Title: In-vitro evaluation of biocompatibility of nanomedicines intented for IV route: focus on cytotoxicity and hemocompatibility tests.

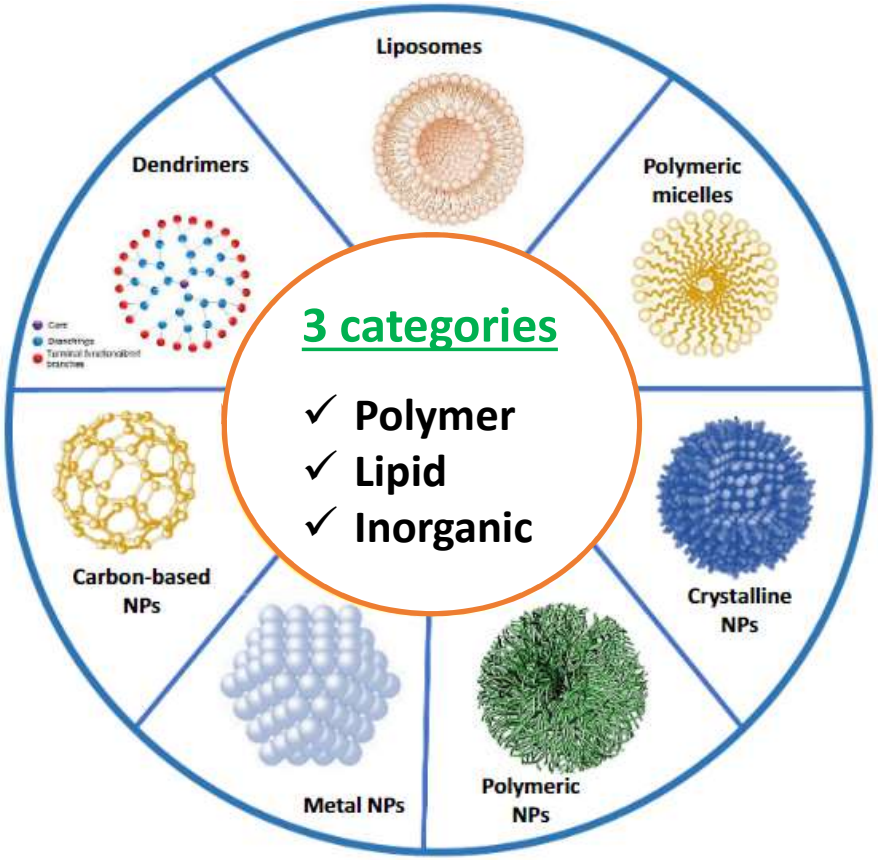
Part of my PhD project in Biomedical and Pharmaceutical sciences.

ILANGALA BOOKA Ange

Promoter : Prof. Phillipe Compère

Co-Promoter : Prof. Patrick MEMVANGA

Nanotechnology based Drug Delivery Systems



- ❑ **More than 50 formulations currently on the market.**
- ❑ **More than 400 formulations currently in clinical trials.**

1. Unique properties of NPs

- ✓ **Small size**: 1 – 1000 nm (overcoming biological barriers)
- ✓ **Ability to efficiently load various drugs**: small molecules and biologics (peptides, protein, nucleic acids etc.)
- ✓ **Large surface-to-volume ratio (50% molecules on the surface):** tunable via external stimuli such as heat, pH, ultrasound etc. « temporal and spatially controlled drug release ».
- ✓ **Tunable surface functionality**: charge, targeting ligands moities etc.

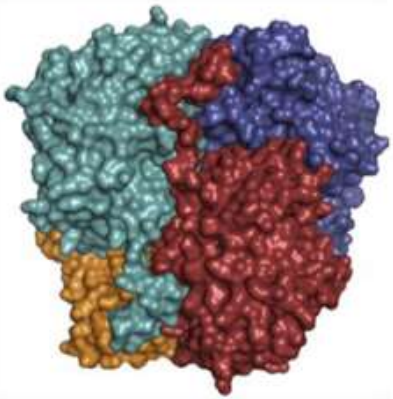
2. Various applications

- ✓ **Bioavailability enhancement:** poorly water soluble and permeable molecules **(BCS II & IV = 90% API)**
- ✓ **Improvement of in-vivo stability of encapsulated drugs:** protection of labile drugs from harsh conditions (pH, enzymatic degradation etc.)
- ✓ **Tuning Pharmacokinetics of encapsulated drugs:** site-specific delivery, transmucosal and intracellular delivery

DESCRIPTION OF MY PhD PROJECT

Tumor targeting liposomes for the intracellular delivery of novel LDHB inhibitors

Targeting LDHB Tetramerization site

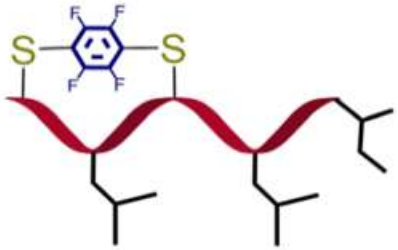


Attractive target for cancer therapy

1. SiHa: Cervical cancer
2. MCF-7: Breast cancer
3. HCT116: Colon cancer

1. **Lactate fueled respiration**
2. **Lactate fueled autophagy**
3. **Lactate induced angiogenesis**

Designing new ligands
« Therapeutic peptides »



LDH disruptor

Sequence of peptides
- ATLKEKLIAPVAEEEATVP
- CTLKCKLI

Pierre Sonveaux, PhD
Professor
University of Louvain (UCLouvain) Medical School
Brussels, Belgium

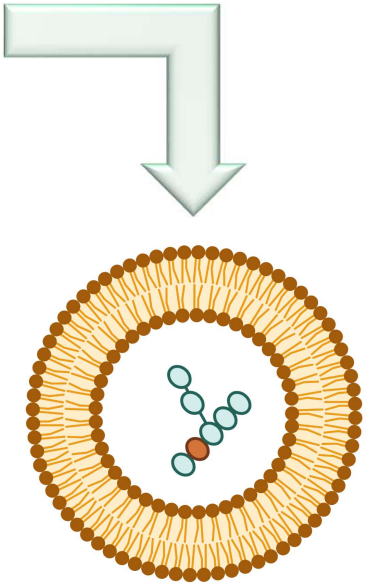
IREC UCLouvain

Therapeutic peptides

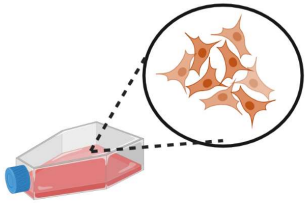
- ❑ High binding affinity
- ❑ Good target specificity
- ❑ Low toxicity and immunogenicity
- ❑ Low of drug-drug interaction

- ❖ Limited *in-vivo* stability
- ❖ Short half-life
- ❖ Poor overall bioavailability
- ❖ Limited to extracellular target

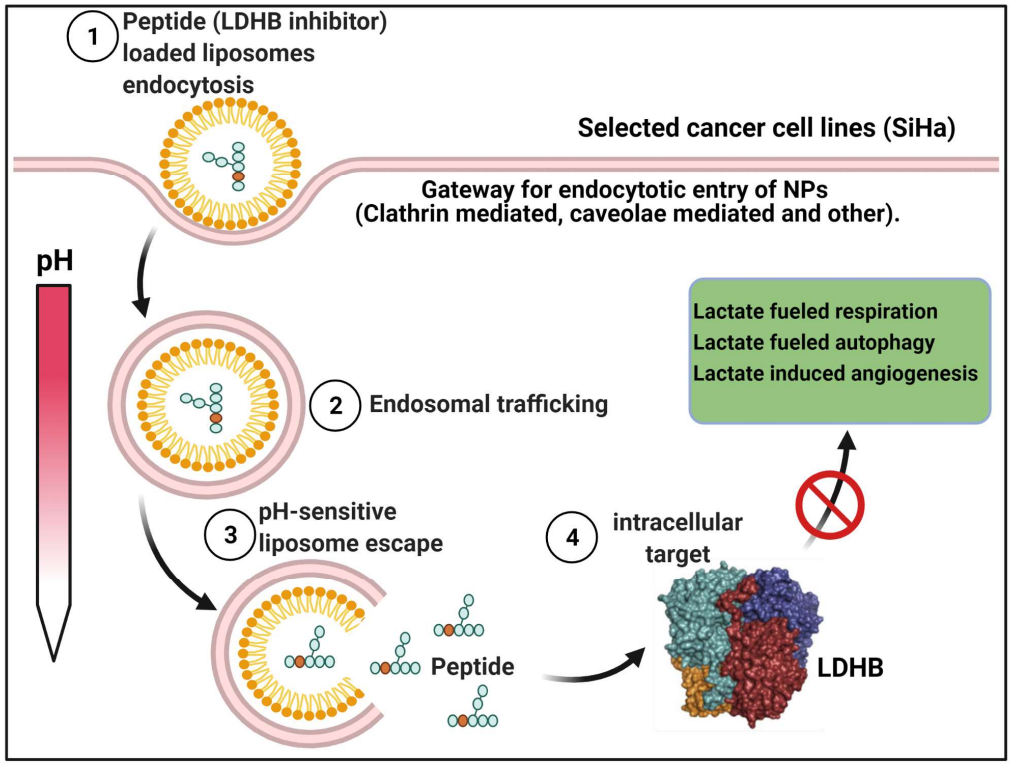
Need for advanced delivery systems for therapeutic peptides

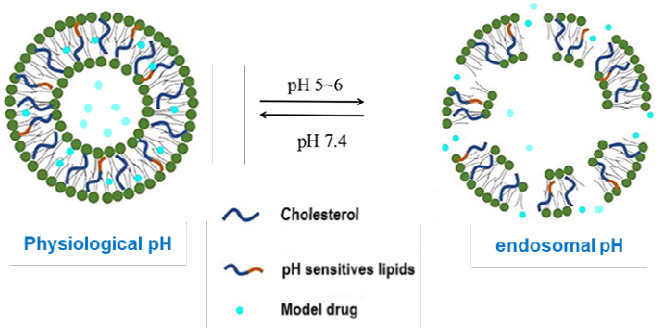


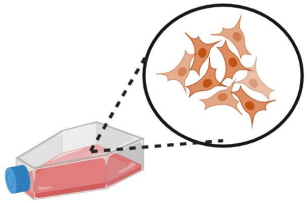
pH-sensitive LIPOSOMES



In-vitro


1 Peptide (LDHB inhibitor) loaded liposomes endocytosis
Selected cancer cell lines (SiHa)
Gateway for endocytotic entry of NPs
(Clathrin mediated, caveolae mediated and other).
pH
2 Endosomal trafficking
Lactate fueled respiration
Lactate fueled autophagy
Lactate induced angiogenesis
3 pH-sensitive liposome escape
4 intracellular target
Peptide
LDHB


pH 5~6
pH 7.4
Physiological pH
endosomal pH
Cholesterol
pH sensitives lipids
Model drug



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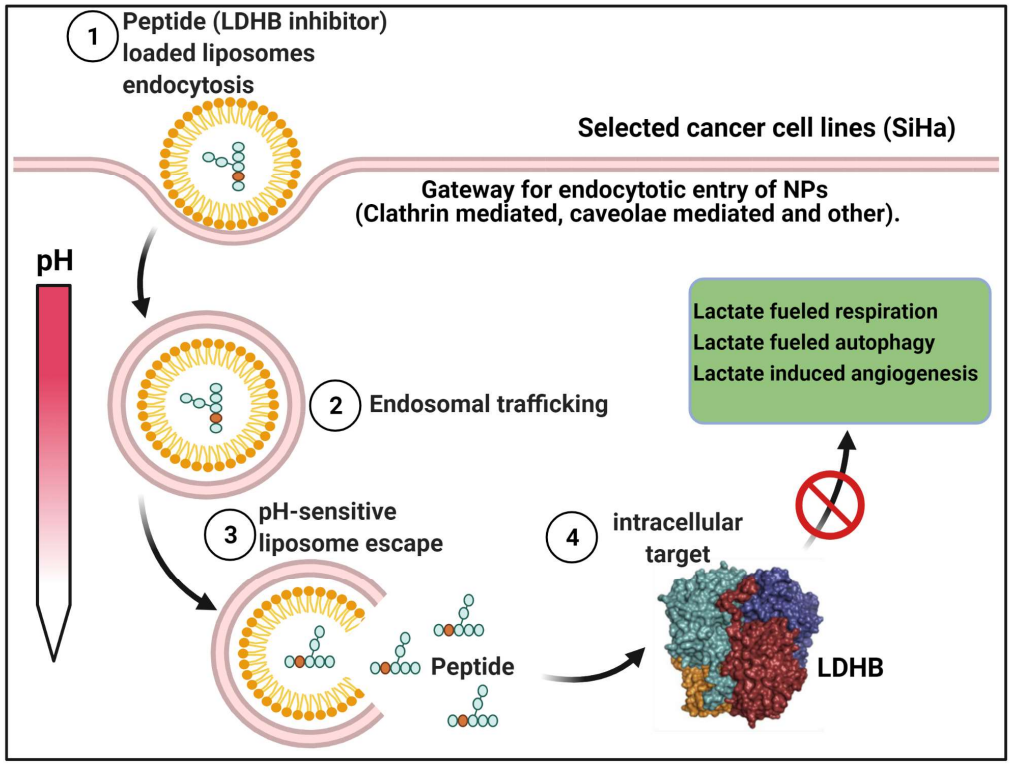
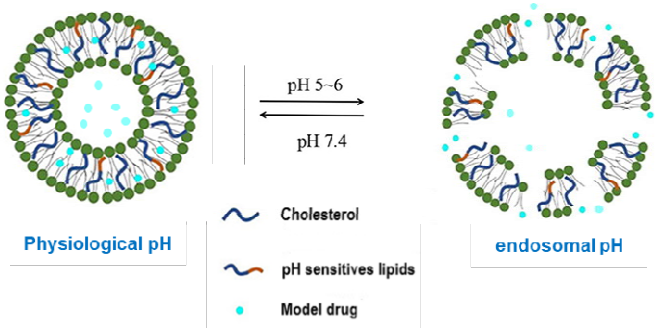
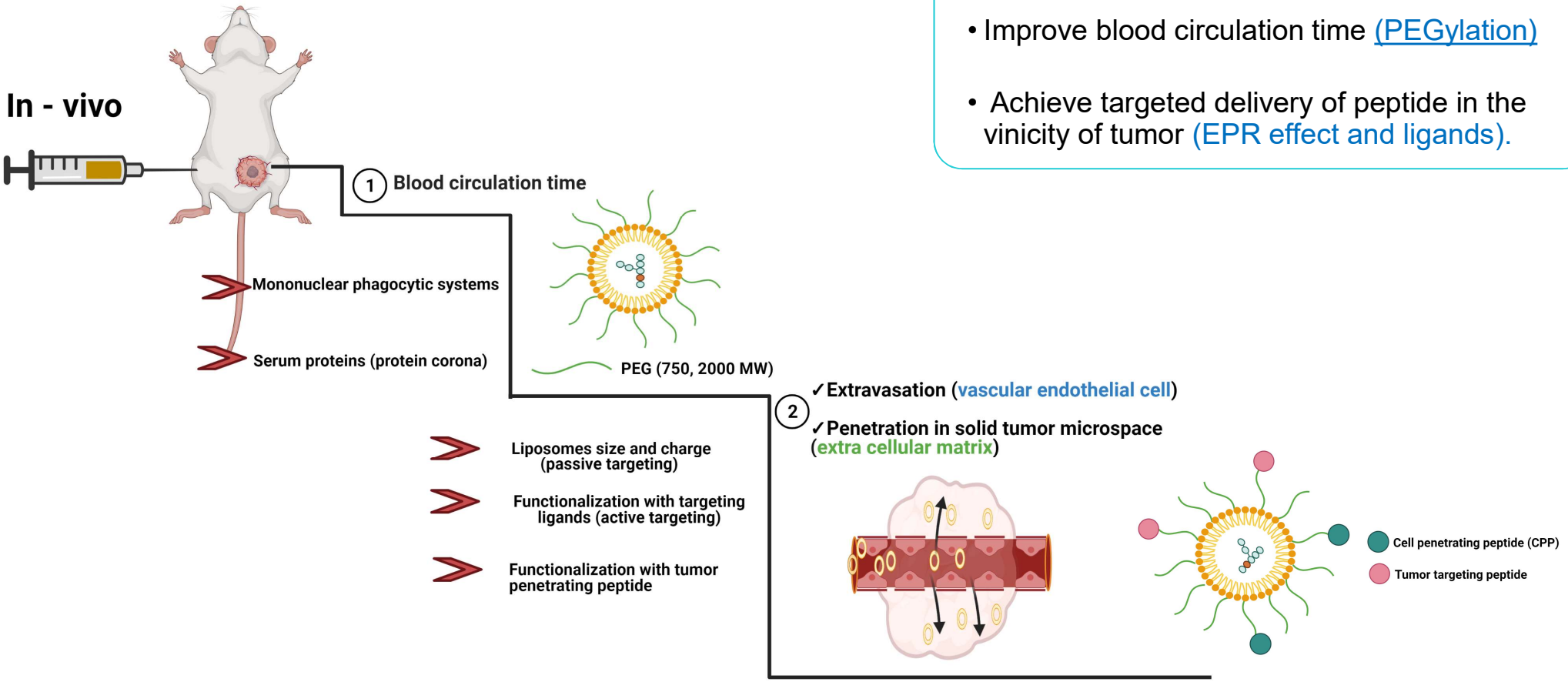




pH sensitive lipids for the design of liposomes

Fusogenic lipids	• 1,2 dioleoyl-sn-glycerol-3-phosphoethanolamine **(DOPE)** • Cholesteryl hemisuccinate **(CHEMS)**
Ionisable lipid in endosomal pH	• 1.2 dioleoyl-3-dimethylammonium-propane **(DODAP), D-Lin-MC3-DMA.**
Switchable lipids in endosomal pH	• **CSL1, CSL2, CSL3**

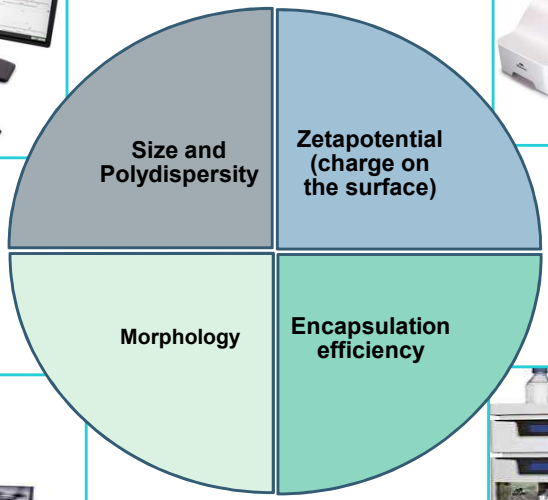
- Increase the metabolic stability of the encapsulated peptides (Proteases)
- Improve blood circulation time (PEGylation)
- Achieve targeted delivery of peptide in the vinicity of tumor (EPR effect and ligands).



Physicochemical characterization of liposomes/understanding the biological response.

Dynamic light scattering (DLS):

- ✓ Size and size distribution
- ✓ Batch quality & δ in manufacture



Electrophoretic light scattering (ELS):

- ✓ Stability (Zetapotential)
- ✓ Biological fate.

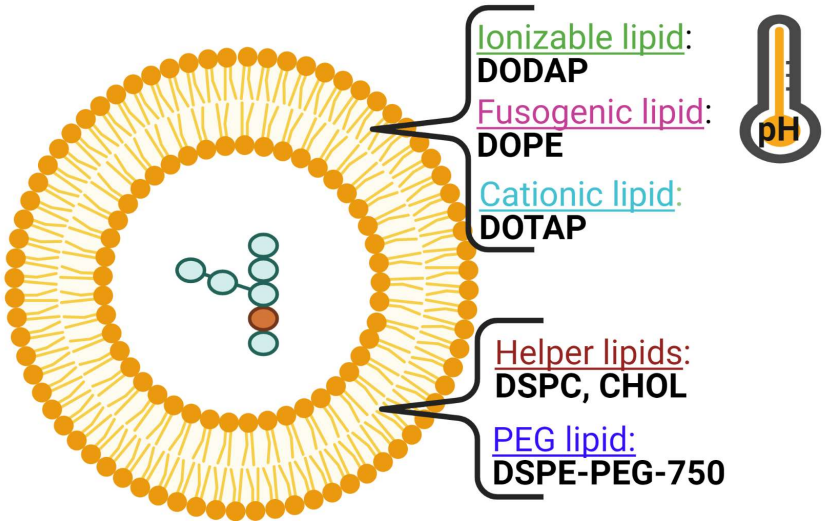
TEM, cryogenic-TEM

- ✓ Shape and detailed structure of liposomes (MLV, ULV)

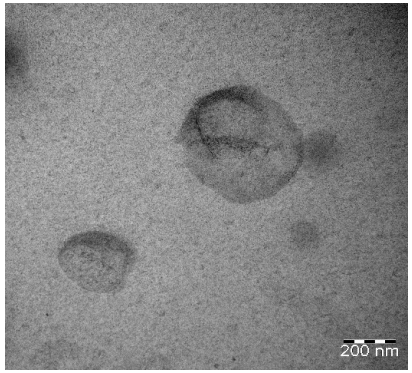
HPLC - UV

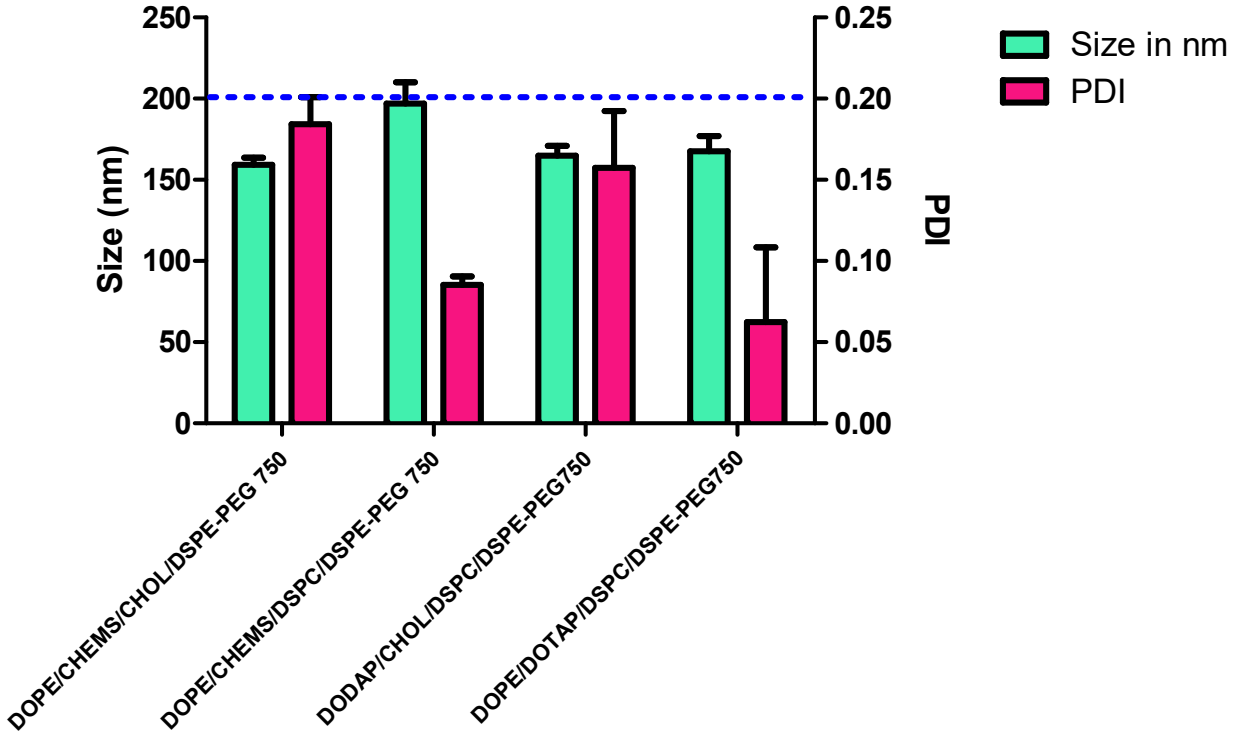
- ✓ Method development and validation for the estimation of peptide in liposomal formulations.

Liposomes formulation design and physicochemical characterization at physiological pH



TEM micrographs





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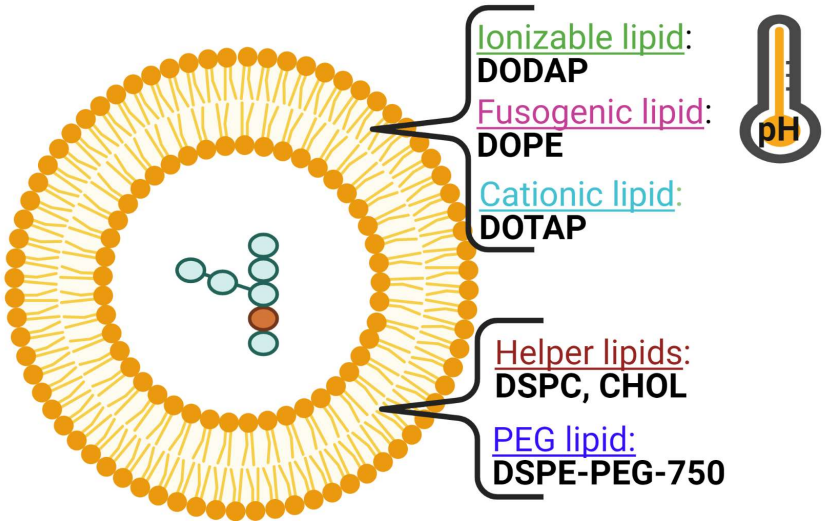
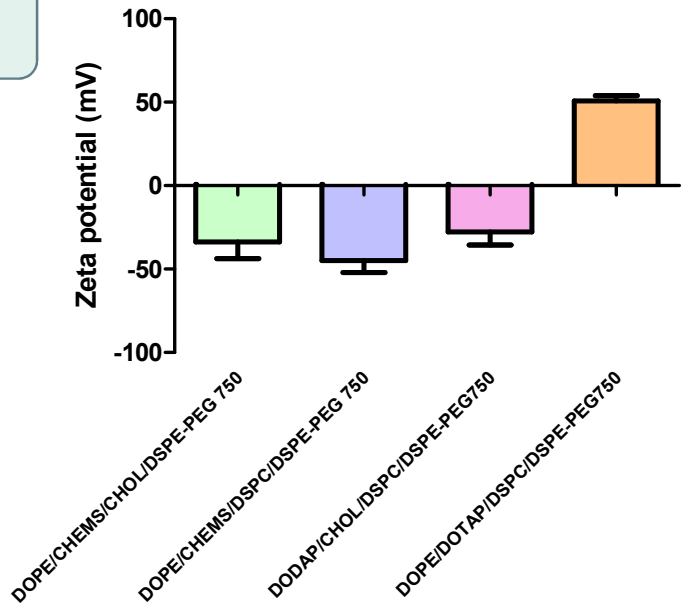
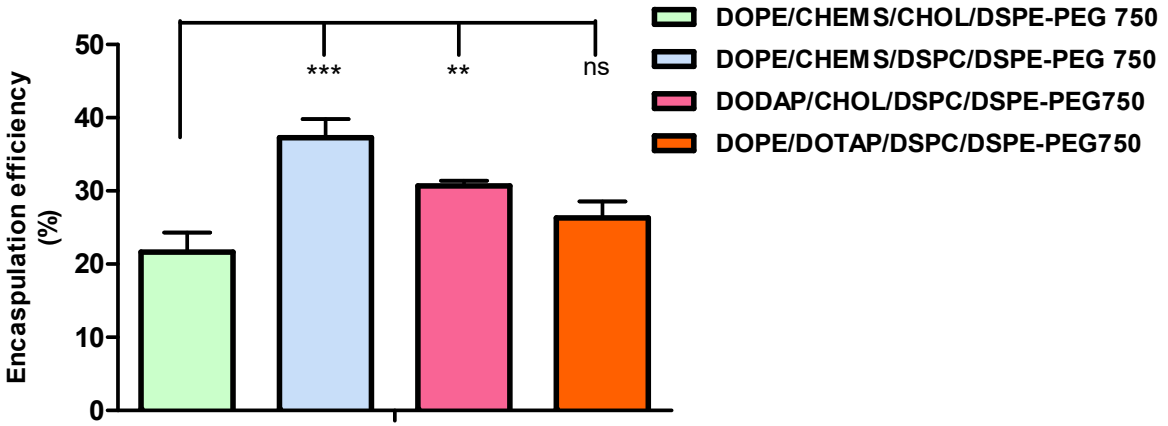




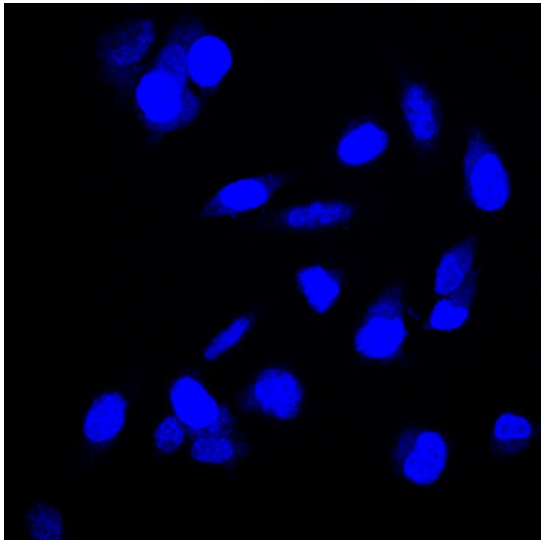
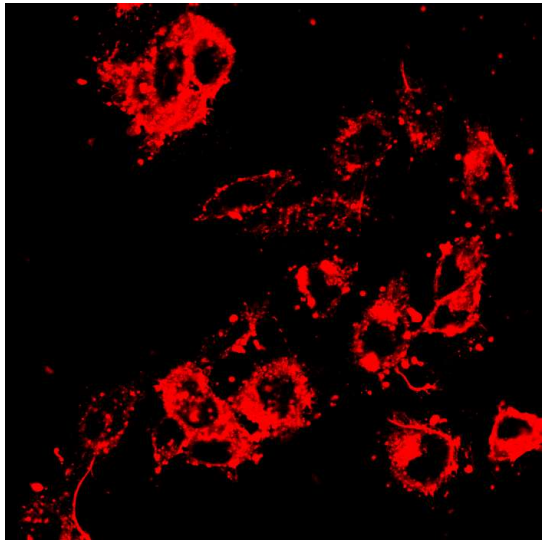
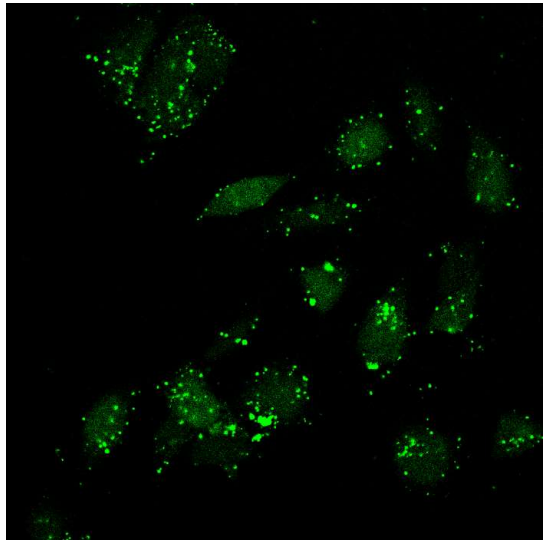
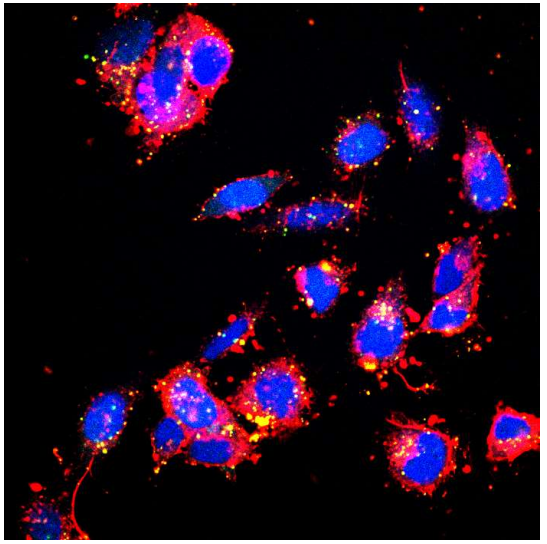


Cell uptake and intracellular localisation/release of the cargo in SiHa cell line

Nuclei staining with DAPI

Rhodamine B labelled liposomes

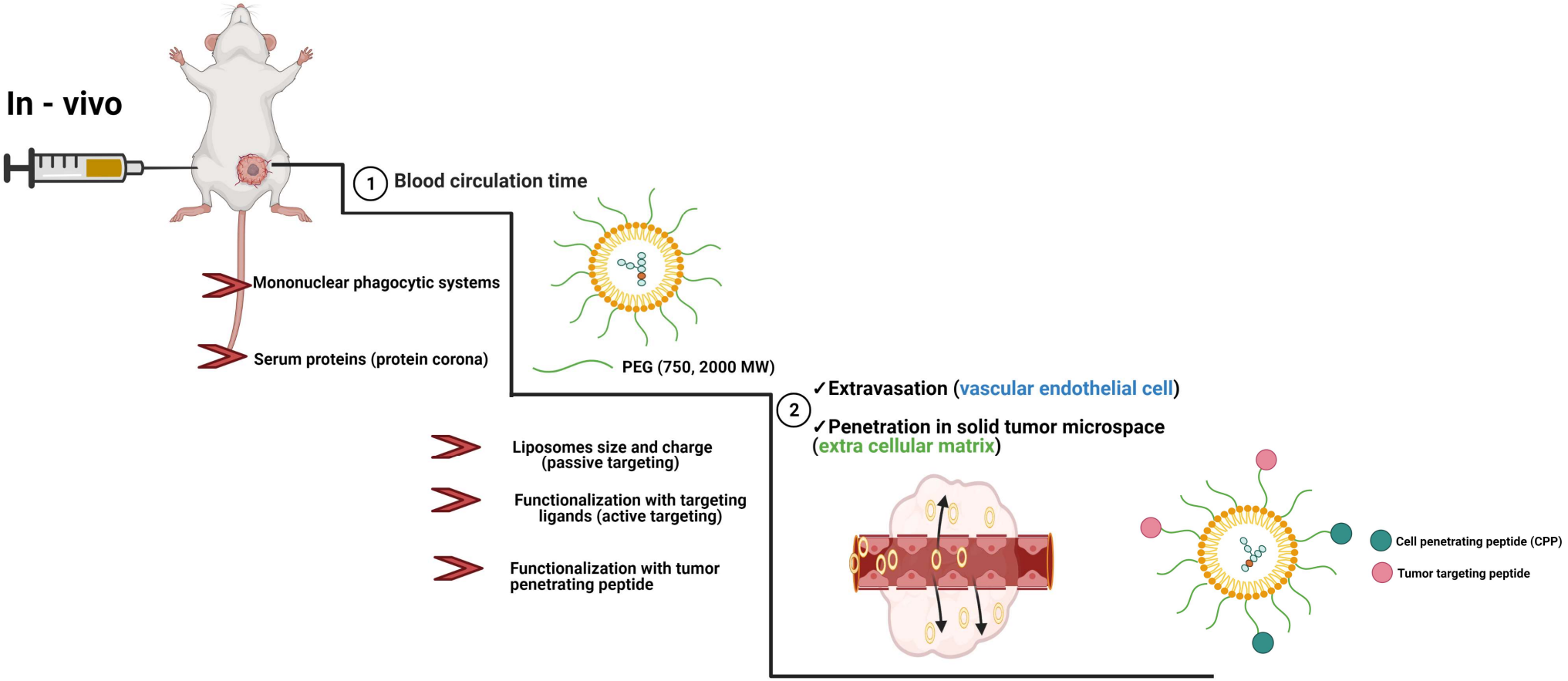
Calcein green fluorescence

Calcein loaded rhodamine Liposomes (DODAP/CHOL/DPSC/PEG-750: 45/20/30/5)

Conclusion: The liposomes are well internalized inside cell (SiHa) as it can be seen in the area around the nucleus (blue). Incubation time: 5 hr

ARE THOSE LIPOSOMES FORMULATIONS BIOCOMPATIBLE?



ARE THOSE LIPOSOMES FORMULATIONS BIOCOMPATIBLE?

What is Biocompatibility?

A key concept in the development of Drug Delivery System contacting the human body.

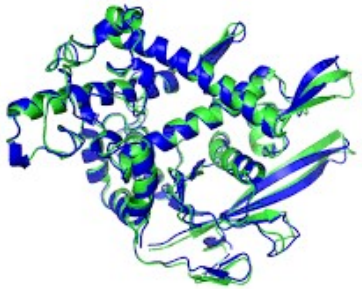
Biocompatibility refers to the ability of a biomaterial/DDS to perform its desired function with respect to a medical therapy, without eliciting any undesired local or systemic effects in the recipient (host).

Understanding the biological response.

Nanoparticles by IV route = expose large surface

Volume		Particle size		Total surface area
1 cm^3	→	1µm	→	6 m^2
1 cm^3	→	1nm	→	6,000 m^2

Interaction (react) with various biological domains



Biomolecules = Proteins, Lipoproteins etc

Cells = endothelial cell, Red blood cells etc

Understanding the biological response.

This physiological reactivity is driven by physicochemical properties.

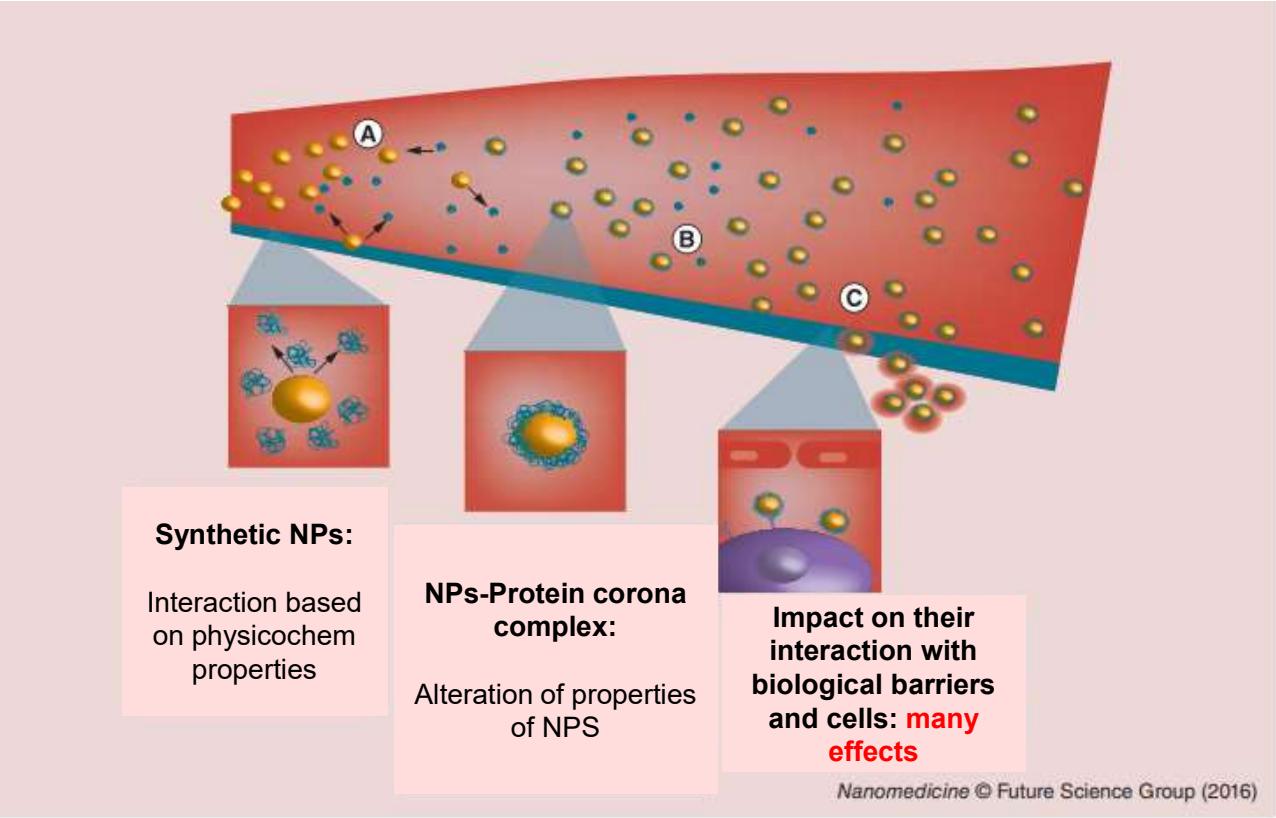
Size and shape
- Size distrubution
- Shape

State of dispersion
- Agglomeration/aggregation

Physico and chemical propeties
- Chemical composition
- Crystalline phase and crystallite size
- Solubility
- Impurities

Surface area and porosity

Surface properties
- Surface composition
- Catalytic properties
- Surface charge
- Adsorption molecules (functional ligands)
- Lipophilicity/hydrophilicity



Claudia Corbo et al, 2016

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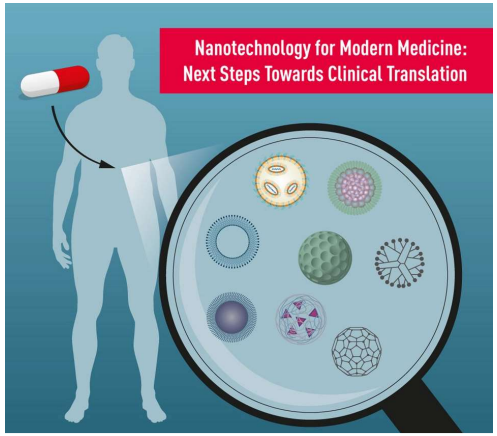

EFFECTS

Serum protein corona formation

- Modification of cellular uptake
- lost of targeting (interact other receptors)
- Aberrent cell tissue response (necrose, apoptosis)

Immune cell recognition

-rapid clearance of NPs
- Allergic reactions

Trigger of harmful hemoreactivity

-hemolysis
-coagulation dysregulation (factors)

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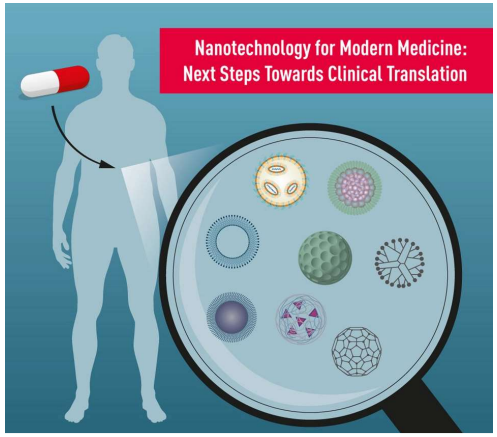

- ❑ All of these may limit their clinical use as drug delivery vehicles for IV administration
- ❑ For any new formulation (nanoformulation) it is mandatory to identify the concentration or dose at which the drug delivery system ceases to be safe for therapeutic use

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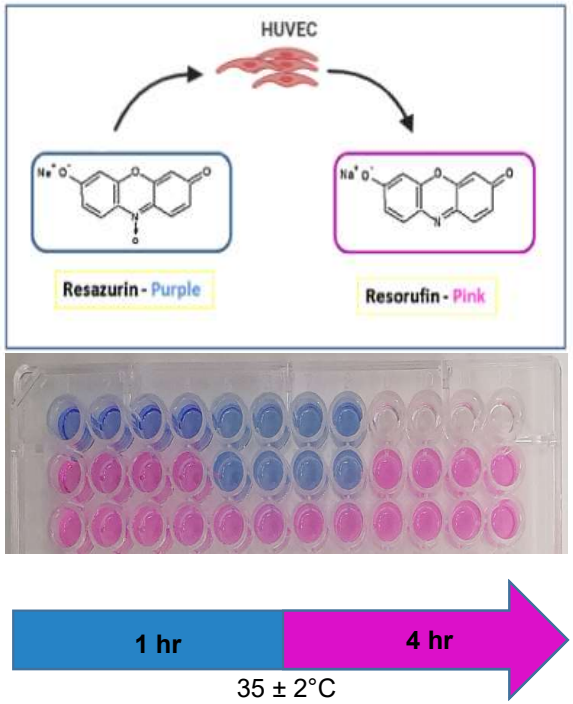
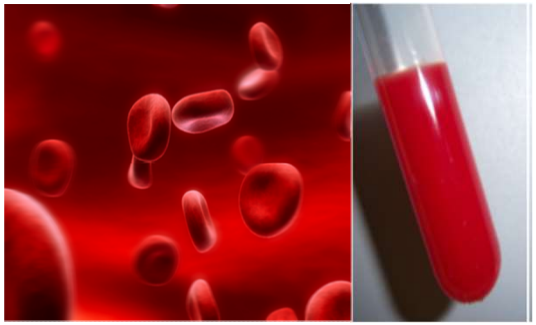
ARE THOSE LIPOSOMES FORMULATIONS BIOCOMPATIBLE?

In-vitro testings: Basically two groups of assessments

Toxicity towards healthy cells



Hemocompatibility



- **Hemolysis**
- **Platelet aggregation**
- **Generation of thrombin**

Cytotoxicity evaulation of Liposomes formulation designed

I. Cytotoxicity

Different in-vitro methods:

Why? Give you also an idea on the mechanism of cytotoxic involved =
destruction of cell membrane, prevention of protein systhtesis, expression of protein etc.
e.i. methods: Alamar Blue, MTS and LDH.

How to choose the cell line?

Based in administration route and likely of NPs to encounter them:
- HUVEC: human umbilical vein endothelial cells
- SiHa: squamous carcinoma cells.

How to culture the selected cell line?

HUVEC: EMB-2 Basal medium + appropriate supplements.
SiHa: DMEM + appropriate supplements.

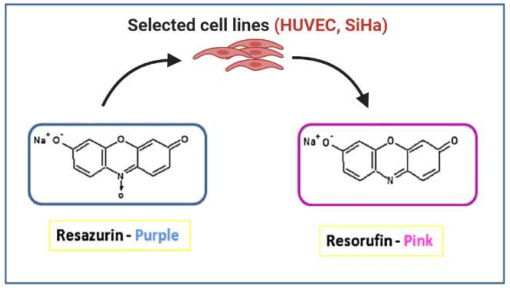
Principle of these cytotoxicity assays

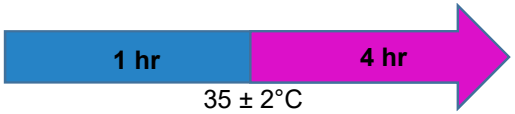
Alamar Blue	MTS	LDH
Fluorimetric assay	Colorimetric assay	Colorimetric assay
Resazurin	Tetrazolium salt	Tetrazolium salt
↓	↓	↓
Pink fluorescent resorufin By mitochondrial enzymes of **living** cells Read at 560/590 nm (excitation/emission)	Colored formazon By mitochondrial enzymes Read at 492 nm Intensity is correlated to number of **viable** cells in the culture	Colored formazon By coupled enzyamtic rx LDH + diaphorases Read at 492 nm Intensity is correlated to the **mortality** cells in the culture

Positive Control: Triton-X 100 (1%)

Negative control: PBS buffer

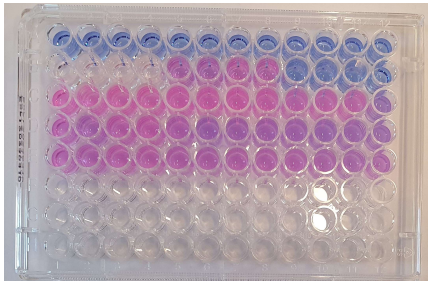
Toxicity against
Human umbilical vein endothelial cells (HUVEC)
and Cervical carcinoma cell lines (SiHa)



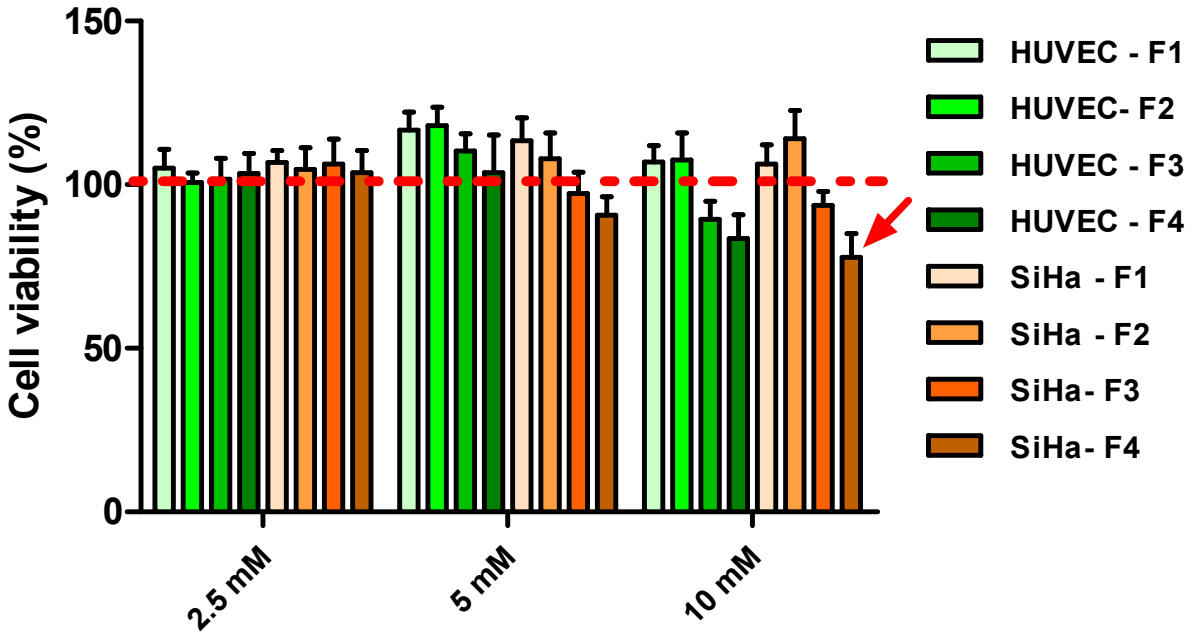


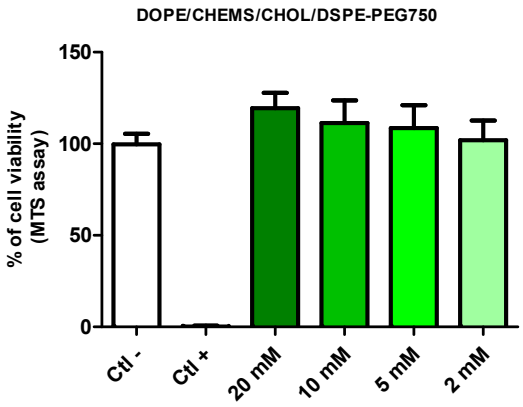
Resazurin vialibility assay

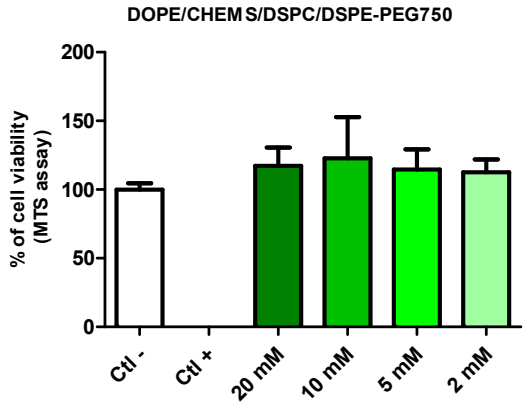
HUVEC



SiHa




DOPE/CHEMS/CHOL/DSPE-PEG750
% of cell viability (MTS assay)
150
100
50
0
Ctl -
Ctl +
20 mM
10 mM
5 mM
2 mM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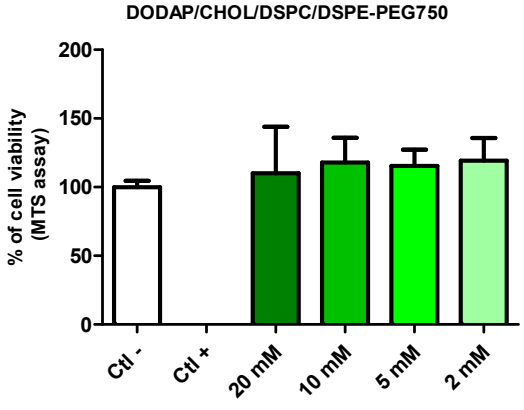
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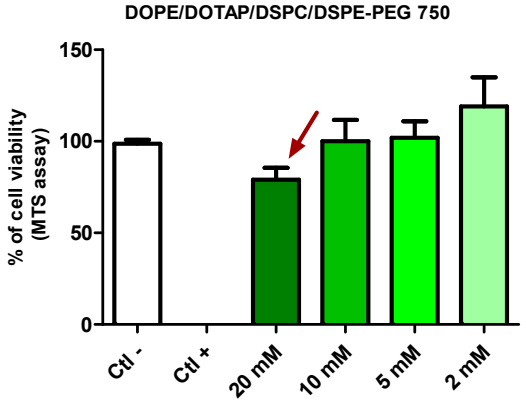
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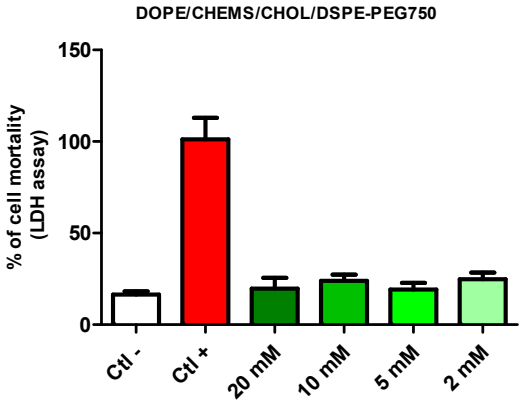
DOPE/CHEMS/CHOL/DSPE-PEG750
% of cell mortality (LDH assay)
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100
50
0
Ctl -
Ctl +
20 mM
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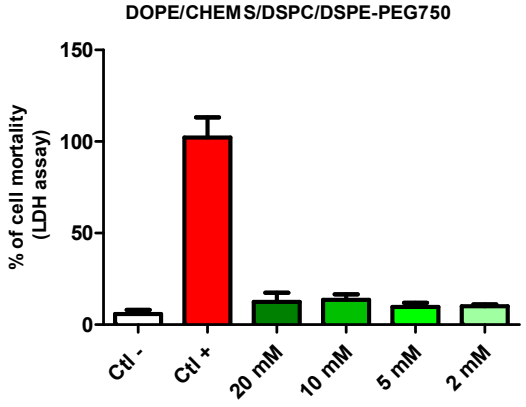
2 mM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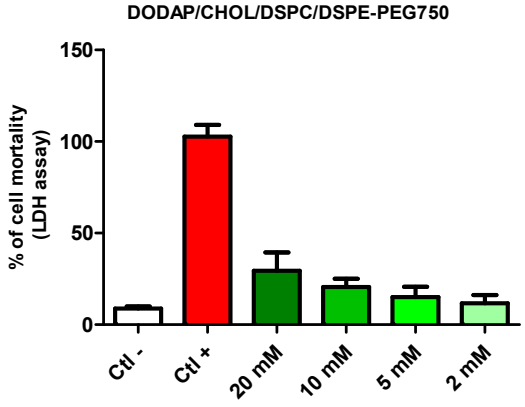
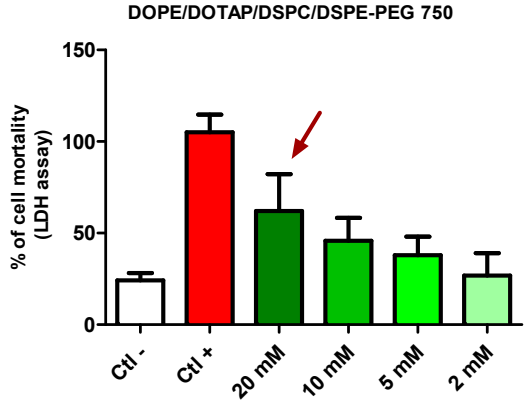
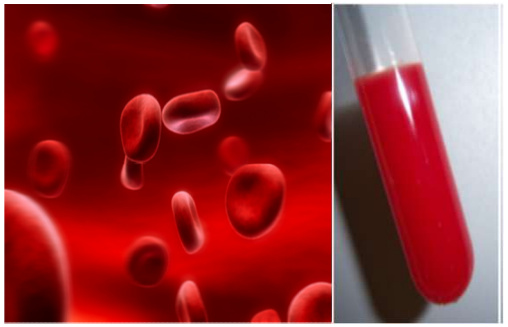
5 mM
2 mM


DODAP/CHOL/DSPC/DSPE-PEG750
% of cell mortality (LDH assay)
150
100
50
0
Ctl -
Ctl +
20 mM
10 mM
5 mM
2 mM


DOPE/DOTAP/DSPC/DSPE-PEG 750
% of cell mortality (LDH assay)
150
100
50
0
Ctl -
Ctl +
20 mM
10 mM
5 mM
2 mM

Hemocompatibility tests



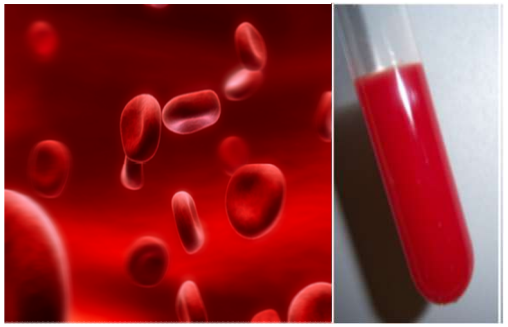
- **Hemolysis**

STEPS

1. 10% whased RBC + liposomes at various conc
2. Incubation at 37°C for 1hr with 5% of CO2
3. Centrifuged and collect supernatant
4. Measure absorbance at 550 nm

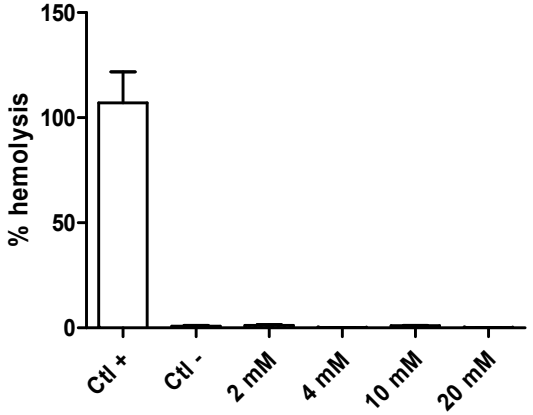
$$\mathbf{H}\ (\%) = \frac{(\text{OD550nm sample} - \text{OD550nmHEPES buffer})}{\text{OD550nm Triton X100 1\%} - \text{OD550nmHEPES buffer}}\ \text{X}\ 100$$

Hemocompatibility tests



- **Hemolysis**

F1



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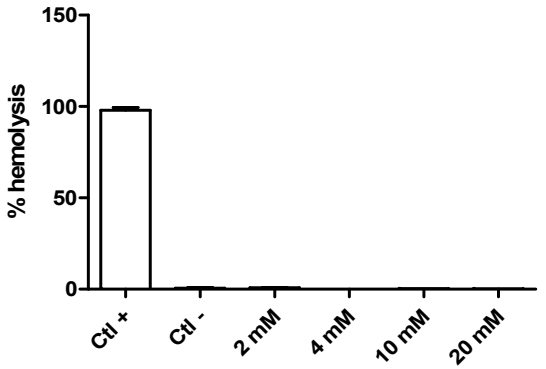

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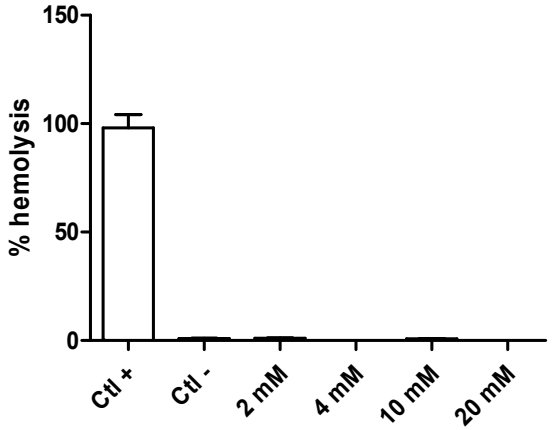

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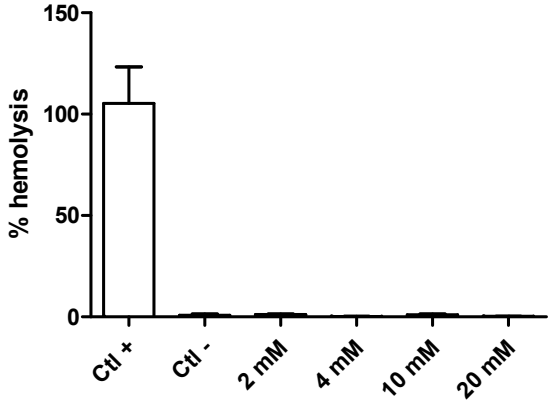
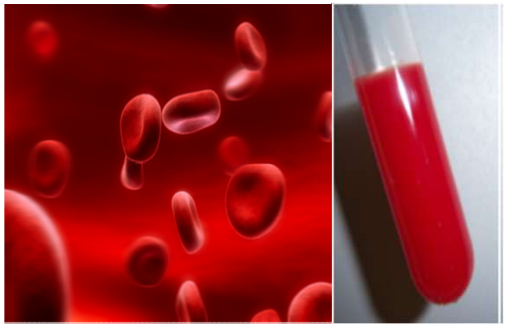


Hemocompatibility tests



- **Platelet aggregation**

STEPS

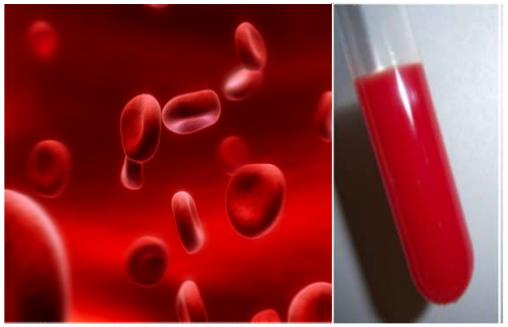
1. Platelet enriched Plasma (PRP) + liposomes at various conc

2. Incubation at 37°C for 1hr

3. Three different inducer of aggregation (ADP, AA, and collagen) were added

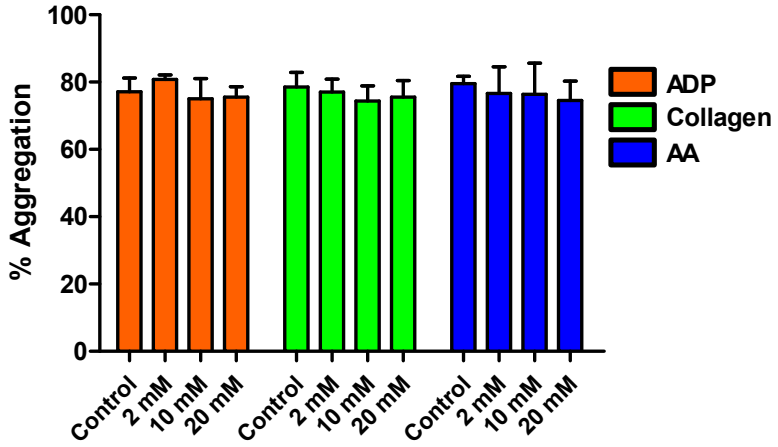
4. Induction of platelet aggregation was meaured by turbidimetry with optical aggregometer at 620 nm

5. % of aggregation was derived by comparison with positive control



- Platelet aggregation

F1



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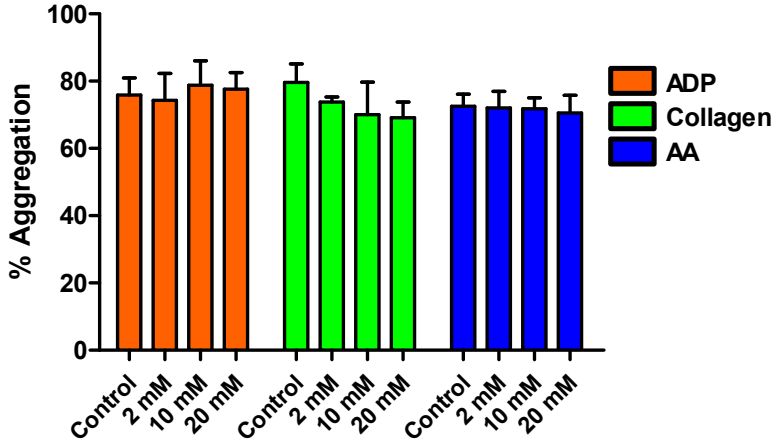

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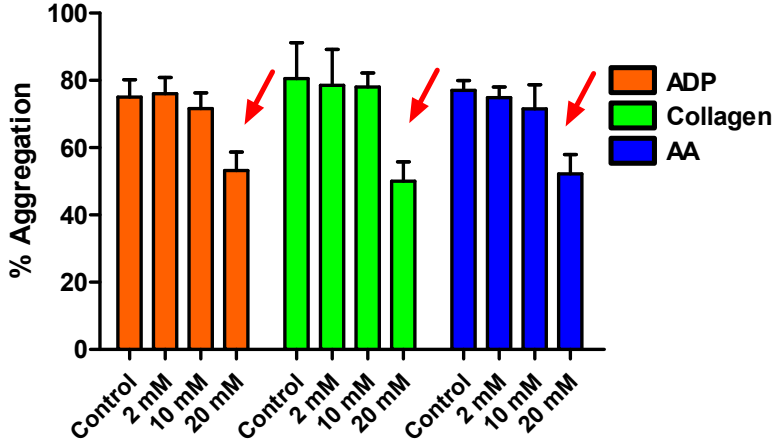

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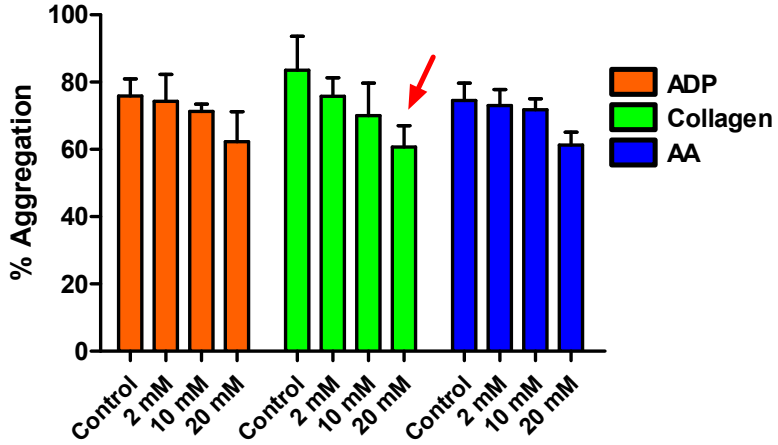
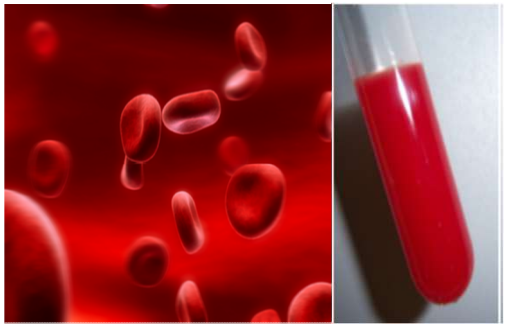
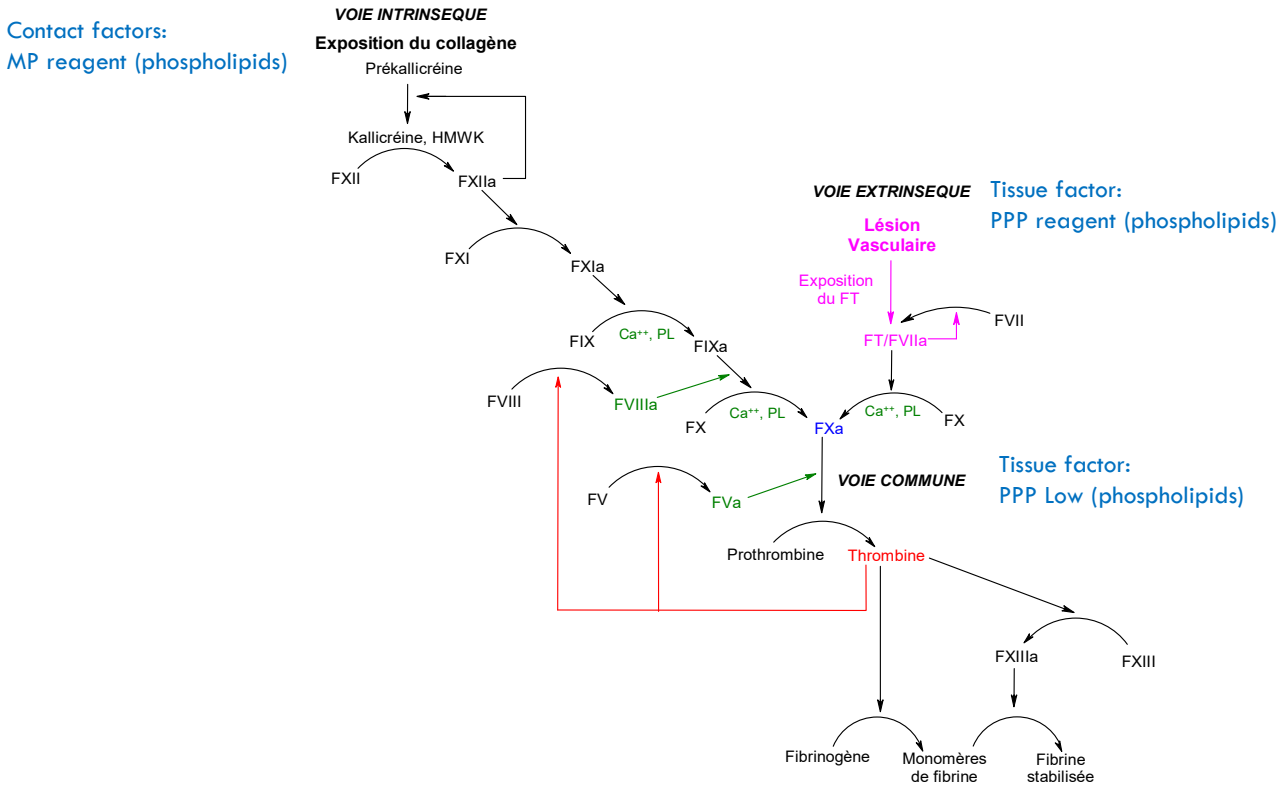


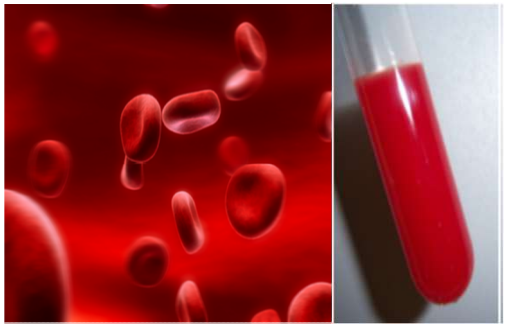
The impact of liposomes on coagulation was studied using the calibrated thrombin generation test (cTGT).



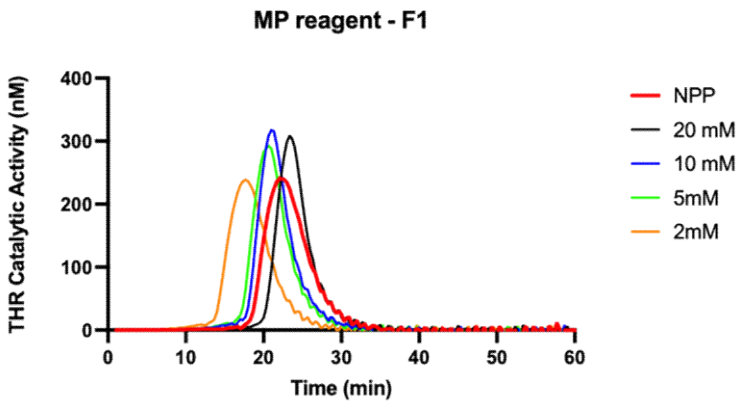
- **Generation of thrombine**

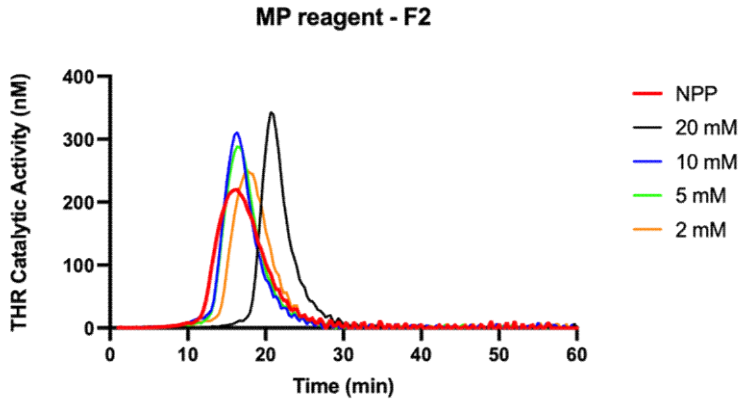
1. the formulations are placed in the presence of normal pool plasma (NPP) + a coagulation activator.
2. The thrombin formed during coagulation will cleave a substrate and make it fluorescent.
3. The results are converted into "thrombin activity“ using Software

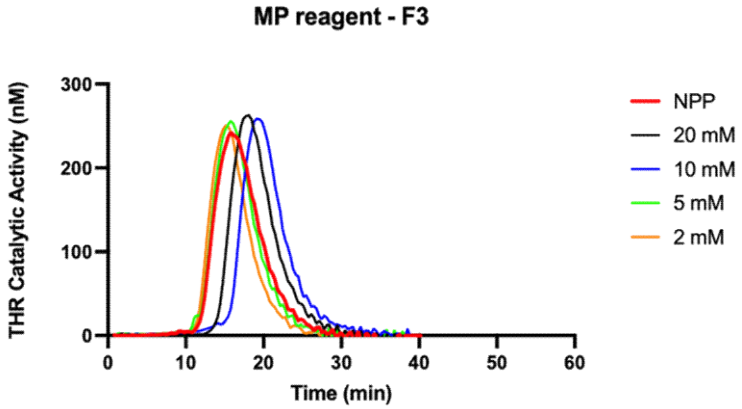


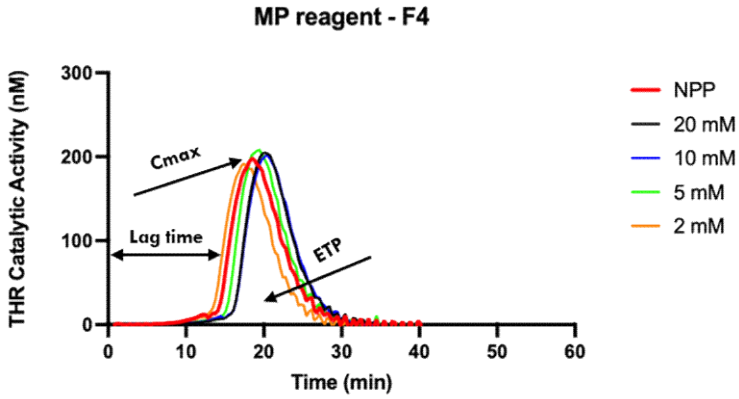


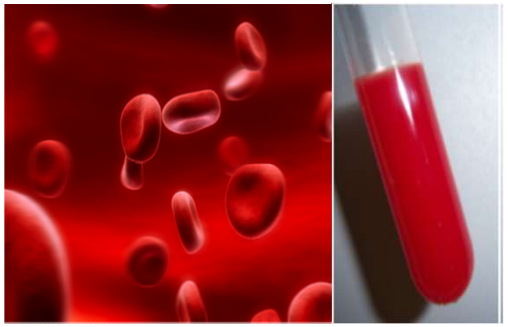
- **Generation of thrombine**



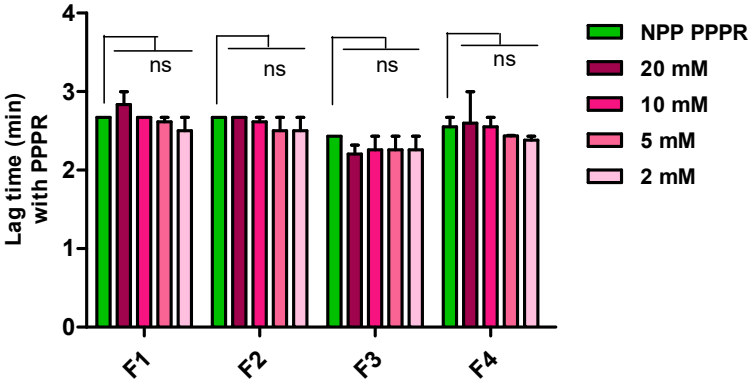


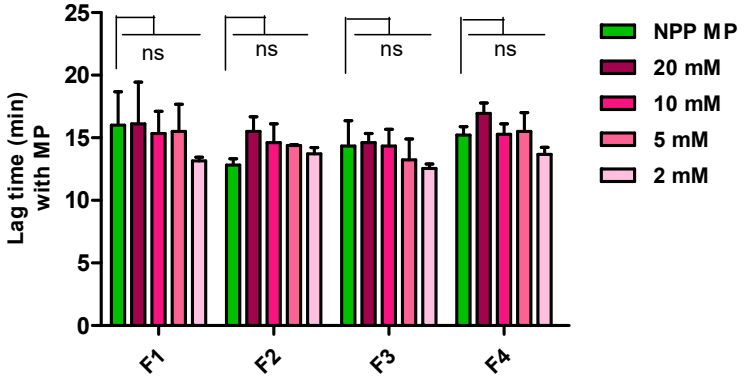


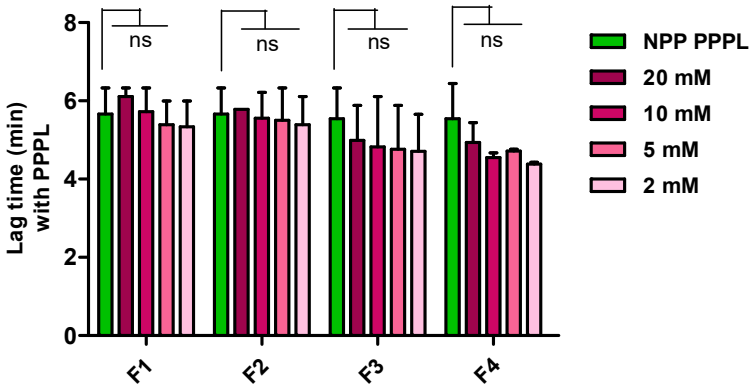




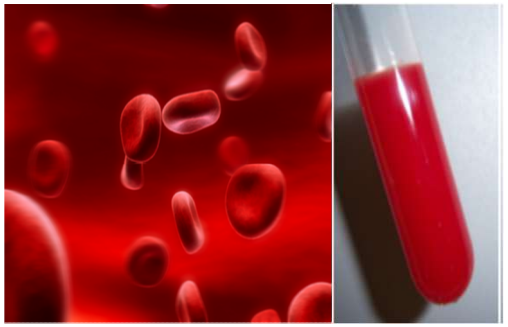
- **Generation of thrombine**



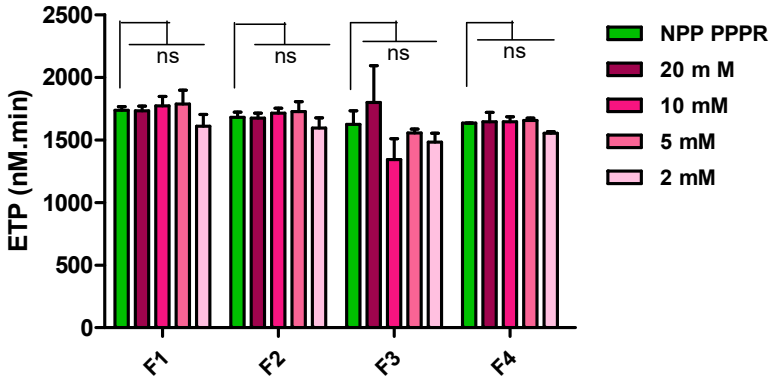


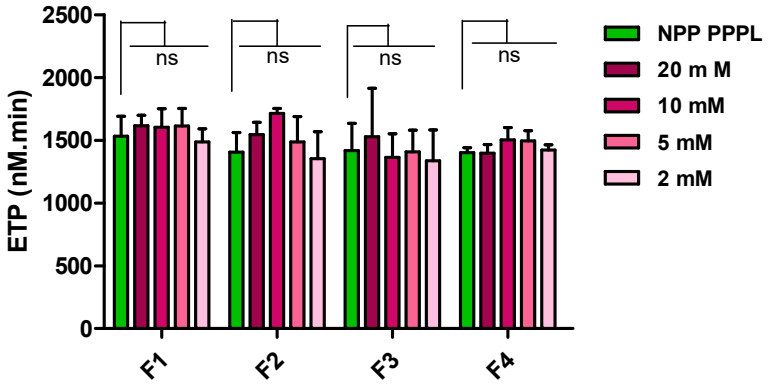


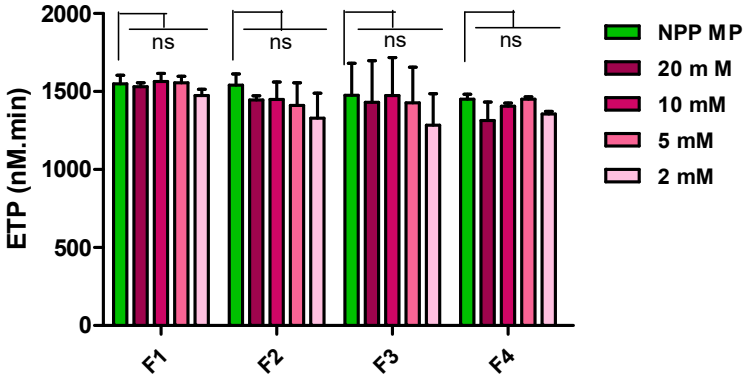
Statistical comparaisons with the negative control were perfomed by using one-way ANOVA, followed by the Dunnett's test.

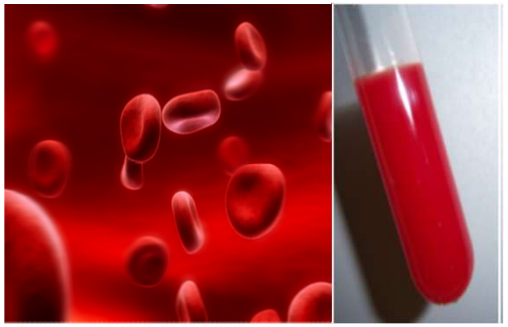


- **Generation of thrombine**

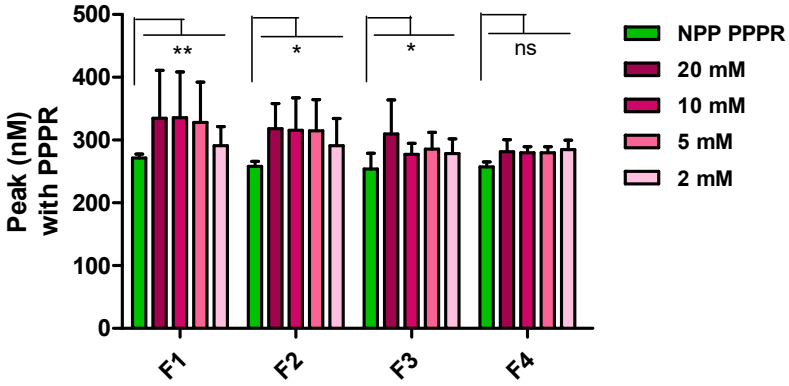


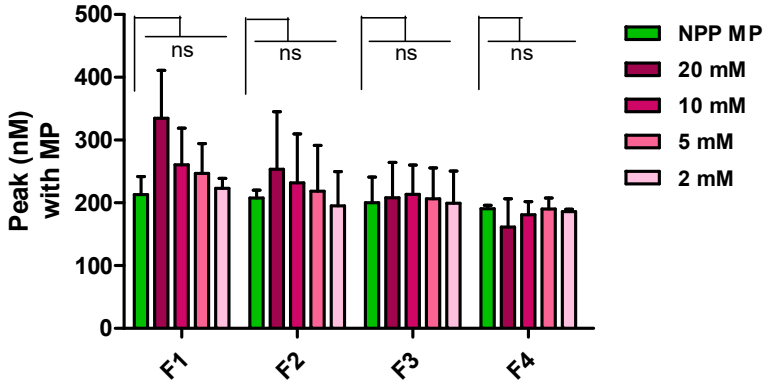


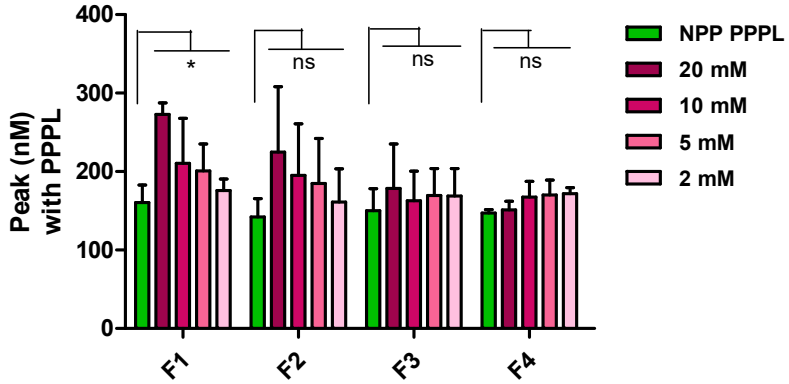




- **Generation of thrombine**







Conclusion

Overall, considering that:

1. The formulated liposomes did not show cytotoxicity towards endothelial cells and SiHa cell, except at the highest concentration (20mM), which is unlikely to be reached in vivo.

2. The formulated liposomes caused no significant changes in platelet activation, negligible disruption of thrombin generation, and the absence of red blood cell lysis.

3. Their intravenous injection can be considered without fearing major alterations of normal blood function (blood homeostasis).

Acknowledgments

LIÈGE université
Center for Interdisciplinary Research on Medicines

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