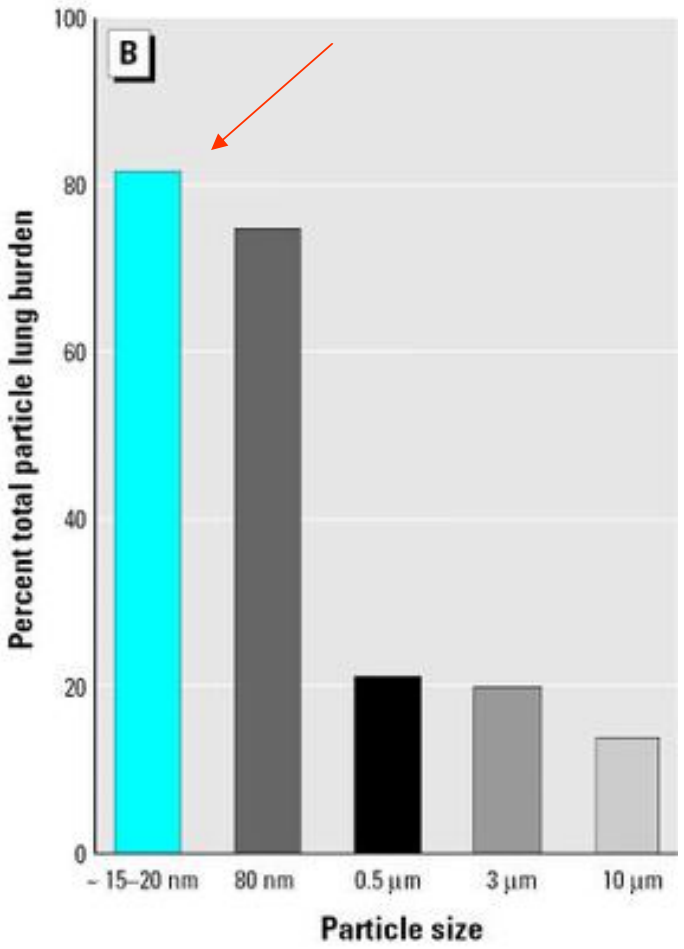
Sputum AM containing inhalated sulfur NPs



Distribution of inhaled particulate of different size



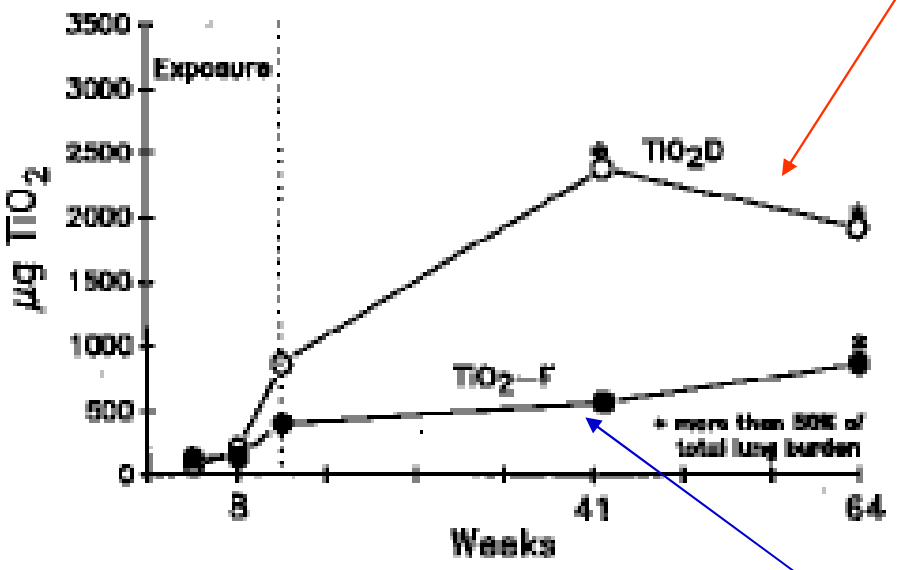
Phagocytosed by AM



Present in epithelial cells and free in the intertitium

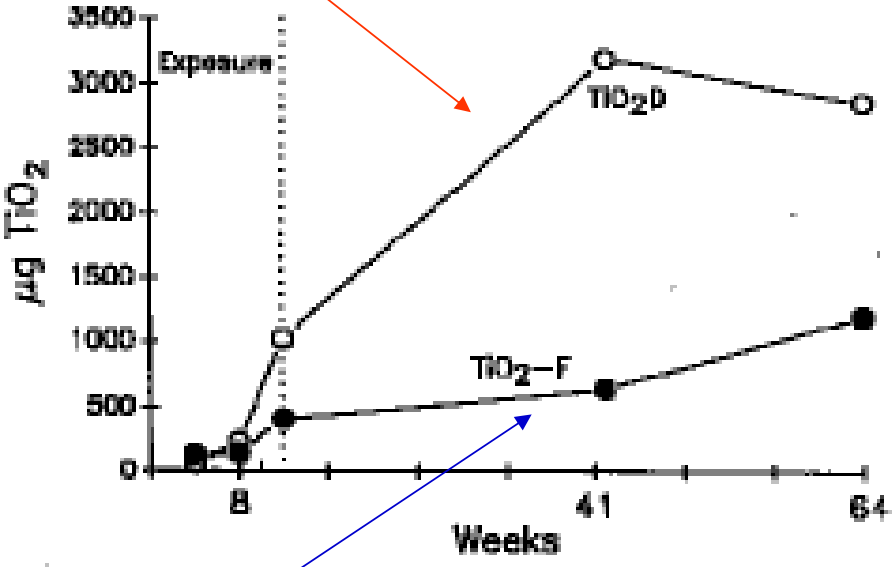
Smaller TiO_2 NPs infiltrates better the interstitium and the lymph nodes

20 nm Ø



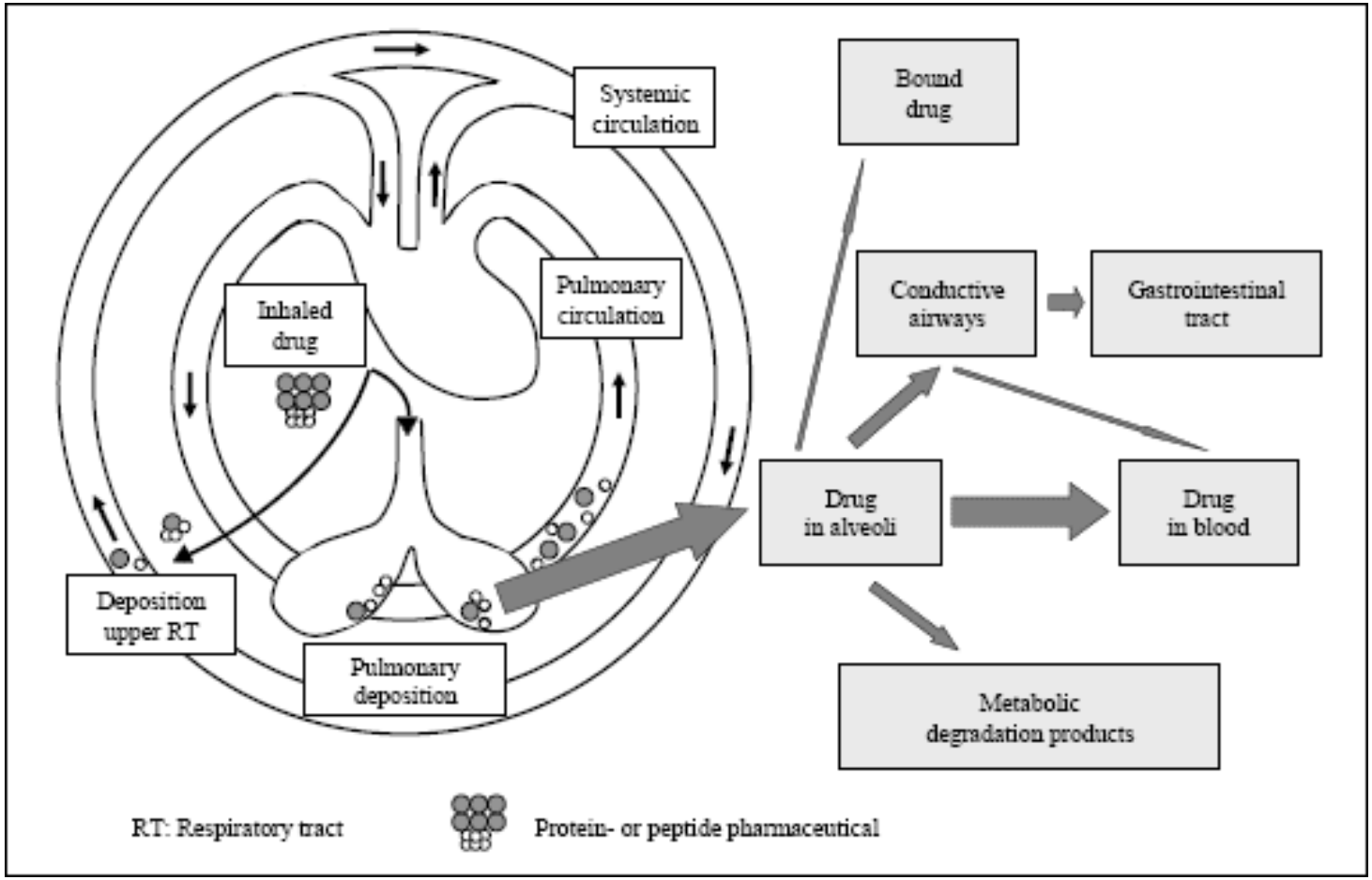
Lung interstitium

250 nm Ø

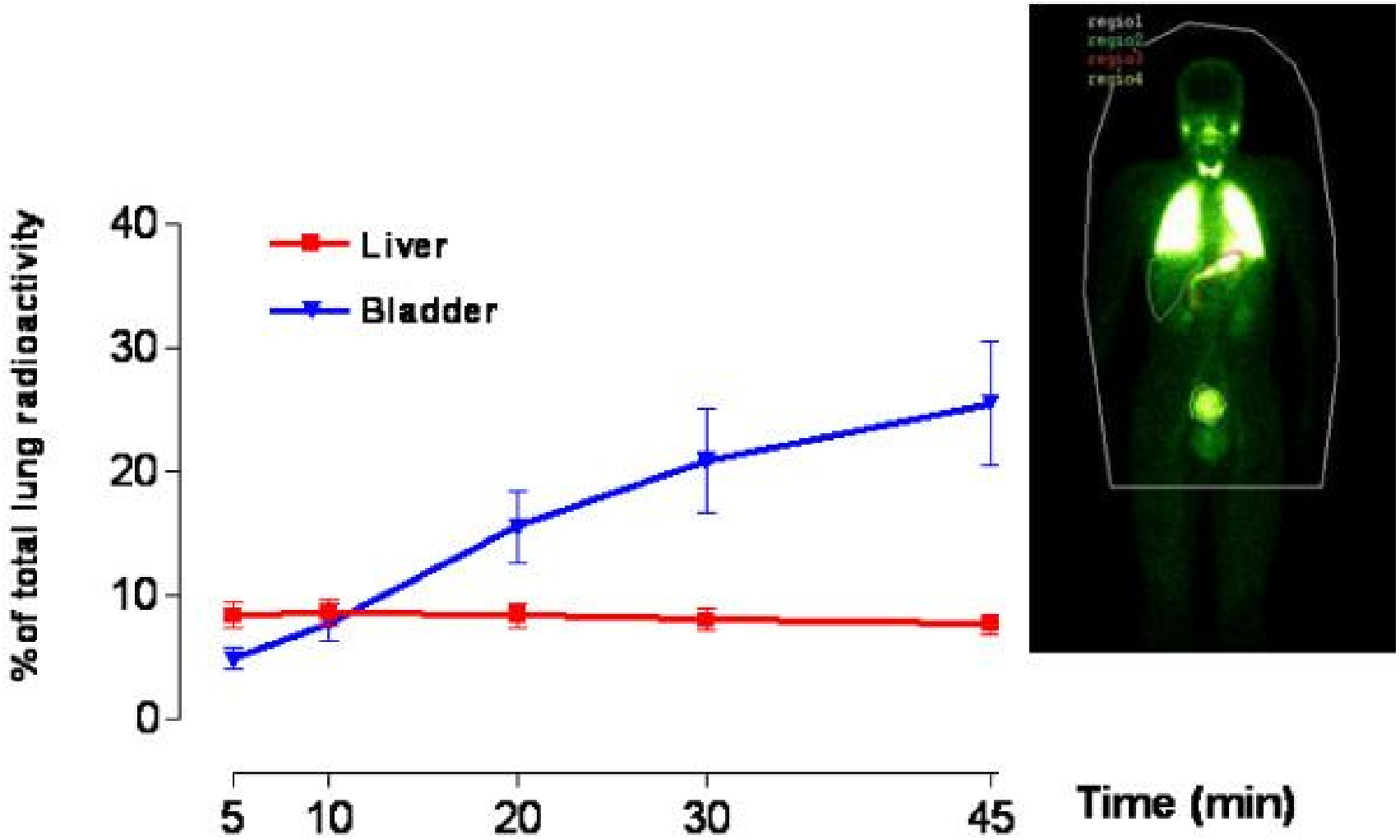


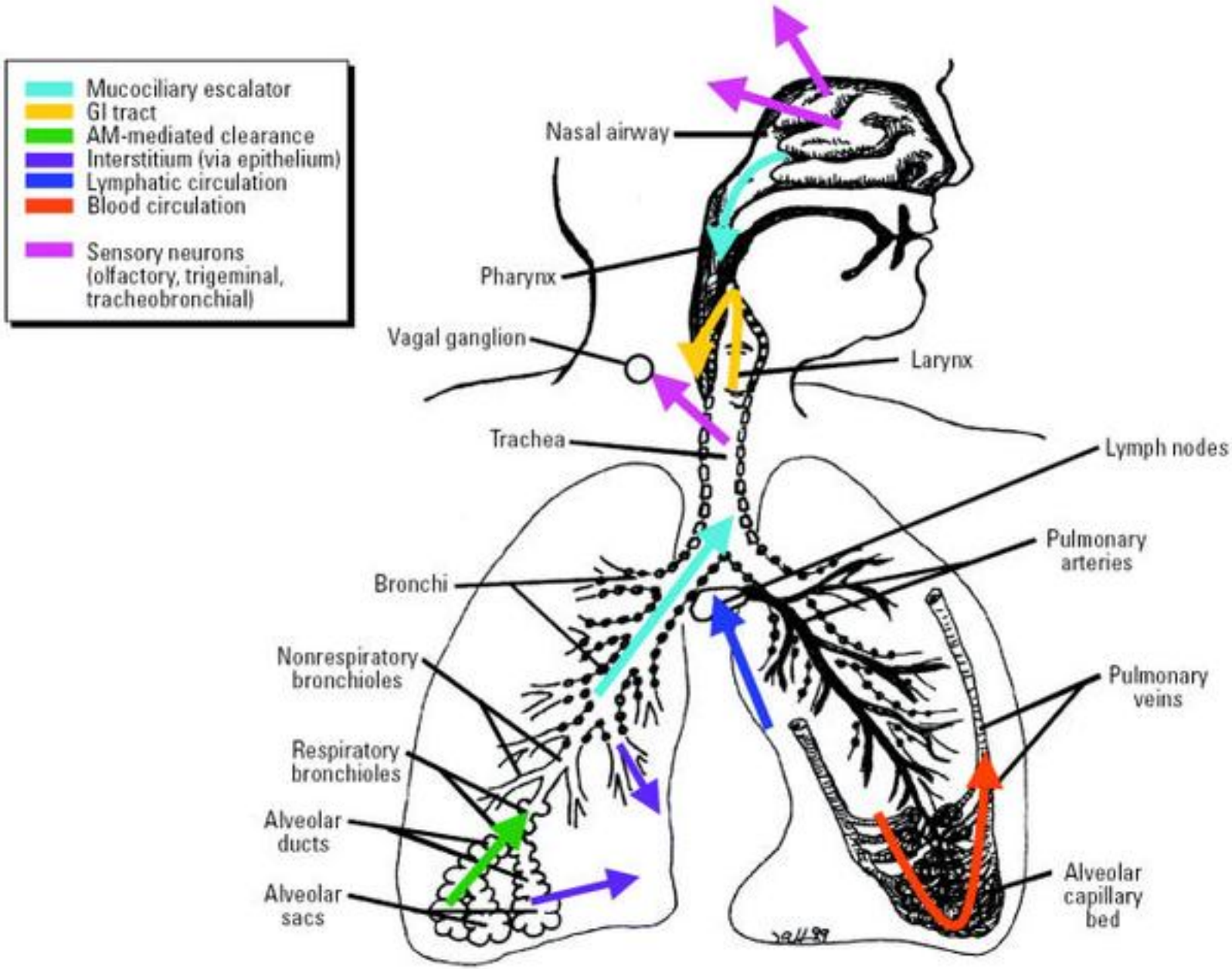
Lung interstitium + regional lymphnodes

Translocation from the lung



Translocation of inhaled ultrafine particles. Time-activity curve over liver and bladder expressed as percent of initial lung radioactivity. Insert, Whole body gamma camera image of 1 representative volunteer recorded at 60 minutes. The radioactivity over the organs is expressed as counts per minute (CPM) per pixel within each region of interest (ROI). The values recorded over the stomach were not included because this radioactivity may also come partly from swallowing of particles deposited in the mouth.




Mucociliary escalator
GI tract
AM-mediated clearance
Interstitium (via epithelium)
Lymphatic circulation
Blood circulation
Sensory neurons (olfactory, trigeminal, tracheobronchial)
Nasal airway
Pharynx
Vagal ganglion
Larynx
Trachea
Lymph nodes
Pulmonary arteries
Bronchi
Nonrespiratory bronchioles
Pulmonary veins
Respiratory bronchioles
Alveolar ducts
Alveolar sacs
Alveolar capillary bed

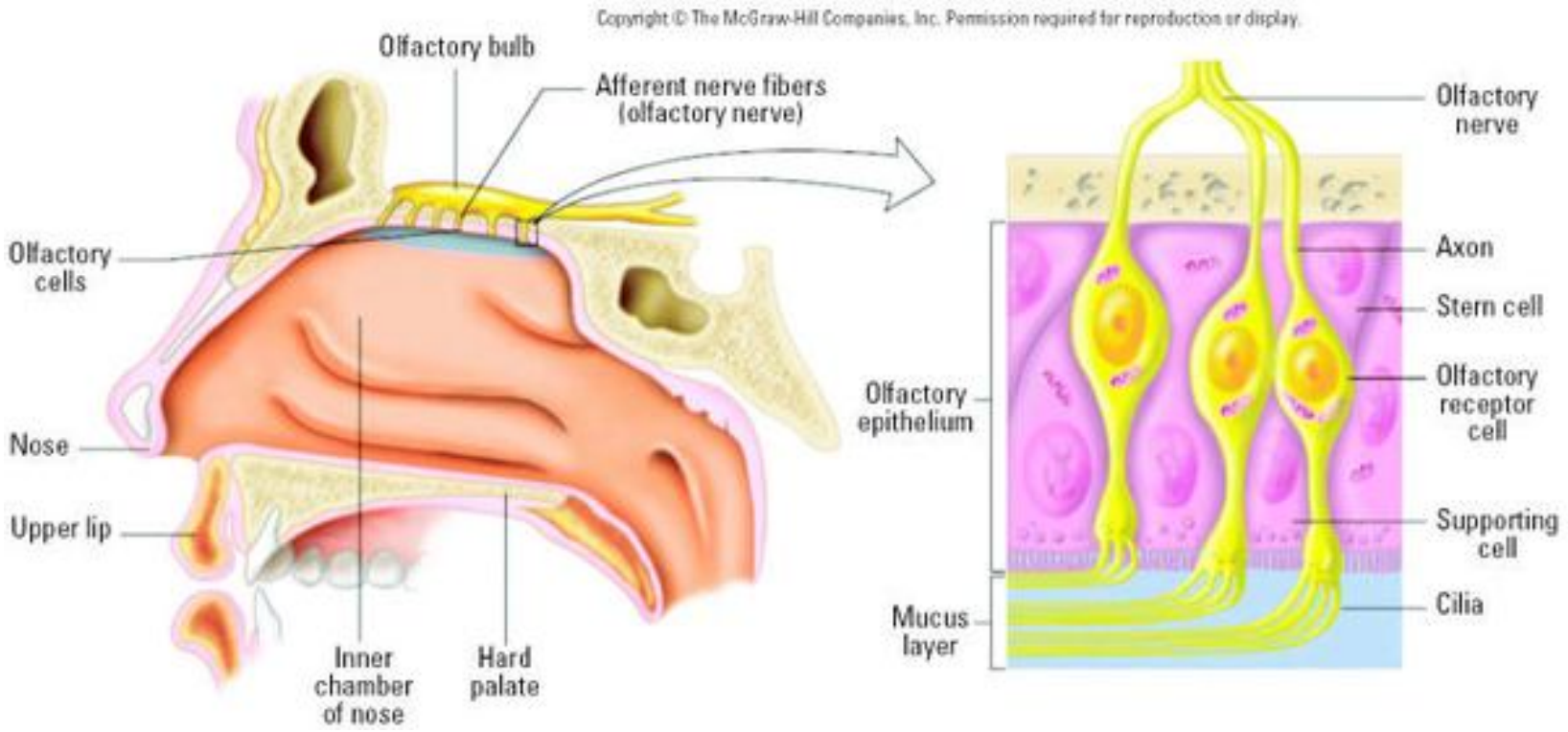
Neuronal uptake and translocation

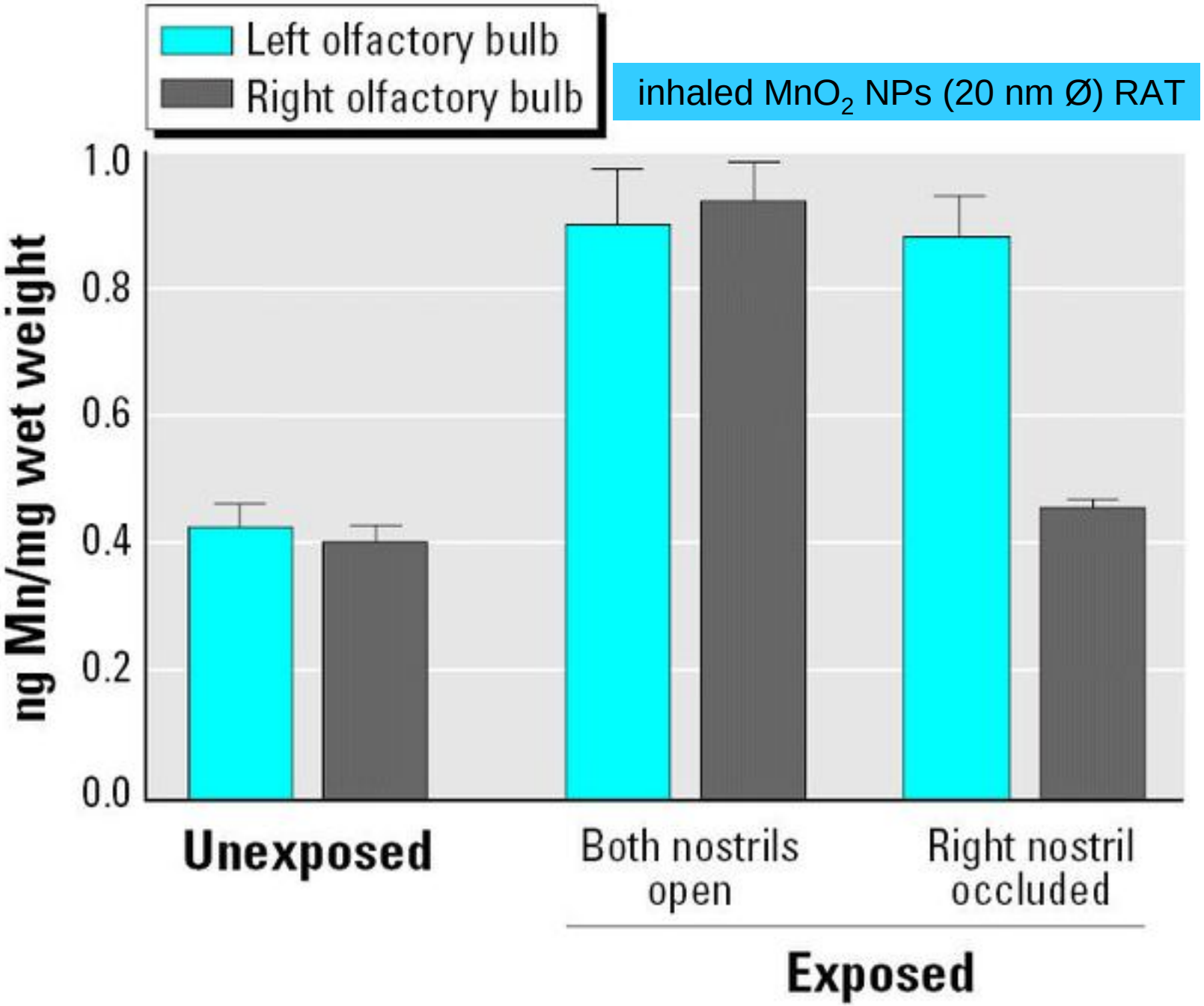
Table 6. Studies of neuronal translocation of UFPs from respiratory tract.

Reference	Study
Bodian and Howe 1941	Olfactory axonal transport of polio virus (30 nm) after intranasal instillation in chimpanzee; transport velocity, 2.4 mm/hr
de Lorenzo 1970	Olfactory axonal transport of 50 nm silver-coated gold after intranasal instillation in squirrel monkey; transport velocity, 2.5 mm/hr
Hunter and Dey 1998	Retrograde tracing of trigeminal neurons from nasal epithelium with microspheres
Hunter and Undem 1999	Rhodamine-labeled microspheres (20–200 nm) translocation via sensory nerves of TB region to ganglion nodosum in hamster after intratracheal instillation
Oberdörster et al. 2004	^{13}C particles (CMD ~ 36 nm) in olfactory bulb after whole-body inhalation exposure in rats

TB, tracheobronchial.

Olfactory cells, bulbs and nerves



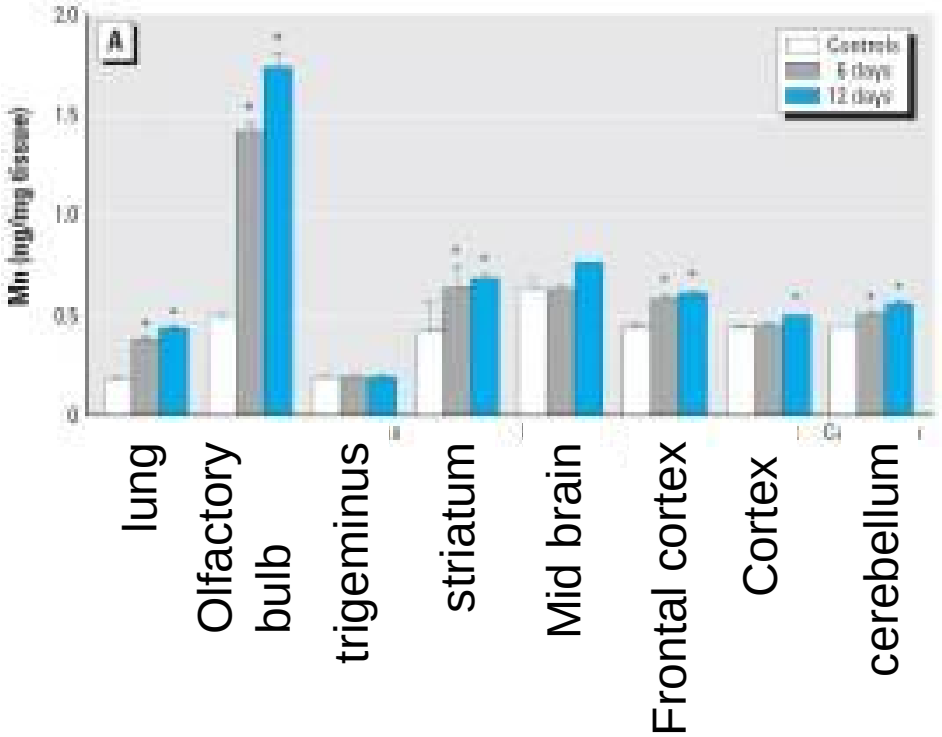

Left olfactory bulb
Right olfactory bulb
inhaled MnO_2 NPs (20 nm Ø) RAT
ng Mn/mg wet weight
1.0
0.8
0.6
0.4
0.2
0.0
Unexposed
Both nostrils open
Right nostril occluded
Exposed

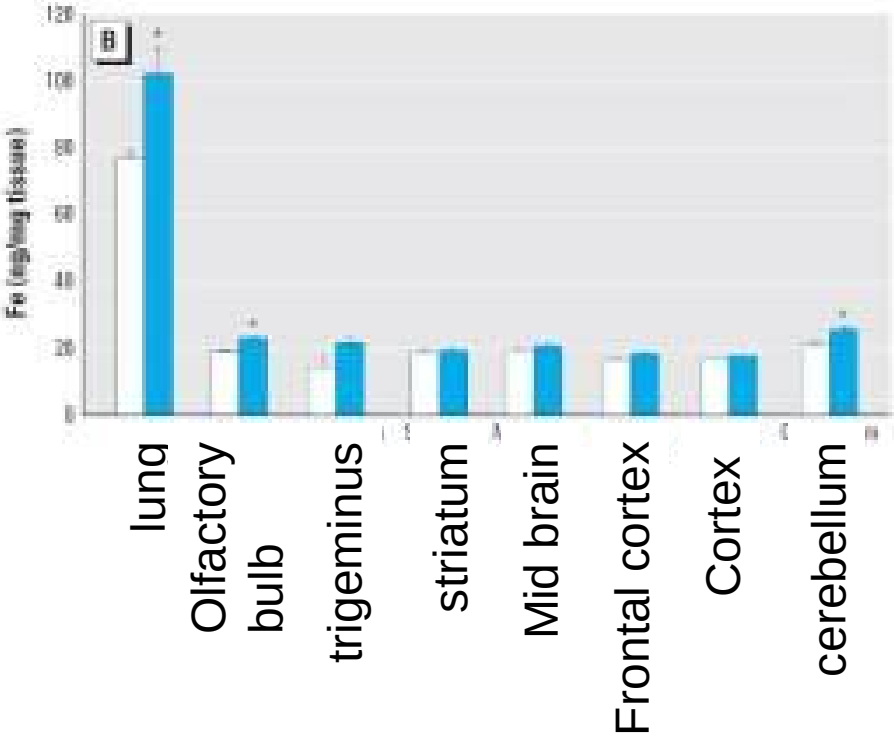
Trasnslocation of MnO_2 NPs to the brain from the olfactory bulb

control

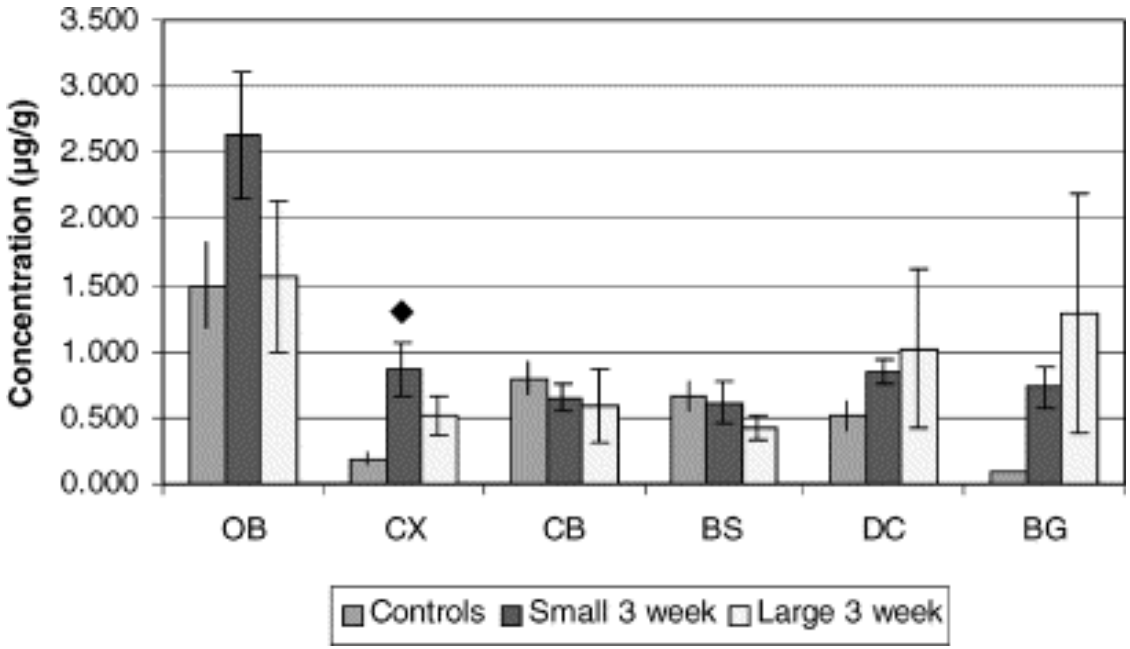
6 days

12 days





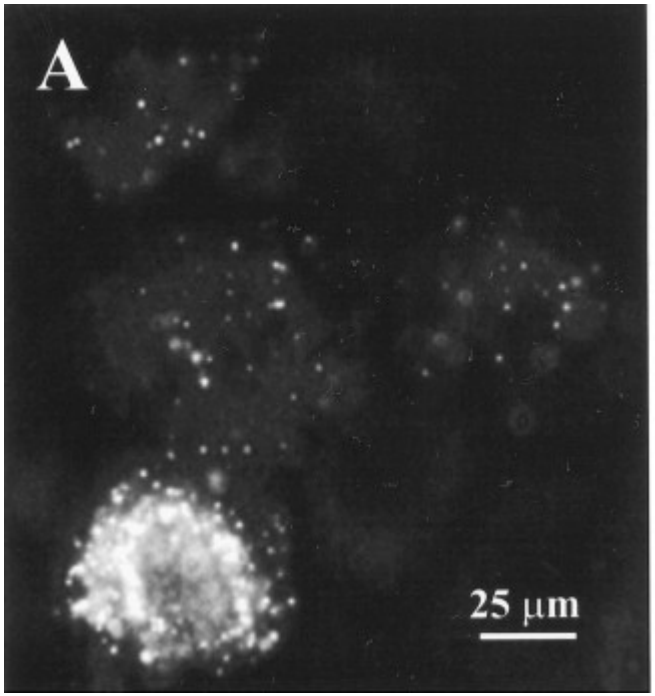
Olfactory bulb and CNS presence of inhaled small (1,3 µM) and large (13 µm) MnO_2 MPs

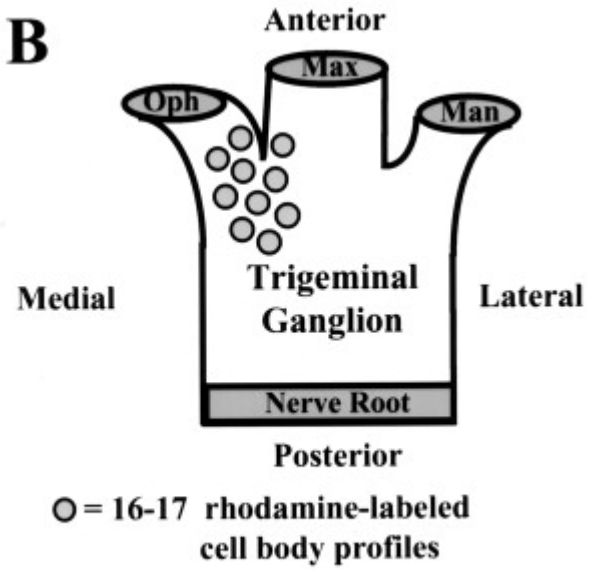


OB: olfactory bulb
CX: cortex
CB: cerebellum
BS: brainstem
DC: diencephalon
BG: basal ganglia

Note: Fila olfactoria axons diameter 100-200 nm

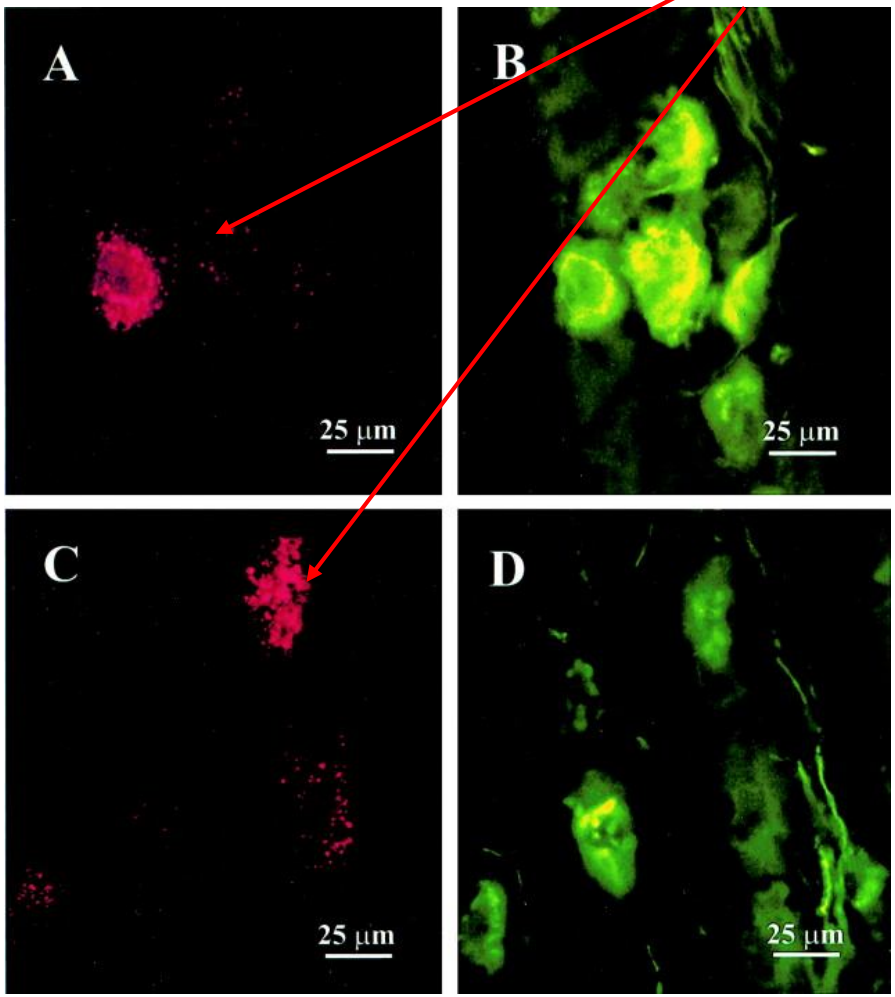
- Intranasally instilled Rhodamine-labelled Latex NPs (20-200 nm Ø) reach the trigeminal ganglion (cranial) via the ophtalmic and maxillary branches of the trigeminus nerve that supply snsory nerve endings in the nasal mucosa

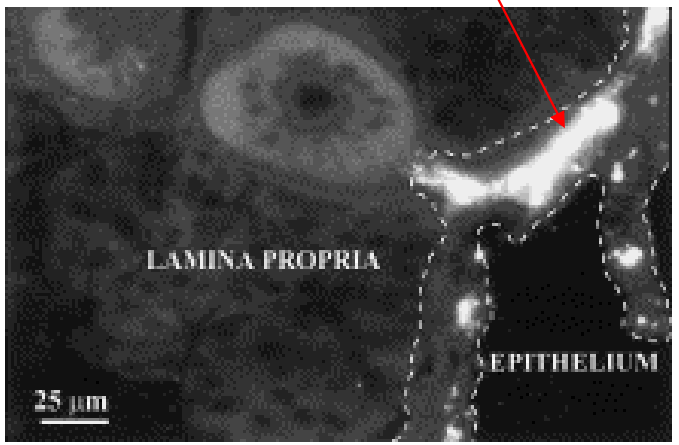




RATS

Latex particles in the nasal eptithelium and in the trigeminus ganglion neurons





murine model vs Humans

- It is possible that inhaled environmental and occupational NPs reach the human brain via the olfactory bulb?

- Caveat: in rat 50% of the nasal nucosa is olfactory, while in humans only 5%!

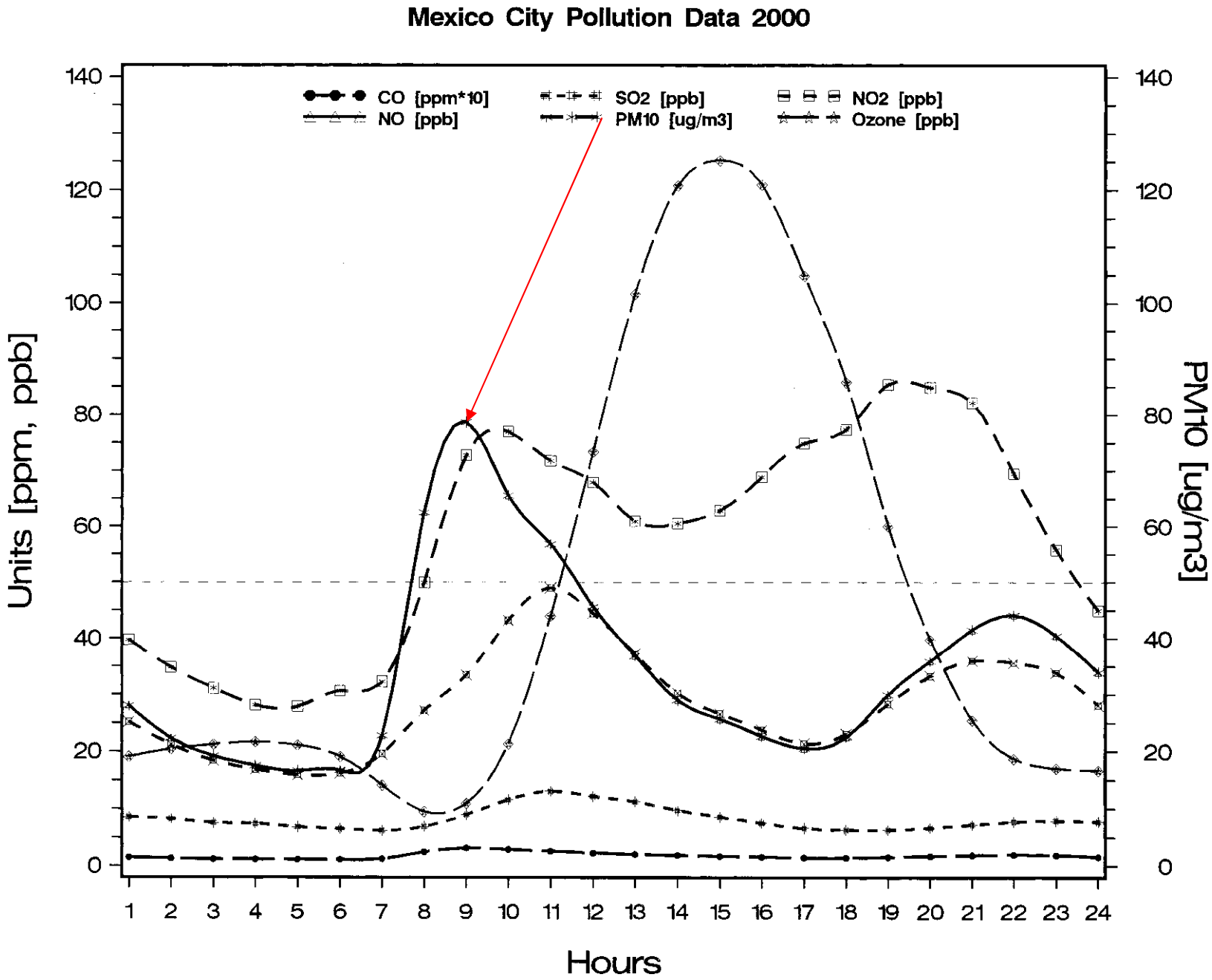
- However, in pig Latex NPs instilled in the trachea reached the ganglion nodosum in the neck area via the vagal nerve

- Cardiovascular effects of inhaled CDNPs (e.g. DEPs) may be due in part to direct translocation of NPs to the neurons of the autonomic nervous system via sensory nerves in the respiratory ract

Link beteween CNS alteration (and neurodegenerative diseases) and air pollution?

- Olfactory mucosa and bulb, cortical and subacortical brain structures neurodegenerated and inflammated in dogs from heavely polluted areas, but not in animals from rural areas (Mexico)

- Inhaled PM10 in polluted areas has been implicated in the Alzheimer’s disease (AD)


Mexico City Pollution Data 2000
CO [ppm*10]
NO [ppb]
SO2 [ppb]
PM10 [ug/m3]
NO2 [ppb]
Ozone [ppb]
Units [ppm, ppb]
PM10 [ug/m3]
Hours
0
20
40
60
80
100
120
140
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
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19
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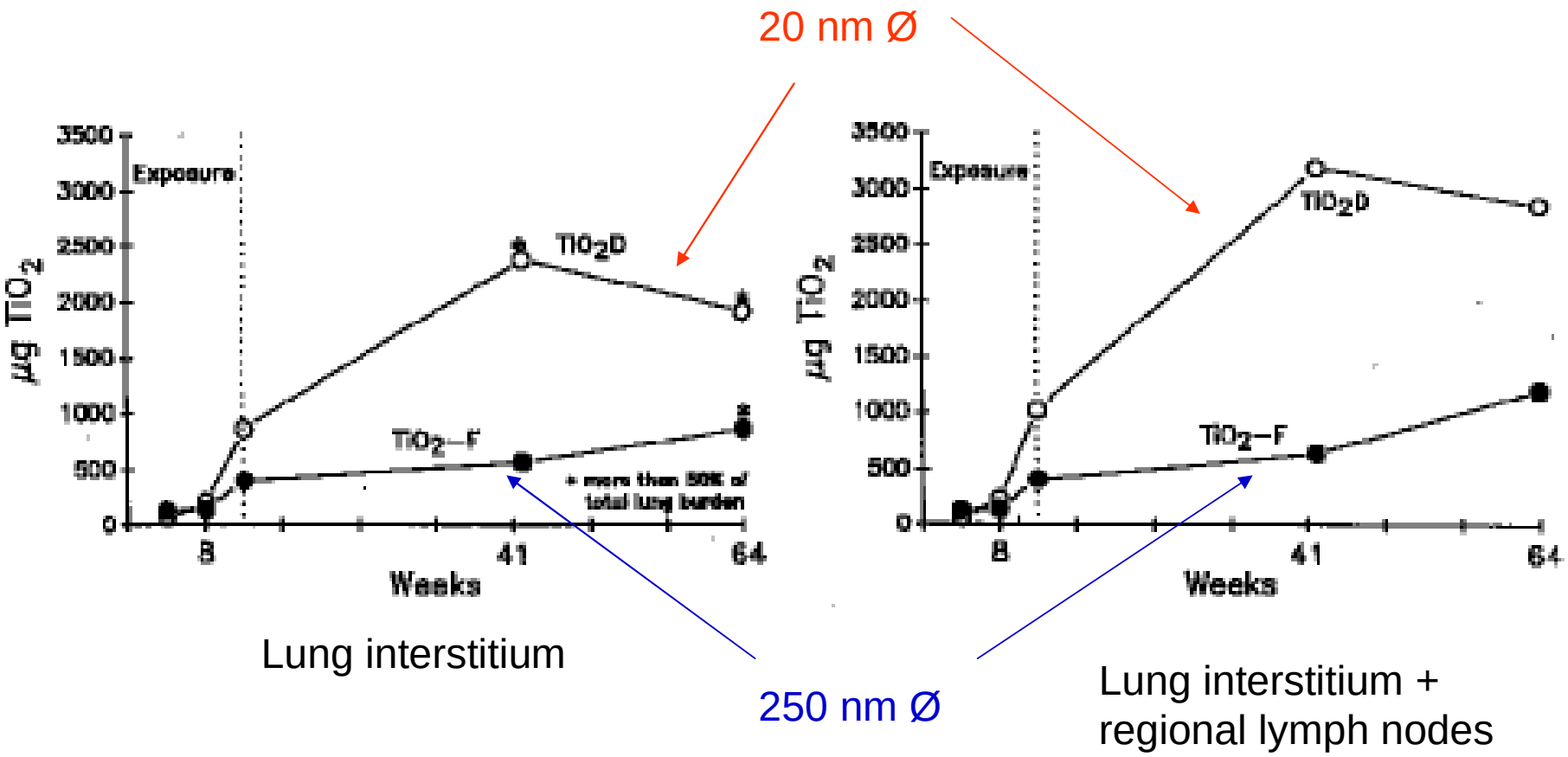
21
22
23
24

Lung translocation to epithelium and interstitium

- As discussed, AM are relatively less efficient in phagocytose NPs (<100 nm Ø) than MPs (1-5 µm Ø)

- NPs (<100 nm Ø) get ready access to the epithelial cells and the underlying interstitial space

Translocation from the lung to the regional lymphatic system (and then to the blood?)



Smaller TiO_2 NPs infiltrates better the interstitium and the lymph nodes

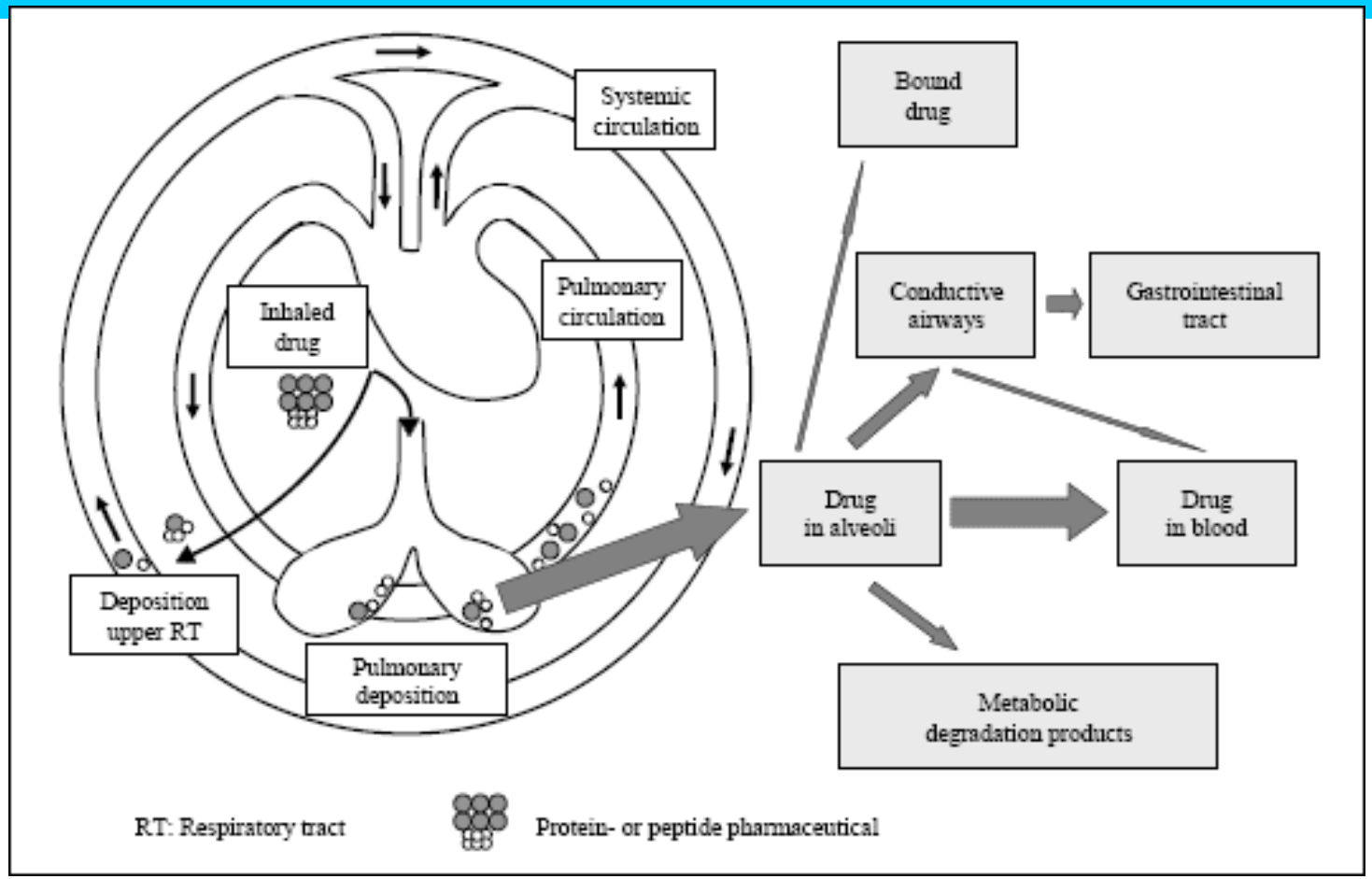
- NPs present in PTFE (Teflon) fumes are found shortly after inhalation (~15 min) in rat lung in the epithelial cells and in the interstitial space

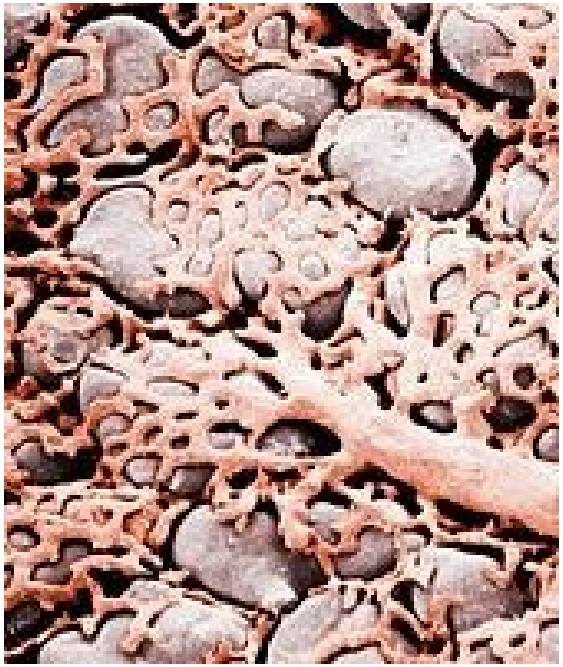
- Inhalation of TiO2 NPs with large size range (12-250 nm Ø): decreasing size resulted in a shift from inflammatory effects in the surface to inflammatory effect in the intertitium, in parallel with an increased interstitial presence

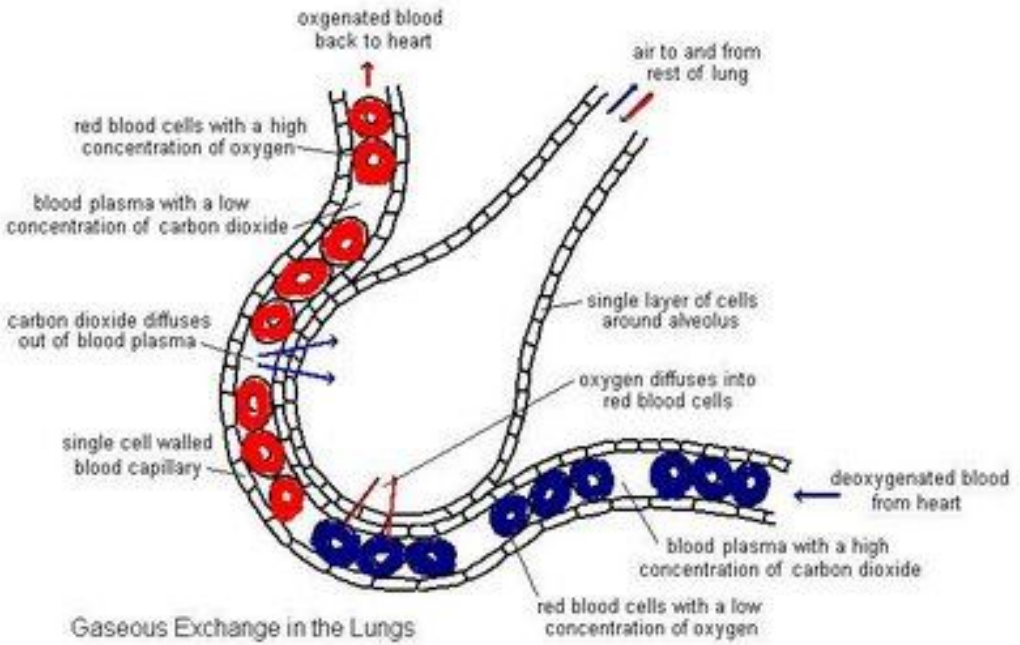
- It is important to consider that interstitial translocation of fine particles is higher in larger species (dogs, non-human primates) than in rodens.

- Translocation data in the murine model may undersestimate what happens human

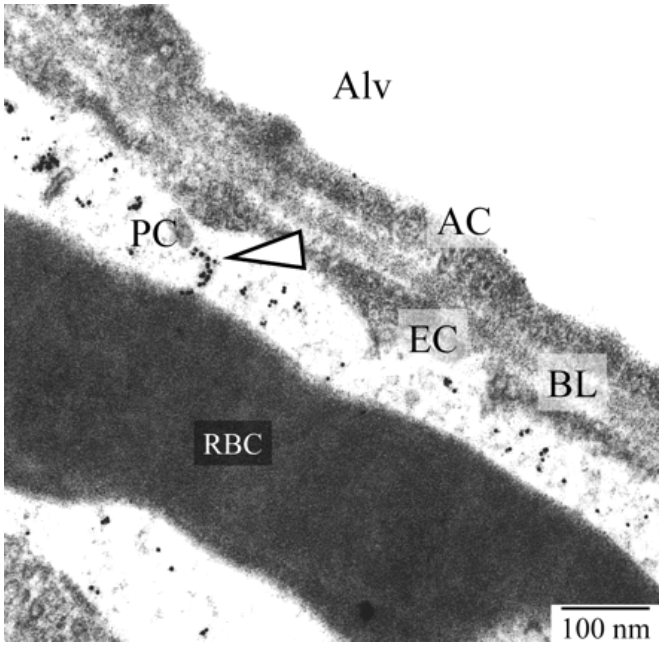
Translocation from the lung to the circulation



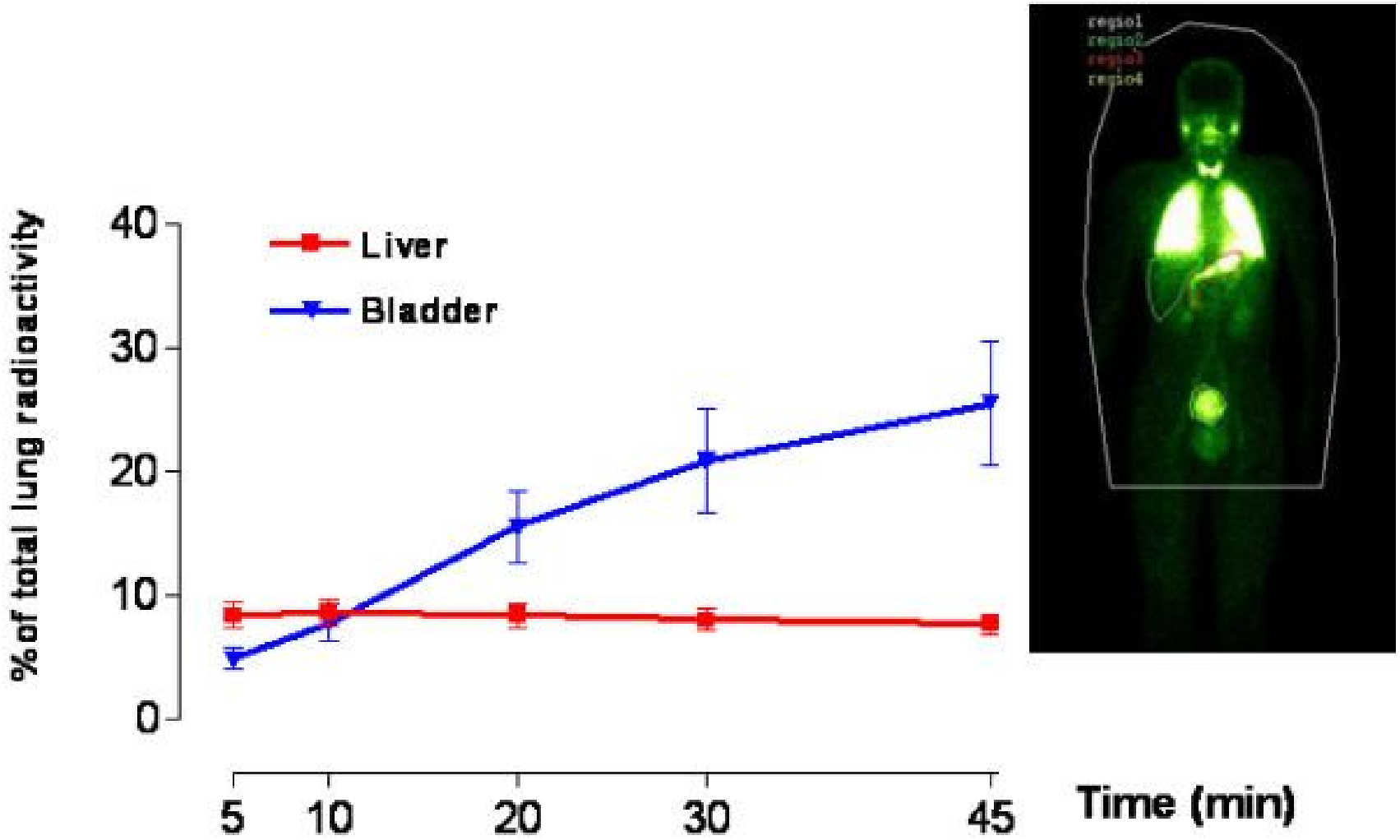




Lung capillaries surrounding alveoli

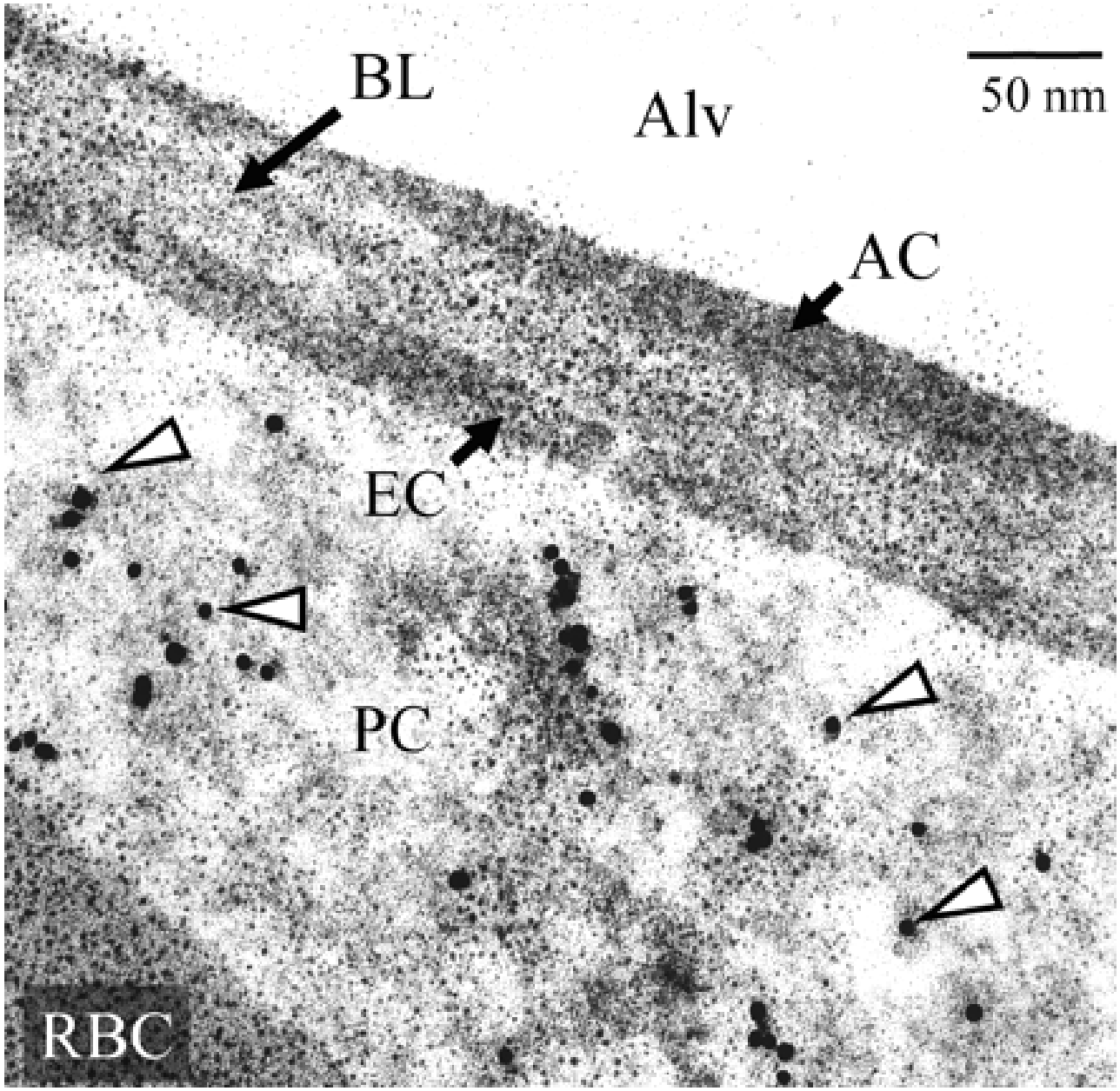


Translocation of inhaled ultrafine particles. Time-activity curve over liver and bladder expressed as percent of initial lung radioactivity. Insert, Whole body gamma camera image of 1 representative volunteer recorded at 60 minutes. The radioactivity over the organs is expressed as counts per minute (CPM) per pixel within each region of interest (ROI). The values recorded over the stomach were not included because this radioactivity may also come partly from swallowing of particles deposited in the mouth.

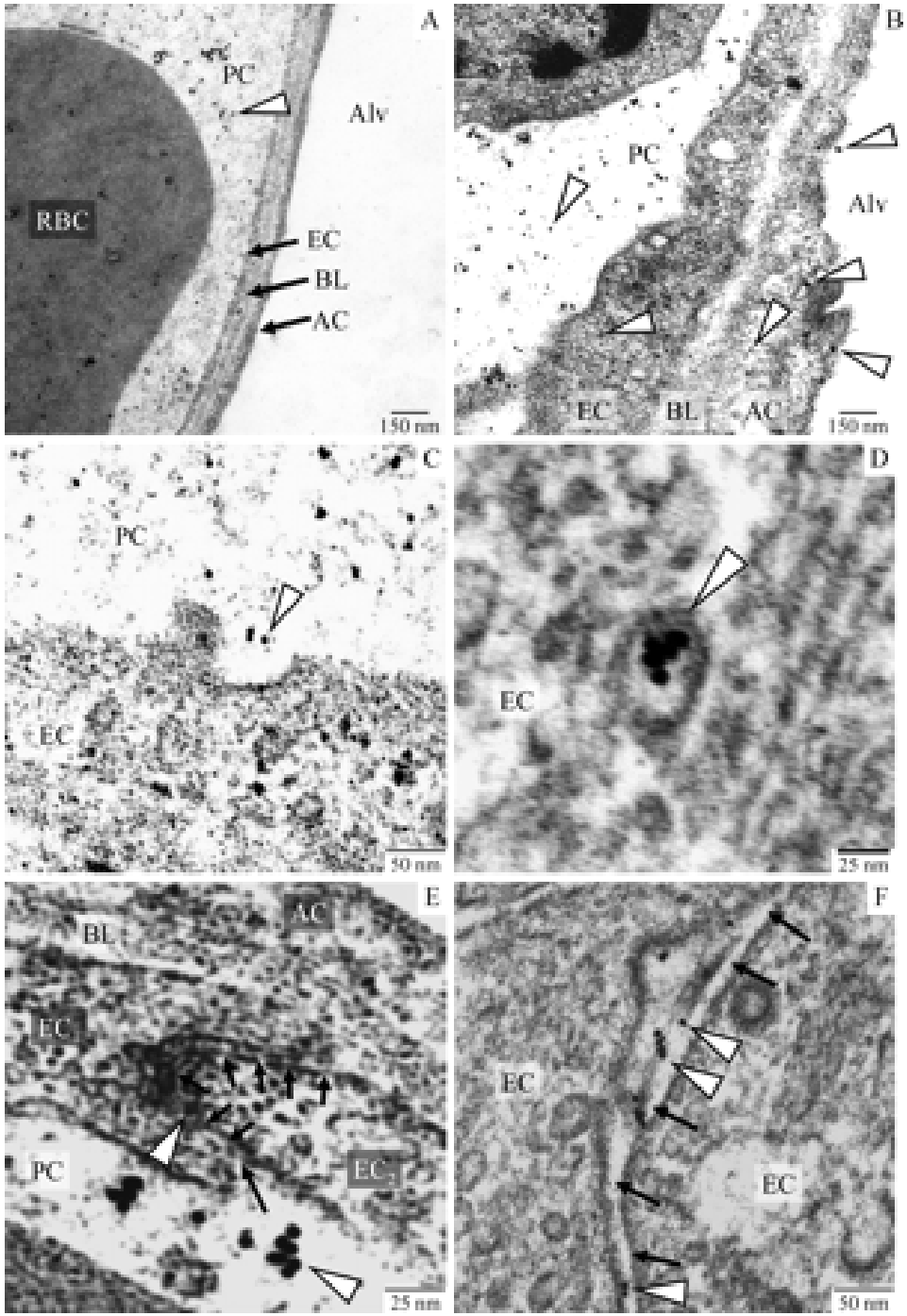


Animal models

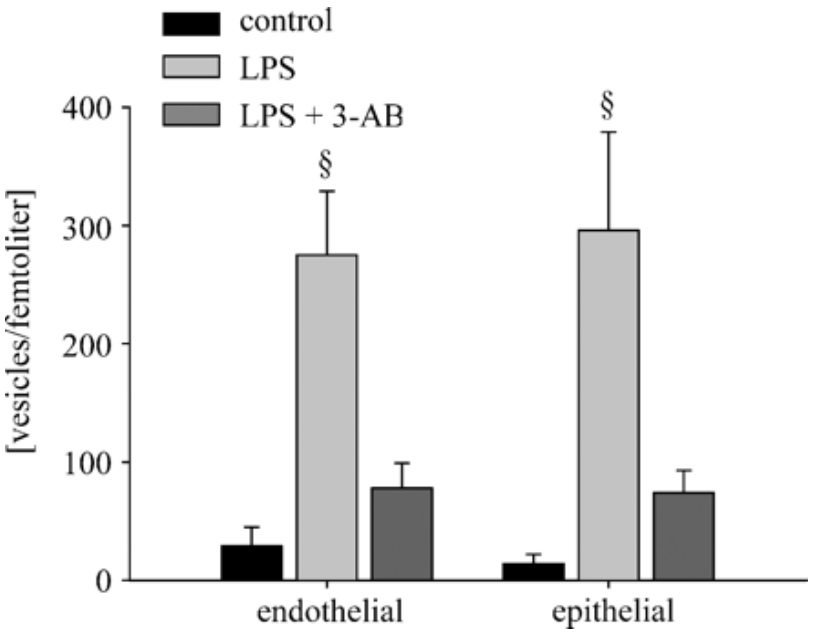
- In 1977 it was first shown that 30 nm Ø gold NPs translocate to the blood from the alveolar epithelium after instillation in the trachea
- Gold NPs were found in large amount in platelets in the lung capillaries
- Can this predispose to platelet aggregation with formation of micro-thrombi?

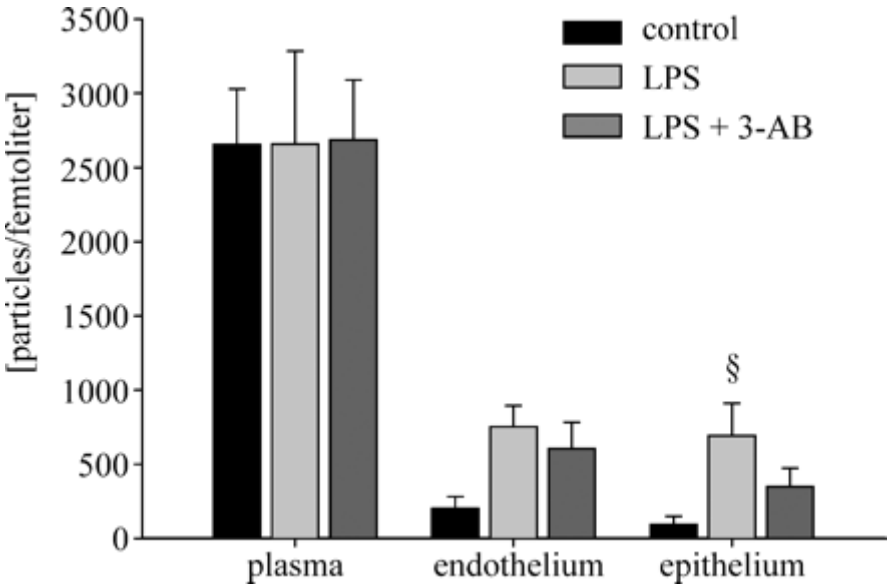


gold NPs at high magnification.The plasma of a capillary contains homogenously dispersed gold particles (arrowheads, diameter 8 nm). Alv, alveolus; RBC, red blood cell; EC, endothelial cell; AC, alveolar epithelial cell; BL, basal lamina; PC, plasma compartment.



- Arrowheads, gold particles; arrows, interendothelial space. Control group (*A*) presented normal microanatomy with an extremely thin gas exchange barrier. After 2 h of LPS infusion, endothelial and epithelial cells showed a boosted endo- and transcytosis activity (*B–D*). Endothelial cells formed caveolae with concentrated gold particles (*C*). The predominant part of transport of gold-labeled albumin across endo- and epithelial cells was by vesicle traffic (*D*). Additionally, interendothelial cell junctions were frequently open for passive paracellular migration of macromolecules (*E, F*).



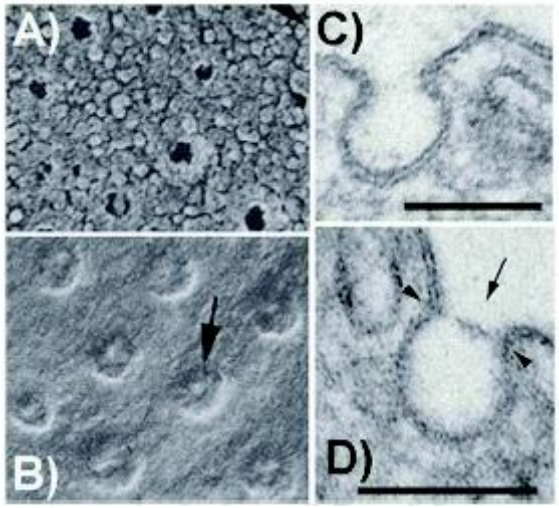


- Number of gold-NPs positive vescicles

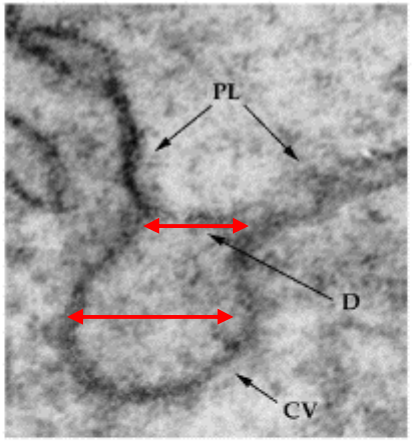
Number of gold-NPs

- Phospholipids and albumin are present in the Alveolar Lining Fluid (ALF)
- Caveolae are abundant endothelial cells of the lung capillary (*while not in brain capillaries!*) and in the type I alveolar epithelial cells
- Caveolae with an opening of ~ 40 nm disappear and reappear in the aveolar epithelial cells with inspiration (alveolus dilatation) and espiration (alveolus restriction)

The caveolae



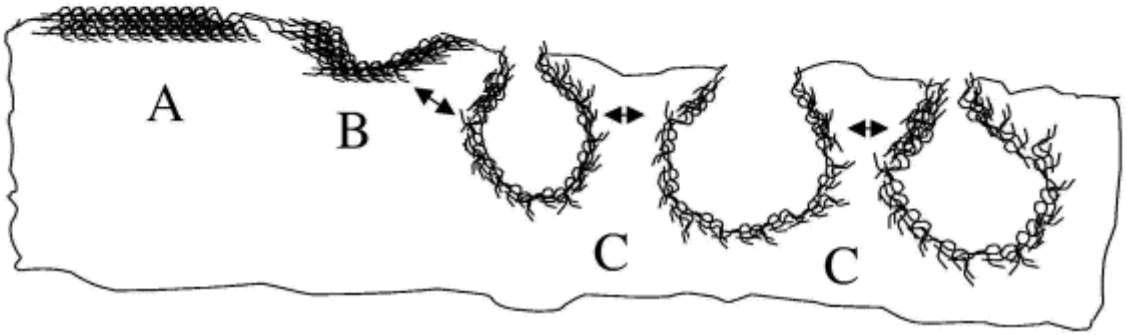
(a)



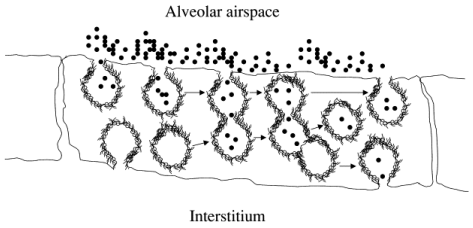
~ 40 nm Ø

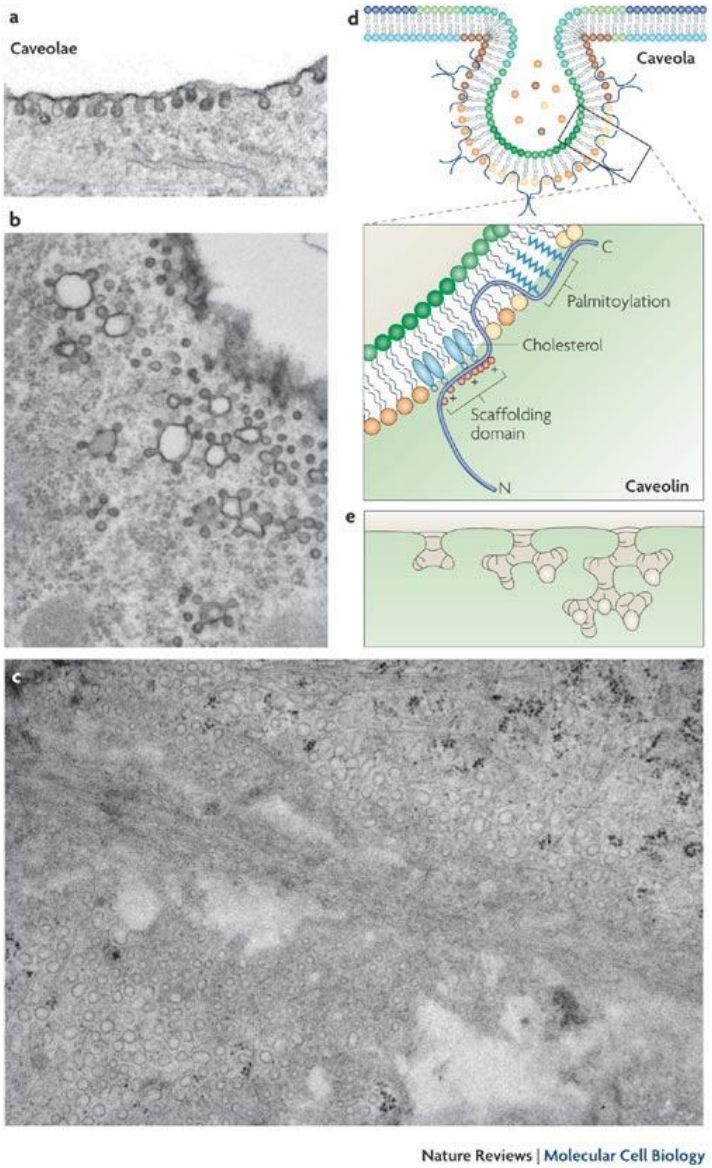
~ 80 nm Ø

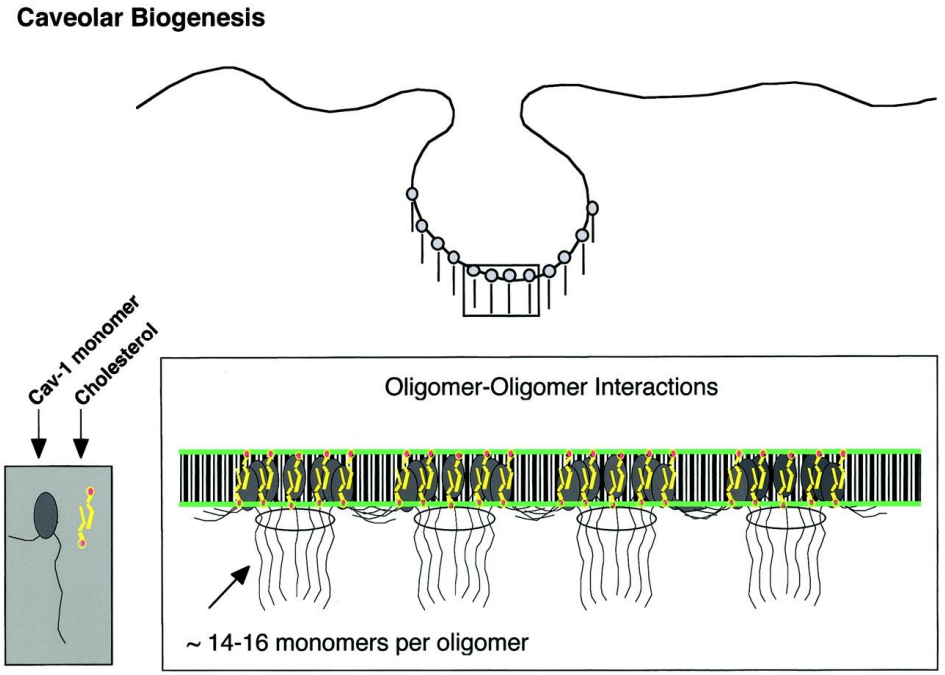
(b)



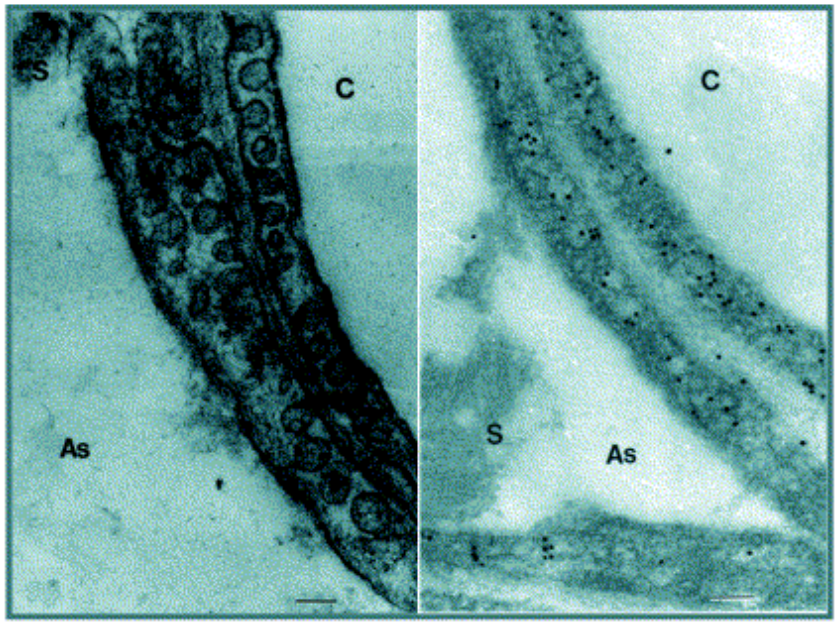
Transcytosis mediated by caveolae







(c)



(d)

Caveolin-1 immunogold staining

Human data

- Data are ambigouous
- One study showed a rapid apperance of ^{99}Tc-labelled carbon NPs (20 nm Ø) in the blood with significant accumulation in the liver (not confirmed by others)

- Considering data in anima models, it is lkely that lung-blood translocation occurs

- The extent is however probably dependent on particel surface characteristics/chemistry and size

Human pathology

- Lung-blood translocation might provide a mechanism for cardiovascular effects of ambient inhaled UFPs (CDNPs like DEPs)

- The other mechanism is the indirect effect of an *acute phase response* (with changes in blood coagulability)

Lung-blood traslocation metallic vs non-metallic NPs

- Metallic NPs (<30 nm Ø) pass rapidly into the circulatory system
- Non-metallic NPs of different size (4-250 nm Ø) pass very little or not al all
- However, both can cross fast the elveolar barrier in subjects suffering from respiratory or circulatory diseases
- Lung inflammation increases the microvaculature permeability to NPs

Table 4. Particle size and surface chemistry-related alveolar–capillary translocation.

Particle size (nm)	Type	Translocation	Localization/effect	Reference
5–20	Gold, albumin coated	Yes	Via caveolae	Mehta et al. 2004
8	Gold, albumin coated	Yes	Via "vesicles"	König et al. 1993
8	Gold, albumin coated	Yes	Via caveolae	Heckel et al. 2004
18	Iridium	Yes[a]	Extrapulmonary organs	Kreyling et al. 2002
30	Gold	Yes	Platelet?	Berry et al. 1977
35	Carbon	Yes	Liver	Oberdörster et al. 2002
60	Polystyrene[b]	Yes	Thrombus, early	Nemmar et al. 2002b Silva et al., in press
60	Polystyrene	?	No thrombus	Nemmar et al. 2002b
80	Iridium	Yes[a]	Extrapulmonary organs	Kreyling et al. 2002
240	Polystyrene, lecithin	Yes	Monocytes	Kato et al. 2003
240	Polystyrene, uncoated	No		Kato et al. 2003
400	Polystyrene	No	No thrombus	Nemmar et al. 2003

?, unknown.
[a]Minimal. [b]Indirect evidence.

GI (Gastrointestinal tract translocation)

- NPs from:

the respiratory ways via the mucociliary escalator

ingested with food, cosmetics or other products

Korean company commercialising a food integrator based on Sulfur NPs

Safety?

Legislation?

examples

- Radiolabelled PEGylated C60 fullerene NPs administered orally in rats: 98% in the feces within 48 hr, 2% in the urine (*via blood*)
- Ultrafine 192 Ir not significantly translocated form GI to blood
- Oral TiO2 Nps (100-500 nm Ø) are found in the liver to some extent (*via blood*)

Colloidal silver

- Blue man: “argyriasis”

GI → blood of Polystyrene beads

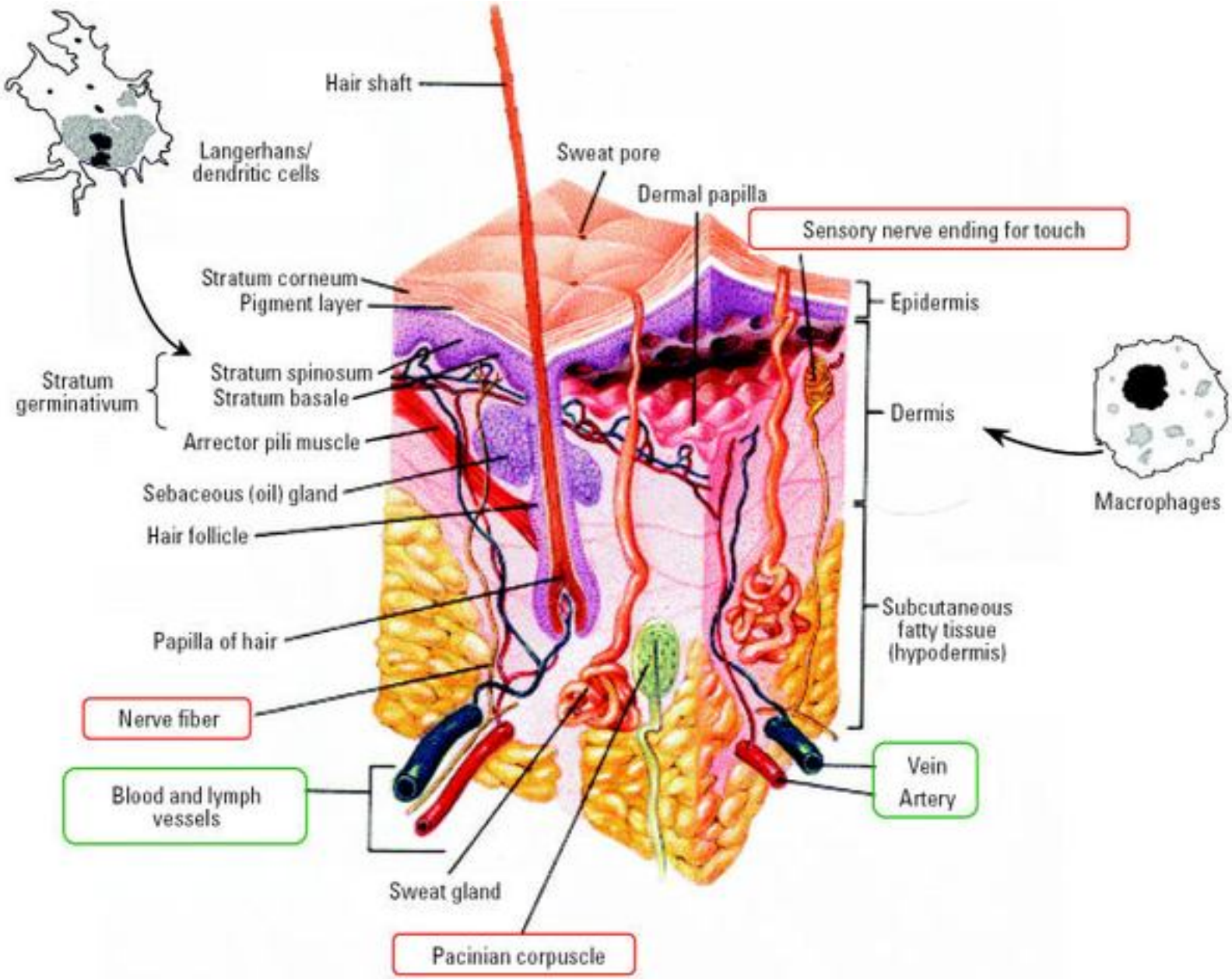
- Oral administrated particles cross the GI mucosa in rat, enter the mesenteric lymph and are found later in blood and variuos other organs

Ø	%
3 µm	~ 0
1 µm	0,8
100 nm	5,8
50 nm	6,6

SKIN

- Epidermis is a very tight barrier for the underlaying dermis: *Stratum corneum* + *stratum pigmetosum* + basal layer

- The dermis is rich in micorvasculatur, lymph vessels, macrophages, Langherans cells (specialised APC dendritic cells) and five differenrts sensory nerve endings)


Hair shaft
Langerhans/
dendritic cells
Sweat pore
Dermal papilla
Sensory nerve ending for touch
Stratum corneum
Pigment layer
Epidermis
Stratum
germinativum
Stratum spinosum
Stratum basale
Dermis
Arrector pili muscle
Sebaceous (oil) gland
Hair follicle
Macrophages
Papilla of hair
Subcutaneous
fatty tissue
(hypodermis)
Nerve fiber
Vein
Artery
Blood and lymph
vessels
Sweat gland
Pacinian corpuscle

- Broken skin is a readly available NPs and MPs portal entry (e.g. large amount of soil particle in inguinal lymph nodes in bare foot people; remember *podoconiosis*)

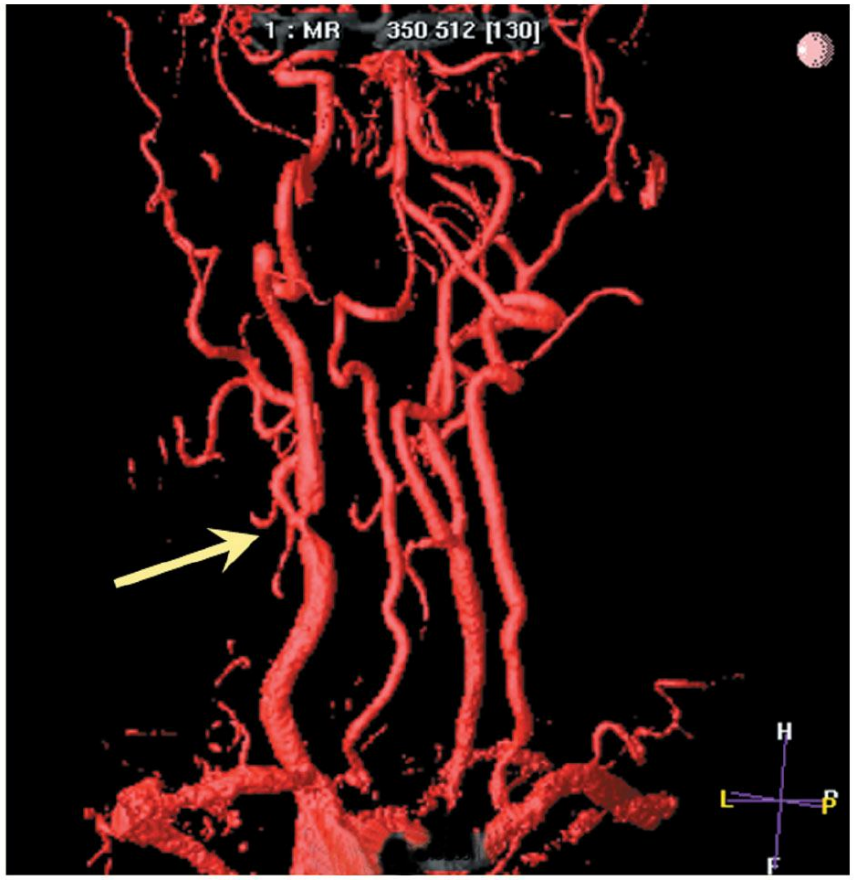
- Flexed skin (but not flat skin) allows the penetration of particles up to 1 mm diameter to the dermis →regional lymph nodes →blood ?

- Near infrared QDs injected intradermally in mice and pigs labelled regional lymph-nodes (useful for imaging?)
- Uptake in dermal macrophages and Langherans cells are likely responsible for lymph-nodes translocation
- Possible immuno-modulatory actions of NPs?
- Neuronal uptake via skin nerve endings (likely)? (remember that Herpes virus uses this route to the dorsal root ganglion..)

Possible effects of NPs in the blood

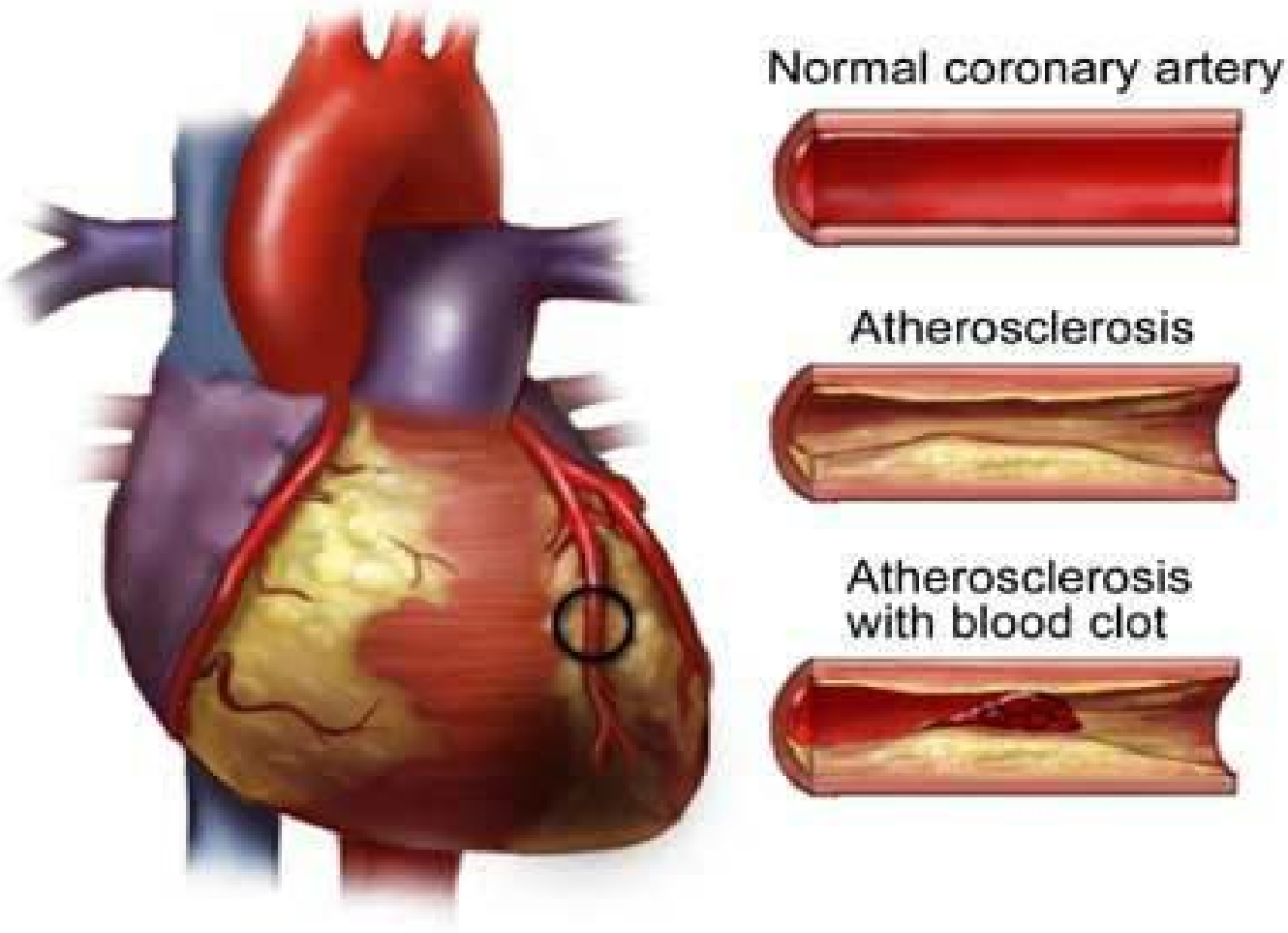
- Atheromatous plaque increase and destabilization (?)
- Platelet activation/aggregation (thrombi)
- Activation of the coagulation cascade
- Interaction with blood cells (red blood cells; leukocytes)
- Proinflammatory effects, hypersensitivity (due to complement activation)

Atheromatous plaque at the right carotid artery bifurcation

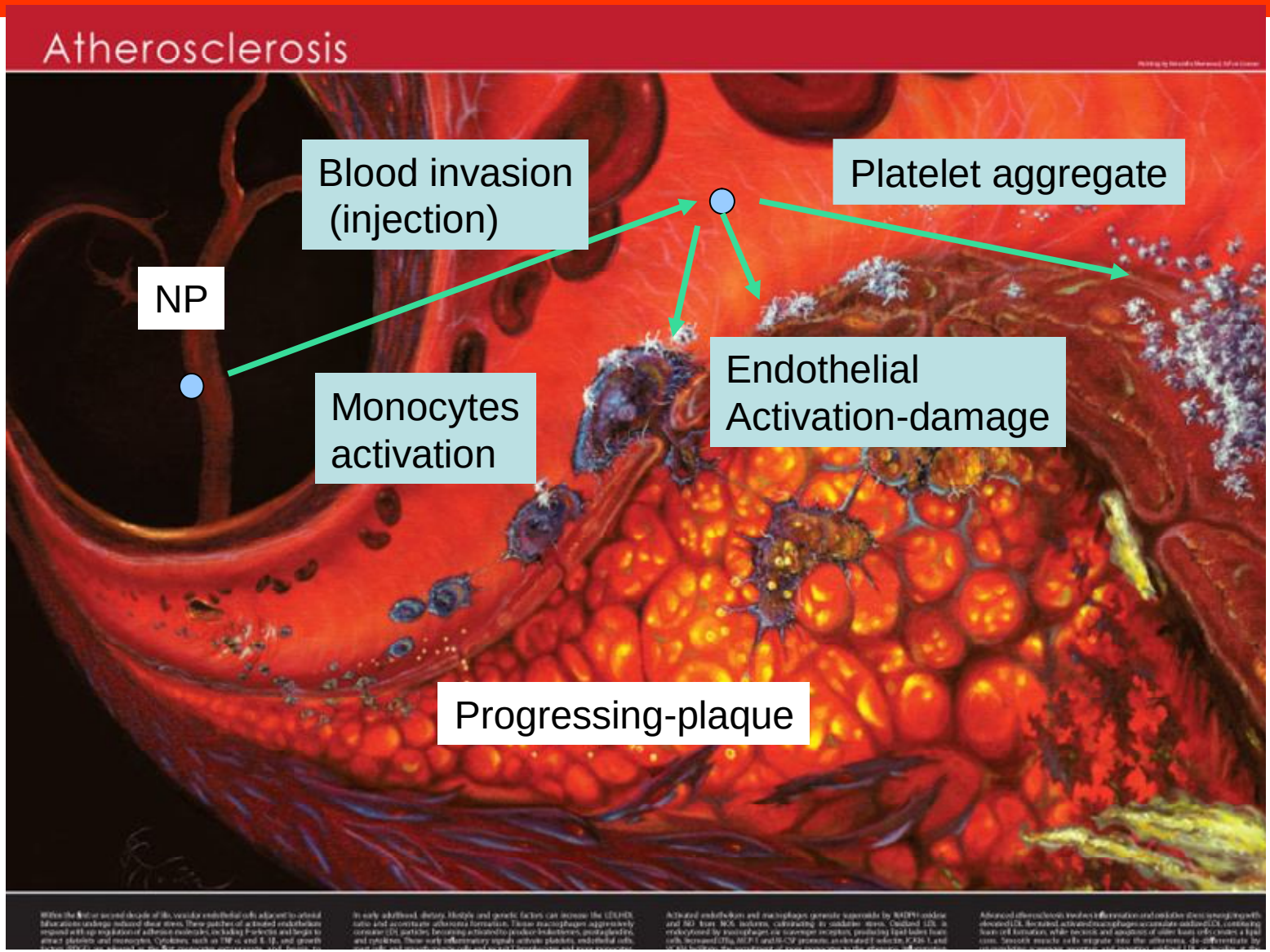


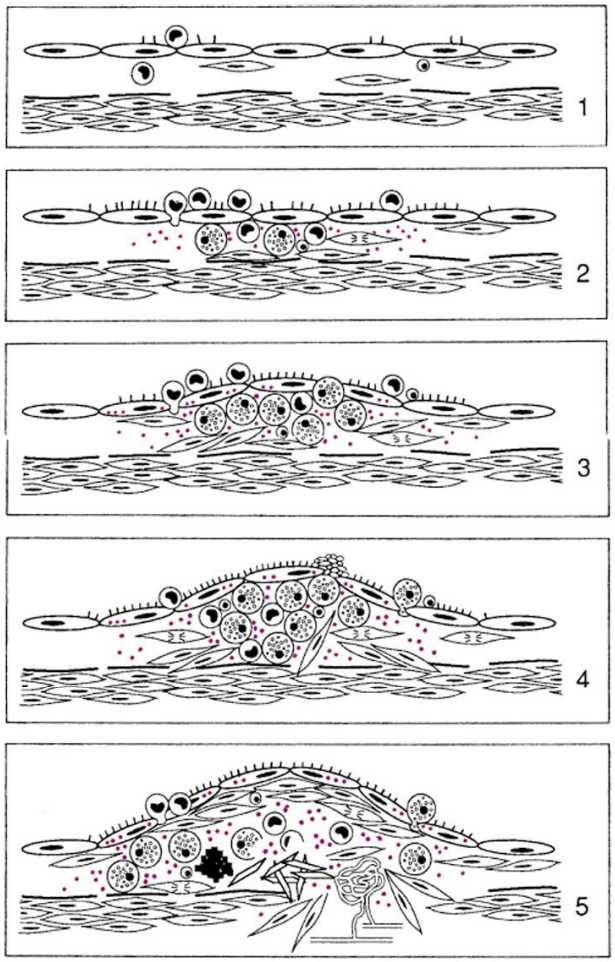
neck

Coronary atheroma



Etheromatous plaque increase and destabilisation is associated with PM inhalation





Platelet Aggregation

Flowing disc-shaped platelet → Rolling ball-shaped platelet → Hemisphere-shaped platelet → Spreading platelet

FIRM, BUT REVERSIBLE ADHESION

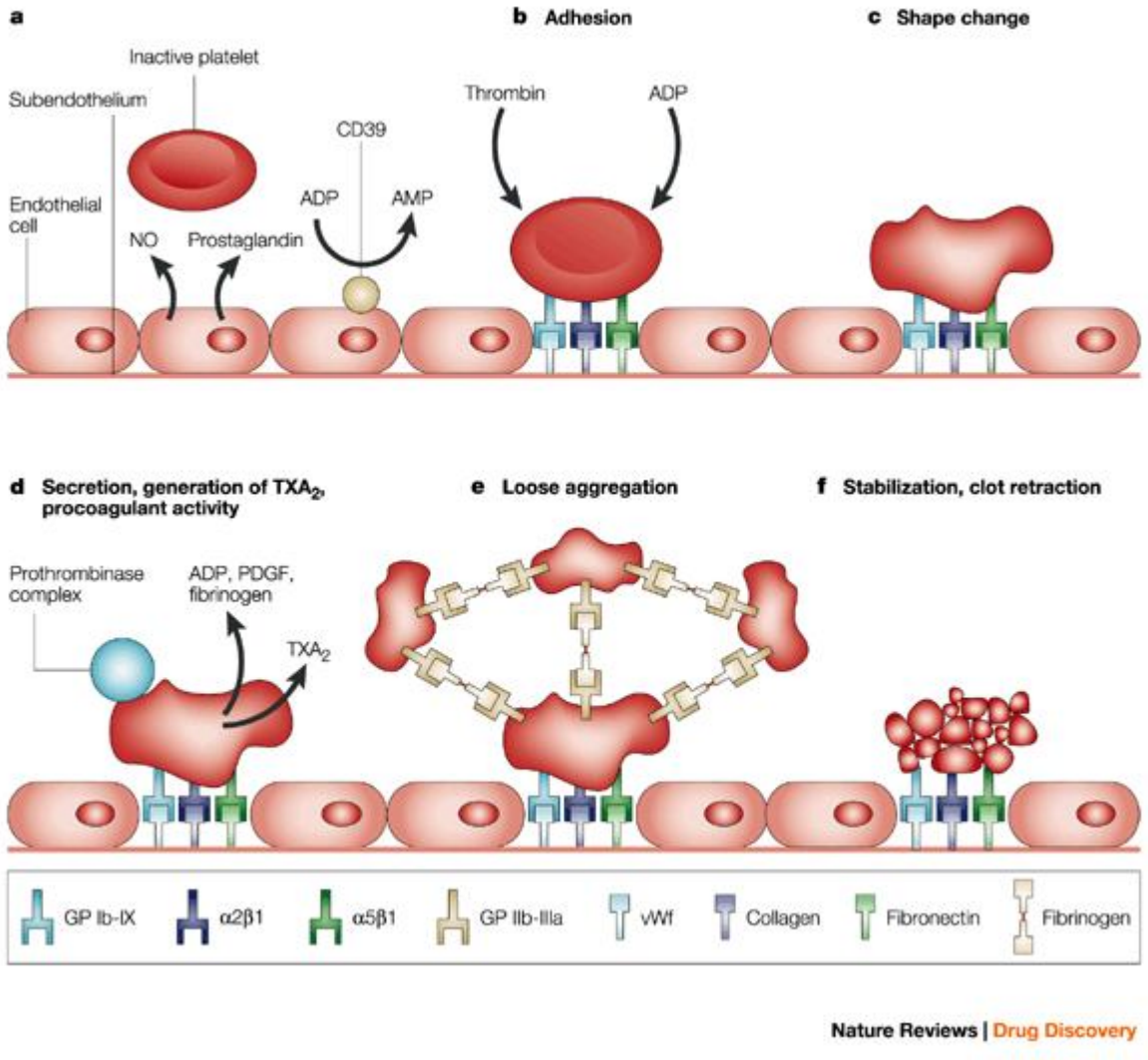
IRREVERSIBLE ADHESION

Scanning electron micrograph of discoid, dormant platelets

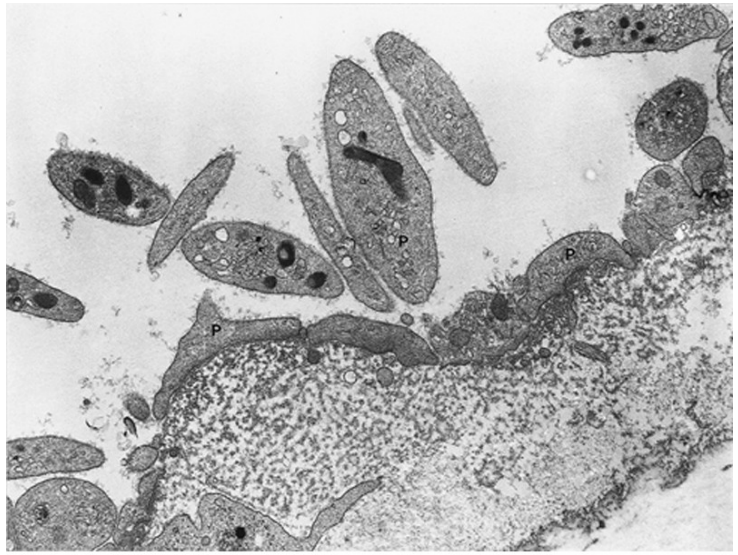
Activated, aggregating platelets illustrating fibrin strands

Adapted from: Kuwahara M *et al. Arterioscler Thromb Vasc Biol* 2002; 22: 329–34.

Platelet activation/aggregation



Resting platelets



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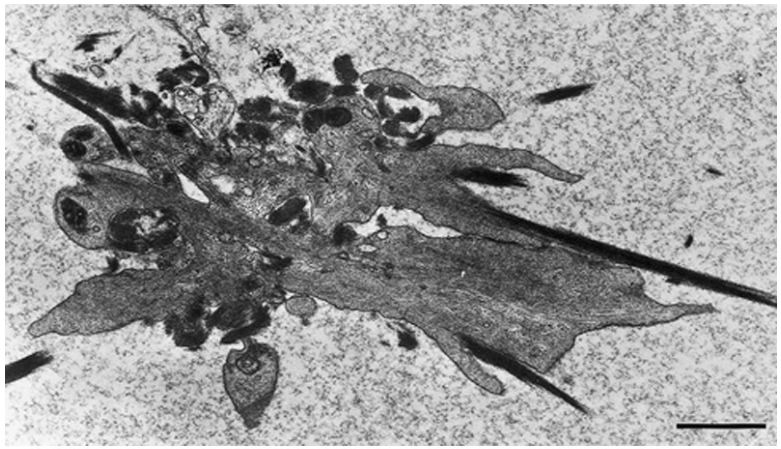

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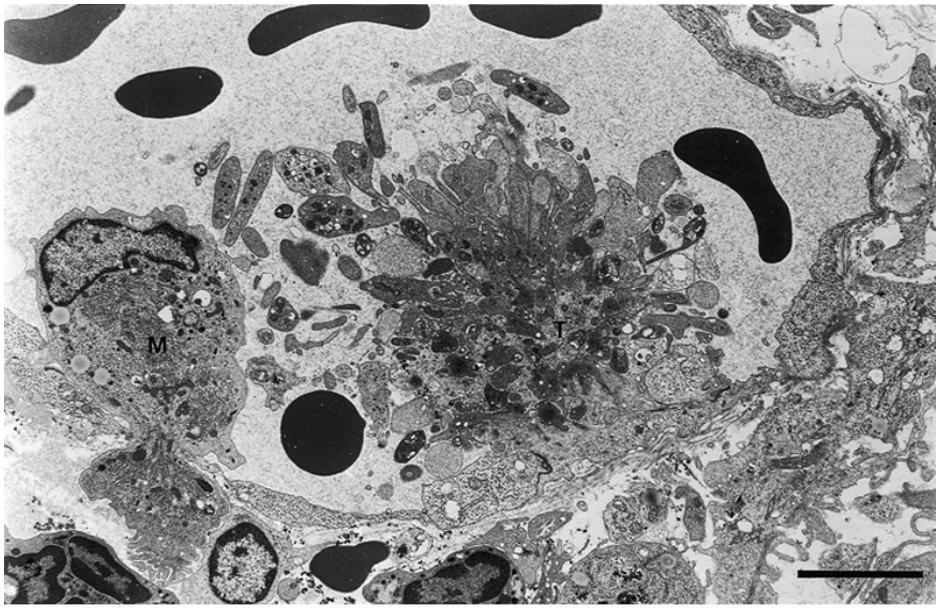
Activated platelets

Platelet aggregation



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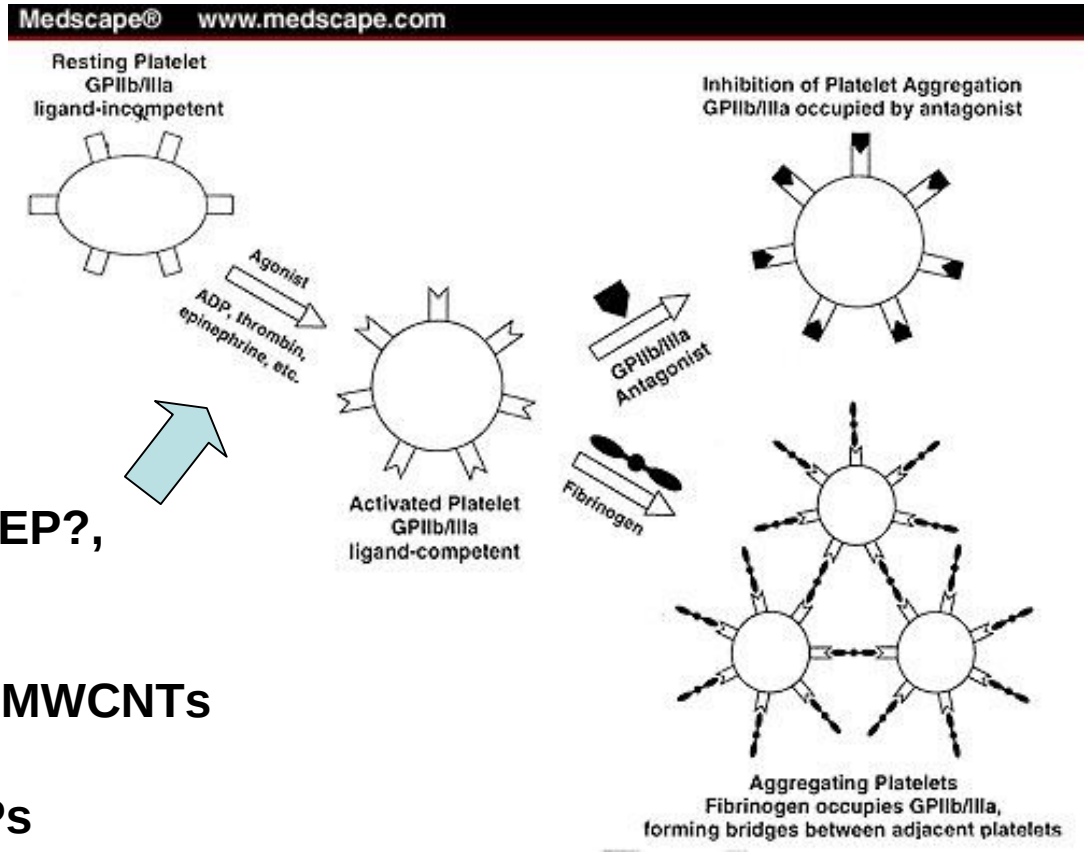
Platelet aggregation



CDNPs (DEP?, CB NPs)

SWCNTs, MWCNTs

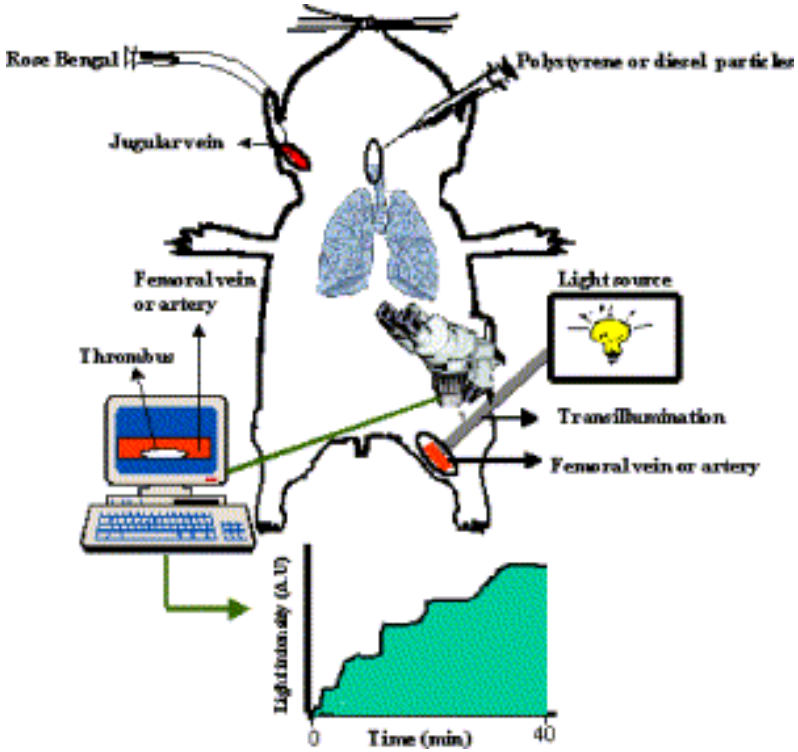
Others NPs

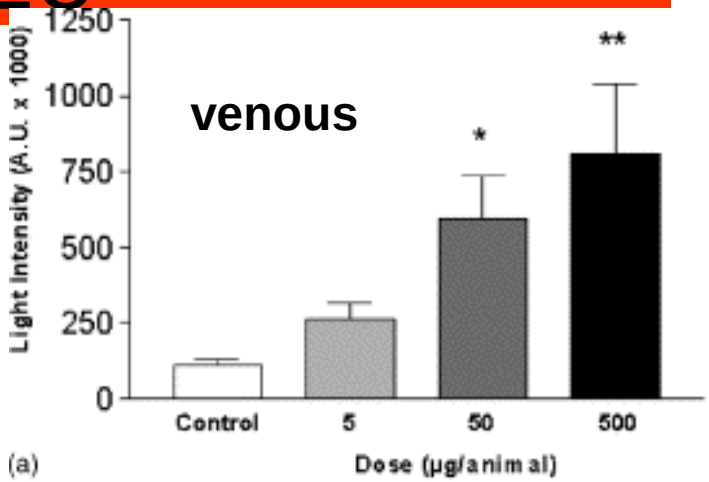
- CB NPs injected in the blood of mice induce platelet aggregation in the hepatic vasculature and pro-thrombotic changes of hte liver epatic endothelium

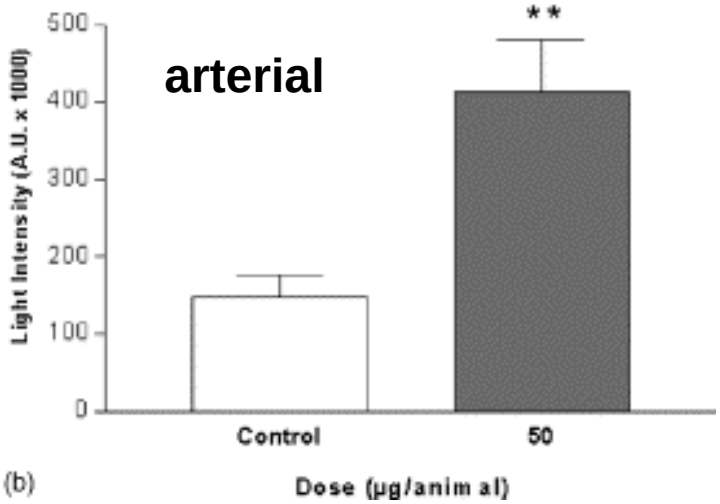
- Implanted material may generate thrombogenic MPs and NPs

- CNTs (but not fullerene C60) induce platelet aggregation

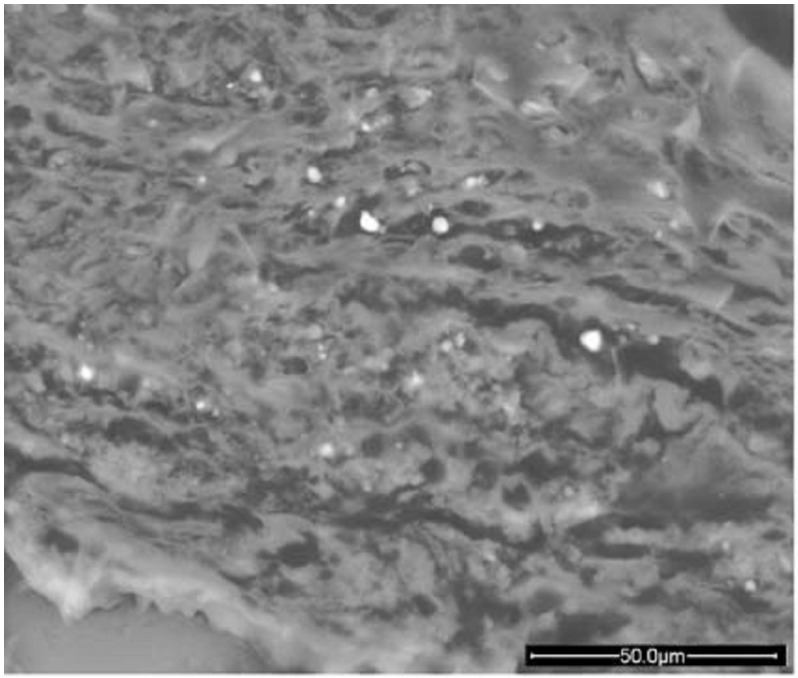
DEPs tracheal instillation increase thrombi size



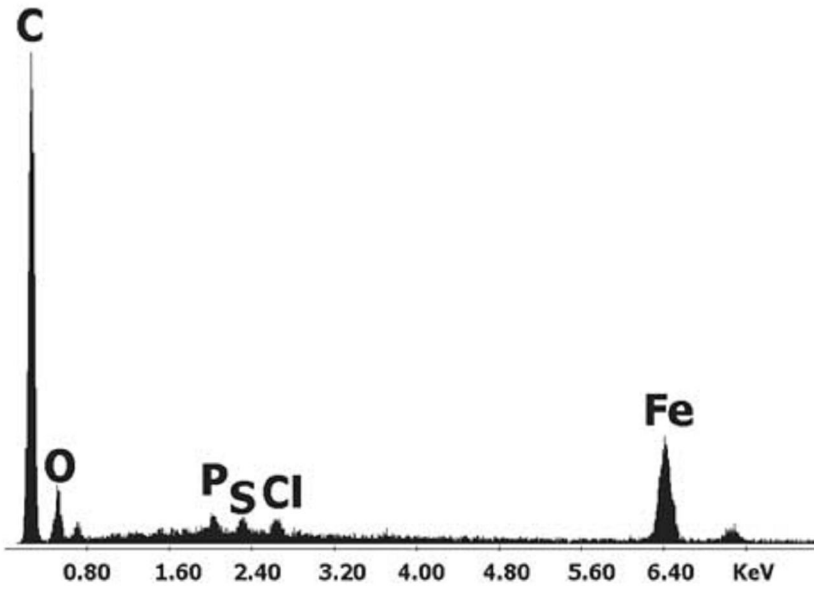




Thrompi in explanted vena cava filters form patients show NPs



(A)



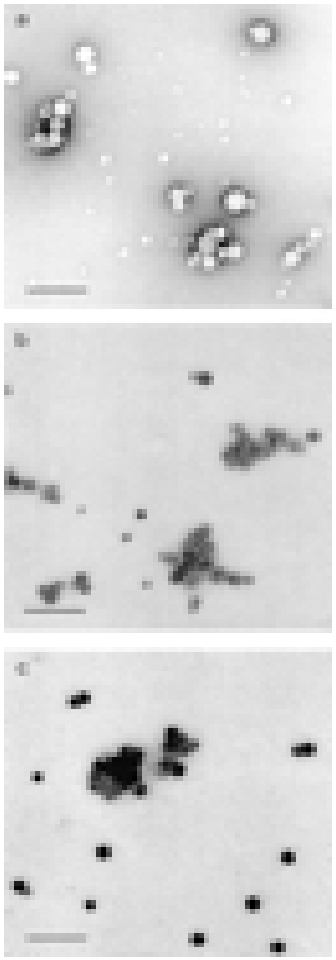
(B)

- (A) Case 11 ESEM image of a section of the thrombus containing micro-and nano-particles disseminated in the thrombus. (B) Case 11 EDS spectrum of the Fe nanoparticles from the debris.

Platelet – NPs interaction

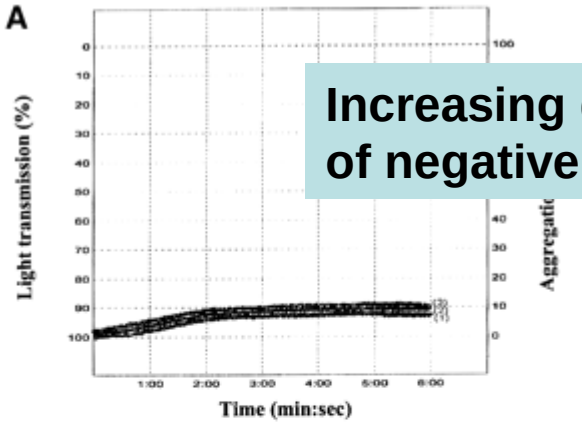
- NPs charge is suggested to play a critical role in NPs-platelet interaction and activation of blood clotting

Exp with polystyrene beads

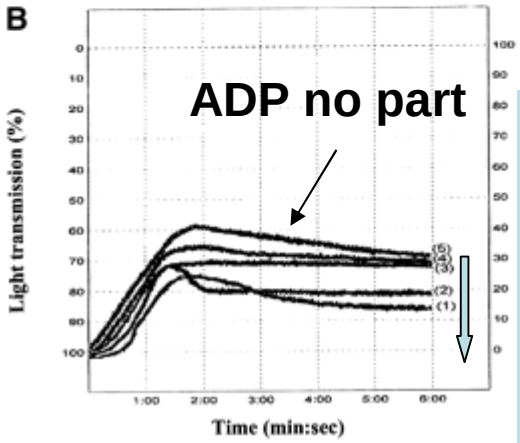


Neutral NPs	No effect on platelet
Negative NPs (carboxylated)	Inhibition of clot formation
Positive NPs (ammine-modified)	Enhanced platelet aggregation

Effect of carboxylate-polystyrene particles on platelet aggregation



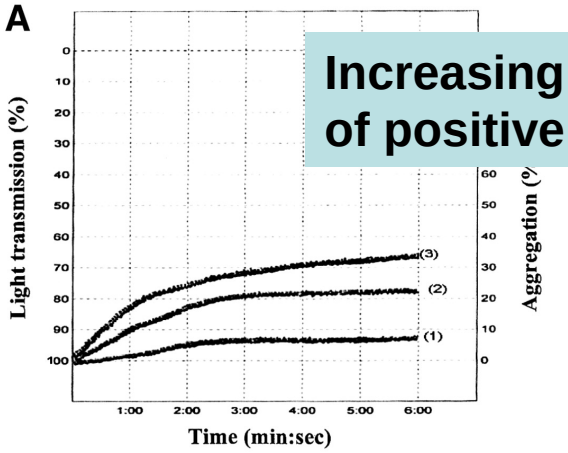
Increasing doses of negative particles



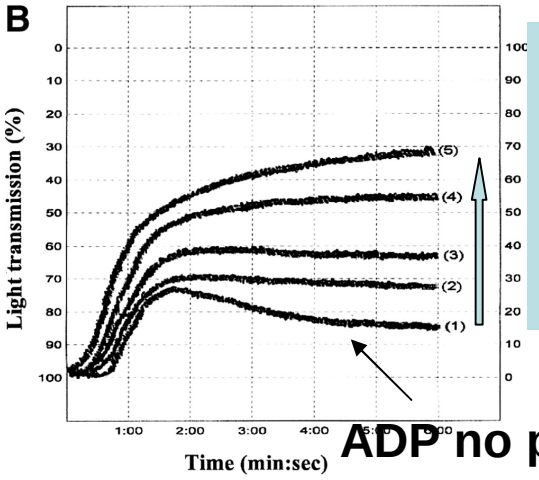
ADP no part

ADP + Increasing doses of negative particles

Effect of aminepolystyrene particles on platelet aggregation

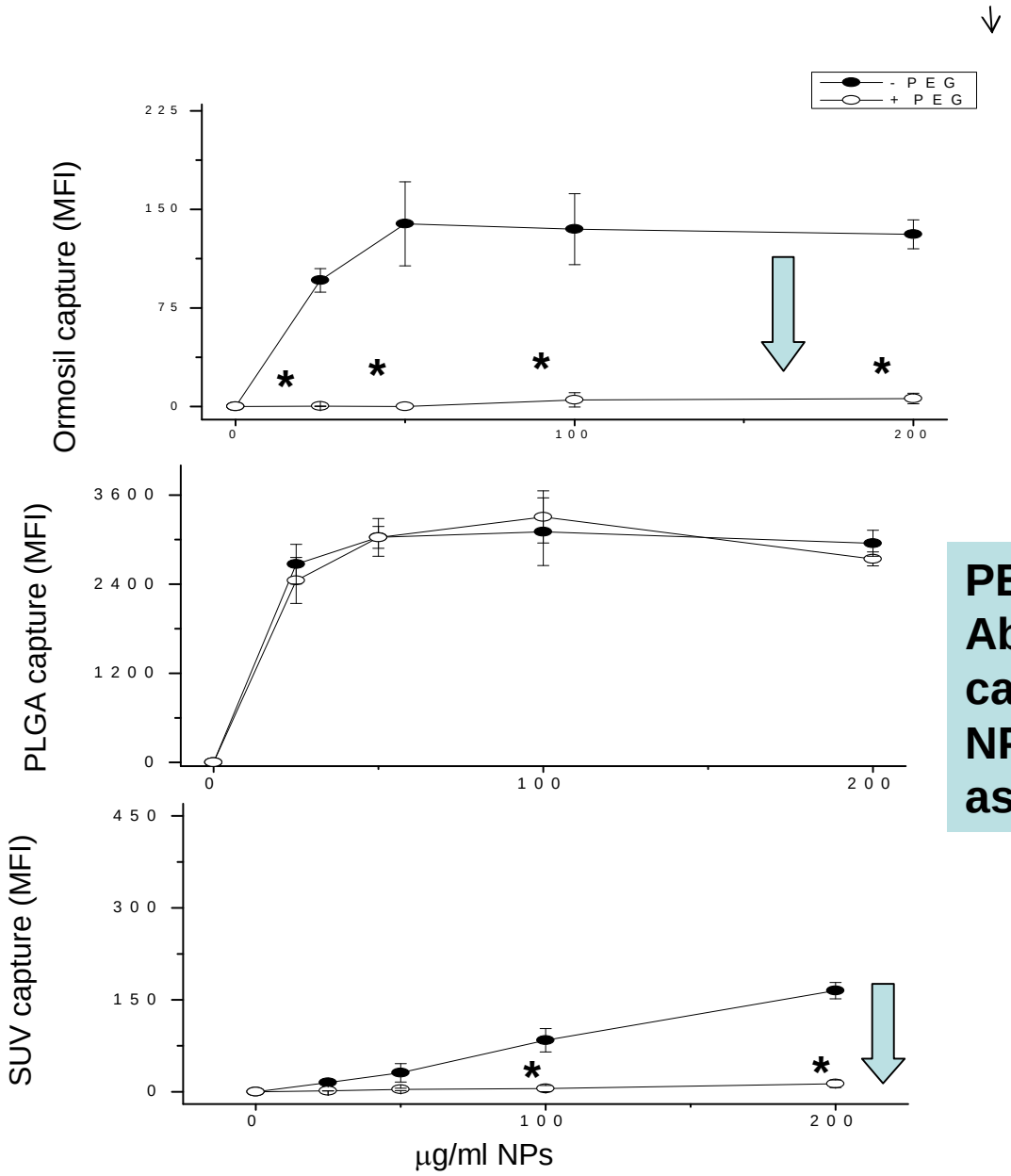


Increasing doses of positive particles



ADP + Increasing doses of positive particles

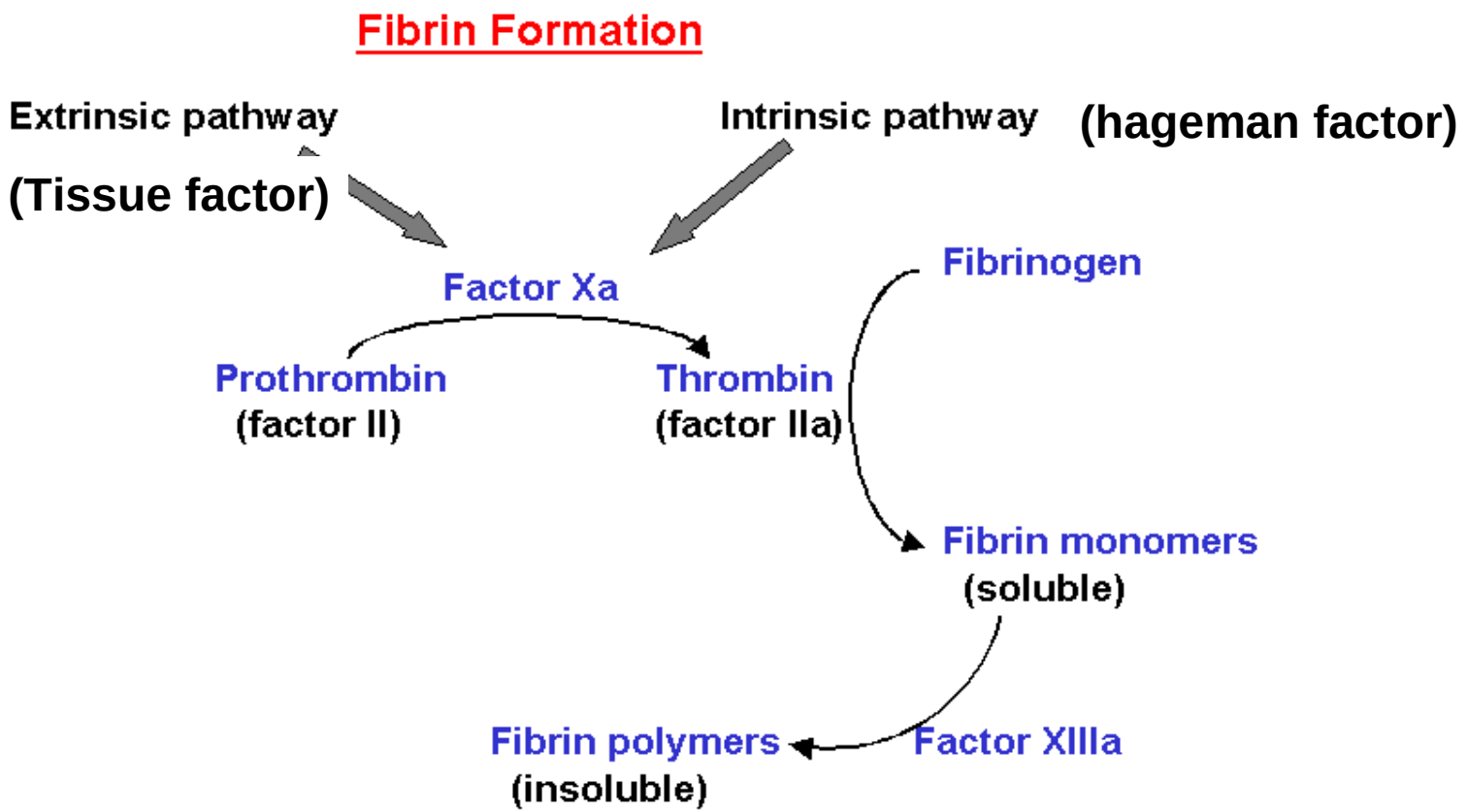
ADP no part



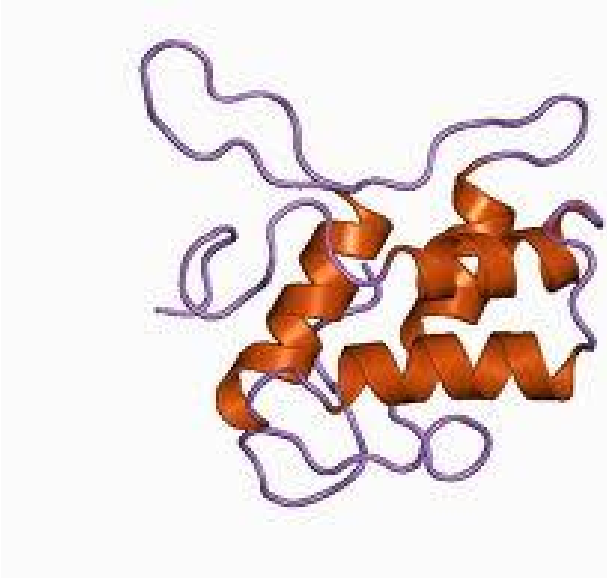
PEG-coating Abolish in some cases NPs-platelets association

Association of some NPs to platelets

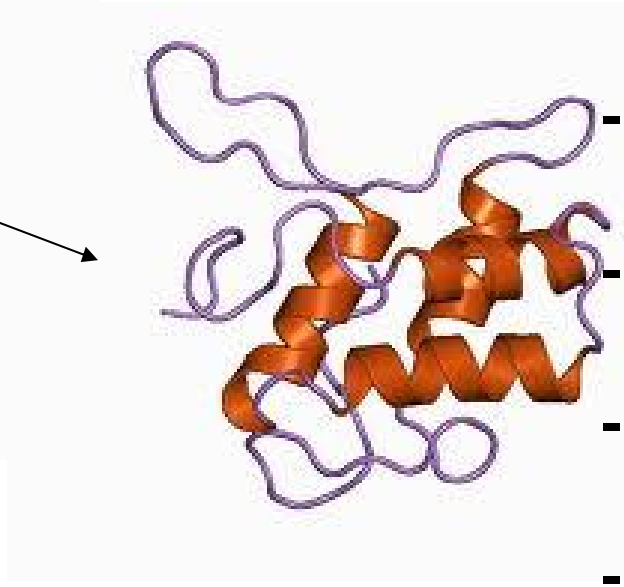
Coagulation pathways



Hageman factor



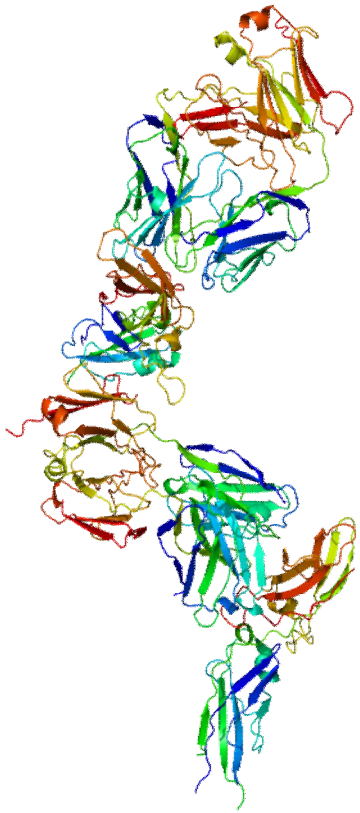
Soluble protein in plasma

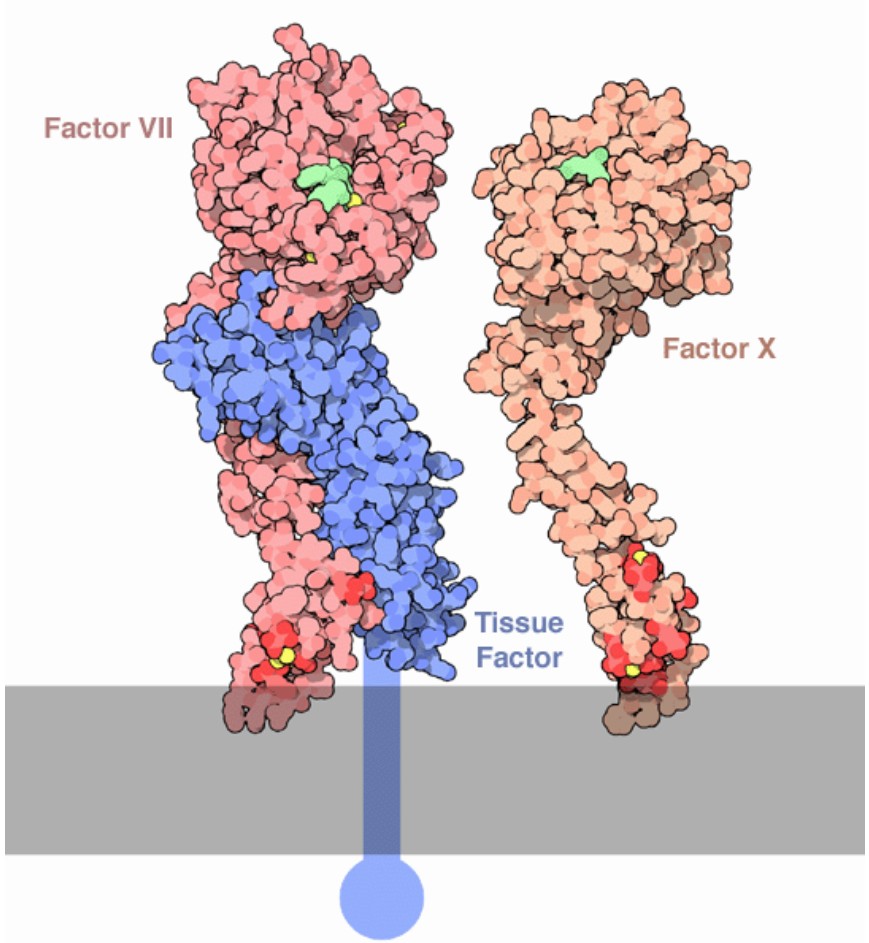


it adsorbes on negative surfaces

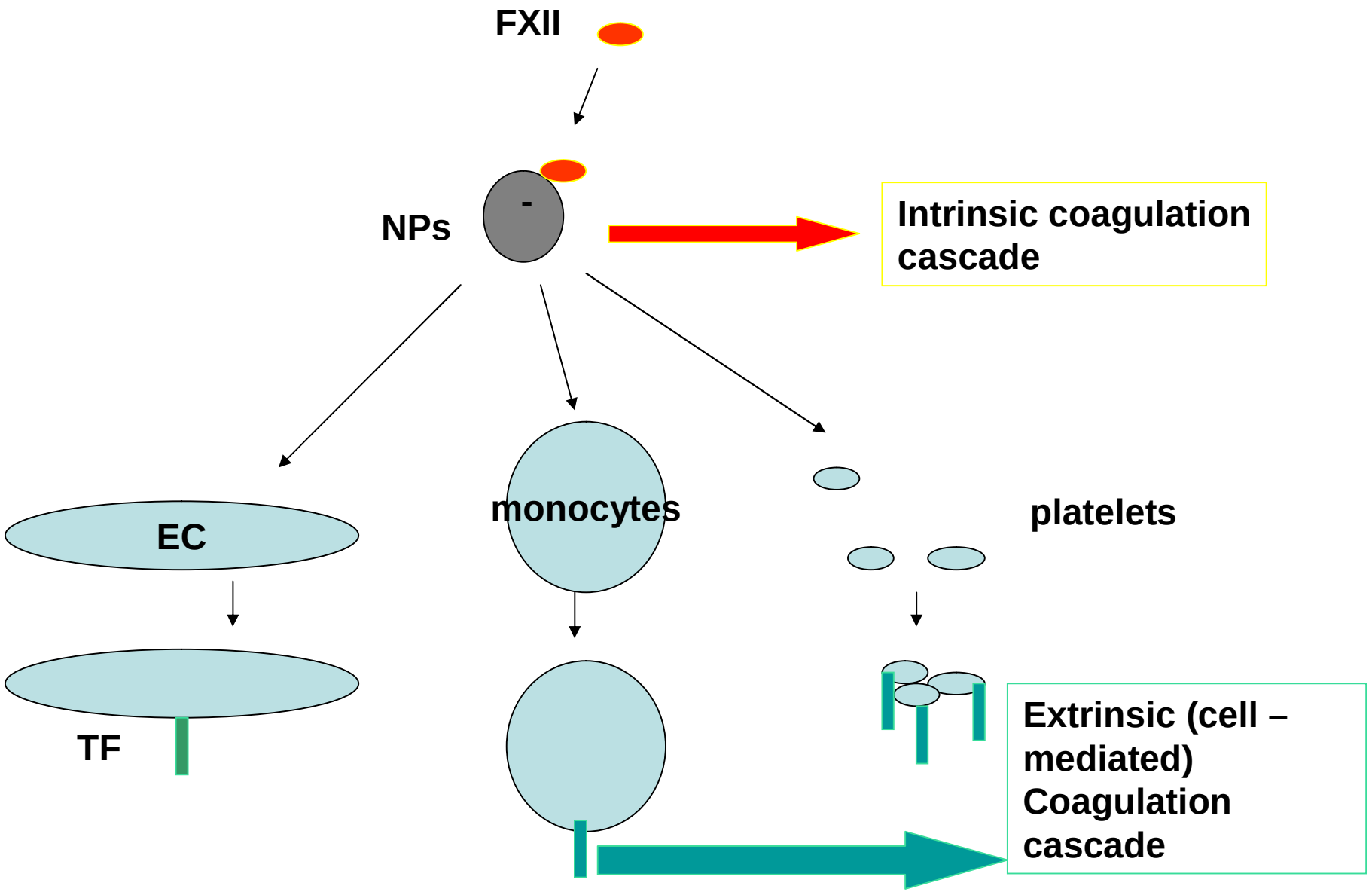
Negative Surface (material, Glass, Collagen exposed Subendothelial Tissue, NPs?

Tissue factor

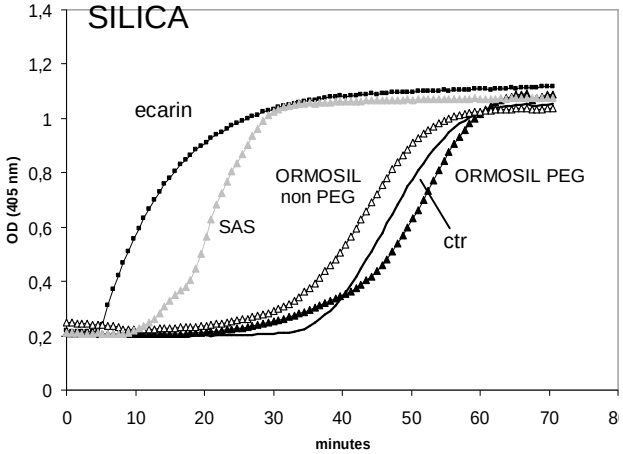




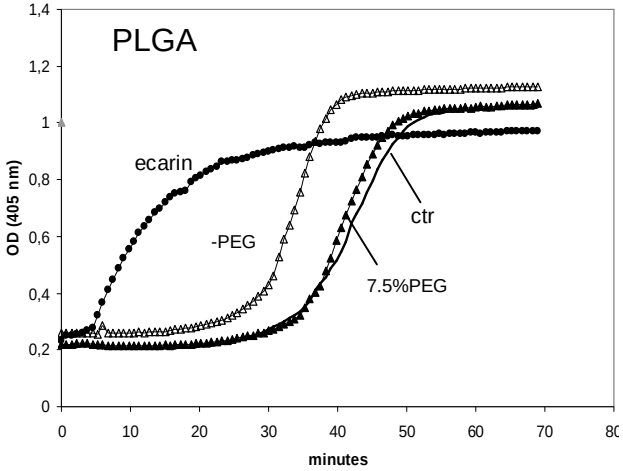
TF **Activated endothelial cells, monocytes, platelets**


FXII
NPs
-
Intrinsic coagulation cascade
EC
TF
monocytes
platelets
Extrinsic (cell – mediated) Coagulation cascade

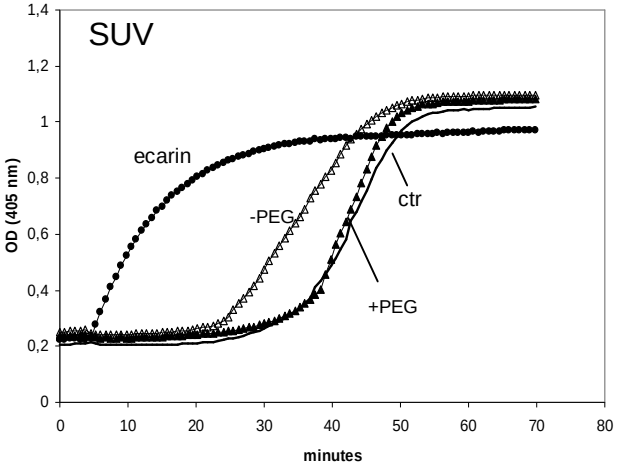
SiO_2 NPs



Poly-lactiv-glycolic-acid NPs



liposomes



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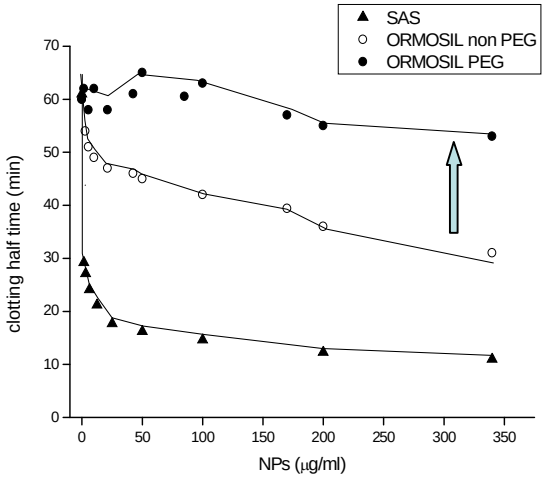
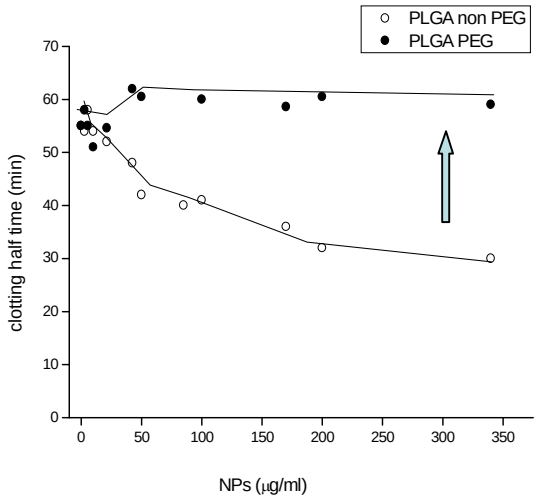
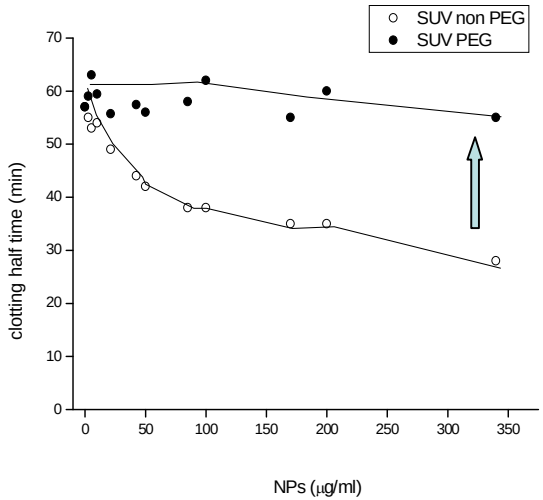
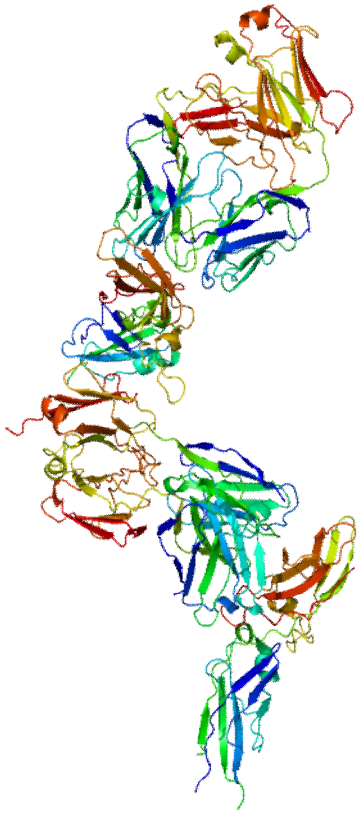




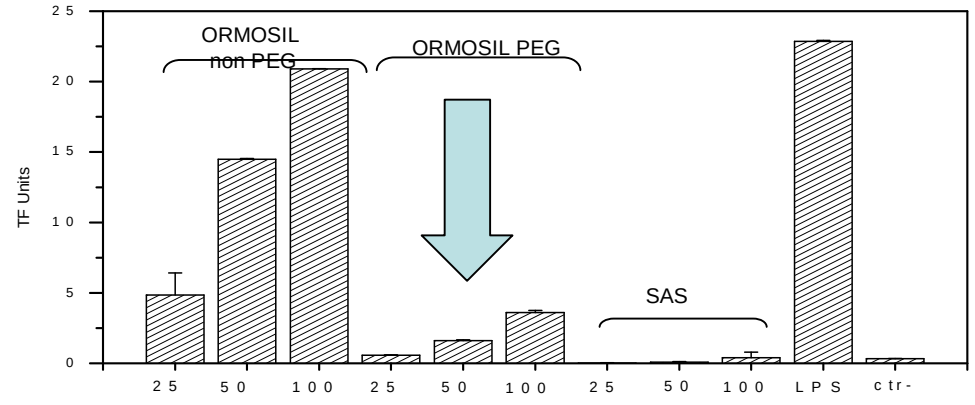


Plasma coagulation due to Hageman factor adsorption

PEG-coating inihibits NPs coagulation via the intrinsic pathway

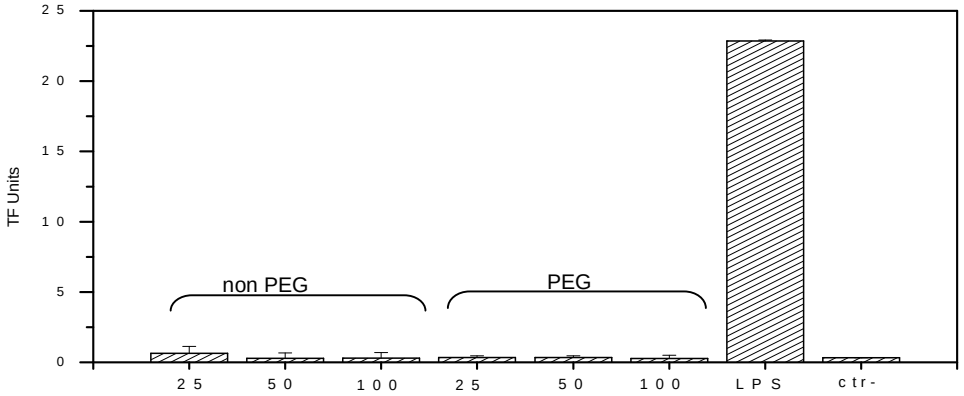


TF induction
In monocytes
By NPs

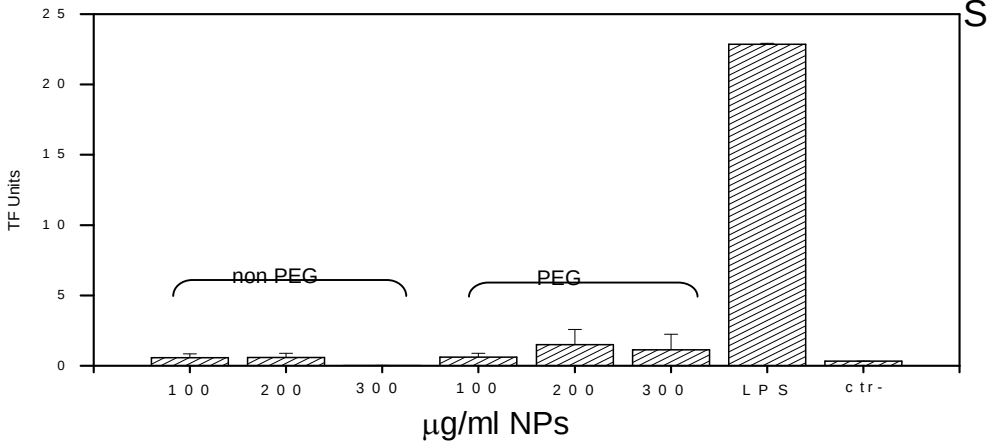


SILICA

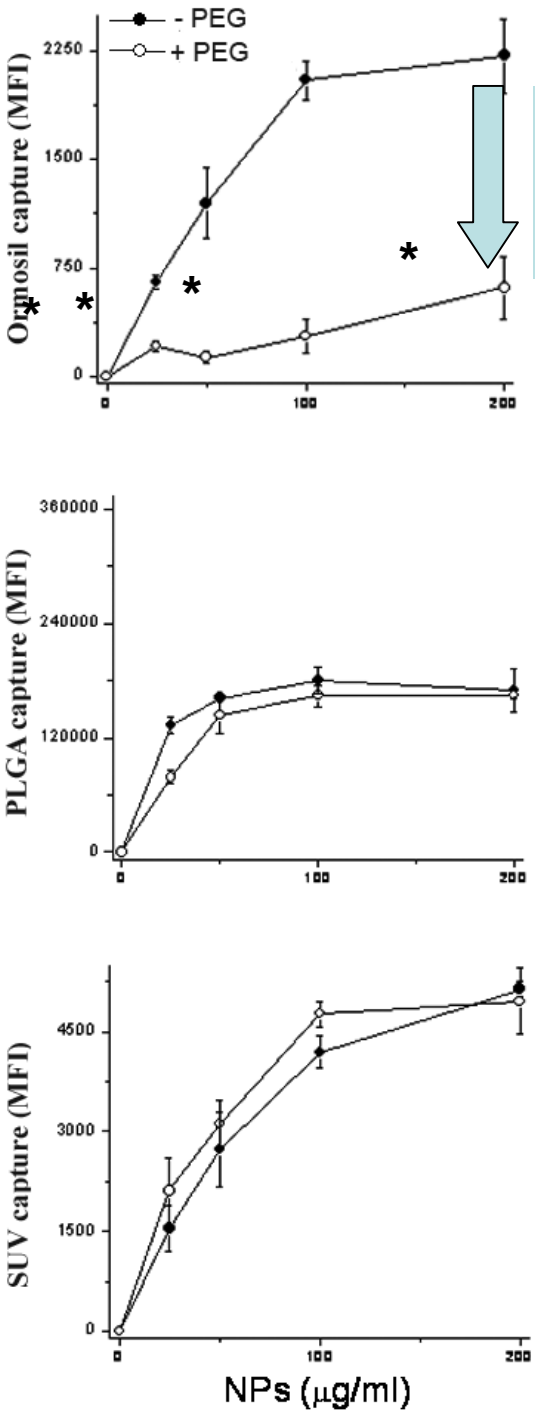
PEG-coating



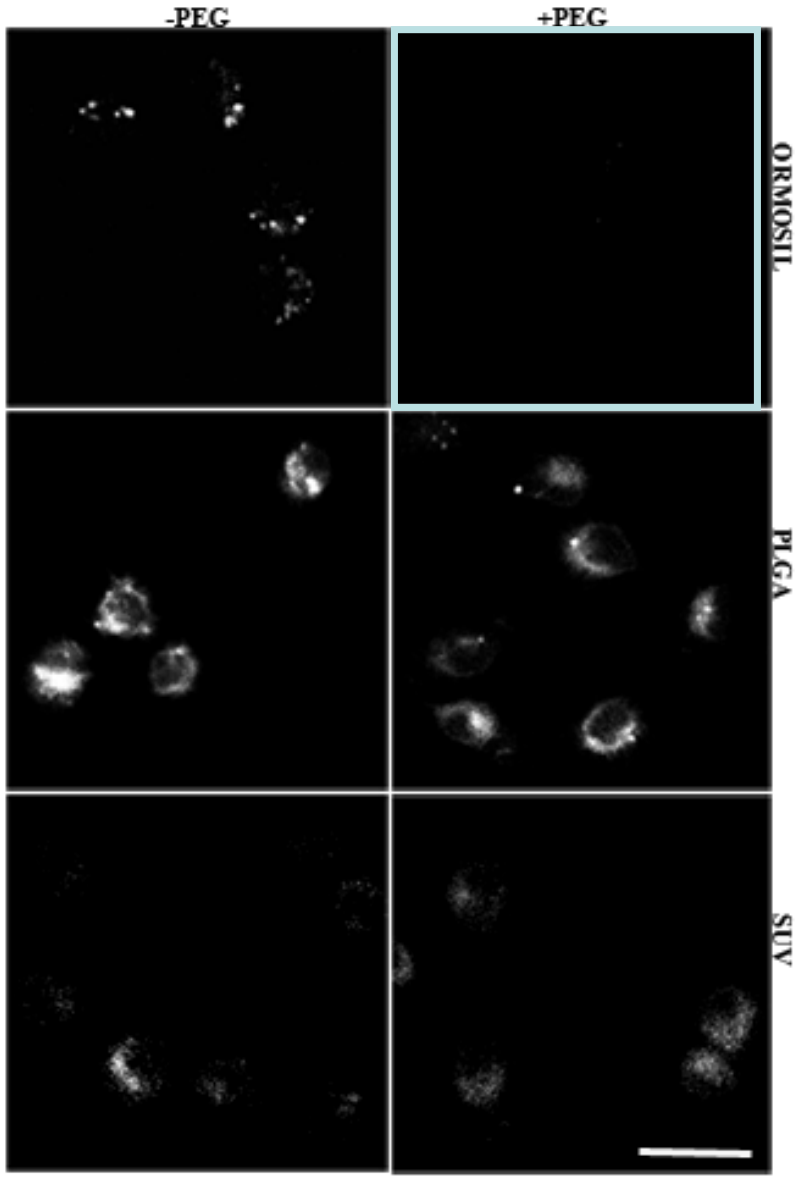
PLGA



SUV



PEG-coated NPs

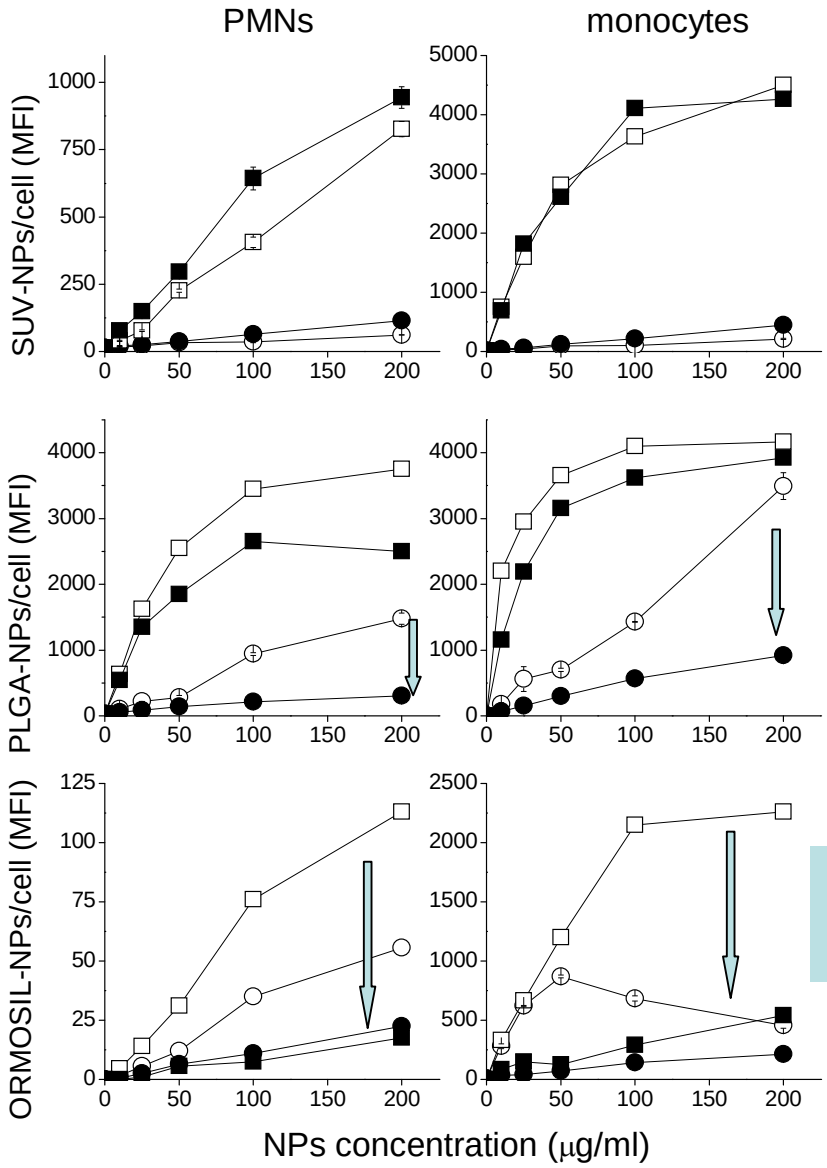


NPs capture by monocytes

NPs-cell association in the blood

- Red blood cells: they have no endocytic activity: adsorption on the surface? Membrane translocation?
- Circulating monocytes and PMNs are professional phagocytic cells: they can capture NPs
- Endothelial cells: cationic NPs are captured 10x more efficiently than negative-neutral ones

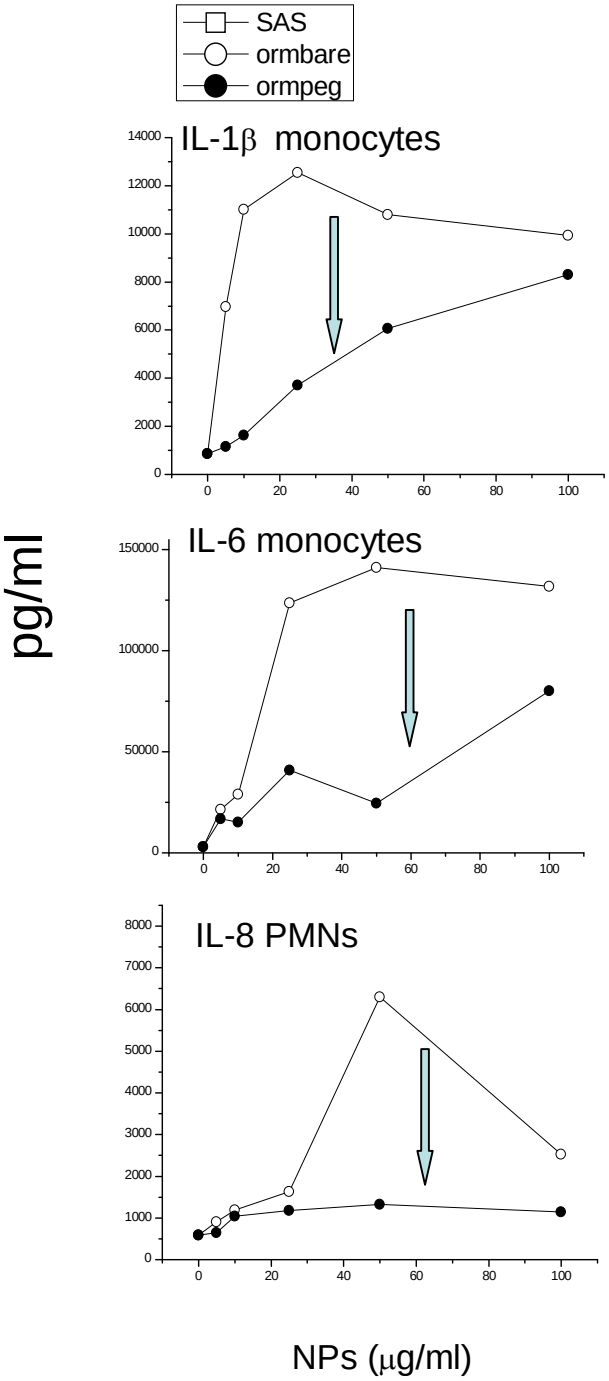
fig 2



Monocytes and PMNs capture different type of NPs

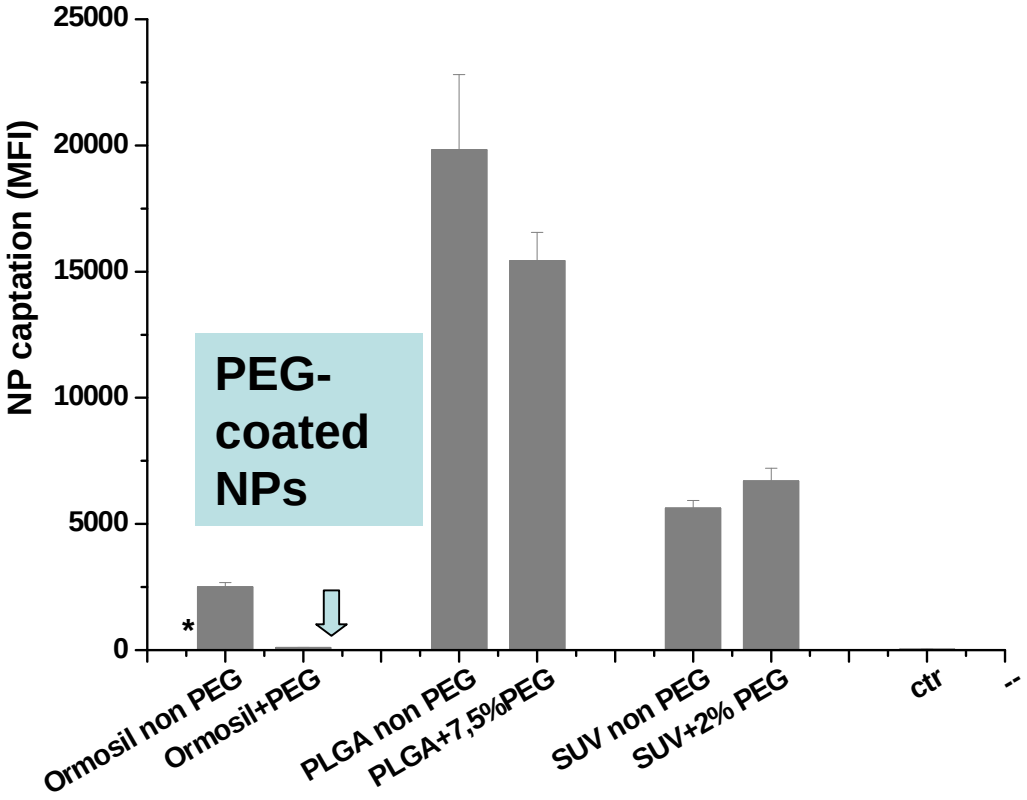
PEG-coated version of some of them are "stealth": reduced capture

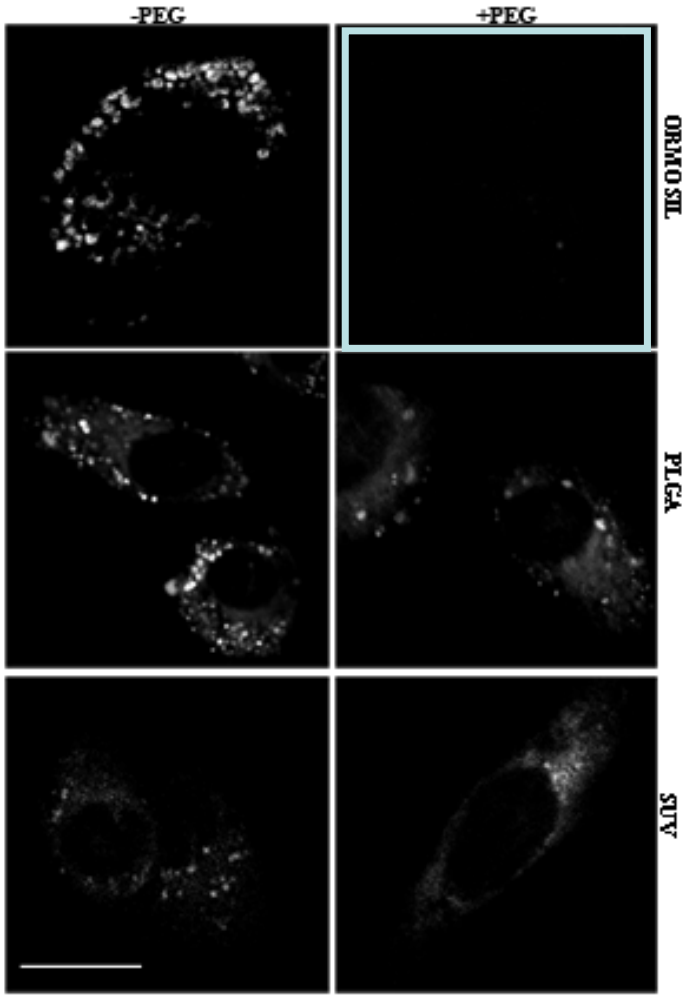
Fig 5



ORMOSIL-NPs interacting with Monocytes and PMNs Can induce proinflammatory Cytokines and chemochines

PEG-coated NPs





NPs capture by endothelial cells in vitro (HUVEC)

Blood-organs translocation

- Endothelial crossing (normal and pathological situations)
- Other organs
- The Reticular Endothelial System (RES)
- Blood-brain barrier crossing

Endothelial barrier

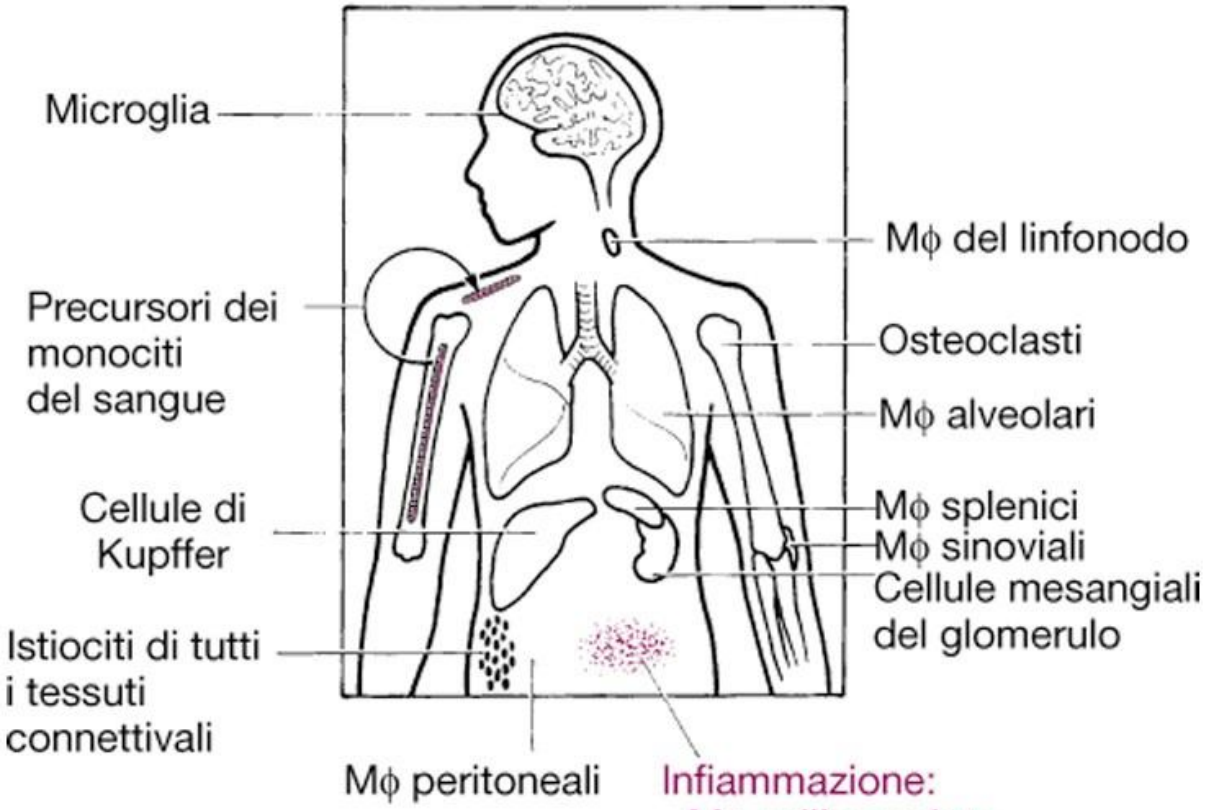
- In general endothelial cells have very tight junctions (~ 2 nm Ø)
- Larger values are however found in certain organs: e.g in the liver the endotheliaum is fenestrated with pores up to 100 nm..
- IMPORTANT: inflammation and tumor alterations leads to an increased endothelial permeability!

injection of NPs

route	First	later
intradermal	Lymphatic vessels	Regional lymph nodes
intramuscular	Neuronal uptake + lymphatic vessels	Ganglia, SNC? + regional lymph nodes
intravenous	Blood: quickly in the systemic circulation	many organs

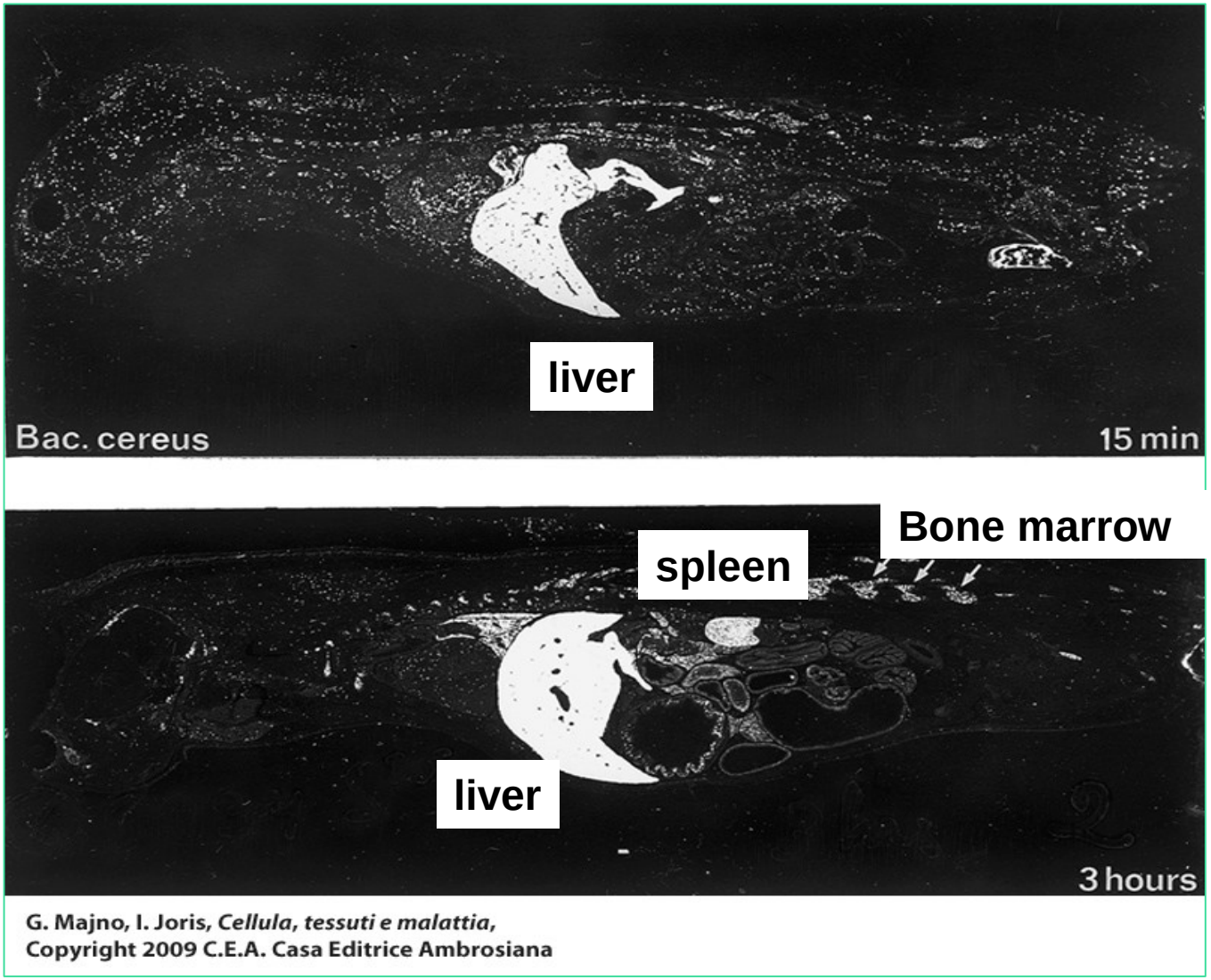
- Several studies with QDs, C_{60} fullerenes, polystyrene beads and plant virus (10-240 nm Ø) injected intravenously indicated translocation to:
- Liver, spleen, bone marrow, lymph nodes, small intestine, brain, lung…
- In general PEG-coating diminish Liver-spleen capture

CELLULE DEL SISTEMA DEL FAGOCITA MONONUCLEATO



Mϕ = macrofagi

Autoradiography of mice with venously injected radioactive bacteria



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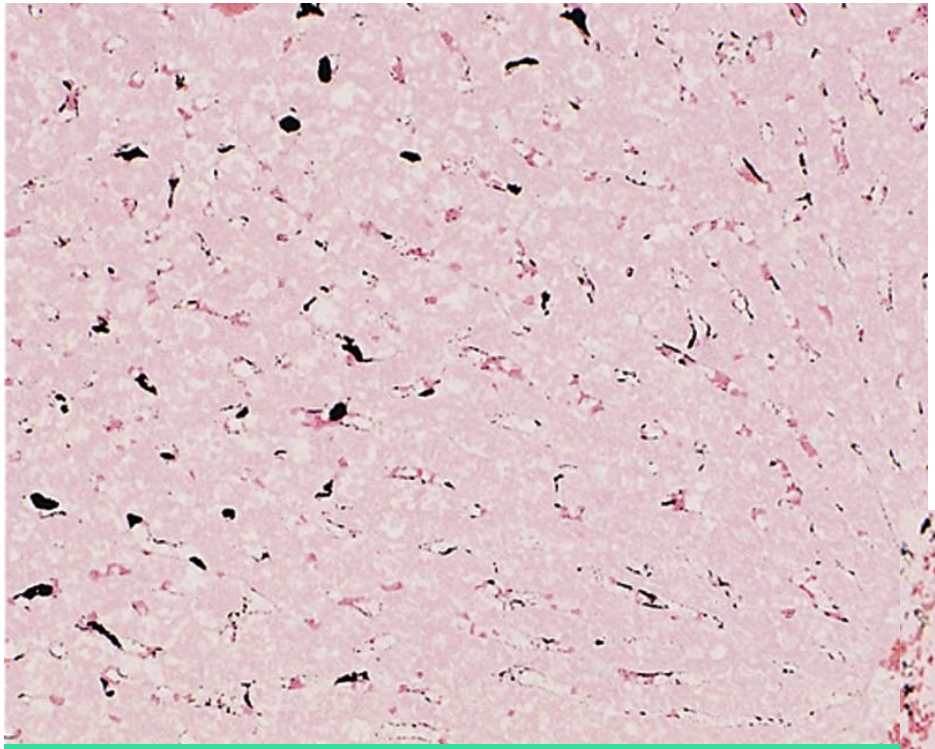
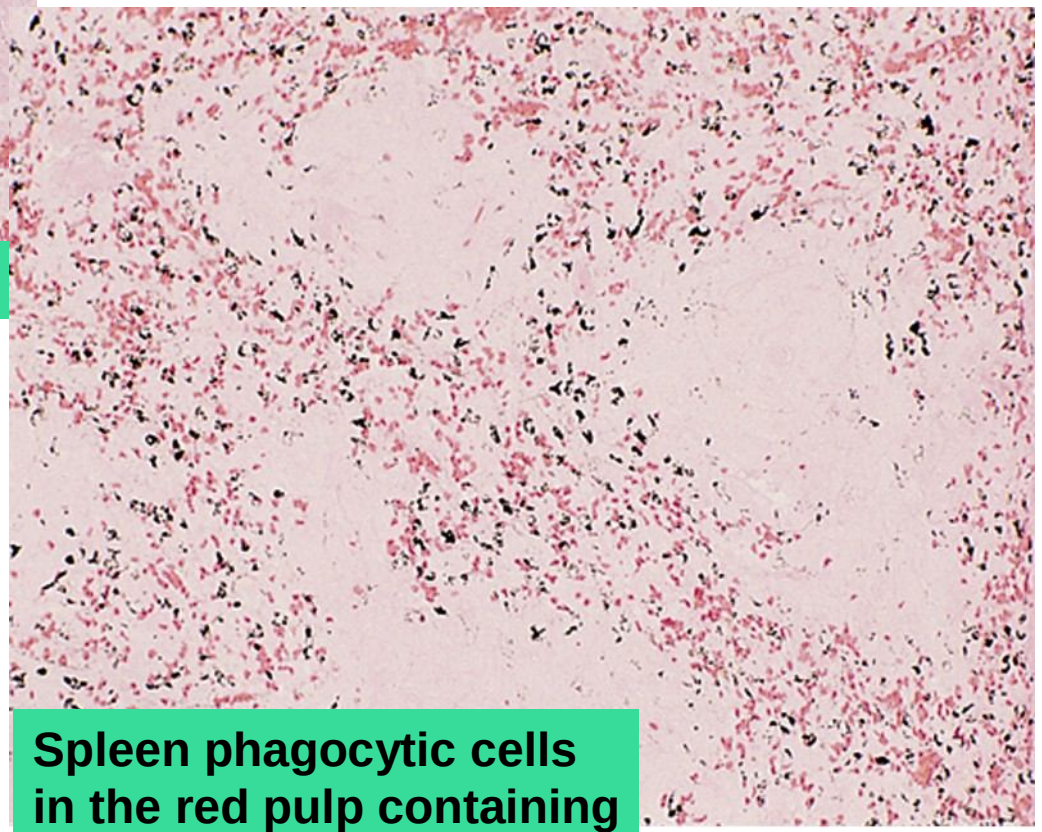
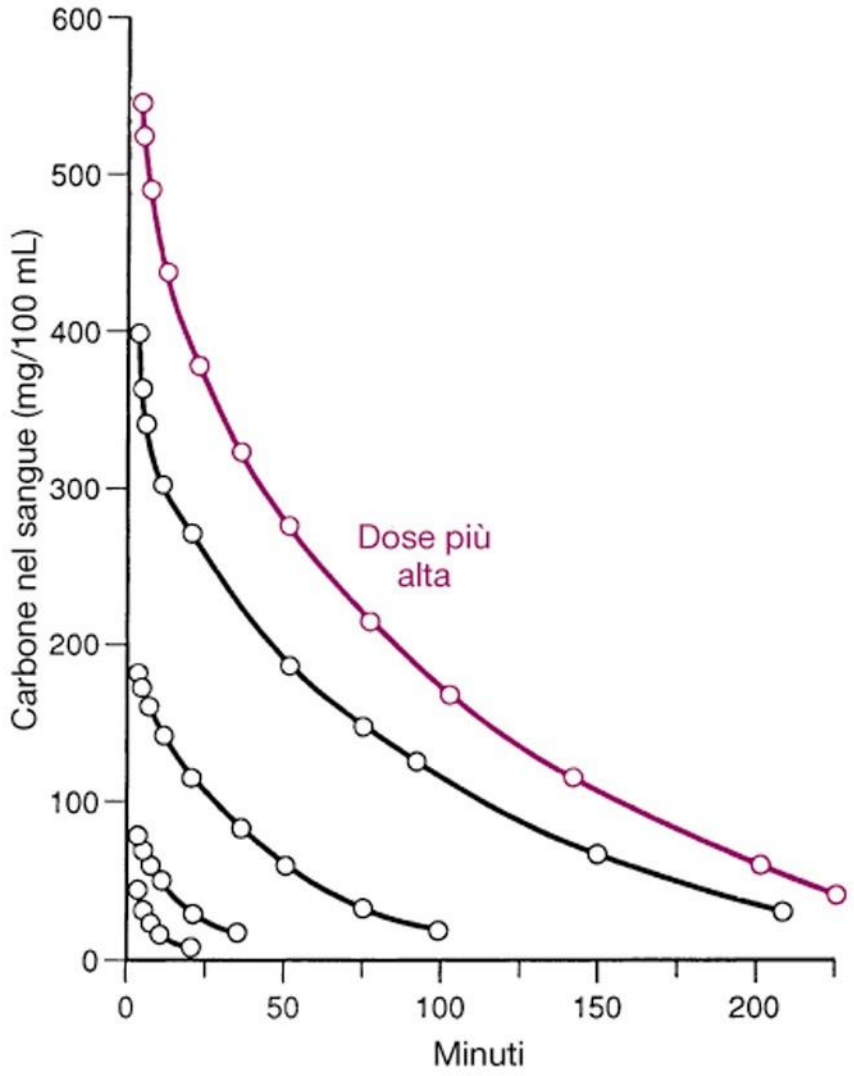


Liver Kupffer cells containing CB NPs



Spleen phagocytic cells in the red pulp containing capture CB NPs



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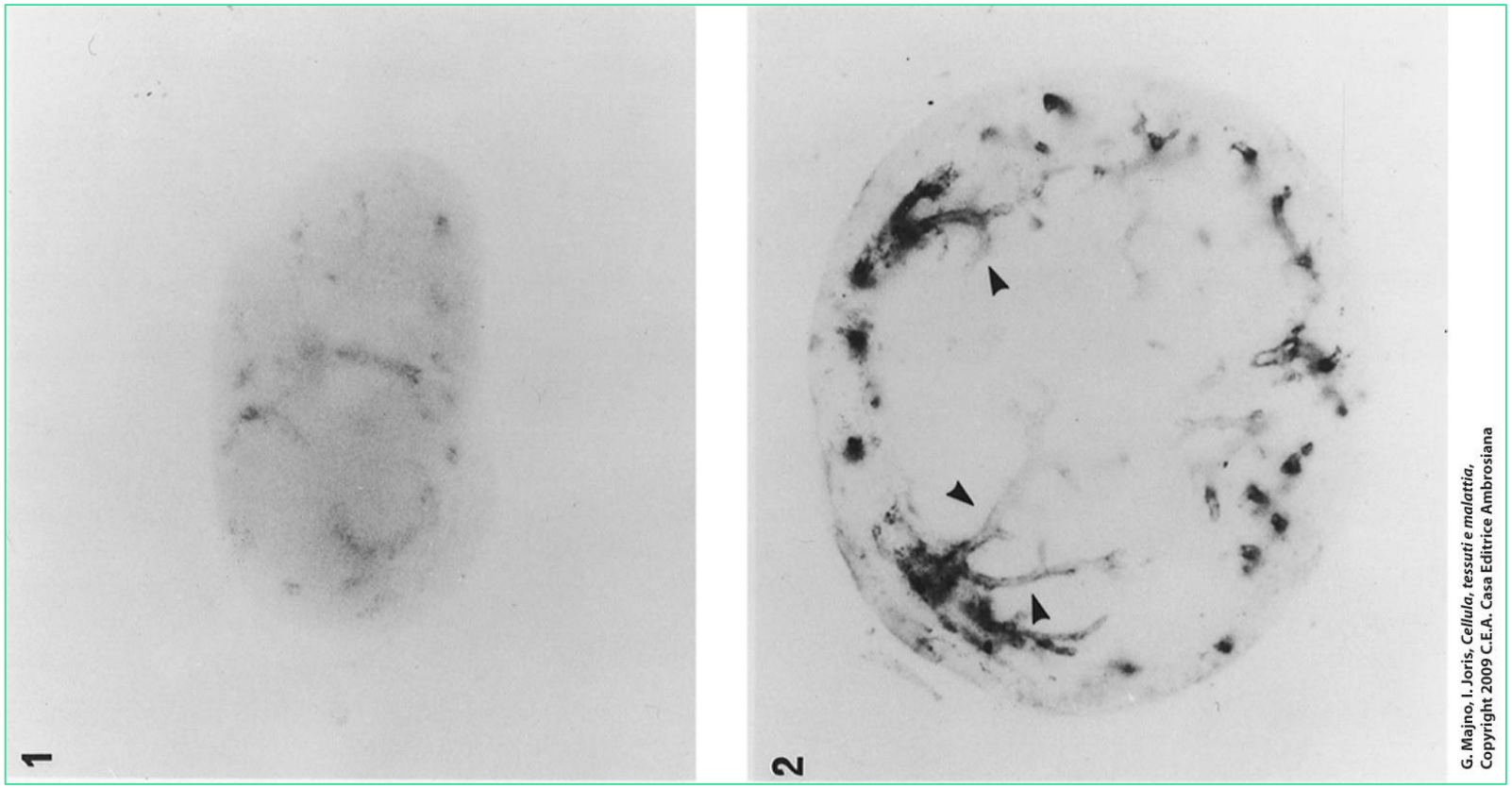
- Clearance rate of CB NPs from the blood due to the RES

noemal

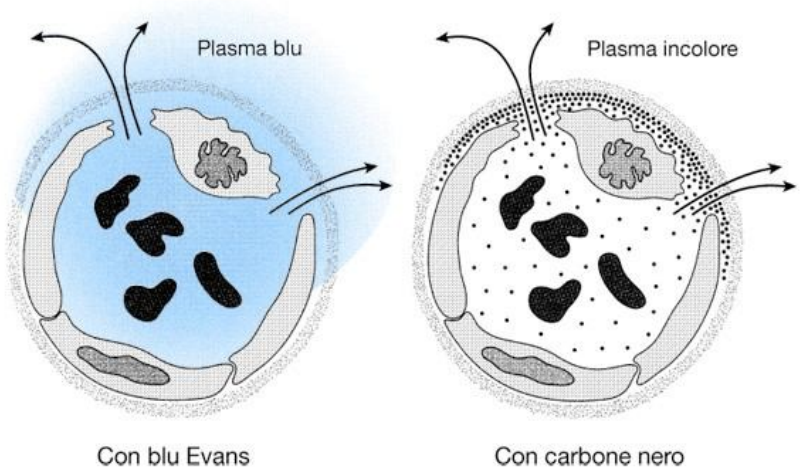
Acute inflammation

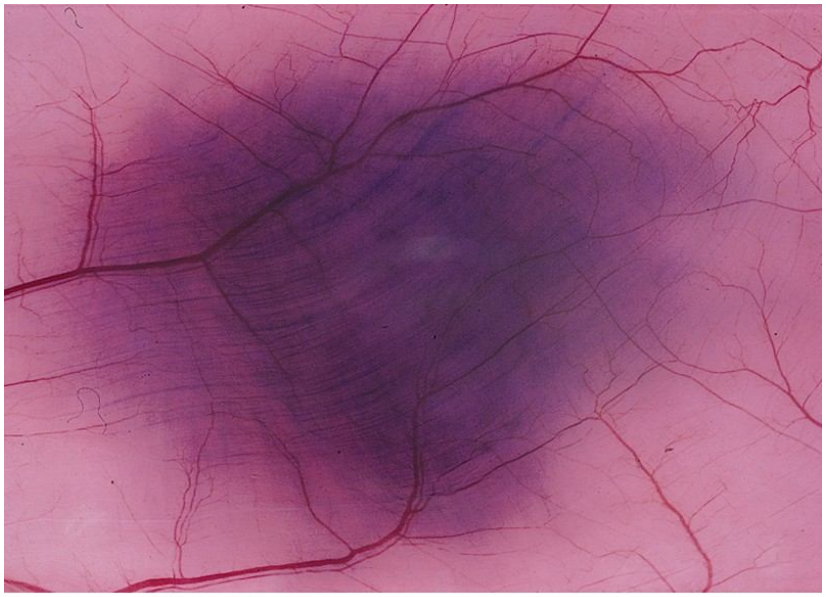


Translocation of CB NPs in the poplitean lymphnodes in a noral rat and in a rat in which the footpad was injected with LPS

The basament membrane is a NPs barrier

- It retains NPs larger than 20-30 nm





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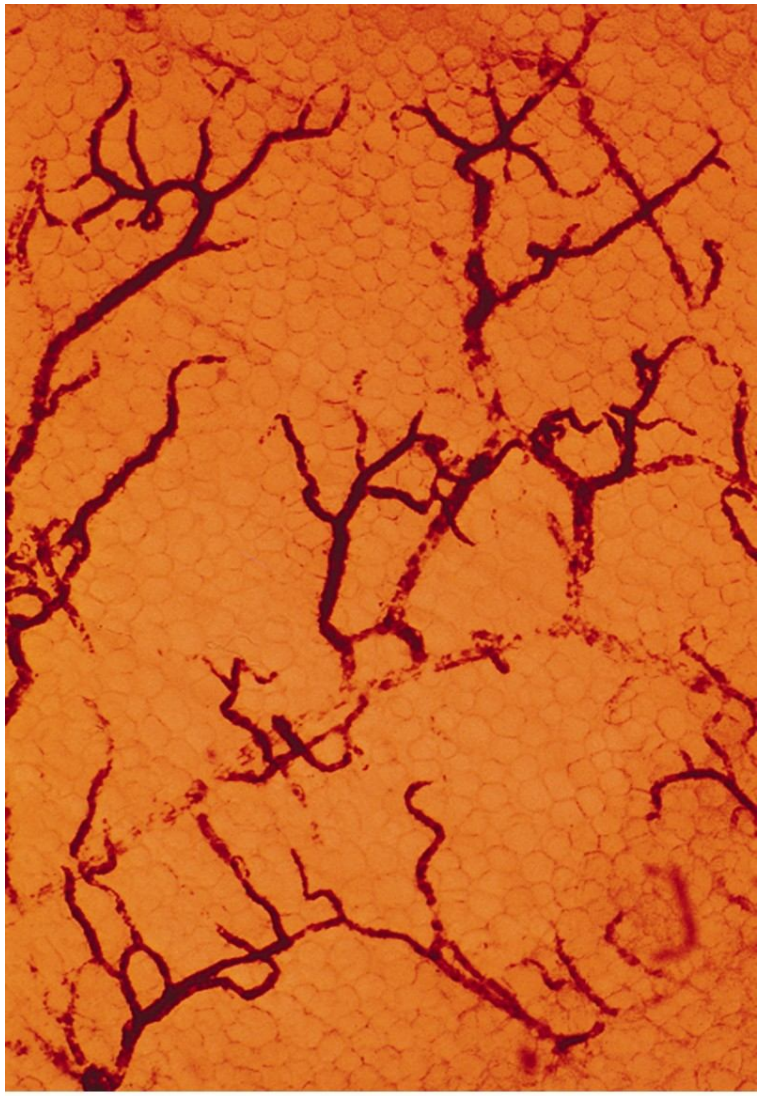
Blue evans bound to albumin freely diffuse in the tissue from inflammed Blood microvessels

CB NPs label the walls of the Inflammed small blood vessels



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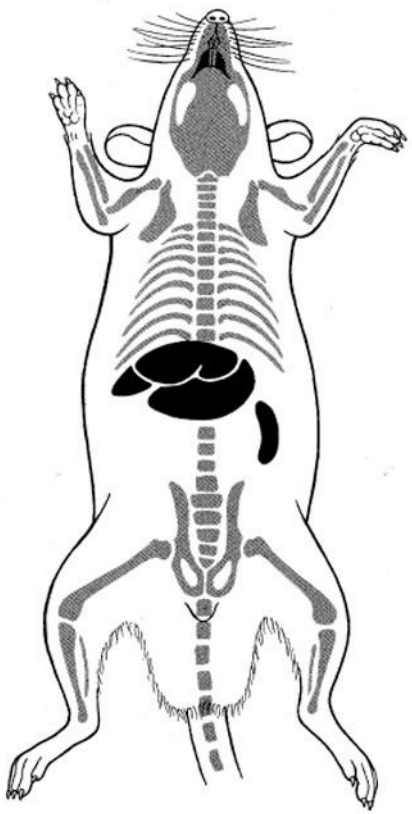
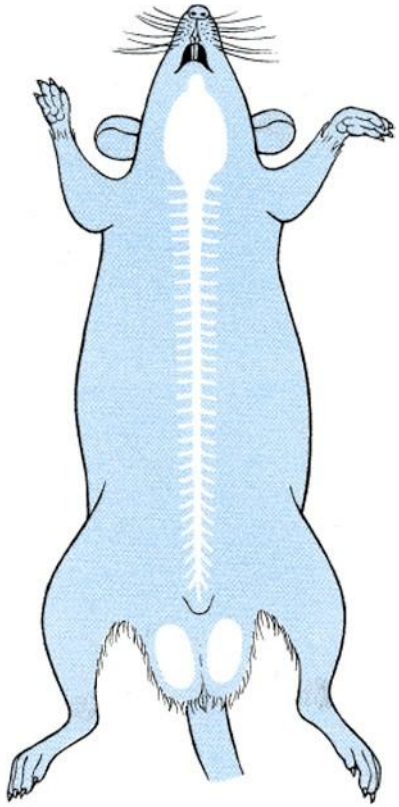
CB NPs distribution

Blue Evans-Albumine distribution





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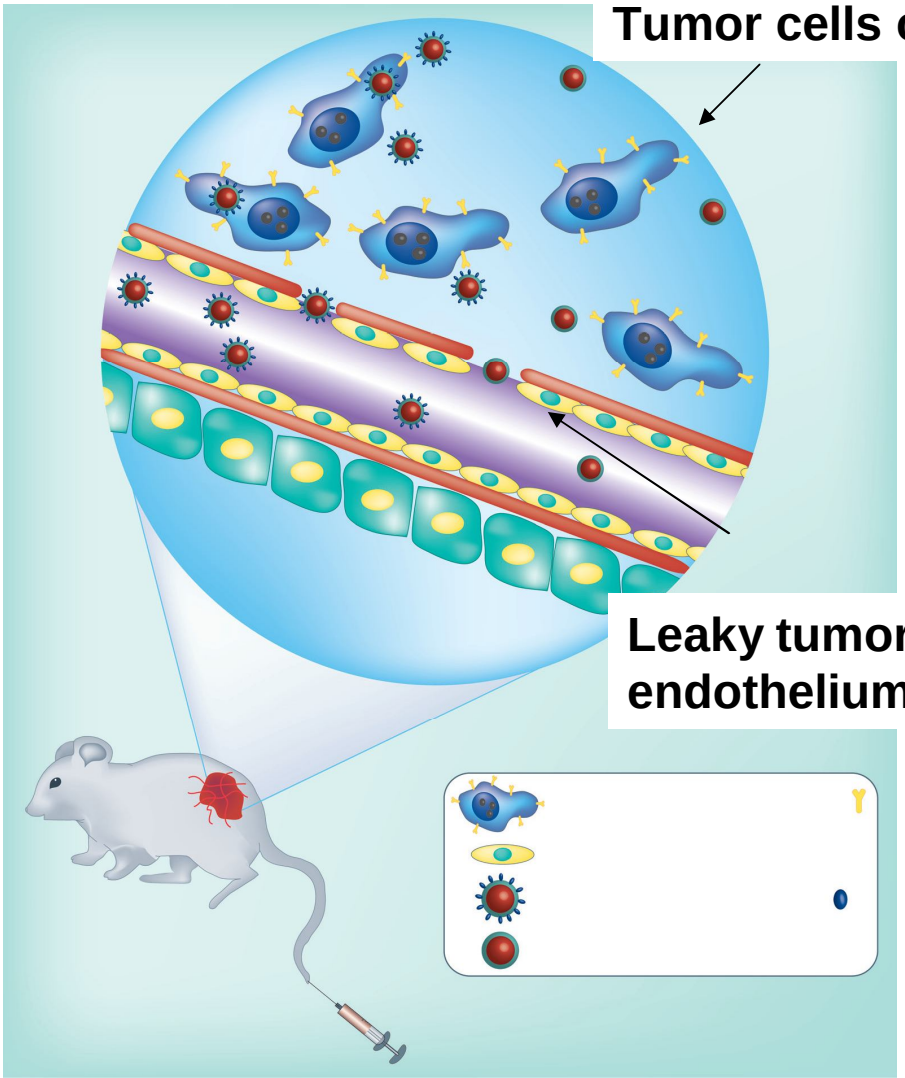
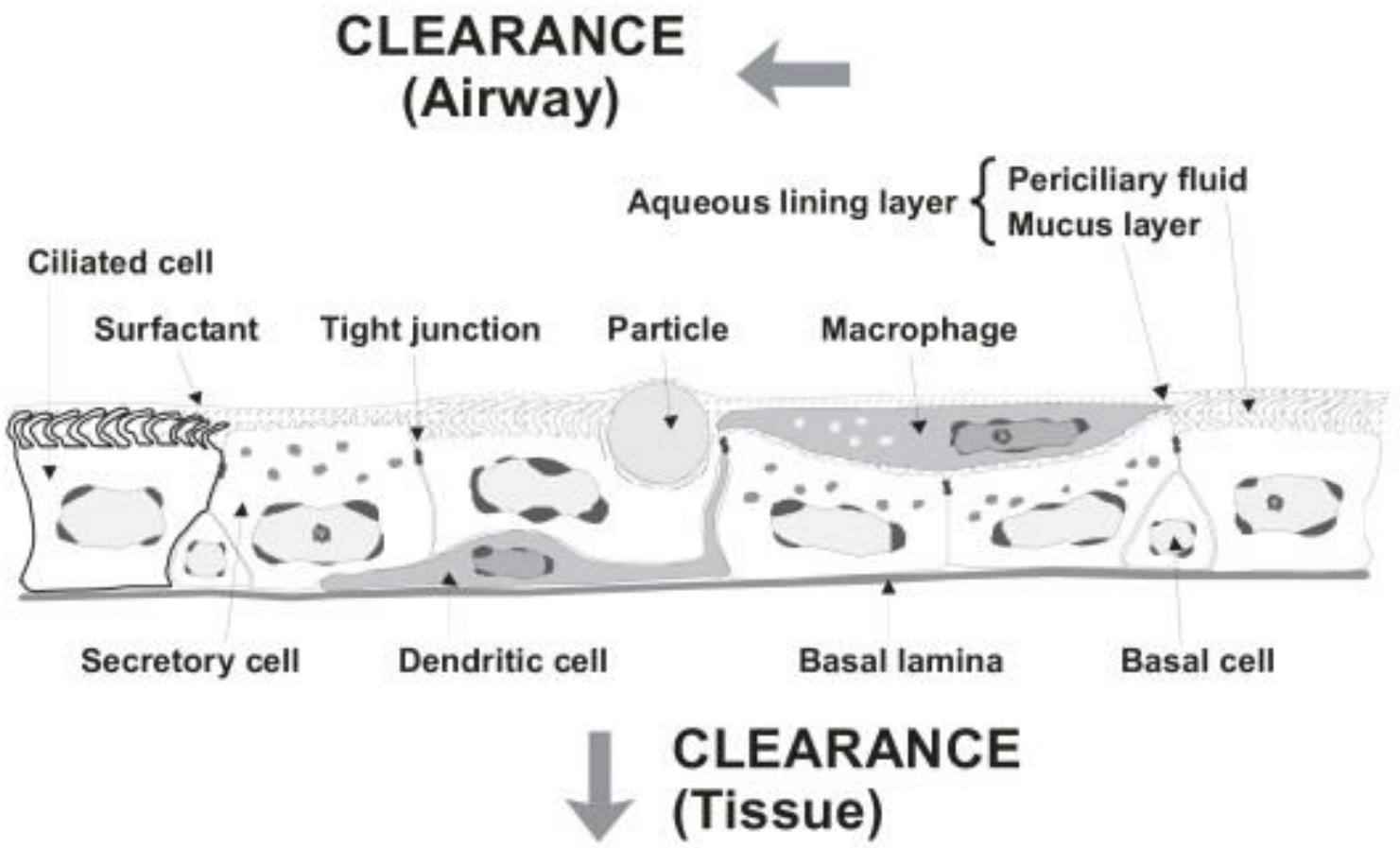
If the NPs does not cross the endothelium
It is captured by the RES: liver, spleen mostly (black) and bone marrow less intensely (grey)

Only small molecules (albumine size) can easely cross the endothelial barrier
With the esception of SNC and teaticles

active and passive tumor targeting is favoured by endothelial and basal membrane alterations



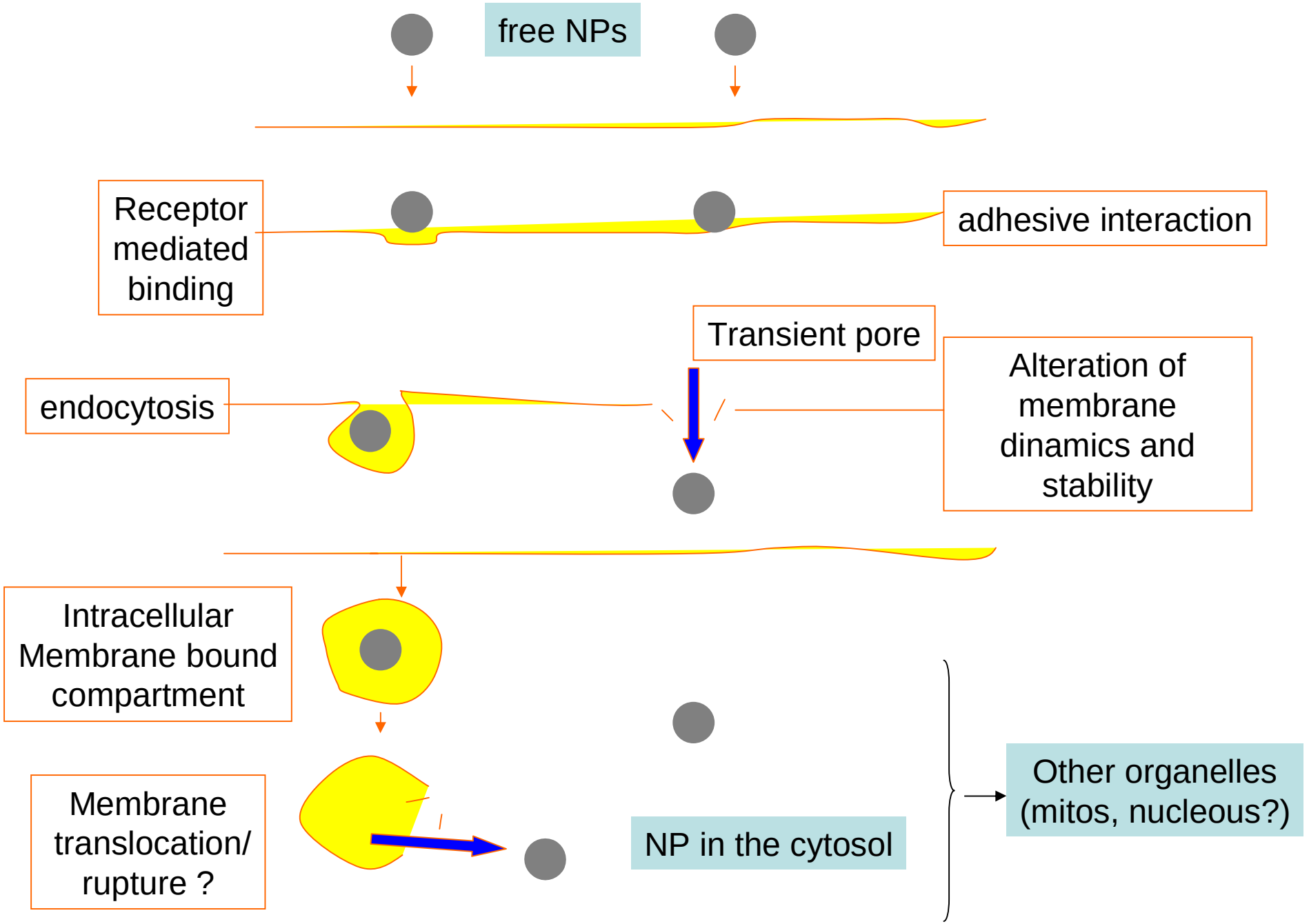
In the passive mode, nanometer-sized particles such as quantum dots accumulate at tumor sites through an enhanced permeability and retention effect. For active tumor targeting, nanoparticles are conjugated to molecular ligands such as antibodies and peptides to recognize protein targets that are overexpressed on the surface of tumor cells such as the EGF receptor, the transferrin receptor or the folate receptor.


CLEARANCE
(Airway)
Aqueous lining layer
Periciliary fluid
Mucus layer
Ciliated cell
Surfactant
Tight junction
Particle
Macrophage
Secretory cell
Dendritic cell
Basal lamina
Basal cell
CLEARANCE
(Tissue)

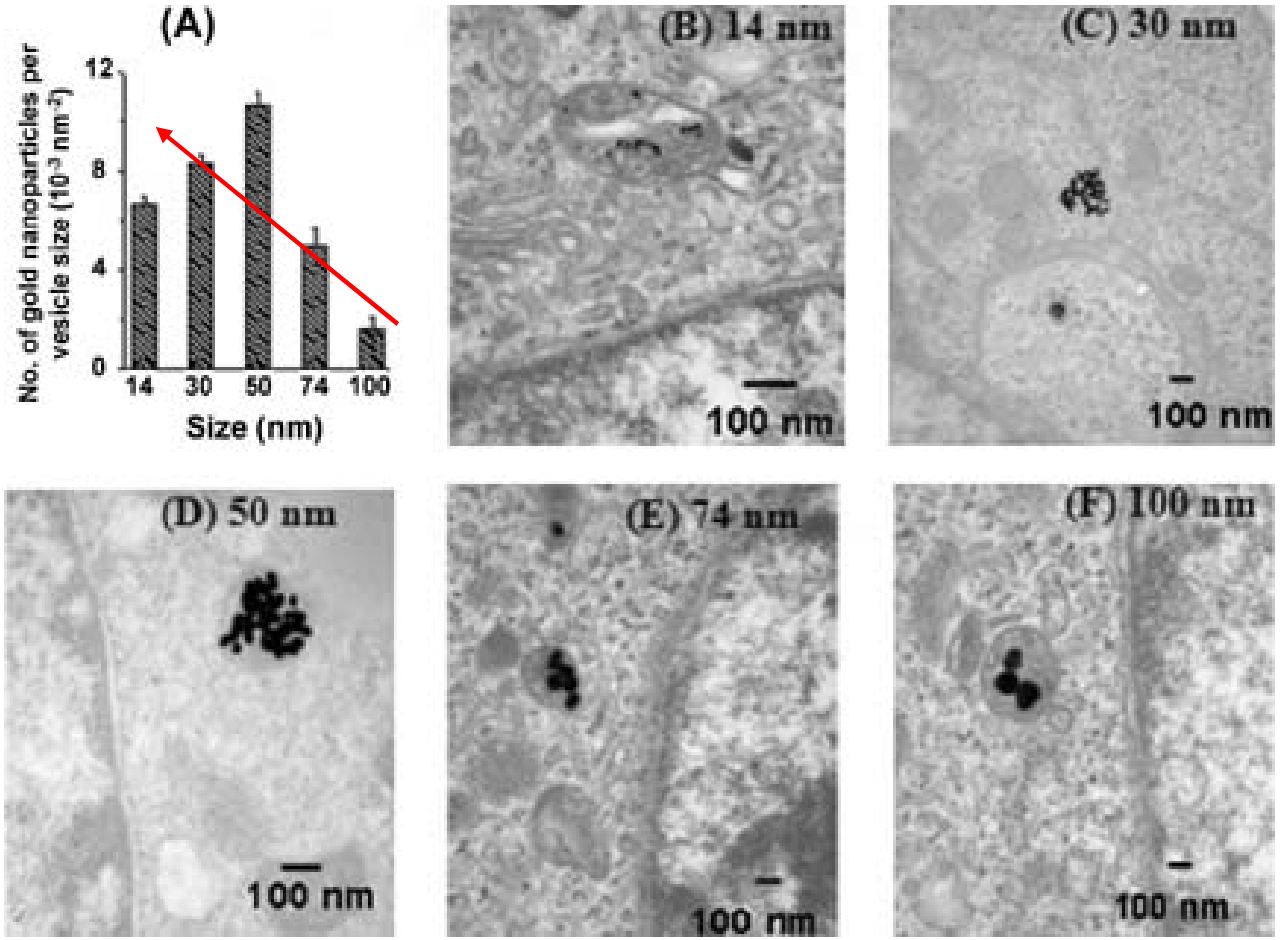
Many obstacles must be overcome by a nanotheragnostic to reach its cellular and intracellular target

- Epithelial layer
- Basal membrane
- Tissue phagocytosis
- Lymph drainage
- RES clearance
- Endothelial layer
- Endothelial basal membrane

• Plasma (and other cellular) membrane

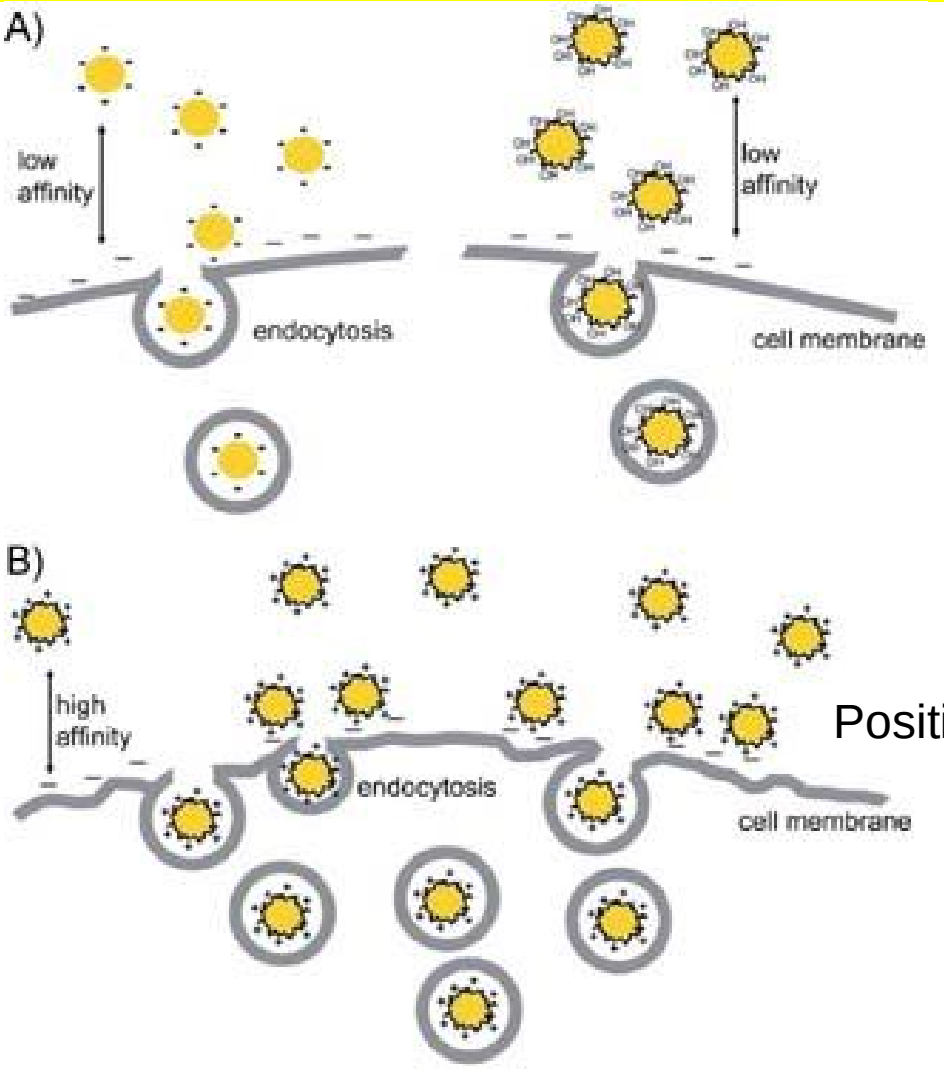

free NPs
Receptor mediated binding
adhesive interaction
Transient pore
endocytosis
Alteration of membrane dinamics and stability
Intracellular Membrane bound compartment
Membrane translocation/ rupture ?
NP in the cytosol
Other organelles (mitos, nucleous?)

Endocytosis and NPs shape



Endocytosis and NPs harge

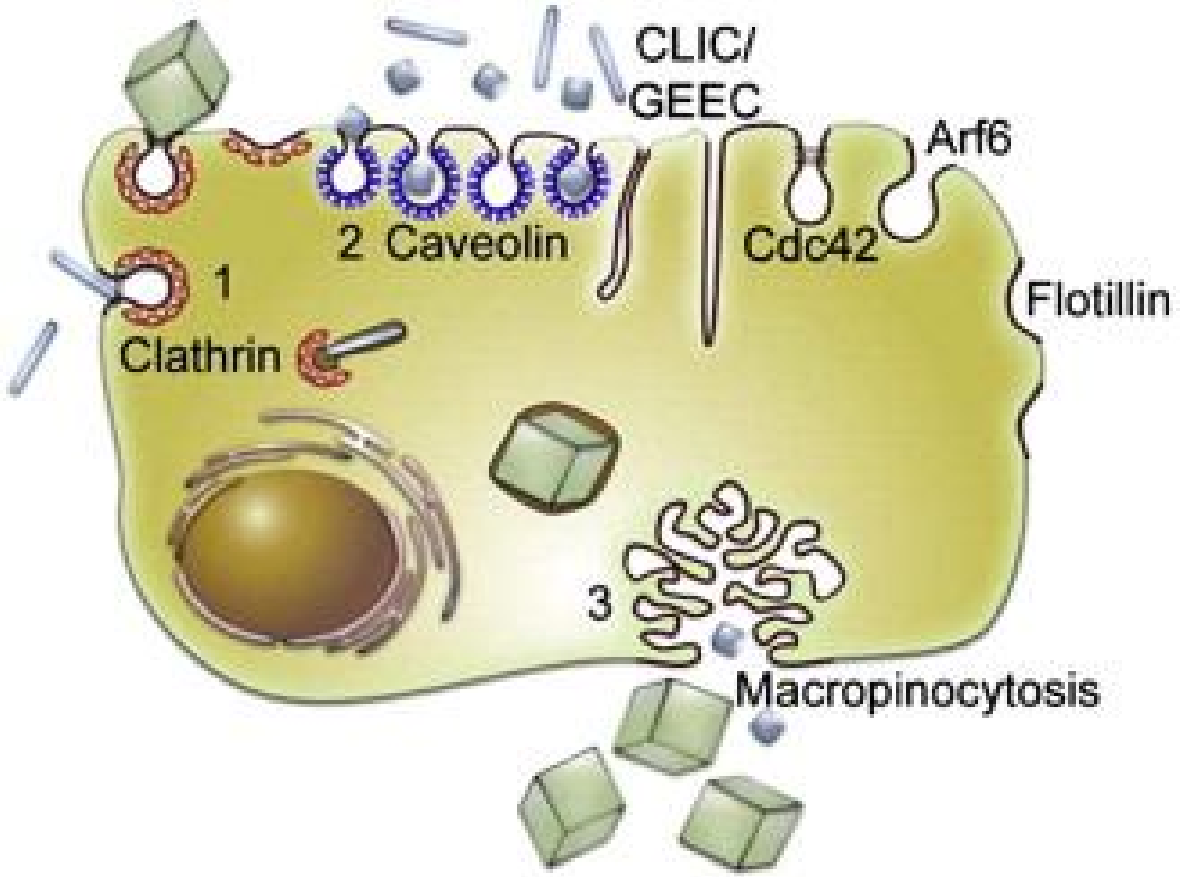
Negative NPs



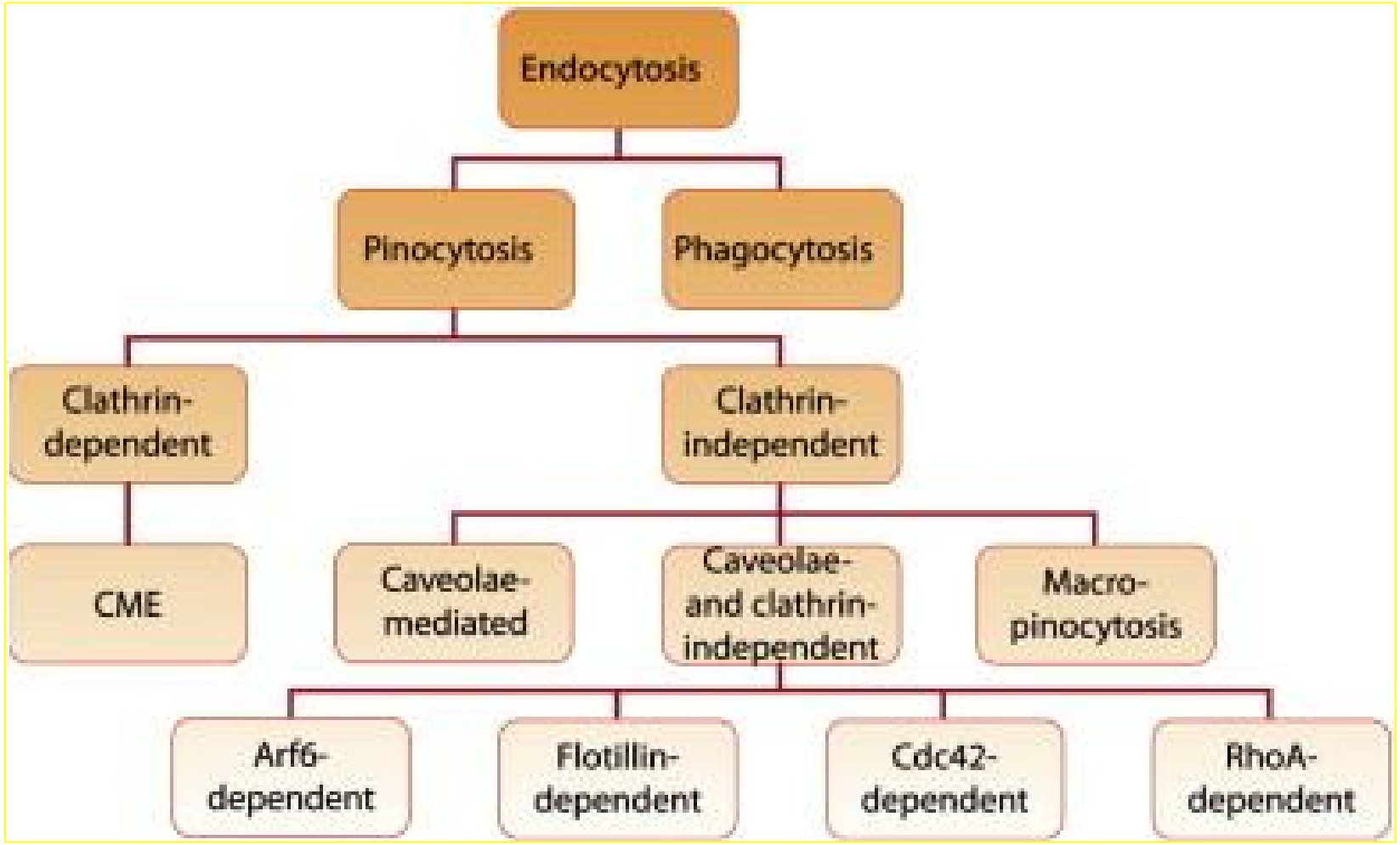
Neutral NPs

Positive NPs: strong uptake

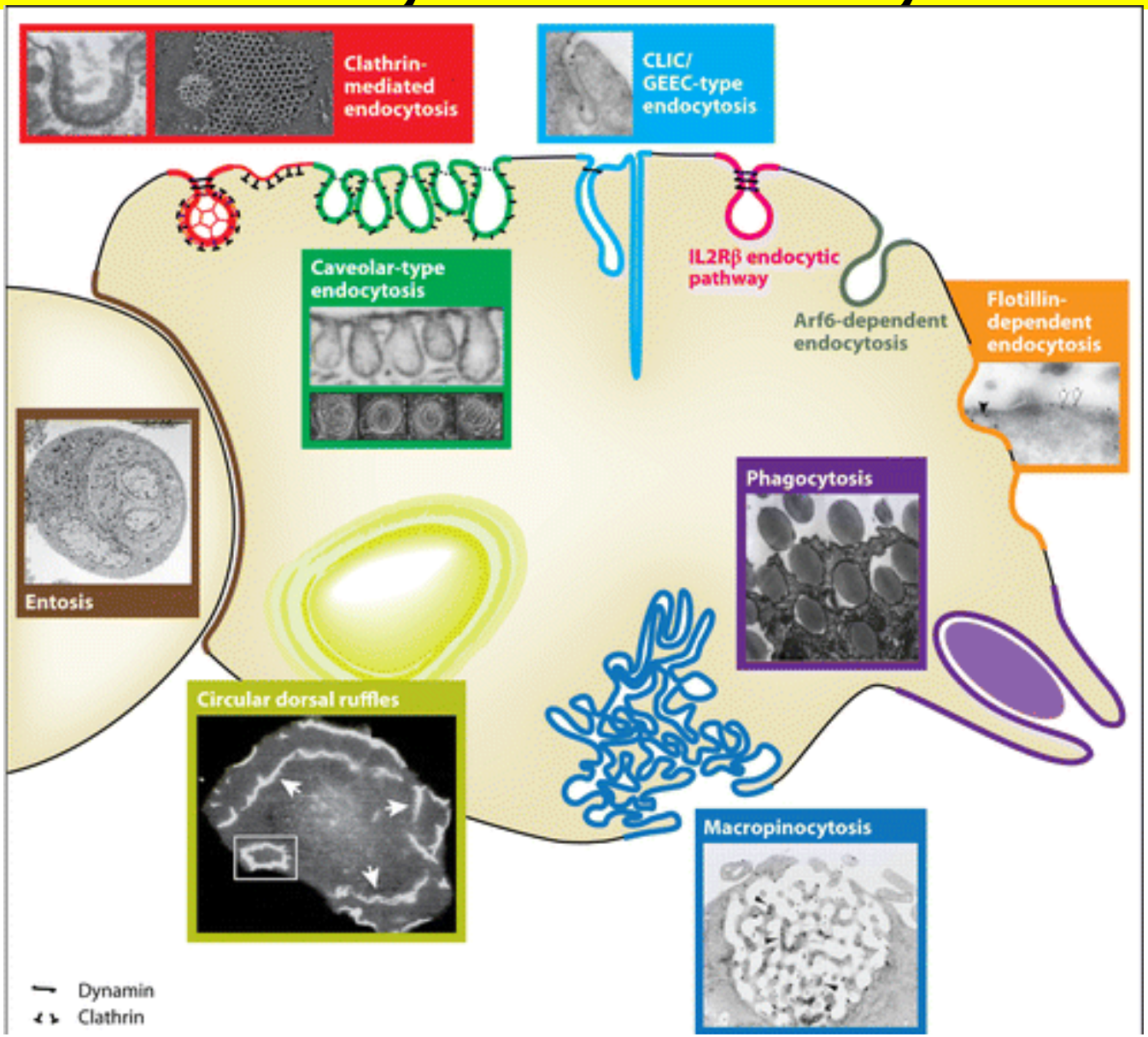
Cell endocytic pathways



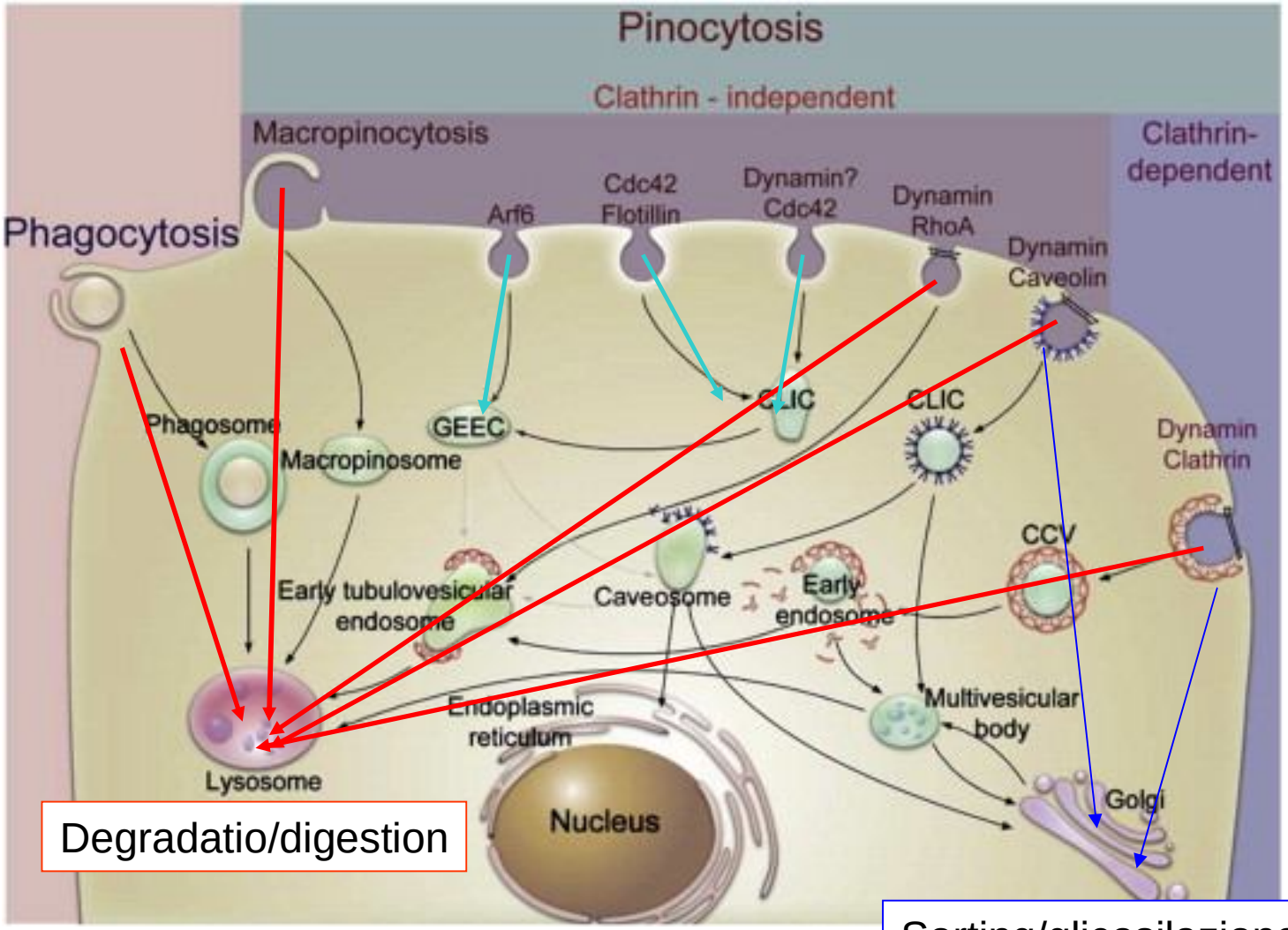
There are several types of endocytosis

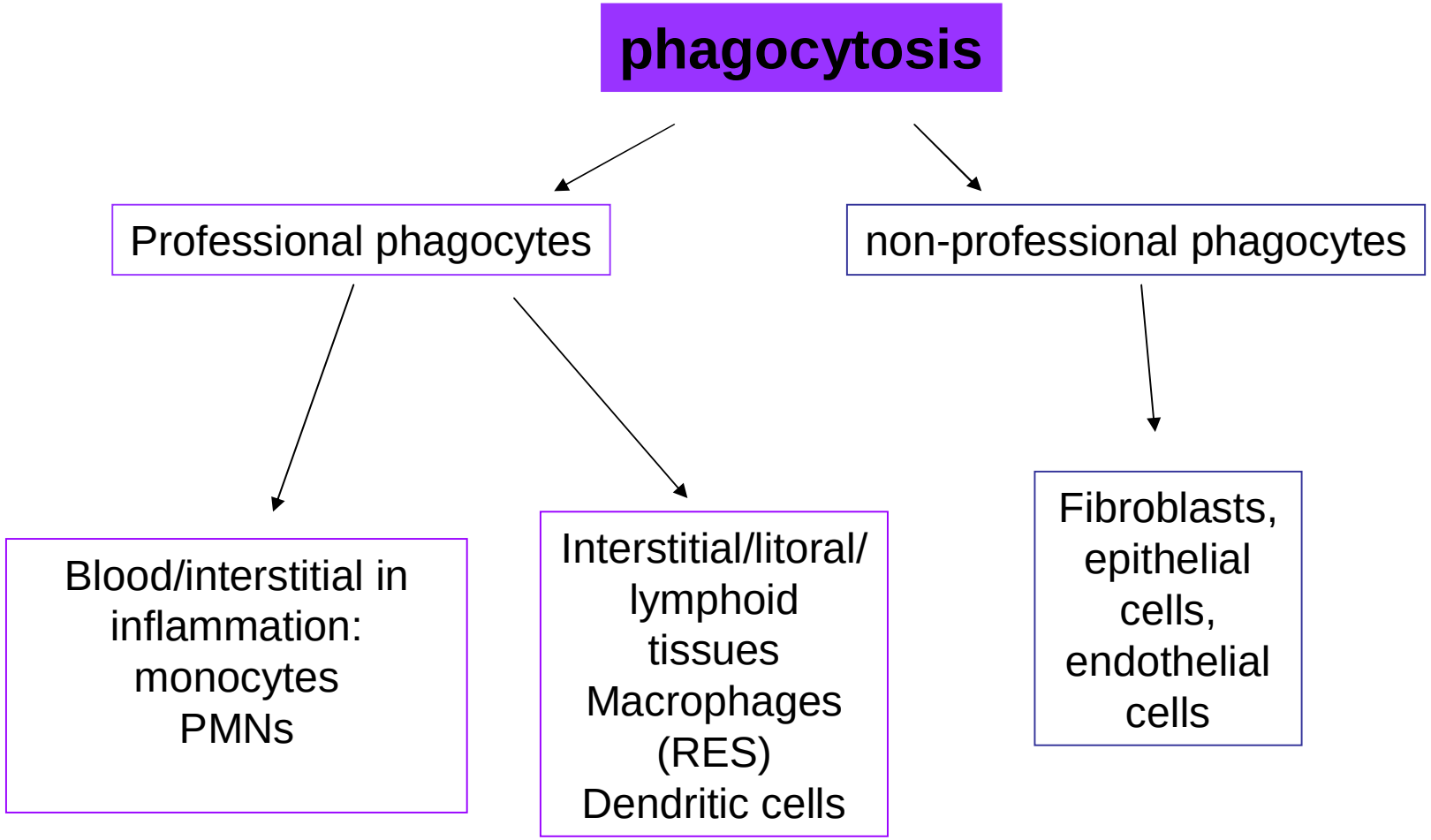


10 different types of endocytosis (but not everyone in every cell…)

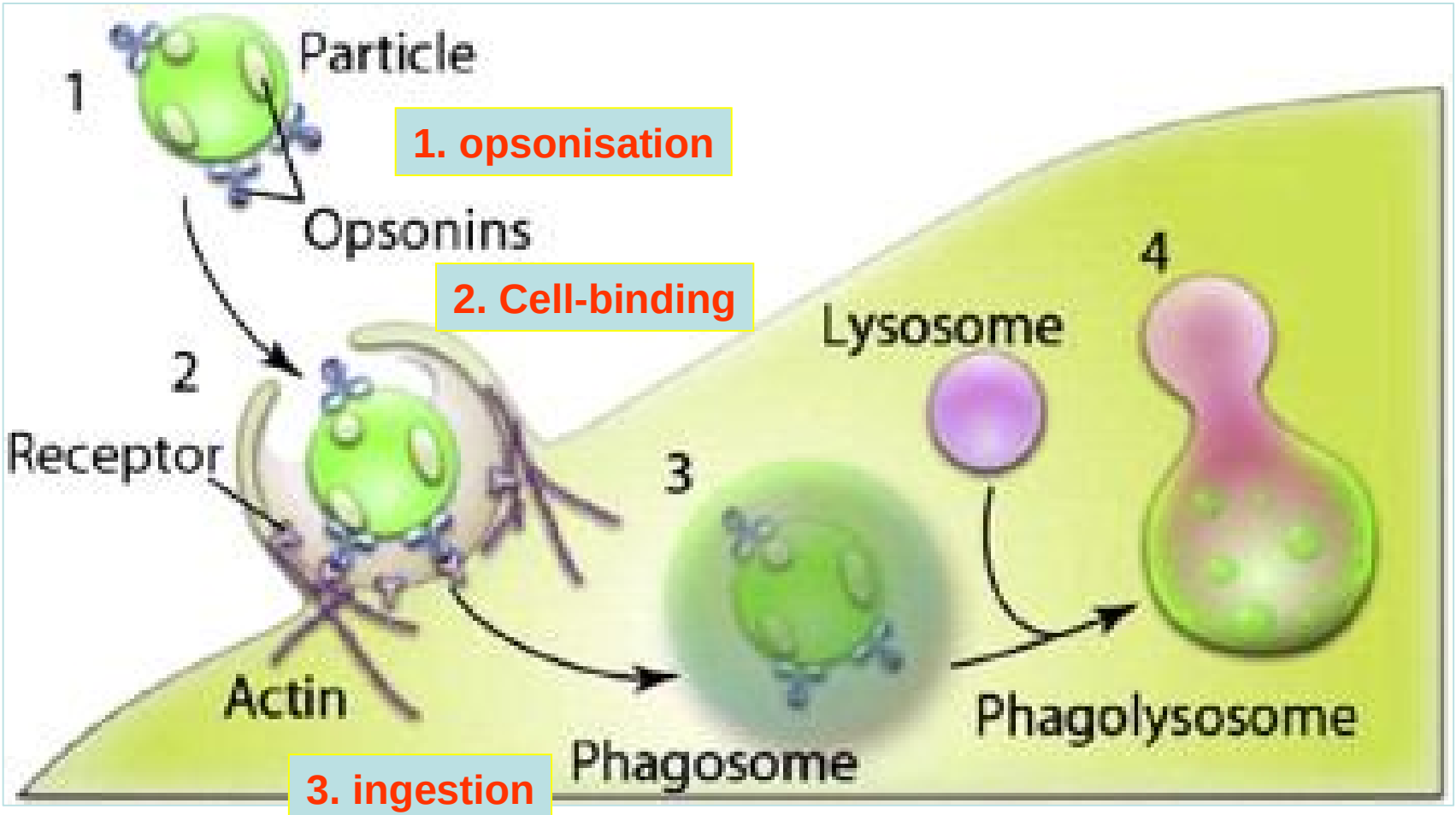


Iintracellular traffic: regulated by specific signals and mechanisms




phagocytosis
Professional phagocytes
non-professional phagocytes
Blood/interstitial in inflammation: monocytes PMNs
Interstitial/litoral/ lymphoid tissues Macrophages (RES) Dendritic cells
Fibroblasts, epithelial cells, endothelial cells

Phagocytosis

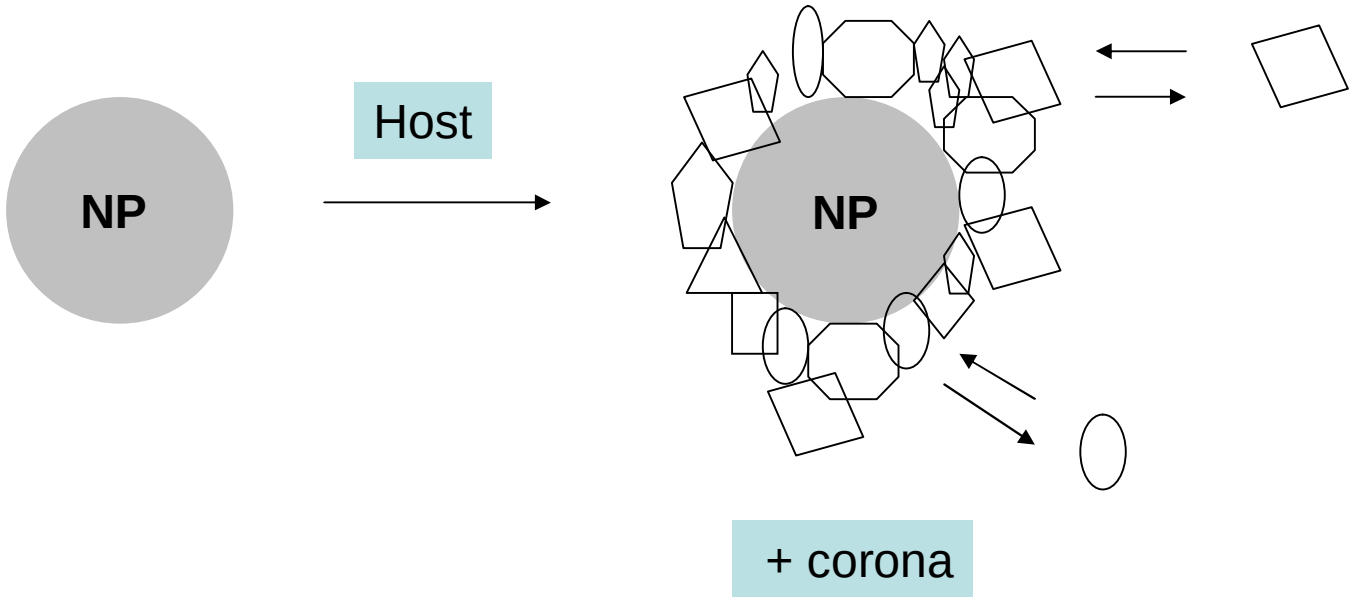


opsonines

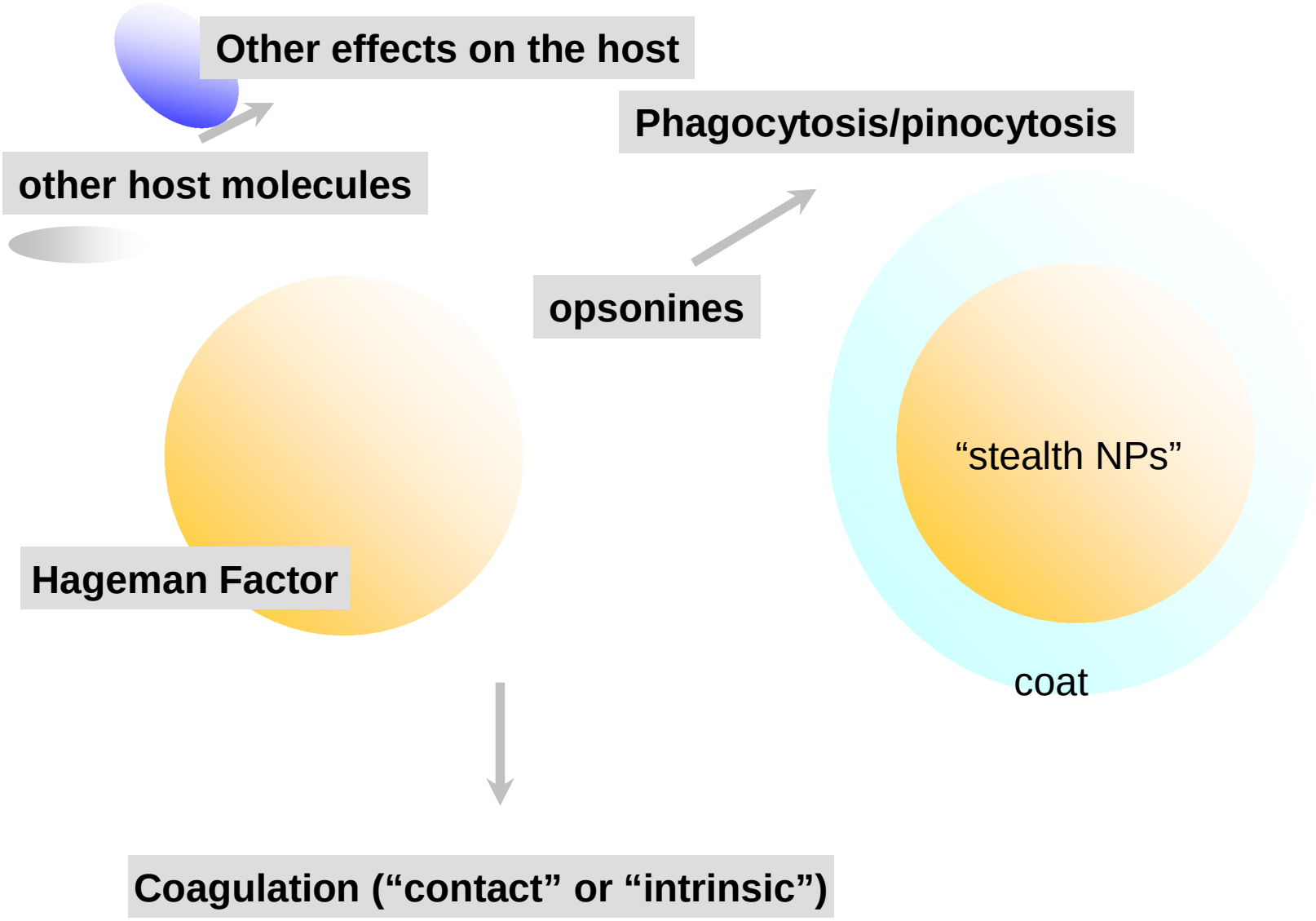
- Innate: complement (C3, C5, C4); blood serum proteins (includign laminin, fibronectin); others
- Adaptative: antibodies

!! Very important concept in NPs cell capture: host molecules surface adsorption: “molecular corona” !!!

Host protein crucial role: they form a dynamic “corona” around the NPs



Many proteins and other molecular components in the host bio-fluids
(plasma, air-way films, mucosa, interstitium, lymph, SNC..)

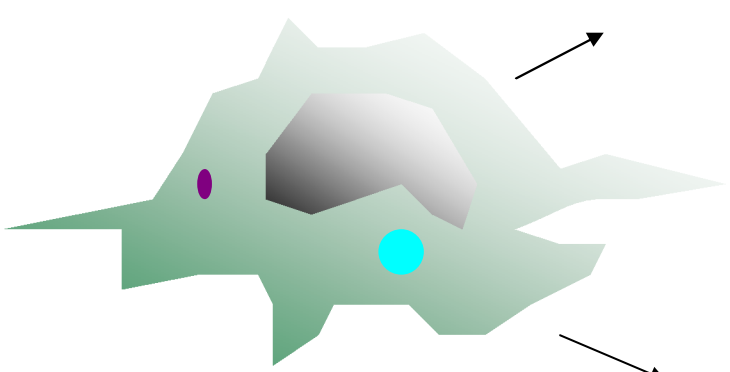


Monocyte
Macrophage
PMN
Dendritic cell

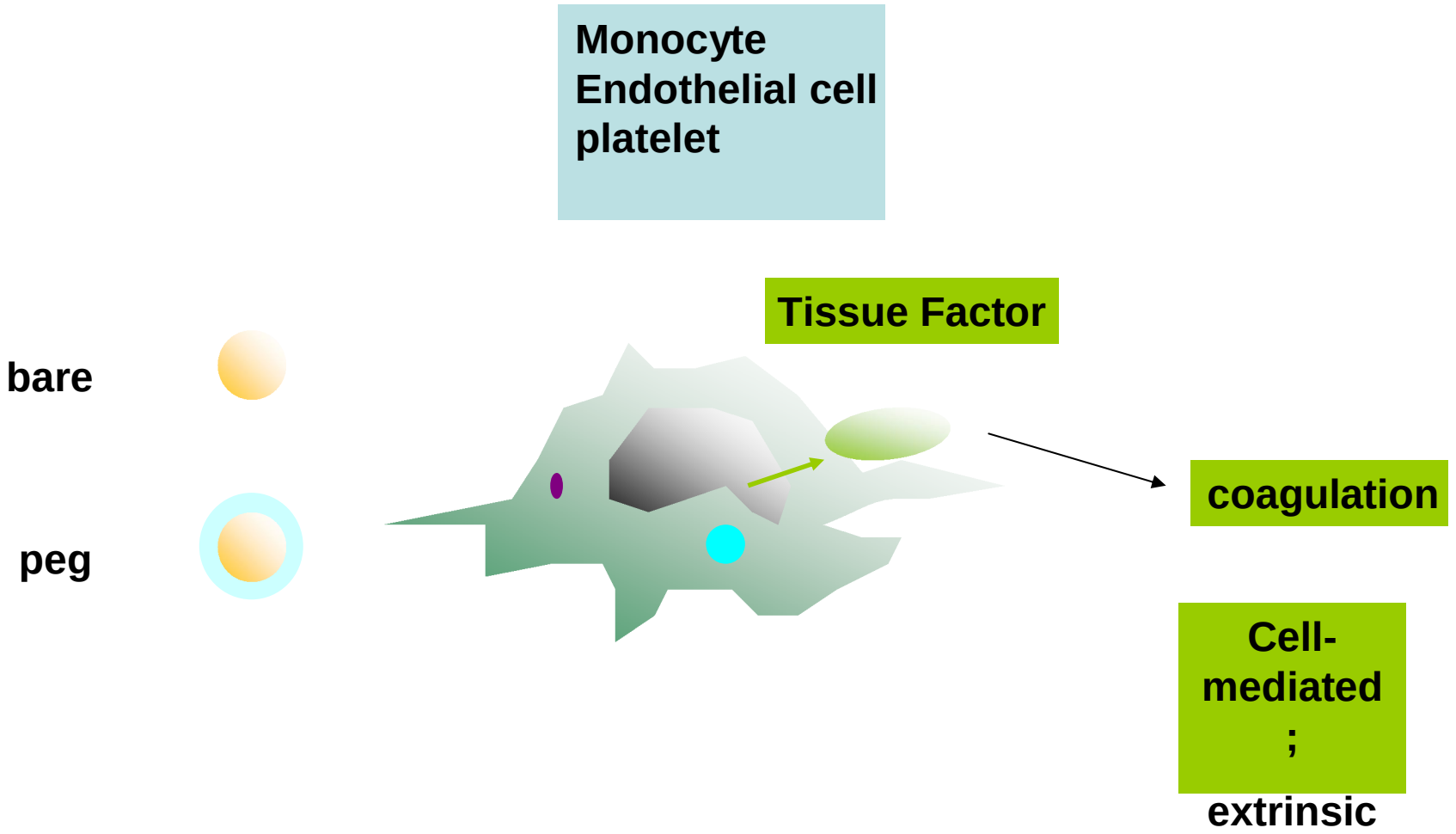
Cell-capture: rapid clearance!!

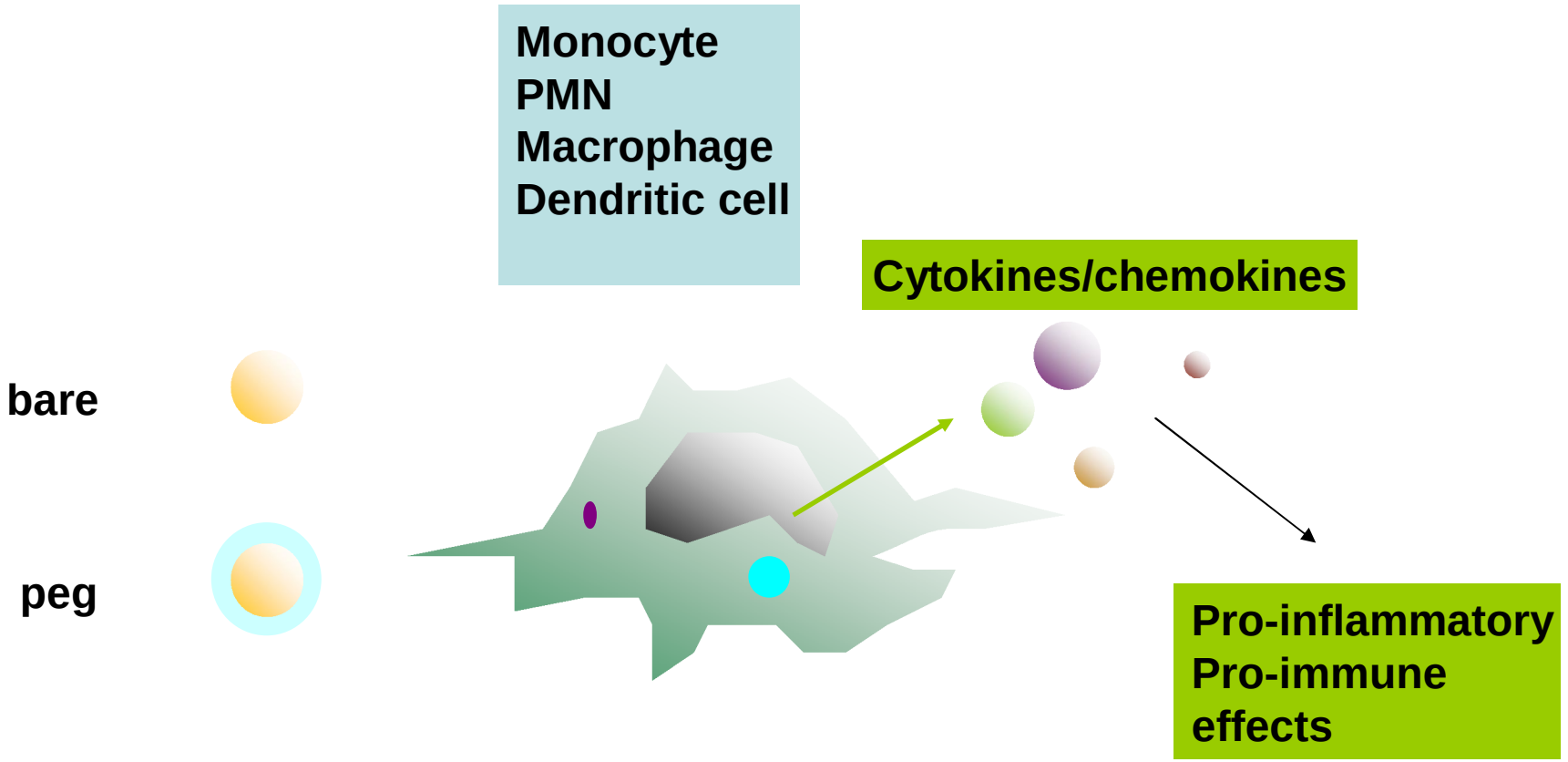
bare

peg

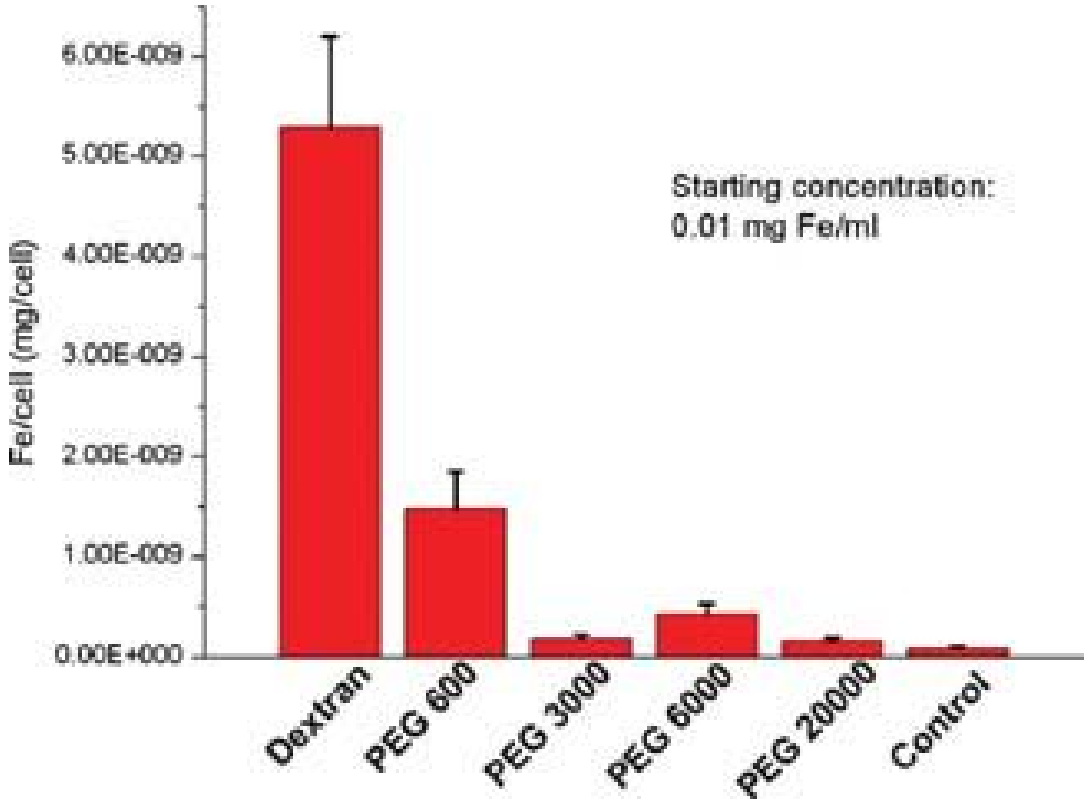


Cytotoxicity ?


Monocyte
Endothelial cell
platelet
Tissue Factor
bare
peg
coagulation
Cell-
mediated
;
extrinsic


Monocyte
PMN
Macrophage
Dendritic cell
Cytokines/chemokines
bare
peg
Pro-inflammatory
Pro-immune
effects

Poly Ethylen Glycol is a good “stealthing agent”



Phagocytes receptors

- Complement receptors, mannose-fructose receptors, scavanger receptors,

- Fc receptors

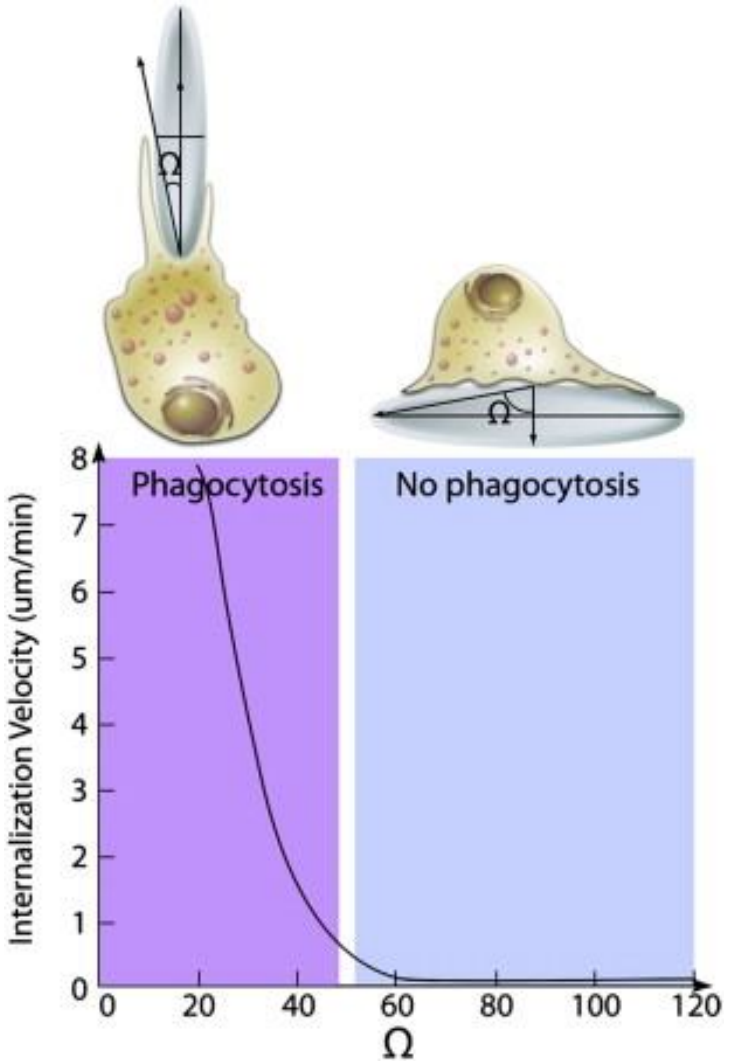
Ingestion, digestion

- Particles with up to 14-20 μm Ø Actin-rearragement and phagosome formation

- Phagosome maturation: fusione with late endosomes + lysosomes = phagolysosome (digestion)

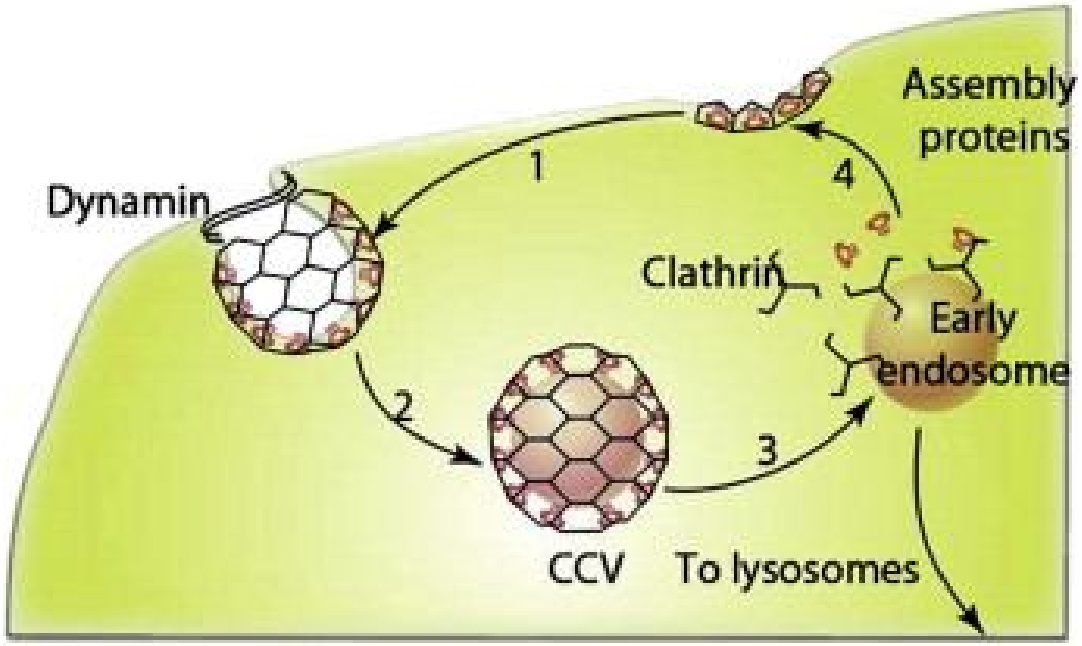
Shape relevance in MPs phagocyotsis

- 1-10 μm MPs with very different shape are all phagocytosed:
- The local contact shape is however important

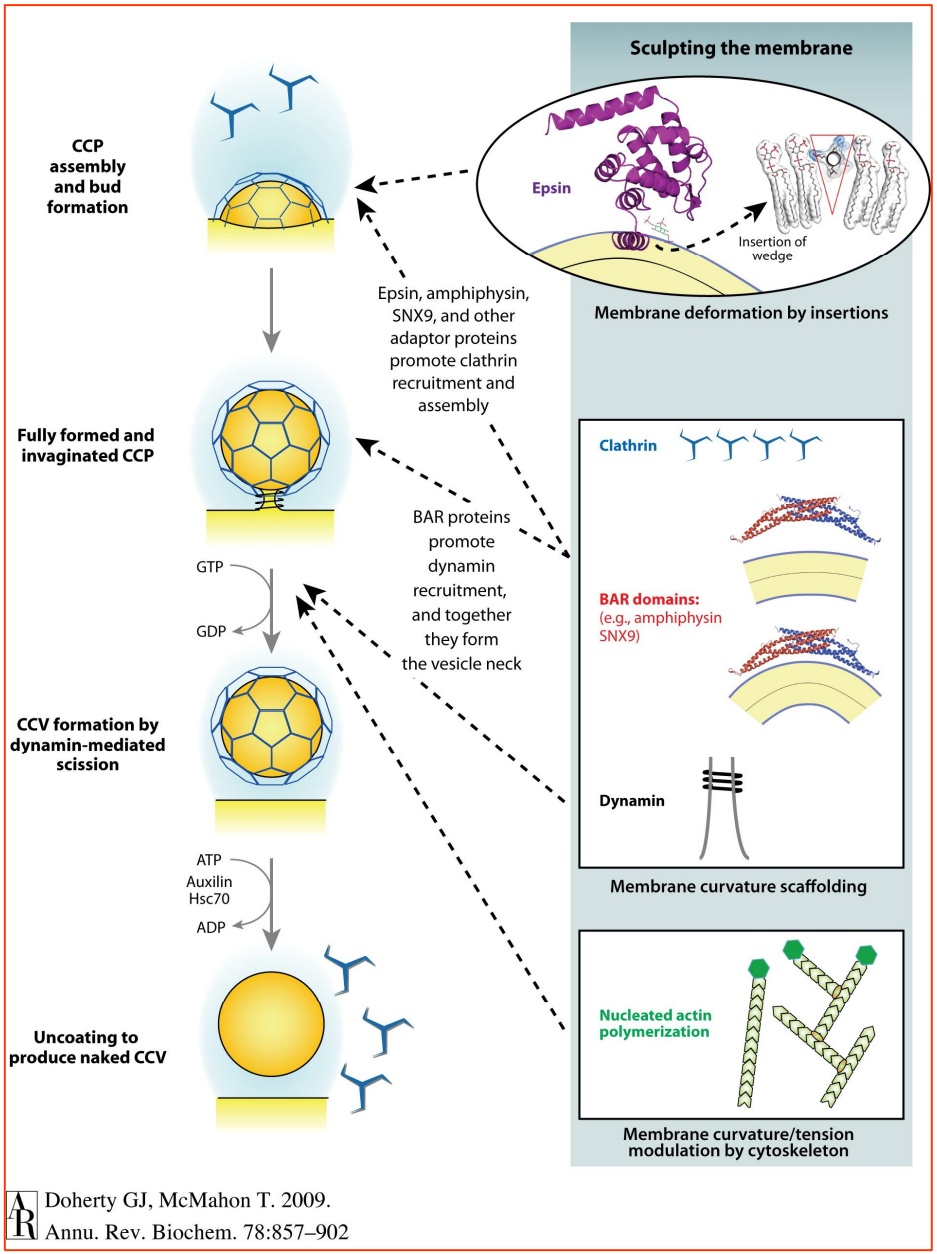


Clathrin Mediated Endocytosis (CME)

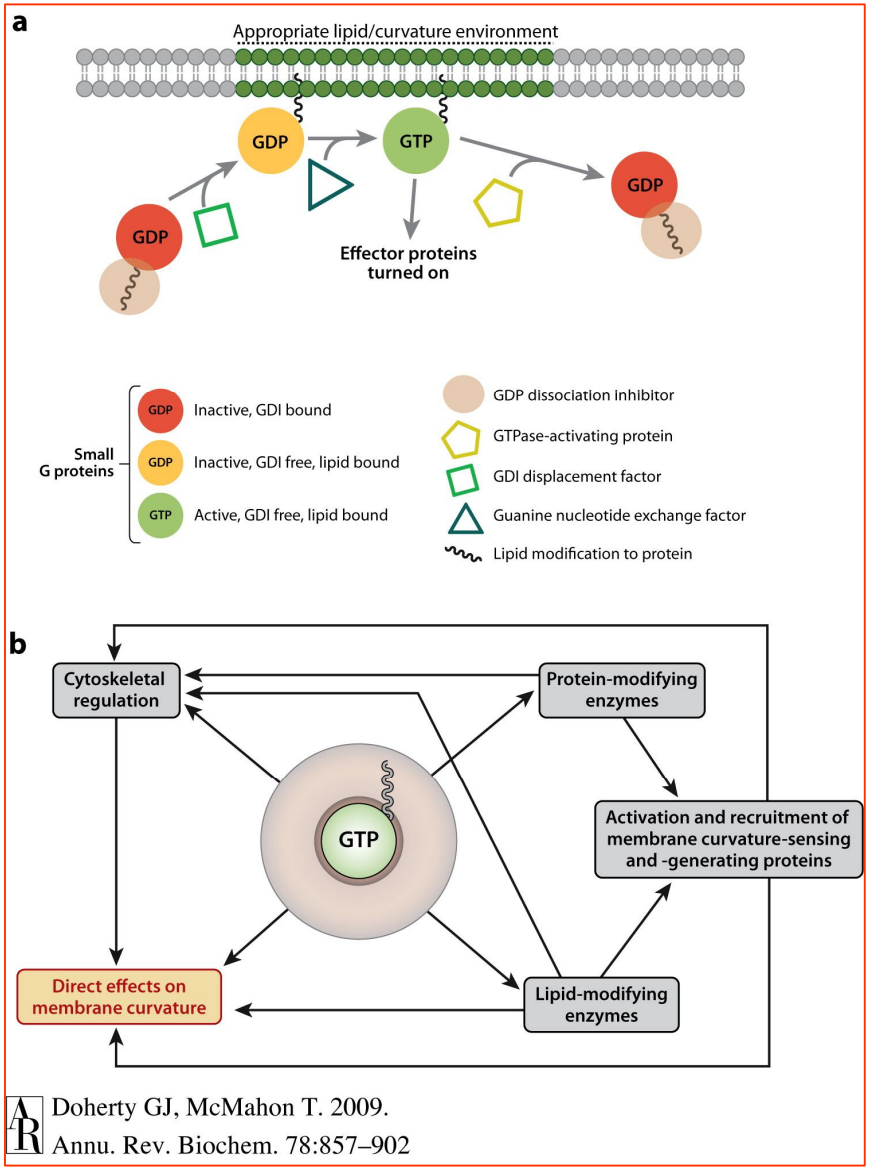
- Some cell receptors like Low Density Lipoprotein receptors (LDL-R) and Transferrin reeptors (Tf-R) localise to coated pits
- The coated vescicles is ~ 120 nm Ø
- Protein involved: clathrin, dynamin, AP180, A-2



Molecular mechanisms in CME

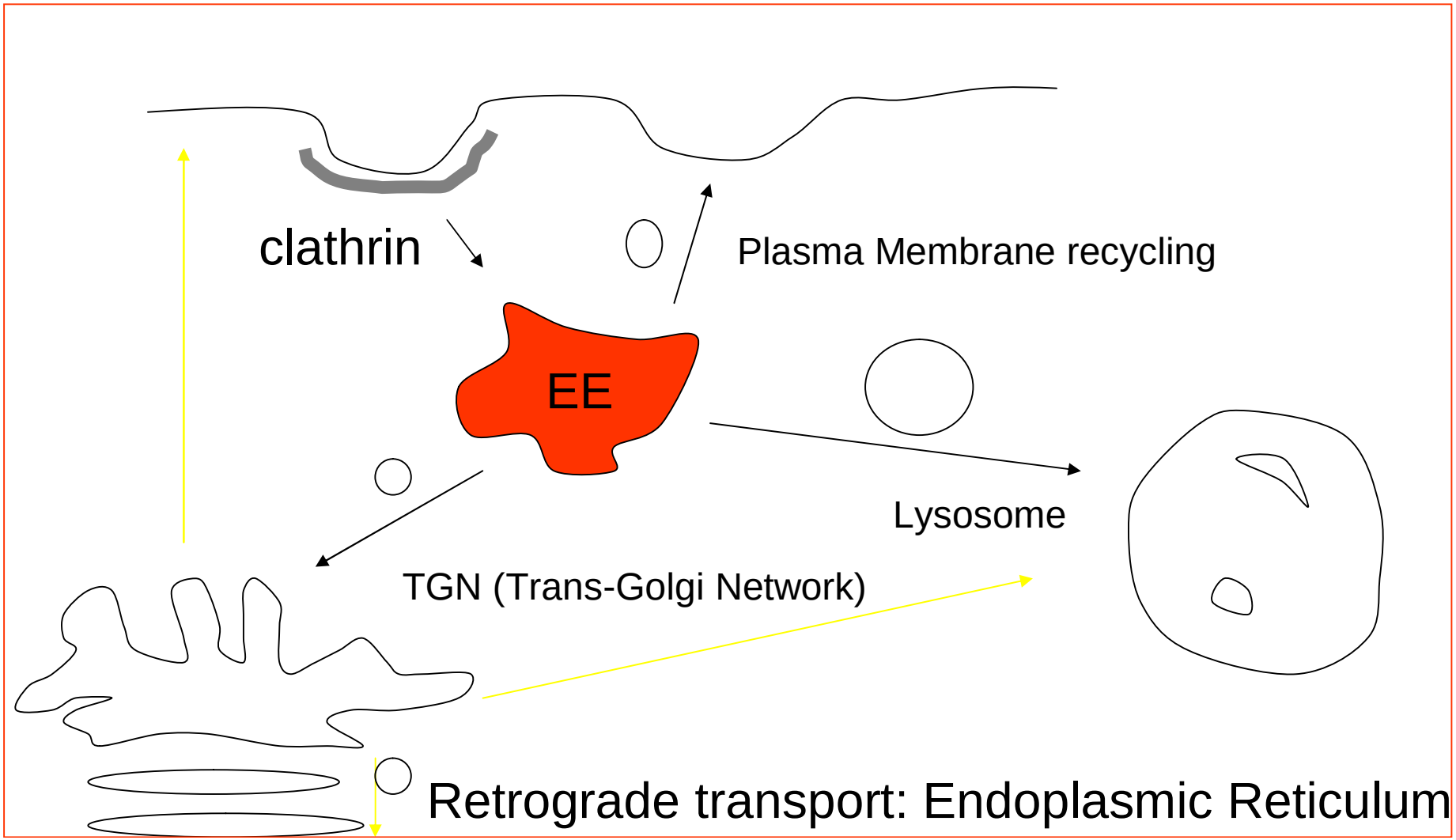


Doherty GJ, McMahon T. 2009.
Annu. Rev. Biochem. 78:857–902



Doherty GJ, McMahon T. 2009.
Annu. Rev. Biochem. 78:857–902

sorting from the Early Endosomal (EE) rab 5 + compartments



Caveolae mediate endocytosis

caveolae are abundant in:
- muscle
- Endothelial cells
- Fibroblasts
- Adipocytes

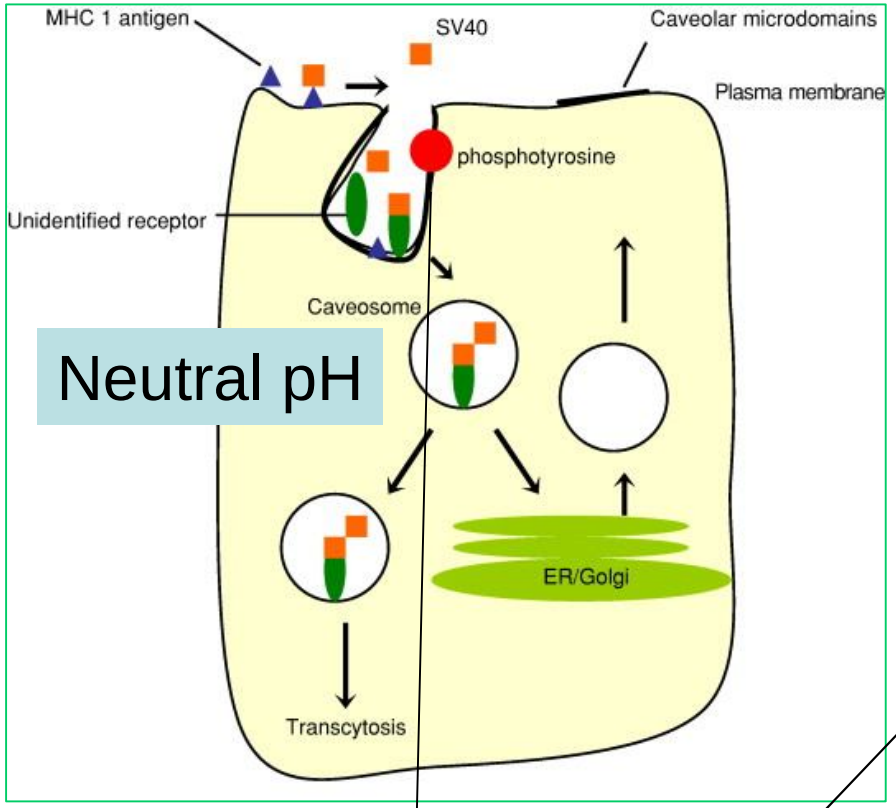
Absent in

- neurons
- Leukocytes

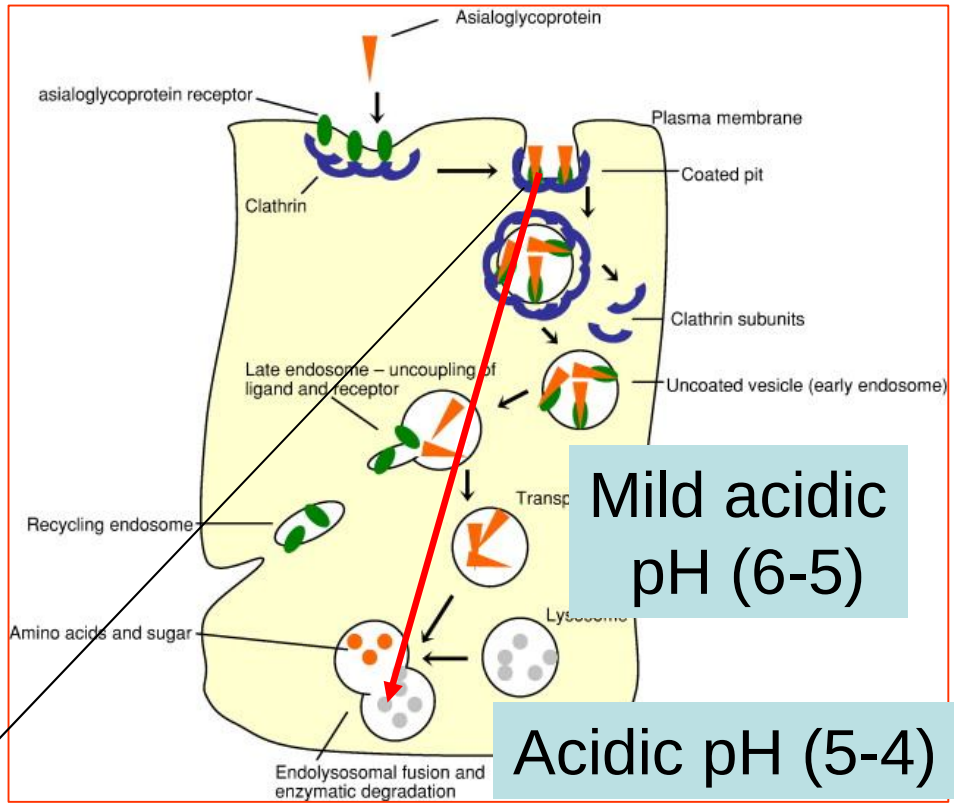
Caveolae (*lat: small caves*)

- Flasked-shaped (60-80 nm Ø)
- They are a subset of *lipid rafts,* the cholesterol-rich plasma membrane regions
- A hair-pin protein called caveolin-1 is necessary for the caveolae biogenesis (caveolin 2 and 3 isophorms present in muscle): it forms the membrane curvature
- Dynamin perfomes the pinching off
- Other proteins: VAMP2 and SNAP for subsequent vescicle fusion events
- The bud caveola fuses with a caveosomes or with special Multi vescicular bodyies (all neutral pH, not acidic!)

Caveolae vs CME in endothelial polarised cells



Neutral pH



Mild acidic pH (6-5)

Acidic pH (5-4)

Transcytosis: endothelial barrier crossing

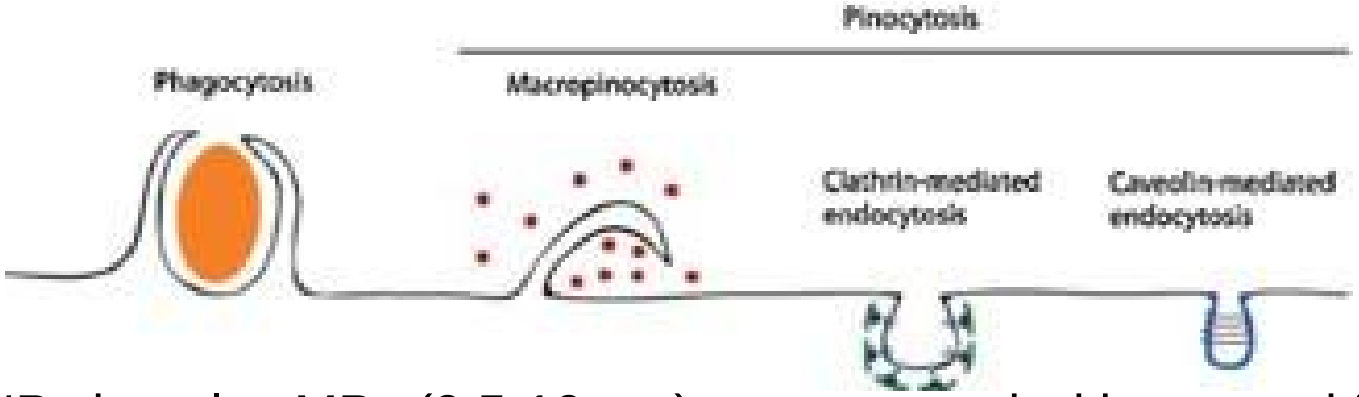
Dead end

Clathrin- and caveolae independent endocytosis

- ~ 90 nm Ø vescicles, or tubulo-vesicular membrane structures
- 4 types: Arf-6 dep., Flotillin dep., Cd42 dep., Rho A dep.
- IL-2R and IgE R are internalised via this way
- They by-pass the rab 5 + EE
- Ex: GPI-anchored proteins are transported to tubulo-vescicular GEECs (GPI-lincked Early Endosomal Compartments)

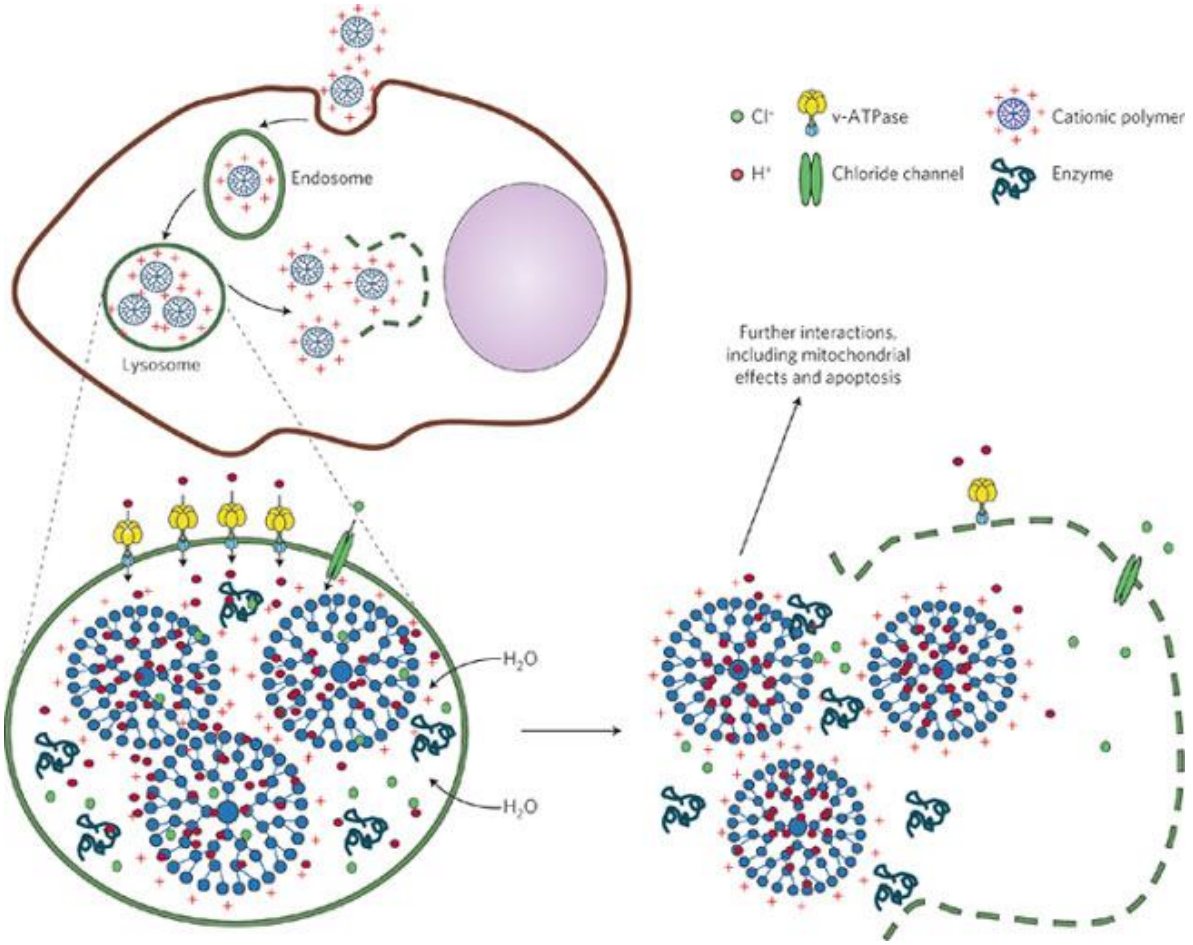
macropinocytosis

- Transient activation of tyr-kinase plasma membrane receptors leads to the formation of membrane ruffles

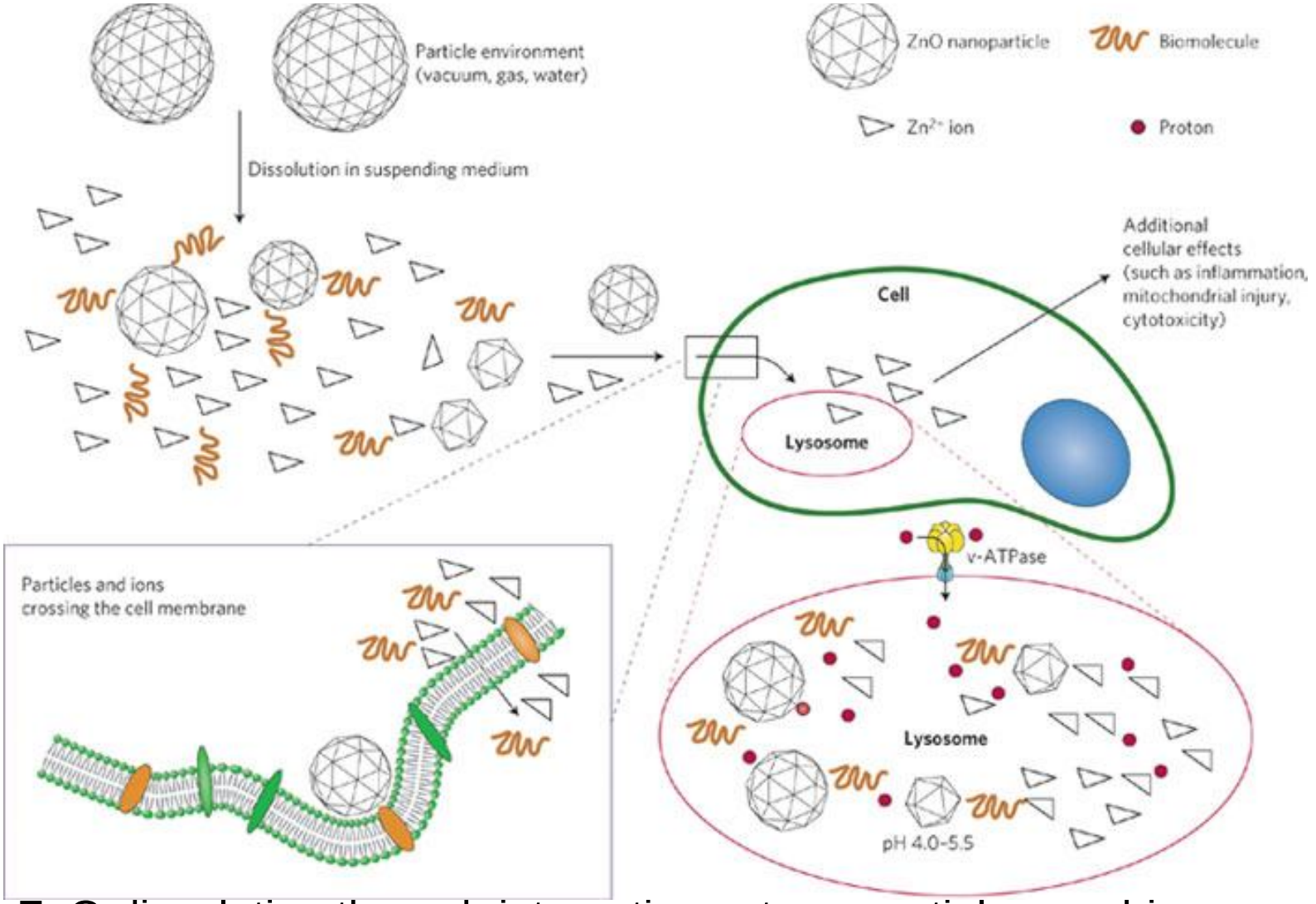


- NPs but also MPs (0,5-10 μm) are captured with no need for cell adhesion/binding

- Present in virtually all cell types except macrophages and brain microvasculature endothelial cells



- Cationic (for example **PEI-coated**) particles bind with high affinity to lipid groups on the surface membrane and are endocytosed in the tight-fitting vesicles. Once these cationic nanoparticles enter into an acidifying lysosomal compartment, the unsaturated amino groups are capable of sequestering protons that are supplied by the v-ATPase (proton pump). This process keeps the pump functioning and leads to the retention of one Cl- ion and one water molecule per proton. Subsequent lysosomal swelling and rupture leads to particle deposition in the cytoplasm and the spillage of the lysosomal content.



- ZnO dissolution through interactions at sequential nano–bio interfaces in the extracellular environment and the acidifying lysosome generates cellular toxicity through the release of toxic Zn2+ ions. Release of Zn2+ in the lysosome and the intracellular environment can induce a series of harmful cellular outcomes, such as lysosomal damage, mitochondrial perturbation, ROS production, excitation of pro-inflammatory cytokine and chemokine production.

Some generalisations (attempt) on NPs endocytosis

Cell type

- *It has a strong influence*: polarisation (epithelial, endothelial, neuron) or not (remember tumour cells are in general less differentiated than normal ones):

1. Which endocytic entry routes are active (consider membrane domains: apical basolateral)?
2. What is the intracellular trafficking after entry (importance of understanding normal and nanomaterial induced intarcellular membrane traffic of NPs)?
3. NPs can alter the normal intracellualr membrane trafficking
4. Previously unknown membrane traffick pathway might be revealed by the use of cell entering NPs
5. Not only the type of cell-entry route is essential but also the qantity of NPs cell load: positive carrelation with cytotoxic effects

Rule: the initial stage of entry does not garantee for NPs final destination!!!!!!

Shape

1. The shape of the MPs (not NPs) contacting the phagocyte is important (frustrated phagocytosis)

2. for NPs only one systematic study with PRINT NPs: difficult to generalise

size

1. It has long been assumed that it plays a paramount role in determining which of different entry routes is used by a given NPs: many postulated that a size comprised between 10 and 100 nm Ø is necessary for cell endocytosis: indeed a small size helps endocytosis
2. However, tehre is no clear size cut off: N/MPs up to 5 µm Ø can enter cells (for µm particles micorpinocytosis is involved)

Problem: the evaluation of the size dependence of a NPs endocytosis is made difficult by the high polydispersity of many nanomaterials

•

Surface charge

1. It is definitly important
2. the majority of reports suggets that positive charged NPs are internalised via CME (e.g. stearylamine-coated PEG-co-PLA; PLLmodified PLGA; NH_2-SNTs; chitosan NPs, PRINT..)

   BUT exceptions are: PEI-based polyplexes; PRINT: multple entry routes

4. Negative charged NPs are more likely to enter via caveolae (e.g. DOXIL®, cl-micelles, QDs)

   BUT xceptions: Carboxylate-modified PolyStyrene (PS)_NPs and negatively harged PLGA-NPs: clathrin- caveolae-independent entry

5. it is wiely believd that positive NPs are endocytosed at a higher rate and extent than corresponding negative NPs (due to repulsion by the negatively charged pm)

BUT striking exception: negative QDs are endocytosed faster than negative and neutral ones

6. it is unclear from the literature if neutral NPs have preferential entry routes

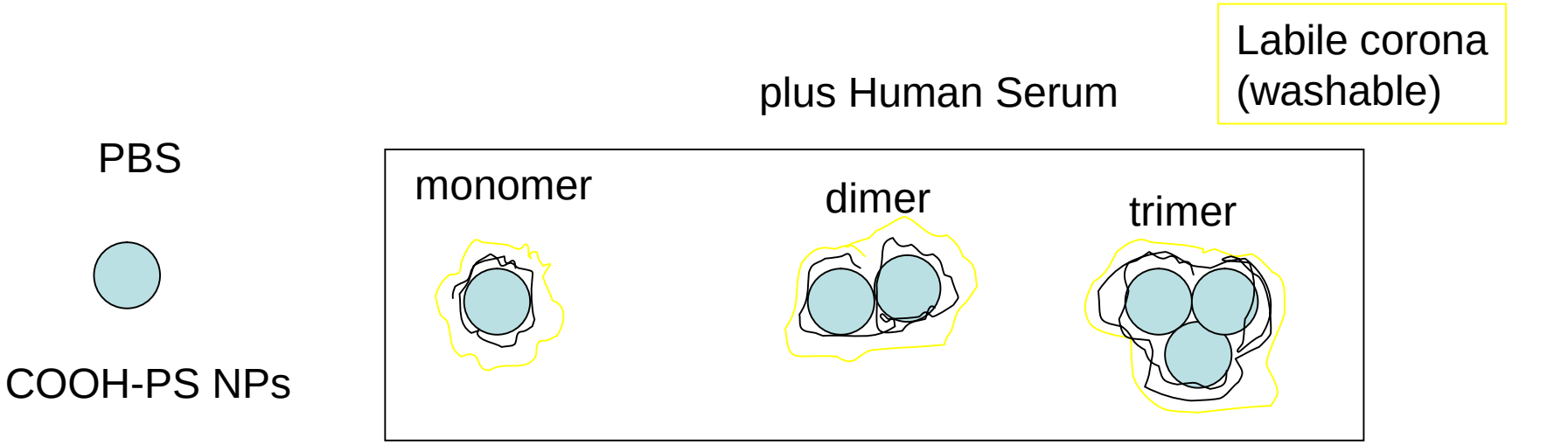
- 7. one important aspect concerns the real charge of a NP after introduction in a orhanismic fluid or even in a cell medium (serum-containing)

NPs corona

- The bare surface of a NPs (or any EMNs) is covered by several bio-molecules drawn from the host corporeal fuids, including a select group of protein: the “corona”
- The corona is likely made by: a *hard* corona: made by slowly exchanging host bio-molecules and by an additional layer of more dynamic and rapidly exchanging bio-molecules

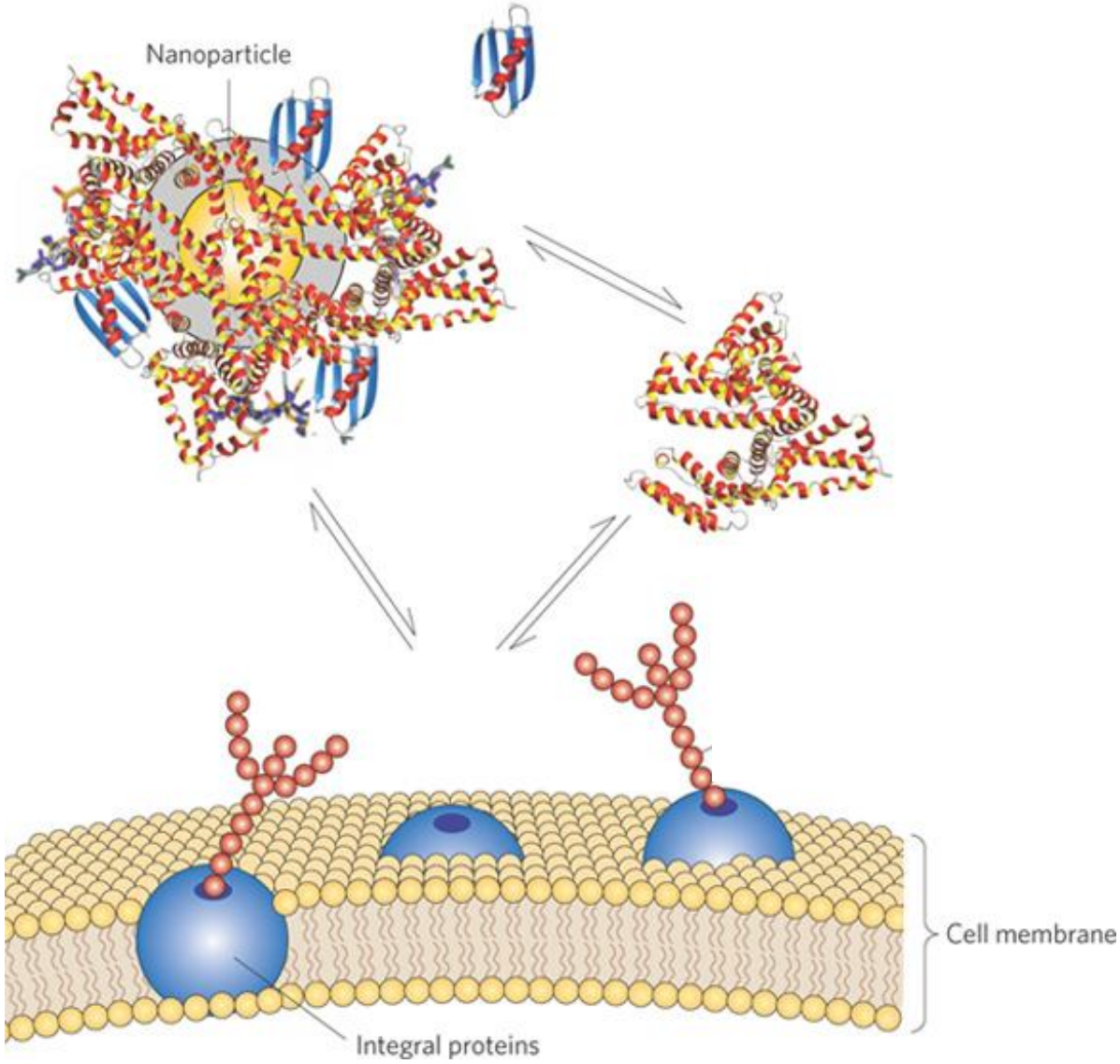
- 100 Ø nm carboxylated NPs (negative) placed in 100 % human plasma (remember! *plus Ca2+ chelating agents to block coagulation…) increase their size due to the formation of a ~20 nm thick protein layer*

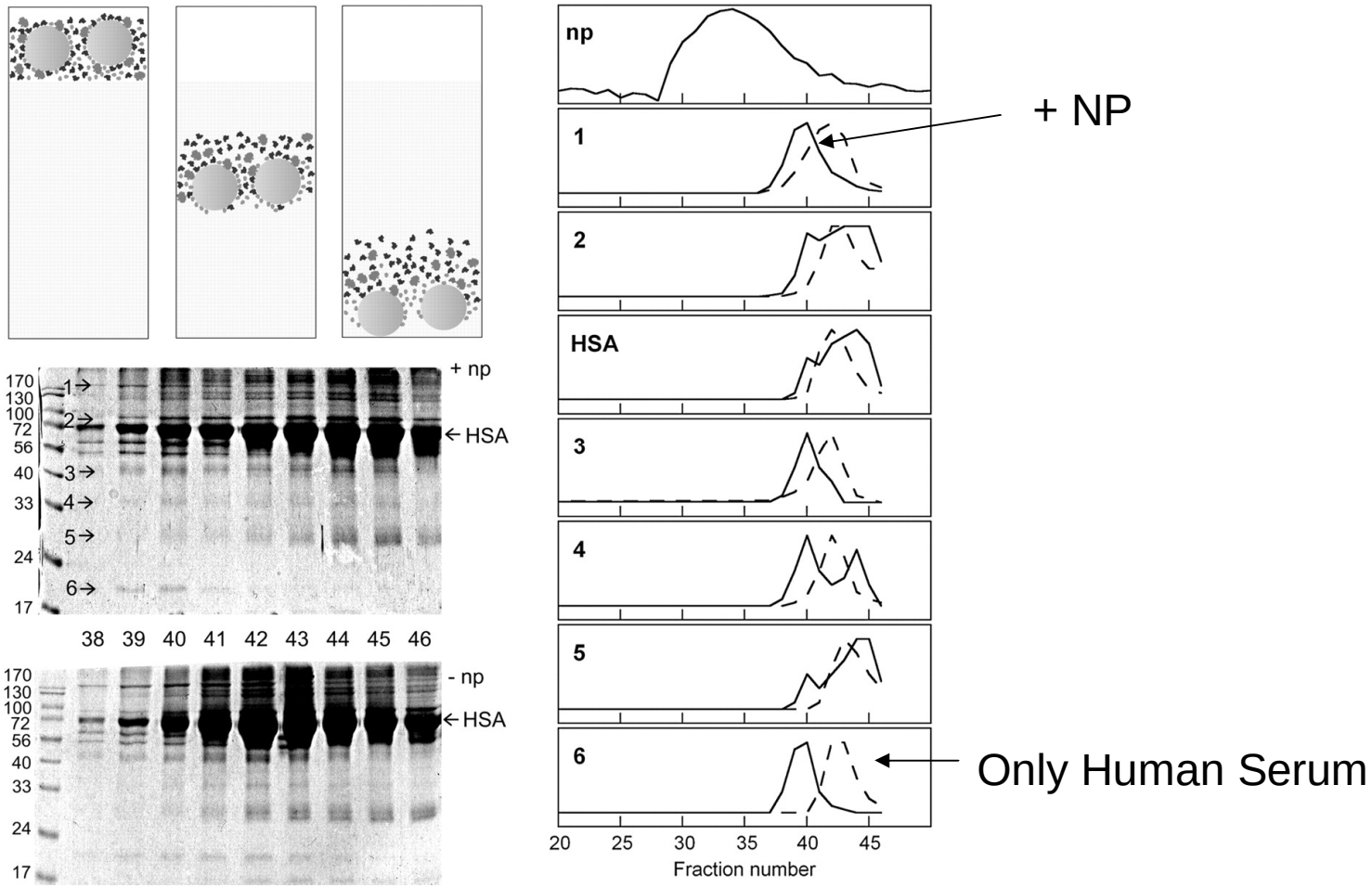
- In full plasma, a rapid “ hard” coat (formed in < 1 hours) is gradually increased by more labile layer with time (up to 5 hours kinetics)
- NPs washings preserve the hard coat,, but eliminates the second recruited components



Monomer Bare (PBS)	0	1	4	6 hours	
Z-pot mv	- 30	-7,8	7,5	9,2	9,2	
	- 30	-8,8	8,0	9,8	8,9	washed
Size (nm)	100	185	183	198	198	
	100	170	172	162	167	washed

- Uptake of a nanoparticle–protein complex by cells depends on:
- 1. whether the cell membrane has receptors for the proteins,
- 2. the proteins are presented in the correct orientation to interact with the receptor
- 3. the nanoparticle-bound protein can compete effectively with the free protein for the receptor





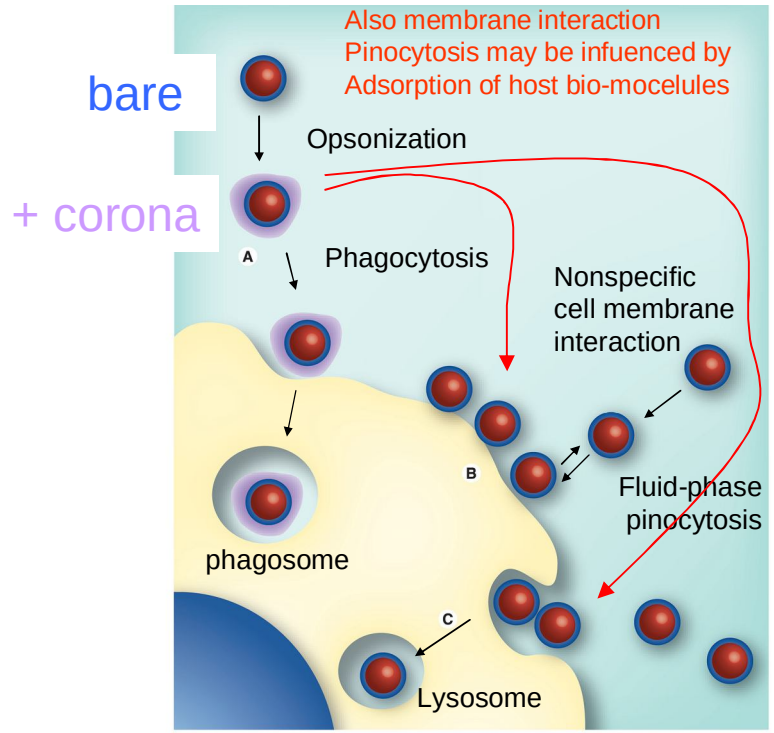
SDS/PAGE of precipitated plasma proteins from a chromatogram (gel-filtration) with 200 nm 50:50 NIPAM/BAM particles (*Middle*) and without particles (*Bottom*).

amine-modified; 3, 100-nm plain; 4, 50-nm plain; 5, 100-nm carboxyl-modified; and 6, 50-nm carboxyl-modified.

- Immunoglobulin
- Ig alpha-1 chain C region X X X X
- Ig alpha-2 chain C region X
- Ig gamma-1 chain C region X X X X X
- Ig gamma-2 chain C region X X X X
- Ig gamma-3 chain C region X X
- Ig gamma-4 chain C region X X X
- Ig kappa chain C region X X X X X X
- Ig lambda chain C regions X X X X X X
- Ig mu chain C region X X X X
- Immunoglobulin J chain X X
- Ig kappa chain V-I region X X X
- Ig kappa chain V-II region X X X X
- Ig heavy chain V-III region X X
- Ig kappa chain V-IV region X X
- Lipoproteins
- Apolipoprotein A-I X X X X X X
- Apolipoprotein A-II X X
- Apolipoprotein A-IV X X X X X
- Apolipoprotein B-100 X X X X
- Apolipoprotein C-I X X X X
- Apolipoprotein C-III X X X
- Apolipoprotein D X X X
- Apolipoprotein E X X X X
- Apolipoprotein F X
- Apolipoprotein L1 X X
- Beta-2-glycoprotein 1
- (apolipoprotein H)
- X X X X
- Clusterin (apolipoprotein J) X X X X X X

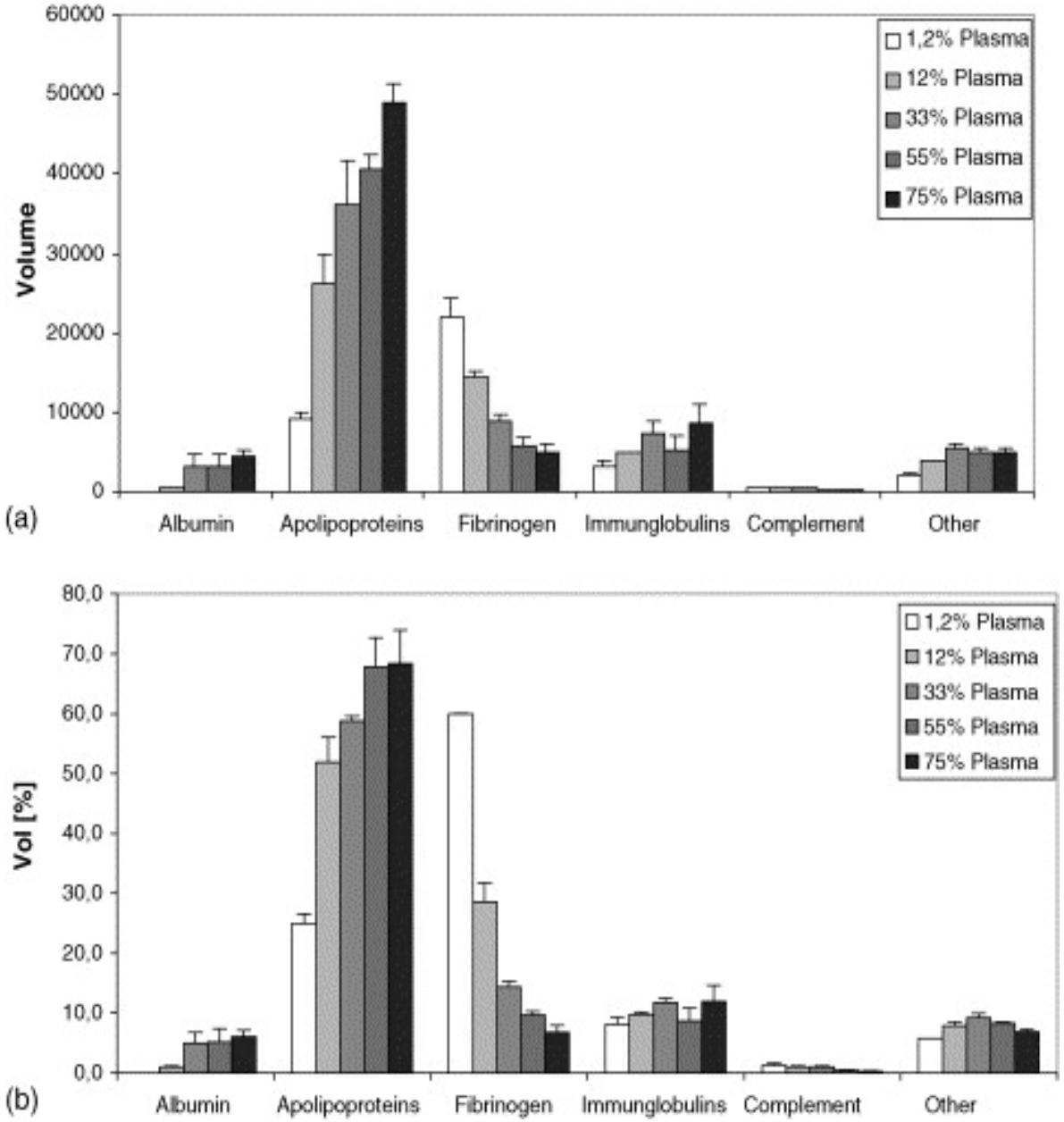
Proteomics of surface modified PS nanoparticels !!!!

- Complement pathways
- Complement C1r X
- Complement C1 s X X X
- Complement C1q X X X X
- Complement C2
- Complement C3 X X X X
- Complement C4 X X X X
- Complement C5 X X
- Complement C6 X X
- Complement C8 X X
- Complement C9 X
- Complement factor B X X X
- Complement factor H X X X X X X
- Complement factor I X
- Complement C4b-binding protein X X X X X
- Plasma protease C1 inhibitor X X X
- Acute-phase protein
- Mannose-binding protein X
- Alpha 1-antitrypsin X X X
- Alpha 1-antichymotrypsin X X X X
- Fibrinogen X X X X X X
- Plasminogen X X X X
- Complement factors (see above) X X X X X X
- Serum amyloid P component X X
- Serum amyloid A X
- Transthyretin X X X
- Coagulation factors
- Fibrinogen (factor I) X X X X X X
- V X
- XI (plasma thromboplastin
- antecedent)
- X X
- Prekallikrein X
- Kininogen X X X X X
- Fibronectin X X X X X
- antithrombin III X X
- Vitamin K-dependent protein S X X X X
- Plasminogen X X X X
- Alpha 2-antiplasmin X X
- *1, 100-nm amine-modified; 2, 50-nm


Also membrane interaction
Pinocytosis may be infuenced by
Adsorption of host bio-mocelules
bare
Opsonization
+ corona
A
Phagocytosis
Nonspecific
cell membrane
interaction
B
Fluid-phase
pinocytosis
phagosome
C
Lysosome

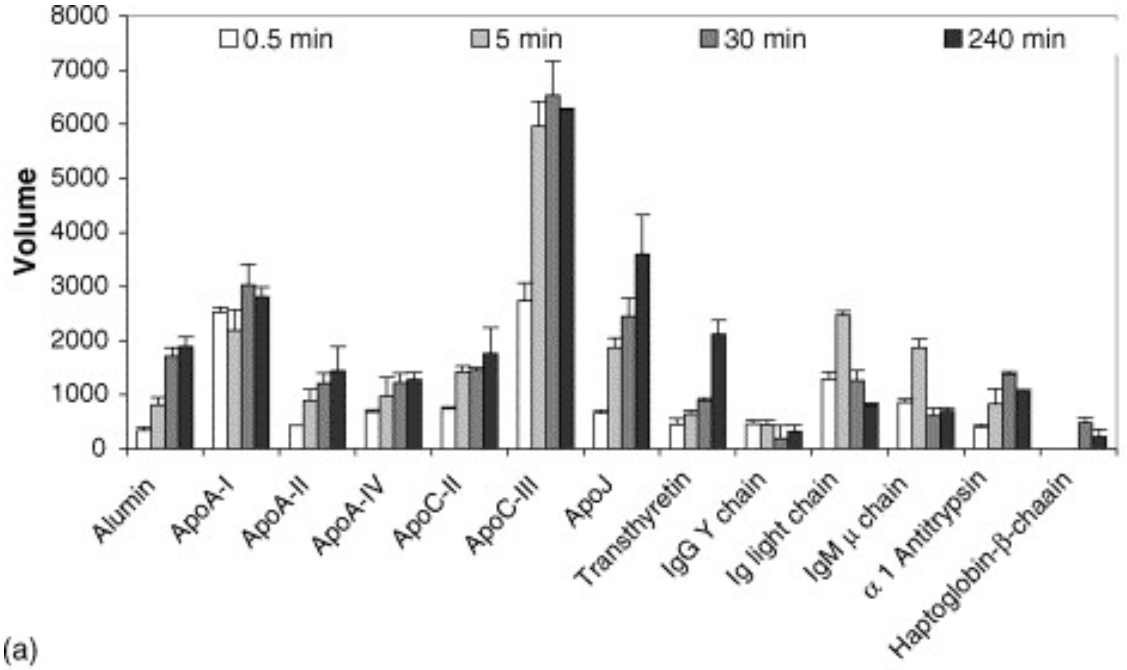
Charcaterisation of the protein/lipid composition of the corona of some NPs

- SLN are particles made from solid lipids (i.e. solid at room temperature and also at body temperature) and stabilized by surfactants.

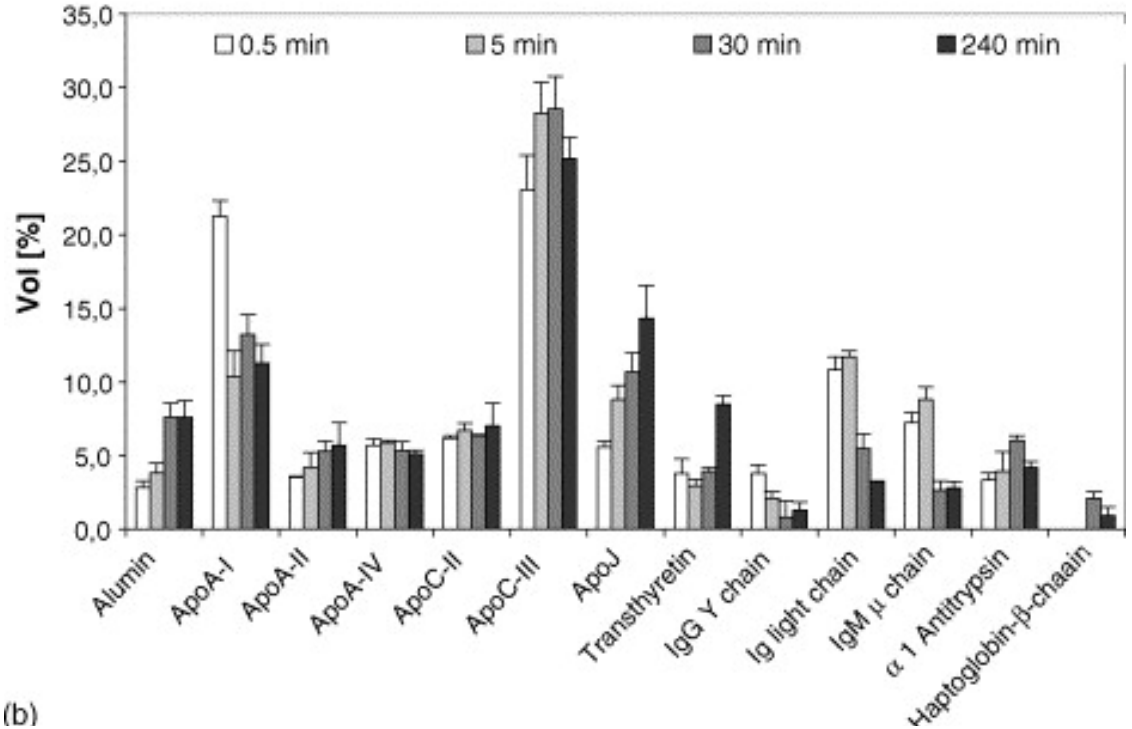


- Plasma proteins

on SLN



(a)



(b)

Apolipoproteins and lipid adsorption: important NPs behaviour?

- Exchange of Apo-I form HDL to NPs?

- In co-polymer NPs of Nisopropylcrylamide and N-t-butylacrylamide: complete Liproteins adsorbed, with the lipid components not only apolipoproteins

Kinetics, Vroman effect

- Plentiful proteins with a lower affinity bind first and are then displaced by less represented high affinity binding proteins
- Such a displacement occurs in seconds or less
- Different serum plasma dilution results in different protein pattern adsorption

Adsorption of plasma proteins on NPs and their behaviour after blood injection (kinetics, RES clearance)

- Adsorption of plasma proteins is the main factor influencing the behaviour of an injected (blood) NPs (first study with drug-nanocarriers)
- e.g. bindign of IgG, complement, fibrinogen promotes phagocytosis and elimination by the RES (Opsonines)
- On the countrary dysopsonines like albumin promote prolonged circulation of NPs

The five minutes “rule”

- A particulate which is efficiently captured by the RES disappears from the blood within ~ 5 min
- It is generally observed that if a NP survives for more than 5 min in the blood it is a long circulating one
- > 90% of cleared NPs are captured by Kupffer cell in the liver

Coating effect on stability in solution

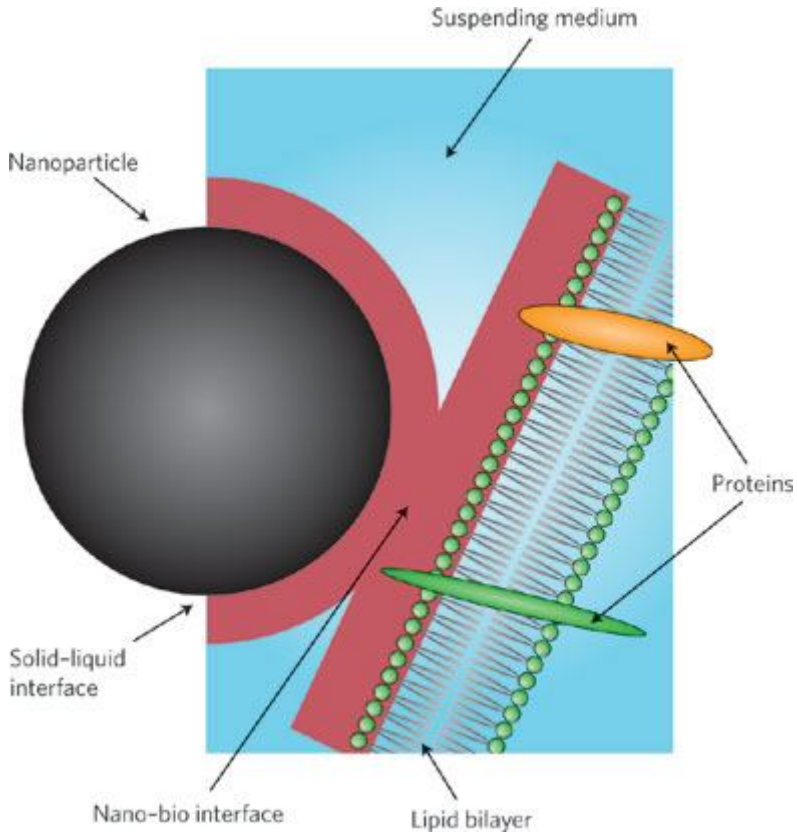
- Stronlgy charged NPs are stable in solution: they do not aggregate due to coulumbian repulsion
- Neutral NPs may be less stable due to van der Waal attractions between NPs surfaces
- Surface coating of NPs with non-ionic polymers strongly reduces interparticulate attractive van der Waal forces

Polymeric coat stabilises NPs thanks to:

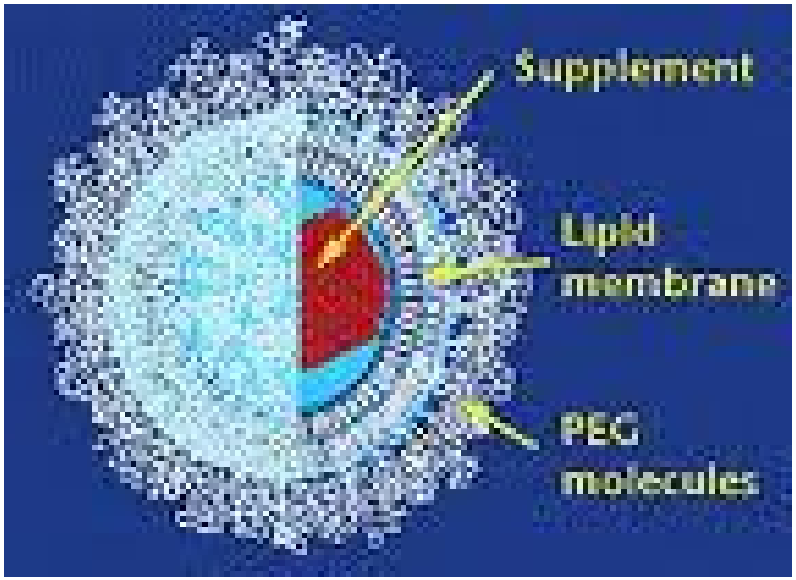
- Volume restriction: loss of conformatioinal entropy when two surfaces approach each other. There is a reduction of the available volume foe each polymer

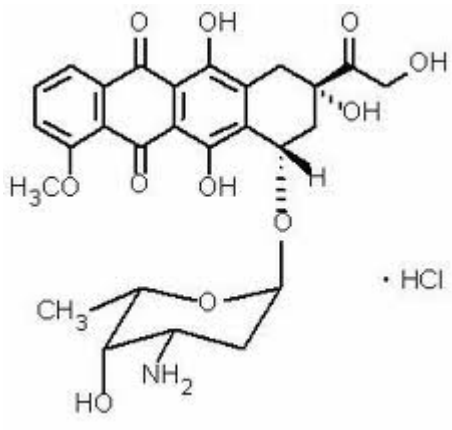
- *Osmotic pressure: the two layers when NPs collide in solution are compressed leading to an increased concentration of the polymeric components which in turn leads to a local solvent influx and increase of osmotic pressure which tend to repulse the two particles*

- *Consider: If the polymer density is high, the contribution of the entropic effect is likely less relevant than the pressure effect*


Suspending medium
Nanoparticle
Proteins
Solid–liquid interface
Nano–bio interface
Lipid bilayer

DOXIL (Caelyx)

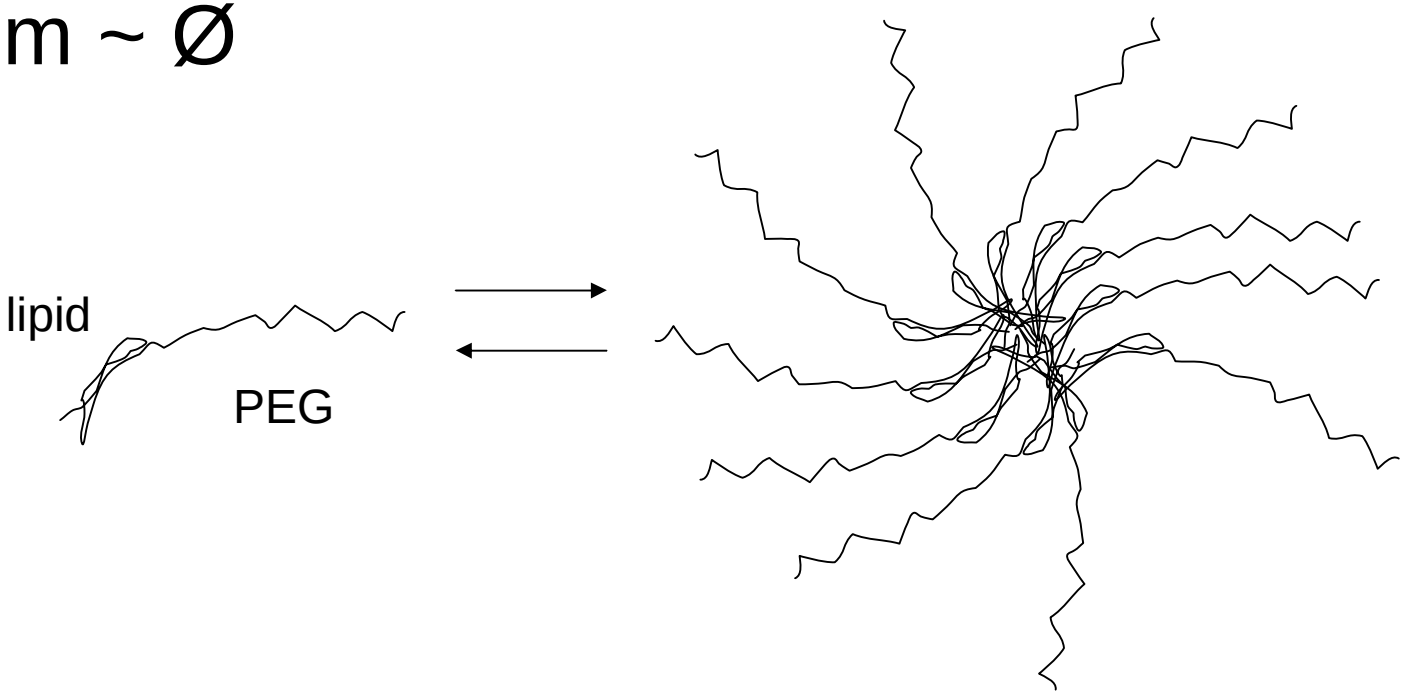




Long circulating NPs

doxorubicin

- PEG-phospholipids conjugates exhibit a low CMC (Critic Micellar Constant) value and form stable micellar structures of 30 nm ~ Ø

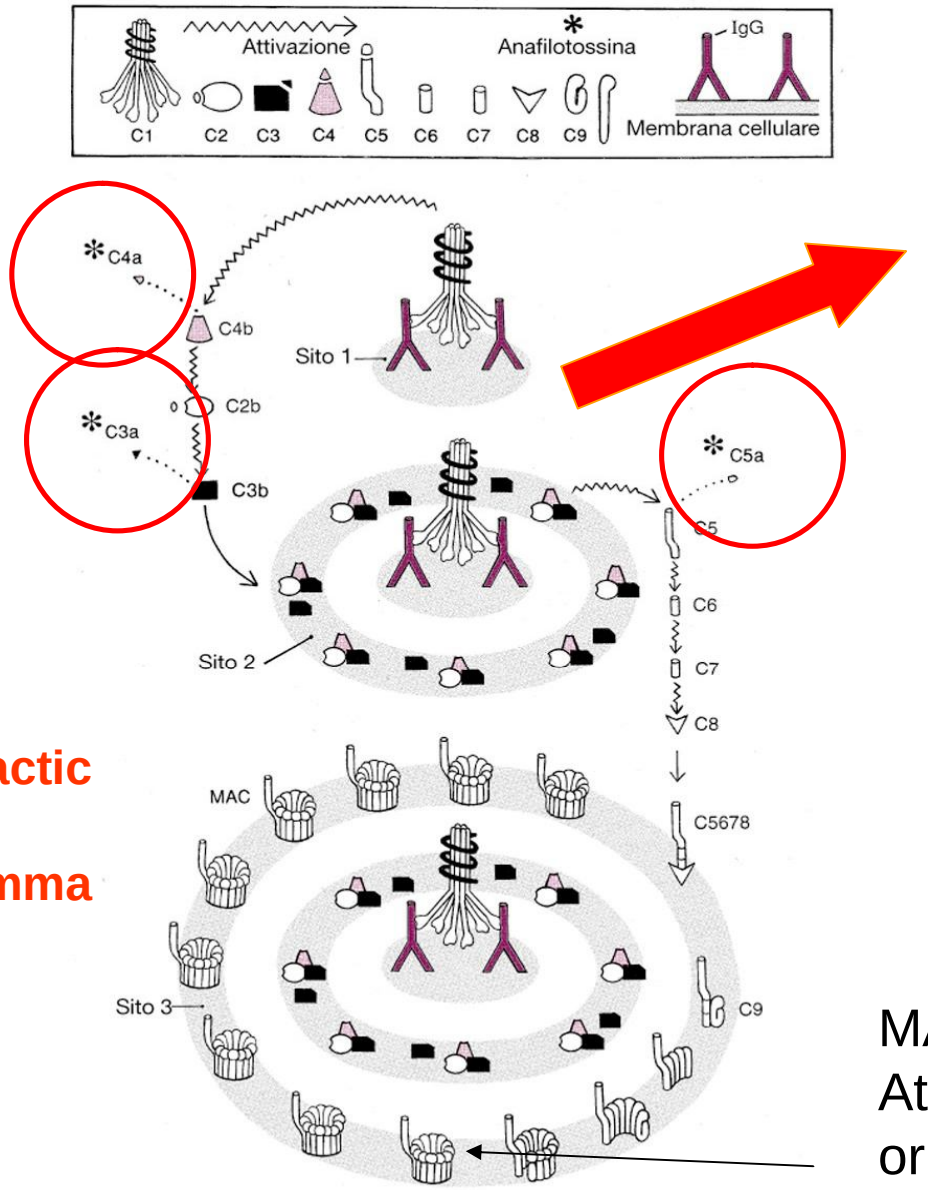


Use of PEG-LP

- PEG micelles used for hydrophobic drug solubilization
- Stabilisation of oil-in-water nanoemulsions
- Inclusion in liposomal NPs (DOXIL)
- Surface adsorption on CNTs to help dispersion in acqeous media

However..

- PEG-bearing nanomedicines may lead to hypersensitivity reactions (HSR) in sensitive individuals
- Pseudoallergy: flushings and circulatory disturbance
- in pig models DOXIL induced pulmunary cardio-pulmunary distress is related to complement activation



Hypersensitivity

Chemotactic - proinflammatory peptides

MAC = Membrane Attacking Complex or C5b-9

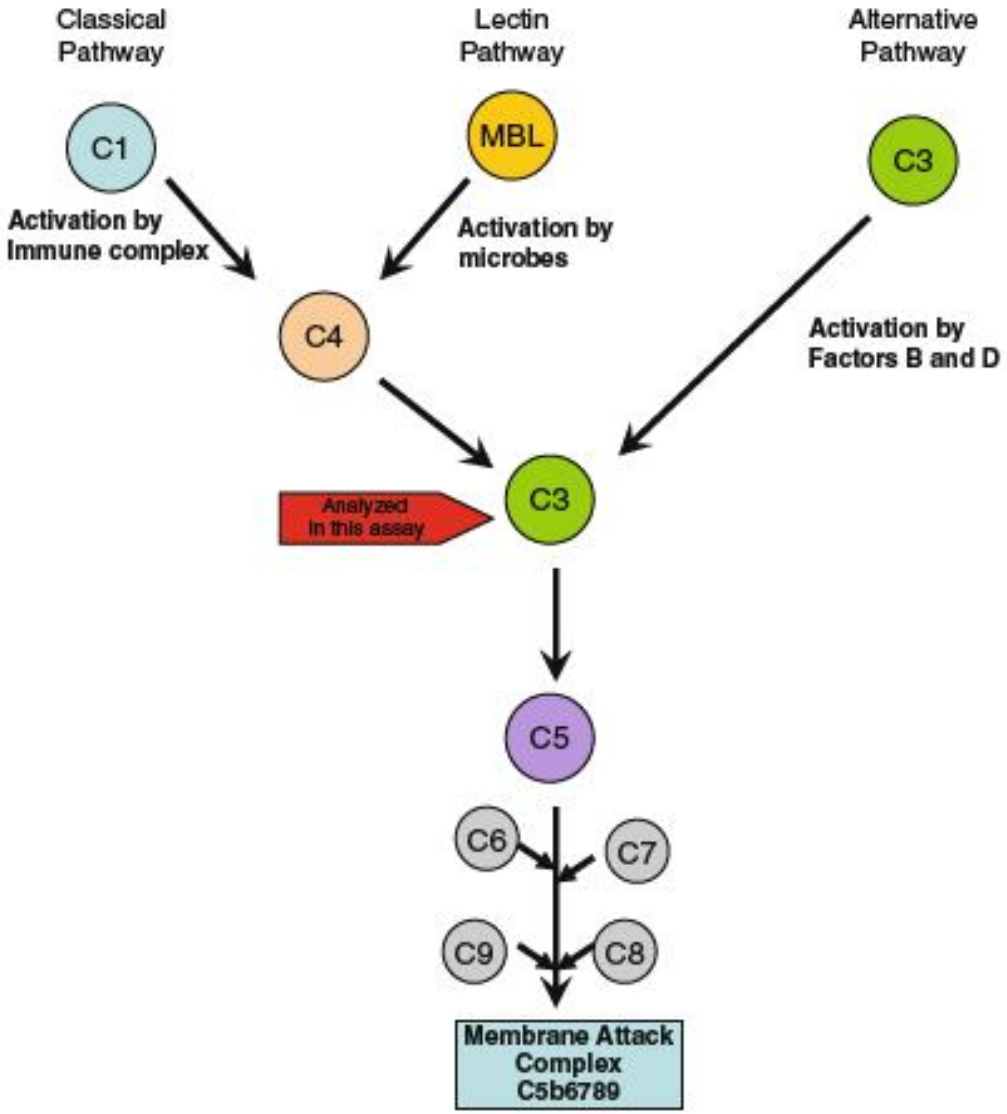
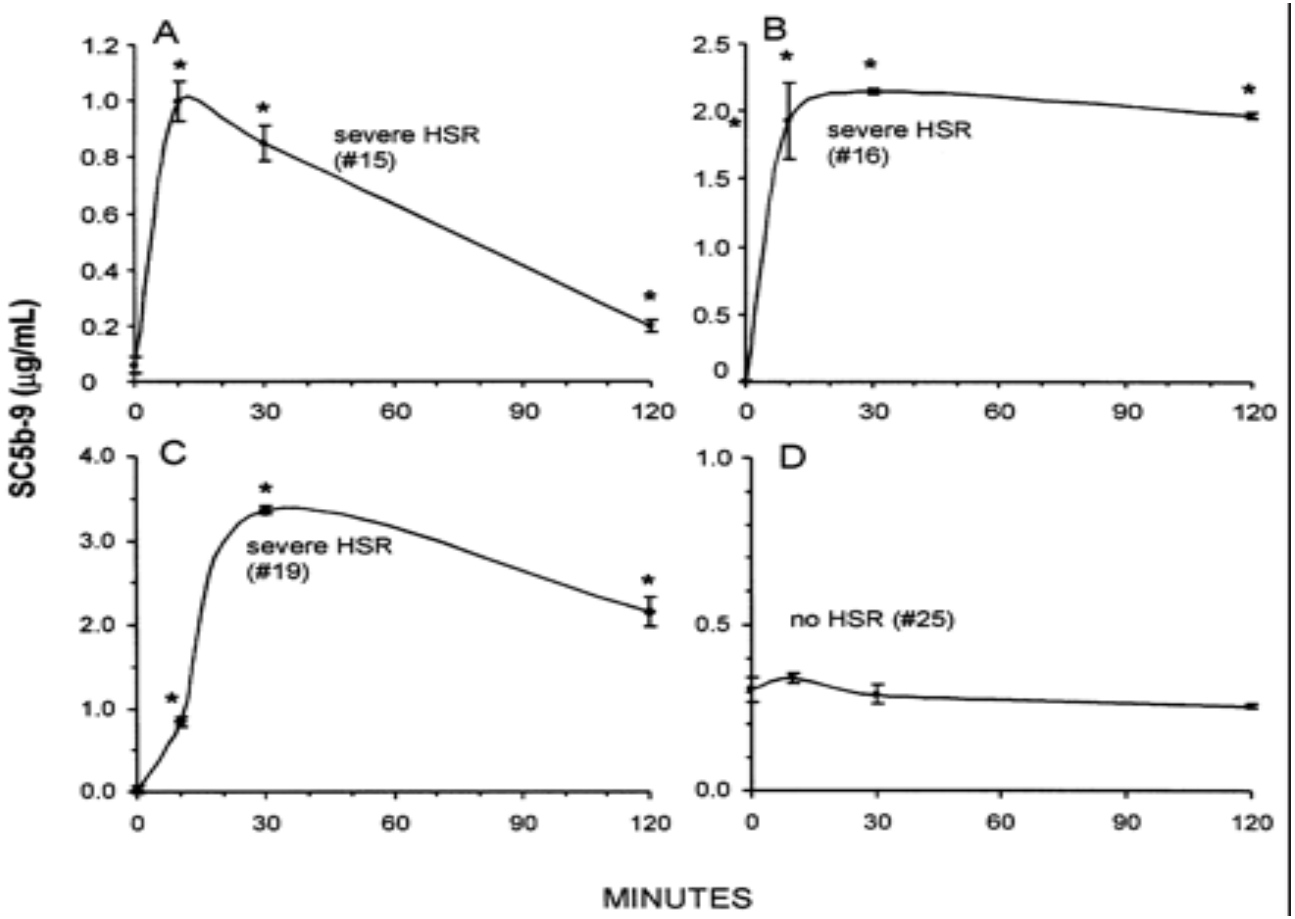


Fig. 1. Complement activation pathways. The antibodies used in this assay detect both full C3 protein and any C3 cleavage products.

Severe HyperSensitivityReaction due to DOXIL relates with complement activation (patient dependent.. Why?)



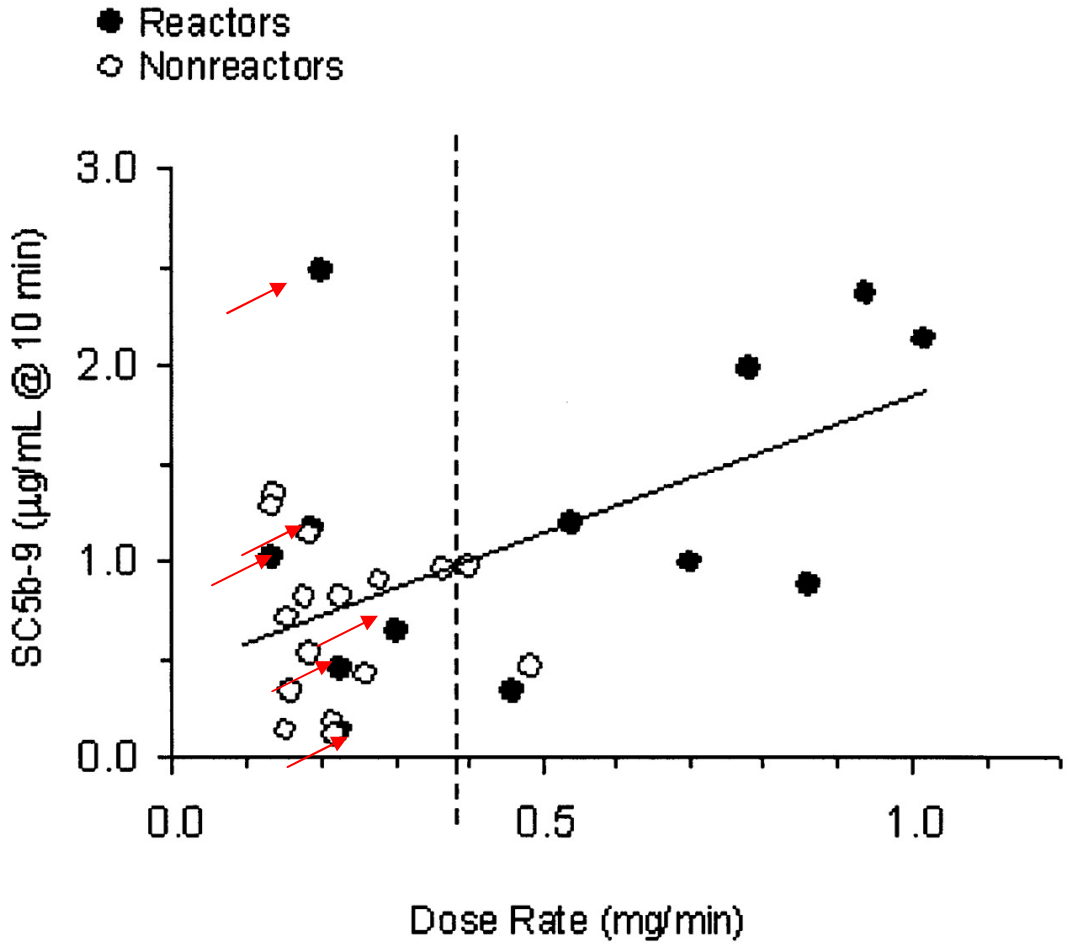
- Time course of Doxil-induced changes in plasma complement terminal complex (SC5b-9) in cancer patients and its individual variation

Doxil dose rate and subject sensitivity

Complement acitvation correlates with increased dose rate

BUT in some individuals

Complement is activated even at low dose rate

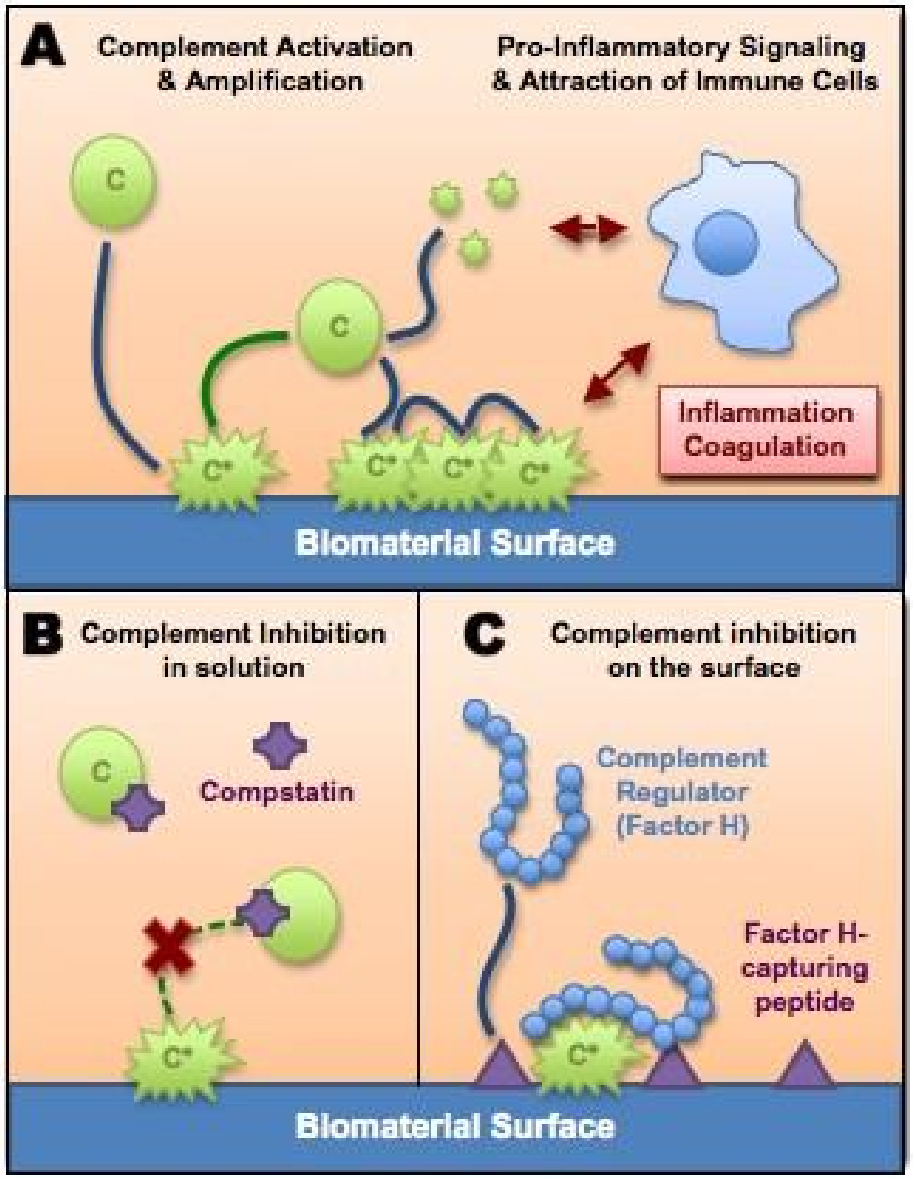


Using other liposomes it could be conlcuded that:

- **Surface functionalization of NPs with certain chemical structures elicits swift complement activation that is initiated by natural IgM antibody and propagated via the classical pathway**

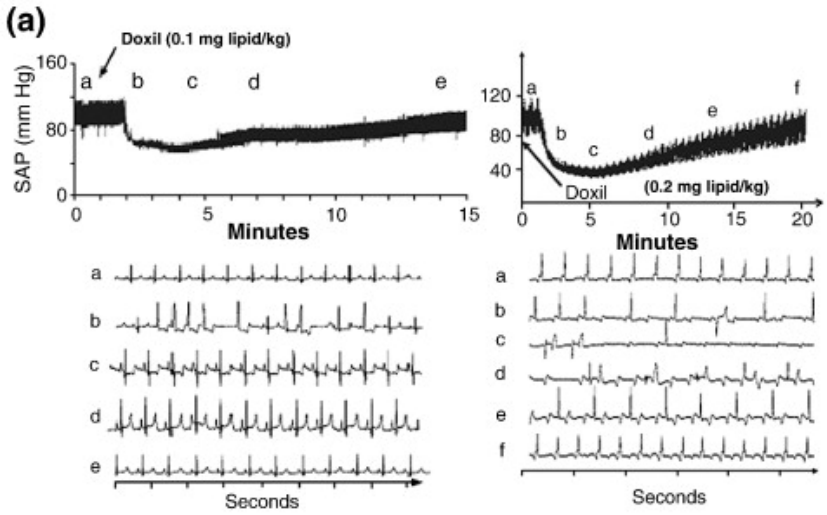
- **The intensity of the response is dependent on the chemical structures of the lipid derivative and not zeta potential effects alone**

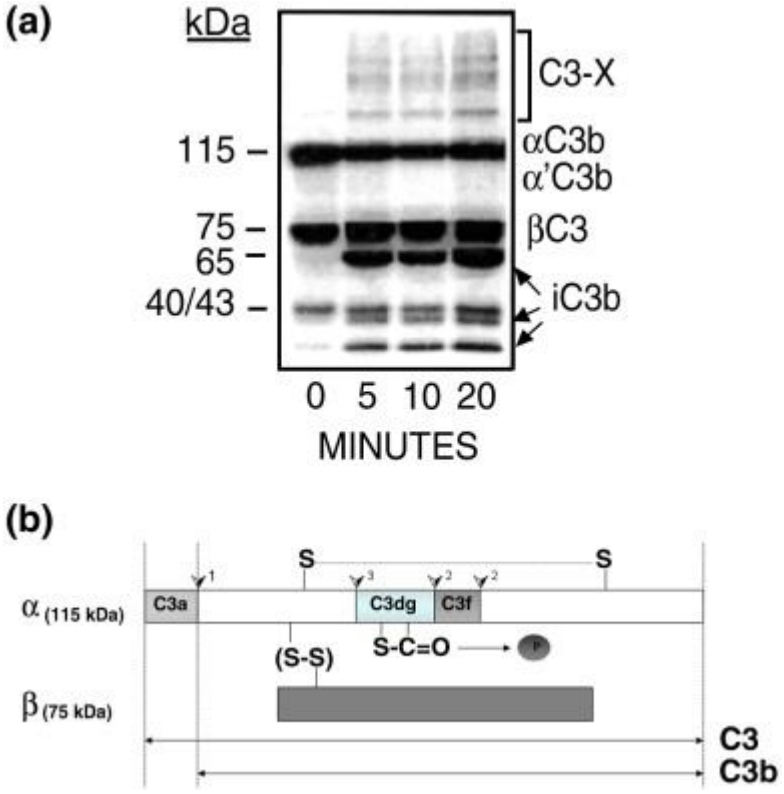
- **the extent of complement activation may be tempered by complement inhibiting regulatory proteins that bind to the surface of NPs**


A
Complement Activation
& Amplification
Pro-Inflammatory Signaling
& Attraction of Immune Cells
C
C
C*
C*
C*
C*
Inflammation
Coagulation
Biomaterial Surface
B
Complement Inhibition
in solution
C
Compstatin
C*
C
Complement
Regulator
(Factor H)
Factor H-
capturing
peptide
C*
Biomaterial Surface

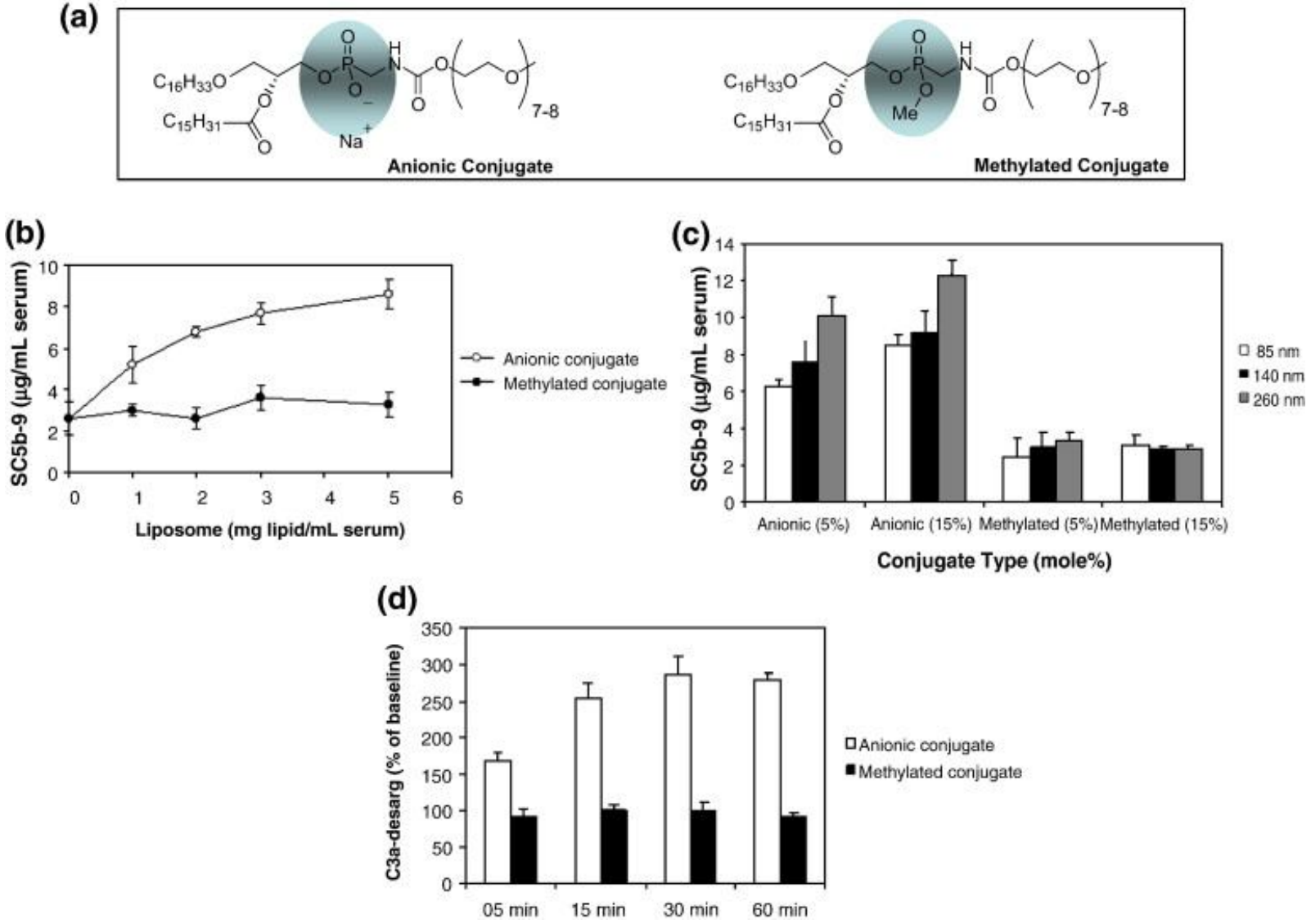
DOXIL in the pig model

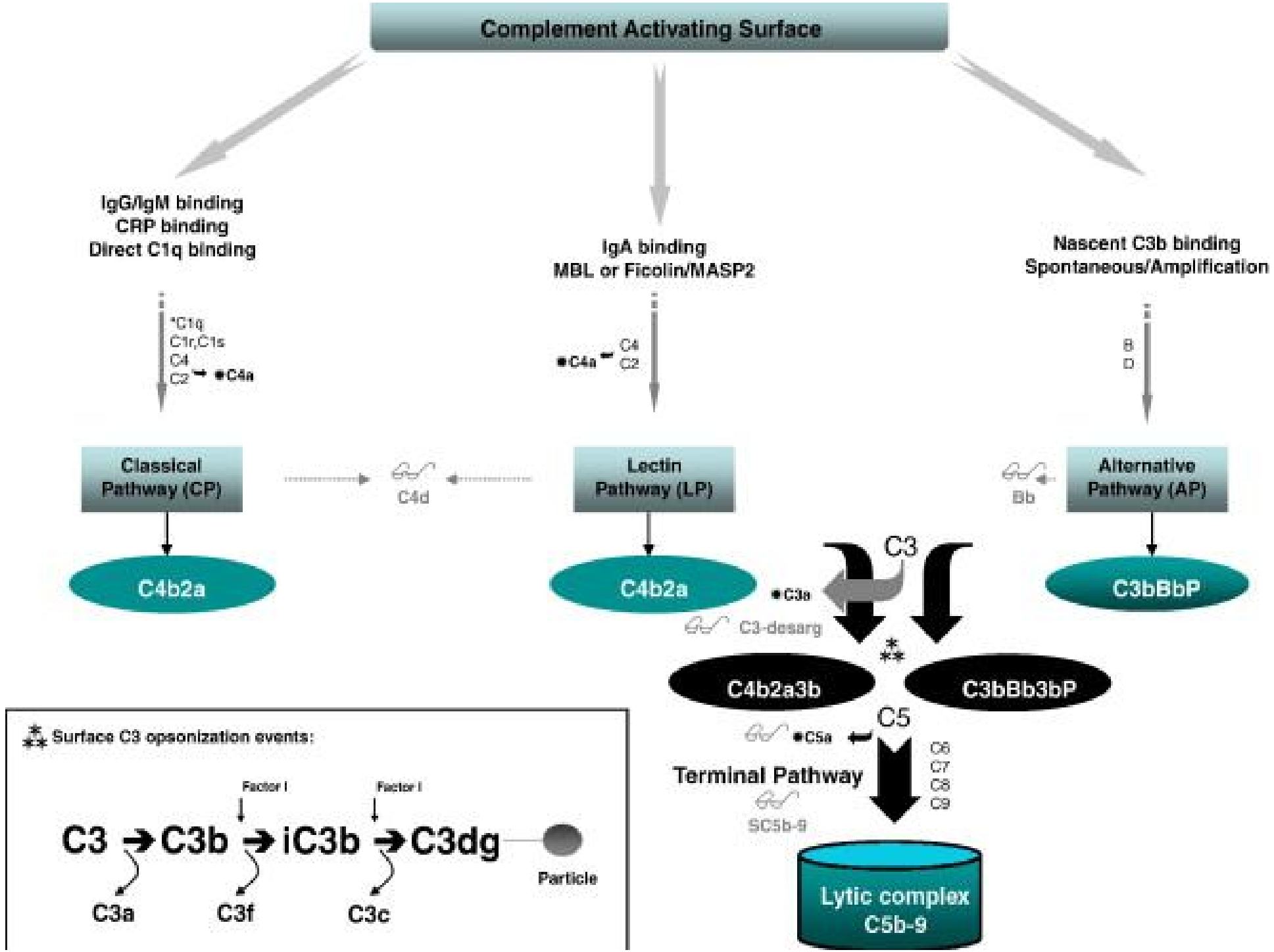
Systemic Arteriolar Pressure



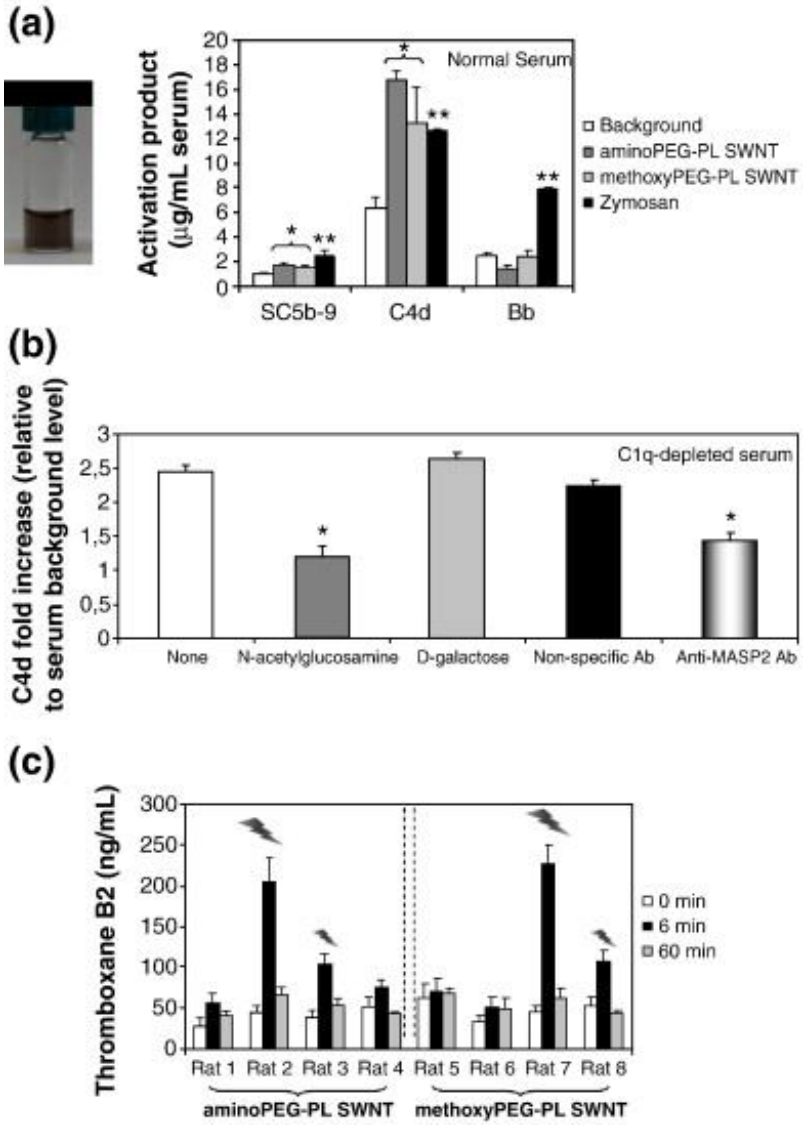

(a)
kDa
115 –
75 –
65 –
40/43 –
C3-X
αC3b
α'C3b
βC3
iC3b
0 5 10 20
MINUTES
(b)
S
S
1
3
2
2
C3a
C3dg
C3f
α (115 kDa)
(S-S)
S-C=O
β (75 kDa)
C3
C3b

SC5b-9 induced by negative and methylated PEGylated derivative

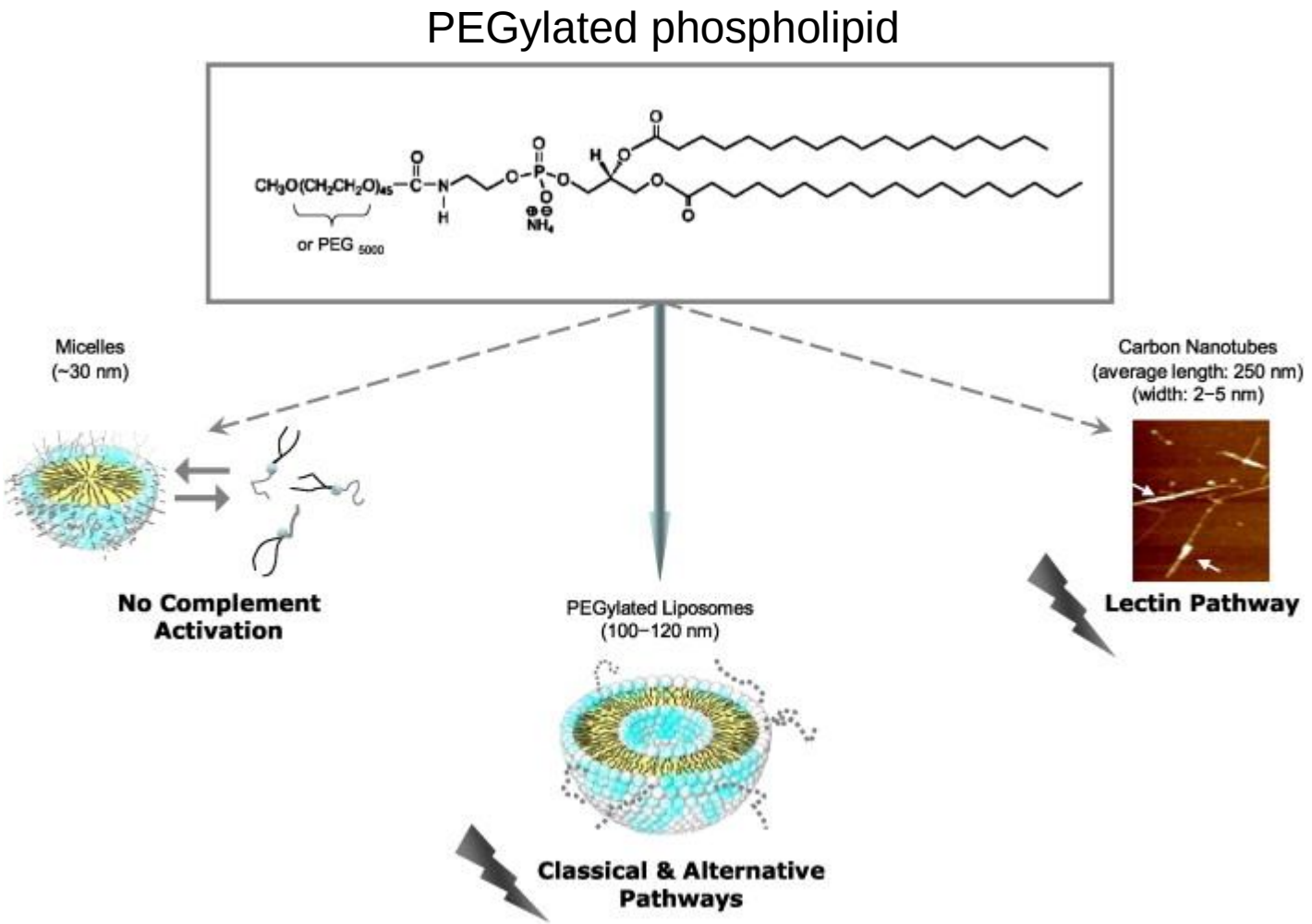



Complement Activating Surface
IgG/IgM binding
CRP binding
Direct C1q binding
IgA binding
MBL or Ficolin/MASP2
Nascent C3b binding
Spontaneous/Amplification
*C1q
C1r,C1s
C4
C2
*C4a
C4
C2
*C4a
B
D
Classical Pathway (CP)
C4d
Lectin Pathway (LP)
Bb
Alternative Pathway (AP)
C4b2a
C4b2a
C3
*C3a
C3-desarg
C3bBbP
C4b2a3b
C3bBb3bP
C5
*C5a
Terminal Pathway
C6
C7
C8
C9
SC5b-9
Lytic complex
C5b-9
Surface C3 opsonization events:
Factor I
Factor I
C3
C3b
iC3b
C3dg
Particle
C3a
C3f
C3c

PEG-CNTs

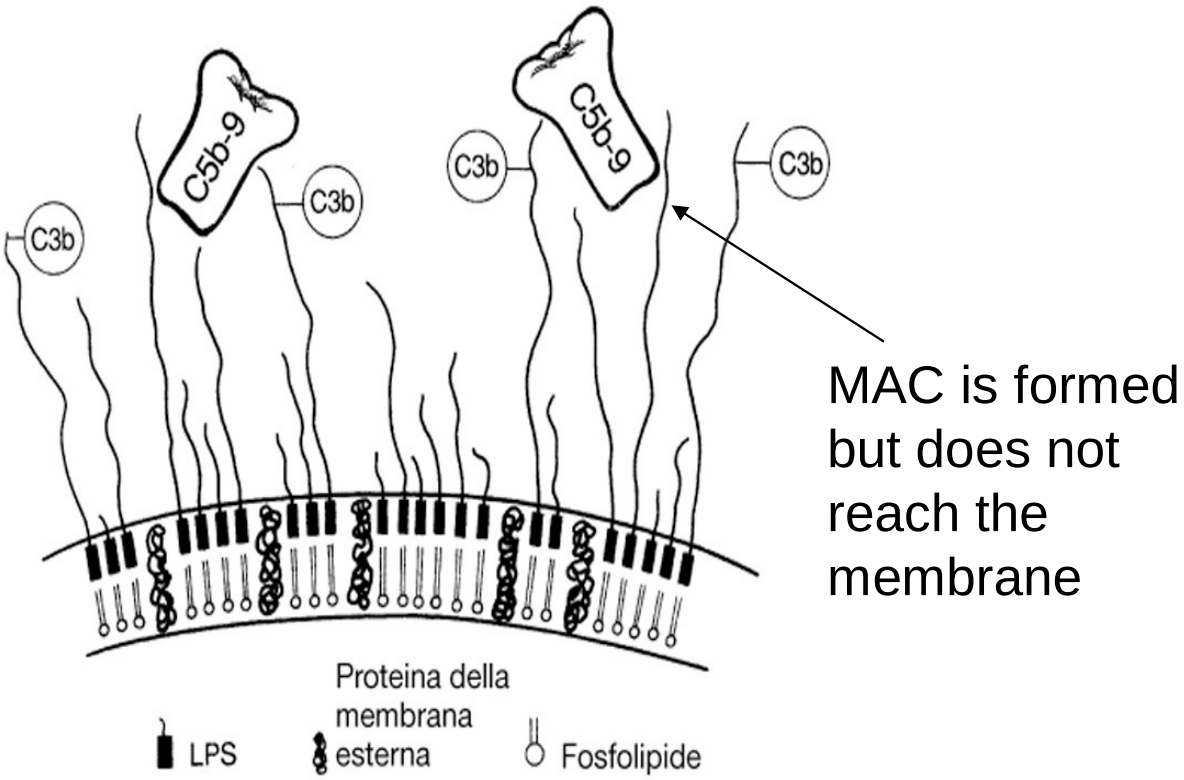

(a)
Activation product (μg/mL serum)
Normal Serum
Background
aminoPEG-PL SWNT
methoxyPEG-PL SWNT
Zymosan
SC5b-9
C4d
Bb
(b)
C4d fold increase (relative to serum background level)
C1q-depleted serum
None
N-acetylglucosamine
D-galactose
Non-specific Ab
Anti-MASP2 Ab
(c)
Thromboxane B2 (ng/mL)
0 min
6 min
60 min
Rat 1
Rat 2
Rat 3
Rat 4
Rat 5
Rat 6
Rat 7
Rat 8
aminoPEG-PL SWNT
methoxyPEG-PL SWNT

Liposomal NPs ad CNTs activate the complement cascade



How can DOXIL be long circulating?

- Liposomal NPs are opsonised but the Complement protein cannot intercat with cell receptors (masked by PEG chains)



G. Majno, I. Joris, *Cellula, tessuti e malattia*,
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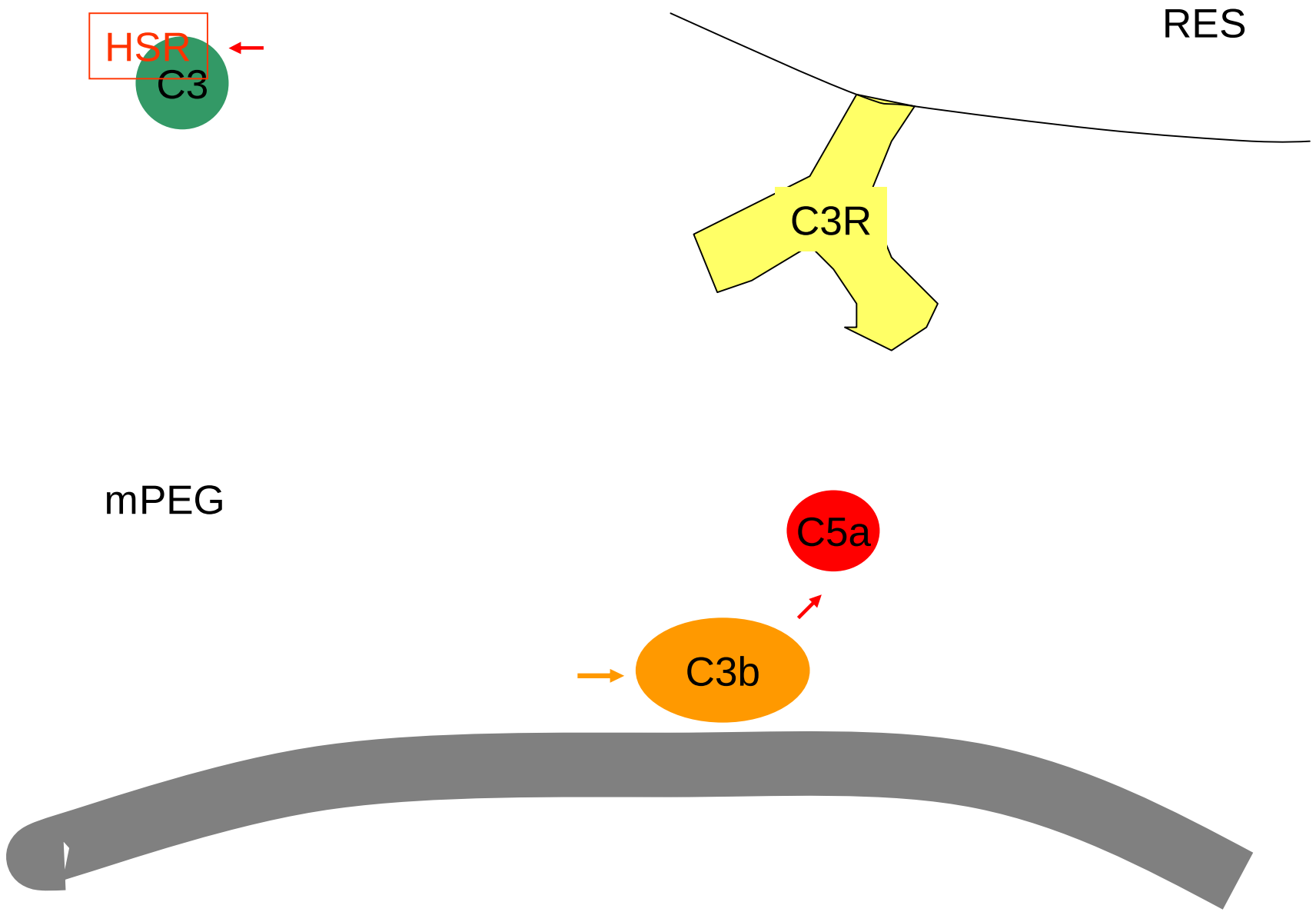
- The saccharidic part of LPS hampers to some extent the complement effects, but does not block complement activation!

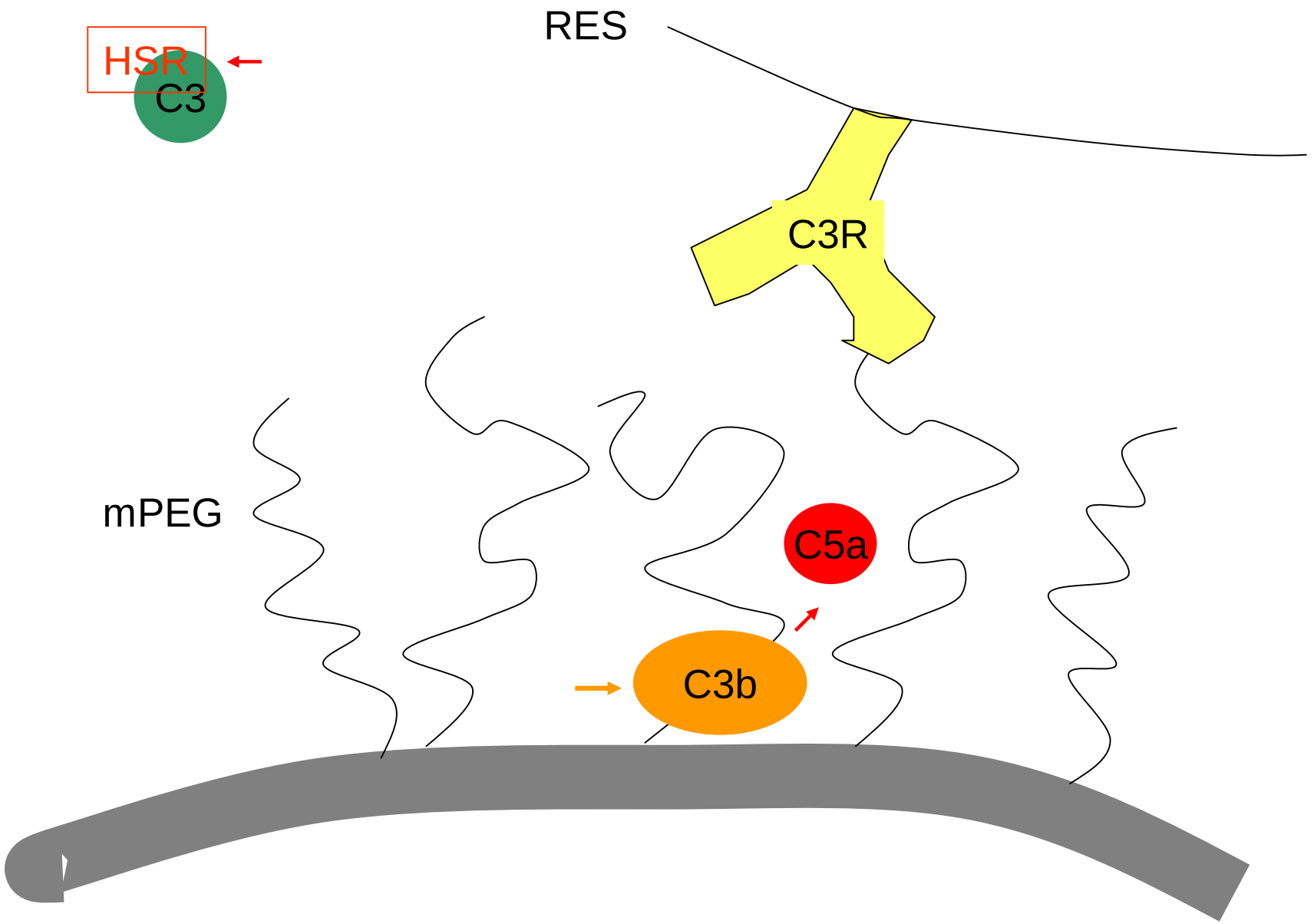
Mechanisms of phagocytosis escape by DOXIL (in spite of complement activation

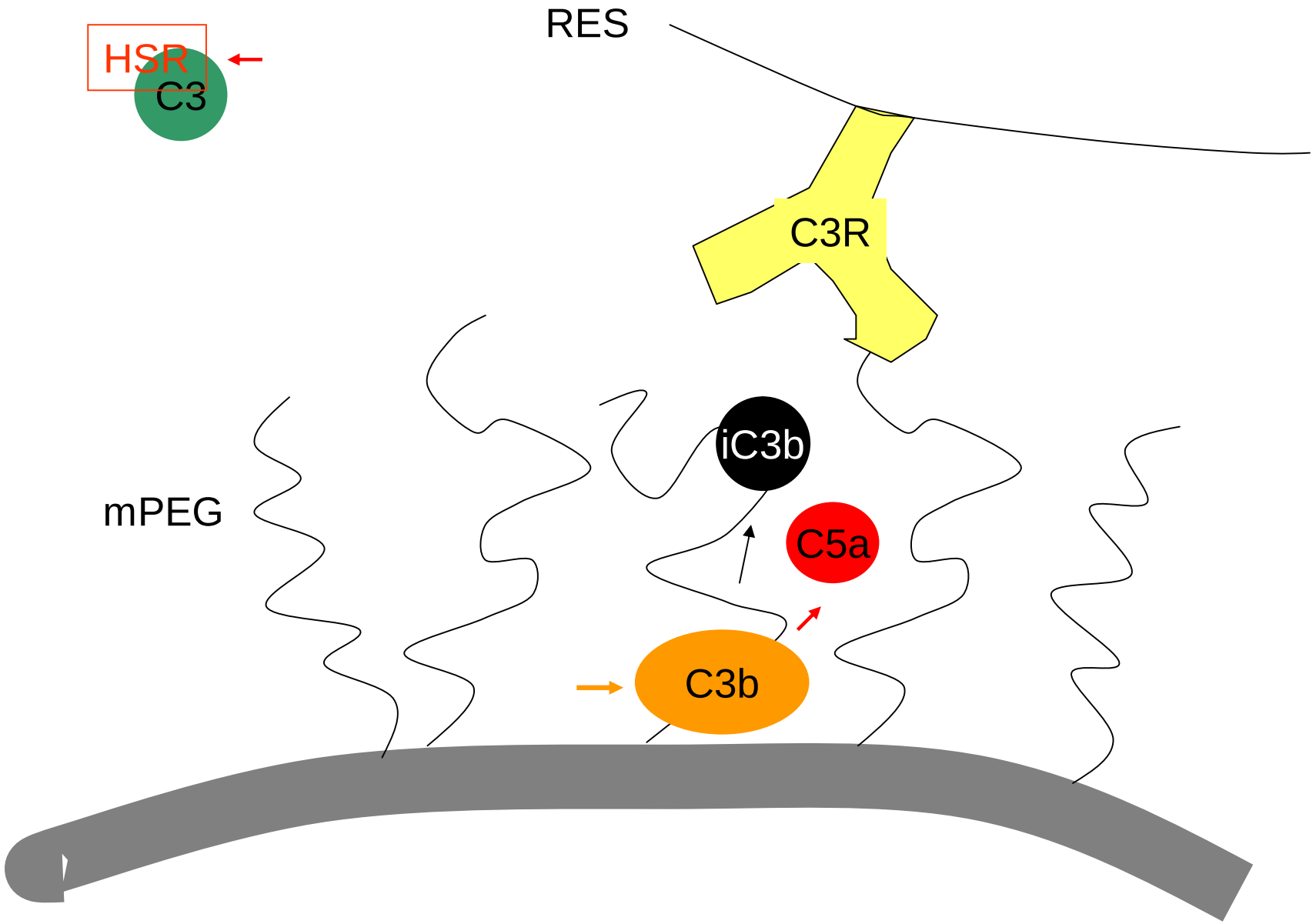
- C3b is fixed but in a cryptic location, inaccessible to the C3b receptor (PEG chains hinder the interaction)

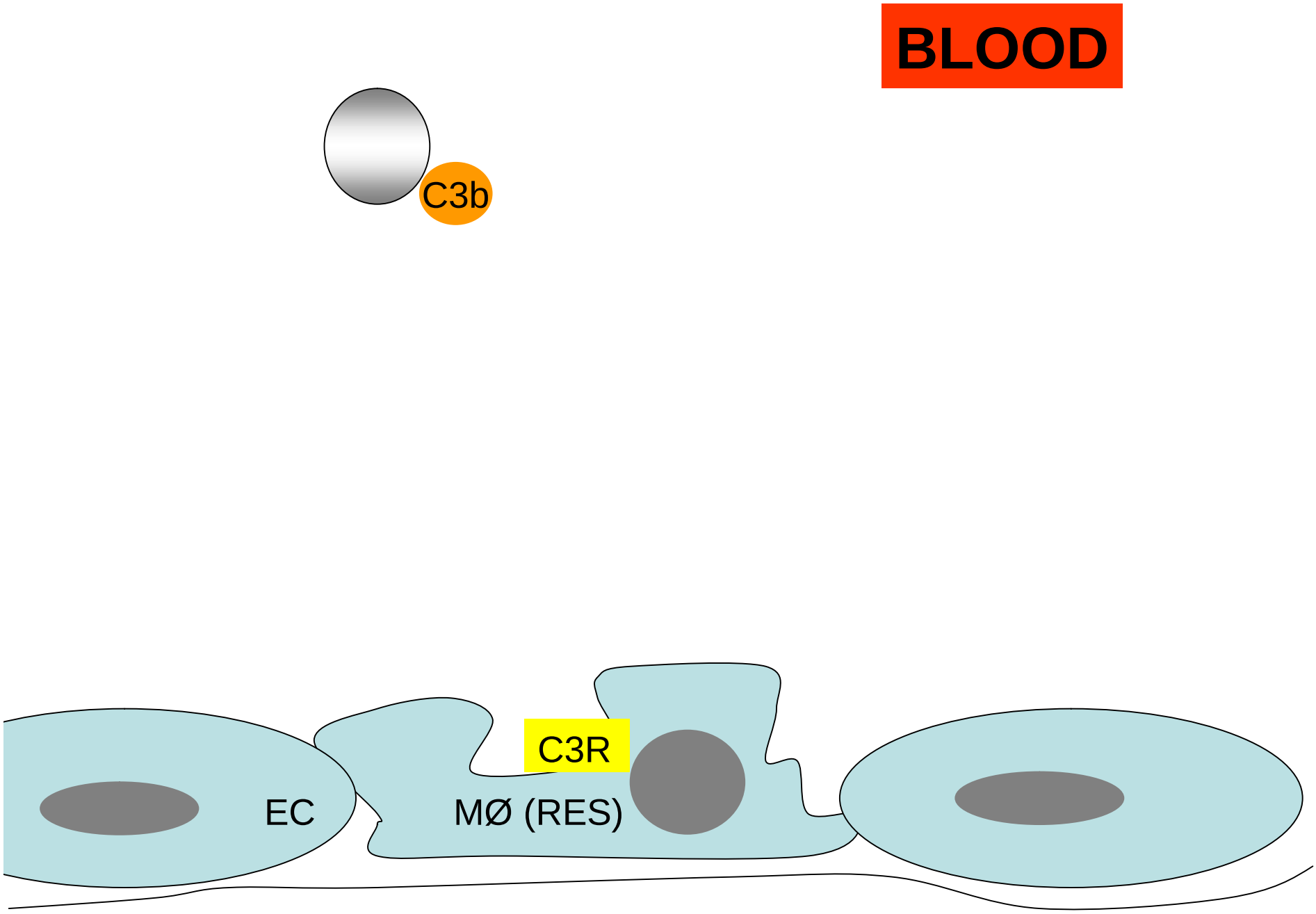
- Degradation of C3b fragments releases products that inhibits the C3b receptor (CR3)

- C3b may interact with CR1 on red blood cells: prolonged criculation due to binding to rbc???


HSR
C3
RES
C3R
mPEG
C5a
C3b


HSR
C3
RES
C3R
mPEG
C5a
C3b

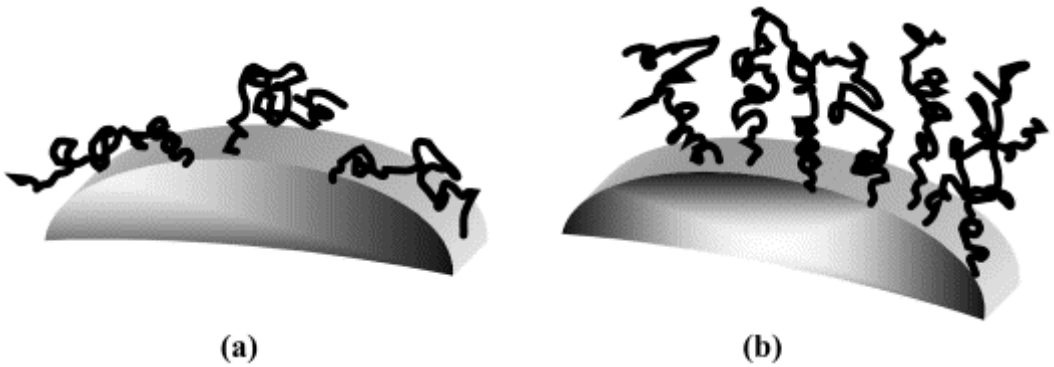

HSR
C3
RES
C3R
iC3b
mPEG
C5a
C3b


BLOOD
C3b
C3R
EC
MØ (RES)

PEG density is important

Low PEG density

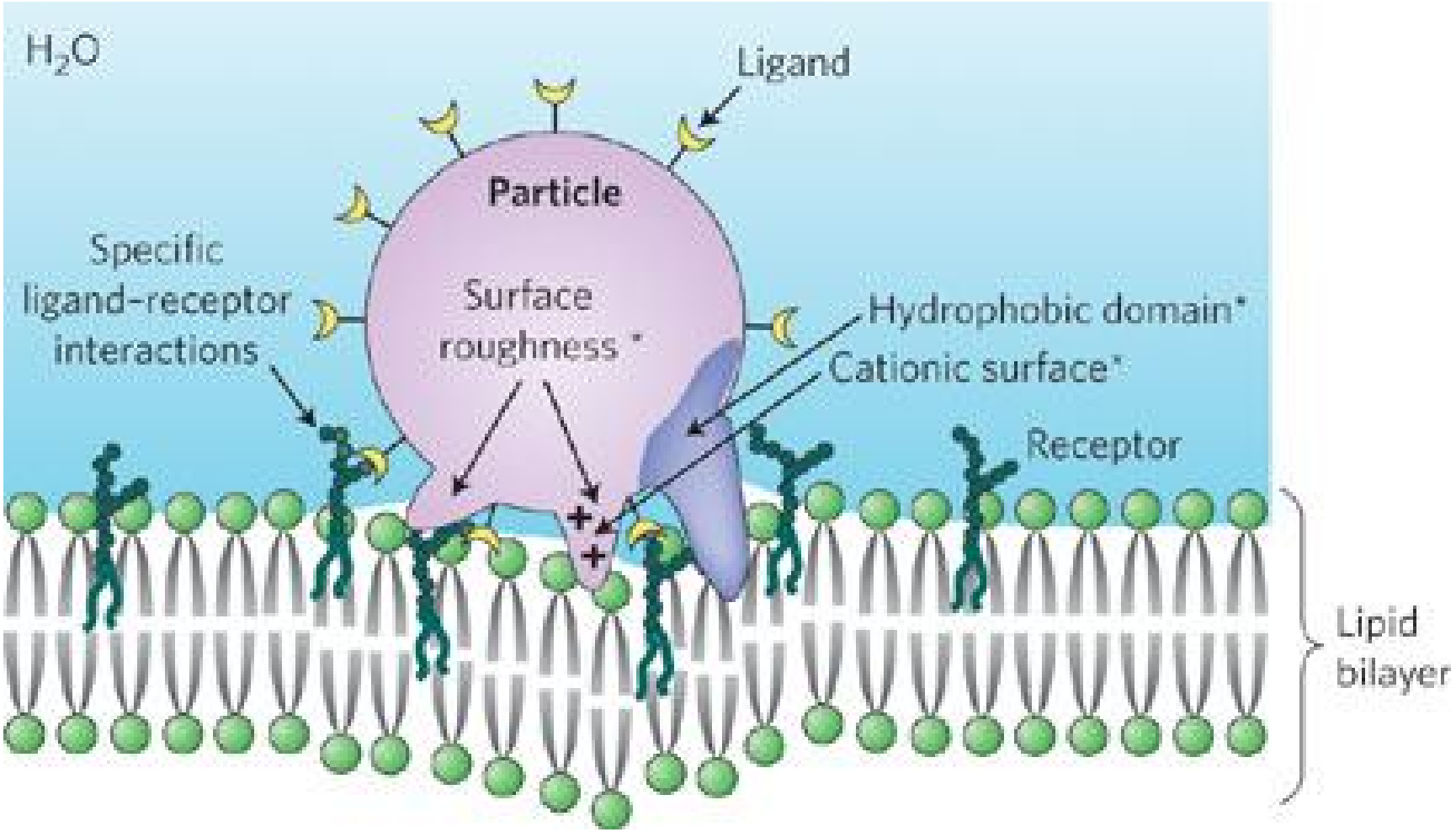
High PEG density



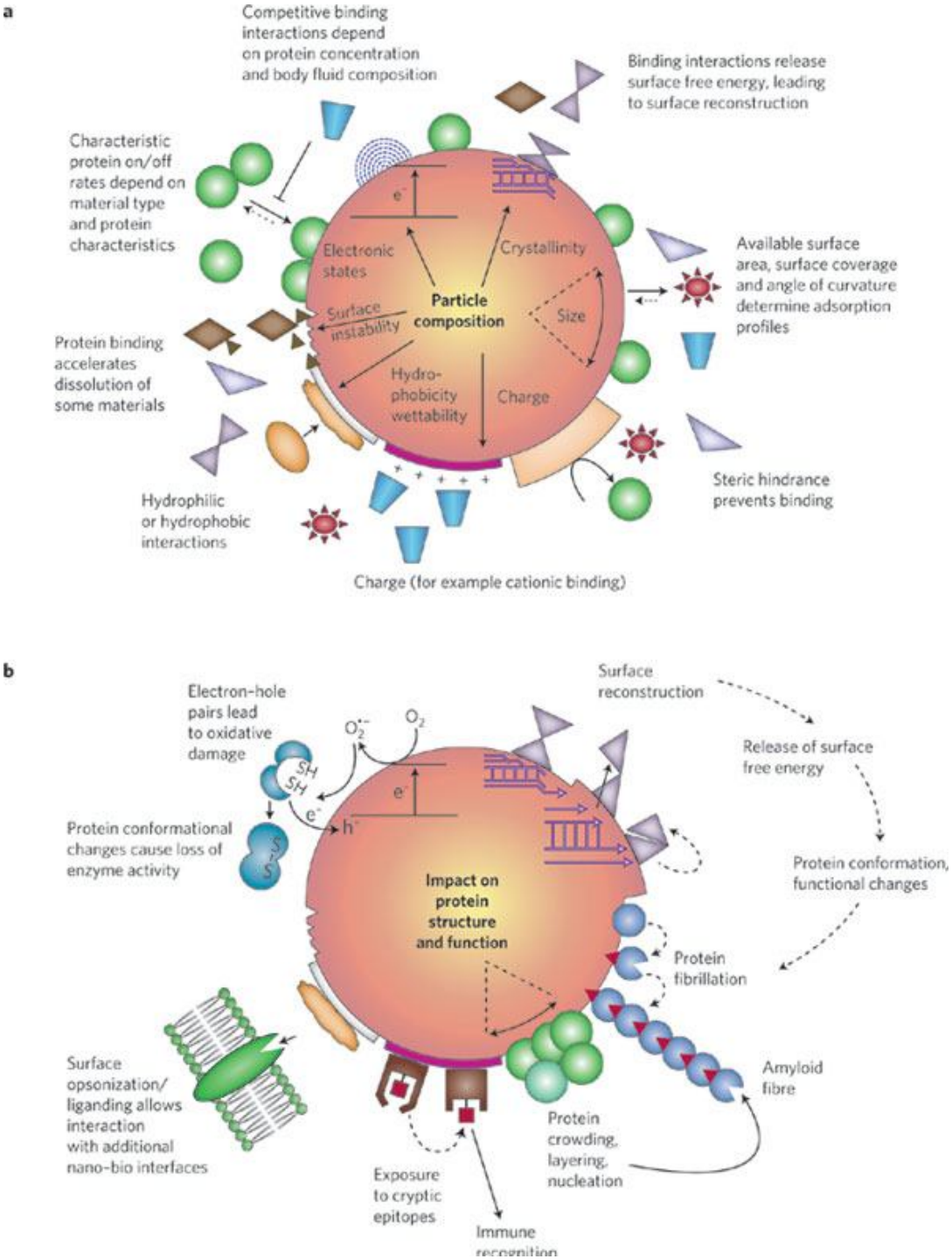
mushroom

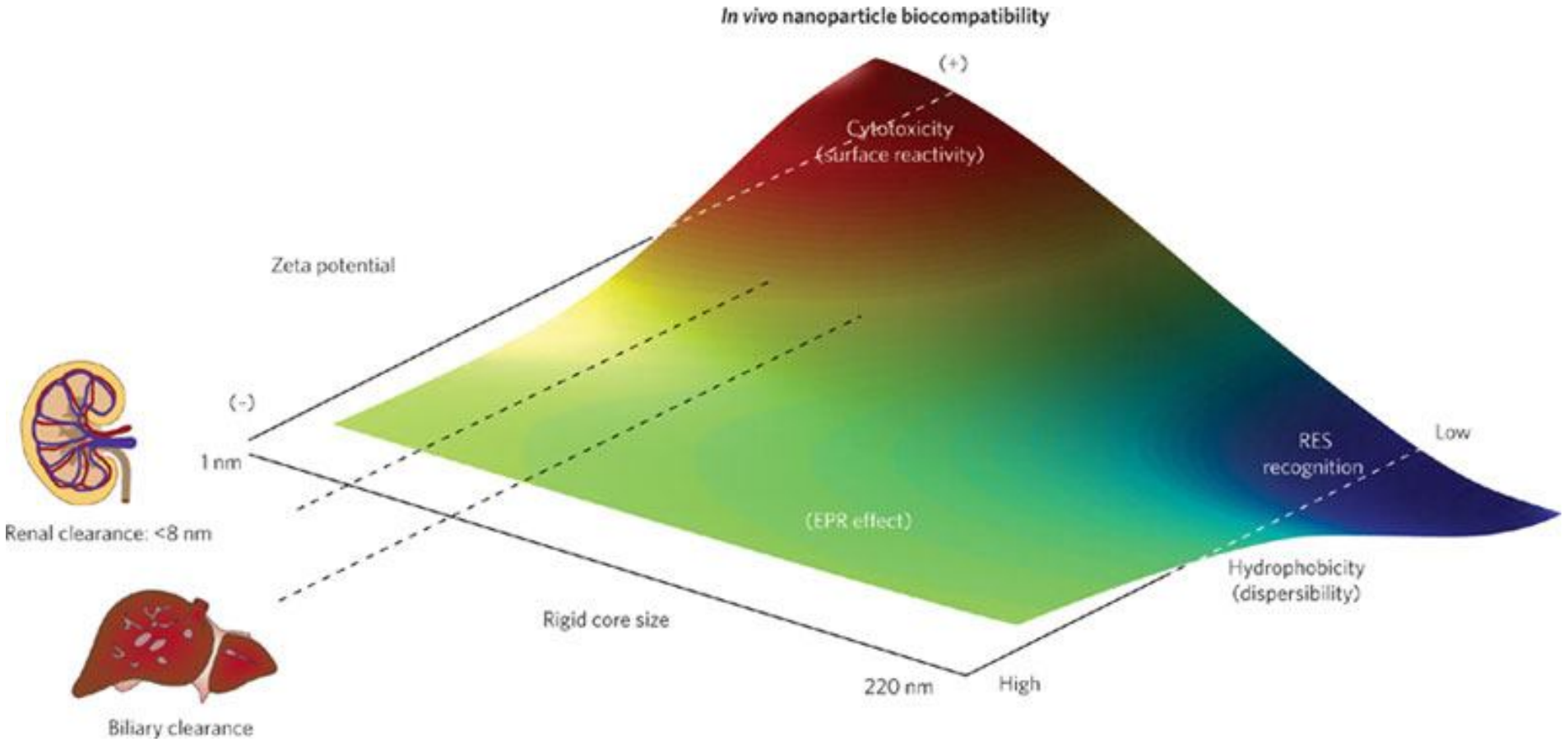
brush

- Fino qua



- The intrinsic nanoparticle characteristics that promote surface binding (roughness, hydrophobicity, cationic charge) generally lead to nonspecific binding forces (marked by asterisks) that promote cellular uptake. In contrast, specific receptor–ligand interactions generally lead to endocytic uptake. A combination of nonspecific binding forces on the surface of spiked particles can lead to direct penetration of the surface membrane without the need to involve endocytic compartments


a
Competitive binding interactions depend on protein concentration and body fluid composition
Binding interactions release surface free energy, leading to surface reconstruction
Characteristic protein on/off rates depend on material type and protein characteristics
Electronic states
Crystallinity
Particle composition
Surface instability
Size
Available surface area, surface coverage and angle of curvature determine adsorption profiles
Protein binding accelerates dissolution of some materials
Hydro-phobicity wettability
Charge
Steric hindrance prevents binding
Hydrophilic or hydrophobic interactions
Charge (for example cationic binding)
b
Electron–hole pairs lead to oxidative damage
Surface reconstruction
Release of surface free energy
Protein conformational changes cause loss of enzyme activity
Impact on protein structure and function
Protein conformation, functional changes
Protein fibrillation
Amyloid fibre
Surface opsonization/ liganding allows interaction with additional nano–bio interfaces
Protein crowding, layering, nucleation
Exposure to cryptic epitopes
Immune recognition



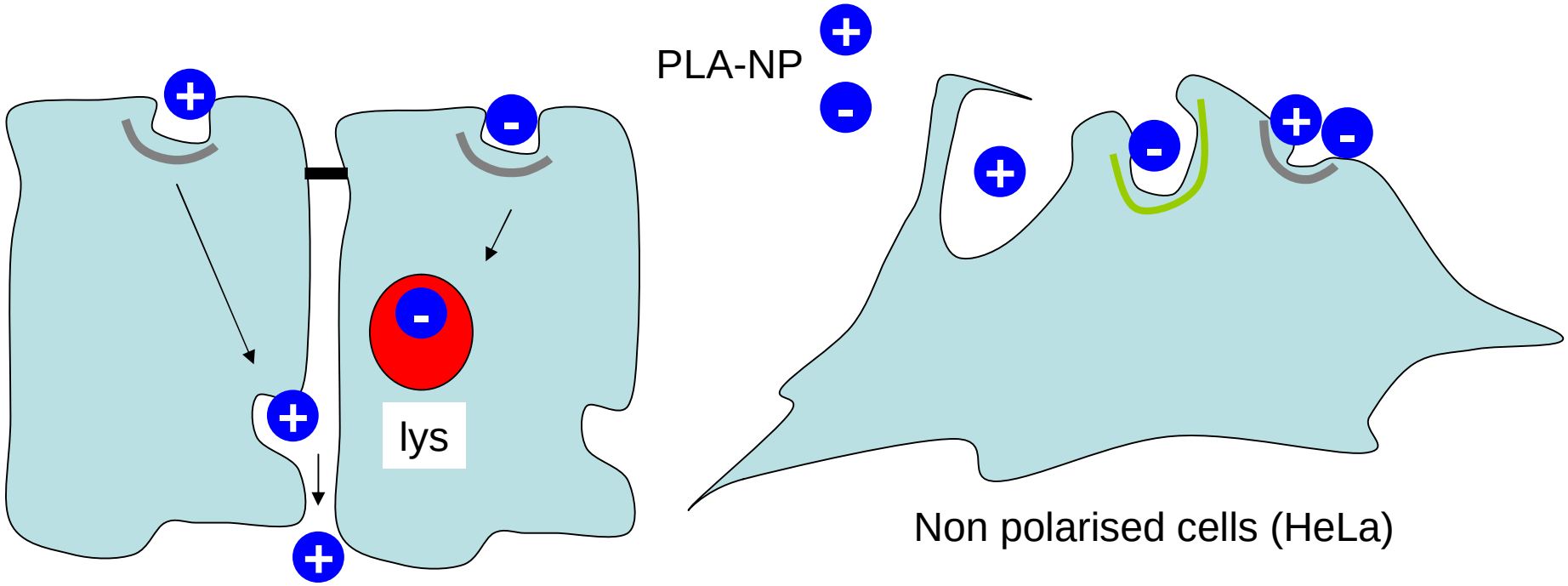
- The main independent particle variables that determine the *in vivo* biocompatibility (colour spectrum) are size, zeta potential (surface charge) and dispersibility (particularly the effect of hydrophobicity). Biocompatibility is reflected in the colour spectrum, with red representing likely toxicity, blue likely safety and blue–green–yellow intermediate levels of safety (in the same order). Cationic particles or particles with high surface reactivity are more likely to be toxic (red hue) than the larger relatively hydrophobic or poorly dispersed particles, which are rapidly and safely (blue hue) removed by the reticuloendothelial system (RES). Particles that promote enhanced permeation and retention (EPR) effects—and are therefore optimal for chemotherapeutic drug delivery to cancers — generally have mid-range sizes and relatively neutral surface charges.

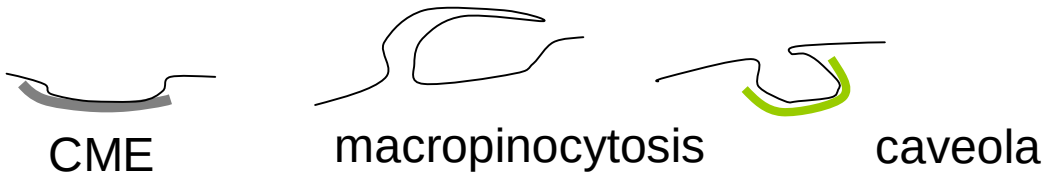
NPs and CME

- PLA NPs (Polylactide) and PEG-co-PLA NPs (Poly(ethylene glycol)- copolymer) : ~ 100 nm Ø
- biodegradable NPs, negatively charged for partial hydrolised PLA, they can be made positive charged by stearyl amine modification
- General feature: + PLA NPs are endocytosed more efficinetly than – PLA NPs

In polarised epithelial cells PLA-NPs use CME (no caveolae), while in non Polarised ones they use CME, caveolae and macropinocytosis

Charge influences very much endocytosis and intracellular sorting

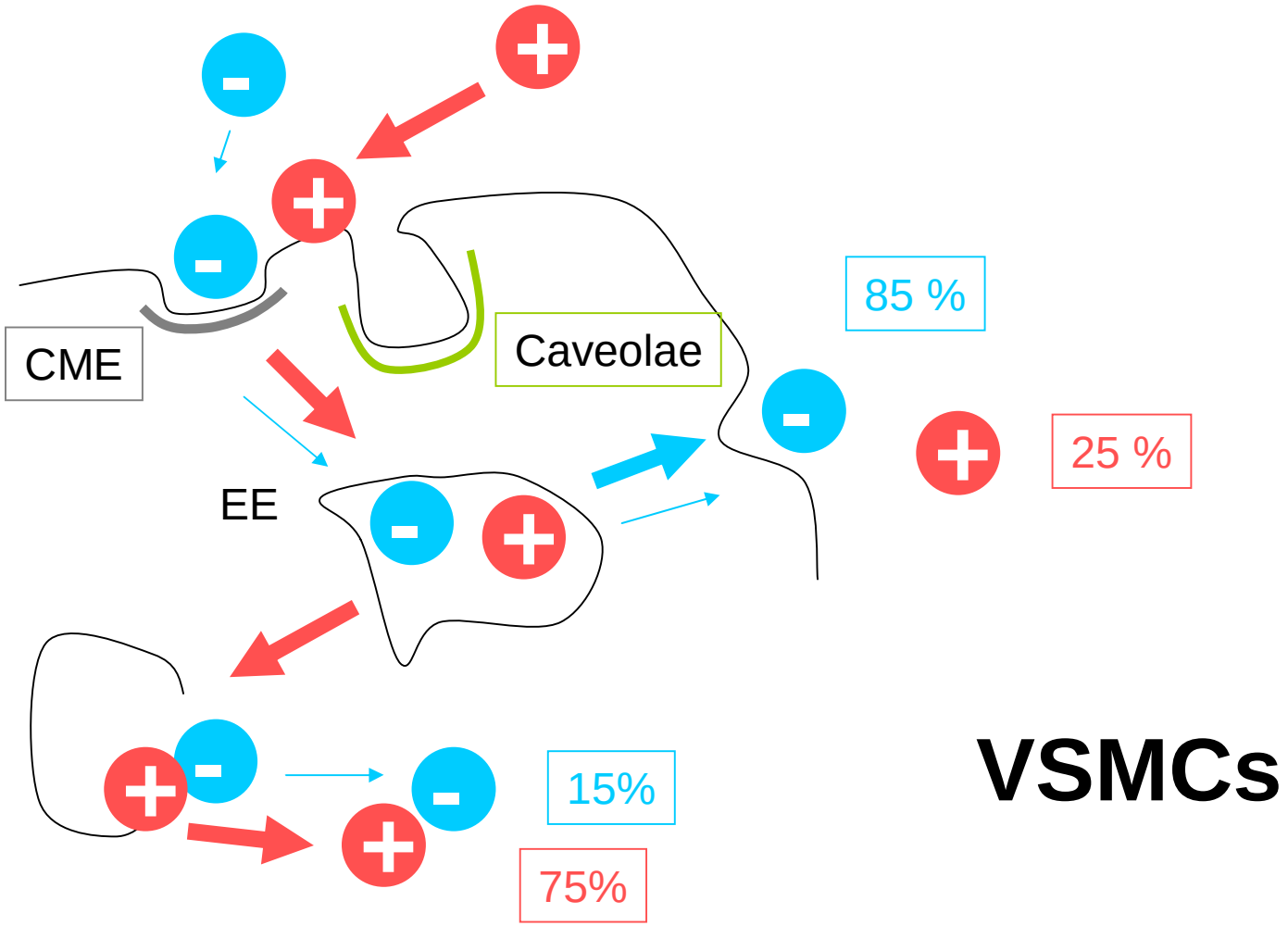




PLGA-NPs and CME

- Poly(lactic-co-glycolic acid), negative and similar to PLA-NPs but larger (~ 300 nm Ø) and heterogenous (polydispersity index 0,2)
- In vascular smooth muscle cells (VSMCs) they enter by CME, while in epithelial corneal cells (rat) they use caveolin- clathrin independent pathways
- They can be made positive by poly-(L-lysine) modification

In vascular endothelial cells Positive PLGA NPs are internalised more efficiently than negative ones and trasnlocate into the cytosol with high efficacy



Silica based nanomaterials and CME

- Template synthesized silica nanotubes (SNTs; 200 nm long, 50 nm large), functionalised with positive charges (amine silane groups) enter via CME
- In cancer cells SNTs have been found to go to lysosomes
- Mesoporous Si-based NPs (~ 100 nm Ø) also enter mesenchimal and fiborblasts via CME (NOT caveolae)

Chitosan NPs

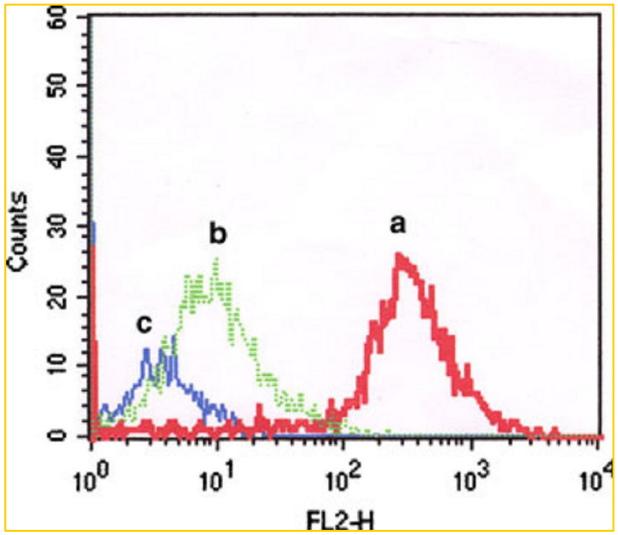
- Cationic NPs (mean Ø 430 nm; highly heterogeneous- polydispersity index 0,5)
- They enter via CME in A549 and CaCo2 cells
- If made hydrophobic they enter via CME + caveolae + macropinocytosis

Surface modified NPs to target CME

- Various NPs were modified with ligands to target specifically the CME; mannose 6 P, Transferrin (Tf), nicotinic acid

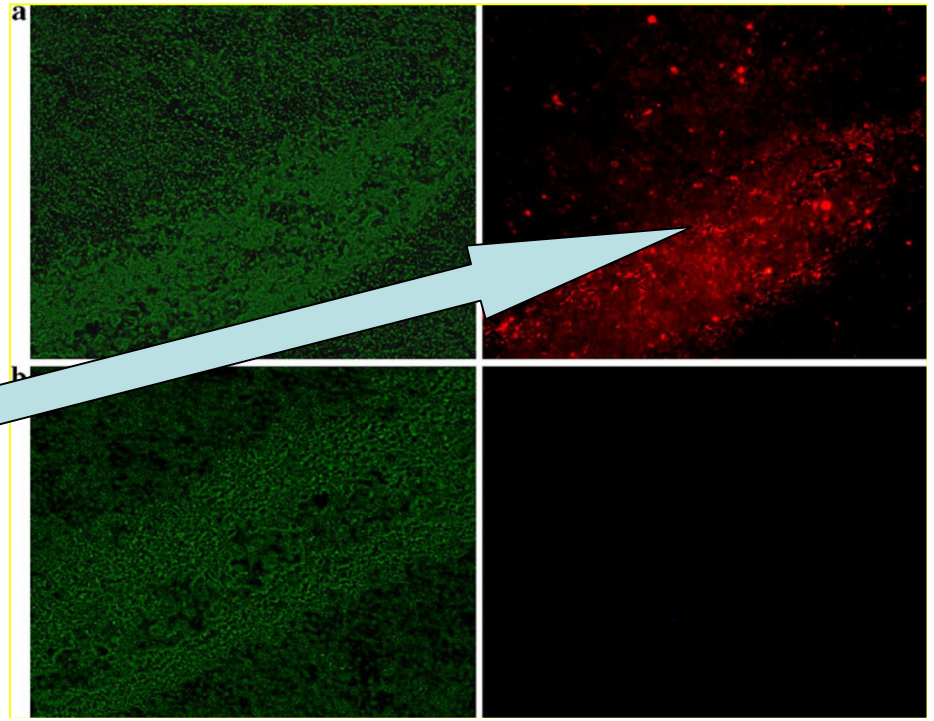
- Important: the intracellular traffic of the NP+ligand may be altered compared to the one of the single ligand alone!

transferrin functionalized poly(ethylene glycol)/poly(lactic acid)



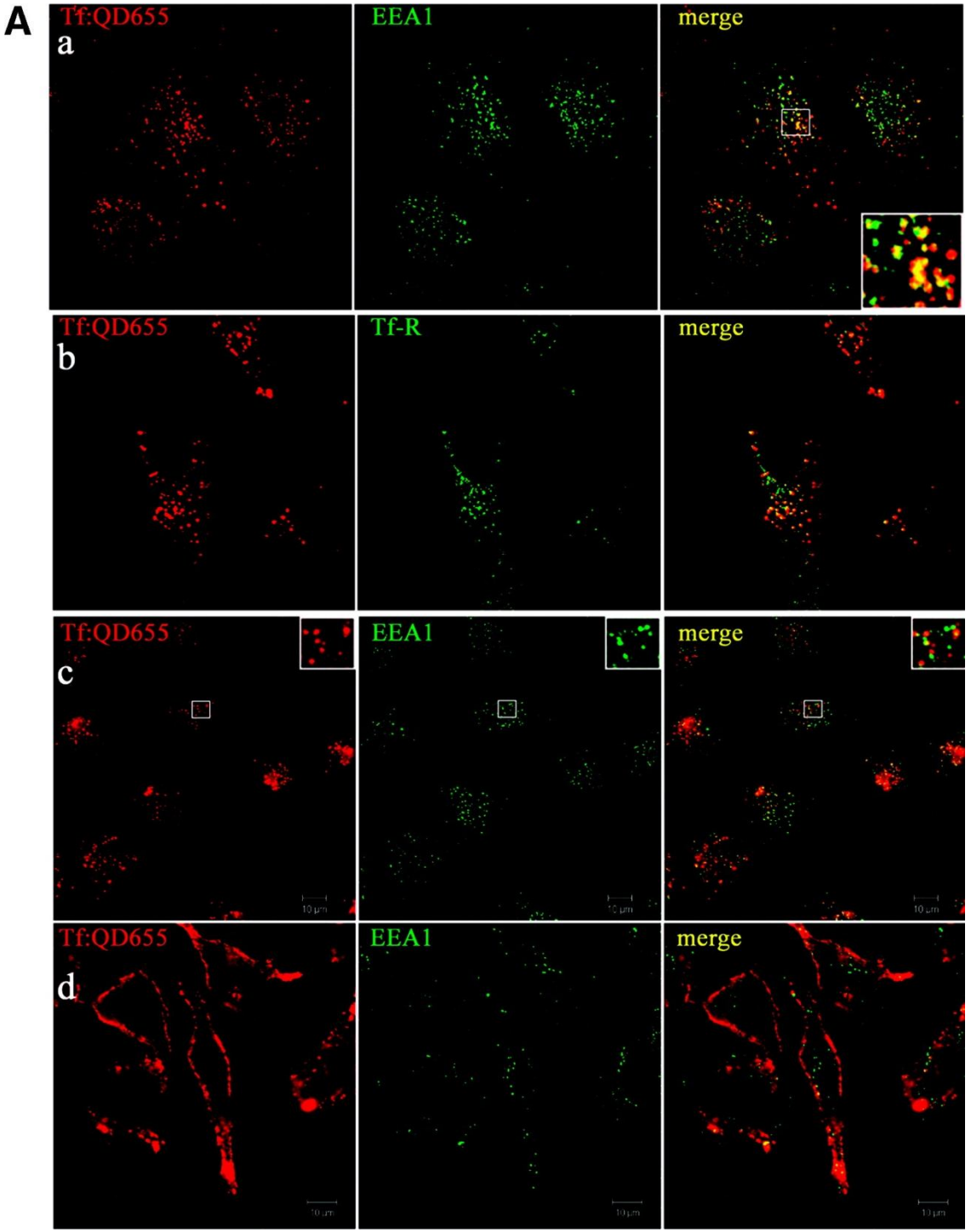
Flow cytometry analysis of C6 cells incubated with Tf-PEGPLA (a), mPEG–PLA (b), or PBS control (c)

Phase contrast and fluorescence micrographs of SD rats' brain frozen sections after nanoparticles injection via tail vein for 24 h. a Tf-PEG–PLA nanoparticles. b B-PEG–PLA nanoparticles. The zone with heavy cell distribution was glioma tumor

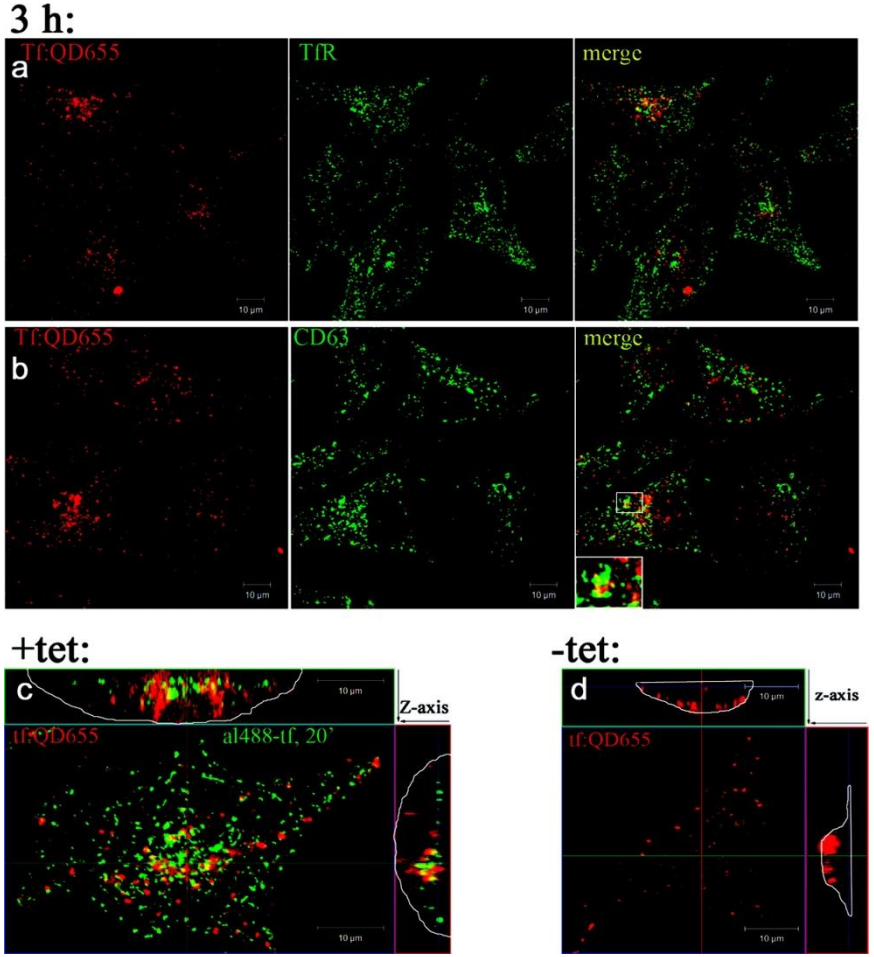


- QDs-Tf (~ 50 nm Ø) enter cells via CME, however they are not recycled back to the plasma membrane or delicvered to lysosomes as Tf: on the contrary they accumulate in another perinuclear compartment..

Intracellular location of transferrin (Tf):quantum dot (QD655) bioconjugates in live HeLa cells.



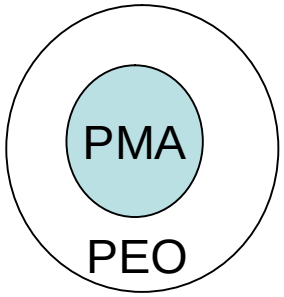
(A) Intracellular location of transferrin (Tf):quantum dot (QD655) bioconjugates in live HeLa cells. The Tf:QD655 bioconjugates were constituted and bound to the cell surface at 4 °C, and then allowed to be internalized into the cells for various times at 37 °C. The cells were then fixed and prepared for immunofluorescence microscopy labelling them with antibodies against EEA1 and Tf-R and with the appropriate secondary ab-Cy2 conjugates. Tf:QDs internalized for 15 min colocalized partly with EEA1 and the TfR (a,b). Endocytosis of the Tf:QD655s for 30 min in the HeLa dynK44A cell line (c,d): Images show the dynamin-dependent uptake of surface-bound Tf:QD655 with normal uptake in control cells (c) and no uptake in the mutant dynamin expressing cells (d, induced for 48 h, −tetracycline). Yellow in the merged images indicates colocalization. Bars, 10 μm. (B) Intracellular accumulation of Tf:QD655 bioconjugates after endocytosis for 3 h in HeLa cells. Tf:QD655s are retained in endosomes that partly colocalize with TfR and CD63 (a,b). Dynamin-dependent endocytosis and accumulation of Tf:QD655 into perinuclear clusters of endosomes in HeLa K44A cells (c,d): Tf:QD655 bound at the cell surface at 4 °C were endocytosed at 37 °C for 2 h, with addition of alexa488-transferrin for the last 20 min. Images shows the *z*,*x* section (upper panel) and the *z*,*y*section (right panel) cutting orthogonally through the cell obtained from *z*-stack image series of the cells. In the uninduced control cells (+tet), the Tf:Qdots were observed in perinuclear endosomal clusters inside the cell. In the cells with induced mutant dynamin (−tet), the Tf:Qdots localized along the periphery of the cell. Bars, 10 μm.


3 h:
a
Tf:QD655
TfR
merge
b
Tf:QD655
CD63
merge
+tet:
c
Z-axis
tf:QD655
al488-tf, 20'
-tet:
d
z-axis
tf:QD655

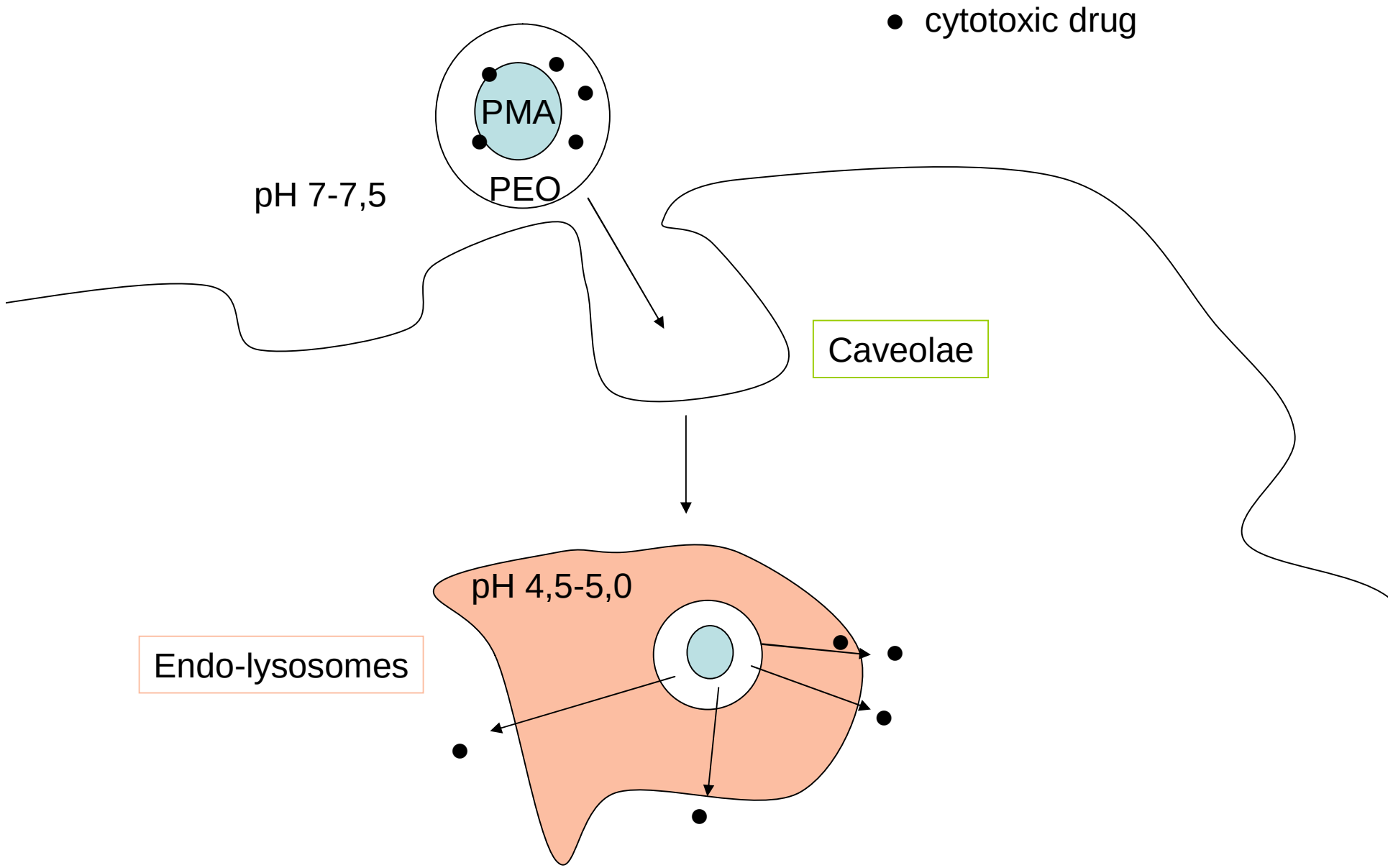
- QDs-Shiga toxin (~ 30 nm Ø), are interanalised via CME. Unlike free Shiga toxin, they do not sort to the TGN but move to endosomes

NPs internalised by caveolae

- PEO-b-PMA: poly(ethylene oxide)-b-poly(metacrylic acid)- also called cl-micellae (core-cross-linked polymeric micellae)

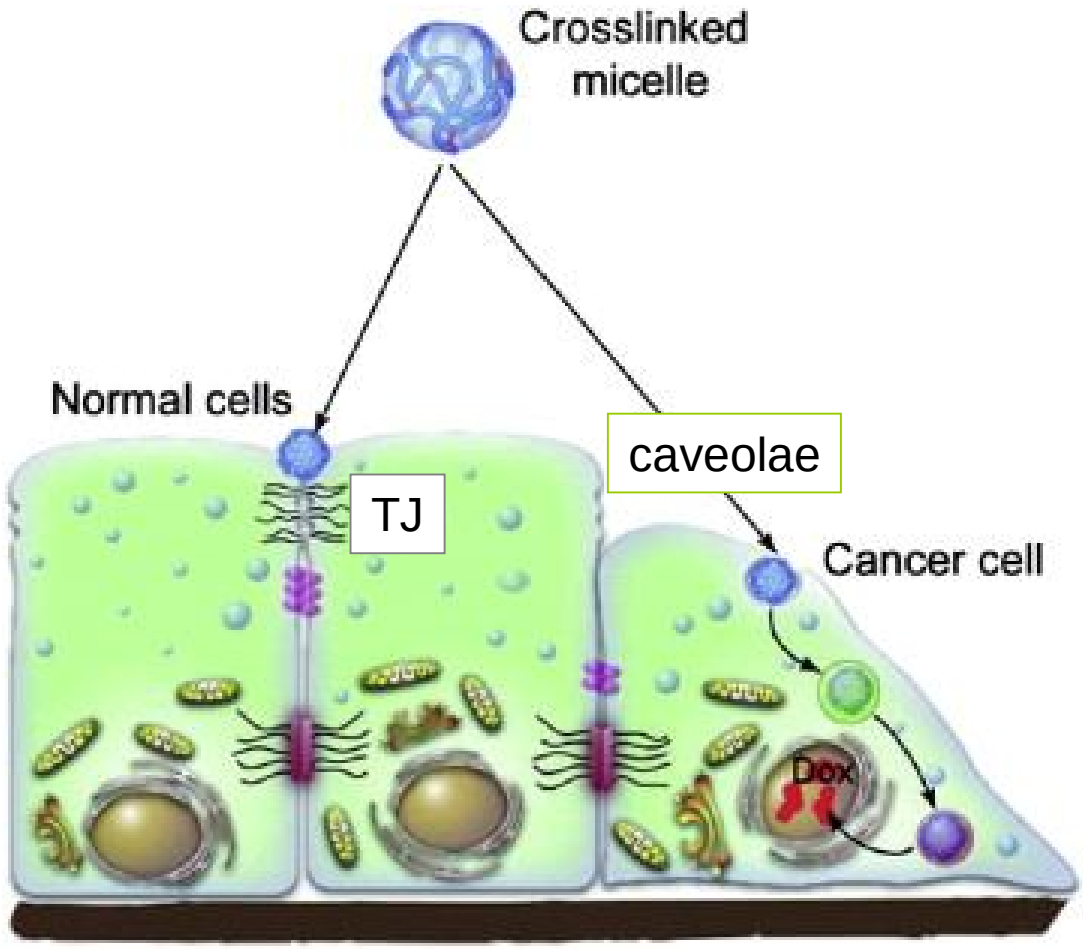


	pH 7,4	pH 5,0
Zeta pot	-18 mV	- 7 mV
Ø (nm)	~160	~ 110
Drug release	no	yes


cytotoxic drug
PMA
PEO
pH 7-7,5
Caveolae
pH 4,5-5,0
Endo-lysosomes

PEO-b-PMA NPs

- In normal polarised cells (no caveolae in the apical membrane domain), cl-micells stuck to the TJs and do not enter celle
- In cancer cells, which lost polarisation, they mostly enter via caveolae
- They reach the lysosomes and release a cytotoxic drug (like doxorubicin) ⟶ cell killing



DOXIL®

- Slightly negative charged PEGylated liposomes (~ 90 nm Ø) containing encapsulated doxorubicin
- Used in the treatment of metastatic ovaria cancer
- DOXIL NPs enter via caveolae and are delivered to lysosomes were the biodegradable liposome release the antitumor drug

Polysiloxane NPs

- Self-assembled nanoparticles (~100 nm Ø) of poly(3-aminopropyl)siloxane (PAPS) modified with stearic acid (anionic fatty acid) and galactose
- This NPs target caveolae in aortic human endothelial cells
- In addition they induce the dissociation of eNOS (the NO costitutive generating enzyme) and iots dissociation from caveolin-1

QDs

- CdSe/ZnS core-shell ellipsoid QDs differently functionally modified:

coating	Ø (nm)	charge	caveolae location Skin cells
PEG	45	neutral	NO
PEG-NH_2	20	positive	NO
Poly (acrylic acid)	18	negative	YES

Abraxane ®

- Nanoparticle made of albumine bound Paclitaxel (~ 130 nm Ø)
- Approved by the FDA for treatment of metastatic/relapsing breast cancer
- Abraxane binds to gp60 the albumine-receptor present in the caveolae of endothelial cells
- After transcytosis is binds to SPARC (Secreted Proteins, Acidic Rich in Cystein) released by the tumor cells → SPARS/Abraxane complexes are taken up by tumor cells → neoplastic cell celective killing

Specific surface modification for NPs targeting to caveolae

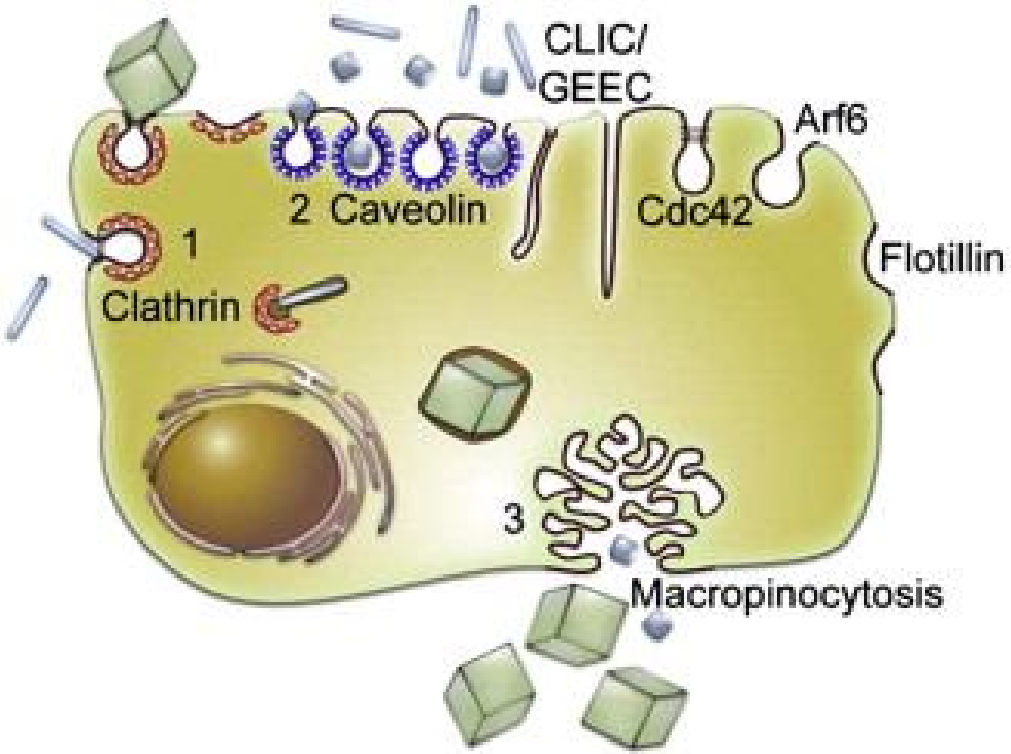
- In vivo proteomic approach of lung cancer caveolae has identified aminopeptidase P (APP) as a caveolae-tumor-specific protein
- Gold NPs coupled to mab to APP accumulate in endothelial cells of the lung vasculature

- Cyclic RGD peptide binds to $\alpha_v\beta_3$ integrin receptor in caveolae
- Thiolated c(RGDDfk)-PEG-b-PLL coploymer Nps for cellualr delivery of DNA
- They enter HeLa cells via caveolae and localise in perinuclear regions (non lysosomes) where DNA transfecion likeky occurs

NPs exploiting multiple entry pathways

- PRINT (Particle Replication in Non-wetting Templates) micro/nanoparticles: made with top-down lithographic fabrication method
- Nearly monodisperse materials of defined shape, size and charge
- Cross-linked PEG based hydrogels obtained by UV copolymerisation in different mols
- They are positive charged NPs (seta potentials + 21/41 mV) entering different endoctic pathway according to shape and dimension
- When acylated (nd made negative) they all shoed negligible cell entrance
-

PRINT entrance

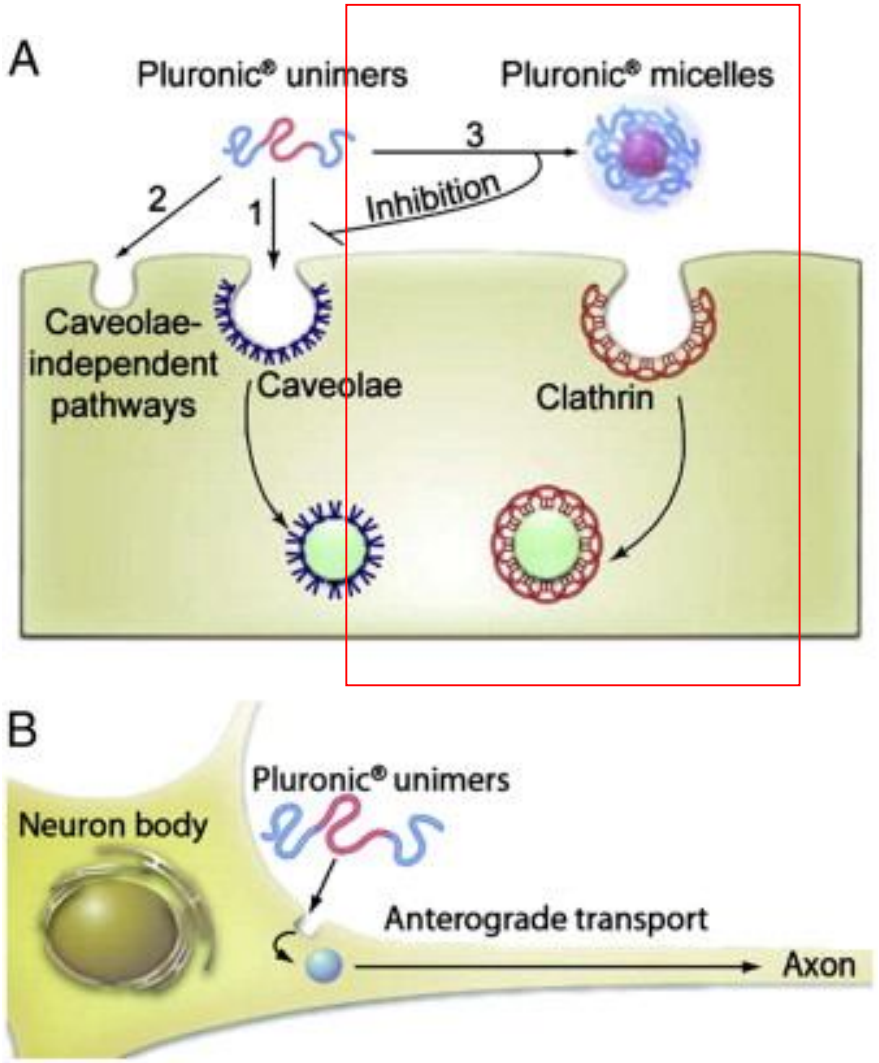


Pluronic block copolymers

- Copolymer of i. poly(ethylene oxide) (PLO) ii. poly (propylene oxide) (PPO) = Pluronic ® p85 (P85)
- Below the Critical Micellar Constant (CMC) the copolymer is a single molecule (unimer)
- Above the CMC it forms aggregated micelles of ~ 150 nm Ø with a core of hydrophobic PPO end a shell of hydrophilic PEO
- They are neutral NPs

Entrance of Pluronic® block polymers in epithelial and neuronal cells

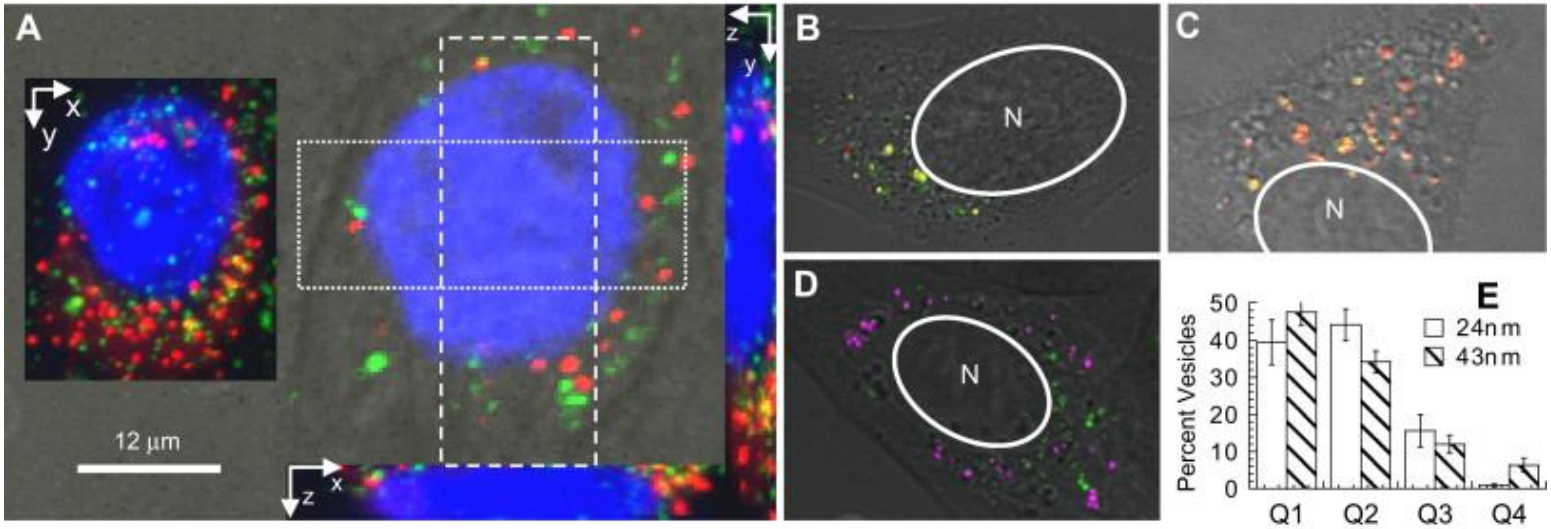
- The unimers enter via caveolae or via clathrin- caveolin- indipendent pathways, if the cell is devoid of caveolae
- In the cell they by pass the endolysosomes and go to the ER and to mitos!
- They enter in brain endothelial cells and in neurons via clathrin/caveolin indep endocytosis (anterograde transported)
- However P85 micelles internalise exclusively through CME, also inhibiting the caveolae mediated endocytosis



PS NPs

- Negatively charged PolyStyrene NPs with different size enter differently in Hela cells:
- 43 nm Ø via CME to the lysosomes
- 24 nm Ø via clathrin- caveolin-independent pathway to a distinct perinuclear compartment (not lysosomes)

Polystyrene NPs of different size



- 24 and 43 nm nanoparticles (NP) are trafficked to spatially distinct intracellular locations within 4 h post-incubation with live cells. 24 nm NP are red, 43 nm NP are green, and the nucleus is stained with Hoechst 34580 (blue).

PAMAM (Poly Amindo Amine) dendrimers

- Repeatedly branched, monodisperse, highly symmetric compounds
- Amine modified cationic dendrimers
- CArboxylate-modified anionic dendrimers

- + Lower-generation dendrimers G2 via CME
- + higher generation G4 via multiple routes

- + dendrimers EE lys (rapidly-20 min)
- - dendrimers to Lys slowly

- Both – and + dendrimers open up TJs in epithelial polarised cells and cross the epithelium via the paracellular route

Non-viral gene delivery/DNA agents

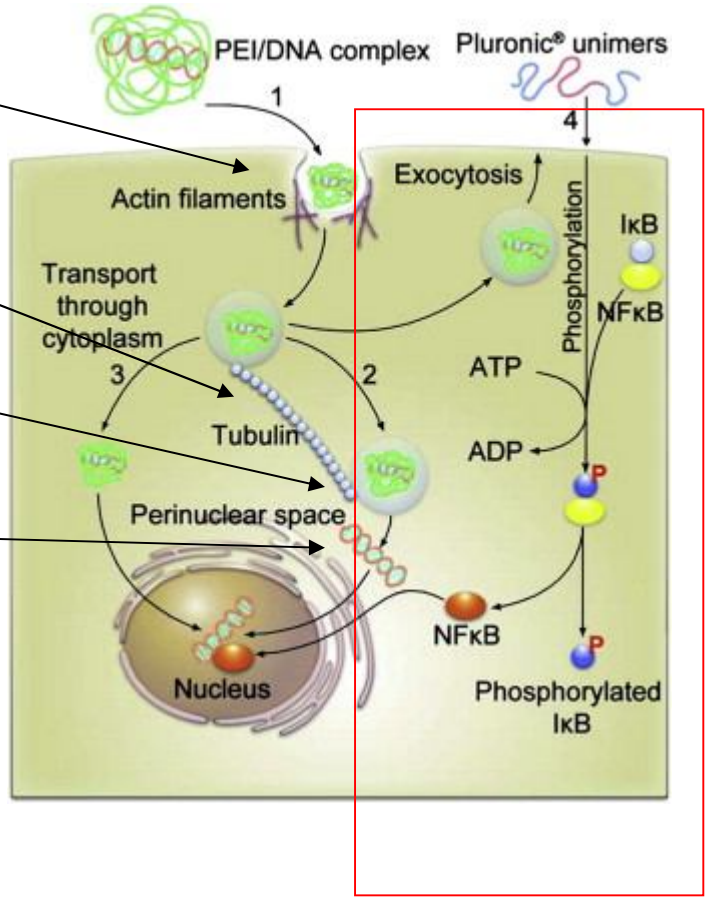
- Cationic lipids and synthetic polycations forms nanosize complex with DNA: *lipoplex* and *polyplex*
- *The endocytic route and intracelular traffic is difficult to study because they undergo multiple structural transition (conflicting report*

Polyethyleneimine (PEI)-based polyplex

- Brached and linear PEI generate polyplex which use CME, caveolae and clathrin-caveolin independent endocytic route (according to the cell type and to… the research group!)

Linear PEI/DNA polyplexes

- NP initially colocalises on the surface with actine
- Later with tubuline
- It moves along the tubule and reachs the perinuclear region (not degradative lysosomes: good)
- DNA translocated there?
- Pluronic® unimers enhance nuclear transport of NPs and also translocated DNA (via NFkB?)



Lipoplex

DOTAP N-[1-2,3-(dioleoyloxy)propyl]- N-N, N tri-methylammonium methyl sulphate

lipofectamine

DMRIE

1,2 – dimmyristoiloxypropyl – 3-dimethyl-hydroxy-ethyl ammoium bromide

CME

caveolae

lysosomes

Surface-modified NPs targetting multiple pathways

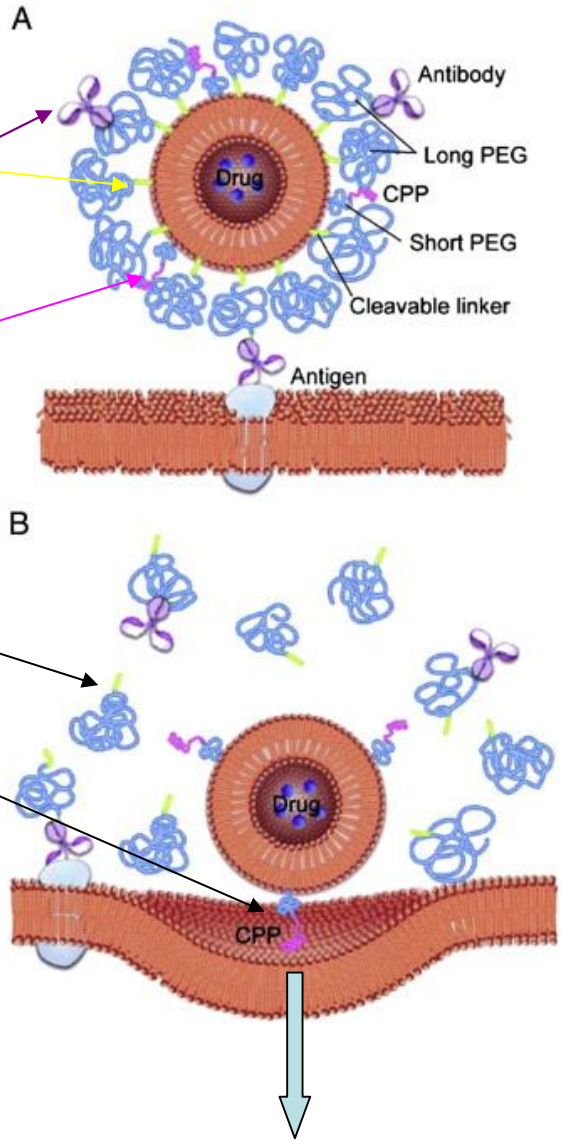
- Various NPs (liposomes, iron, polyplexed DNA/siRNA) were modified with small cationic peptides (10-30 aa long)Cell-Penetrating Peptides (CPPs) or Protein Translocation Peptides (PTPs)

- Examples of CPPs: TaT (from HIV); penetratin; transportan; poly-arginine; rabies virus glycoprotein (RVG) peptide

PEGylated-PE liposomal NPs

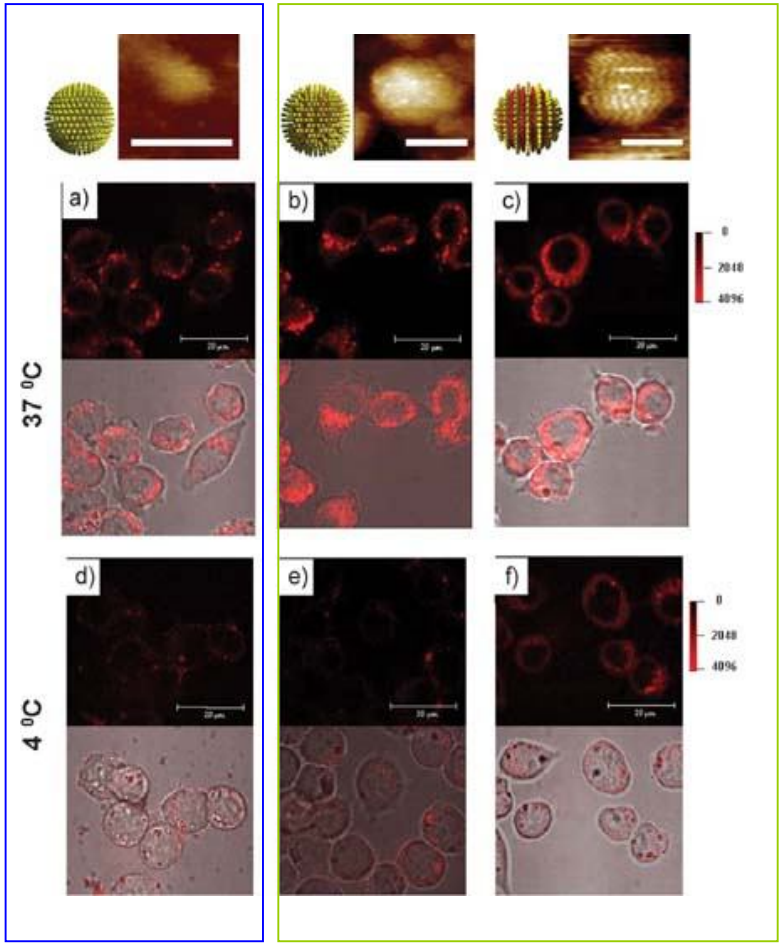
- The liposome is made stealth by cleavable surface PEG chain
- A tumor specific mab confer cancer cell targetting
- The CPP Tat is linked to a shorter PEG chain on the liposomal surface (normally covered by larger PEG)
- After cell binding the liposome is peeled of PEG by hydrolases, and TaT directely mediate membrane fusion
- The drug cargo is delivered in the cell cytosol

- The NPs can also enter cells via macropinocytosis

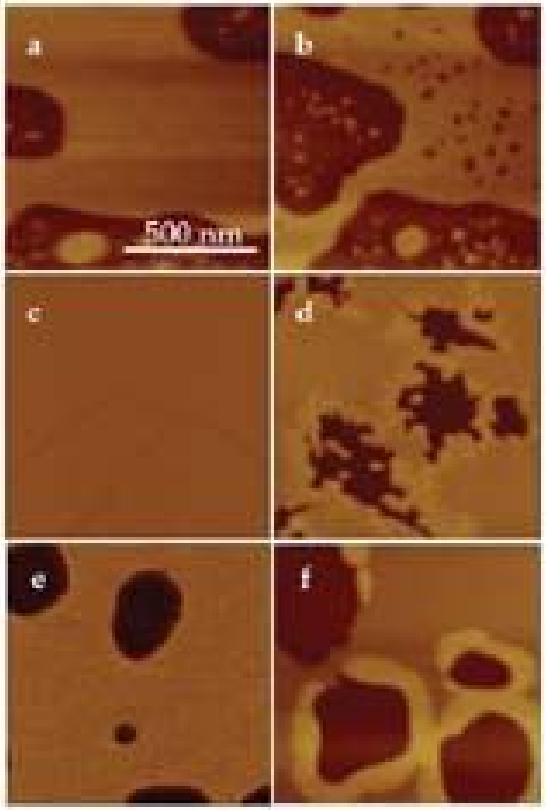


Role of spatial arrangement on the NPs particle in cellular uptake or direct translocation

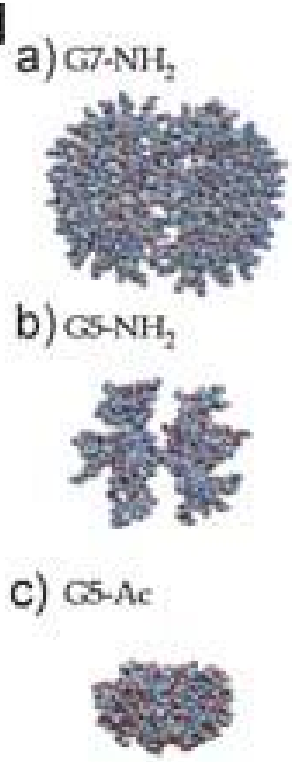
- NPs covered with an amphiphilc layer homogeneously distributed are endocytosed
- When the coat forms regolar patterns it induces cytosolic traslocation



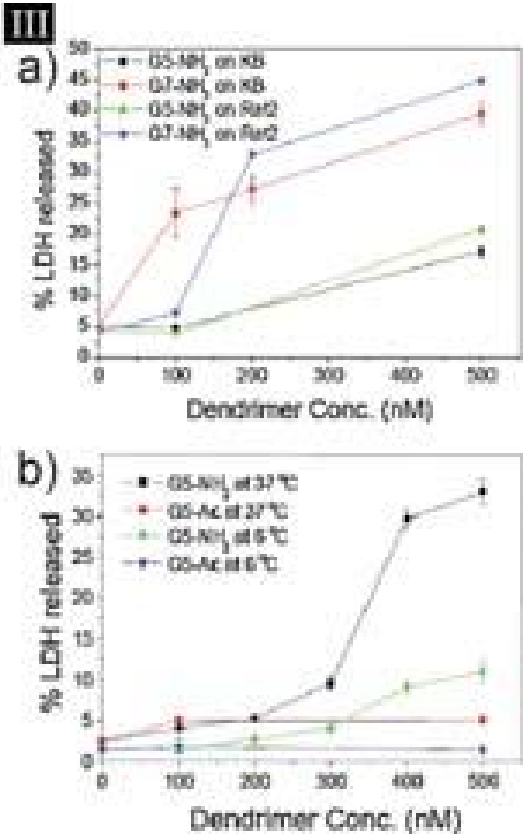
I

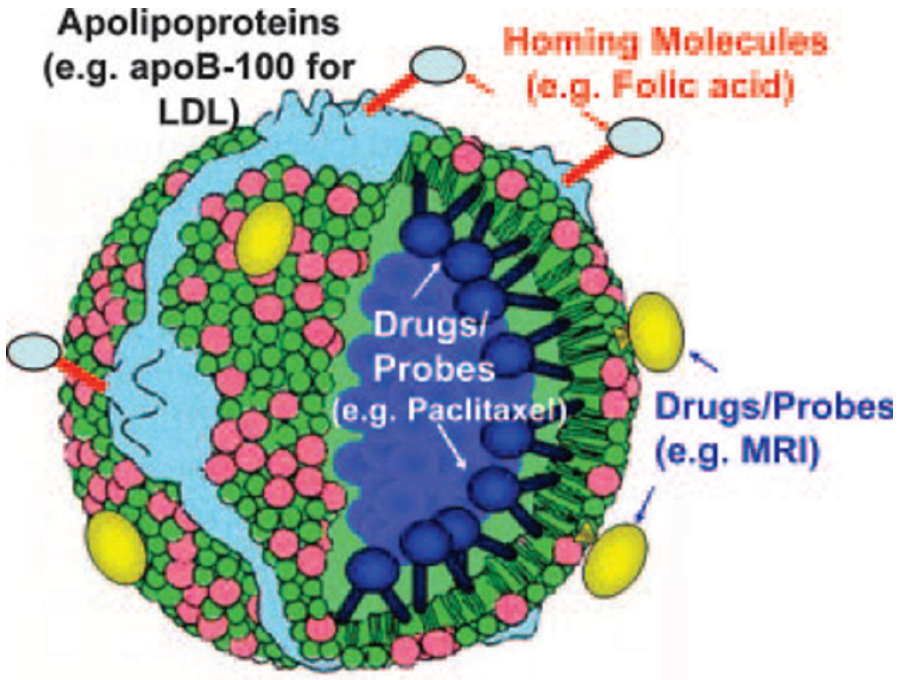

a
b
500 nm
c
d
e
f

II


a) G7-NH2
b) G5-NH2
c) G5-Ac

III


a)
% LDH released
Dendrimer Conc. (nM)
b)
% LDH released
Dendrimer Conc. (nM)

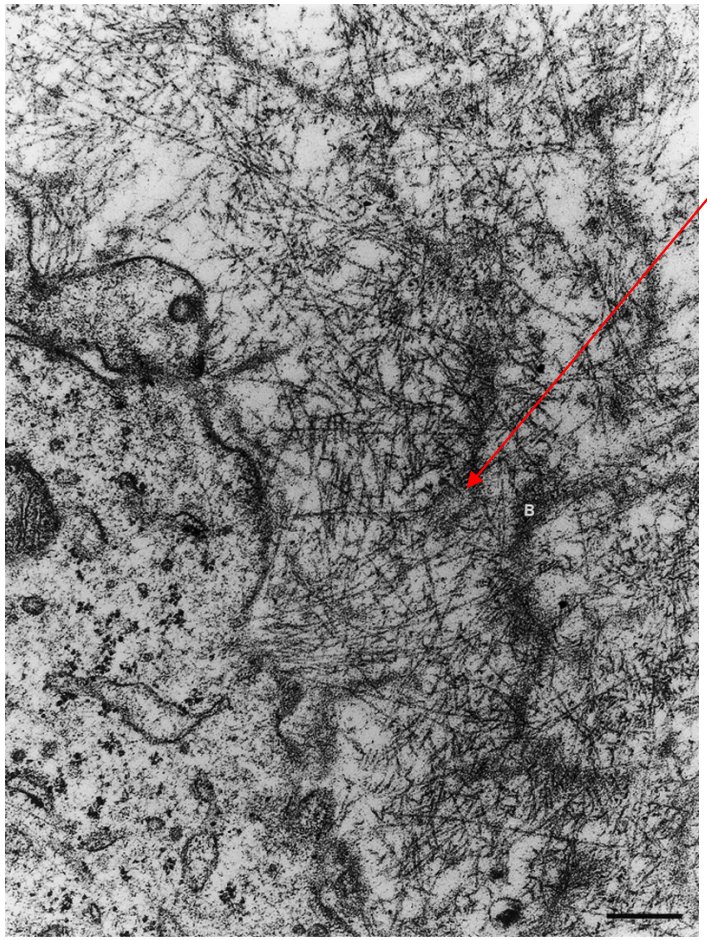

Apolipoproteins
(e.g. apoB-100 for
LDL)
Homing Molecules
(e.g. Folic acid)
Drugs/
Probes
(e.g. Paclitaxel)
Drugs/Probes
(e.g. MRI)

Proteins-NPs relation

- So far we concentrated on the effects of host bio-molecules (opsonines, complement, corona in general) on NPs behavior

- There is also the reverse condition: the effect of NPs on the interacting (host) proteins

Can NPs favour amyloid fibrille nucleation? (related to important human pathologies)



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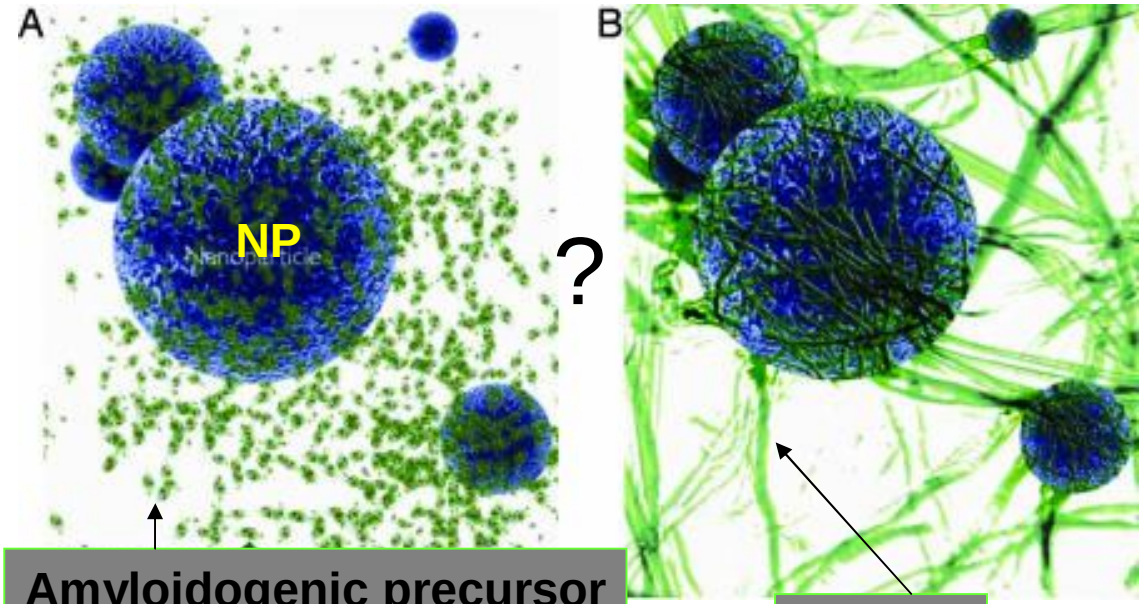
Bar: 500 nm

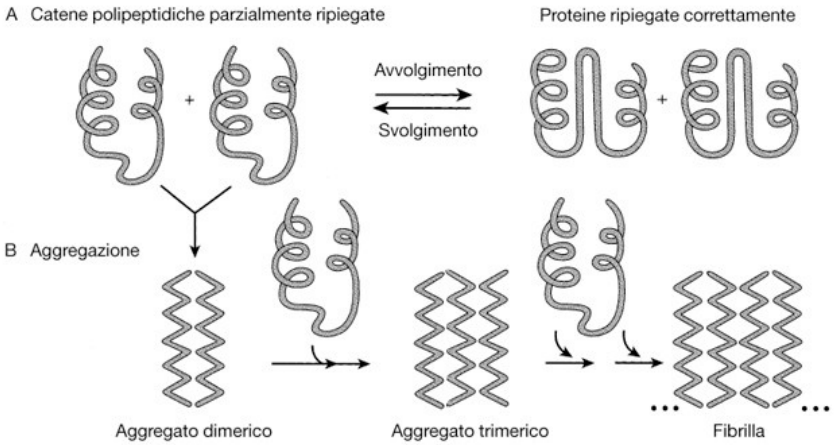
Amyloid fibrilles (7-10 nm Ø; length undefined µms)

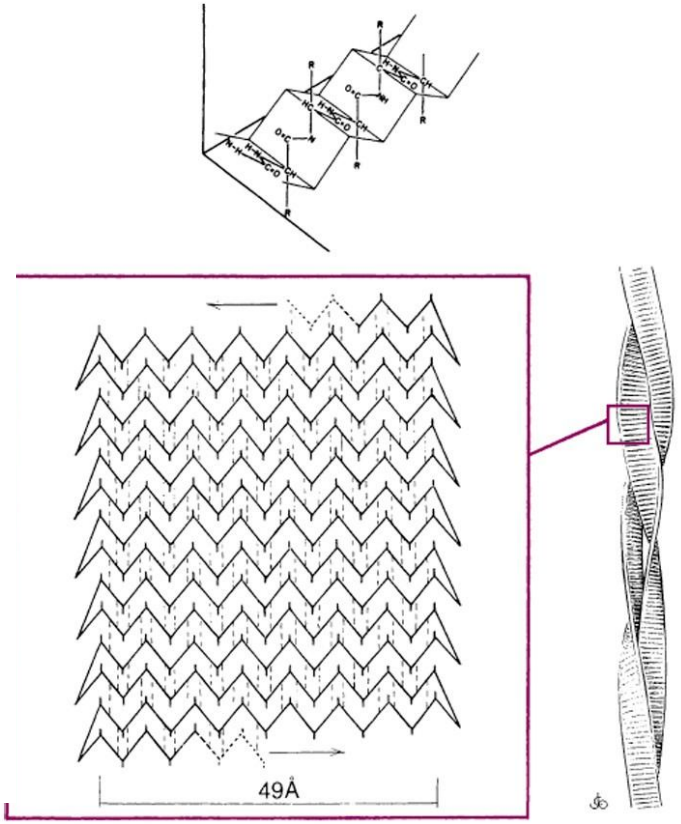


Amyloidogenic precursor

fibrilles

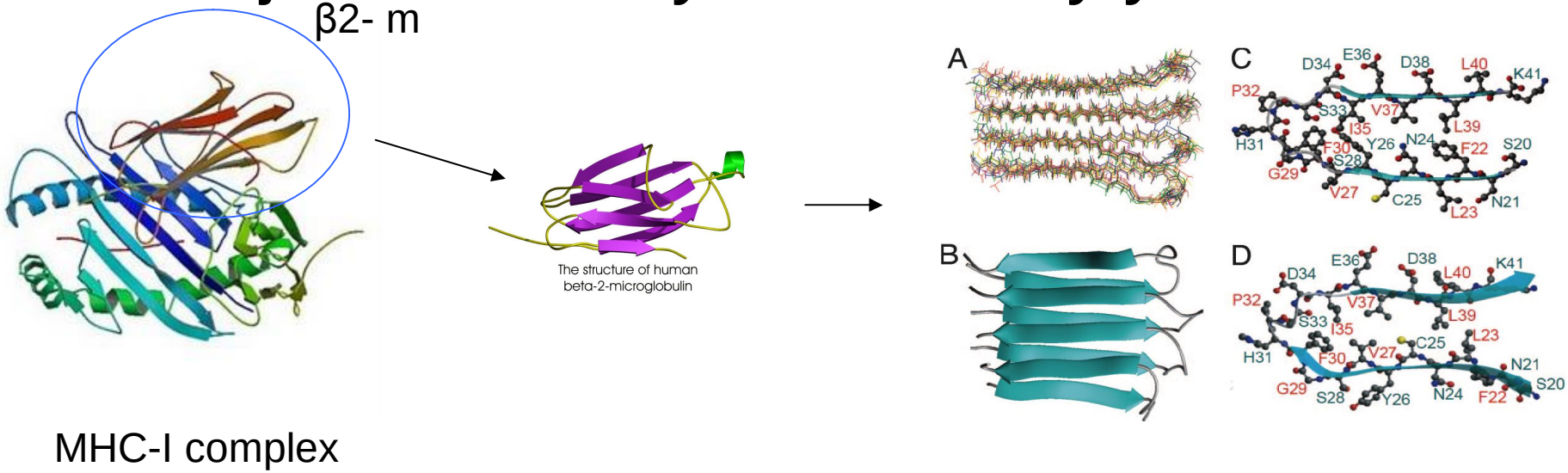
Amyloidosis: a protein “conformational” disease





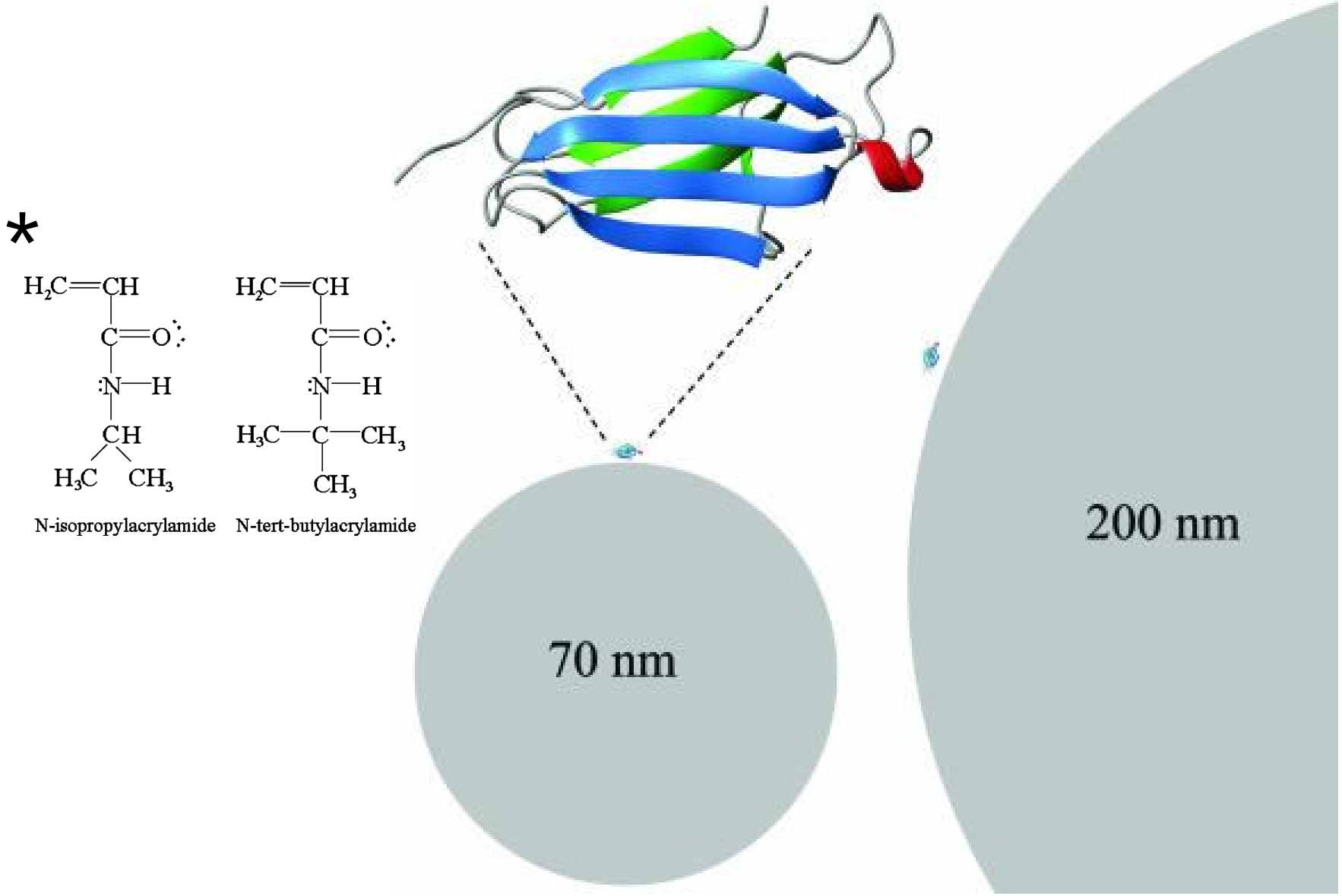
Note: The amyloid fibrilles are endogenous pathogenic nanostructure!!

- β2- microglobulin (β2-m) fibrillation occurs on the surface of cerium oxide, copolymer NPs and ~~C~~NTs amyloid fibrilles *in vitro*
- β2- m are known to induce iatrogenic systemic amyloidosis in indivdual subjected to dialysis for many years

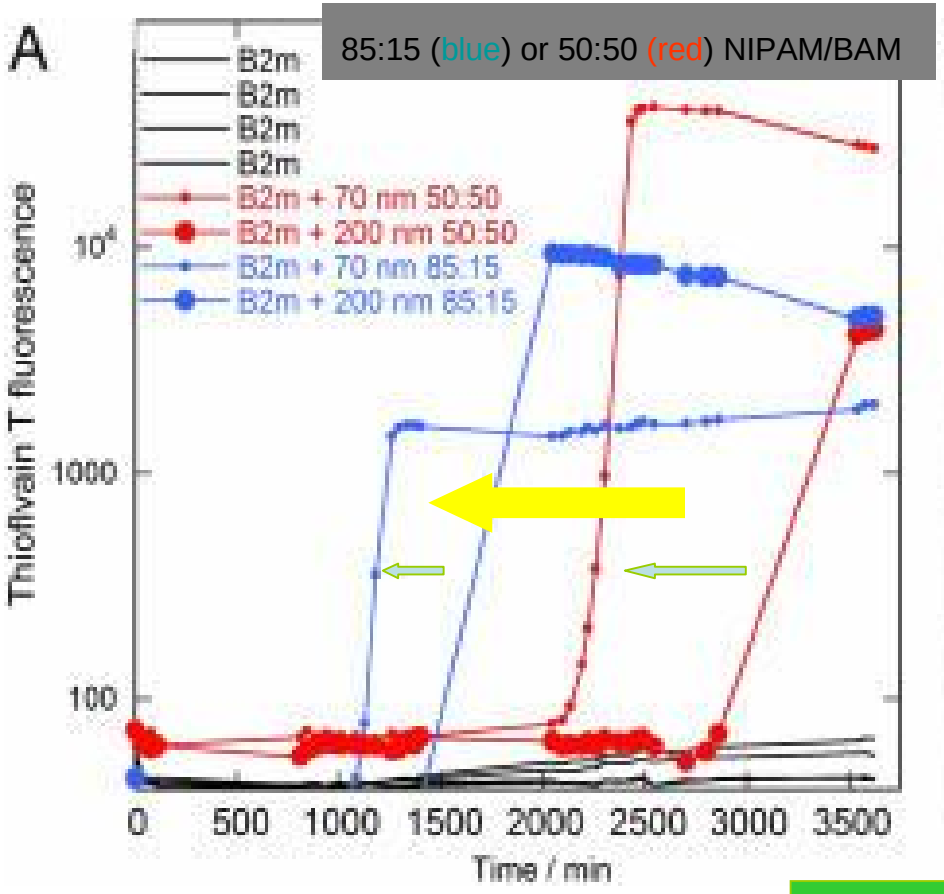


MHC-I complex

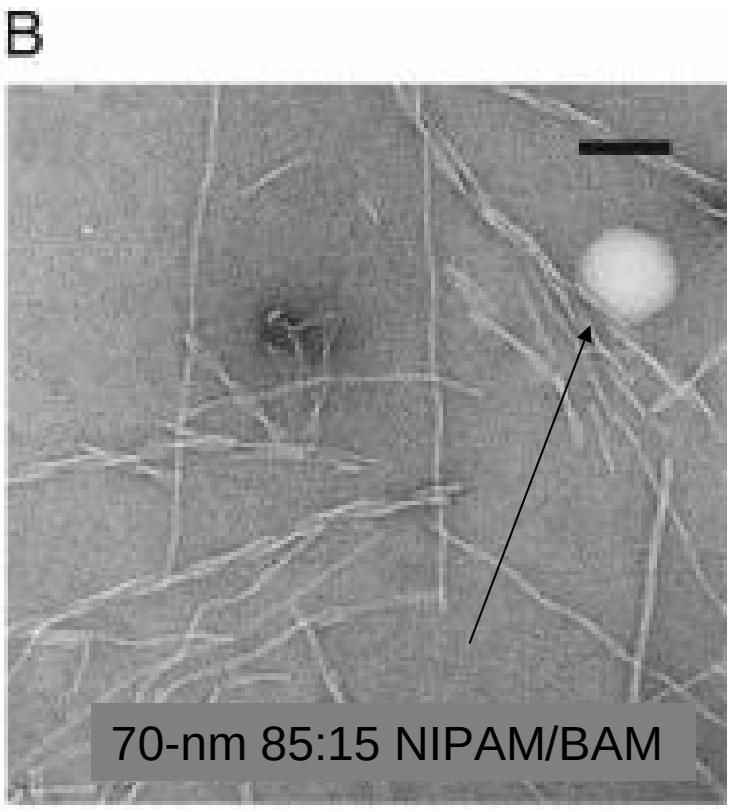
Size comparison of the interaction of monomeric β2m with NIPAM/BAM* nanoparticles



β2m fibrillation in the presence of NIPAM (*N*-isopropylacrylamide)/BAM (*N-tert*-butylacrylamide) nanoparticles



10 ug/ml NPs



smaller

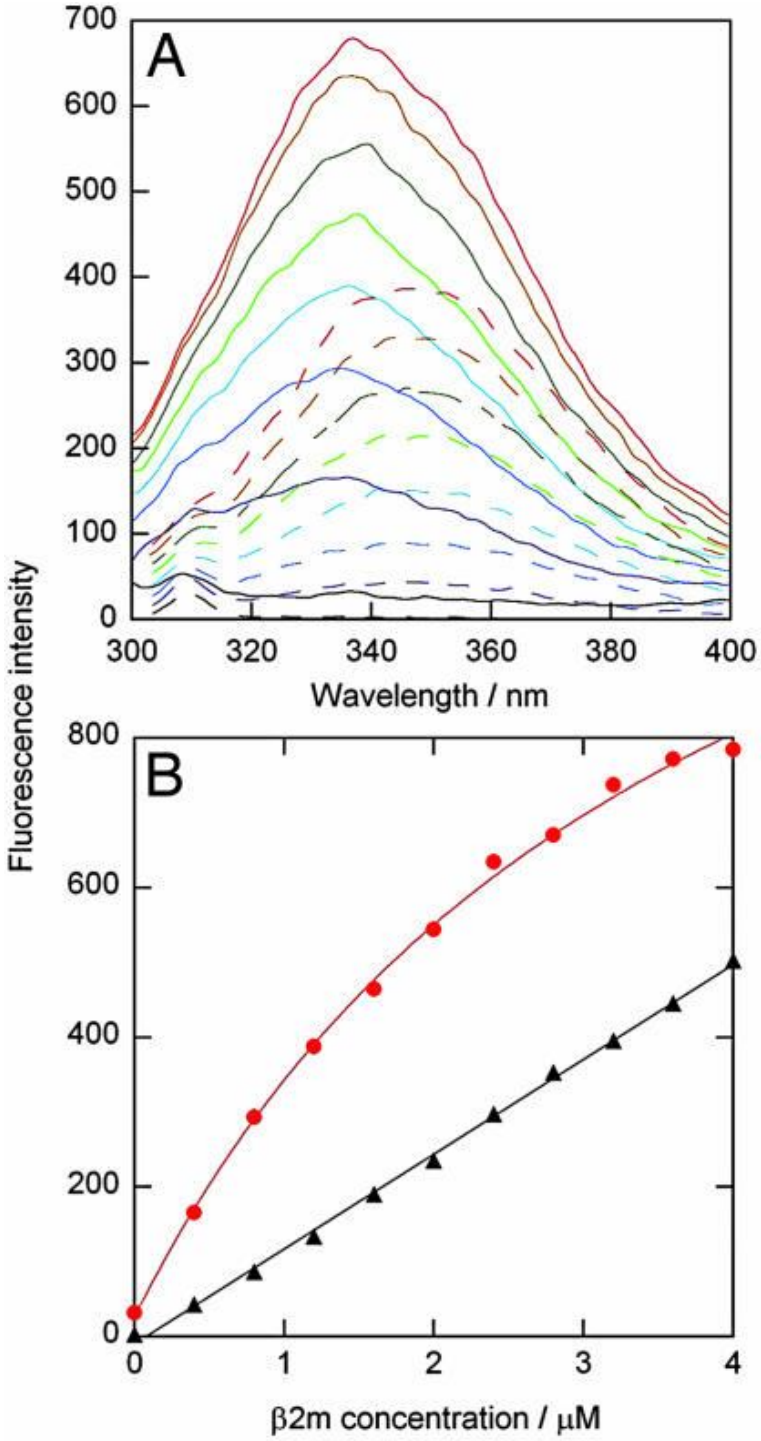
More hydrophilic

Change in β2m conformation in the

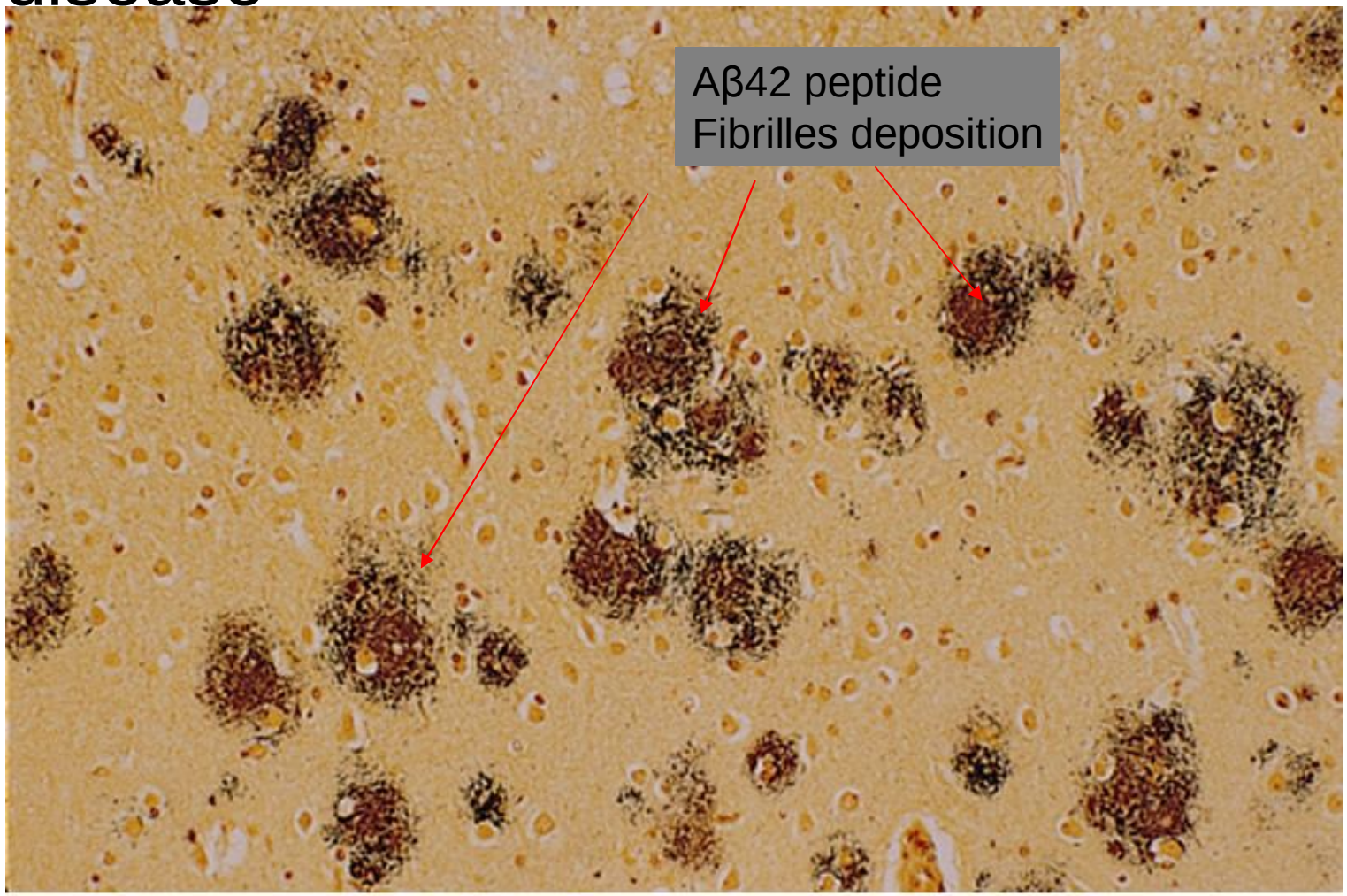
presence of nanoparticles

- A: Trp fluorescence spectra of β2m titrated into buffer (dashed lines) and into a solution with 70-nm 50:50 NIPAM/BAM nanoparticles (solid lines).

- B: Fluorescence intensity at 335 nm versus β2m concentration. β2m titrated into buffer (black triangles) fitted by a straight line, and β2m titrated into 70 nm 50:50 nanoparticles (red filled circles)



- Brain senile plaques in Alzheimer's disease



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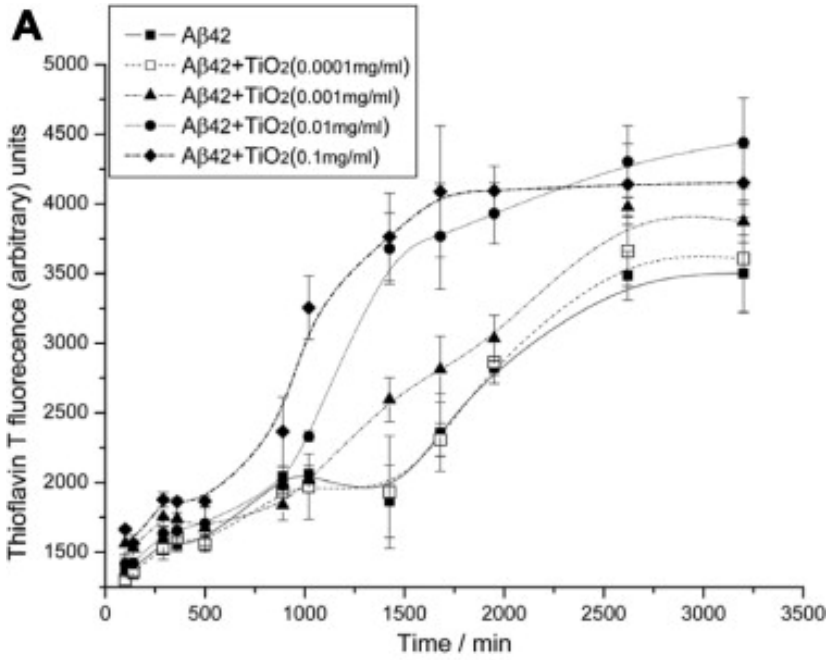
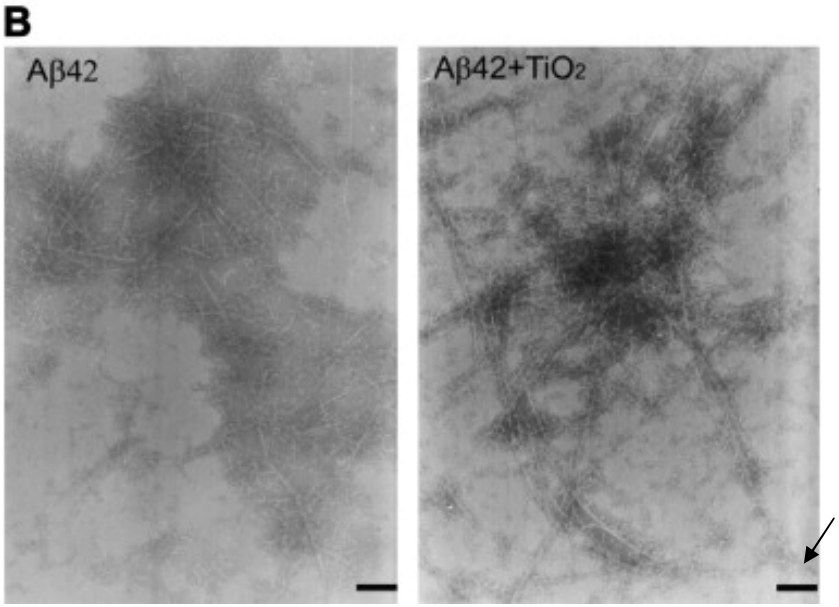
$Ti0_2$ NPs catalyse the fibrillar nucleation of Aβ42 (the precursor of amyloid in Alzheimer's disease)

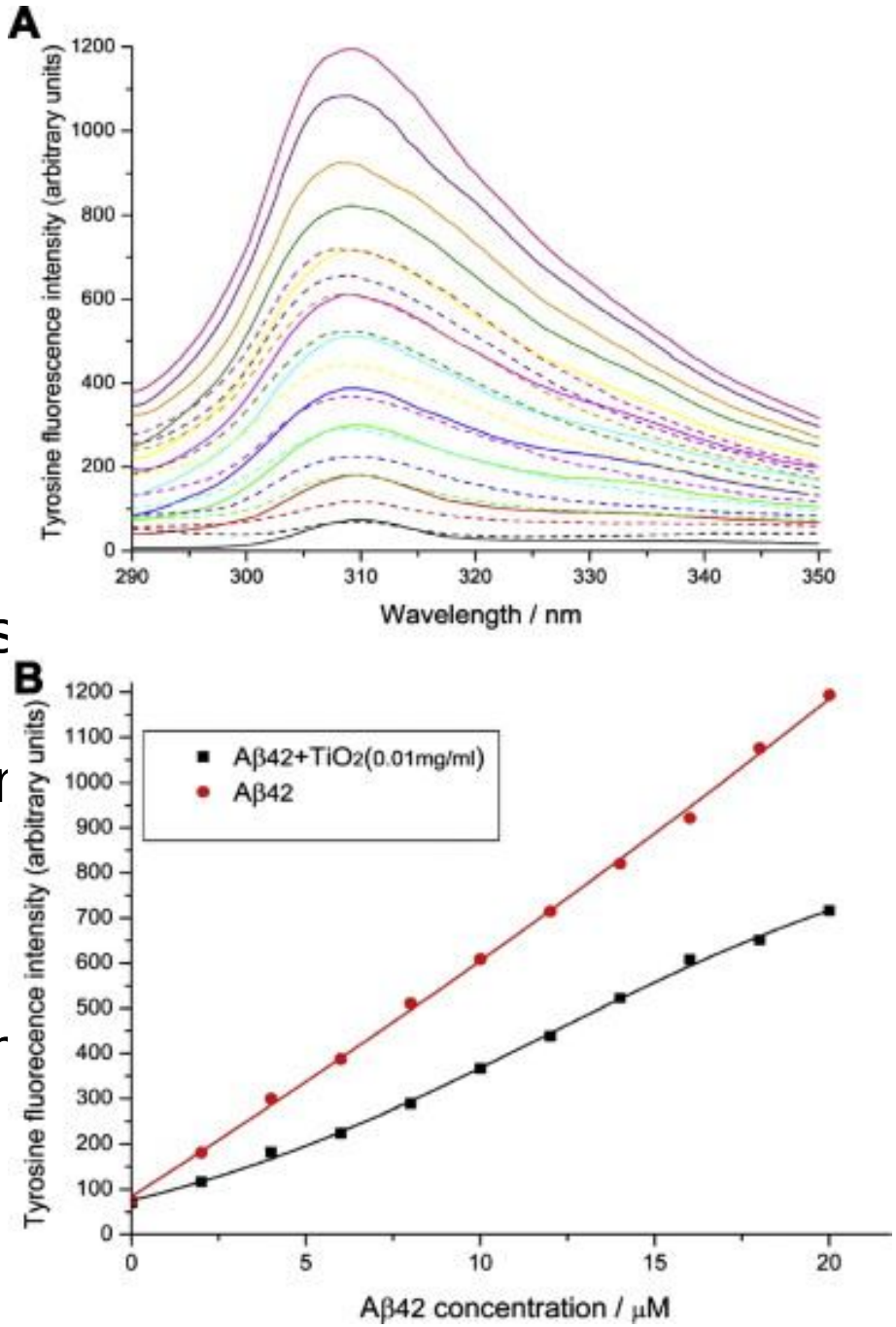




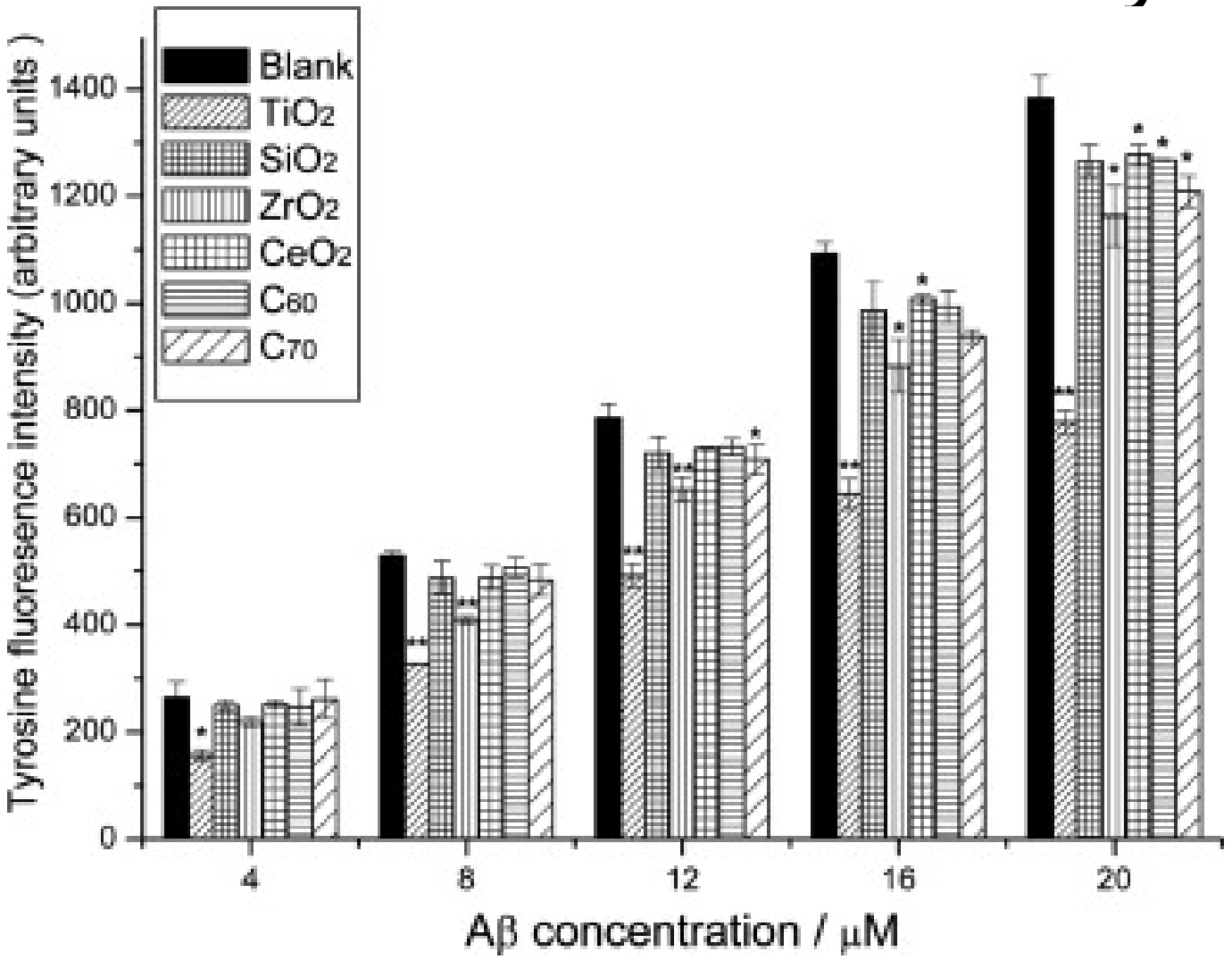
Bar= 100 nm

$Ti0_2$ NPs catalyse the nfibrillar nucleation of Aβ42 (the precursor of amyloid in Alzheimer's disease)

- fluorescence spectra of Aβ42 titrated into buffer (solid lines) and into a solution with TiO2 nanoparticles (0.01 mg/ml, das lines).
- Fluorescence intensity at 309 r versus Aβ42 concentration. Aβ42 titrated into buffer (red filled circles) and titrated into TiO2 nanoparticles (0.01 mg/m black filled squares).

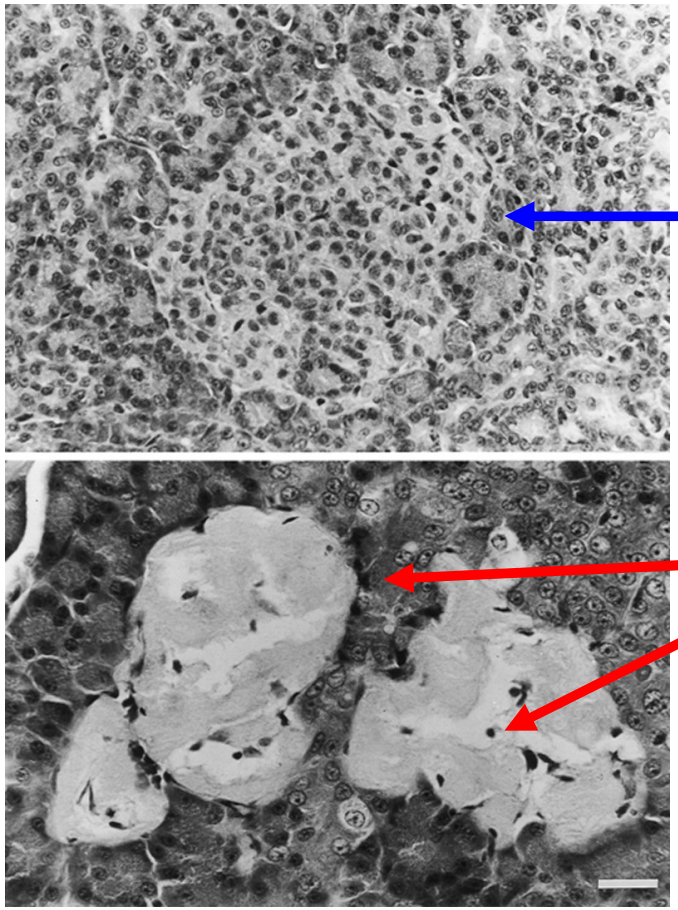


Also other NPs have a (minor) Ab fibrille nucleation activity



Islet Amyloid Polypeptide

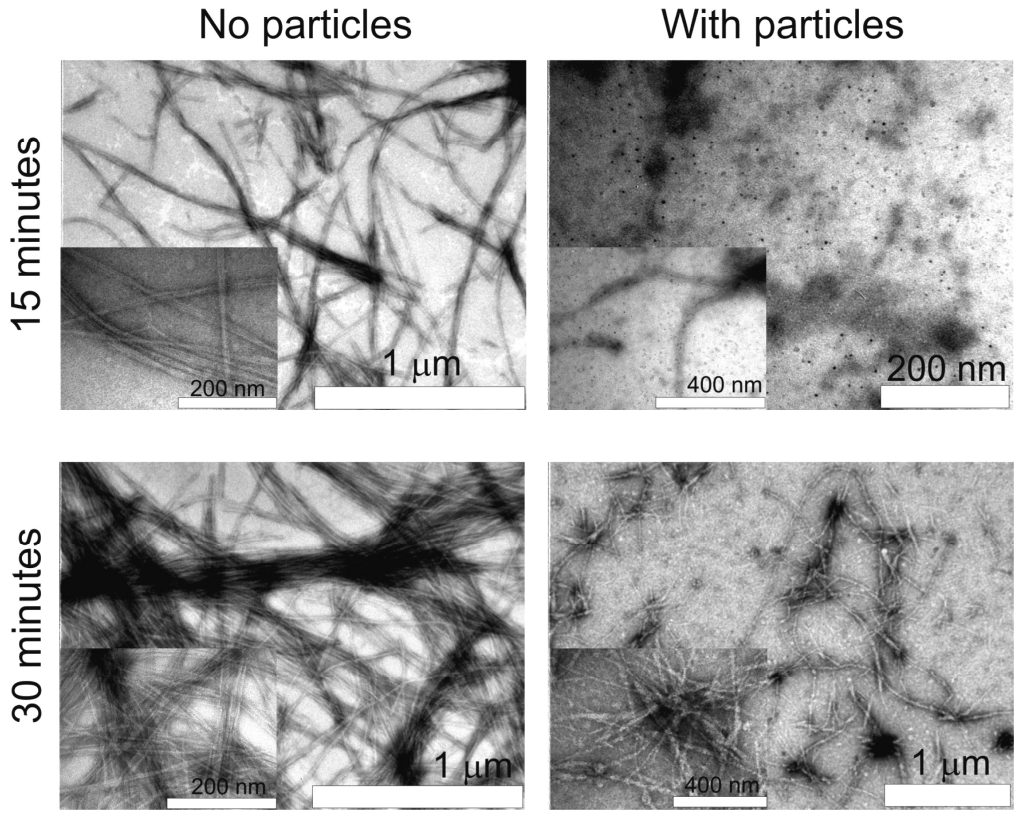
Pancreatic islets



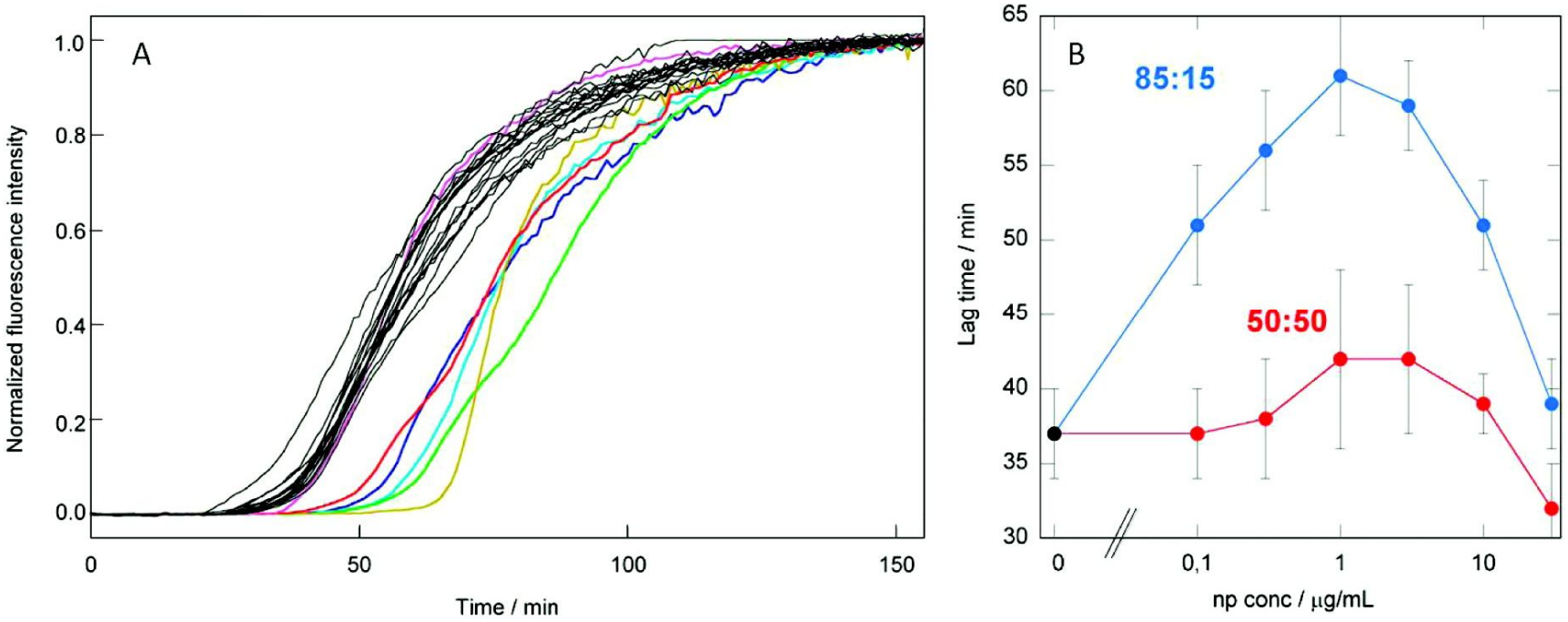
normal

with amyloid fibrillar deposition of IAPP in **diabetic patients**

NiPAM:BAM NPs inhibits IAPP (islet amyloid polypeptide) fibrille formation



TEM images of IAPP samples in the presence and absence of particles (85:15 NiPAM:BAM, 10 µg/mL) for two different time points, 15 and 30 min, along the fibrillation process of 12.8 µM IAPP in 1% DMSO in 20 mM TRIS, 100 mM NaCl, 0.02% NaN3, pH 7.5.



(A) Variation of ThT fluorescence intensity with time in the absence (black) or presence of 85:15 NiPAM:BAM nanoparticles at (dark blue) 0.1, (light blue) 0.3, (green) 1, (yellow-green) 3, (red) 10, and (purple) 30 µg/mL. 12 µM IAPP in 20 mM TRIS, 100 mM NaCl, 0.02% NaN3, pH 7.5.
(B) Lag time versus nanoparticle concentration for (blue) 85:15 and (red) 50:50 NiPAM:BAM nanoparticles

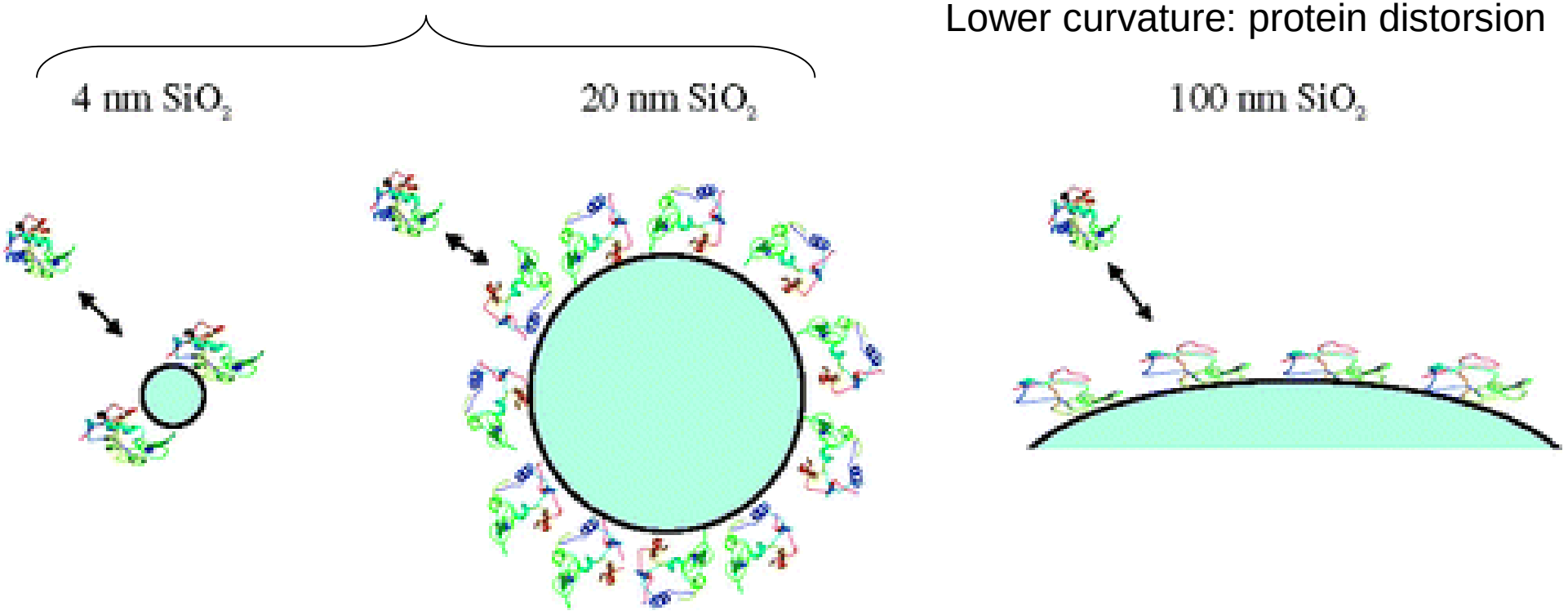
Alteration of enzyme structure and activity

- SiO_2 NPs have been shown to unfold a critical α-helixes essential for the catalytic activity of chicken egg lysozime
- Oxidation of –SH into SS bridges in *glycerol aldehyde phopshate dehydrogenase* with loss of function
- ROS may also oxidase -SH on proteins and enzymes with loss of function

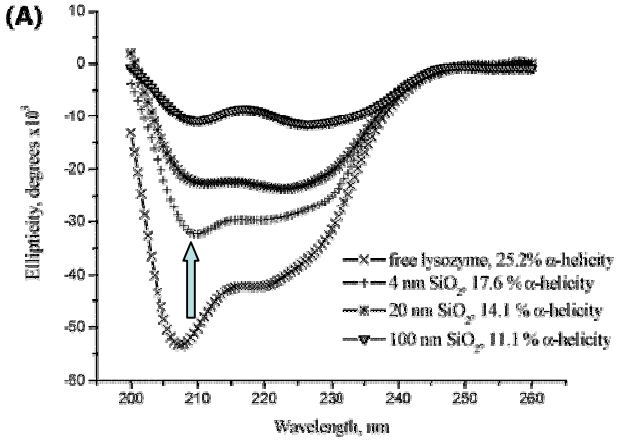
chicken egg lysozime adsorption on SiO_2 NPs: larger NPs alter the conformation more efficiently then the smaller ones

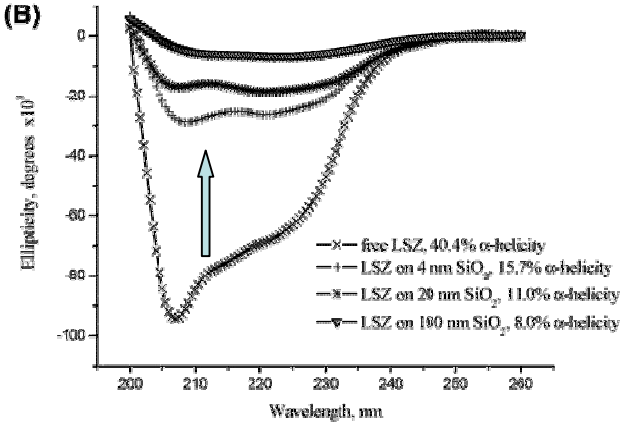
Curvature too intense

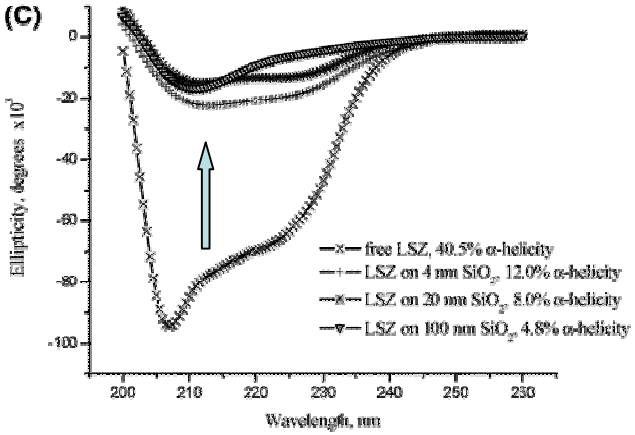
Lower curvature: protein distorsion



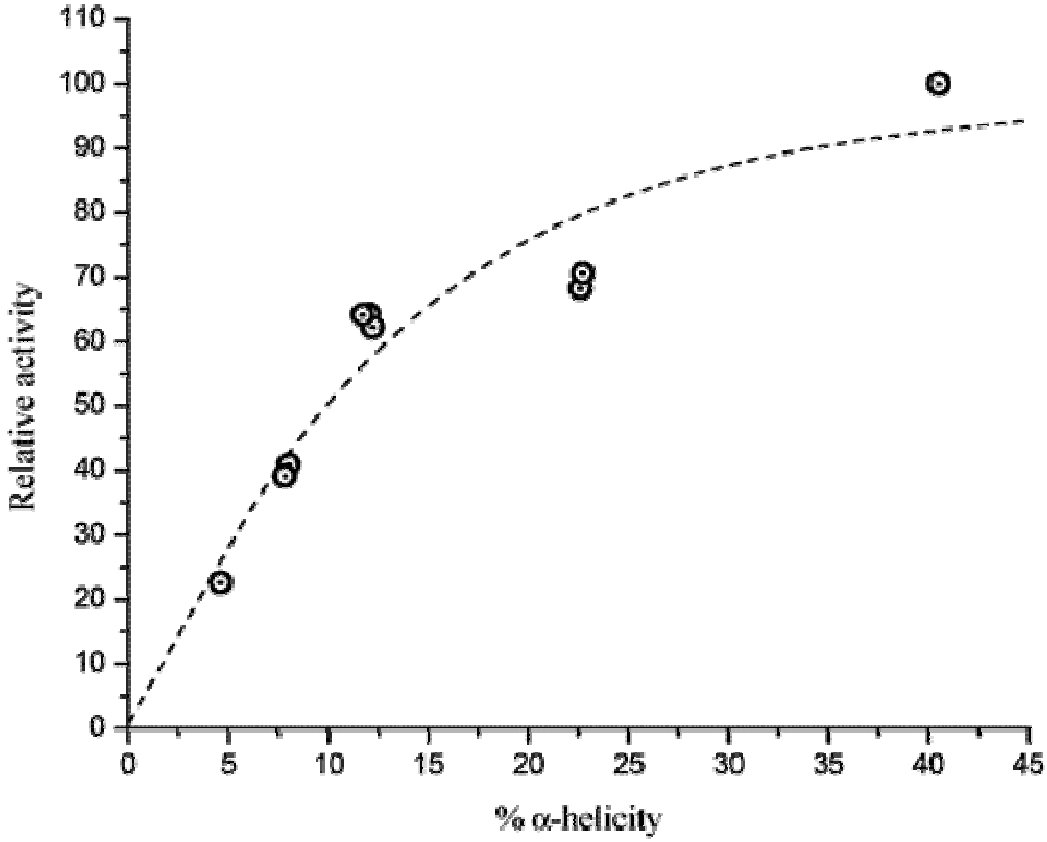
α-helixes disruption by SiO2 NPs





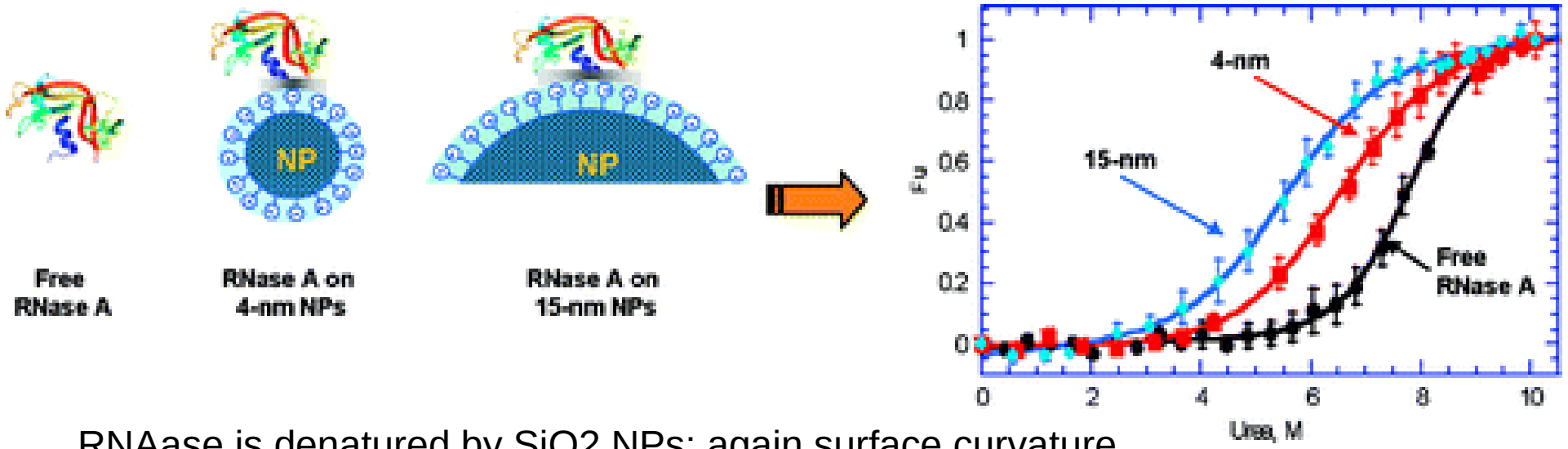


Loss of Lysozime activity



Exposure of cryptic epitopes in proteins

- Epitopes normally not exposed in a native soluble antigen can become exposed when the antigen is particle bound immune response to self antigen



RNAase is denatured by SiO2 NPs: again surface curvature is relevant to favour protein NPs interaction

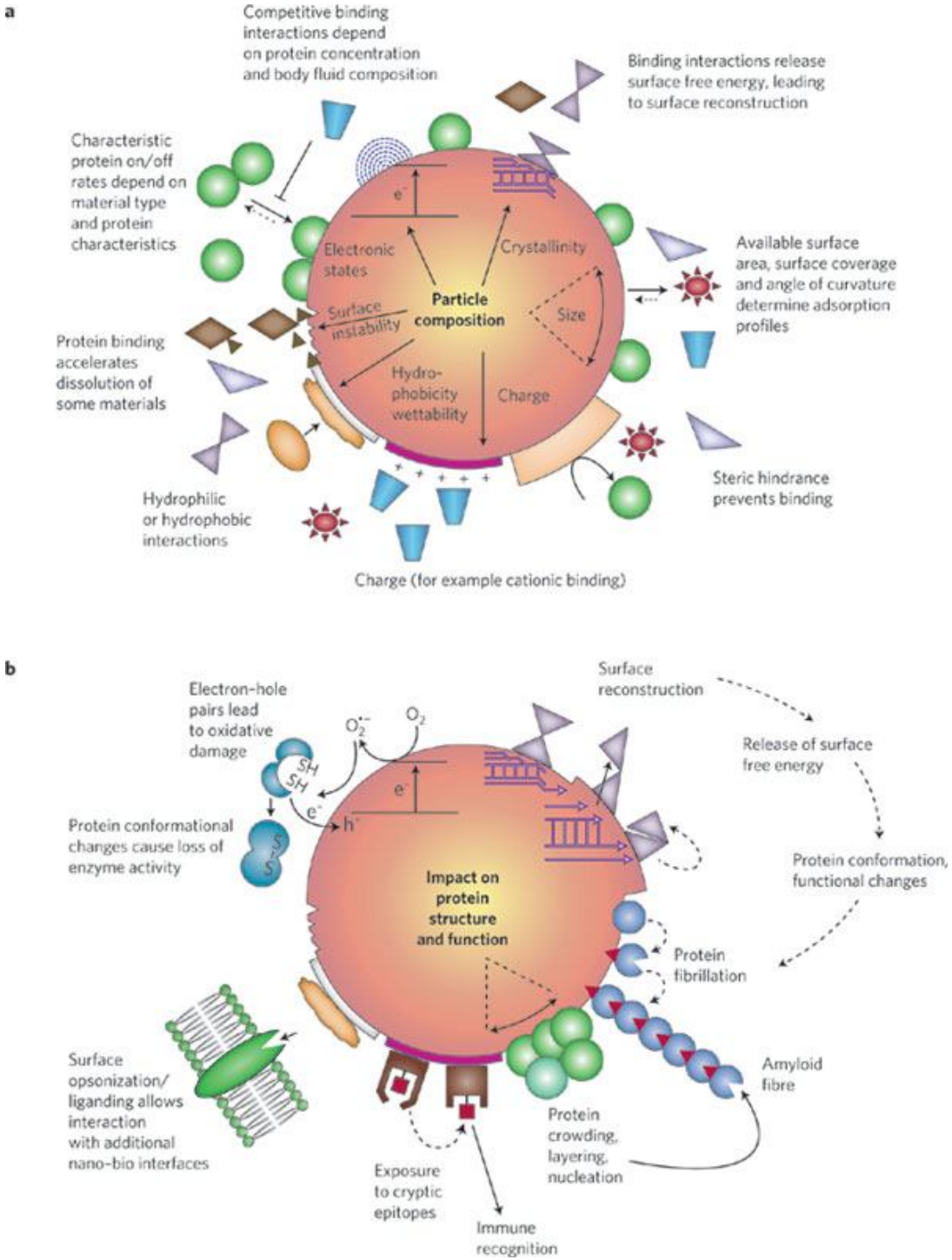
The main independent particle variables that determine the *in vivo* biocompatibility

1, size 2. zeta potential (surface charge) and 3. dispersibility (particularly the effect of hydrophobicity).

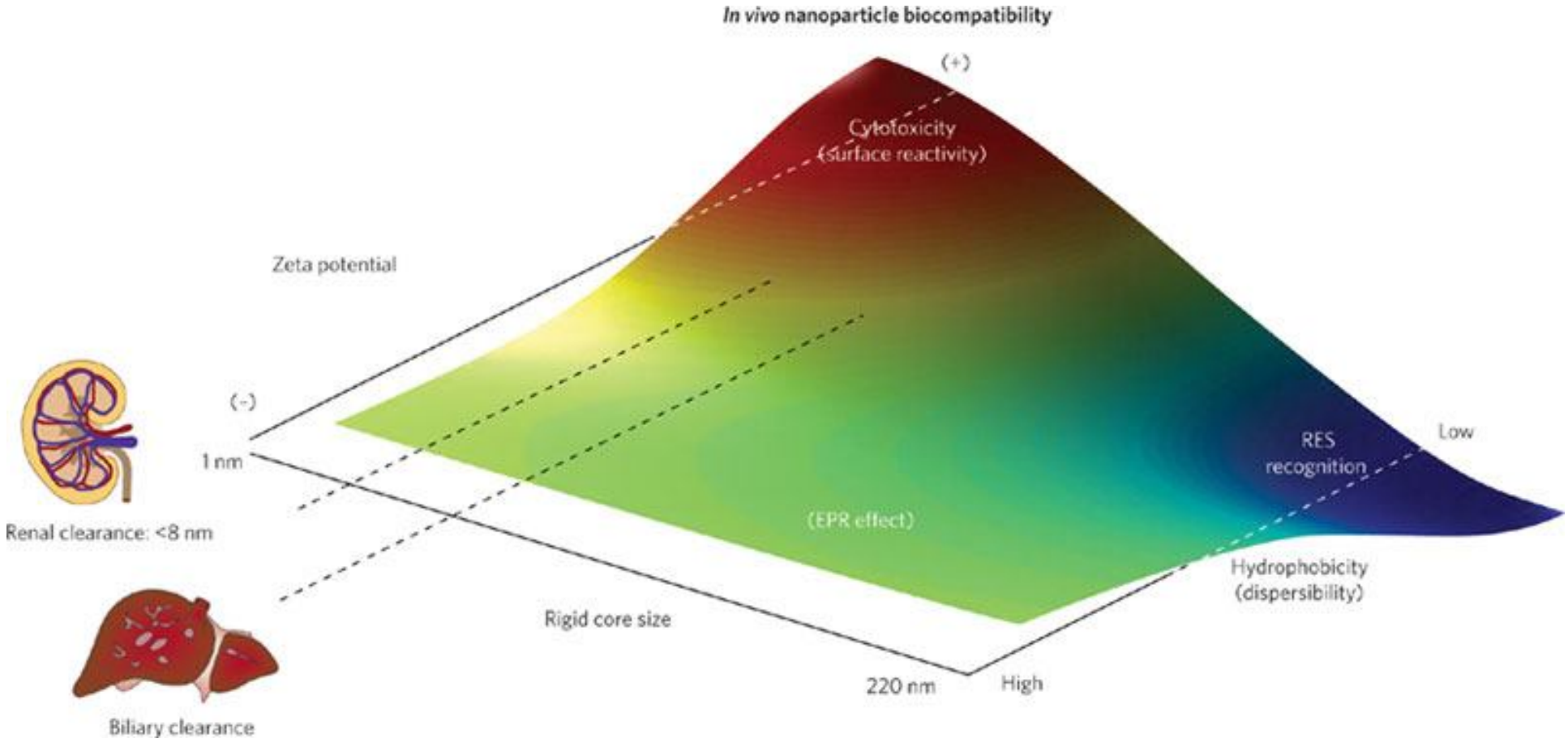
Cationic particles or particles with high surface reactivity are more likely to be toxic than the larger relatively hydrophobic or poorly dispersed particles, which are rapidly and safely removed by the reticuloendothelial system (RES).

Particles that promote enhanced permeation and retention (EPR) effects—and are therefore optimal for chemotherapeutic drug delivery to cancers — generally have mid-range sizes and relatively neutral surface charges.

A comprehensive summary of NP/bio-molecules reciprocal effects



The host-NP interaction “surface”



- Earliest successfull vaccines were live-attenuated or inactivated pathogen formulations (pertussis, sabin polio, measles, Respiratory Syncitial Virus, rotavirus..)

- From our poin of view these are “naturally” occuring particles/carrier (in the µm and nm ranges) with their load of immunogenic antigens

- These corpuscolated vaccines had however adverese secondary effects due to their complex nature (many different and not controlled components)

- Genomics and proteomics today allow to identify a wide renge of vaccine candidate

- Purified, single antigenic molecules are safer than old particulated vaccines
- BUT: they have a shorter half-life in vivo and not always they are processed and presented by immune cells (antigen presenting cells)

- The first report on the use of purified antigens dates to 1924 (Ramon G.)
- Interestingly, many particulated matter increased when co-injected with the AG the immune response
- Agar, tapioca, starch, oil, lechitin and saponine

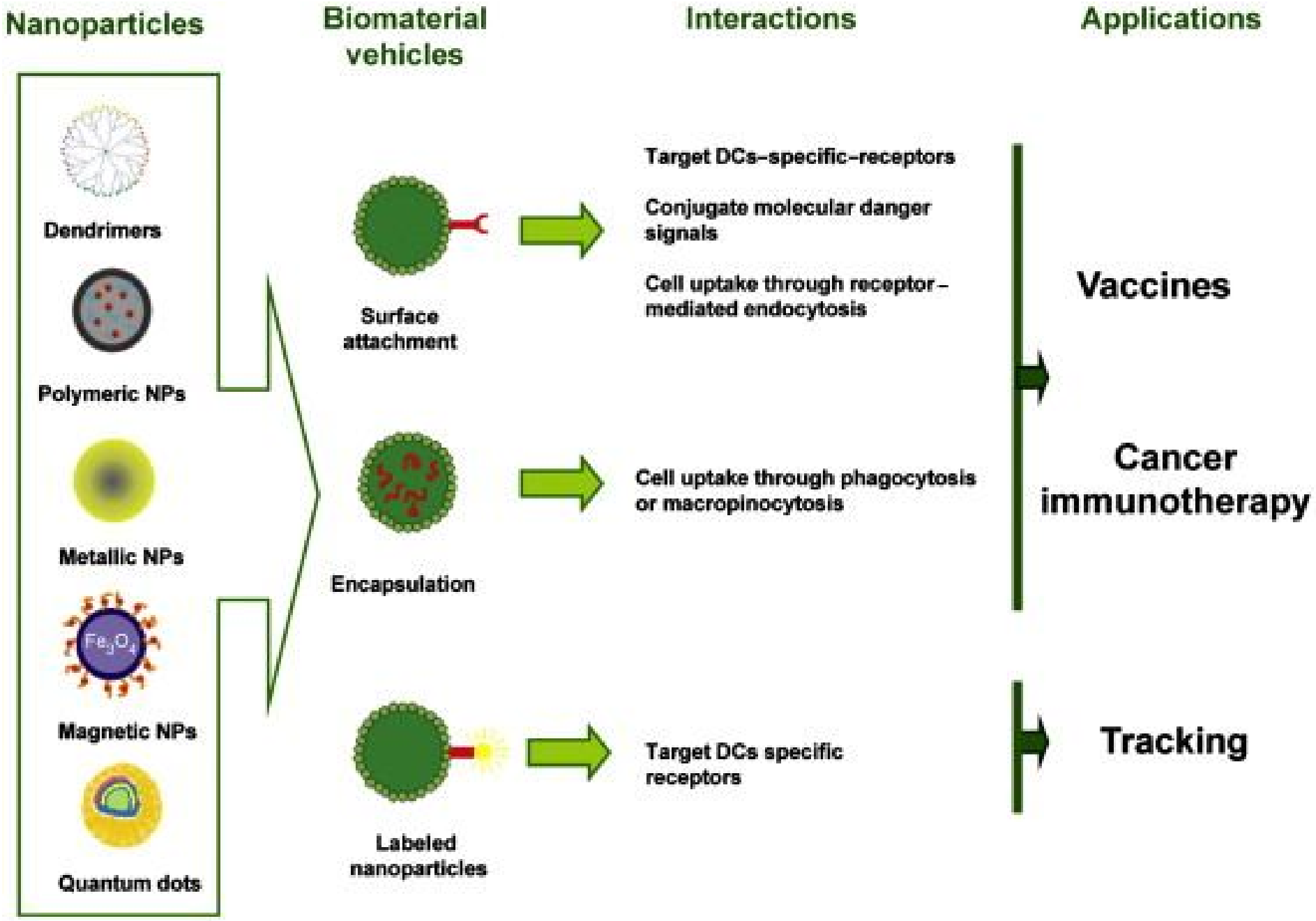
- Various mixtures of such particelles, emolsions or micells could increase the immunogenicity of purified tetanus and diphtheria toxoids: they were called adjuvants

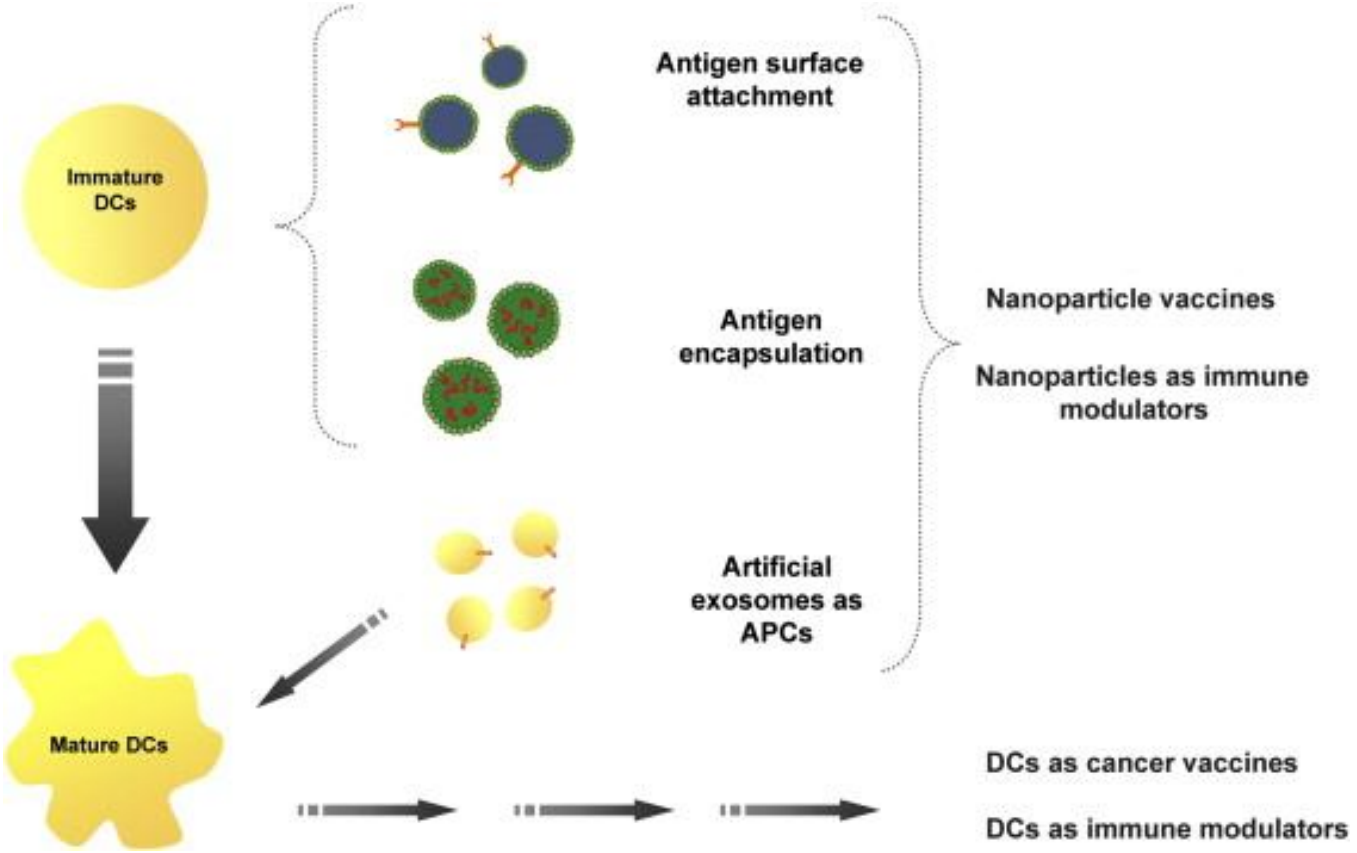
NPs for vaccines or adjuvats

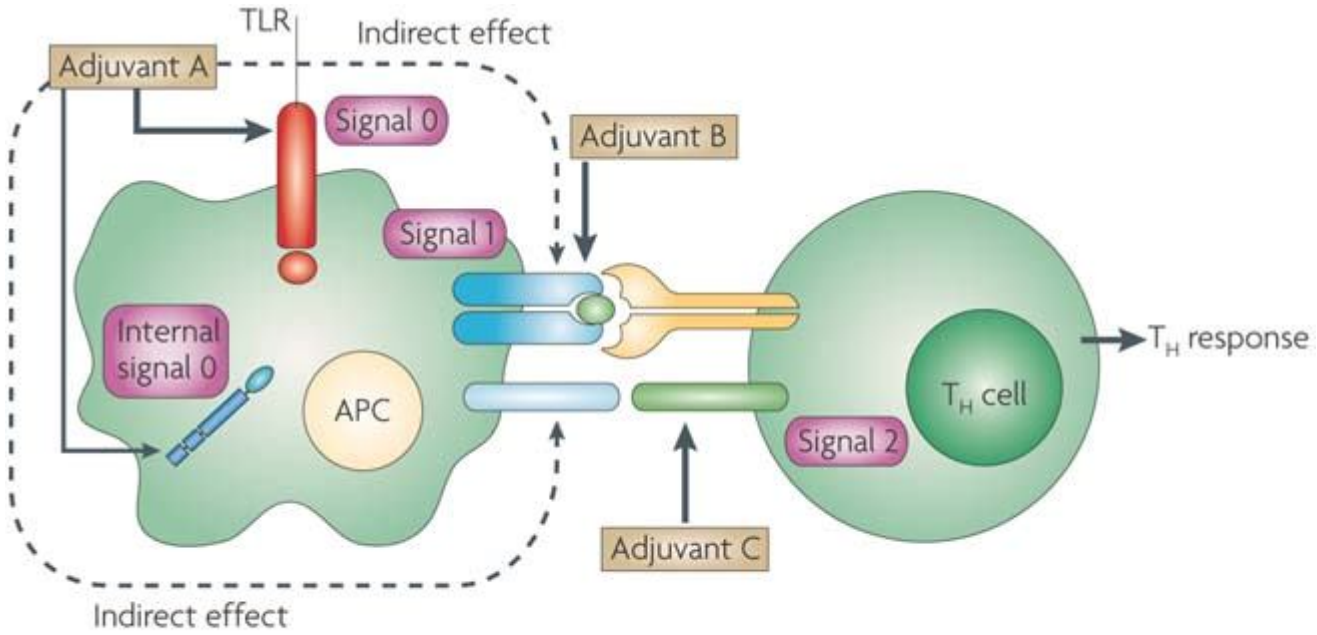
- injected intradermally or intramuscular

  The targets (macrophageic or DCs antigen presenting cells) are close to the injection site, with few fisical or cellualr barrier or obstacles

- or via tre respiratory digestive mucosal tract: the target is the MALT, enderlying the epithelial mucosa: only one barrier to overcome (but remember that in the gut there is a special gate for probing the mucosa antigen composition: the M cells)


Nanoparticles
Biomaterial vehicles
Interactions
Applications
Dendrimers
Polymeric NPs
Metallic NPs
Fe_3O_4
Magnetic NPs
Quantum dots
Surface attachment
Encapsulation
Labeled nanoparticles
Target DCs–specific–receptors
Conjugate molecular danger signals
Cell uptake through receptor–mediated endocytosis
Cell uptake through phagocytosis or macropinocytosis
Target DCs specific receptors
Vaccines
Cancer immunotherapy
Tracking


Immature DCs
Antigen surface attachment
Antigen encapsulation
Artificial exosomes as APCs
Nanoparticle vaccines
Nanoparticles as immune modulators
Mature DCs
DCs as cancer vaccines
DCs as immune modulators



Nature Reviews | Microbiology

- Normally NPs for other uses need to avoid immune cells (be stealth)

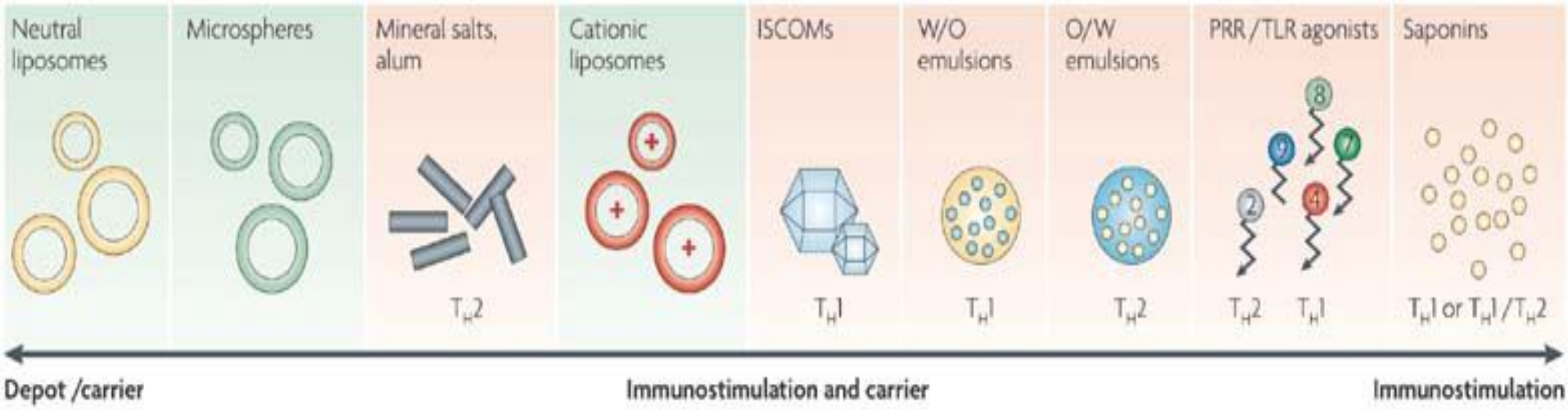
- In these case we have the opposite aim: favour more specific capture by APCs

Type of nano-micro Particolate for vaccine application

- Particle emulsions
- Encapsulating particulate carriers
- Naturally self-assembling virus-like particles (VLPs)
- Solid carrier particles

Particle effect	Specific outcome	Reference
Modulate innate immune response	• Polystyrene, PLGA and alum particles stimulate release of IL1β and IL18 *in vitro* when presented in concert with toll like receptor ligand	[5•]
Modulate quality and quantity of antigen presentation: depot effect	• Enhanced antigen-specific Th1 specific cytokine profile	[28••]
	• Antigen-specific cytotoxic T cell response	[45]
	• Choice of MHC pathway controlled through particle dissolution rate	[48••]
	• Extended antigen presentation	[47, 4]
Targeting dendritic cells and cell compartments	• Use of amphipathic polymer to target dendritic cells	[43••]
	• Use of surface charge to target intracellular compartments	[50•, 41, 9]
Enhance uptake of antigen	• Use of positive surface charge to increase uptake of particles by APC	[50•, 51]
	• Increase antigen uptake by dendritic cells through particle entrapment	[9••]

Current Opinion in Microbiology



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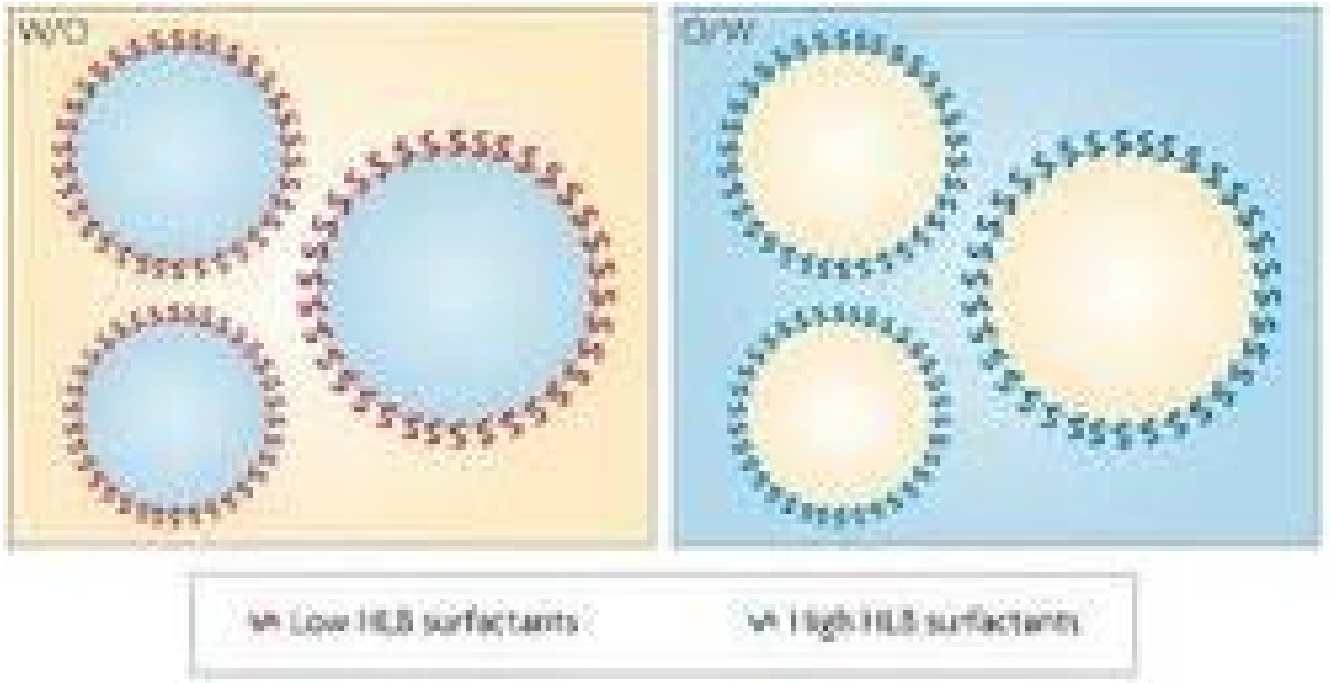
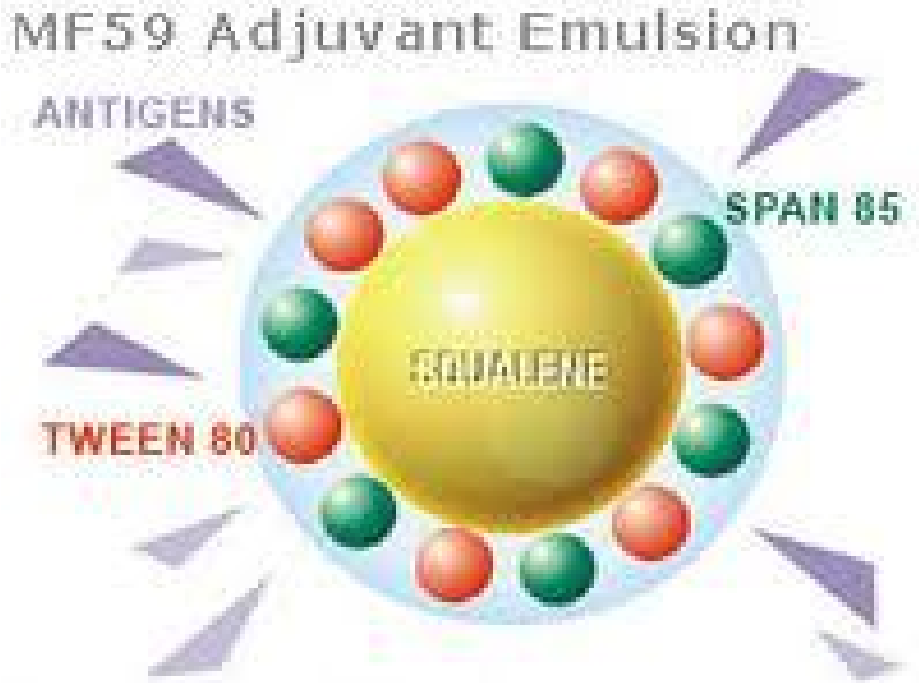
emulsions


W/O
O/W
Low HLB surfactants
High HLB surfactants
Nature Reviews | Microbiology

Particle emulsion

- Water-in-oil (W/O) Freund endocytosis are instable, viscous and give strong local reactions (granulomas)
- MF59 is a oil-in-water nanoemulsion (200-300 nm Ø) based on squalene and detergents. Biodegradable, less viscous, non toxic
- MF59 is endocytosed by iDCs, and then induces per se cytokine production, DCs maturation (adjuvant)



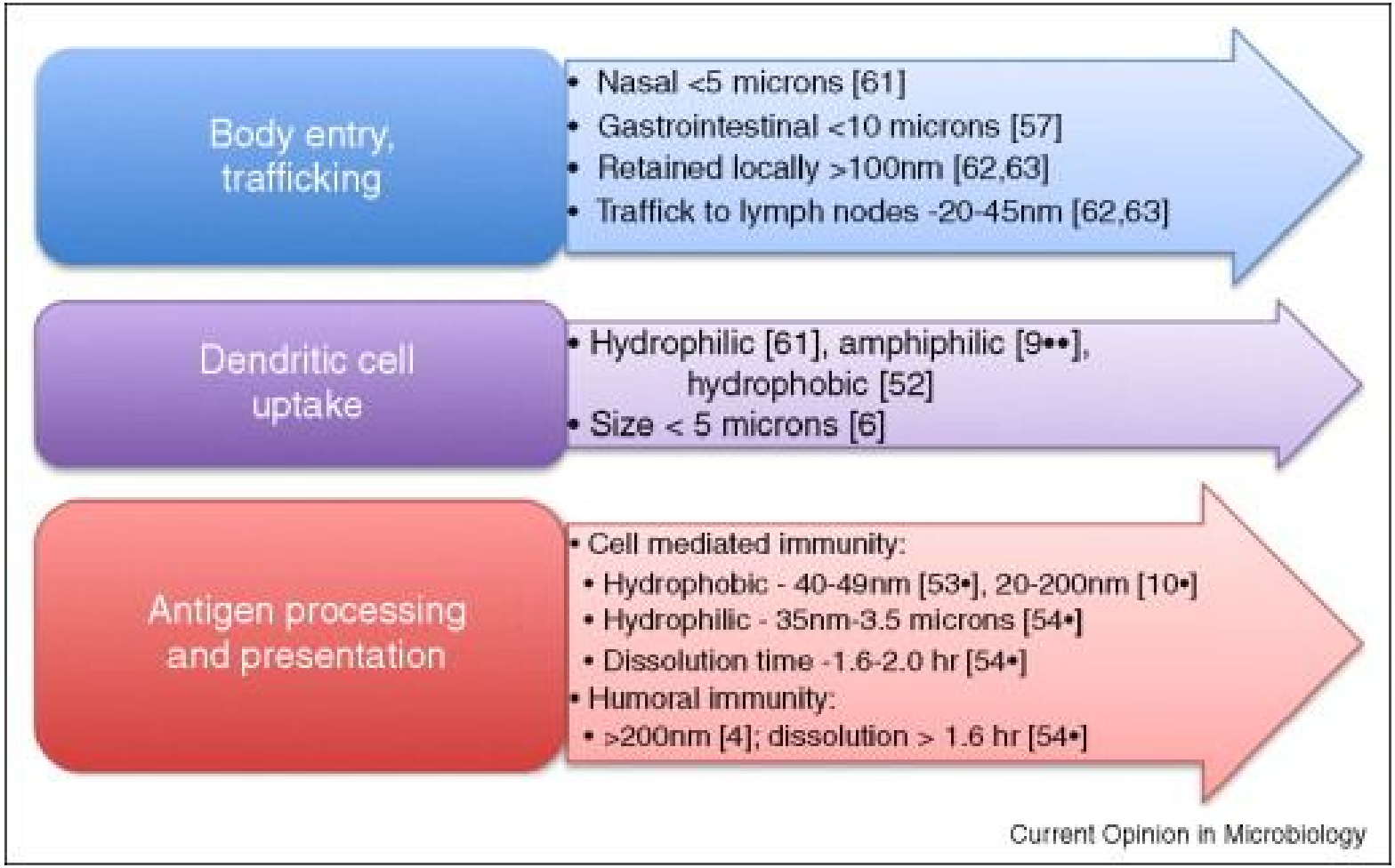
Source: Novartis Vaccines

Encapsulating particulate carriers

- Biodegradable polymeric carriers ~ Ø> 200 nm

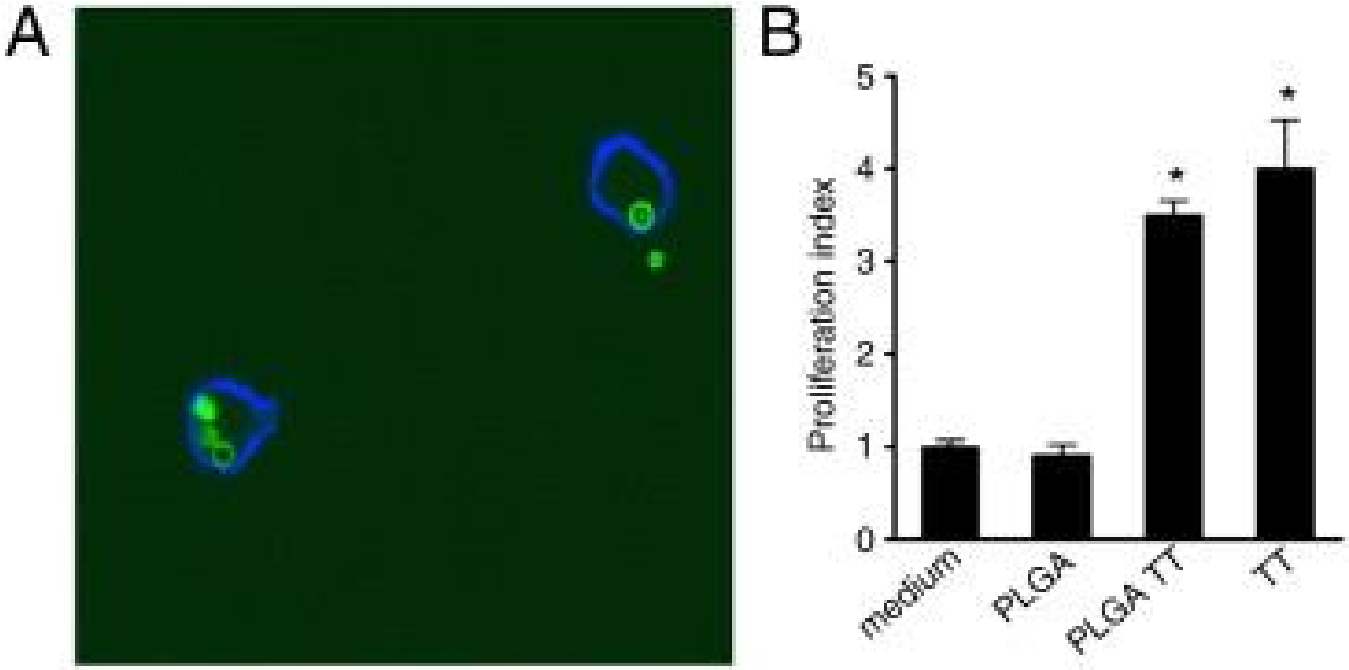
- PLG polylactide-co-glycolides
- PLGA poly(D,L-lactic-coglycolic acids)
- PLA poly (D,L-lactide)
- POE poly(ortho esters)

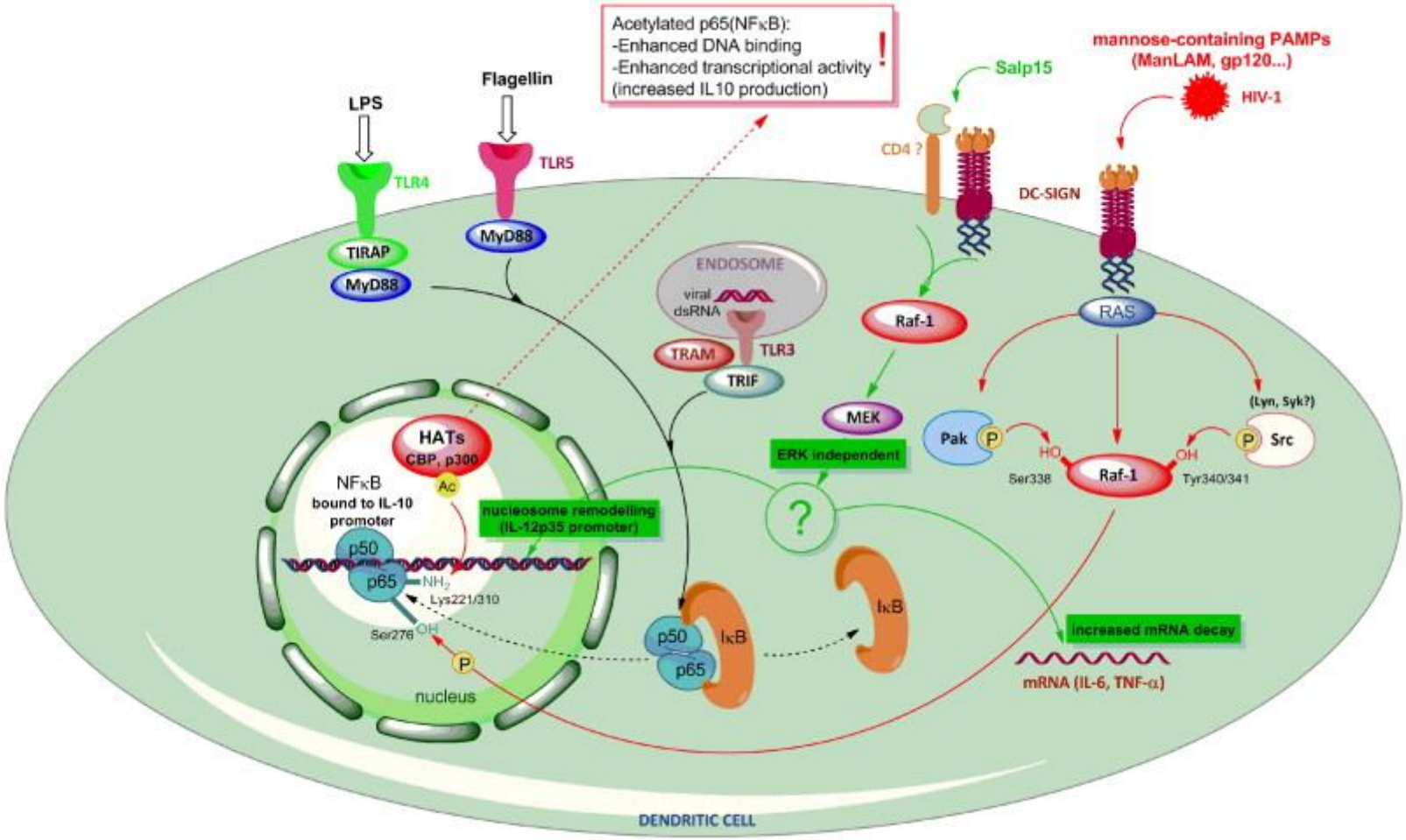
- The antigen is protected from extracellular enzyme degradation, and is gradually released by the entrapping matrix
- They are captured very efficiently by DCs
- Problem: lack of stability during the procedure of antigen encapsulation, storage and storeìage release
- Alternative: adsorption on the polymer surface after synthesis, positivly modified version were tested for improved delivery

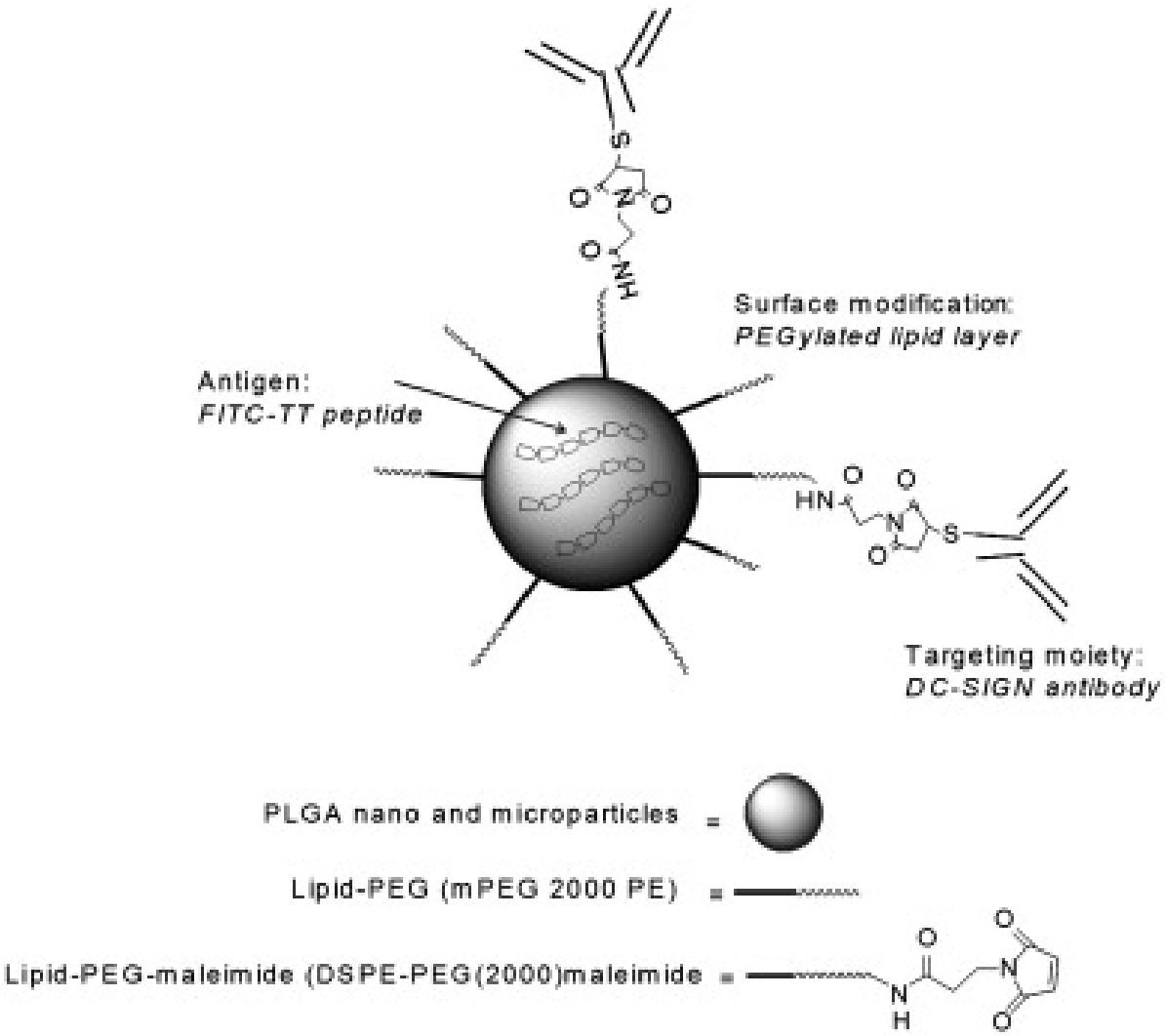

Body entry, trafficking
• Nasal <5 microns [61]
• Gastrointestinal <10 microns [57]
• Retained locally >100nm [62,63]
• Traffick to lymph nodes -20-45nm [62,63]
Dendritic cell uptake
• Hydrophilic [61], amphiphilic [9••], hydrophobic [52]
• Size < 5 microns [6]
Antigen processing and presentation
• Cell mediated immunity:
• Hydrophobic - 40-49nm [53•], 20-200nm [10•]
• Hydrophilic - 35nm-3.5 microns [54•]
• Dissolution time -1.6-2.0 hr [54•]
• Humoral immunity:
• >200nm [4]; dissolution > 1.6 hr [54•]
Current Opinion in Microbiology

Targeted PLGA nano- but not microparticles specifically deliver antigen to via DC-SIGN in vitro

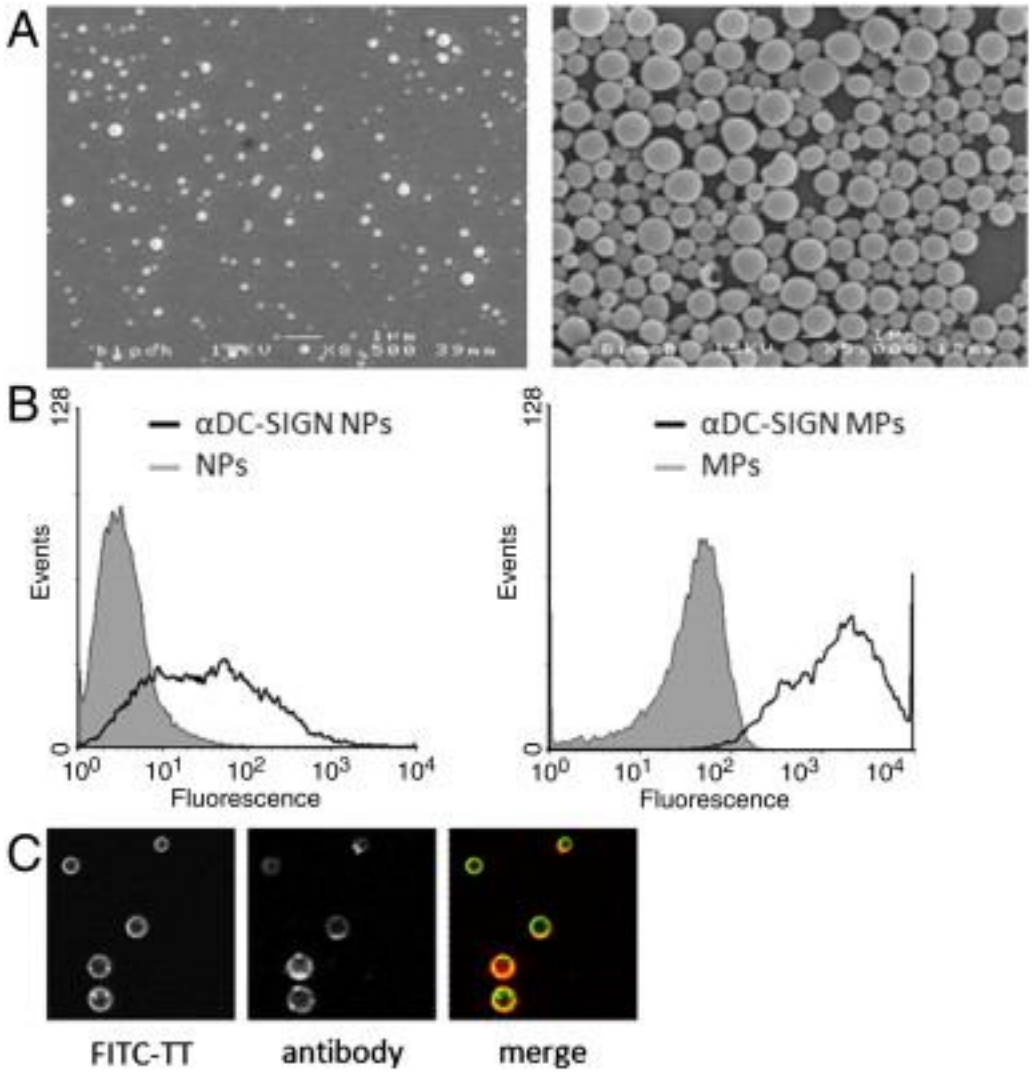
Uptake of PLGA MPs with FITC-TT peptide by DCs results in antigen presentation

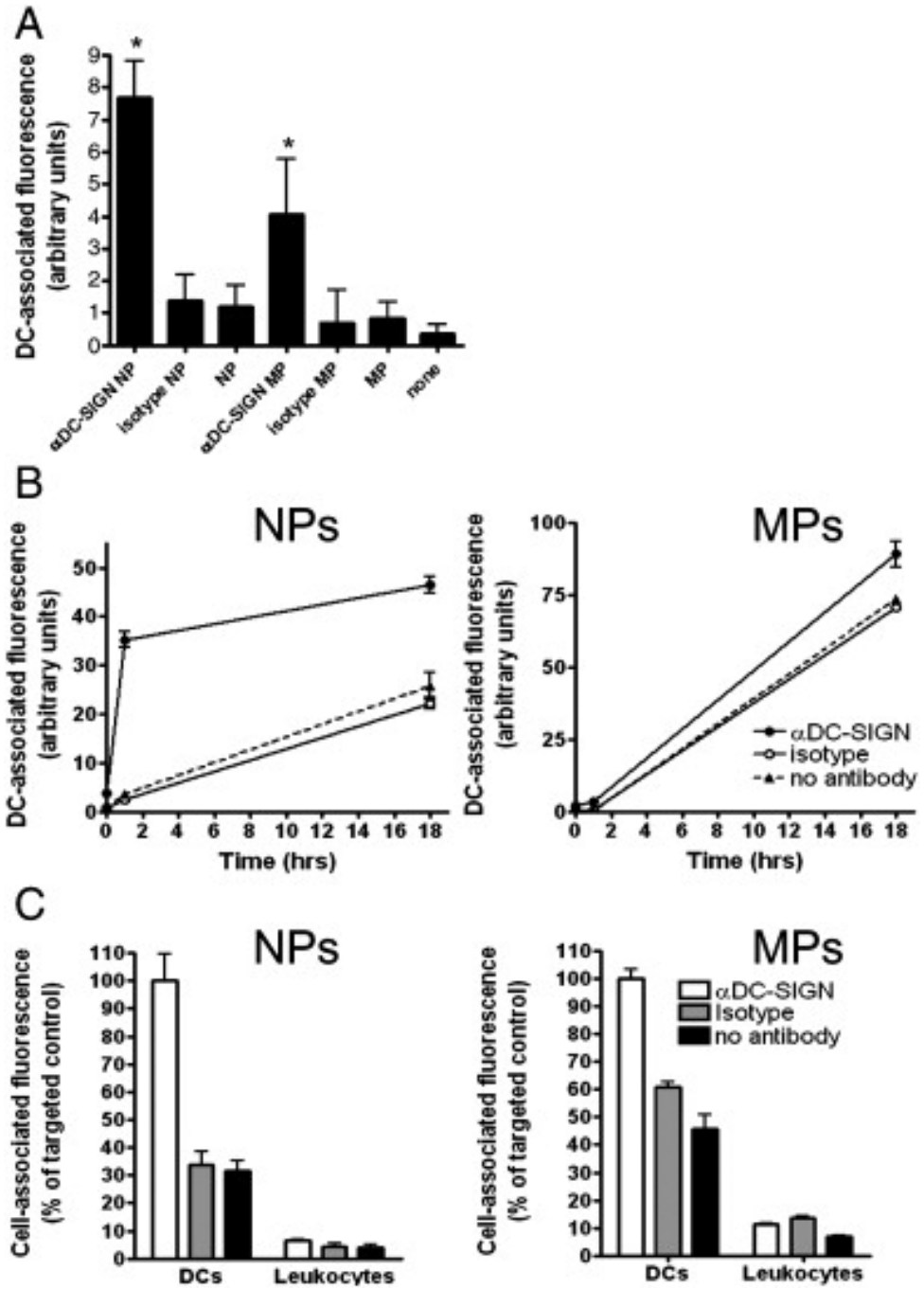



Acetylated p65(NFκB):
-Enhanced DNA binding
-Enhanced transcriptional activity
(increased IL10 production)
!
LPS
Flagellin
TLR4
TLR5
MyD88
TIRAP
MyD88
ENDOSOME
viral dsRNA
TRAM
TLR3
TRIF
Salp15
CD4 ?
DC-SIGN
mannose-containing PAMPs
(ManLAM, gp120...)
HIV-1
RAS
Raf-1
MEK
ERK independent
?
Pak
P
HO
Ser338
Raf-1
OH
Tyr340/341
P
Src
(Lyn, Syk?)
HATs
CBP, p300
Ac
NFκB
bound to IL-10
promoter
nucleosome remodelling
(IL-12p35 promoter)
p50
p65
NH2
Lys221/310
Ser276
OH
P
nucleus
p50
p65
IκB
IκB
increased mRNA decay
mRNA (IL-6, TNF-α)
DENDRITIC CELL

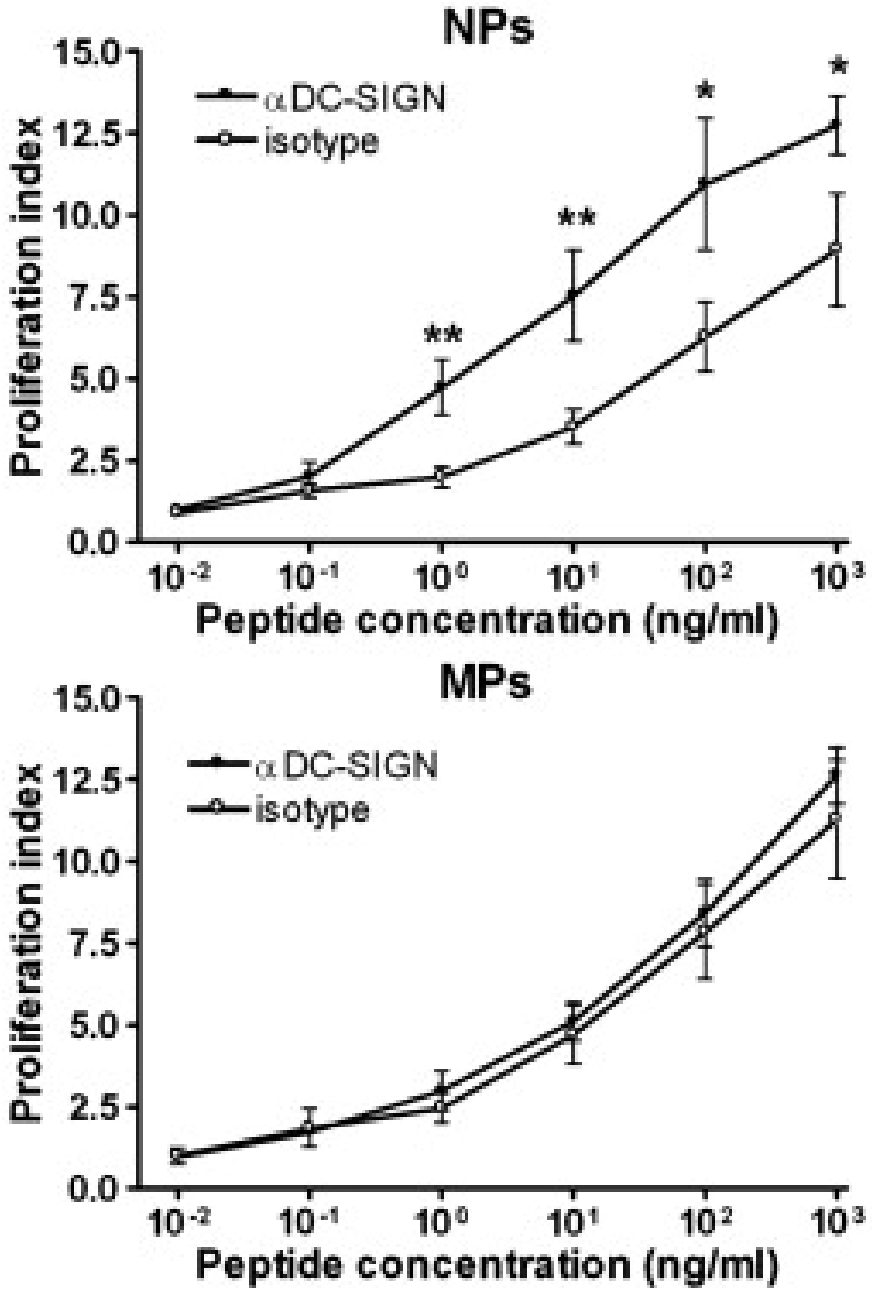

Surface modification:
PEGylated lipid layer
Antigen:
FITC-TT peptide
Targeting moiety:
DC-SIGN antibody
PLGA nano and microparticles =
Lipid-PEG (mPEG 2000 PE) =
Lipid-PEG-maleimide (DSPE-PEG(2000)maleimide =

Antibodies to αDC-SIGN are introduced on the surface of PLGA NPs and MPs





- Interactions of targeted NPs and MPs with DCs and blood leukocytes



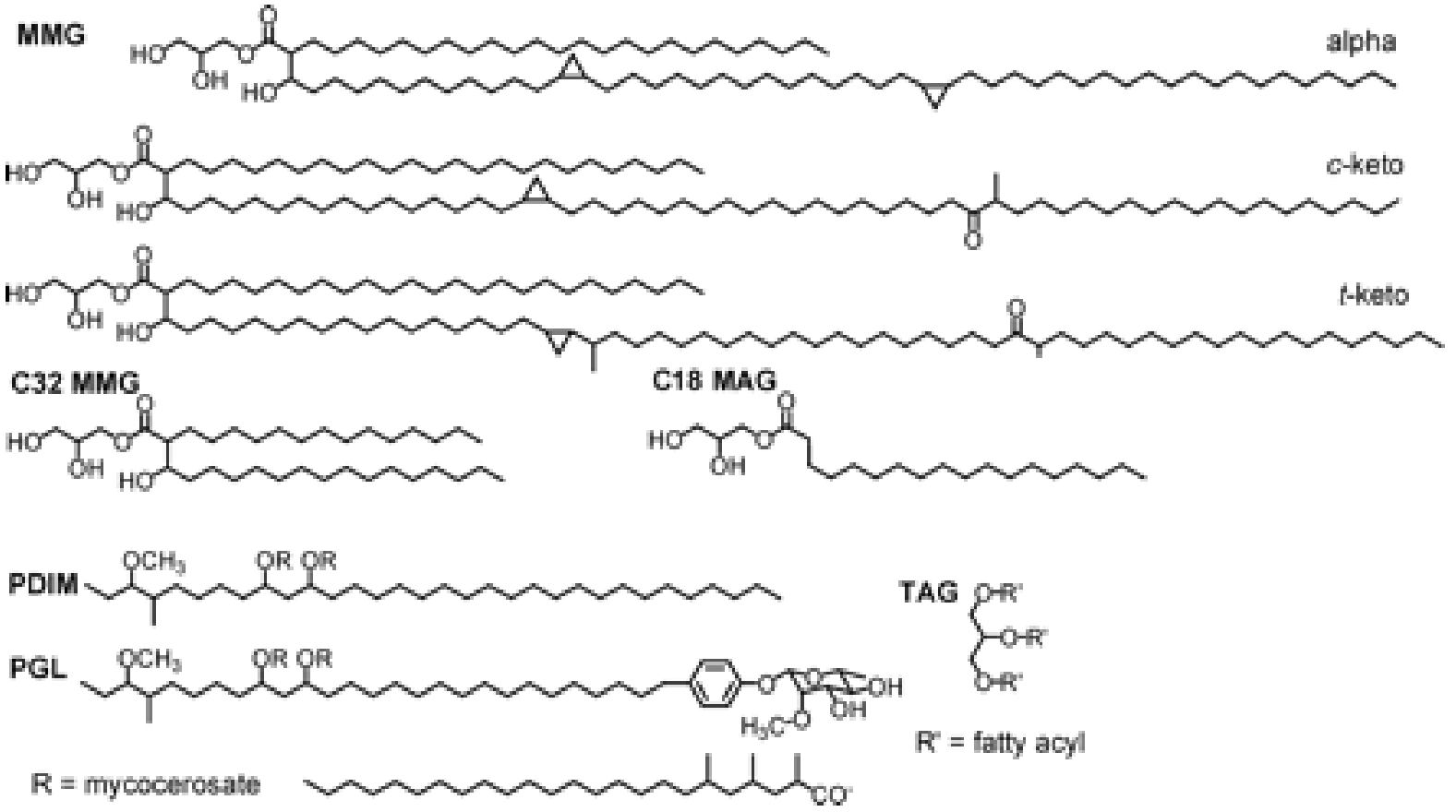
- Specific targeting of NPs to enhances antigen presentation

- Degradable polymeric NPs mediate both MHC-I and MHC-II dependent immune resposes

liposomes

- Size ~ 100-250 nm Ø depending on the type
- They protect antigens by rapid intracellular degradation by APCs: prolonged intracellular half-life
- They are teken up by APS and induce preferntially MHC-I dep immune responses
- Liposomes containing immunostimulating (adjuvants) micorbial glycolipids were also formulated: they carry antigens in DCS aand aslo induce their maturation
- However: poorly stable during storage, limited shelf-life, sterility problems

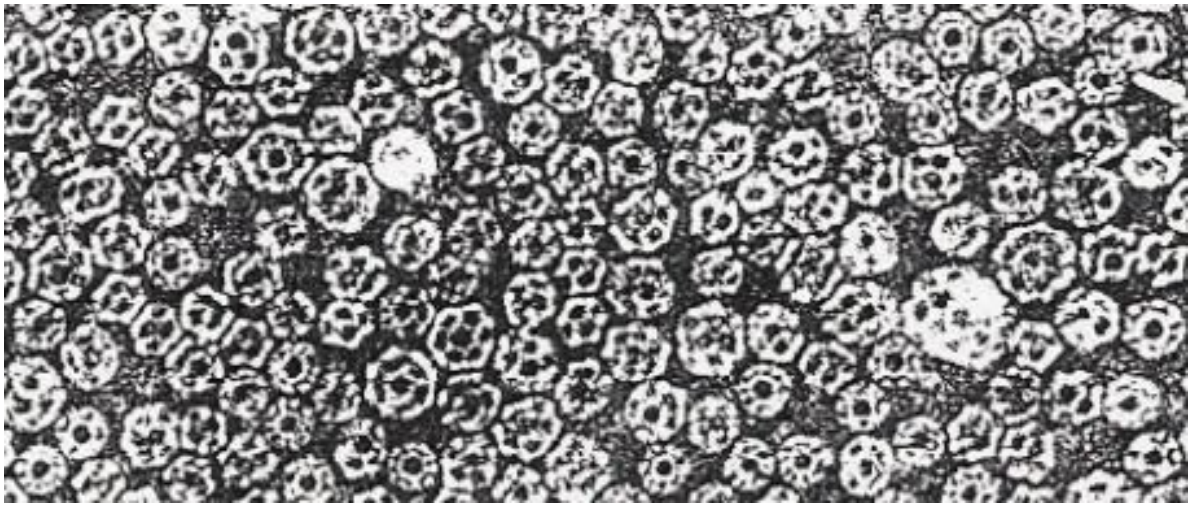
Structures of apolar lipid purified from *M. bovis* BCG Copenhagen

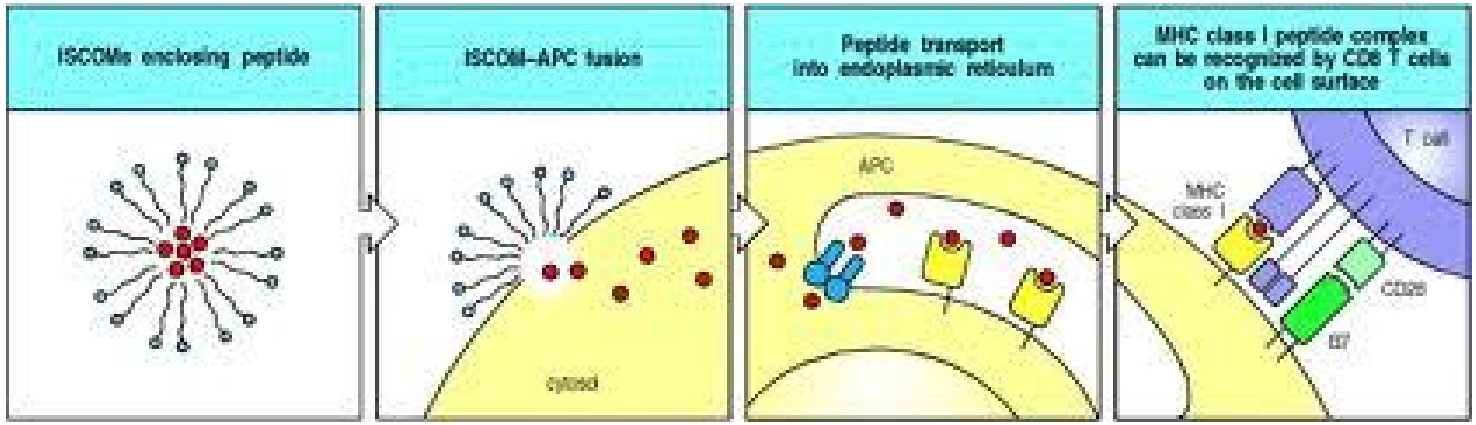


ISCOMs

- micellar NPs (~ 40 Ø nm)
- Purified fractions from Quillarja saponaria (saponines) + cholesterol + phospholipids + cell membrane antigens (viral preferentially)
- The lipophilic part promote endocytosis by monocytes, macrophages and DCs
- Saponine is highly immunomodulating
- Both MHC-I and MHC-II responses are induced
- The net negative charge of ISCOMs ensures the colloidal stability of the suspension and the easy delivery of positive charged antigens
- Very stable: in saline pH 6,3 3 months at 37°C, 2 years at 0°C- it resists repeated freezing and thawing cycles

ISCOMs




ISCOMs enclosing peptide
ISCOM–APC fusion
Peptide transport into endoplasmic reticulum
MHC class I peptide complex can be recognized by CD8 T cells on the cell surface
cytosol
APC
T cell
MHC class I
CD28
B7

Naturally self-assembling virus-like particles

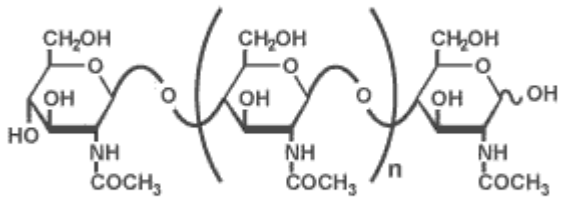
- Non-replicating virus-like particles
- It is difficult to incorporate antigens without destabilizaing the particles
- Necessary to test every new type for secondary effects

Solid carrier particles: artificial “more micro then nano”-sized vaccines

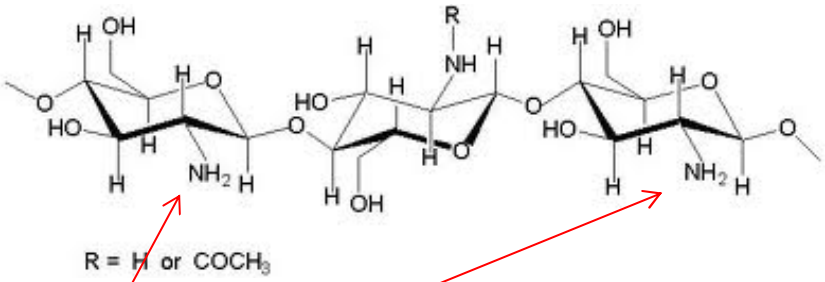
- Chitosan
- Gold
- SiO2
- polystyrene

- Chitosan is a mucopolysacharide closely related to cellulose. Chitosan is obtained by deacetylation of chitin, the major compound of exoskeletons in crustaceans.

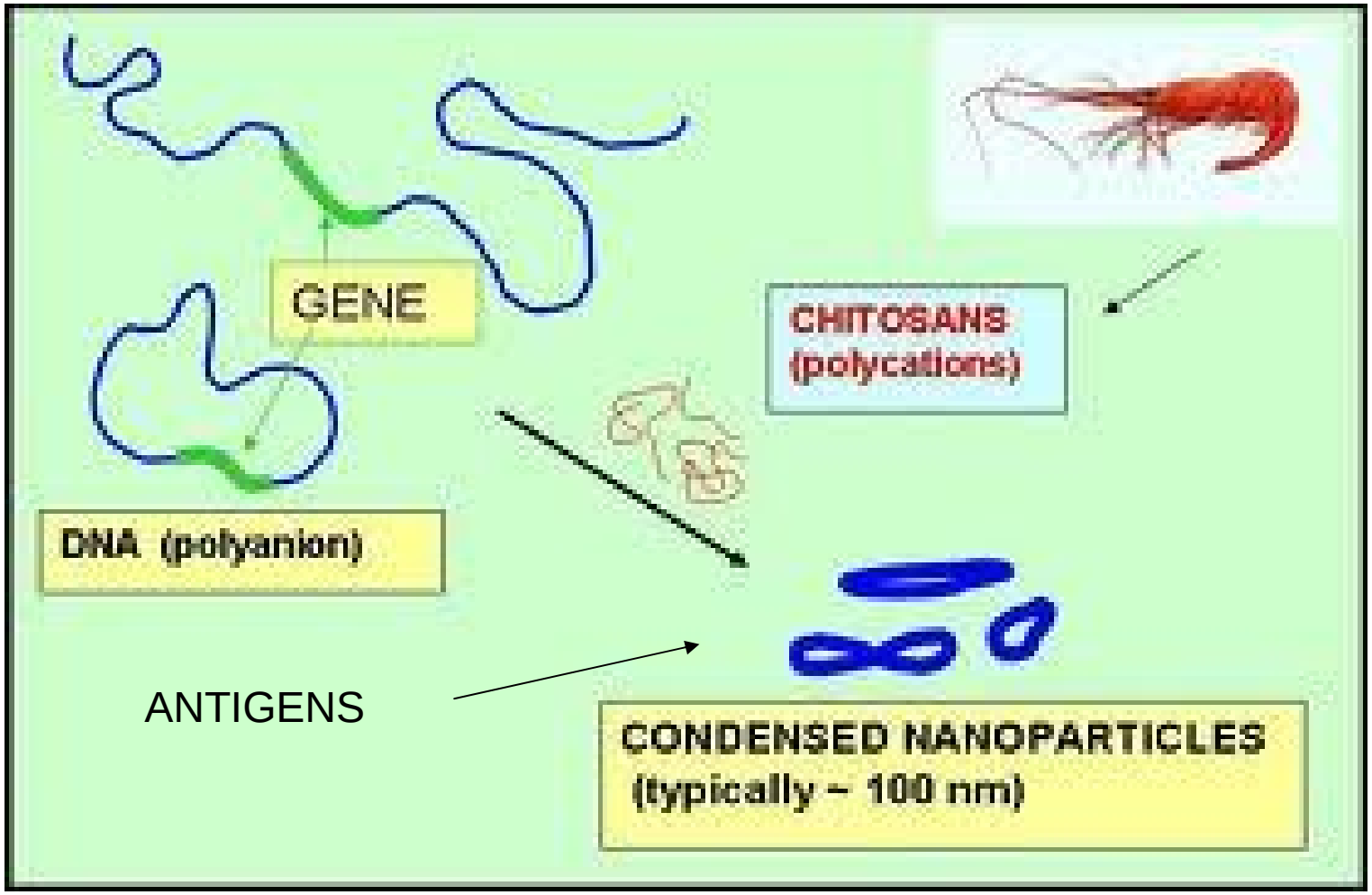
chitin



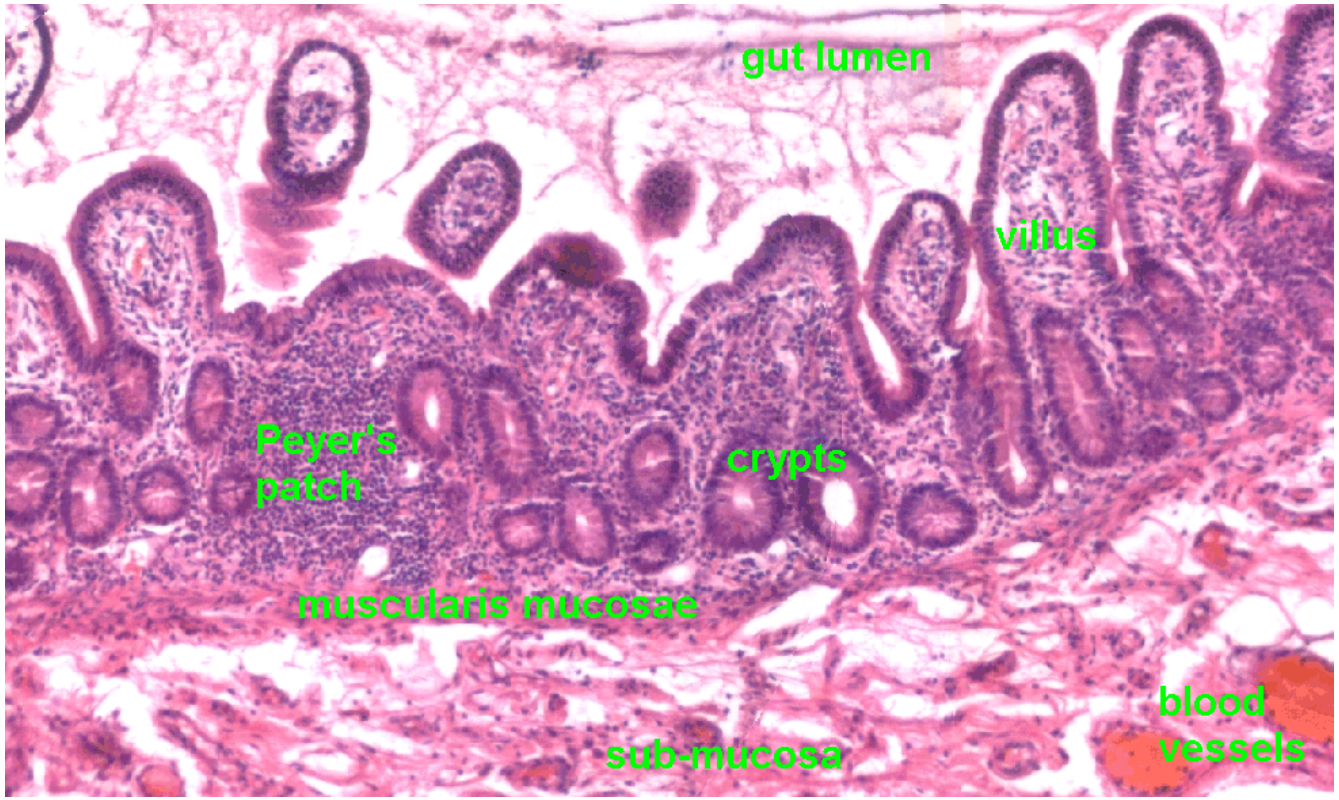
chitosan

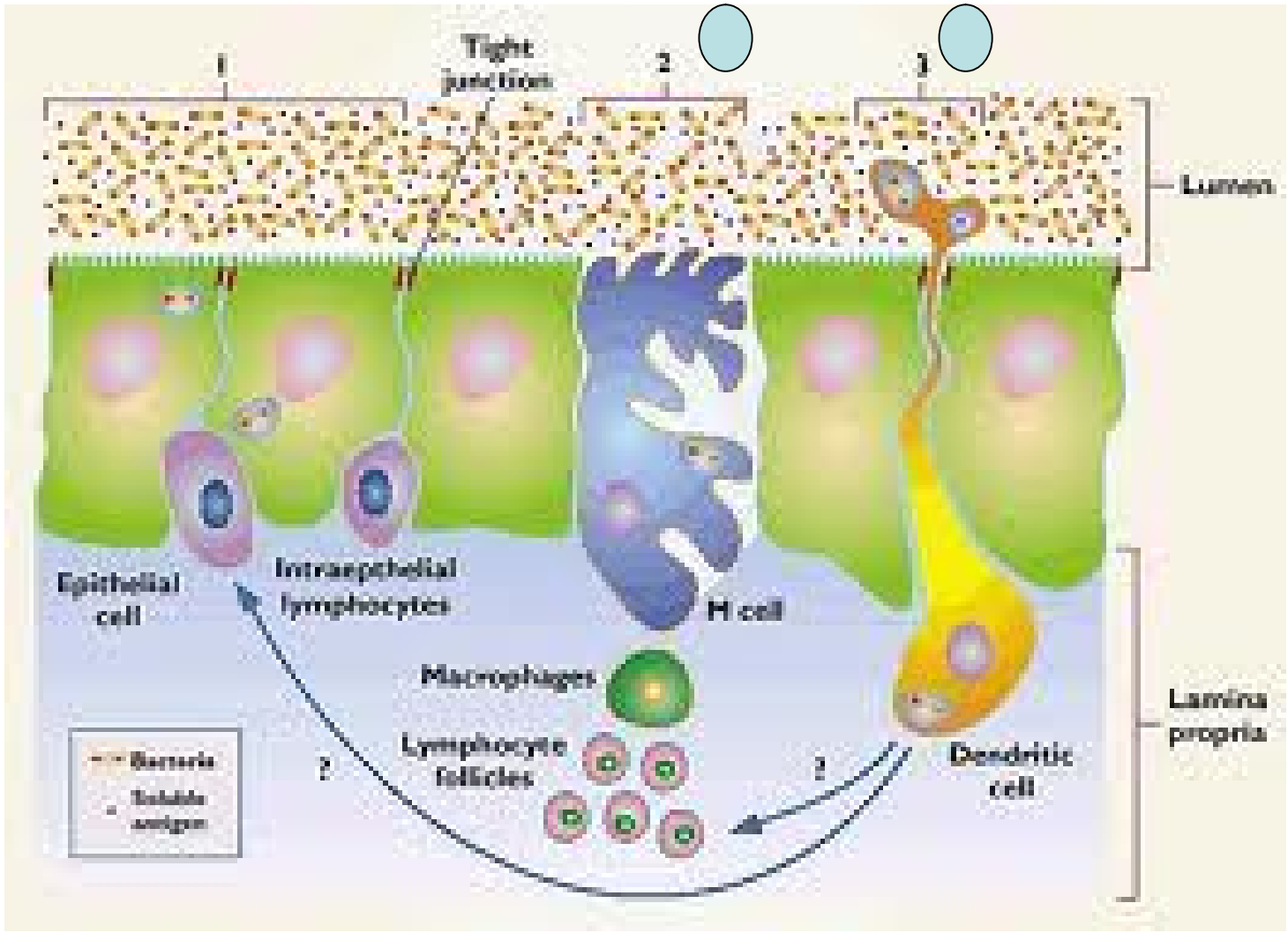


Positive charges at physiological pH


GENE
CHITOSANS
(polycations)
DNA (polyanion)
ANTIGENS
CONDENSED NANOPARTICLES
(typically ~ 100 nm)

- Chitosan is a cationic naturally occuring biodegradable polysaccharide
- Interesting MPs of chitosan are taken up by M cells in the gut Peyer’s patches (GALT)
- Promising candidate for oral vaccine
- Intranasal chitosan + AG inhances mucosal and systemic immune responses to AG


gut lumen
villus
Peyer's patch
crypts
muscularis mucosae
blood vessels
sub-mucosa


1
Tight junction
2
3
Lumen
Epithelial cell
Intraepithelial lymphocytes
M cell
Macrophages
Lymphocyte follicles
Dendritic cell
Lamina propria
?
?
Bacteria
Soluble antigen

- AG bound to Polystyrene beads (0,5-2 µm Ø) are efficiently presented via MHC-I and activate CD8 CTL

- Gold MPs (1-3 µm) used for gene-gun applications DNA immunisation

Particle-associated antigen capture

- iDCs in peripheral tissues have a variety of endocytic pathways, used to screen their environments for foreign antigen

- Particles < 150 nm normally captured by CME, caveolae or by clathrin- caveolae-indep endocytosis
- Particles > 0,5 µm by macropinocytosis (not very specific sampling of the extracellular fuid)

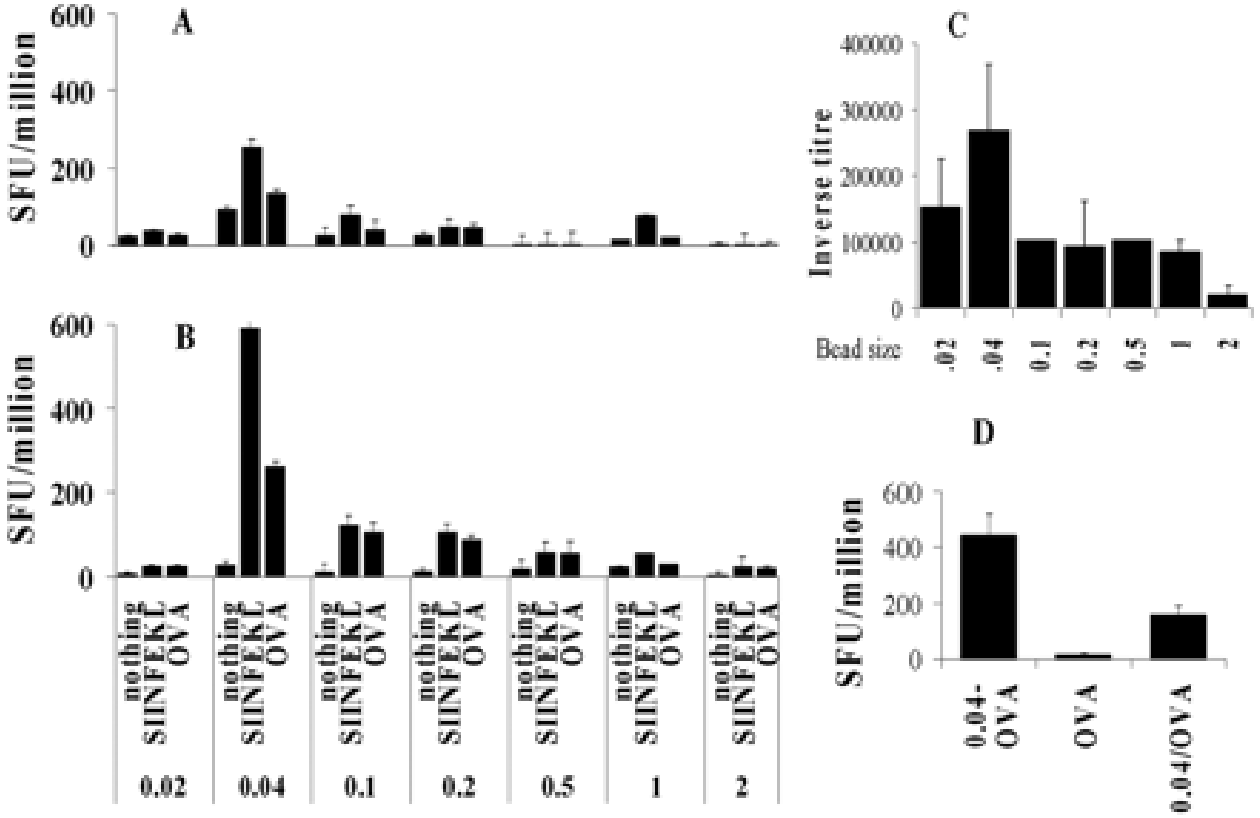
- iDCs are the only cells that constitutively express macropinocytosis
- Macrophages (and endothelial cells) show marcopinocytosi only if stimulated
- mDCs (competent for antigen presentation to T lymphocytes) strongly down regulate micopinocytosis

- Phagocytosis is a more specific uptake of particles (normally µm) which requires a tight contact with the plasma membrane, via receptor. Only present in professional phagopcytes (macrophages and DCs from teh point of view of immune response)

- Virus-sized particulates (20-200 nm Ø) are endocytosed by micropinocytosis and preferentially ingetsed by DCs
- Larger size partulates (0,5 µm or larger) are captured by iDCs or activated macrophages by macropinocytosis
- 5-20 µm particulates (bacterial-cell size) are phagocytosed primarily by macrophages

Immunogenicity: size relevance

- A study with Polystyrene particles
- Ø < 0,5 µm, and in particular 40-50 nm NPs are more efficient in inducing CD8 CTL (cytotoxic) and CD4Th1(cell mediated) response
- Ø > 0,5 µm tend to induce a CD4Th2 (antibody response) immunity


A
B
C
D
SFU/million
600
400
200
0
nothing
SIINFEKL
OVA
0.02
0.04
0.1
0.2
0.5
1
2
Inverse titre
400000
300000
200000
100000
Bead size
.02
.04
0.1
0.2
0.5
1
2
0.04-OVA
OVA
0.04/OVA

- In vivo studies showed that
- NPs 40-100 nm Øare taken up more efficiently by DEC205+ DCs,
- MPs of 1 µm Ø by F4/80+ macrophages

- Charge effect:

- Positive particles are captured more efficiently by both macrophages and DCs