Chitosan nanobubbles as a combined chemo-immunotherapy strategy against melanoma

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NEXT-GENERATION DELIVERY INNOVATIONS

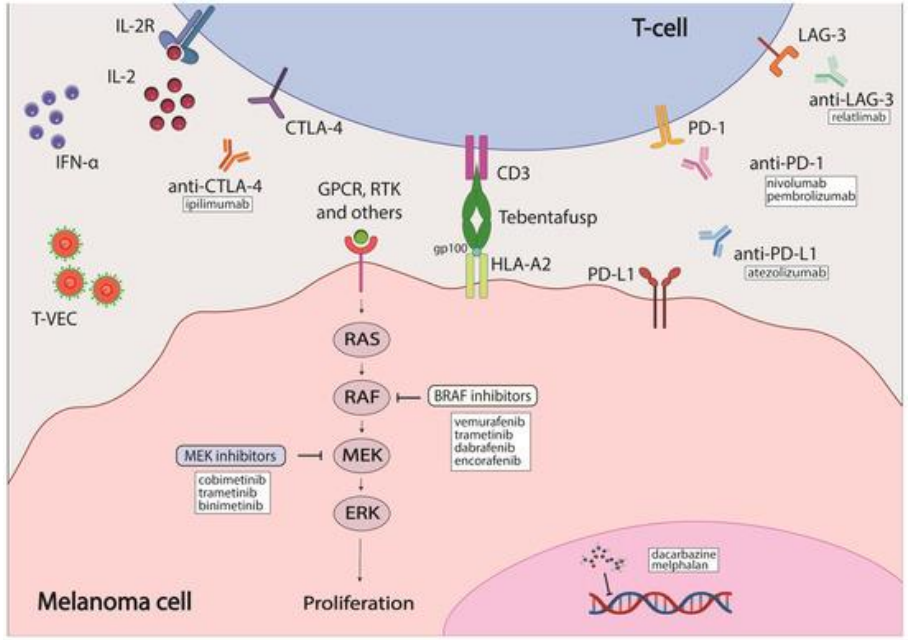
THERAPEUTIC OPTIONS FOR MELANOMA TREATMENT

Cutaneous melanoma is the most lethal form of skin cancer and the advanced disease is poorly responsive to conventional chemotherapy and radiotherapy

Targeted therapy with BRAF/MEK inhibitors and immunotherapy using immune checkpoint inhibitors, capable to increase the anti-tumor immune response by counteracting the tumor-mediated immune suppression, has significantly improved outcomes in melanoma treatment

However, high rate of non-responder patients and occurrence of resistance

Novel valuable alternatives are needed, including combinations of conventional chemotherapies and immunotherapies

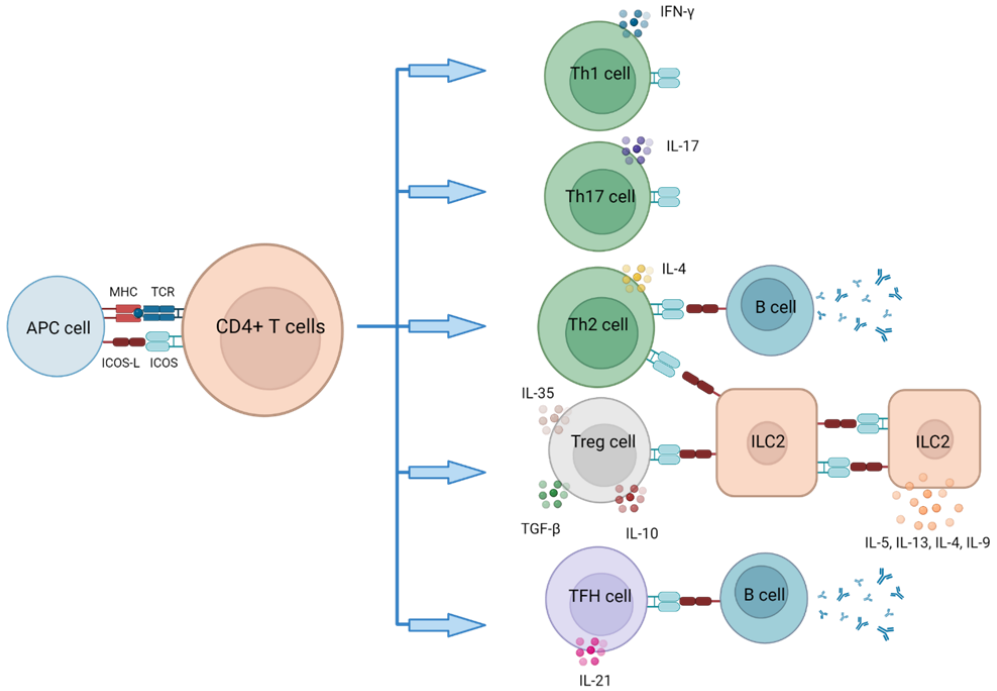


Fateeva et al., Cancers 2024, 16(8), 1571

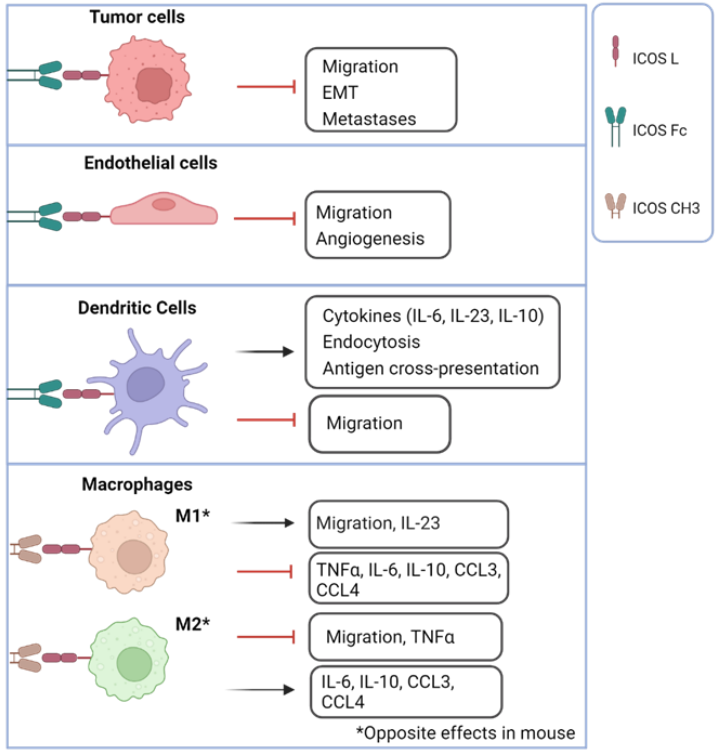
INDUCIBLE T CELL CO-STIMULATOR / ICOS LIGAND SIGNALING

ICOS, inducible T cell costimulator, is an immune checkpoint protein belonging to the CD28 family, expressed on activated T cells.

ICOS costimulation potentiates the effector and regulatory functions of several types of T cells



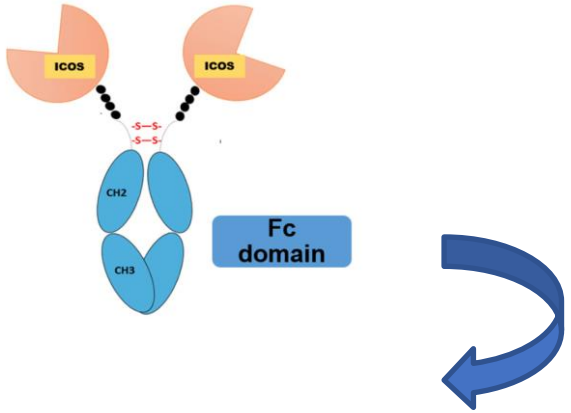
Reverse signaling triggered by ICOS-mediated stimulation of ICOSL



Alves GF, et al., Front Immunol. 2022 Sep 2;13:992614.

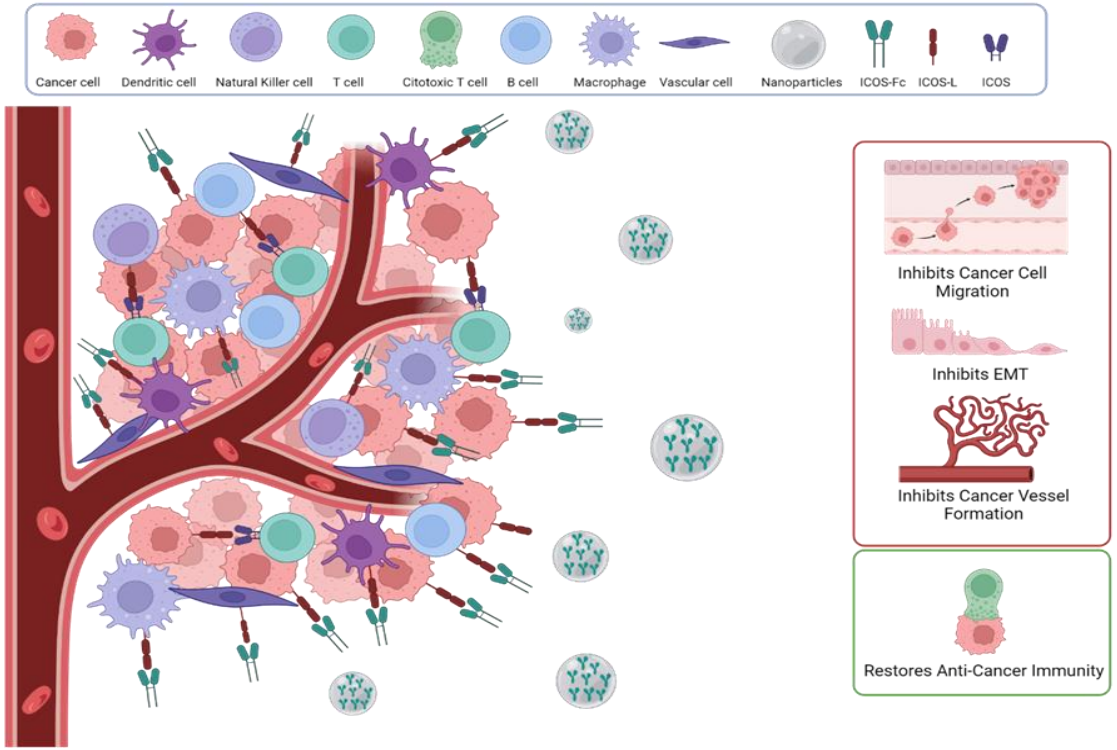
ICOS-Fc ANTICANCER ACTIVITY

ICOS-Fc: recombinant protein composed of the extracellular portion of ICOS fused to the Fc portion of IgG1



Strong anti-tumor activity upon its encapsulation in nanodelivery systems

In vivo effects of ICOS-Fc triggering on tumors



Clemente N, et al., J Control Release. 2020 Apr 10;320:112-124. Monge C et al., Nanomaterials (Basel). 2022 Nov 28;12(23):4233

ICOS-Fc LOADED IN β-CYCLODEXTRIN NANOSPONGES

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Immunotherapy of experimental melanoma with ICOS-Fc loaded in biocompatible and biodegradable nanoparticles

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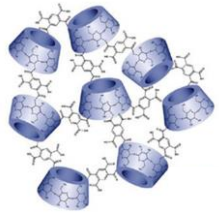
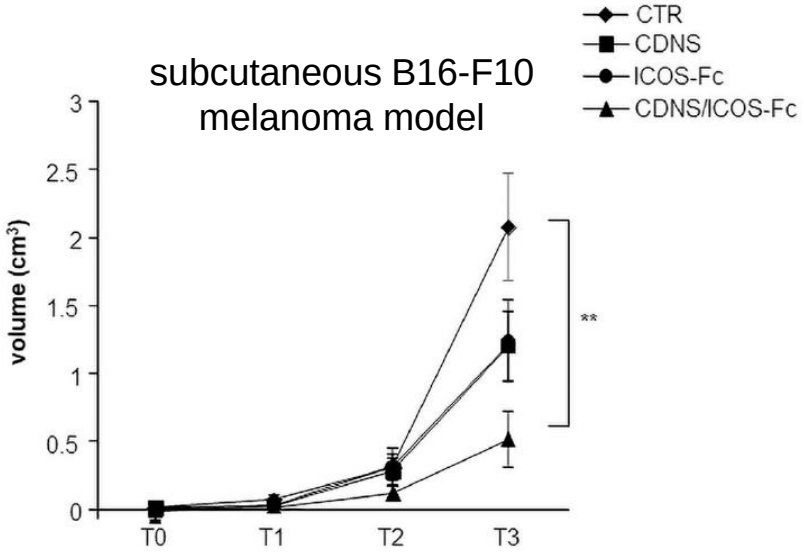
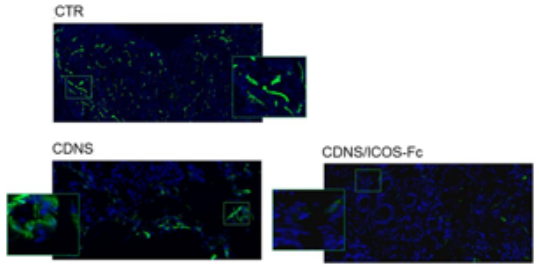


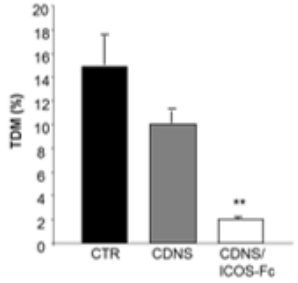
β-CYCLODEXTRIN NANOSPONGES



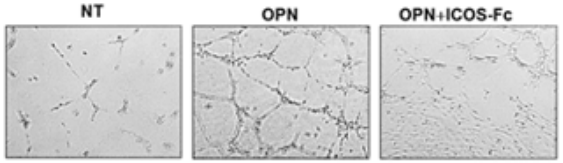
Effect of ICOS-Fc loaded in NS on tumor vascularization

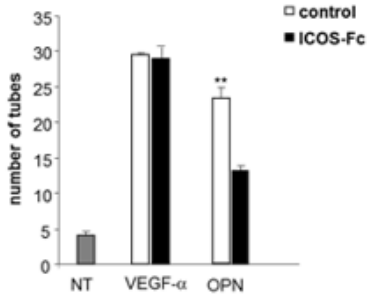


Microphotographs of immunofluorescence staining for anti-CD31 of tumors tissue sections



Tumor microvessel density (TMD) as the CD31-positive area on the tumor sections





In vitro tubulogenesis assay on HUVEC cells

ICOS-Fc loaded NS inhibited tumor angiogenesis, as shown by the decreased tumor vascularization detected in mice treated

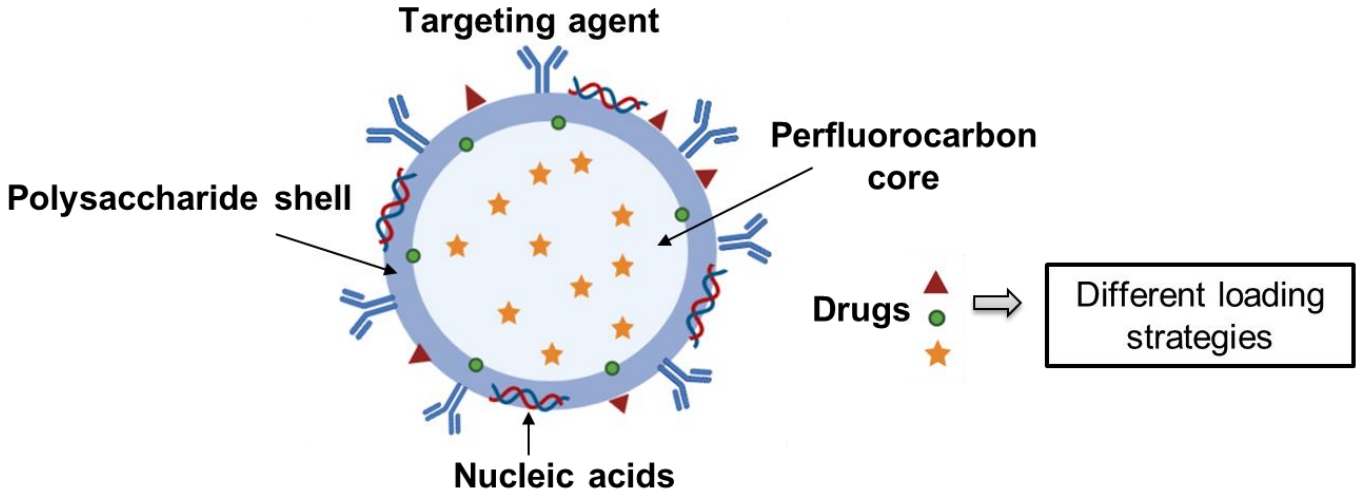
AIM OF THE WORK

Design of polysaccharide nanobubbles as a nanoplatform to combine chemotherapy and immunotherapy for the treatment of melanoma

Development of NBs loaded with paclitaxel and decorated with the immunomodulatory agent ICOS-Fc

POLYSACCHARIDE-SHELLED NANOBUBBLE TECHNOLOGY

Nanobubbles are spherical core/shell structures filled by a gas or vaporizable compounds, comprising a gas core and a polymer shell



➢ **Multifunctional drug delivery platform:**

- Delivery of drugs, gas and genetic material (anticancer, antiinfective, oxygen, DNA, siRNA)
- Stimuli-triggered release (US, ESW)
- Active/passive targeting
- Echogenic properties
- Theranostic system

Argenziano M, et al. Nanomedicine (Lond). 2025 Feb;20(3):255-270
Argenziano M, et al. Drug Deliv Transl Res. 2022 Aug;12(8):2007-2018

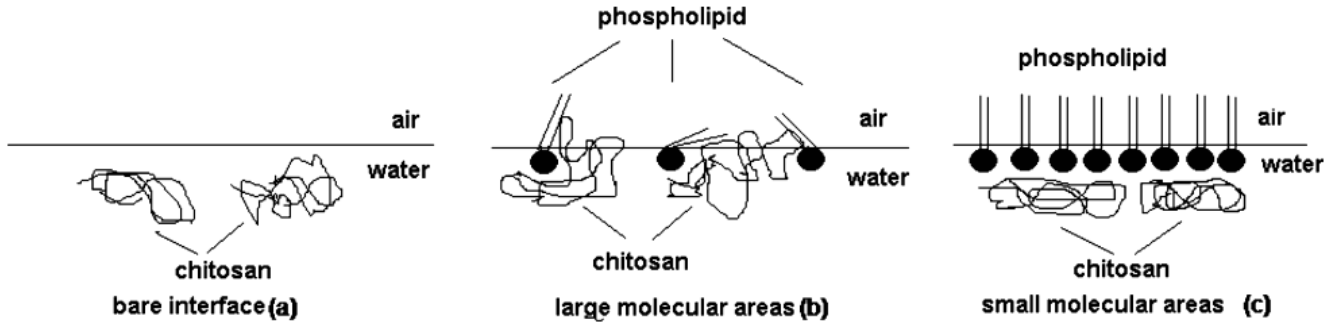
POLYMER-SHELLED NANOBUBBLE STRUCTURE DESIGN

Phospholipids/co-surfactants monolayer to decrease the interfacial tension

The surfactant selection considered the capability in reducing the perfluorocarbon interfacial tension → phospholipid molecules:

- Dimyristoylphosphatidylcholine (DMPC) → C14 alkyl chain lenght
- **Dipalmitoylphosphatidylcholine (DPPC)** → C16 alkyl chain lenght
- Distearoyllphosphatidylcholine (DSPC) → C18 alkyl chain lenght

Phospholipid interfacial monolayer can adsorb charged polymers



Interaction of Chitosan with lipid monolayer

Helder et al Langmuir, 2005, 21,5475;
Pavinatto F. et al., Biomacromolecules 2007, 8

BIOPOLYMERS FOR NB SHELL MANUFACTURING

- **Evaluation of polysaccharide/lipid film interaction**

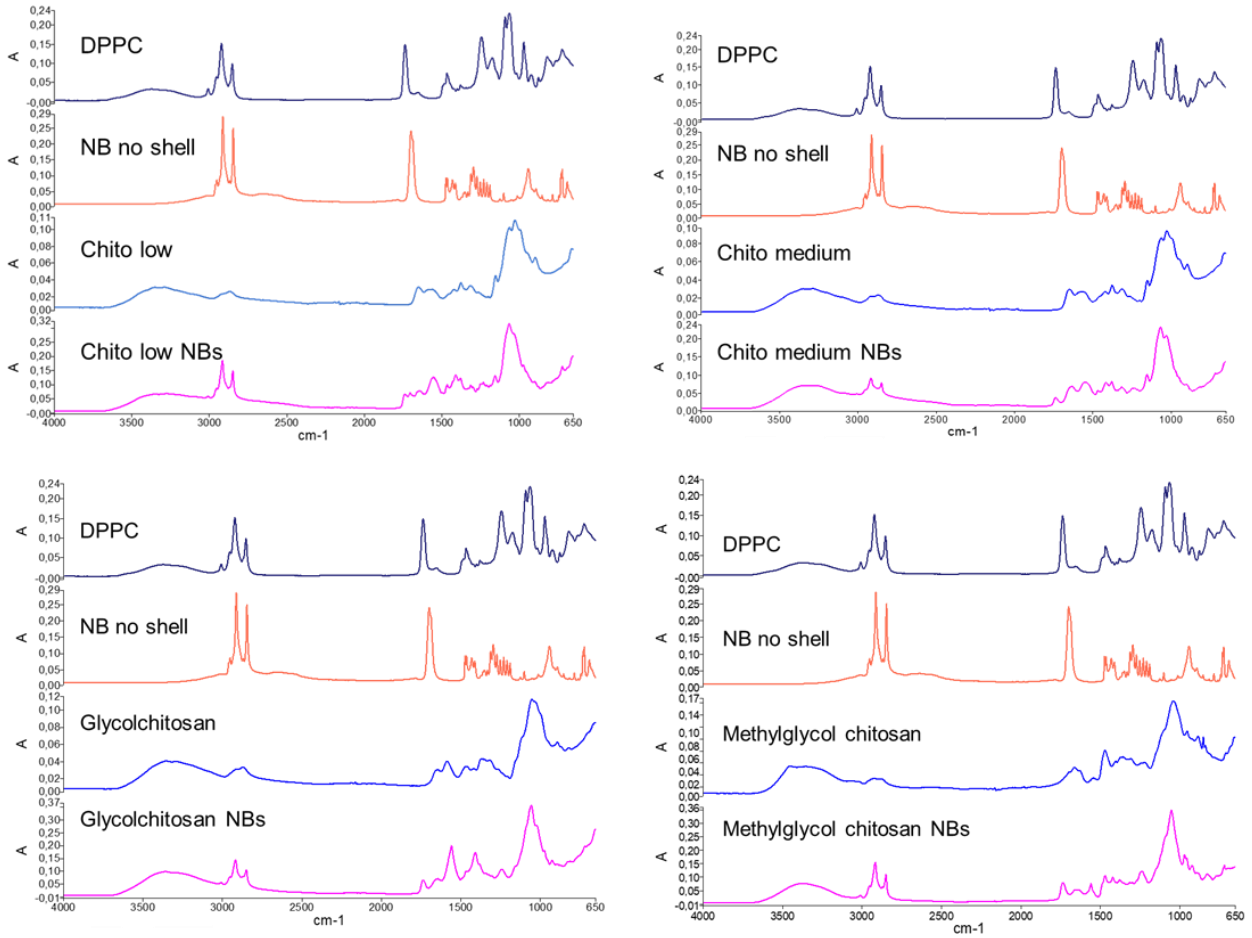
Calculated surface pressure (π*) of polysaccharide-shelled nanobubbles

Sample -	Surface presssure (π*) (mN/m)
Chitosan low MW	- 5.27
Chitosan medium MW	- 9.74
Glycolchitosan	- 4.64
Methylglycolchitosan	- 12.44
Dextran sulfate	- 9.47
Dextran-DEAE	- 12.98

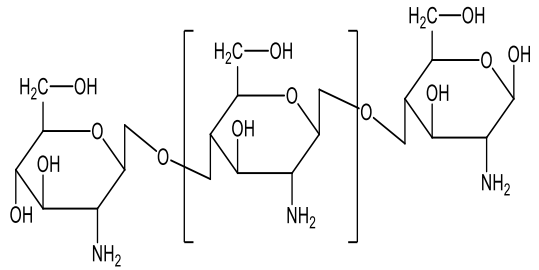
Negative π* value: polymer interact and increased the packing of lipid molecules at the oil-water interfaces

- **Physico-chemical parameters**
- **Stability**
- **Biocompatibility**

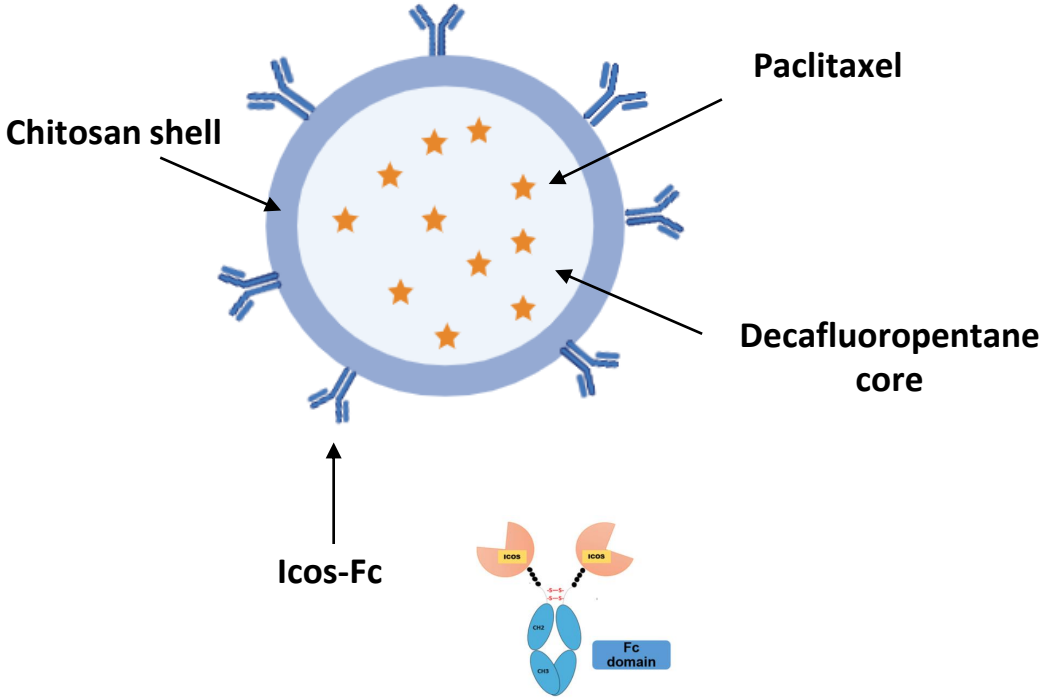
FTIR analysis

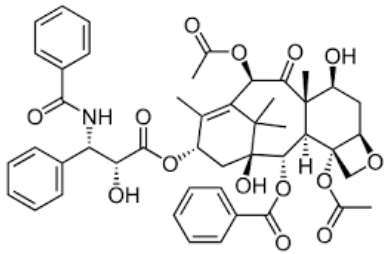


PACLITAXEL-LOADED ICOS-Fc DECORATED NB FORMULATIONS



Chitosan
(low molecular weight,
DD (%)75–85, 50–190KDa)

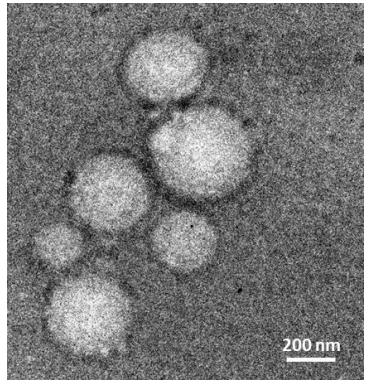




Paclitaxel (PTX)
MW: 853.9 g/mol
logP: 2.5

- NB conjugation with ICOS-Fc via ECD/NHS coupling chemistry

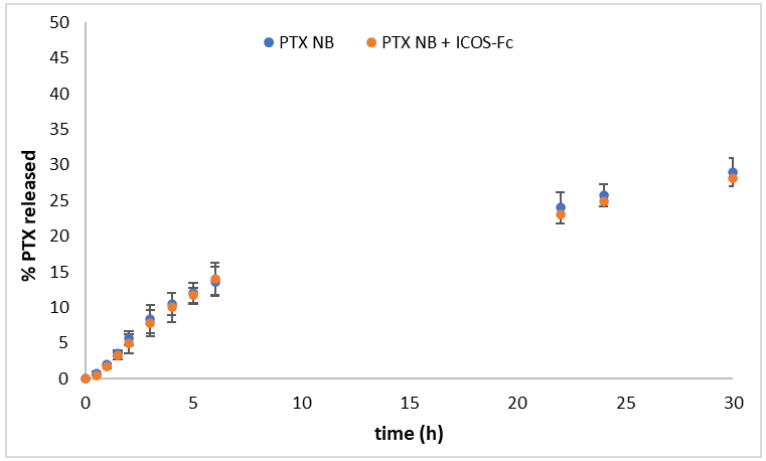
IN VITRO CHARACTERIZATION OF NB FORMULATIONS



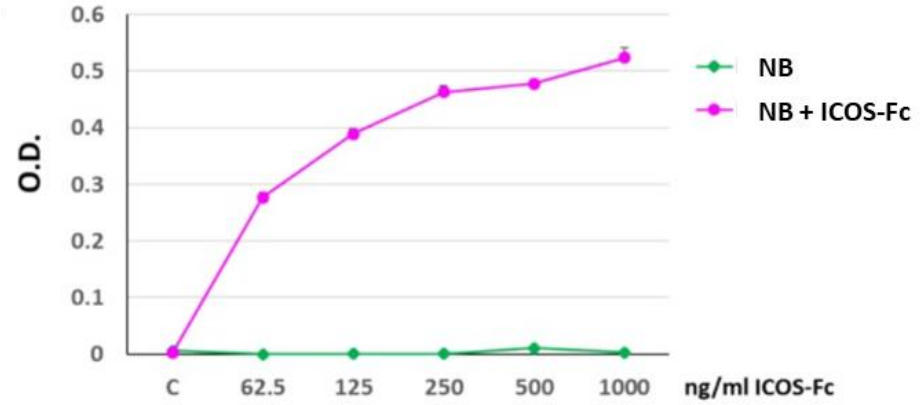
TEM image of NBs

	Average diameters ± SD (nm)	Polydispersity index ± SD	Zeta potential ± SD (mV)
empty NB	305.3 ± 10.6	0.20 ± 0.02	32.14 ± 1.40
PTX-NB	310.7 ± 11.8	0.22 ± 0.03	30.90 ± 1.25
NB + ICOS-Fc	304.5 ± 8.5	0.21 ± 0.02	26.86 ± 1.58
PTX-NB + ICOS-Fc	308.2 ± 9.4	0.22 ± 0.02	27.05 ± 1.06

DRUG LOADING: 5.10%
ENCAPSULATION EFFICIENCY: 95%



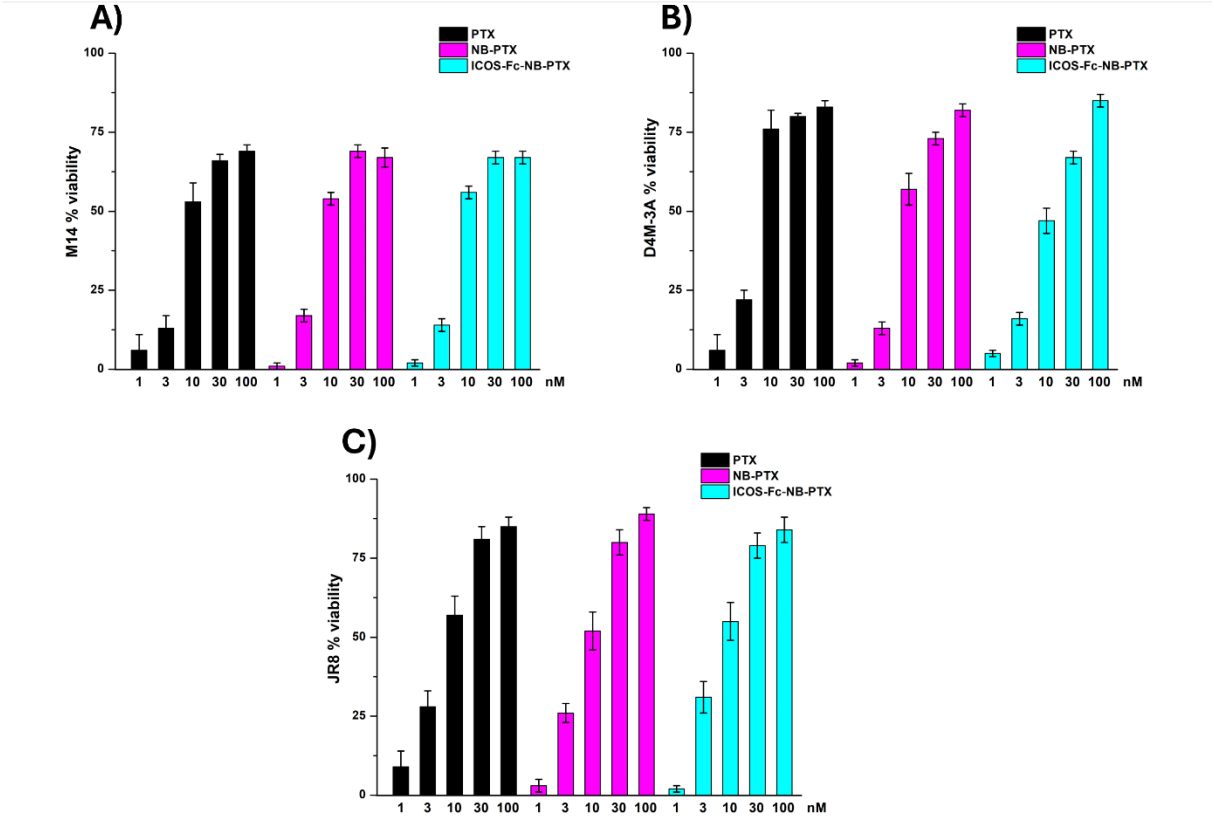
Slow and prolonged PTX *in vitro* release profile with no burst effect

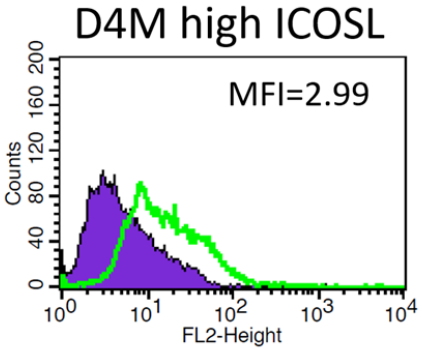


The presence of ICOS-Fc on NB surface has been assessed by ELISA, by using a ICOSL-HIS coated plate.

IN VITRO CELL EXPERIMENTS

- Evaluation of NB effect on **cell viability** of human JR8 and M14 melanoma cells and mouse D4M-3A melanoma cells, carrying the BRAFV600E mutation, by MTT assay





ICOSL expression tested in D4M-3A cells by flow cytometry

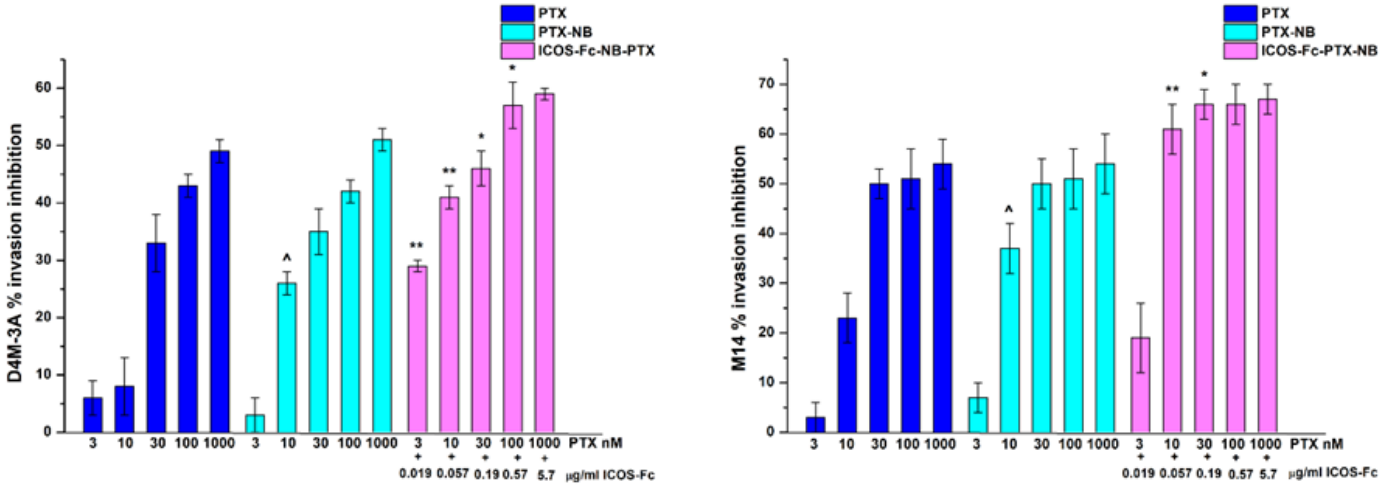
ICOSL levels > 2MFI-R → cut off level to detect the ICOS-Fc effect *in vitro*

Maintenance of PTX cytotoxicity after its encapsulation in NBs

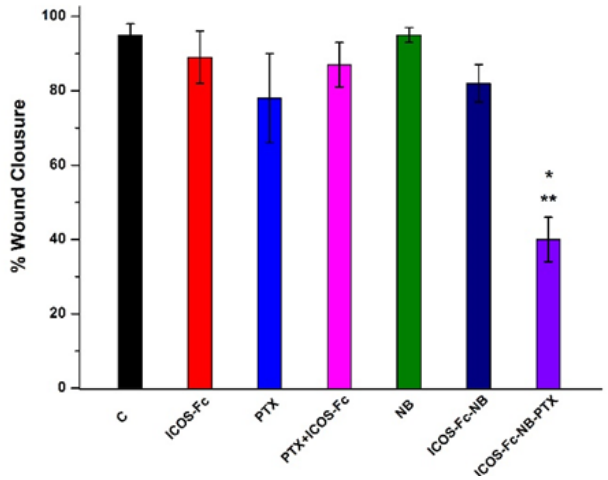
IN VITRO CELL EXPERIMENTS

- Evaluation of NB effect on **cell invasion and migration** by Boyden Chamber and wound healing assays on M14 and D4M-3A melanoma cells

Invasion assay



Wound healing assay

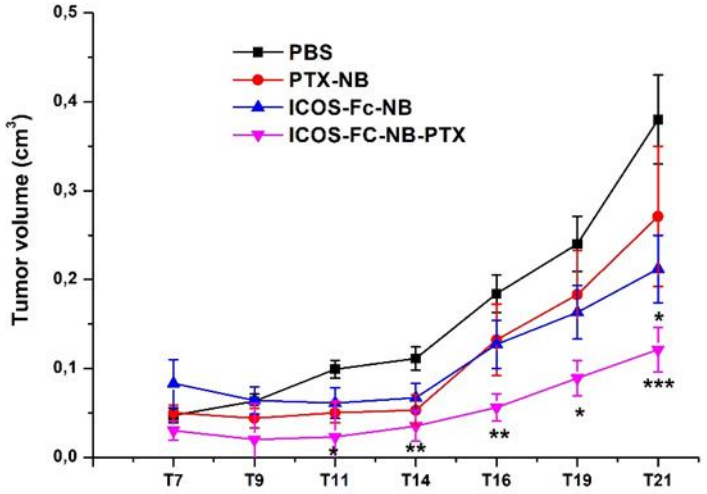


Higher inhibition of cell invasion and migration for PTX-loaded NBs decorated with ICOS-Fc

IN VIVO THERAPEUTIC EFFECT OF ICOS-Fc DECORATED PTX-NBs

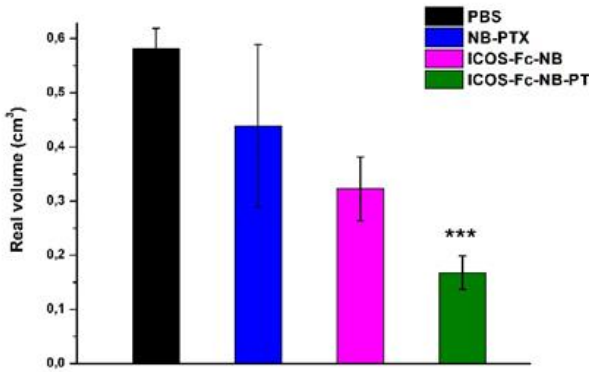
- i.v. injection of PTX free or loaded in NBs decorated or not with ICOS-Fc in C57/BL6 mice injected subcutaneously with D4M-3A cells ($1x10^6$) expressing low levels of ICOSL

Effects of the NB treatments on tumor growth time progression *in vivo* and on final tumor weight and volume measured *ex vivo*



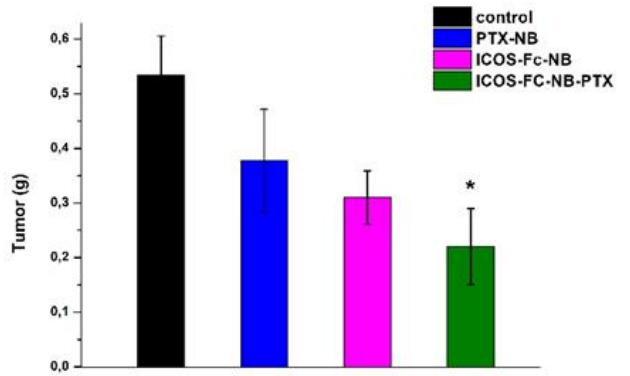
* p<0.05; ** p<0.005; *** p<0.0005

TUMOR VOLUME



*** p<0.0005

TUMOR WEIGHT



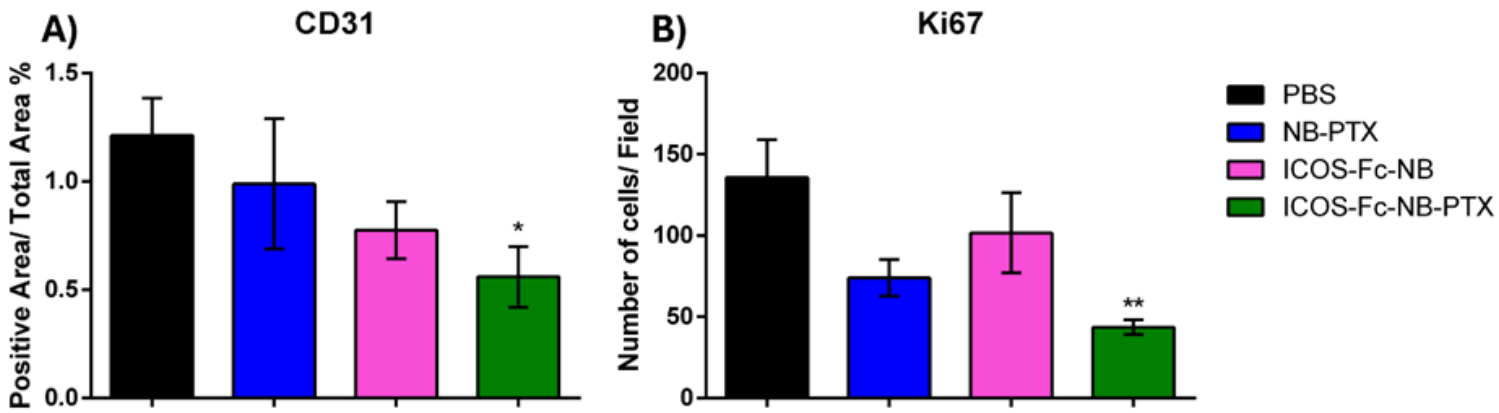
* p<0.05

PTX-loaded NBs decorated with ICOS-Fc significantly decreased the tumor growth compared to the free drugs

Additive anti-neoplastic effects when co-delivered in NBs

EFFECT OF ICOS-Fc DECORATED PTX-NBs ON PROLIFERATION AND ANGIOGENESIS MARKERS

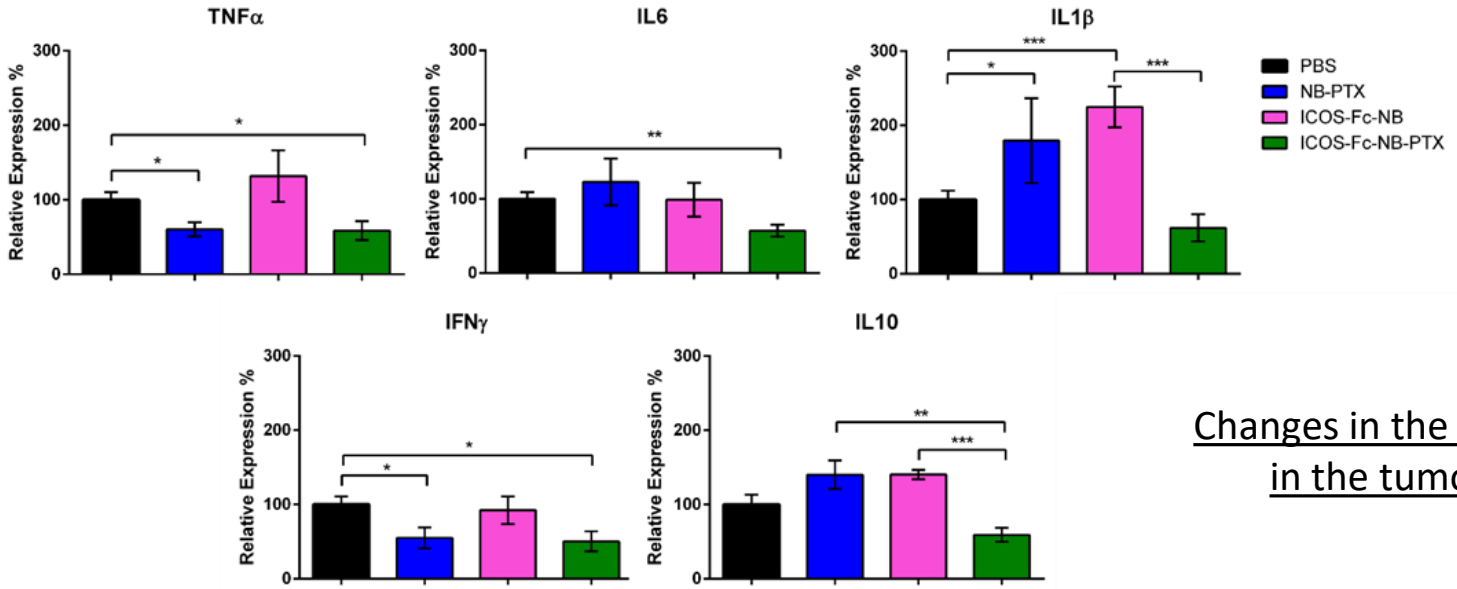
- Immunohistochemical analyses performed in the excised tumors to assess the number of $Ki67^+$ tumor cells marking proliferating cells, and $CD31^+$ blood vessels



Lowest expression of the proliferation marker Ki-67 and the lowest development of CD31+ blood vessels in tumors of mice treated with ICOS-Fc decorated PTX-NBs

EFFECT ON MODULATION OF THE TUMOR MICROENVIRONMENT IMMUNE COMPONENTS

- Expression of several cytokines was assessed at the mRNA level by RT PCR in lysates obtained from the tumor masses



Changes in the cytokine expression pattern in the tumor mass after treatment

* p<0.05; ** p<0.005; *** p<0.0005

Low levels of IL-10 and IL-6 induced by treatment with ICOS-Fc decorated PTX-NBs → impact in decreasing the immunosuppression and the angiogenesis mediated by these cytokines respectively

CONCLUSIONS

- ✓ Development of new chitosan nanobubble system with physico-chemical and stability properties suitable for the co-delivery of paclitaxel and ICOS-Fc
- ✓ The combined treatment with PTX loaded into chitosan NBs surface decorated with ICOS-Fc displays a strong anti-neoplastic activity *in vivo* against a mouse melanoma model using the BRAF-mutated D4M-3A cell line
- ✓ PTX-loaded NBs decorated with ICOS-Fc are highly effective in suppressing tumor growth, by inhibiting tumor cell proliferation and vessel formation
- ✓ Inhibitory effects on the immunosuppressive tumor microenvironment

Chitosan nanobubbles might represent a promising tool for the co-delivery of immunomodulatory molecules in support of chemotherapeutics

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Thanks for your kind attention

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