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Assessment of nanobiodevice toxicity 

in vitro and in vivo

Deliverable 5.2.2

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Section 1 – Overview

We present here the second report on Task 5.2 which consists in assessing the in vitro toxicity of components of the nanodevice and in a second step, the toxicity of the whole device in vivo on animals. The first deliverable, written in August 2011 (M19) presented the first data on the cytotoxicity associated with liposomal and BSA nanoparticles using two cancer cell lines: folate receptor negative (MDA-MB 435) and positive (MDA-MB 468) and a human skin fibroblast cell line BJ5Ta. The aim was to prove that the nanoparticles produced per se, prior to incorporating drugs/siRNAs, do not affect the viability of the cells. We also aimed to establish the concentration at which the nanoparticles are not cytotoxic to be used in further studies.

1.1. Objectives

This deliverable presents in vitro toxicity data on various non target cells of different origin (hepatic, renal, pulmonary, monocytic and fibroblastic) associated with liposomal and BSA nanoparticles, containing or not anti-inflammatory drug inside and folate at the surface. A comparison is made with the cytotoxicity of liposomes on a cellular model of activated macrophages, which represents a target cell for the nanobiodevices.

1.2. Problems encountered

As the M12 deliverable for task 5.2. was only provided at M19, the M24 deliverable for task 5.2. was also slightly delayed and provided at M26 instead of M24. It is recalled that, task 5.2. is highly dependent on the advancement and productions of WP1 (folate-based nanobiodevices) and WP2 (antibody bispecific-based nanobiodevices).

Section 2 – Work performed

2.1. Preparative meetings carried out

Last year, in April 2011, INERIS had a meeting with UMINHO in Braga and defined an action plan for toxicity testing: first experiments were carried out in vitro in order to limit the use of animal testing. Cytotoxicity were assessed on various non target cells using routine cytotoxicity tests (namely metabolic tests such as SRB, MTT assay and derivatives). INERIS proposed to evaluate the cytotoxicity using the metabolic assay Alamar blue™ on four different cell lines: pulmonary cells (A549), hepatic cells (HepG2), renal cells (Caki-1) and a monocytic cell line (THP-1). MTS and SRB tests were performed at UMINHO on the BJ5ta cell line (human fibroblasts). Oxidative stress was assessed by measuring the presence of...
reactive oxygen species using the DCFH-DA probe assay (UMINHO). Inflammatory properties were also tested by dosing cytokines (TNF-α, IL-1β, IL-6 and IL-8) in cell culture media (INERIS). Further *in vitro* experiments will be performed in order to assess the hemolytic properties *in vitro* and the genotoxicity (INERIS).

### 2.2. Experimental details and results

Experiments were carried out on two kinds of nanospheres (BSA-based or liposomes LP), containing or not folate (FA) at their surface and anti-inflammatory drug (celecoxib or CORM-2) inside. The toxicity of these nanospheres was assessed on non target cells from various origin, as described in 2.1. section.

Cell viability was assessed by Alamar blue™ assay (INERIS), MTS or SRB assays (UMINHO). Alamar blue™ is a colorimetric assay detecting the reducing environment that indicates metabolic active cells. It is based on the reduction of resazurin (that is blue in color and virtually non-fluorescent) into resorufin, a compound that is red in color and highly fluorescent. Oxidative stress was assessed by measuring the presence of reactive oxygen species using the DCFH-DA probe assay (UMINHO).

In a second step, the pro-inflammatory potential of the nanospheres was tested at INERIS by measuring the release, in the cell culture medium, of 4 cytokines involved in inflammation (TNF-α, IL-1β, IL-6 and IL-8).

Immunostaining of THP-1, A549, HepG2 and Caki-1 with human FαR2 antibody (Exbio) revealed a positive staining for A549 only, suggesting the presence of the folate receptor on this cell line (figure 1).

![Fluorescent microscopy](image)

**Figure 1.** Fluorescent microscopy shows the presence of folate-receptor (in green) on the membrane of A549 cells (incubation with human FαR2.Clone24cl.IgGI from Exbio and then with anti-mouse IgG (Fc specific) F’ (ab’) 2 fragment-FITC antibody produced in goat. Référence: F2772 (Sigma Aldrich)). Nucleus are stained in blue by Hoechst (INERIS).
A. Cytotoxicity and oxidative stress results

A.1. BSA-based nanoparticles

The cytotoxicity of BSA-based nanoparticles was assessed on THP-1 and A549 cells (INERIS) and BJ5ta (UMINHO).

For THP-1 cells, the profile of cytotoxicity for the 3 formulations tested is the same. No cytotoxicity is observed for 3h and 24h of exposure. For 48h of exposure, significant cytotoxicity is noted at concentrations of 5 mg/mL (Figure 2).

![Cell viability graphs](https://example.com/cell-viability.png)

**Figure 2.** Cytotoxicity of BSA nanospheres on THP-1 cells assessed by Alamar blue™ assay. Values are the mean mean ± SD of 2 independent experiments. * significantly different from control value (p<0.05, Dunett’s test) (INERIS).
A549 cells seem to be more sensitive to BSA nanospheres with a cytotoxicity noted after 24 h and 48 h exposure for a concentration of BSA of 2.5 and 5 mg/ml (Figure 3). As for THP-1 cells, the profile of cytotoxicity for the 3 formulations tested is the same.

![Figure 3](image.png)

**Figure 3.** Cytotoxicity of BSA nanospheres on A549 cells assessed by Alamar blue™ assay. Values are the mean ± SD of 3 independent experiments. * significantly different from control value (p<0.05, Dunett’s test) (INERIS).

The IC50 for A549 cells (calculated by Graph Pad Prism software) are reported in the table below:

<table>
<thead>
<tr>
<th>IC50 (mg/mL)</th>
<th>BSA-FA</th>
<th>BSA-Celecoxib</th>
<th>BSA-FA-Celecoxib</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h</td>
<td>2.6 ± 0.3</td>
<td>1.8 ± 0.2</td>
<td>1.6 ± 0.8</td>
</tr>
<tr>
<td>48h</td>
<td>2.4 ± 0.3</td>
<td>2.0 ± 0.3</td>
<td>1.2 ± 3.0</td>
</tr>
</tbody>
</table>

Table 1: IC50 values of the 3 formulations of BSA nanospheres for A549 cells (INERIS).
The formulations with encapsulated celecoxib as expected tend to be a little more cytotoxic than the BSA-nanospheres without anti-inflammatory drug inside. The presence or absence of folates at the surface does not modify significantly the cytotoxicity of BSA-nanospheres containing celecoxib, suggesting that the presence of folate receptor is not involved in the greater sensitivity of A549 cells.

The encapsulation of different concentrations of CORM-2 was tested, and the best condition was chosen (UMINHO). The cytotoxicity associated with BSA nanospheres containing CORM-2 was analyzed in normal human skin fibroblasts by the MTS assay (metabolic test) and the induction of oxidative stress with the DCFH-DA probe. The range of nanoparticle concentration tested span from 75 to 900 µg/ml and no associated toxicity was detected (Figures 4 and 5).

**Figure 4: Cytotoxicity** of nanosphere formulations with and without CORM2 in normal human fibroblasts (BJ5TA cell line, telomerase immortalized) using MTS assay. (UMINHO)

**Figure 5: Fluorescence signal indicating residual oxidation of the DCFH-DA probe by free radicals in human fibroblasts (BJ5TA) exposed to different nanosphere concentrations (n=1).** (UMINHO)

Similarly, we observed that nanospheres containing CORM-2 seem not to induce oxidative stress in human skin fibroblasts, in any of the timepoints tested (Figure 5).
The encapsulation of different concentrations of Celecoxib was also tested. The cytotoxicity associated with optimized BSA nanospheres containing celecoxib was analyzed as for nanospheres with encapsulated CORM2. The range of nanoparticle concentration tested span from 75 to 900 µg/ml (Figures 6 and 7).

![Graph showing cell viability of different concentrations of nanospheres formulations, with and without celecoxib](image)

**Figure 6:** Cytotoxicity of different concentrations of nanospheres formulations, with and without celecoxib, in normal human fibroblasts (BJ5TA cell line, telomerase immortalized) using MTS assay (UMINHO).

![Graph showing fluorescence signal intensity](image)

**Figure 7:** Fluorescence signal indicating oxidation of the DCFH-DA probe by free radicals in human fibroblasts (BJ5TA) exposed to different nanosphere concentrations (n=1) (UMINHO).

Nanospheres containing celecoxib seem not to induce cytotoxicity and or oxidative stress in human skin fibroblasts, in any of the timepoints tested.

### A.2. Liposomes

UMINHO encapsulated celecoxib in liposomes in a lipid:drug molar ratio 5:1, and the presence of the drug confirmed by mass spectrometry and cytotoxicity assessed by MTS and SRB assays. In order to determine the oxidative stress caused by exposure to liposomes, with and without celecoxib, DCF assay was performed. This method measures the levels of intracellular reactive oxygen species (ROS). The results obtained demonstrate that
liposomes without and with encapsulated celecoxib do not induce oxidative stress in BJ5 human fibroblasts (data not shown).

UMINHO has proceeded towards the encapsulation of CORM-2, a carbon-monoxide releasing molecules. CORM-2 and liposomal formulations with encapsulated CORM-2 do not present higher cytotoxicity in human skin fibroblasts up to 72h of contact. The results obtained by DCF assay demonstrated that liposomes with encapsulated CORM-2 do not induce oxidative stress (data not shown).

Similar results were observed by INERIS on non target cells of various origin (lung : A549; monocytic : THP-1, liver : HepG2 and kidney : Caki-1). Whatever the cell line or the liposome formulations (with or without folates at the surface and with or without anti-inflammatory drug inside), no significant cytotoxicity was noted for concentrations up to 0.82 mg/mL. A slight metabolic boost is often noted at the highest concentration (0.82 mg/mL) for the four cell lines. A typical result is given in figure 8.

![Figure 8](image.png)

**Figure 8.** Cytotoxicity of liposomes containing folates at the surface and CORM-2 inside on THP-1 cells assessed by Alamar blue® assay. Values are the mean ± SD of 3 independent experiments (INERIS).

UMINHO proceeded towards the production of cationic liposomes for subsequent encapsulation of siRNA. The cationic phospholipid DOTAP was introduced and various ratios of phospholipid and cholesterol were tested. All formulations demonstrated to be stable over time (Figure 9).
Figure 9. Particle size (nm) of different cationic liposomal formulations, along time (UMINHO).

UMinho tested the cytotoxicity of these formulations using BJ5ta human fibroblasts and although cytotoxicity is commonly one the major disadvantages of cationic liposomes, these formulations showed low cytotoxicity (Figure 10).

Figure 10. Cell viability of human skin fibroblasts line BJ5ta, after 72h of contact with liposomes formulations with and without celecoxib, determined by A) MTS and B) SRB assays (n=2) (UMINHO).

To make a comparison, the cytotoxicity of liposomes on mouse zymozan-activated macrophages is presented below (work done by COCHIN). The functional effects of liposomes samples obtained from UMINHO and loaded with Celecoxib were checked in vitro using the zymozan-activated macrophage cellular model. To do this, COCHIN set up a new assay using the xCELLigence RTCA system, a technology based on impedance measurement which has been developed to allow the dynamic monitoring of immunotoxic compounds with target cells. This real-time analysis represents an appropriate and powerful alternative to end-point classical assays on cultured cells (MTT,
BrdU, adhesion assay, etc.). COCHIN first set-up the optimum culture conditions to be used with mouse zymosan-elicited macrophages. Results are shown in Fig. 11, indicating that $10^5$ cells/well are optimal. Interestingly, a significant growing phase could be noticed, likely corresponding to a cell proliferation (a described behavior of M2 macrophages).

![Graph showing cell growth over time with different conditions](image)

**Figure 11.** xCELLigence analysis of zymosan-elicited macrophages (COCHIN).

COCHIN then checked various concentrations of the different liposome suspensions with or without folate and with or without Celecoxib (CORM liposomes were not included in this study). A typical result with 80 µg/ml is shown in Fig. 12. It suggests that "empty" liposomes have a moderate, but significant impact on cell growth, increased in the presence of Celecoxib; mainly, the two folate-containing devices showed much more potent effects. Nevertheless, we did not observe a difference between these two samples, related to the presence of the drug. Similar curve profiles were obtained with a higher liposome concentration (160 µg/ml, not shown).

![Graph showing cell growth over time with different conditions](image)

**Figure 12.** xCELLigence analysis of zymosan-elicited macrophages incubated with the same concentration (80µg/ml) of different liposome formulations (COCHIN).
B. Pro-inflammatory properties

The pro-inflammatory potential of the BSA-based nanobiodevices was assessed by measuring various cytokines (TNF-α, IL-1β, IL-6 and IL-8) in the cell culture medium of A549 or THP-1 cells exposed for 1, 3 or 24h to the different formulations.

In order to validate the dosage, the possible interactions of BSA-based nanobiodevices and the different cytokines were evaluated by incubating for 1h a known amount of cytokines with 0.625 mg/ml of BSA-based nanobiodevice. No interactions were detected between the BSA-based nanobiodevice and each of the 4 cytokines (Figure 13), allowing us to assess the pro-inflammatory properties of the devices by dosing the cytokines in the cell culture medium.

![Graphs showing cytokine concentrations](image)

**Figure 13:** Absence of interaction between cytokines and BSA-based nanobiodevice containing celecoxib inside (BSA2). Known amounts of cytokines were incubated for 1h with 0.625 mg/ml of BSA2 in culture medium and dosage of the cytokine was then performed by ELISA. The same quantity of cytokine was measured after incubation in the presence or absence of BSA-based nanobiodevice, suggesting no interference for the ELISA dosage of cytokines between the BSA-based nanobiodevice and the cytokines. Values are means ± SD for 2 independent experiments.

Pro-inflammatory properties of the different formulations of BSA-based nanobiodevices (containing or not folates at the surface and celecoxib inside) were assessed by incubating
cells for 1, 3 or 24 h with 0.156 or 0.625 mg/ml of FBN and measuring the quantity of various cytokines in the cell culture medium by ELISA.

**Figure 14**: Pro-inflammatory properties of BSA-based nanobiodevices after 24h incubation with A549 or THP-1 cells (BSA1 : with folates at the surface, BSA2 : with celecoxib inside, BSA3 : with folates and celecoxib). LPS is a positive control. Values are means ± SEM for 2 independent experiments.
No significant differences were noted between the secretion of cytokines of the control cells and those incubated with the different BSA formulations, for all the timepoints tested. (See Figure 14 for 24h of exposure).

Section 3 – Conclusions and assessment

These two nanospheres (BSA or liposome-based) display a low or no cytotoxicity on the non target cell lines studied, whether or not they have a folate-receptor on their membrane. The presence of folates at the surface or the encapsulation of an inflammatory drug inside do not modify the cytotoxicity of the nanobiodevices.

Under our experimental conditions, these nanospheres show also no significant oxidative or pro-inflammatory properties on the non target cell lines studied.

A slight cytotoxicity of liposomes (materialized by a reduced cell growth) was noticed on mouse zymozan-activated macrophages with the most potent effect for formulations with folates at the surface (with or without celecoxib inside).