H2020-ITN THERACAT (765497)

Work Package Number	WP6	**Task Number**	T6.2	**Deliverable Number**	D6.2	**Lead Beneficiary**	TAU
Deliverable Title	Results of the biocompatibility tests for the catalysts						
Contractual Delivery Date	31/12/2019	**Nature**	Report			**Dissemination Level**	CO
Actual Delivery Date	19/12/2019	**Contributors**	TAU				

Overview/Abstract

We have successfully evaluated the biocompatibility of the polymeric carriers synthesized and characterized by ESR2 (WP3-4) performing proliferation/viability assays (MTT) and red blood cell (RBC) Lysis test. In the next step, the most promising nanocarriers in terms of prodrug encapsulation or catalytic efficiency will be selected to determine their ex vivo and in vivo biological activity, including tumour accumulation, biodistribution in healthy organs, safety profile (WBC count, neurotoxicity, blood chemistry), and antitumor activity (WP5-6).

Explanation for large delay in submitting deliverable

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1. Introduction

The field of nanomedicine comprises the utilization of nanoparticles for diagnostic and therapeutic purposes. In general, nanoparticles are used as delivery vehicles for therapeutic agents, e.g. small molecules, proteins, peptides, and nucleic acids.[1,2] During the past two decades, a plethora of nano-based drugs has been designed to treat various diseases such as neurological disorders, diabetes, infectious diseases, allergy and specially cancer.[1,3] A growing number of nanomedicines have received regulatory approval such as Doxil and Abraxane and many more show promise for future clinical translation. In this context, it is important to evaluate the safety of nanocarriers in order to achieve biocompatibility and desired activity. However, it is unwarranted to make generalized statements regarding the safety of nanoparticles, since they show different morphologies and are based on several materials including lipids[4], metal[5,6], silica[7], polymers[8] and proteins[9], and some of them can display undesired toxicity.
Therefore, the safety of the vehicles developed under the THERACAT-ITN in charge of delivering the prodrugs and the catalyst to the tumor site will need to be assessed. The best vehicle candidates will be further chosen to trigger the biorthogonal deprotection of the prodrug and consequently the antitumoral activity.

2. Objective

Our main goal is to understand the biological activity of the nanocarriers that will be used both to encapsulate drugs or to coordinate the metallic catalyst. The evaluation of their biocompatibility will allow us to discard the candidates which present cytotoxic effects and to choose the inert and most promising carriers in terms of prodrug encapsulation and catalytic efficiency that will further be evaluated by *in vivo* experiments.

3. Methodology

Cell lines. MCF-7 human breast cancer cells and and EMT6 murine mammary adenocarcinoma were obtained from the American Type Culture Collection (ATCC). MCF-7 and EMT6 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg/mL Penicillin, 100 U/mL Streptomycin, 12.5 U/mL Nystatin (PSN), and 2 mM L-glutamine (L-Glu). Cells were grown at 37°C; 5% CO_2.

MTT test. MCF-7 cells were plated onto a 96-well plate (4000 cells/well) in DMEM supplemented with 10% FBS, 2 mM L-glutamine and incubated for 24 h (37°C; 5% CO_2). Then, cells were treated with the different nanocarriers provided by ESR2 at several concentrations. Following 72 h, amount of viable cells was assessed by modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinium bromide (MTT) assay. Thirty µL of 3 mg/mL MTT solution in PBS were added to the wells and incubated for 4-6 h, the medium was then replaced by 200 µL of dimethyl sulfoxide (DMSO) to dissolve the formazan crystals formed, incubated for 20 min at 37°C. Absorbance of the solution was measured at 560 nm by SpectraMax® M5 plate reader (Molecular Devices LLC., Sunnyvale,

California, USA). Percent of viable cells was normalized to the viability of non-treated cells (100% viability).

Red blood cell (RBC) lysis assay. RBC solution 2% (m/v) from five C57Bl6 mice was incubated with serial dilutions of each type of nanocarriers for 1 h at 37°C. The highest nanocarrier's concentration was 5 mg/mL. Sodium dodecyl sulphate (SDS) was used as a positive control and dextran (Mw 70 kDa) as a negative control. Following centrifugation, the supernatant absorbance was measured at 550 nm using a SpectraMax M5e plate reader (Molecular Devices, CA, USA). The results were expressed as percentage of hemoglobin released by % (m/v) of Triton-X100 (100% lysis).

Internalization assay in 3D tumour spheroids. We created a 3D spheroid co-culture system of tumour cells with stromal cells in thick Matrigel as we previously described for our unique hanging-drop spheroids[10]. Briefly, cell suspension of tumour cells, alone or in co-culture with stromal cells (80,000 cells/mL) was prepared DMEM supplemented with 0.24 w/v% methyl cellulose. Cells were deposited in 25 µL droplets on the inner side of a 20 mm dish and incubated for 48 h at 37°c when the plate is facing upside down to allow for spheroid formation. Spheroids were then embedded in Matrigel, seeded in a 96-well plate and monitored for micelles internalization using EVOS FL Auto cell imaging system (ThermoFisher Scientific). The internalization assays are being carried out with the inert and most promising micelles, in terms of prodrug encapsulation and/or catalytic efficiency. The micelles labelled with Cy5 are incubated until 24 h with the spheroids and the internalization is assessed at different time points.

4. Results of the biocompatibility tests for the micellar carriers

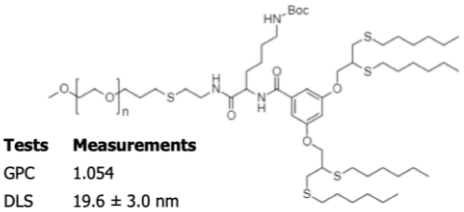
Six different micellar polymeric carriers were provided by ESR2. Four non-degradable polymers (C6, C7, C8 and C12) and two degradable polymers (6x6Cd and 4x8Cd) were synthesized (**Figure 1**). All the micelles have similar hydrodphilic block (5 kDa mPEG).

Cell viability of MCF-7 cells when expossed to several micellar systems. We evaluated the potential toxicity of each micelle type in terms of cellular proliferation using MTT assay. None of the non-degradable micelles (C6, C7, C8 and C12) showed cytotoxicity up to 1 mg/mL (**Figure 2**). However, the 4x8Cd to showed some cytotoxic effects at concentrations higher than 0.01 mg/mL.

RBC Lysis test. We also tested the potential ability of each type of micelle to lysate RBC in order to avoid any undesirable effect when going through *in vivo* experiments. None of the carrier induced the lysis of the red cells making them biocompatible for iv injections (**Figure 3**).

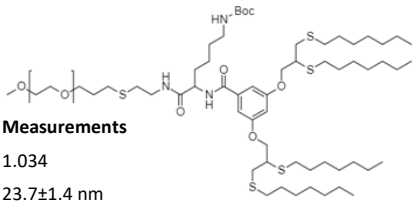
***Ex vivo* 3D tumour models**. We created 3D spheroids from EMT6 cells co-cultured with stromal cells and currently we are monitoring the ability of each nanocarrier candidate, covalently conjugated to Cy5, to internalize into tumour spheroids during the first 24 h. The C12 micelles internalized into EMT6 3D spheroids in Matrigel within 24 h (**Figure 4**).

PEG-C6 Hybrid



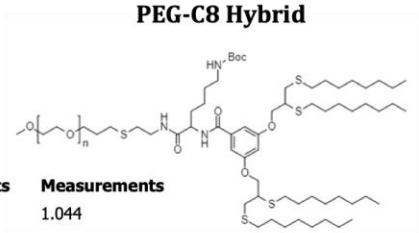
Tests	Measurements
GPC	1.054
DLS	19.6 ± 3.0 nm
CMC	4±2 μM

PEG-C7 Hybrid



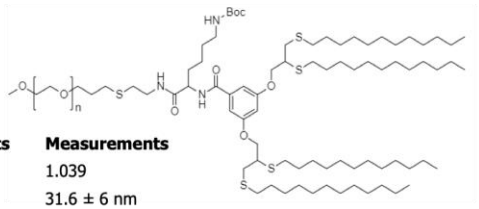
Tests	Measurements
GPC	1.034
DLS	23.7±1.4 nm
CMC	4±2 μM

PEG-C8 Hybrid



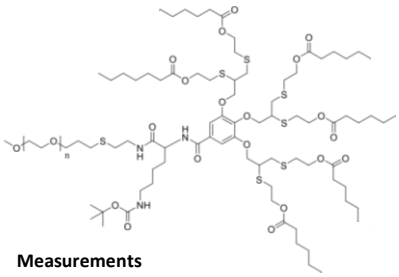
Tests	Measurements
GPC	1.044
DLS	29.9 ± 3.2 nm
CMC	4±1 μM

PEG-C12 Hybrid



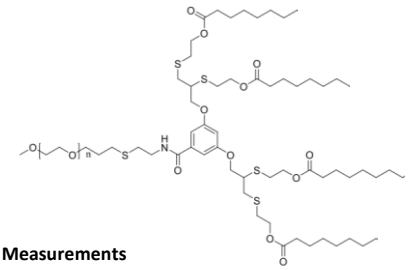
Tests	Measurements
GPC	1.039
DLS	31.6 ± 6 nm
CMC	3±1 μM

Deg 6x6Cd



Tests	Measurements
DLS	20.4.±1.2 nm

Deg 4x8Cd



Tests	Measurements
DLS	18.9±1.6 nm

Figure 1. Chemical structures and characterization of the non-degradable (C6, C7, C8 and C12) and degradable (6x6C and 4xC8) micelles provided by ESR2. All the micellar carriers show high monodispersity with hydrodynamic diameter that ranges between ca. 19 nm and 32 nm.

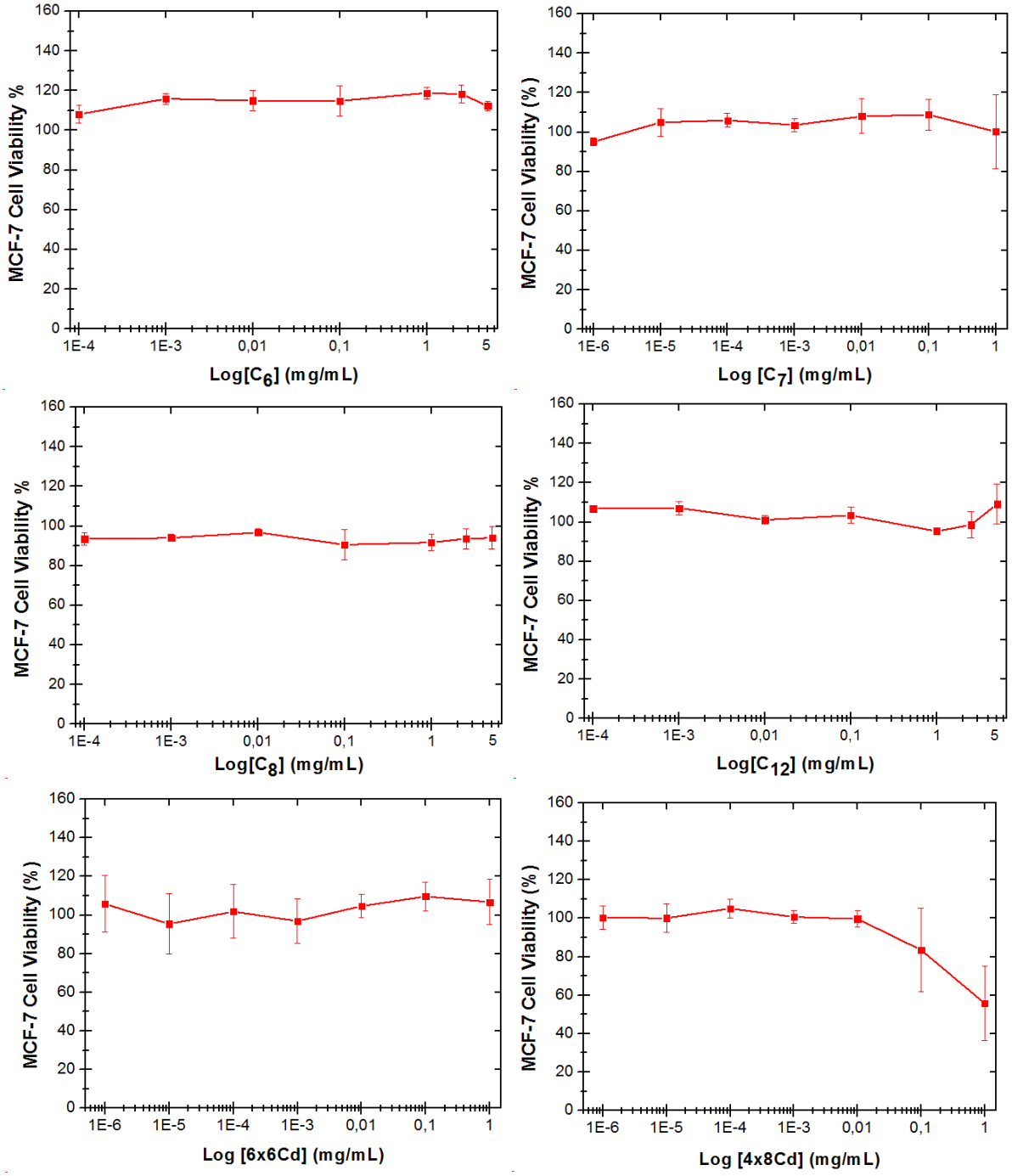


Figure 2. Cell viability of MCF-7 cells assesed with MTT test after incubation with each micelle. None of the non-degradable micelles (C6, C7, C8 and C12) show cytotoxic effects up to 1 mg/mL whereas the 4x8 Cd starts to show cytotoxicity at concentrations higher than 0.01 mg/mL. Mean ± SD (N = 3 independent experiments).

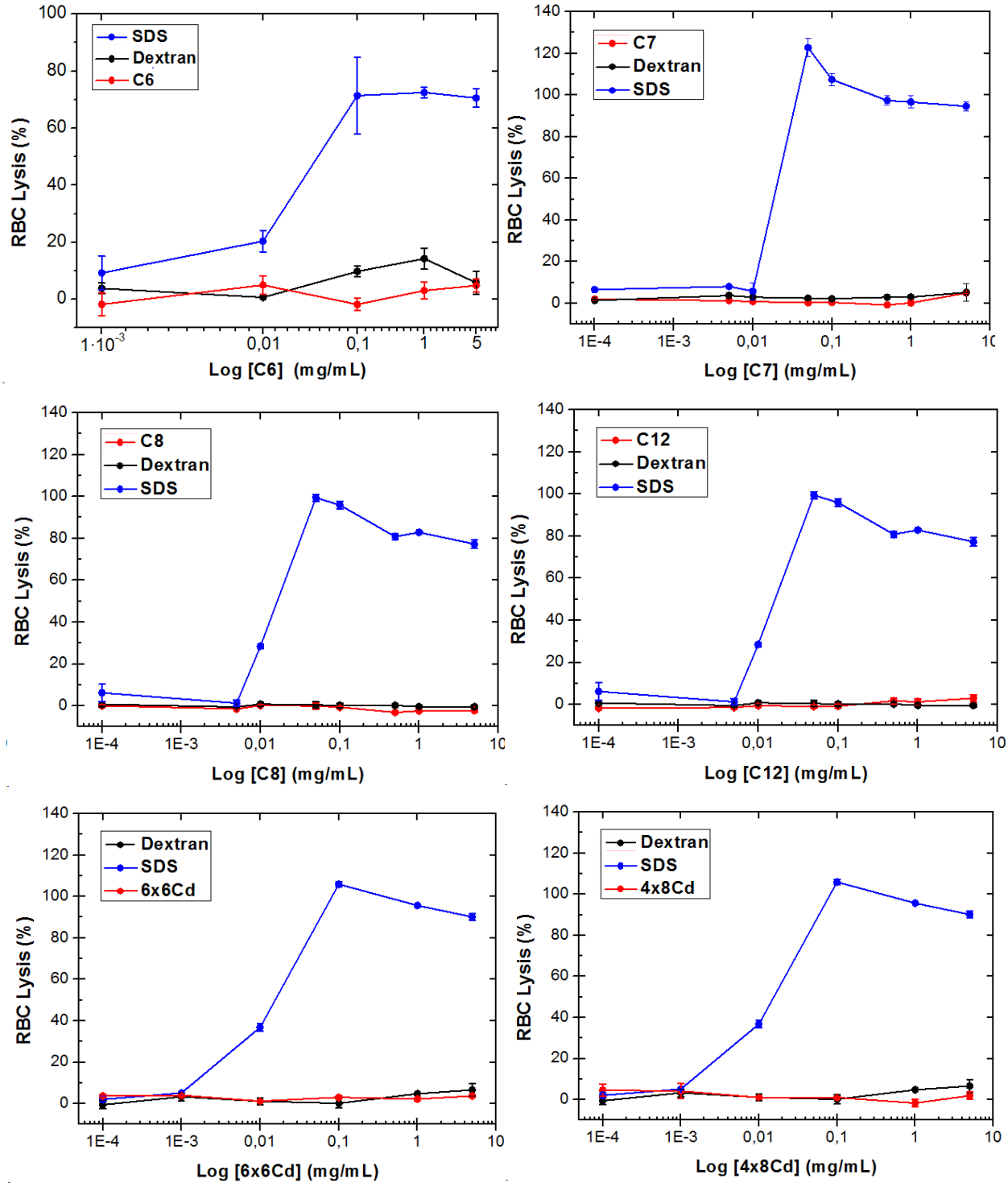


Figure 3. RBC Lysis assay following 1 h incubation. None of the polymeric micelles (C6, C7, C8, C12, 6x6Cd and 4x8Cd) promoted RBC lysis at concentrations up to 5 mg/mL. Sodium dodecyl sulphate (SDS) was used as a positive control and dextran as a negative control. Mean ± SD (N = 3 independent experiments).

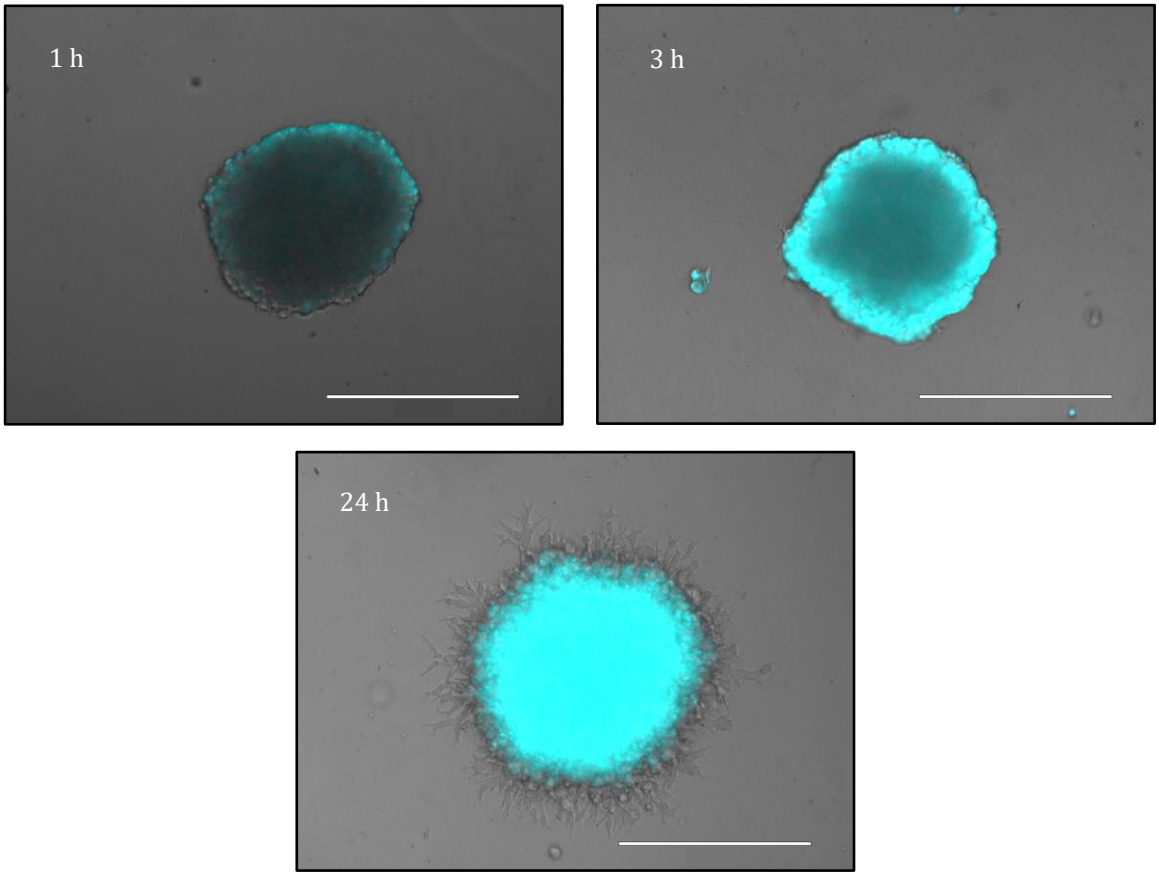


Figure 4. Internalization on 3D EMT6 tumor model. C12-Cy5 micelle completely internallizes into EMT6 3D spheroids within 24 h of incubation. Scale bar 400 µm.

5. Summary

We have successfully assessed the biocompatibility of several nanocarriers, which at the next step will be loaded with the catalysts and prodrugs. Once we achieve the encapsulation of the prodrugs and coordination the catalyst (WP 3-4), the compounds will be evaluated *in vivo* regarding their biological activity, tumour accumulation, biodistribution in healthy organs, safety profile (WBC count, neurotoxicity, blood chemistry) and antitumor activity (WP 5-6).

6. References

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