namur nanosafety centre

INTEGRATED PLATFORM

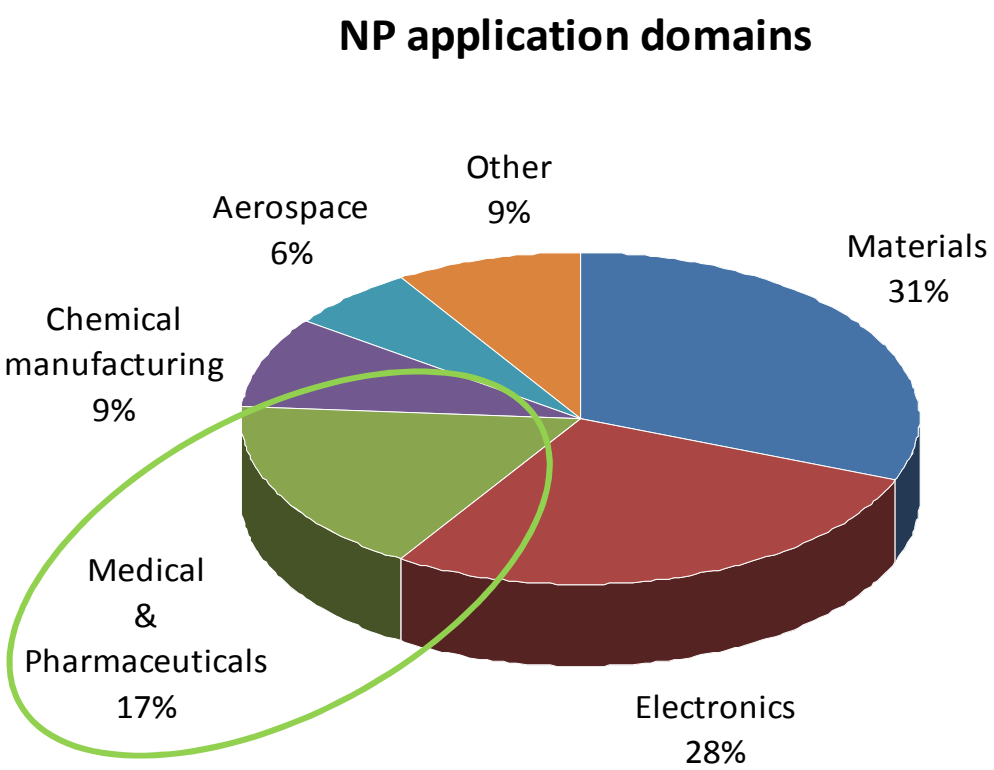
Guidelines proposal for nanomaterials hemocompatibility

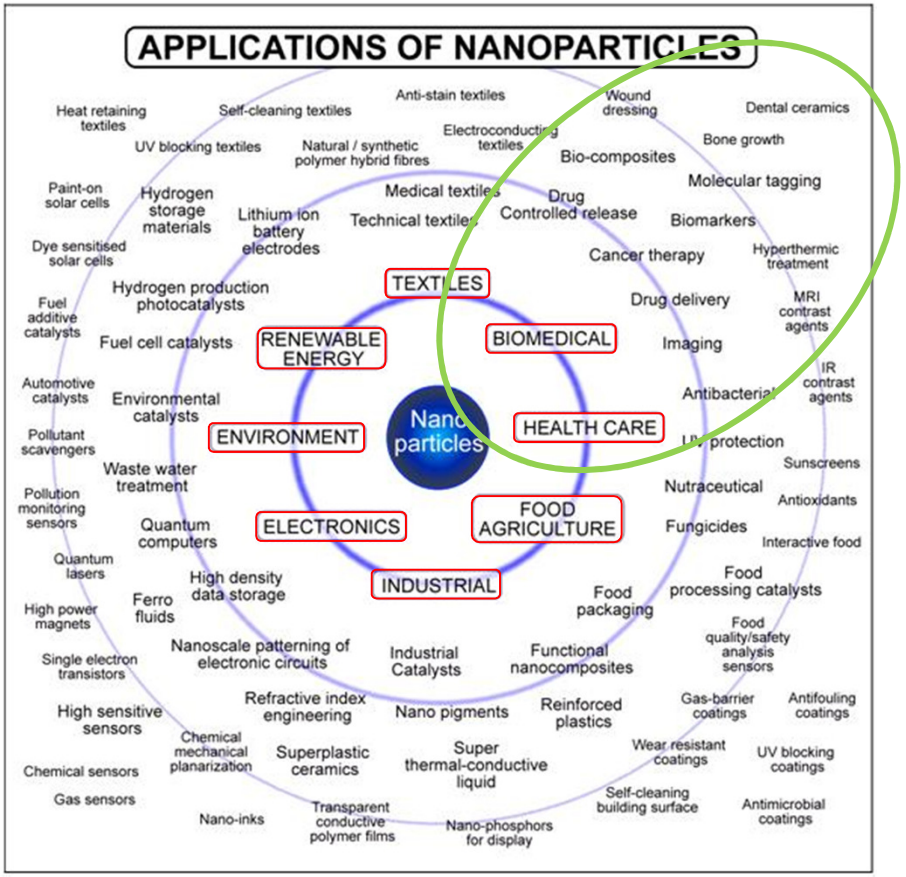
Julie Laloy

December 1st, 2014

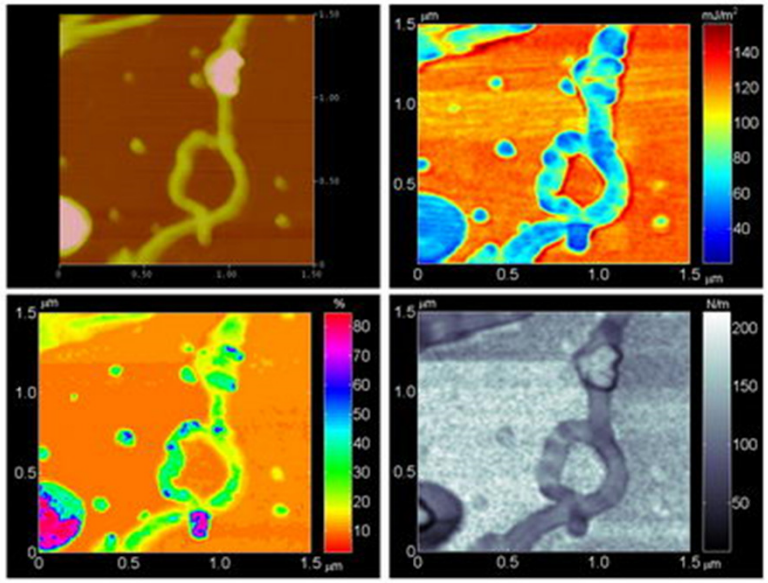
Nanotek & Expo 2014 – San Francisco

Why studying hemocompatibility?





Why studying hemocompatibility? Applications in nanomedicine

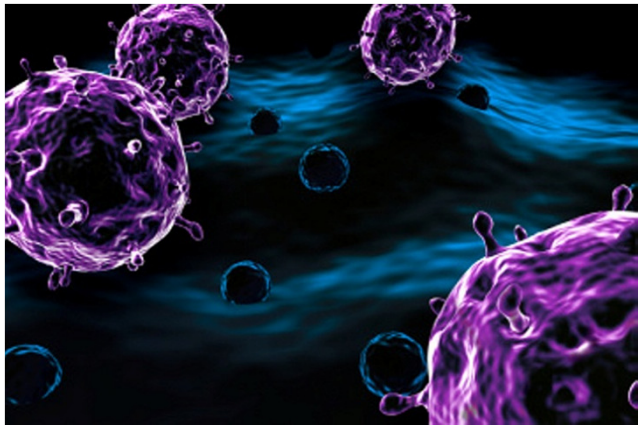


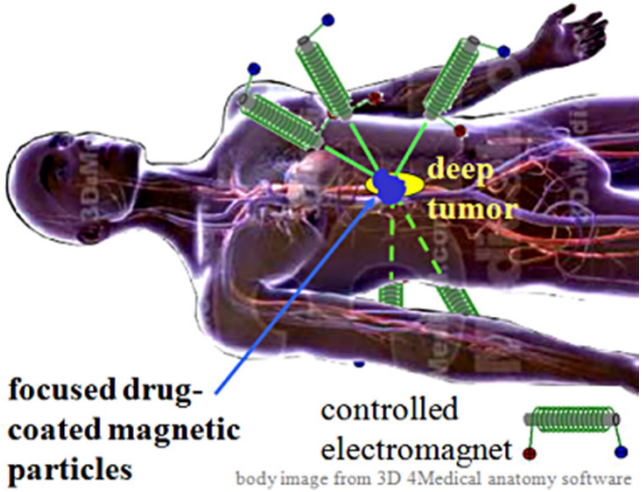
Imaging

Drug delivery

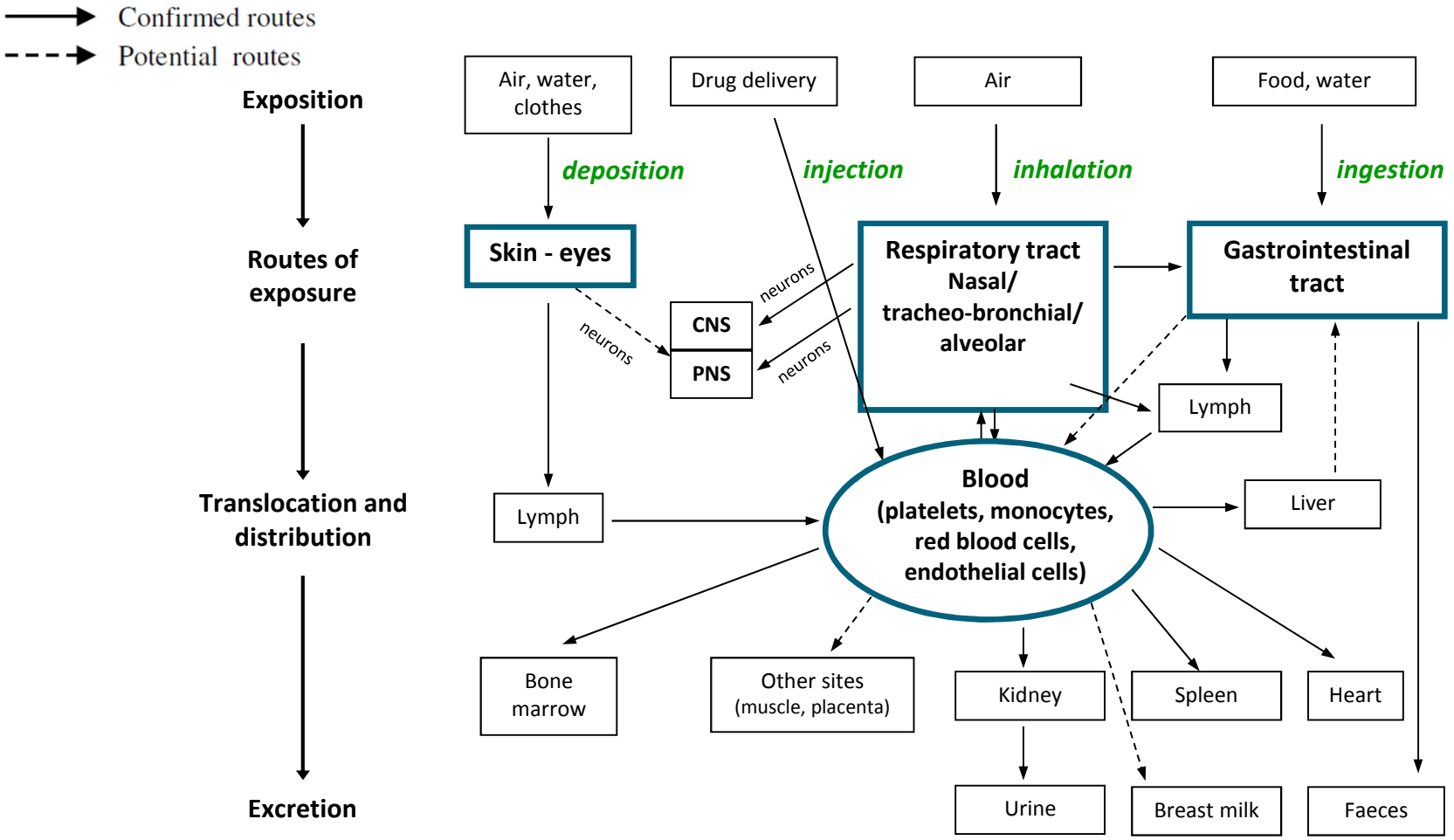
Targeting

Diagnostic agent





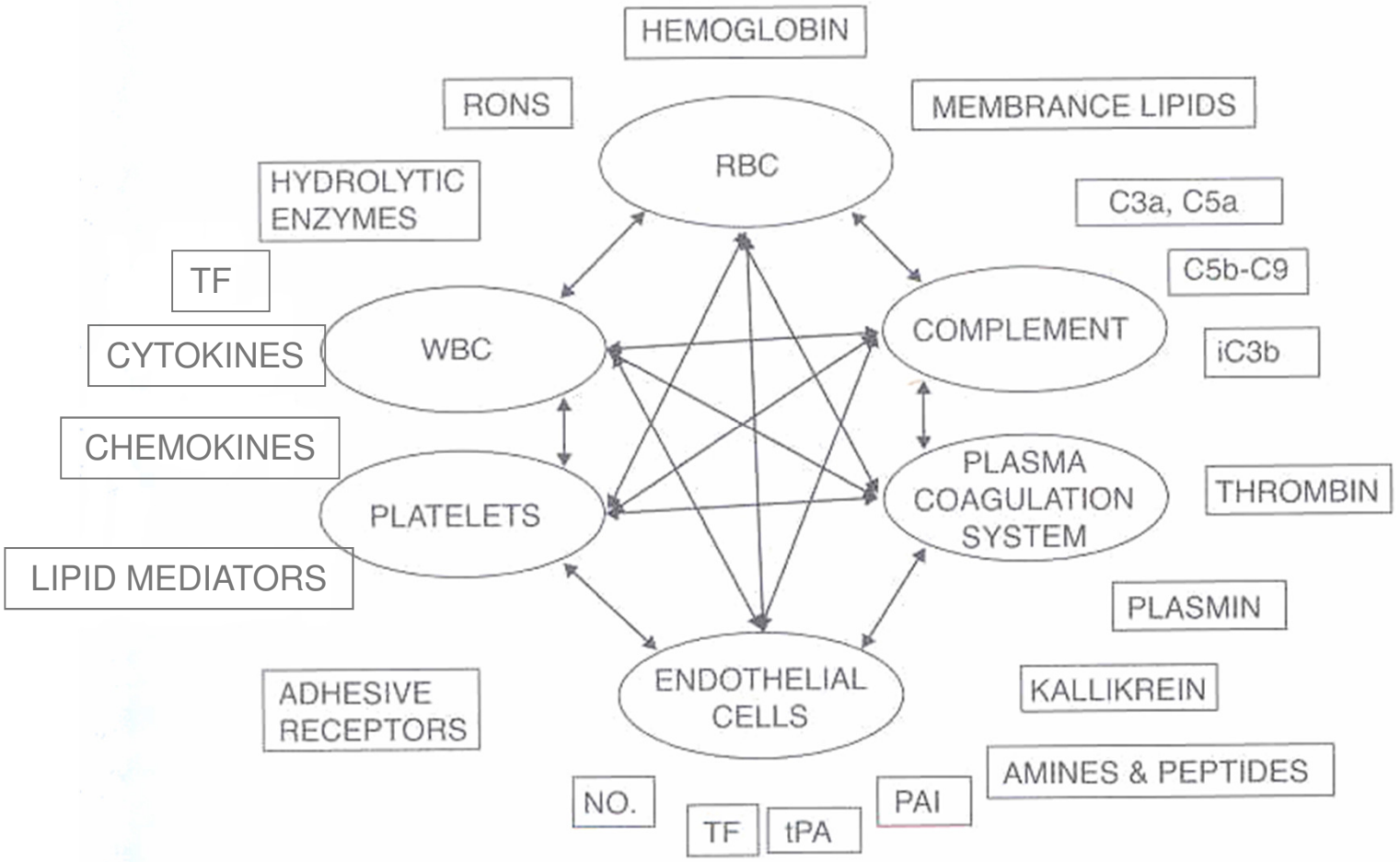
Why studying hemocompatibility?



No OECD or other guidelines available
Limited data from the literature

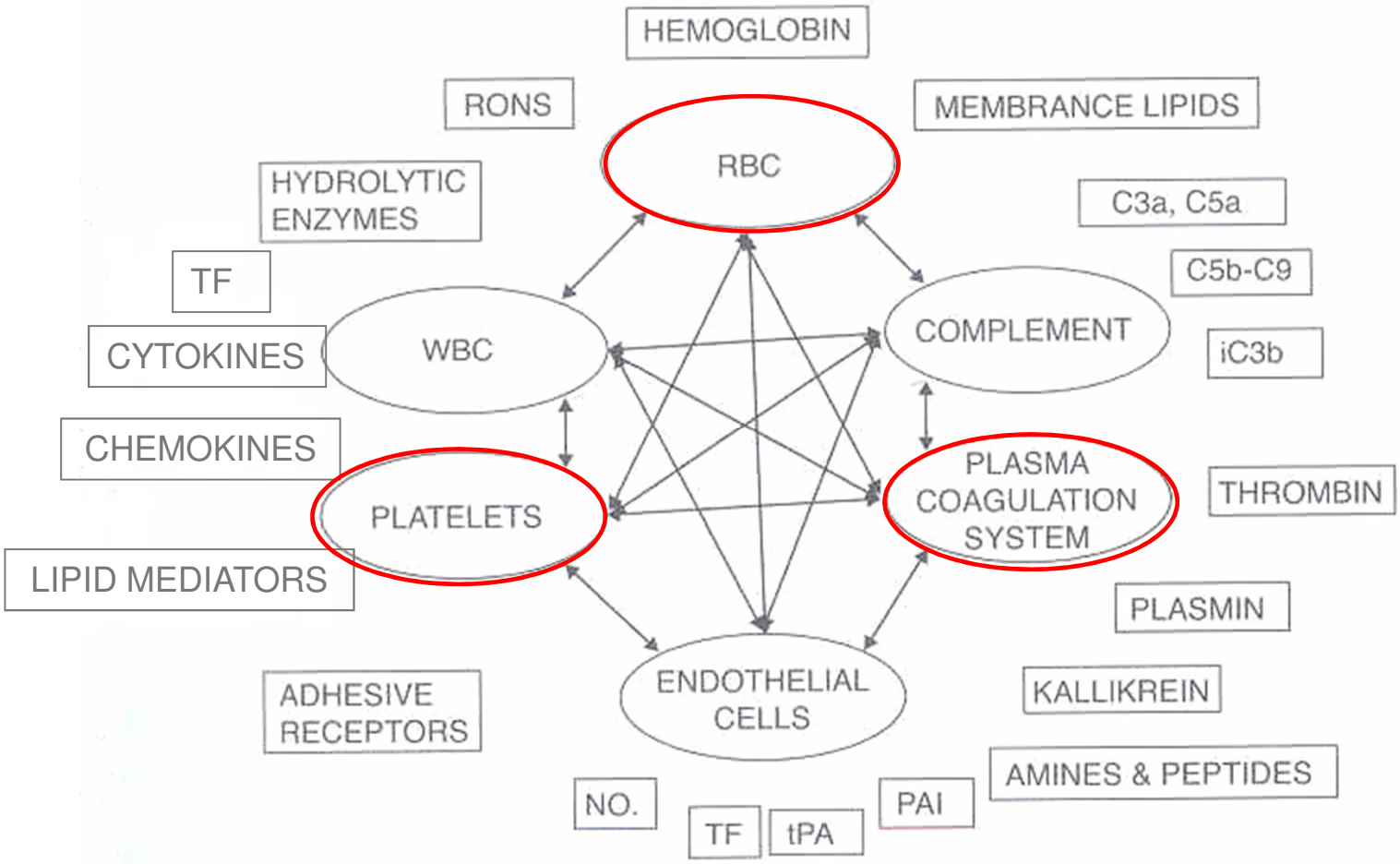
Oberdörster et al., **2005a** (*Review*)
Oberdörster et al., **2005b** (*Review*)
Nel et al., **2006**

Human hemocompatibility
NP impact on …



From « Nanotoxicity » SC Sahu and DA Casciano – Ed Wiley - 2009

Human hemocompatibility
NP impact on …



From « Nanotoxicity » SC Sahu and DA Casciano – Ed Wiley - 2009

Human hemocompatibility
NP impact on …

- Red blood cells hemolysis
- Haemostatic system
  - Primary haemostasis (Platelets aggregation)
    - Light transmission aggregometry (Turbidimetry)
    - Impedance aggregometry (Multiplate® analyzer)
    - Platelet function analysis (PFA-100®)
    - Shear stress (Impact-R®)
    - Electron microscopy (FEG-SEM, TEM)
  - Secondary haemostasis (Coagulation)
    - Chronometric assays (Clotting time assays)
    - Chromogenic assays (Thrombin generation tests)
  - Fibrinolysis
    - Rotem®
    - Euglobulin clot lysis time

Nanoparticles recommanded by OECD for toxicological assessment

Fullerenes (C_{60})	Aluminium oxide (Al_2O_3)
Single-walled carbon nanotubes (SWCNT)	**Titanium oxide (TiO_2)**
Multi-walled carbon nanotubes (MWCNT)	**Cerium oxide (CeO_2)**
Silver nanoparticles (AgNP)	**Zinc oxide (ZnO)**
Iron nanoparticles (FeNP)	**Silicon dioxide (SiO_2)**
Gold nanoparticles (AuNP)	Nanoclays
Dendrimers	

→ NPs selected due to their worldwide distribution and their numerous applications

OECD, **2010**

NPs identification and characterization

Identification & related information	- Name - Molecular structure - Morphology - Use - Catalytic activity - Production process
Physicochemical properties & material characterisation	- Chemical composition - Agglomeration/aggregation state - Solubility, density, porosity - Crystal structure - Particle size and its distribution - Specific surface area - Zeta potential - Catalytic activity - Redox potential - Free radical inducer

→ Importance of NP physicochemical characterization for inter-studies comparison and understanding of their related toxic effects

Example of Silver NPs

Highly bactericide, highest commercialised NPs

Consumer applications :
Anti-odor socks
Disinfectants, deodorants
Water purificants
Cosmetics
Food contact materials (refrigerator surfaces, storage bags…)

Biomedical applications :
Coated on cardiovascular and neurosurgical catheters, orthopedic and cardiovascular implants, surgical instruments
Wound and burn dressings
Bone substitute biomaterials
Perspectives for drug delivery systems and targeting

Example of Silver NPs

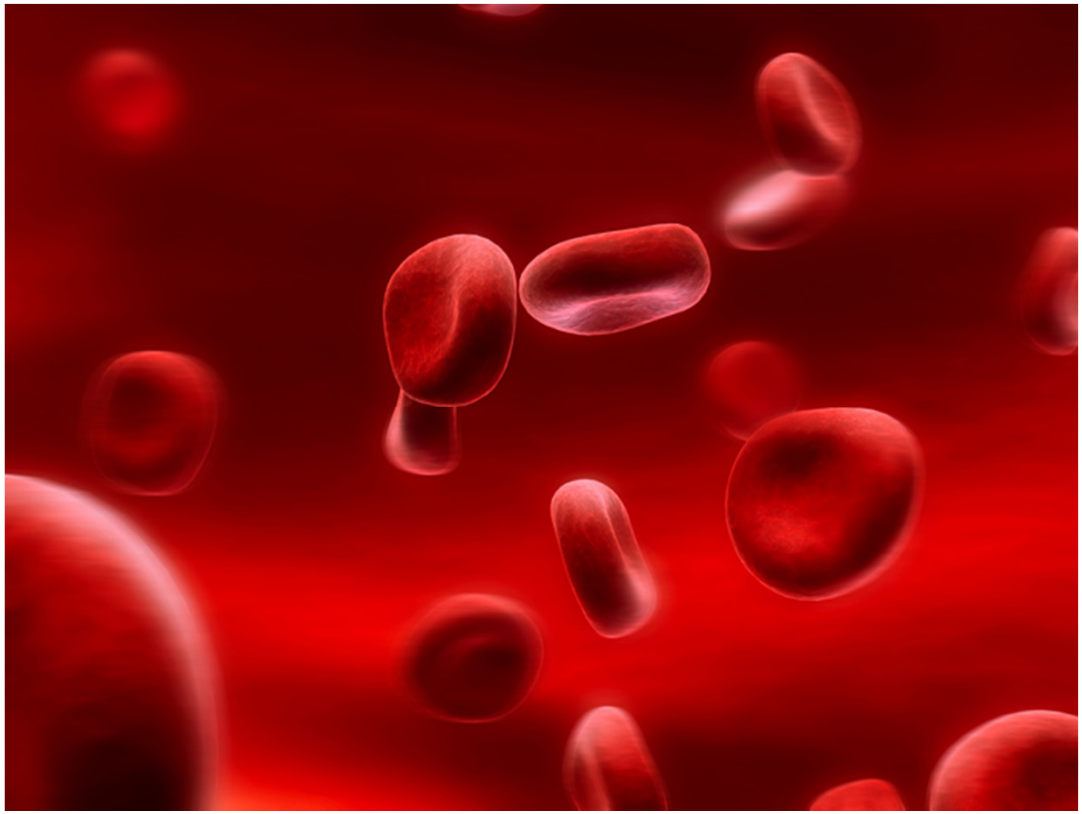
Since 19th century : use of « colloidal silver » to treat various diseases. These are silver nanoparticles.

Regular consumption of a large-particle silver compound is a likely causative agent of argyria

Are silver NPs hemocompatible?

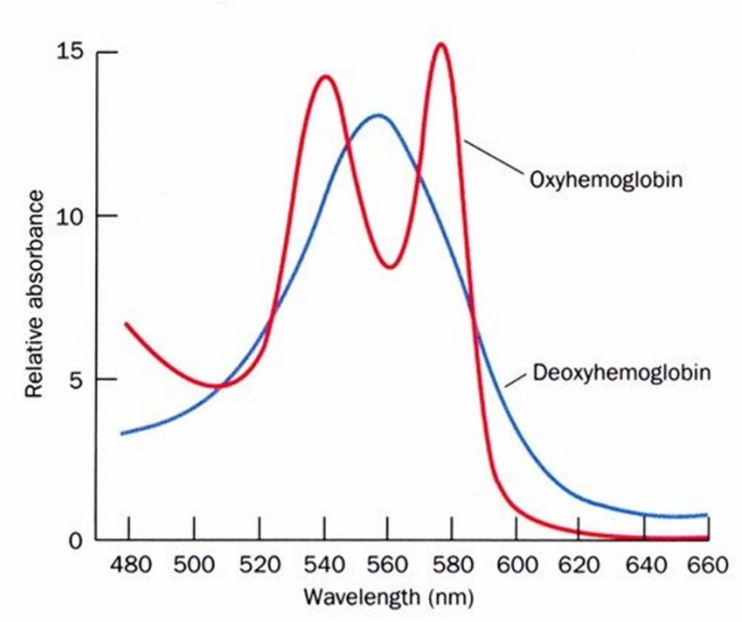
Hemolysis : definition

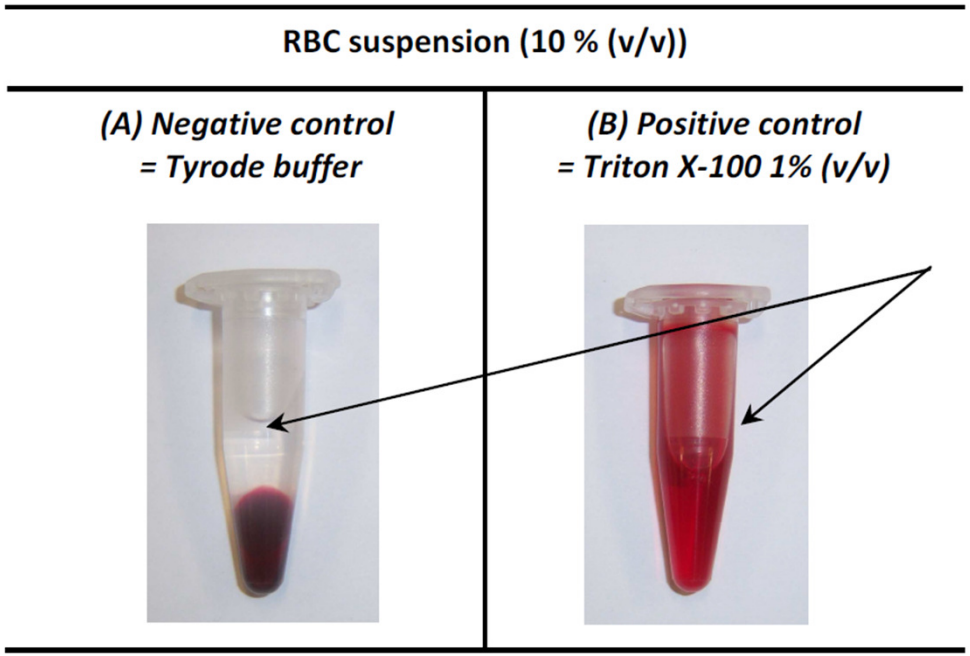
- Red blood cells hemolysis : breakdown of the membrane
- Anemia, hypoxia…
- Major factor to be studied



Hemolysis : test

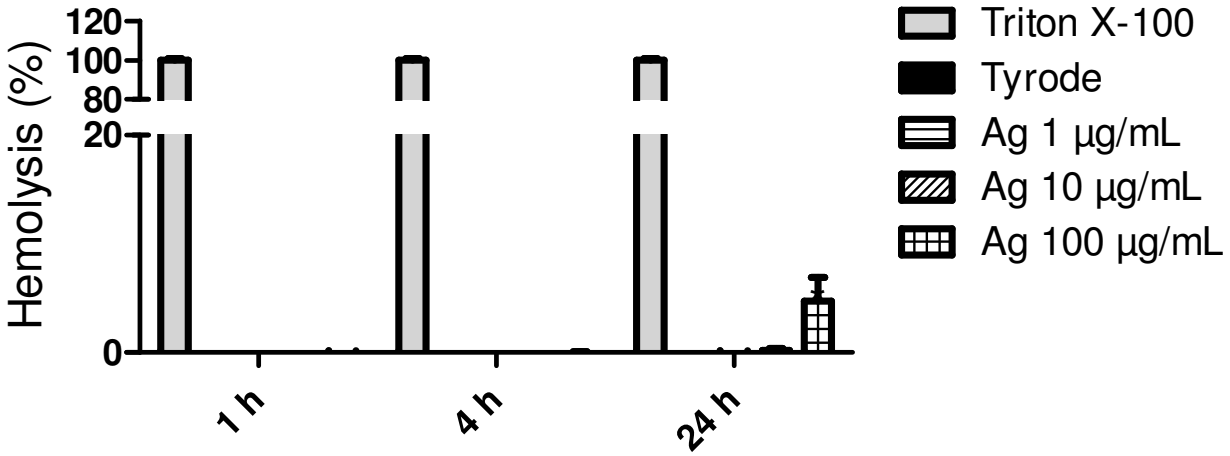
Red blood cells in contact with NPs, centrifugation, $OD_{550\ nm}$ measurement of the supernatant (hemoglobin)



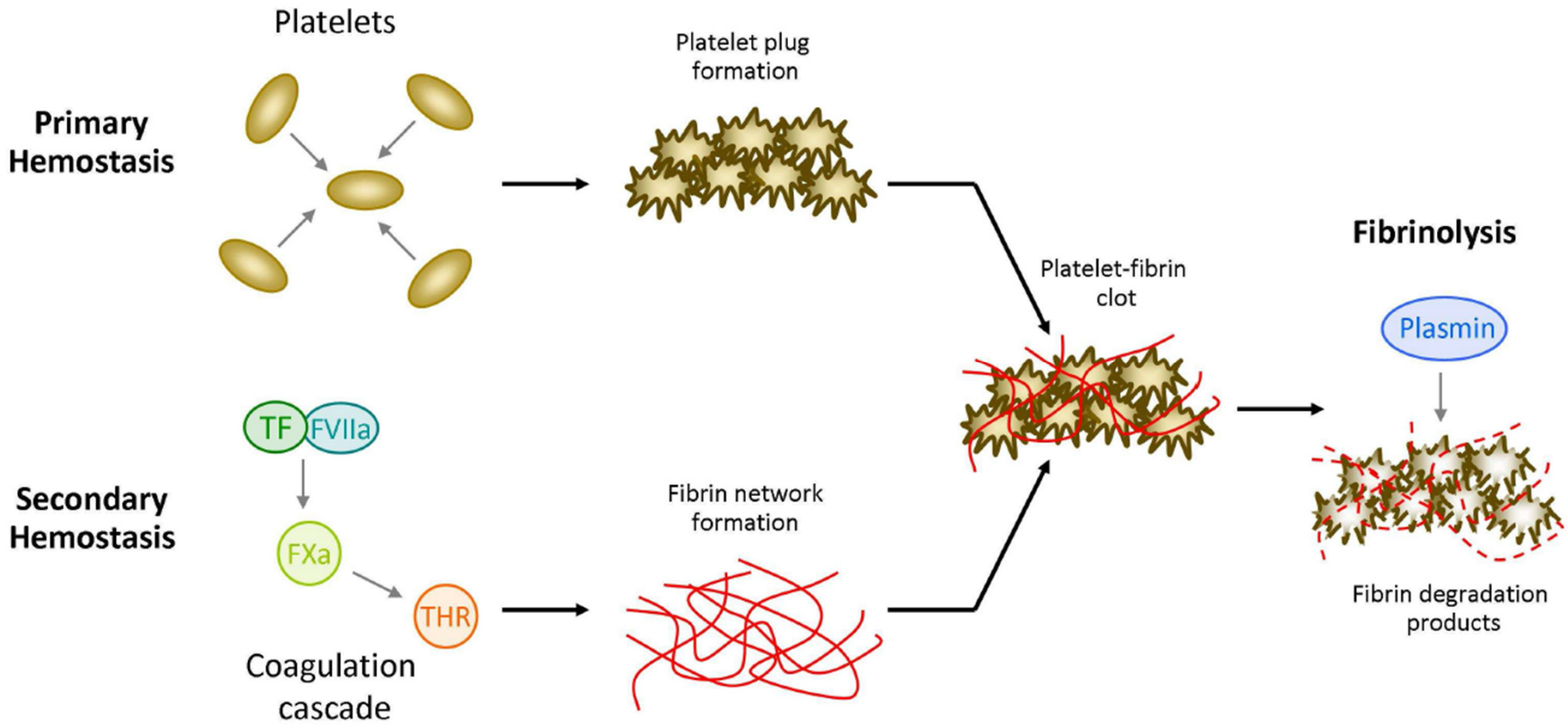


Hemolysis : test

- Mix RBC 10% (v/v) (190 µL) and NP (10 µL)
- Shaking @ rt for 1h
- Centrifugation 5 min @ 10,000 g
- $OD_{550\ nm}$ measurement of the supernatant

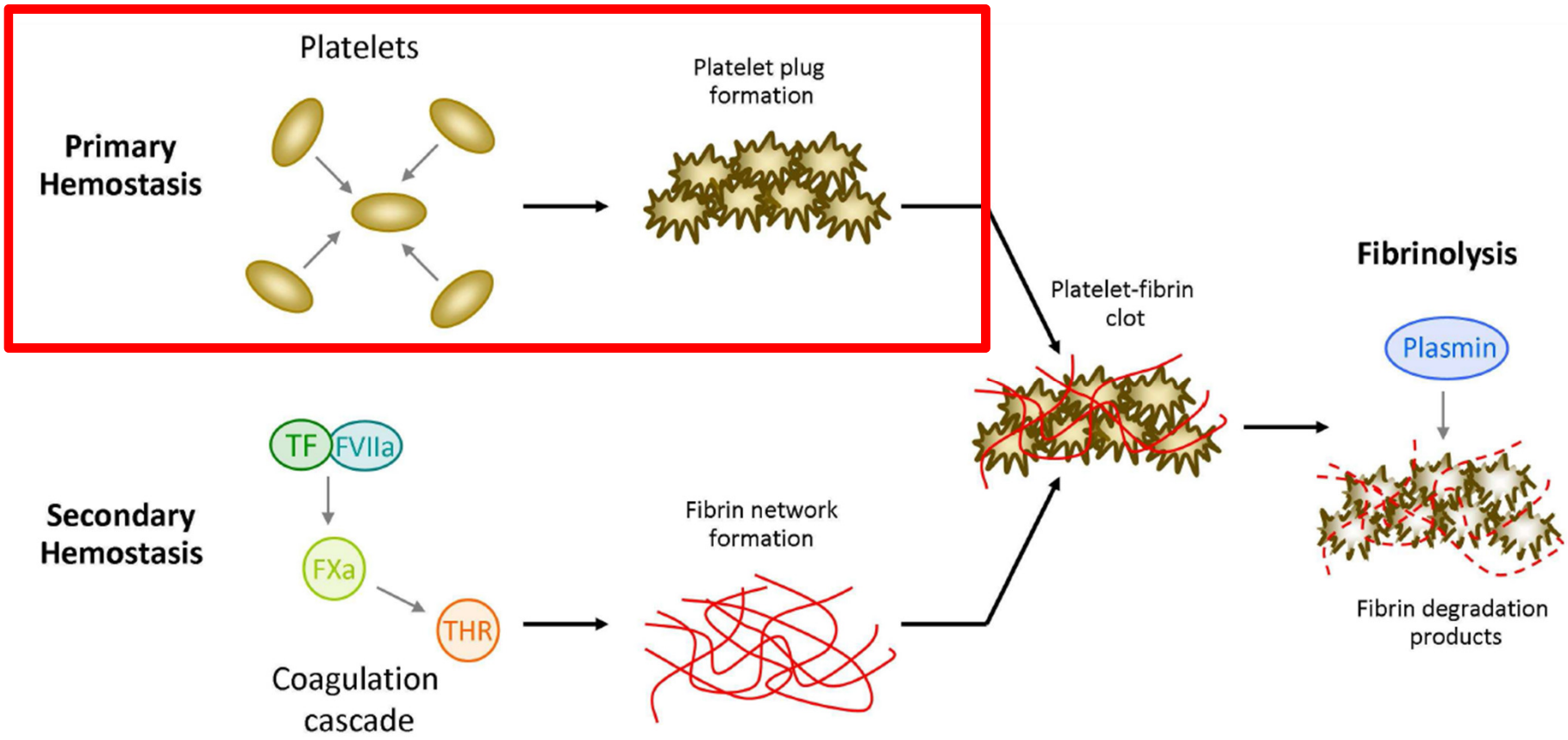


Haemostatic system : definition



Steps of haemostasis : Primary haemostasis - Secondary haemostasis - Fibrinolysis

Primary haemostasis : definition



Steps of haemostasis : Primary haemostasis - Secondary haemostasis - Fibrinolysis

Primary haemostasis : assays

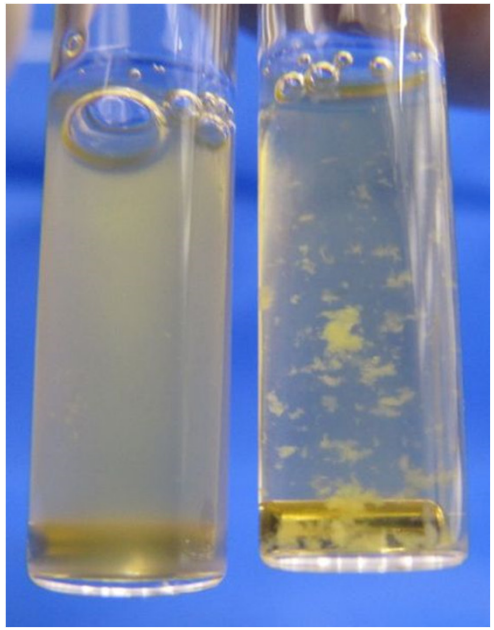
- Assessment of six functional tests to study the potential impact of NMs on platelet functions (adhesion/activation/aggregation)

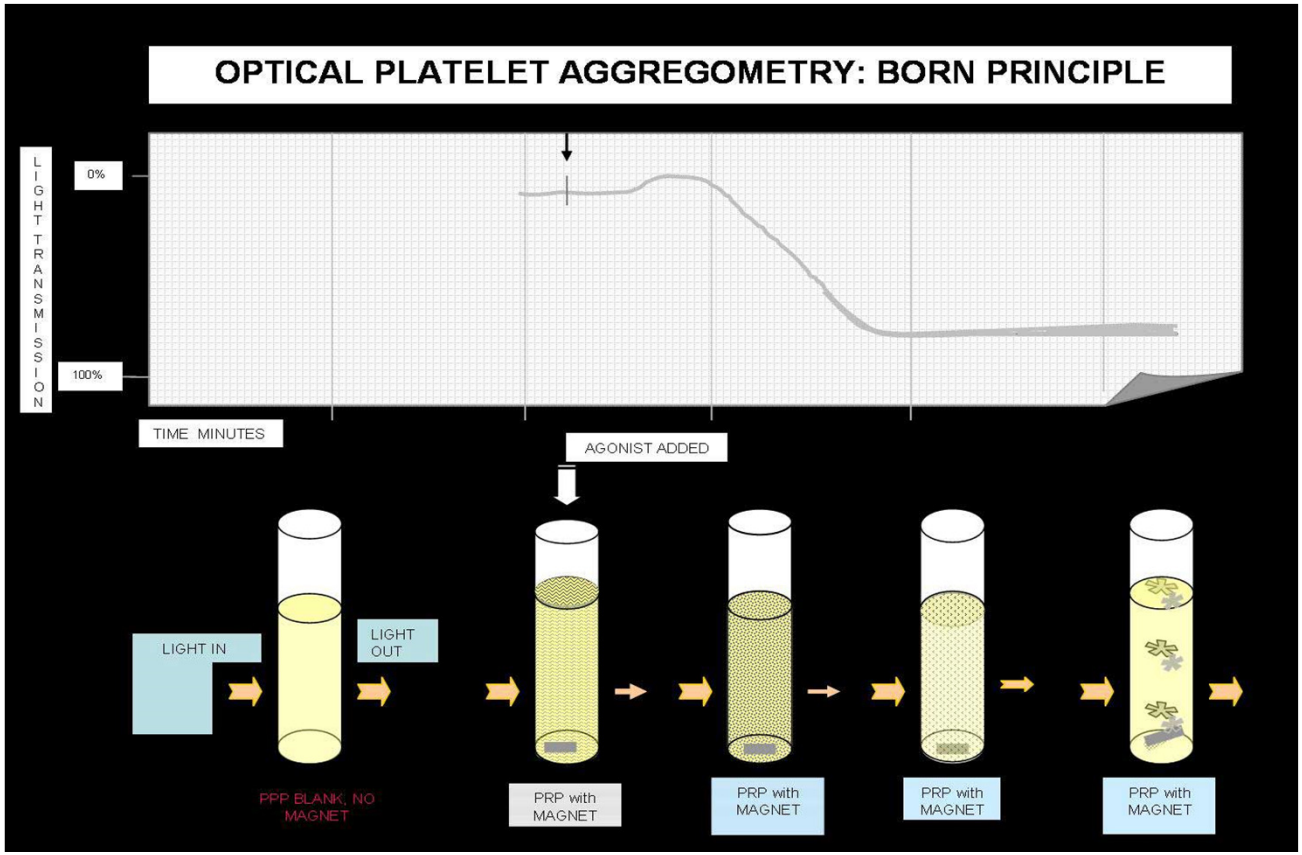
- Techniques known as « quantitatives » :
  - Light transmission aggregometry (LTA)
  - Impedance aggregometry (Multiplate®)
  - Platelet function analysis (PFA-100®)
  - Shear stress (Impact-R®)

- Techniques known as « qualitatives »:
  - Contrast microscopy,
  - Scanning (FEG-SEM) or transmission (TEM) electron microscopy

Light transmission aggregometry : principle

Turbidimetry measure : spectrophotometer (620 nm)





http://www.practical-haemostasis.com/

Light transmission aggregometry : protocol

Principle

Platelet-rich plasma (PRP) preparation (3.10^5 PLT.µL^{-1})

PRP stirred at 1,000 rpm (37°C)

+ NP suspension

+ inducer (ADP / collagen / AA)

+ NP suspension + inducer (ADP / collagen / AA)

$OD_{620\ nm}$ measurement

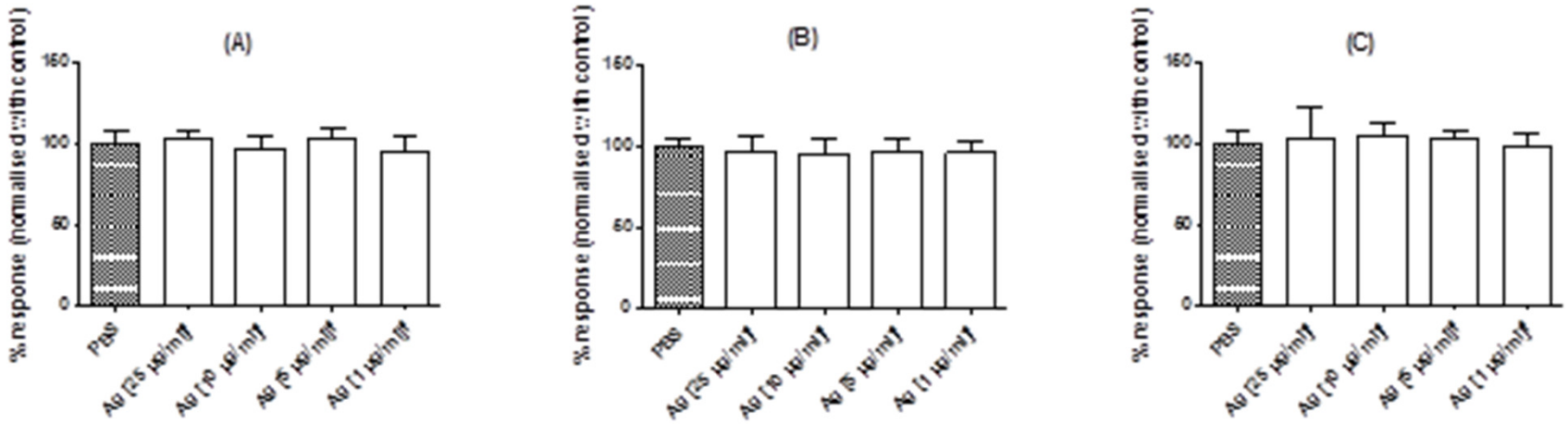
Spontaneous Aggregation
(Pro-aggregating activity)

Check platelet response

Induced Aggregation
(Anti-aggregating activity)

Primary hemostasis : LTA

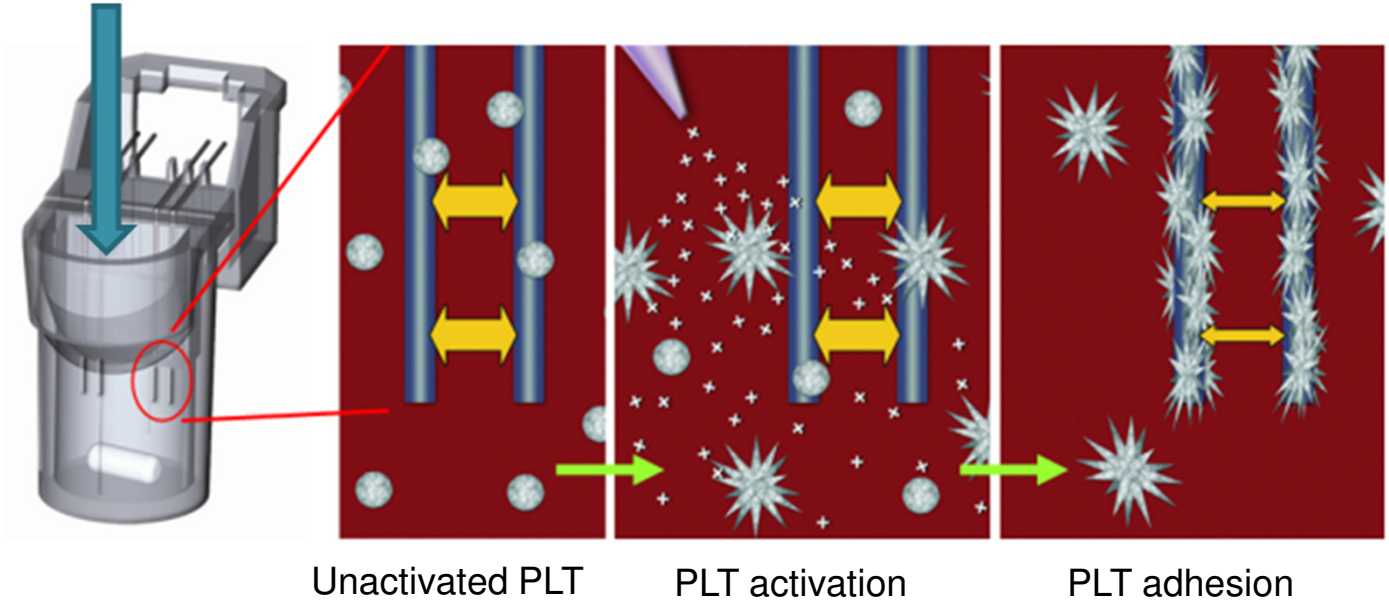
Light transmission aggregometry : no effect



Impedance aggregometry : Principle and interference

Whole blood impedance aggregometry

Electrode resistance enhanced by adhered platelet aggregates



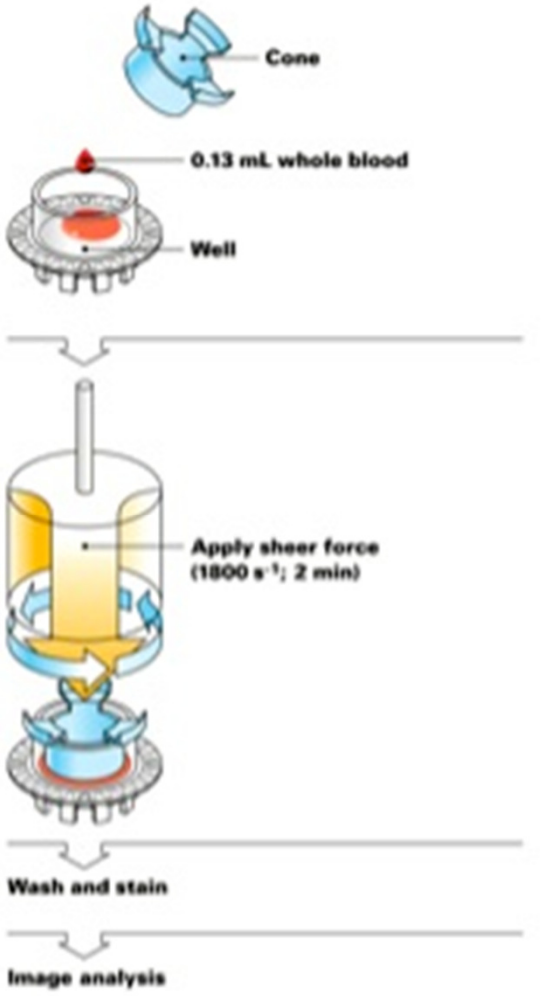
→ Tyrode buffer and NPs alone increase impedance

→ High variability

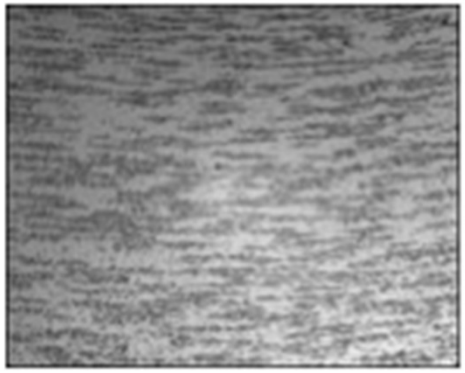
→ Adsorption of NPs on electrodes

Impact-R® : principle

Image analysis
Monitoring
Platelet
Adhesion
Cone & Plate
Technology



Platelet adhesion and aggregation

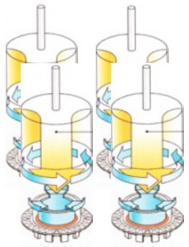


Impact-R® : protocol/ principle

180 µL of citrated whole blood

20 µL of
- NP
- collagen
- β_2-glycoprotein I

2 min
37°C



108,000 rpm
2 min

i) NaCl 0.9% washing

ii) May-Grünwald staining
iii) Drying

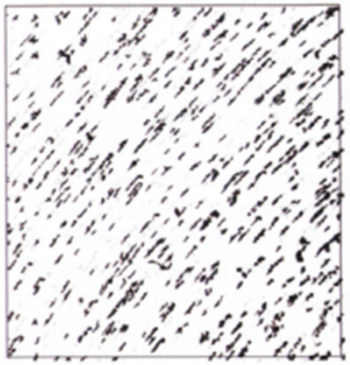
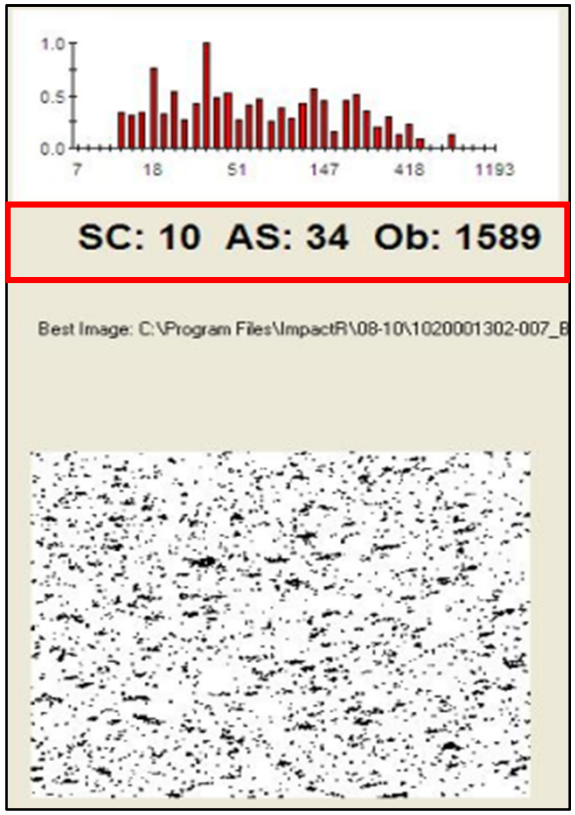
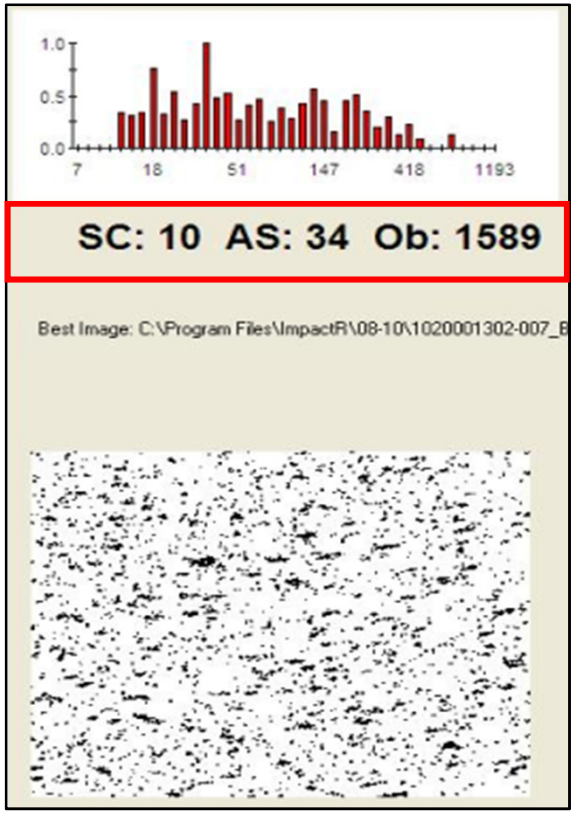


Image scanning
& analyzing (n = 7)



Impact-R® : principle



Results are expressed as :

SC (%) : surface covered
= platelet adhesion

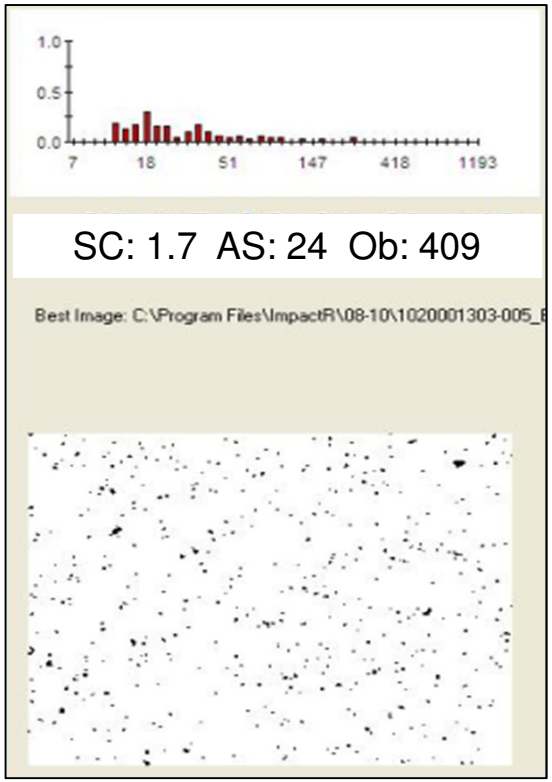
AS (μm^2) : average size of aggregates
= platelet aggregation

Ob (Ob) : number of objects
= platelet aggregation

Impact-R® : protocol

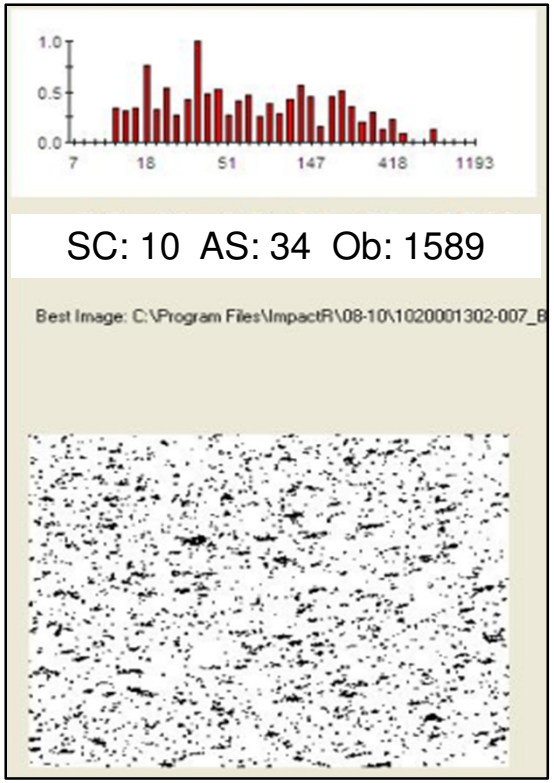
Validation of the technique using an inducer (collagen) and an inhibitor (β_2-**glycoprotein I) of platelet function and a negative control (tyrode buffer).**

+ β_2-glycoprotein I (2.2 µM)

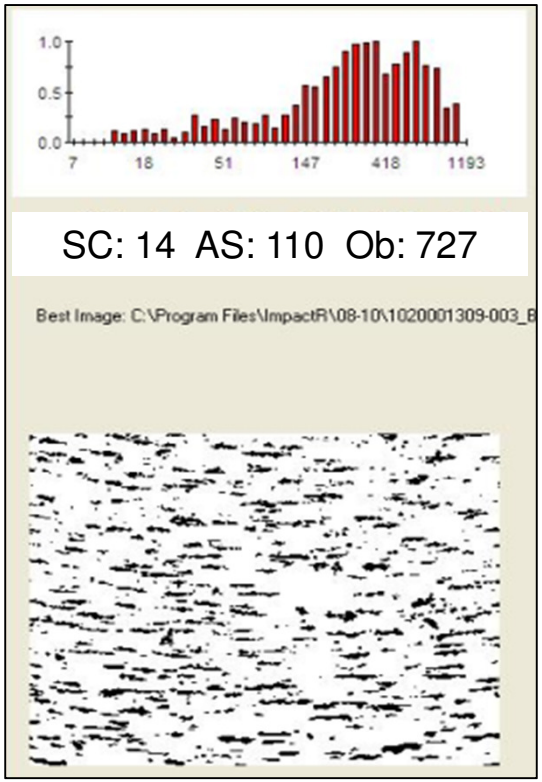


Anti-aggregating control

Citrated whole blood



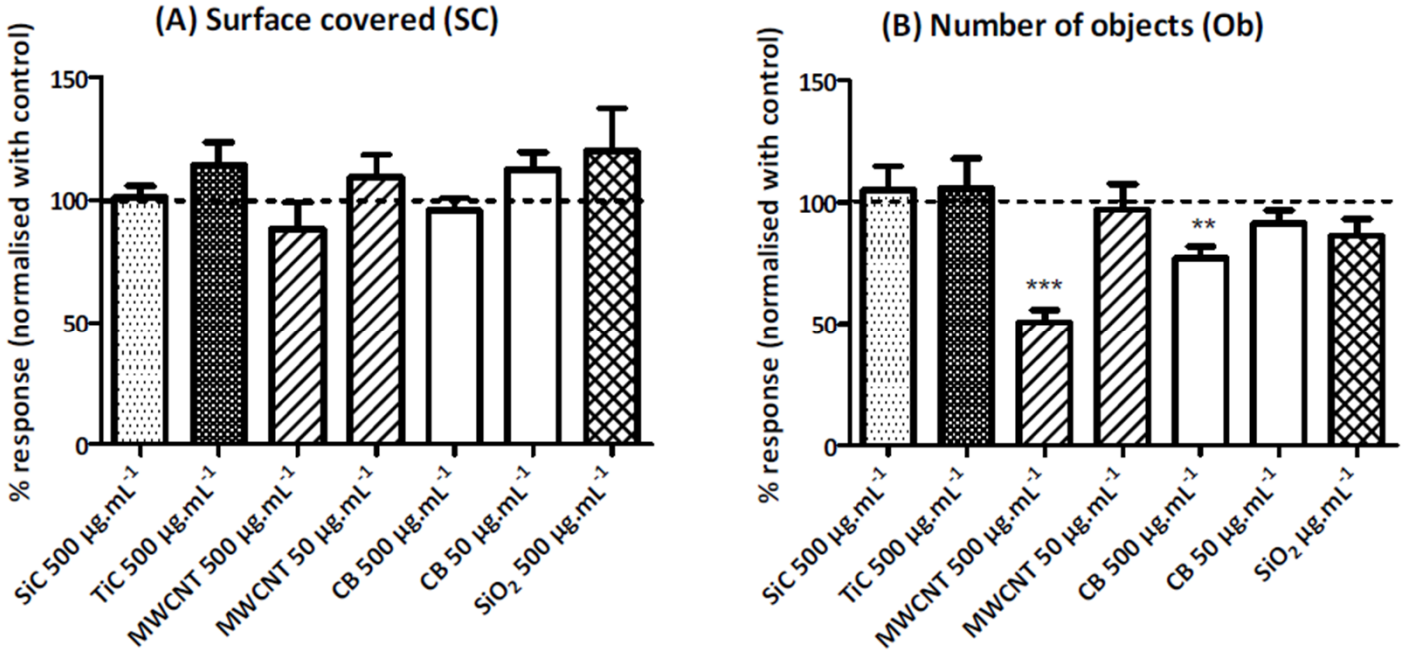
+ collagen (3.2 µg.mL^{-1})



Pro-aggregating control

Impact-R® : results

NP impact on platelet adhesion (SC) and the number of objects (Ob)



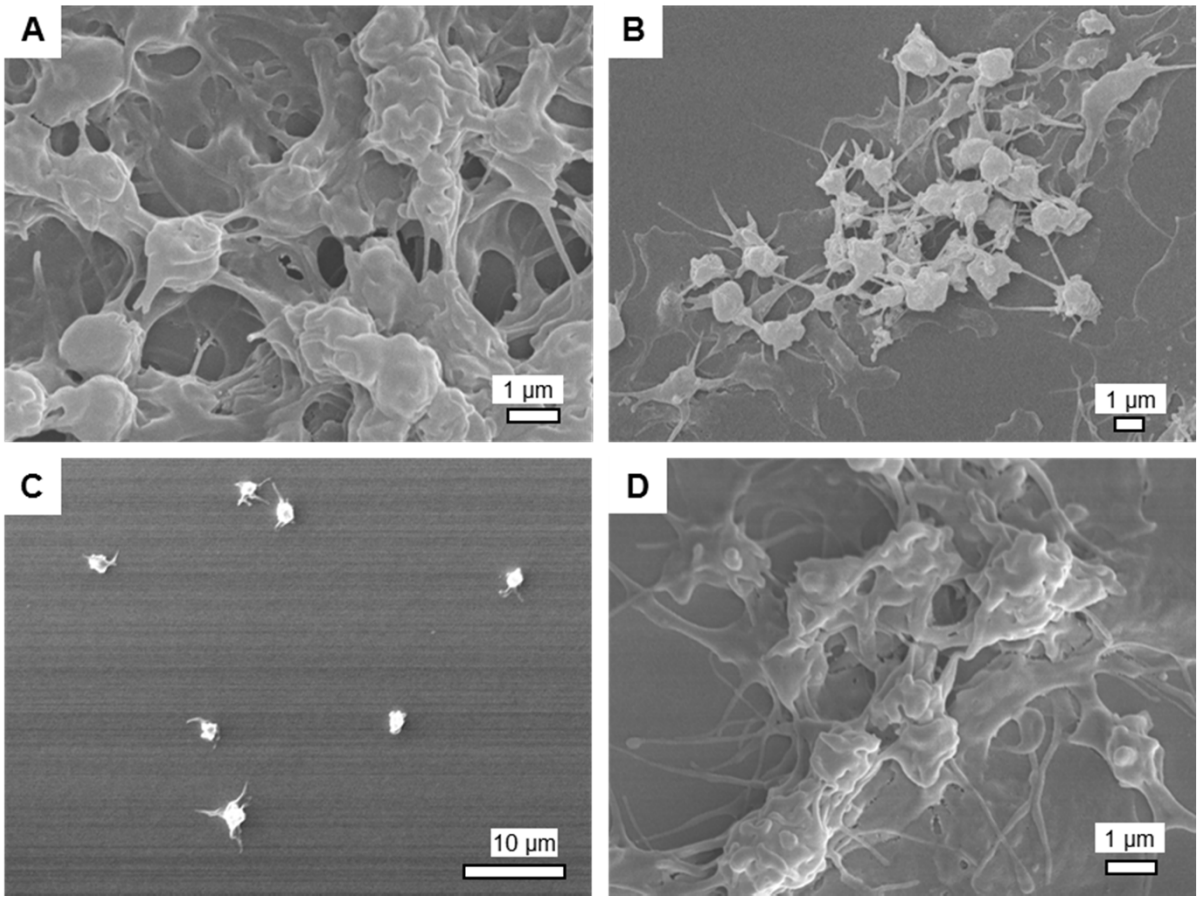
→ **MWCNT** and **CB** had an impact on **platelet aggregation** at 500 µg.mL^{-1} by reducing the number of aggregates without affecting the surface covered. This effect disappeared at 50 µg.mL^{-1}

→ SiC, TiC and SiO2 had no effect on Ob indicating that these 3 NPs had no impact on platelet function.

Scanning electron microscopy : validation

Whole blood alone

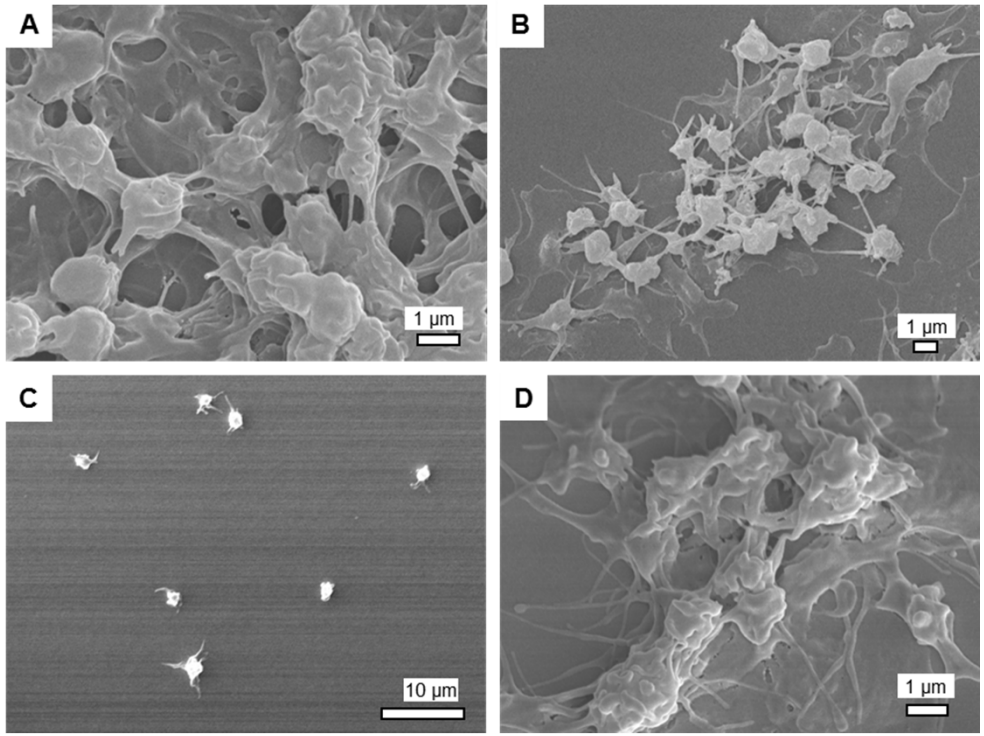
Whole blood with β2-glycoprotein I



Whole blood with tyrode buffer

Whole blood with Collagen

Scanning electron microscopy : validation



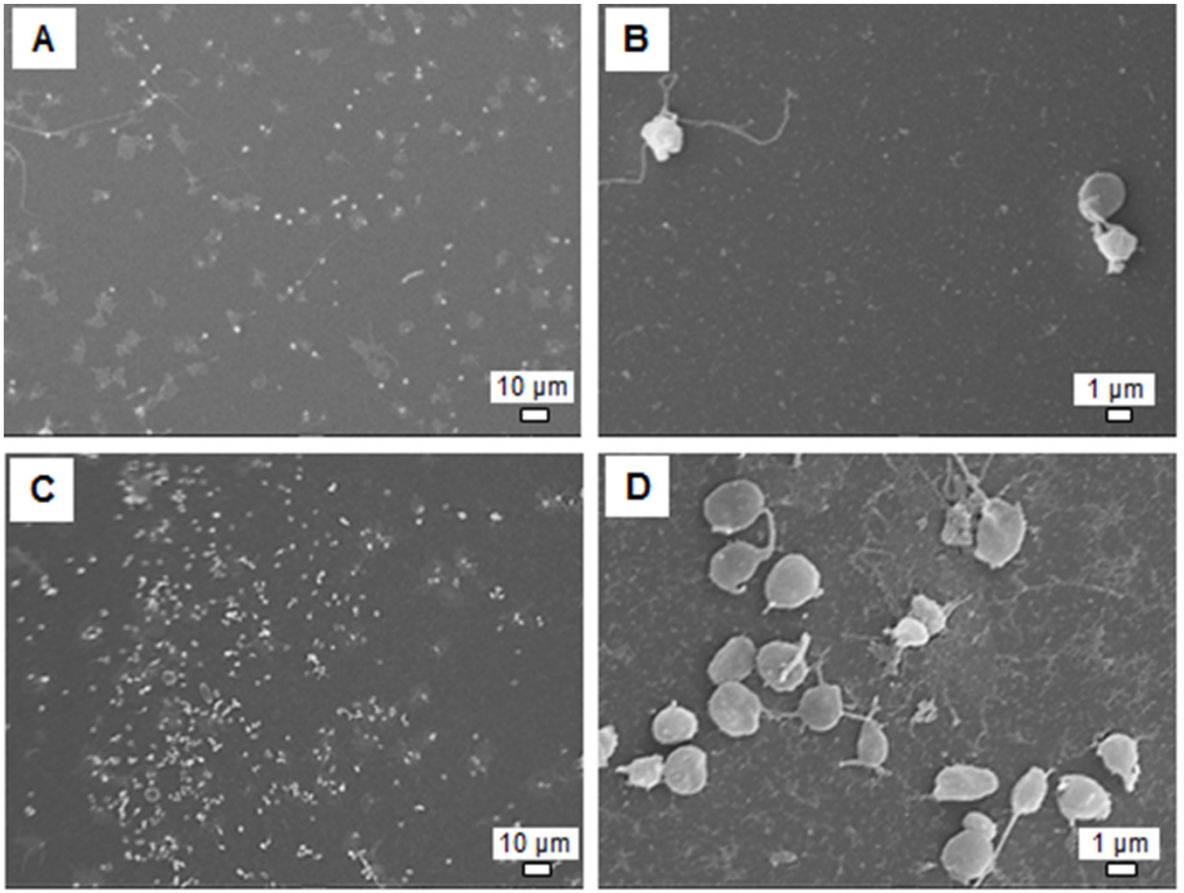
No clear difference can be observed between whole blood (A) and whole blood with the addition of collagen (D).

Tyrode buffer had no effect on platelet function (C).

A reduction of platelet adhesion was observed with β_2-glycoprotein I (B).

Primary hemostasis : LTA

SEM pictures of platelets adhesion without (A-B) or with Ag NPs at a final concentration of 50 µg/mL (C-D) : increased of adhesion



Conclusion of primary haemostasis

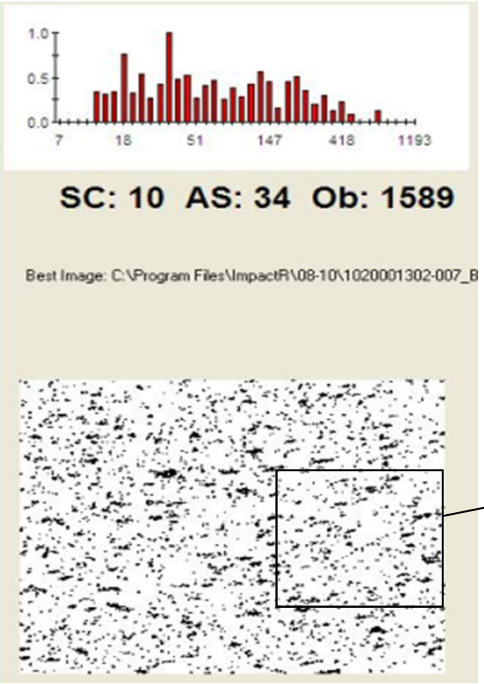
Nanotoxicology. 2013 Apr 15. [Epub ahead of print]

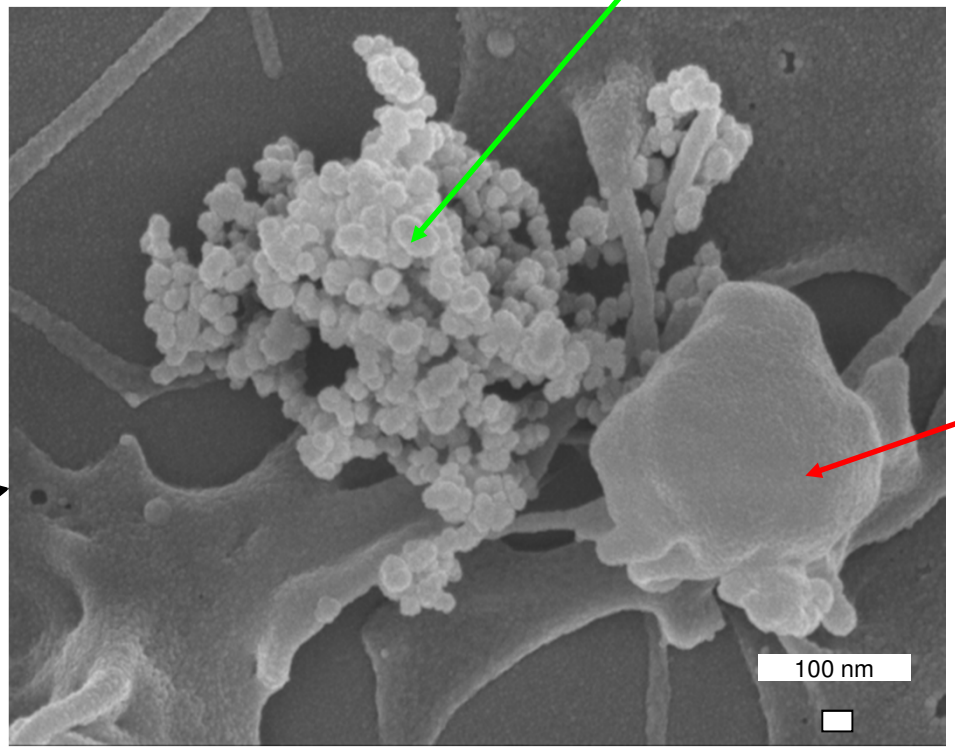
A comparison of six major platelet functional tests to assess the impact of carbon nanomaterials on platelet function: A practical guide.

Laloy J, Mullier F, Alpan L, Mejia J, Lucas S, Chatelain B, Toussaint O, Masereel B, Rolin S, Dogné JM.

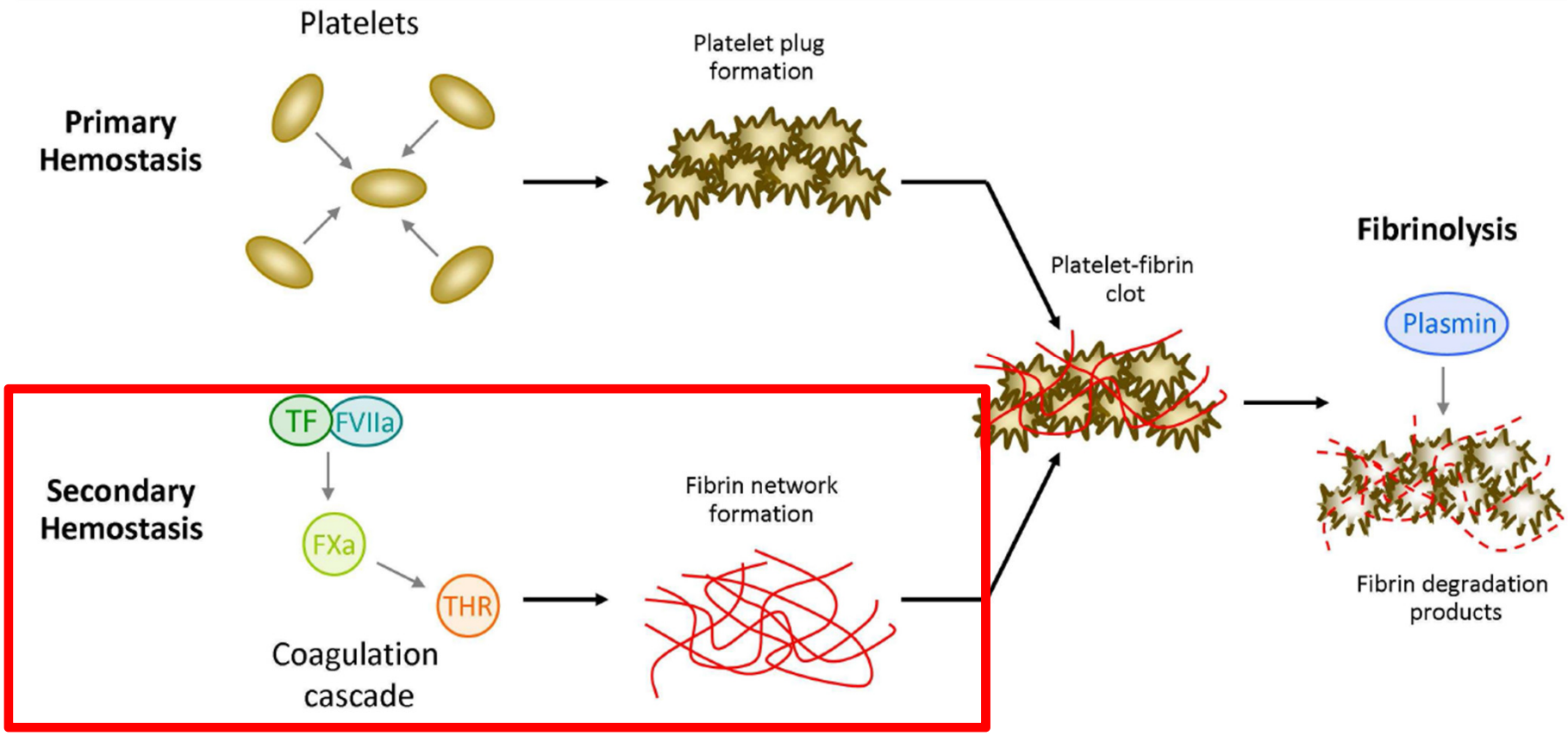
Department of Pharmacy, Namur Nanosafety Center (NNC), NAmur MEdicine & Drug Innovation Center (NAMEDIC), Namur Thrombosis and Hemostasis Center (NTHC), University of Namur (FUNDP) , Namur , Belgium.

The Impact-R® method used in combination with FEG-SEM can be recommended for the evaluation of NP impact on platelet function



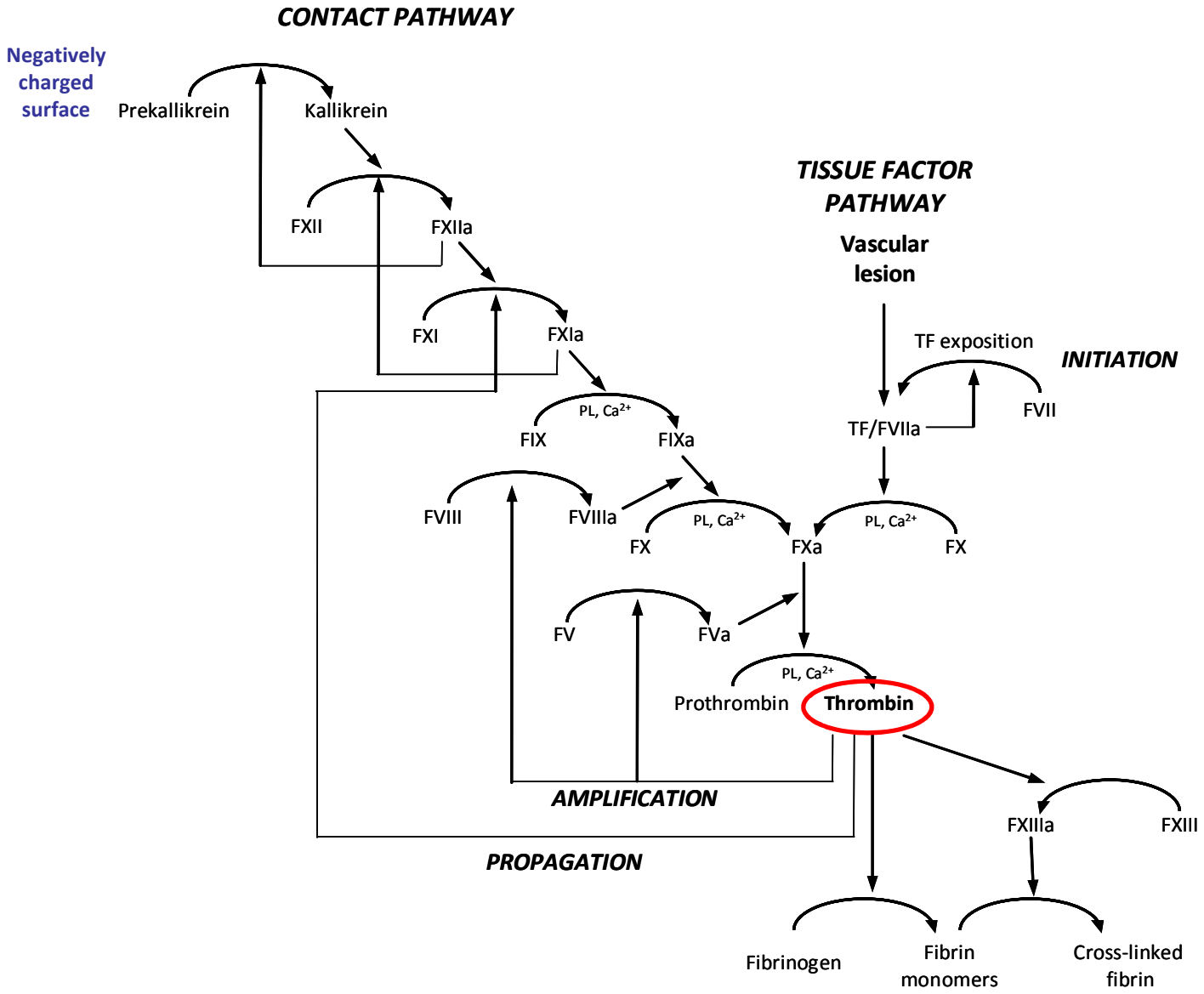


Haemostatic system : definition



Secondary haemostasis consists in the formation by the coagulation cascade of a fibrin network that stabilizes the platelet plug.

Coagulation cascade



Thrombin generation assays

- Thrombin-antithrombin (TAT) complexes
- Chromogenic thrombin generation assay

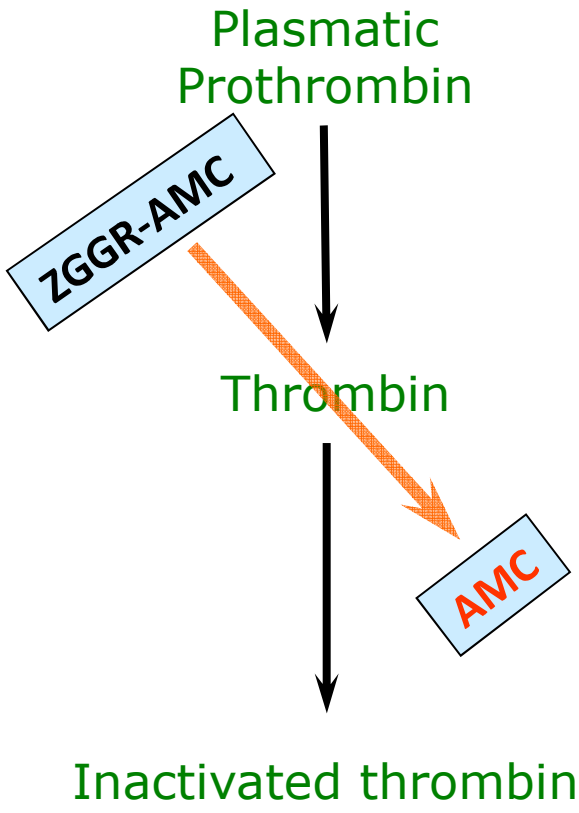
} Light absorbance detection

- Flurorometric calibrated thrombin generation test (cTGT)

} Assay selected

Thrombin generation assay : principle

Thrombin activity measurement - cTGT



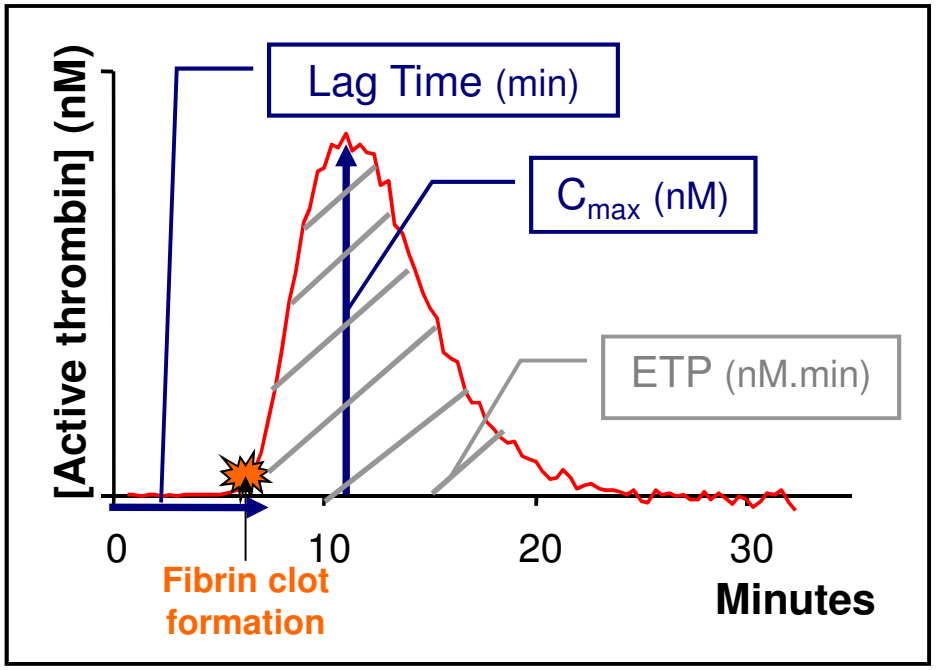
- A thrombin-specific fluorogenic substrate is added to clotting plasma
- Thrombin hydrolyzes AMC from substrate (*Z*-GGR-AMC)
- No linear relation between thrombin activity and fluorescence

  → thrombin calibration curve

Hemker et al., **2002**
Hemker et al., **2003**
Robert et al., **2009**

Secondary hemostasis - cTGT

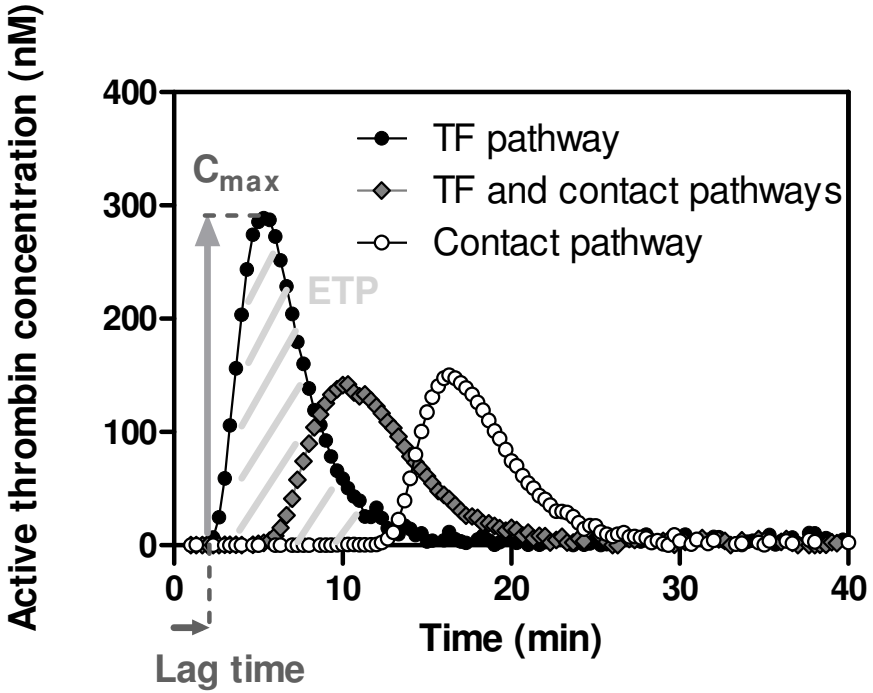
The acquired data are automatically processed by the Thrombinoscope® software to give thrombin generation curves and measurement parameters as lag time, C_{max}, ETP et T_{max}



C_{max} = maximal concentration;
Lag time = time for fibrin clot formation;
ETP = endogenous thrombin potential

Hemker et al., 2002
Laloy et al., 2012

Thrombin generation assay : control parameters

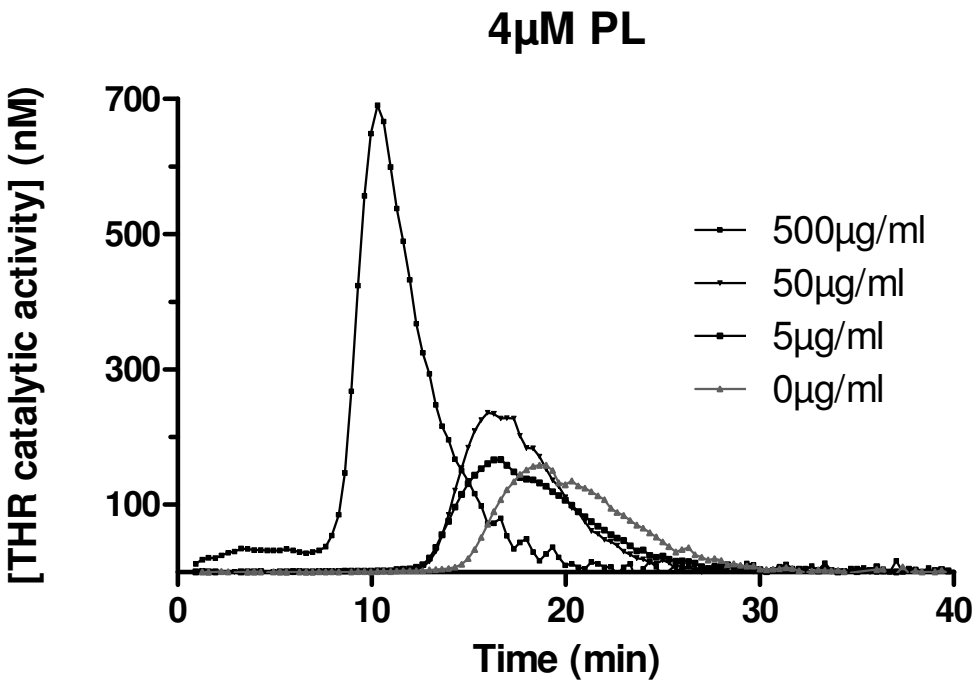


	Lag time (min)	C_{max} (nM)	ETP (nM.min)
TF pathway	2.5	335	1650
TF and contact pathways	6.4	156	1262
Contact pathway	13.7	164	1146

C_{max} = maximal concentration;
Lag time = time for fibrin clot formation;
ETP = endogenous thrombin potential

Secondary hemostasis - cTGT

Procoagulant activity



Laloy et al., 2014
Laloy et al., 2012

Conclusion of secondary haemostasis

Nanotoxicology. 2012 Mar;6(2):213-32. doi: 10.3109/17435390.2011.569096. Epub 2011 Apr 13.

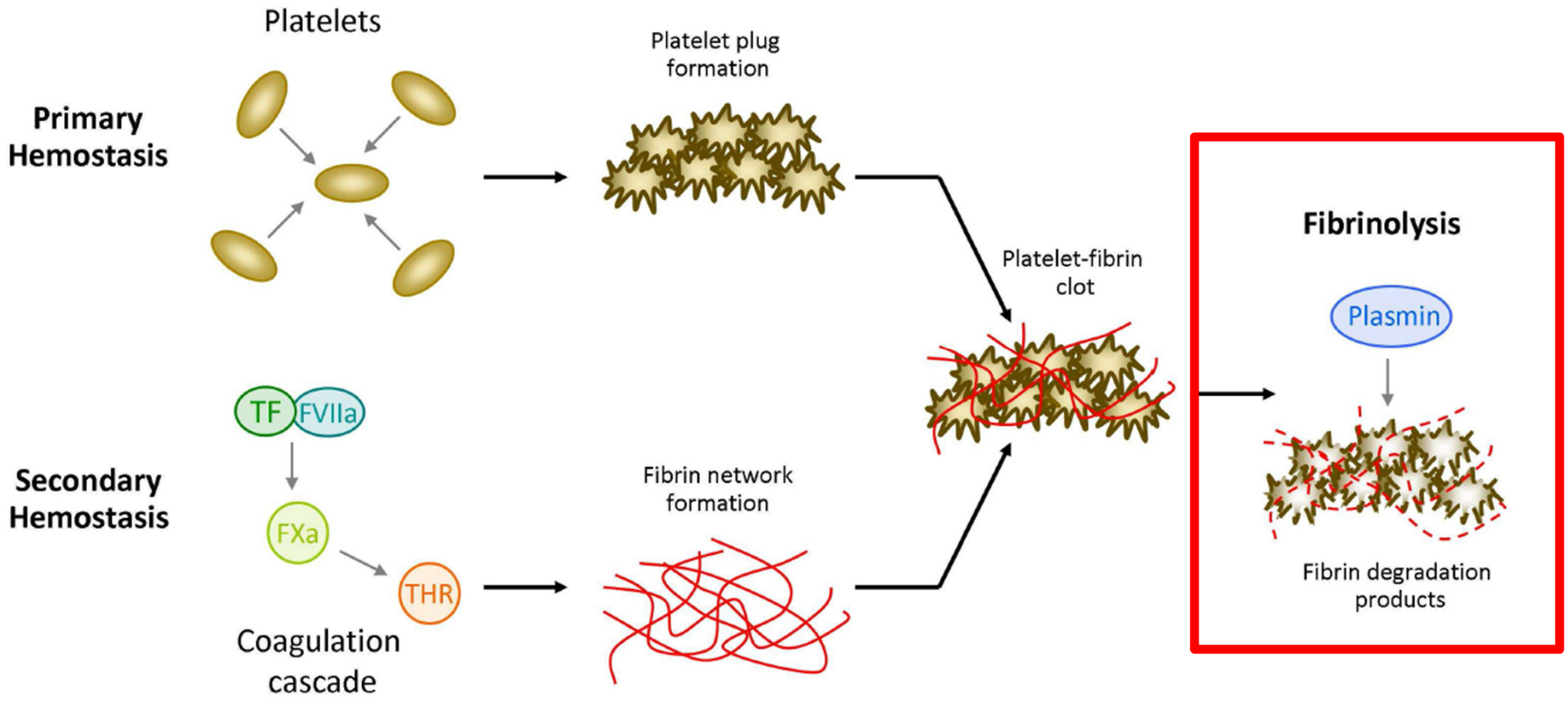
Validation of the calibrated thrombin generation test (cTGT) as the reference assay to evaluate the procoagulant activity of nanomaterials.

Laloy J, Robert S, Marbehant C, Mullier F, Mejia J, Piret JP, Lucas S, Chatelain B, Dogné JM, Toussaint O, Masereel B, Rolin S.

Department of Pharmacy, Drug Design & Discovery Center (D3C), Namur Research Institute for Life Sciences (NARILIS), Namur Thrombosis and Hemostasis Center (NTHC), FUNDP-University of Namur, Namur, Belgium. julie.laloy@fundp.ac.be

"Thus, the cTGT appears as a reference assay to investigate the nanoparticle (NP) procoagulant activity in human plasma."

Fibrinolysis : definition



Fibrinolysis is the process by which fibrin is removed from damaged blood vessels.

Fibrinolysis : assays

Overall assays

- Euglobulin clot lysis time (Von Kaulla)
- Rotem®

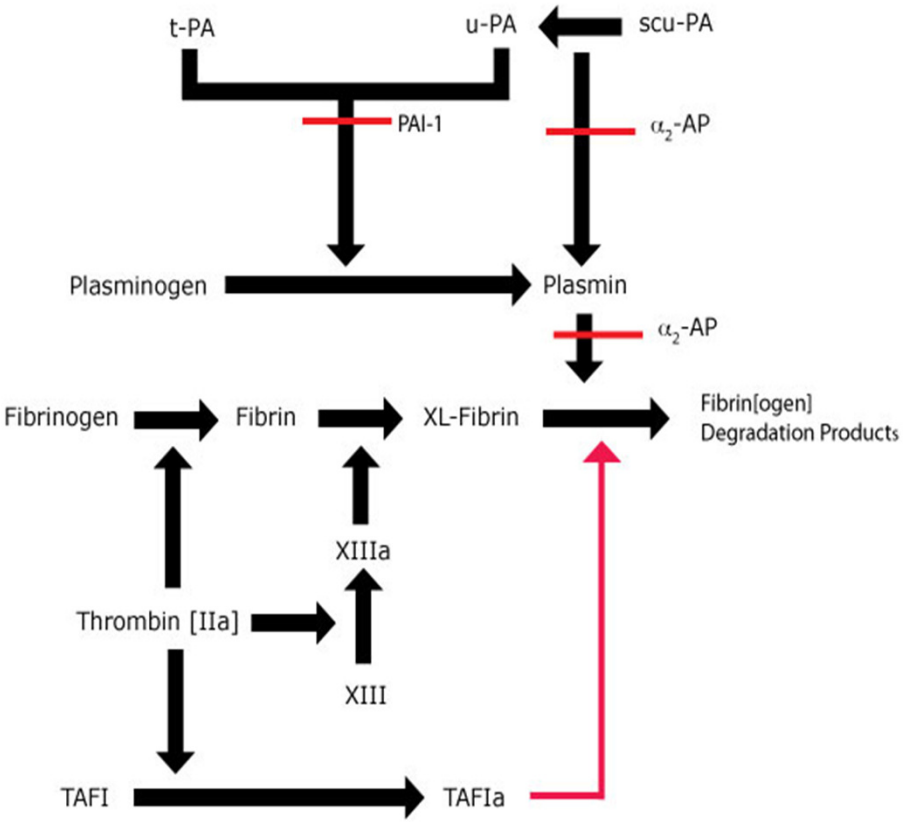
Specific assays

- D-Dimer Assay
- Plasminogen Assay
- Alpha-2-Antiplasmin (Alpha-2-Plasmin Inhibitor) Assay
- Plasminogen/antiplasmin Assay
- T-PA Assay
- PAI-1 Assay
- TAFI Assay

Specific assays could not be used *in vitro*.

Euglobulin clot lysis time

Principle : Acidification and dilution of plasma causes precipitation of euglobulins (fibrinogen, activator of plasminogen, plasminogen) → quicker evalution of fibrinolysis.

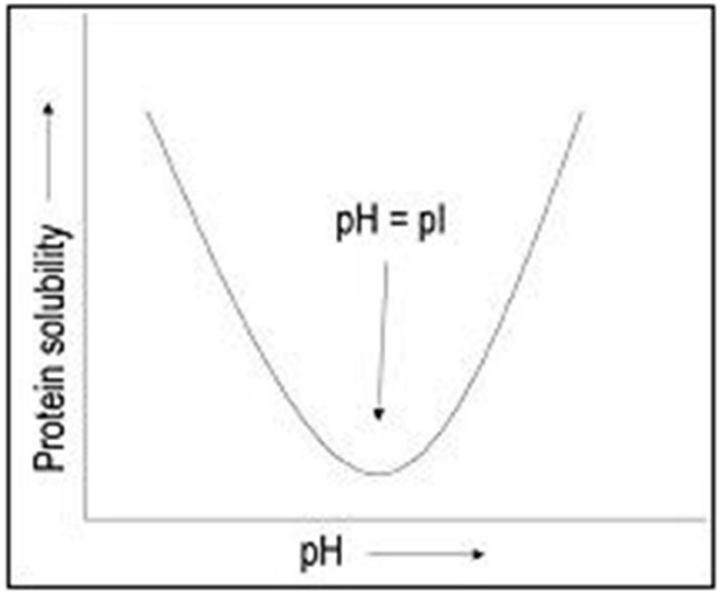


Key: t-PA - tissue plasminoge activator; u-PA - urinary plasminogen activator; XL-Fibrin - cross-linked fibrin; TAFI - Thrombin Activatable Fibrinolytic Inhibitor.

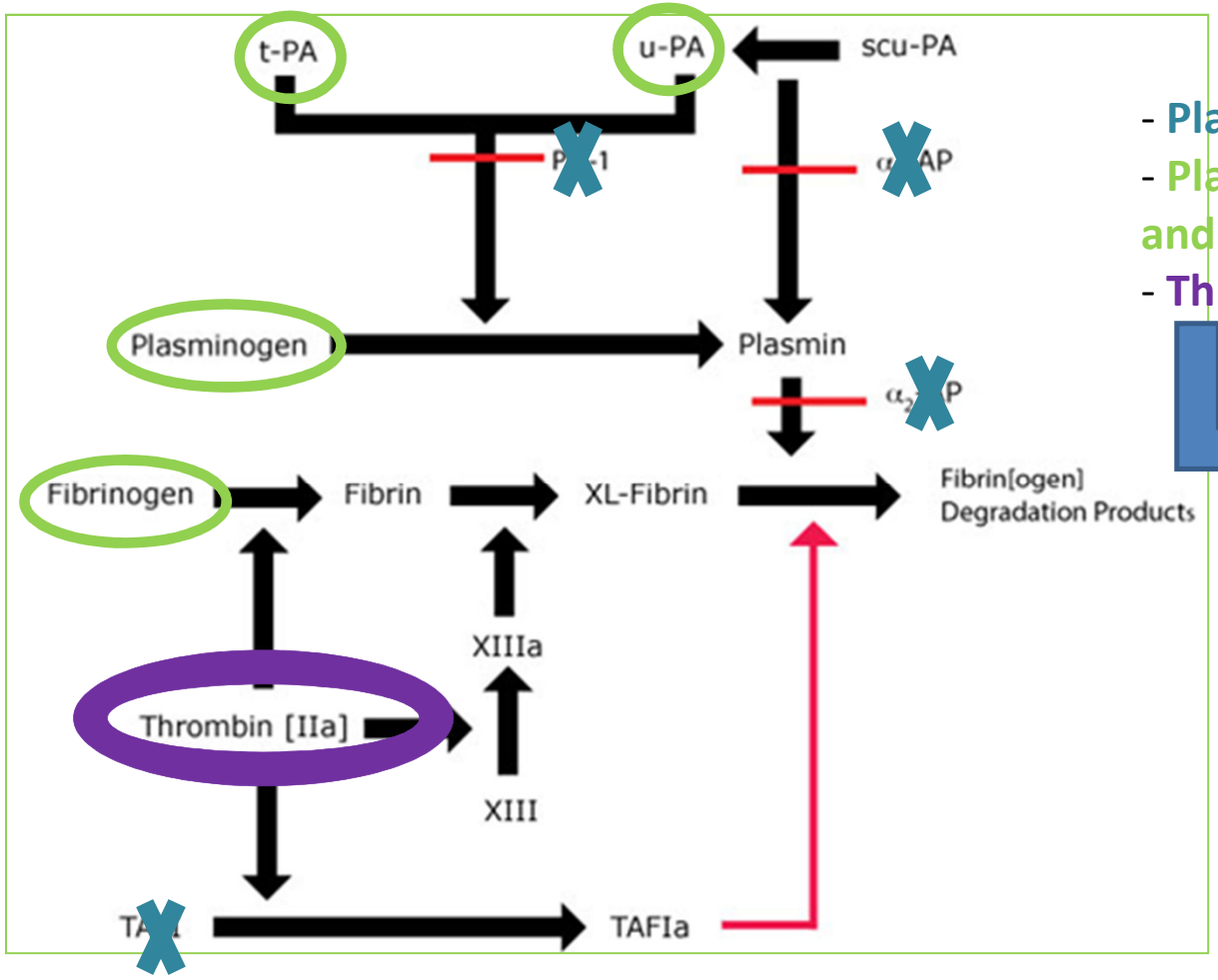
Euglobulin clot lysis time : principle

Plasma collected, diluted with acetic acid and incubated on ice for 15 minutes and then centrifuged.

A **precipitate** forms [the euglobulin fraction of plasma] which contains **plasminogen, plasminogen activators [primarily t-PA] and fibrinogen**.



Euglobulin clot lysis time : principle



- **Plasminogen inhibitors eliminated**
- **Plasminogen, plasminogen activators and fibrinogen preserved**
- **Thrombin added**

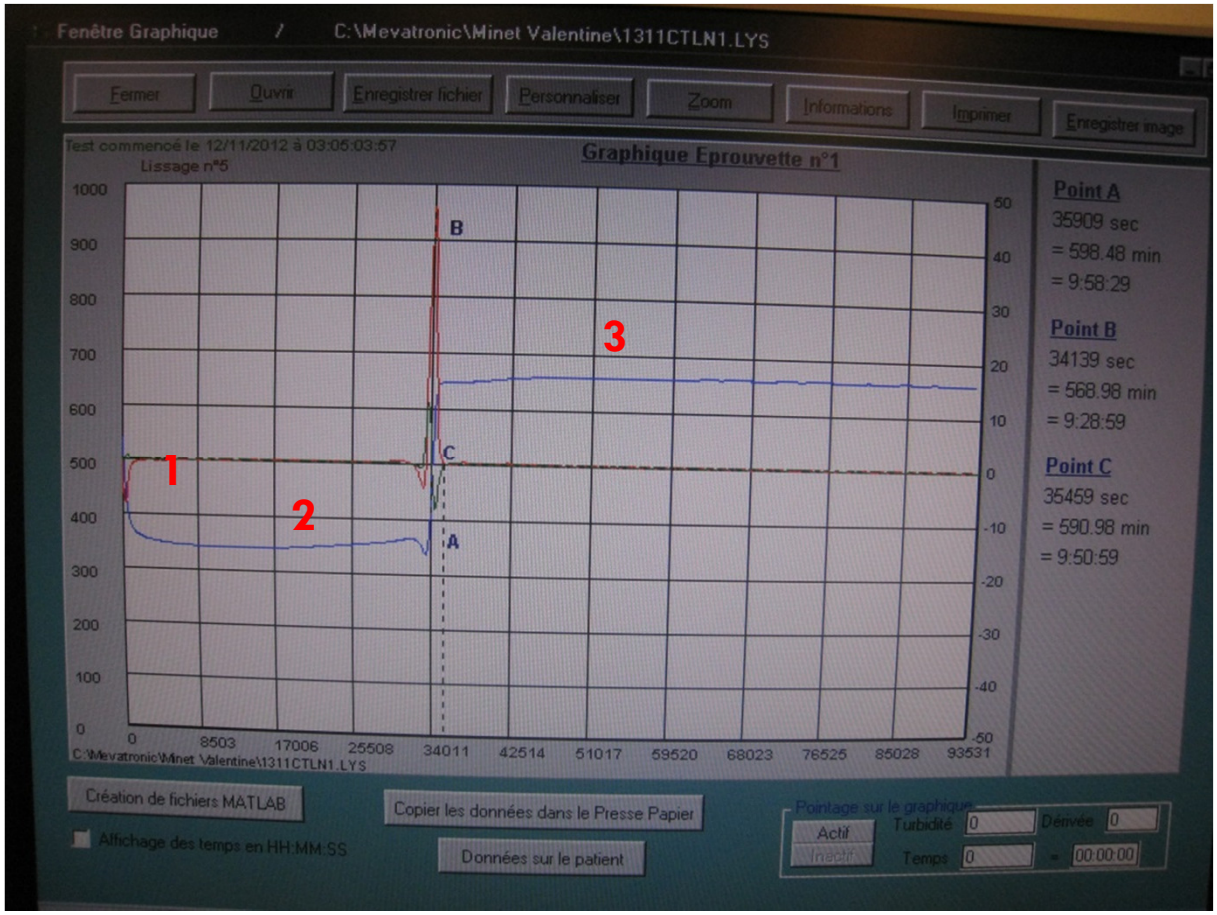
Differential separation in order to accelerate fibrinolysis

Euglobulin clot lysis time : principle

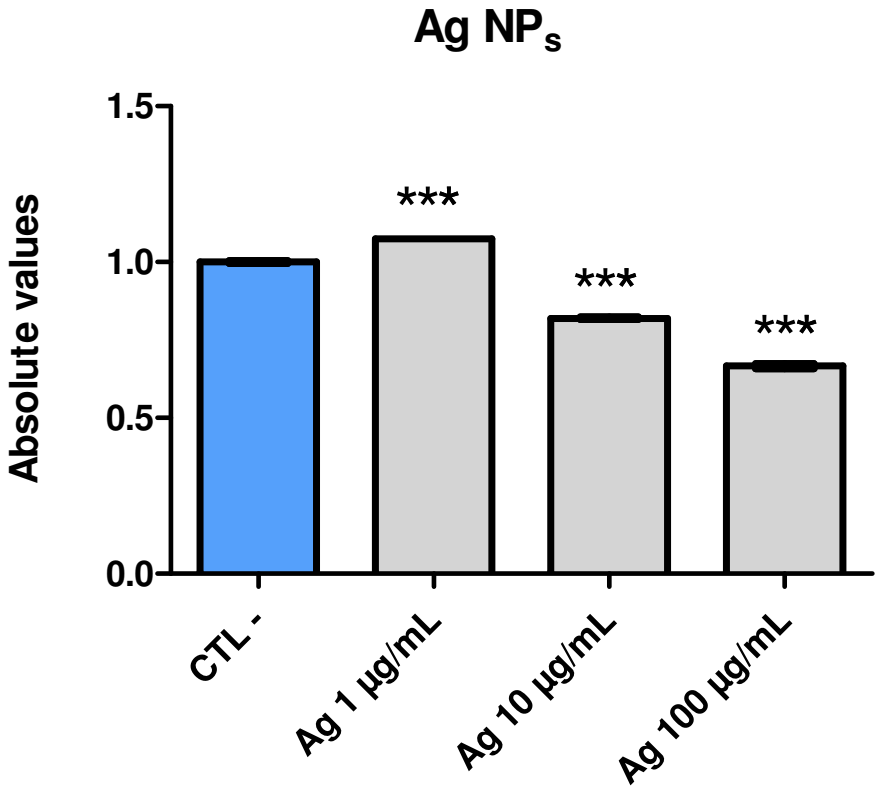
Precipitate of euglobulins diluted and placed in a cuvette.
Thrombin is added.
The machine measures the time to clot lysis by infrared (890 nm)

Reference :

- Normal : 210 – 540 min
- Hyperfibrinolysis : < 30 min
- Hypofibrinolysis : > 540 min



Euglobulin clot lysis time : results

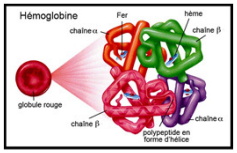


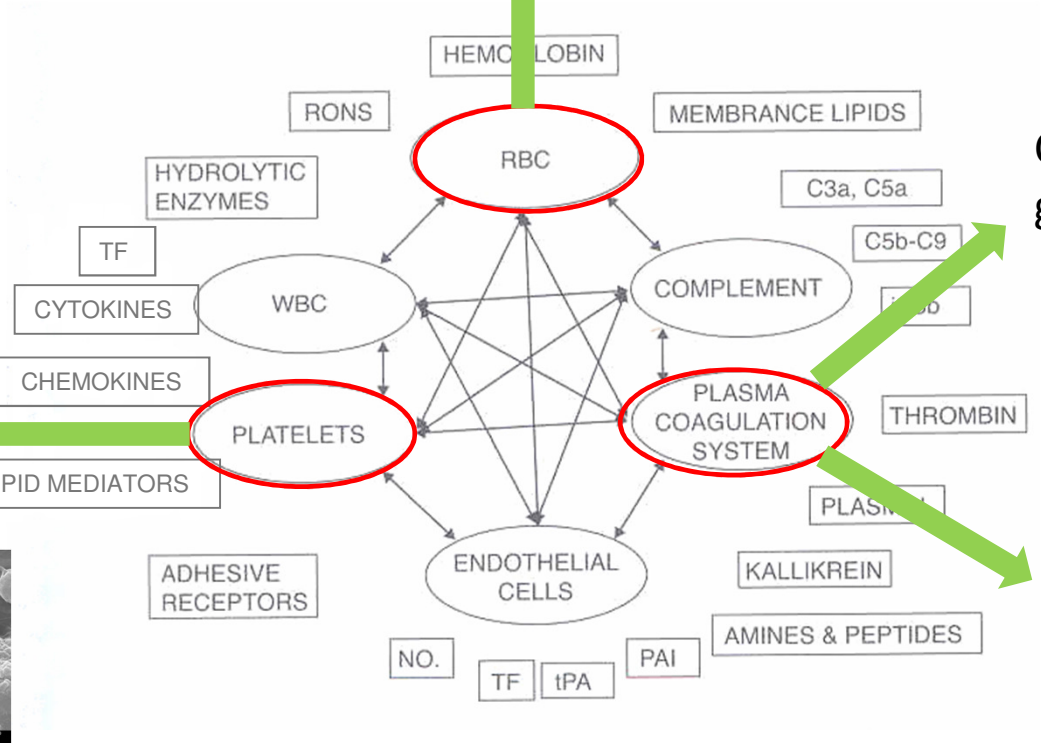
n≥2, t-test performed

Decrease of lysis time

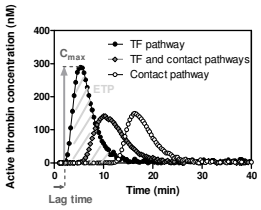
Human hemocompatibility
NP impact on …

$OD_{550\ nm}$ haemoglobin released

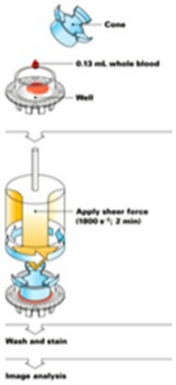


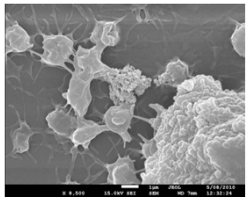


Calibrated thrombin generation test

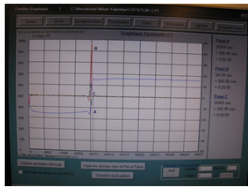


Impact-R® + FEG-SEM



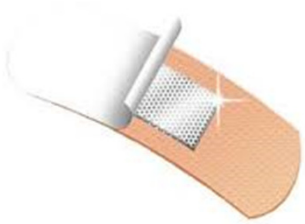


Euglobulin clot lysis time



Conclusions

- Silver NPs :
  - hemolytic potential
  - procoagulant activity
  - Induction of platelet adhesion
- Impact on applications :
  - Local exposure : benefits (platelets, cicatrisation…)
  - Systemic exposure : Safety margins



Nanomaterials and Nanotechnology

Impact of Silver Nanoparticles on Haemolysis, Platelet Function and Coagulation

Original Research Article

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Thank you for your attention!

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Département du Développement Technologique

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SEVENTH FRAMEWORK PROGRAMME

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