PROJECT FINAL REPORT

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Content

NANOFOL

Folate-based nanobiodevices for integrated diagnosis/therapy targeting chronic inflammatory diseases

1.1. Executive summary

NANOFOL developed a new diagnostic/therapy approach using folate-based nanobiodevices (FBN) able to provide a new type of cost efficient treatment for chronic inflammatory diseases such as Rheumatoid Arthritis with low side effects that will constitute a more advantageous solution than current therapies.

NANOFOL has accomplished the overall objectives established in the initial plan. The consortium defined several possibilities for the global nanodevice strategy, in order to reach delivery technology products with therapeutic potential. The consortium produced FBN (liposomal, protein-based (BSA, HSA) and PLA (poly (L-lactic acid)) nanoparticles) with encapsulated anti-inflammatory drugs and siRNAs Lock Nucleic Acid (LNA), that were biologically active, non cytotoxic and capable of specifically targeting folate receptor (FR)-positive cells namely macrophages.

Folate receptor β(FRß) was described as a marker of activated macrophages in joints affected by RA, but its expression in other tissues is largely unknown. In order to study FRß-positive macrophages in detail, we generated mouse monoclonal antibodies (mAbs) against FRß and confirmed their specificity by several immunocellular and immunobiochemical methods using various cells and tissues. Our analysis of macrophages differentiated *in vitro* from blood monocytes revealed that FRß is strongly upregulated in certain subsets of macrophages that can be distinguished by other surface markers as well as by functional properties. To select for relevant macrophage markers to specifically target disease-associated macrophage subsets by bispecific antibody-based nanodevices, we screened for surface proteins associated with FRß in the different subsets by immunoprecipitation and mass spectrometry.

To measure *in vivo* uptake of FBN and therapeutic activity in a pathological context, arthritis was induced in DBA/1 mice. Mice receiving FA-containing liposomes show a better accumulation of the nanodevice, mainly in the paws. The therapeutic activity of these liposome nanodevices loaded with the anti-arthritis drug methotrexate (MTX) clearly indicate that having MTX in liposomes improves the treatment as compared with the soluble drug. Our results showed that this clinical benefit is further increased when liposomes contain peptide-folate. NANOFOL nanodevice containing FA represents an efficient drug delivery system in rheumatoid arthritis.

A key finding of the project has been the fact that the folate-derived nanodevices exhibit outstanding pharmacokinetics relative to MTX in its current form. In current therapy, MTX is used once a week and is supplied in excess, however, through rapid elimination, it is largely removed from the body over 1 to 2 days with the first 24 h being associated with side-effects including lethargy. Our data show that the NANOFOL nanodevice is a promising vehicle for delivering MTX to a target organ.

The NANOFOL nanobiodevices targeting activated macrophages may be an interesting theranostics solution, i.e., simultaneous diagnosis and treatment of the site of inflammation in RA patients. The production of a validated, stable, specific FBN with incorporated imaging

agent and therapeutic agent (drugs or siRNAs) by the NANOFOL consortium will have many applications in all inflammatory diseases where activated macrophages contribute to the disease process.

1.2. Context and project objectives

It is estimated that inflammatory diseases affect more than **80 million people** worldwide leading to suffering, economic loss and premature death. Considering life expectancy in Europe, these numbers are expected to increase in the next 20 years. Moreover, **studies** have shown that disorders such as **rheumatoid arthritis (RA)** can shorten **life span by 10** years.

The treatment of chronic inflammatory disorders, including RA, remains a challenge for the medical and scientific community. The emergence of new drugs creates new options though it also entails **high costs, complicated drug administration**, **allergic reactions** and potentially **fatal side effects**.

Therefore more efficient strategies have to be identified in order to improve inflammatory disease treatment while decreasing the side effects with an improved cost-benefit ratio.

Nano-enabled drug delivery systems will take therapy of chronic inflammatory disorders to a new level by creating a new, highly specific and efficient strategy, with reduced treatment costs.

NANOFOL project aimed to develop nanobiodevices targeting Folate Receptors (Folate Based Nanodevices - FBN) for chronic inflammatory diseases. The major advantage of FBN is to target a cell population implicated in chronic inflammatory diseases (activated macrophages) and specifically deliver encapsulated agents.

The NANOFOL project adopted a specific **risk amelioration strategy** to attain objectives in a **step-by-step approach** in order to gradually improve the concept - specificity, stability, side effects and efficacy - ensuring **reduced animal testing** and **high human safety**.

The NANOFOL overall objectives were:
- Design, development and production of **nanobiodevices** directly targeting effector cells
- Proof of concept *in vitro* and *in vivo* of a folate based nanodevice targeting activated macrophages in chronic inflammation not affecting bystander cells
- Proof of concept *in vitro* and *in vivo* of a nanodevice containing a bispecific antibody (against folate receptor and another macrophage marker) targeting activated macrophages in chronic inflammation not affecting bystander cells
- Proof of concept of FBN delivery therapeutic agents (by small interfering ribonucleic acid molecules (siRNA) or lipophylic molecules) targeting inflammatory signalling pathways
- *In vitro* and *in vivo* testing of cellular toxicity caused by the novel nanobiodevices in cells other than activated macrophages
- Experimental design that will enable **minimal animal experimentation**.
- Development of a **strategy to assess potential life cycle risks** ensuring safe nanobiodevice-mediated delivery.
- Setting-up better citizen awareness on nanomedicine-based therapies and training activities.

1.3. Main S&T results/foregrounds

1.3.1. WP1 - Folate-based nanobiodevices (Leader: UMINHO)

WP1 focused on the design, production and functionalisation of folate based nanobiodevices (FBN) containg drugs or siRNAs and/or imaging agents.

The first step of this work package consisted in the identification of the capsule requirements for an effective functioning. Fusion proteins were designed, composed of domain peptides from surfactant protein (SP) covalently linked to folate at the N-terminal.

The next steps were the construction of nano-vehicles based on lipids, proteins (BSA, HAS), or poly-lactic acid (PLA) with folate at their surface, incorporating an imaging (diagnostic) agent and/or a therapeutic agent (either a pharmacological drug or siRNA).

A full characterization of the vehicles was achieved, in coordination with WP3 -WP5, in order to determine which are the nanodevices most capable of fulfilling their intended use without causing toxicity.

The objectives of this WP mainly aim at:
- Finding the best peptide sequence containing folate to stabilise nanobiodevices
- Reaching optimal conditions to encapsulate drugs and imaging/contrasts agents
- Obtaining optimal conditions to encapsulate siRNA
- Reaching optimal characteristics of FBN which fulfils the application requirements to the *in vitro* and *in vivo* studies.

The main achievements were:

Study on vehicle requirements

Initially, the main requirements for a successful vehicle for theranostics of RA were defined: from physico-chemical characteristics compatible with intended oral delivery or intravenous application, to specific design features (including molecules to be included in the produced vector) intended to promote access to inflammation sites and uptake specificity by activated macrophages.

Vehicle construction was also contemplated taking into account marketed liposomal formulations, production processes, and products and suppliers.

Construction of folate peptide complexes

At UMINHO, several peptide constructs with and without folate, were designed. These peptides were intended for use as stabilizing agents and anchorage of other molecules, such as Folic acid and fluorescent dyes (e.g. Oregon green 488) which were covalently bound to the terminal. These constructs were incorporated into liposomal formulations.

At TUG and UMINHO, folic acid was also coupled covalently to BSA, PEG and DSPE-PEG. TUG also produced HSA -PEG-FA conjugates. These conjugates were used to produce protein-based nanoparticles, as a possible alternative to liposomal folate-based nanodevice.

Production and characterization of folate-based nanodevice (FBN)

The next step was to construct nano-vehicles based on lipids, proteins (albumin), or poly-lactic acid (PLA) with folate at their surface, incorporating an imaging (diagnostic) agent and/or a therapeutic agent (either a pharmacological drug or siRNA).

A full characterization of the vehicles was achieved, in coordination with WP3 -WP5, in order to determine which are the nanodevices most capable of fulfilling their intended use without causing toxicity. Stealth liposomes and albumin particles were produced (figures 1 and 2). [1; 2]
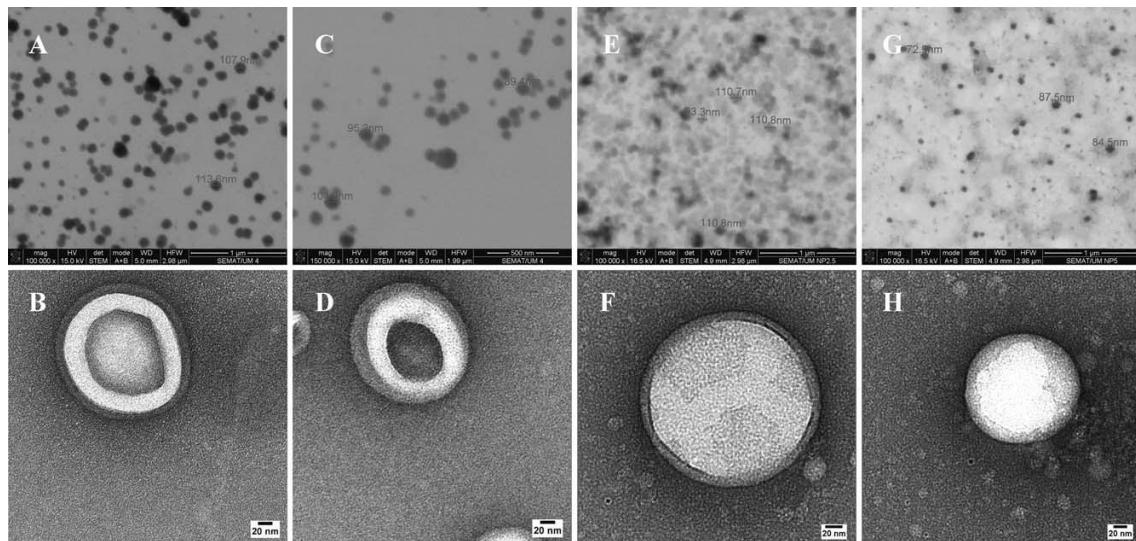
Figure 1: Inclusion of DSPE–MPEG or PEGylated surfactant yields spherical vesicles with narrower size distribution. Representative (A) STEM and (B) TEM images of liposomes containing 5% DSPE–MPEG; (C) STEM and (D) TEM images of liposomes containing 10% DSPE–MPEG; (E) STEM and (F) TEM images of BSA nanoparticles with 2.5 mg mL_1 of PEGylated surfactant; (G) STEM and (H) TEM images of BSA nanoparticles with 5 mg mL_1 of PEGylated surfactant.[1]
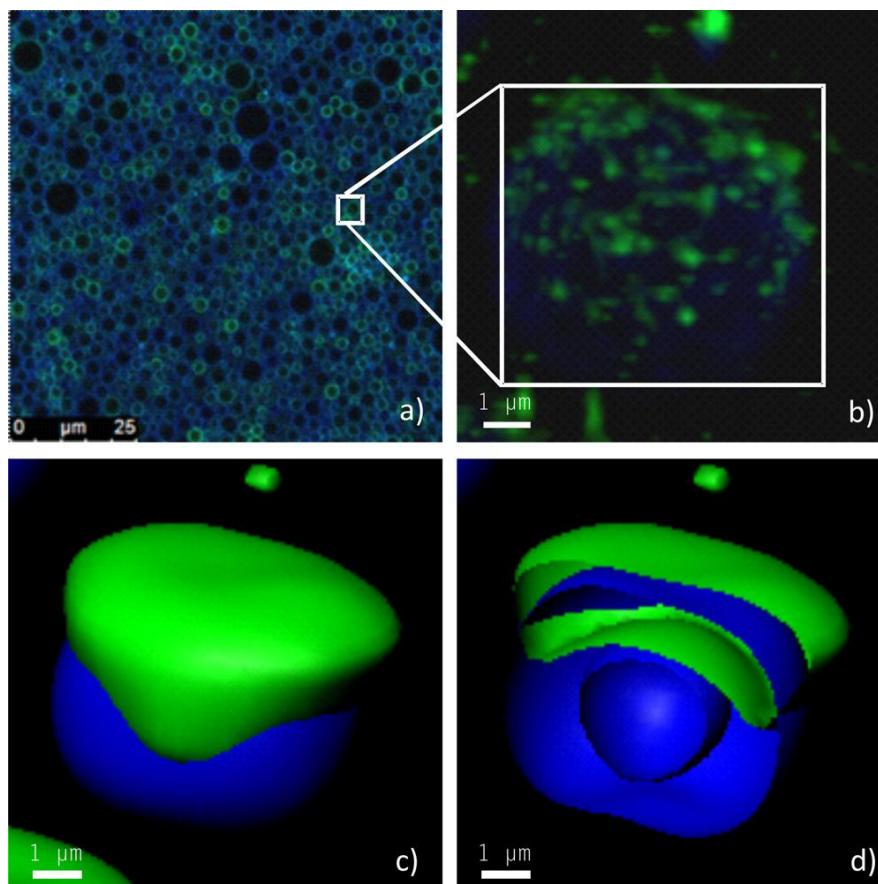
Figure 2: CLSM image (a) and 3D reconstruction (b–d) of FITC-labeled HSA nanocapsules carrying fluorescent red labeled FA on the capsule surface. Blue: HSA, green: FA. [2]

Several liposomal formulations were tested in order to obtain specific targeting and the desired bioactivity in folate receptor (FR) – positive cells. Importantly, liposome formulations were engineered in order to efficiently produce a biological effects in activated macrophages.

Special phospholipid components were included in FBN to promote drug release at the endosome after endocytosis.

Liposomal FBN were sucessfully produced and showed to be efficient in the encapsulation and specific delivery of both hydrophobic and hydrophilic drugs with anti-inflammatory (celecoxib, CORM-2) and antineoplastic properties (methotrexate) in FR-expressing cancer cell line. Furthermore, high specific uptake in joints of arthritic mice was observed as well as an a major beneficial effect on clinical score (WP3).

Encapsulation of siRNA, namely of LNAs specifically targeting transcripts identified as being overexpressed in FRβ expressing activated macrophages (in WP3) was also achieved using the optimized liposomal FBN in the final semester of the project. Effectiveness of these formulations is being evaluated *in vitro*.

The production of microsized PLA capsules was also attempted for FA-mediated delivery. Using microfluidics PLA microcapsules were developed based on an oil-in-water emulsion, where the organic phase contained solvent, polylactic-acid (PLA), fish oil and the hydrophobic drug celecoxib, with folate coupled to PLA, prior to capsule formation. With the selected materials it is not possible in practice to create vehicles with sizes in the nanoscale in a robust and reliable way using the chip technology to deliver a industrial production system for the production of these FBN's.
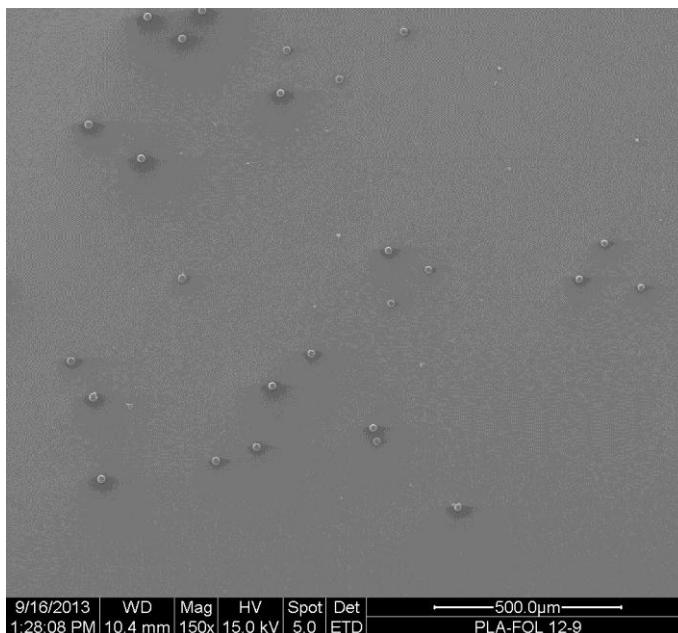
Figure 3: SEM image showing the monodispersity of the produced PLA-folate microcapsules.

References:
[1] Liposome and protein based stealth nanoparticles. E. Nogueira, A. Loureiro, P. Nogueira, J. Freitas, C.R. Almeida, J. Härmark, H. Hebert, A. Moreira, A.M. Carmo, A. Preto, A.C.Gomes and A. Cavaco-Paulo. *Faraday Discuss*. (2013) 166, 417-429.
[2] A. Rollett, T. Reiter, P. Nogueira, M. Cardinale, A. Loureiro, A. Gomes, A. Cavaco-Paulo, A. Moreira, A.M. Carmo, G.M. Guebitz. *International Journal of Pharmaceutics* 427 (2012) 460-466.

This work package was mainly focusing on two main strategies for the generation of antibody-based bispecific nanodevices targeting activated macrophages:

1. One strategy was to couple chemically conventional intact monoclonal antibodies (mAbs) on the surface of the nanodevices.
2. The second strategy focused on the production of genetically engineered mono- and bispecific recombinant antibodies, which were able to integrate into the lipid bilayer of the nanodevices without the need of chemical cross-linkers.

To approach the goal of the bispecific device, a main part of the work was to comprehensively characterise the phenotype and the function of the cells we intended to target, i.e. folate receptor β (FRβ)-positive macrophages, to characterise the FRβ itself, and finally to identify a suitable macrophage-associated target antigen as second target. The second target has to be localised in close proximity to FRβ on the surface of activated macrophages so that both antigens can be simultaneously recognised by the uniquely designed bispecific nanodevice.

Characterisation of the FRβ-positive macrophages and identification of the suitable macrophage-associated second target antigen

Methotrexate has been successfully used for therapy of rheumatoid arthritis (RA) for decades despite the fact that the precise mechanism of its action has not been fully elucidated. However, the main effect of methotrexate seems to be inhibition of folic acid metabolism. Since the β isoform of the folate receptor (FRβ), which is suggested to mediate transport of both folic acid and methotrexate into cells, is markedly overexpressed on the surface of activated macrophages present in RA synovia, these activated macrophages have been proposed as a suitable cell target. We considered therapeutic improvement by understanding these cells and FRβ better. This knowledge should allow specific delivery of methothexate and other drugs to these macrophages for their specific elimination or reprogramming, and thus to avoid toxic effects in healthy tissues.

In order to study FRβ-positive macrophages in detail, EXBIO with the help of MUW generated mouse monoclonal antibodies (mAbs) against FRβ and confirmed their specificity by several immunocellular and immunobiochemical methods using various cells and tissues:

- FRβ-overexpressing cell lines
- primary human macrophages differentiated and activated *in vitro* (protocol developed by the NANOFOL consortium).

In parallel, MUW analysed macrophages differentiated *in vitro* from blood monocytes and found that FRβ is strongly upregulated in certain subsets of macrophages that can be distinguished by surface markers as well as by functional properties. To specifically target the RA-associated FRβ macrophage subset by bispecific antibody-based nanodevices, MUW screened for surface proteins associated with FRβ in the different subsets. Indeed, by immunoprecipitation and mass spectrometry, MUW identified several candidate proteins in the vicinity of FRβ in the different macrophage types. These FRβ-associated macrophage markers therefore represent valid second target antigens. As an additional discovery, MUW gained information about the functional consequence resulting from association of FRβ with these second target antigens, which shed light on novel functions of FRβ and as consequence novel therapeutic interventions via FRβ in activated macrophages.

Preparation of HSA-nanocapsules functionalized with mAbs

For the establishment of a bispecific nanodevice one strategy was to use the previously developed human serum albumin (HSA) nanocapsules as template. As proof of concept TUG/BOKU linked on their surface folic acid and additionally a mAb targeting as second target a FRβ-associated molecule on activated macrophages. We selected a mAb against MHC class II molecules. A pre-step for this experiment was the screening for a suitable method to covalently couple a mAb on HSA nanocapsules.

HSA nanocapsules with a diameter of ca. 500 nm and a narrow size distribution were prepared using a sonochemical process avoiding toxic cross linking agents and emulsifiers [1]. Afterwards, four different methods were tested for the covalent linking of a mAb on the capsules. We found out that for successful coupling a spacer is necessary; we used a PEG spacer, namely polyethyleneglycol with a spacer length of 3000 Da. The procedure for this coupling experiment is shown in Figure 4. With several methods like confocal laser scanning microscopy (CLSM) and enzyme-linked immunosorbent assay (ELISA), TUG/BOKU confirmed the presence of the mAb on the surface of the capsules. Furthermore, MUW demonstrated the specific binding activity of the mAb-HSA nanocapsules to MHC class II molecules on living cells by flow cytometry. These results were published recently in the International Journal of Pharmaceutics [2].
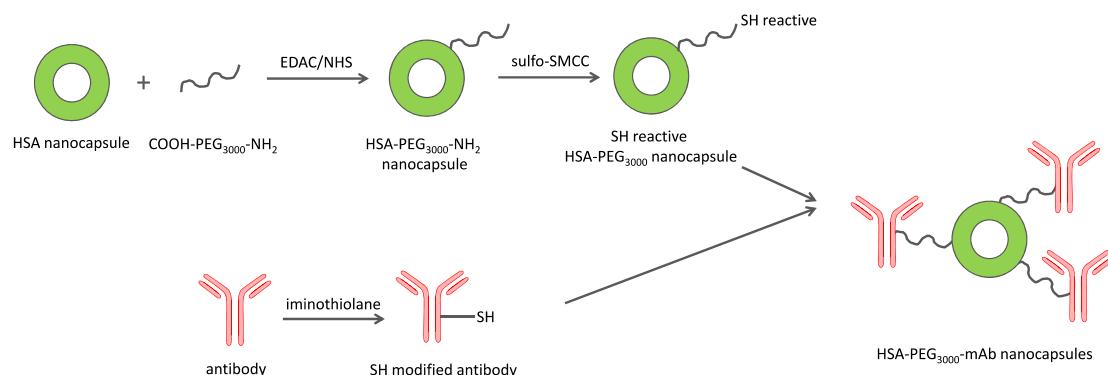
Figure 4: Reaction scheme of chemical coupling of mAb on the surface of HSA-nanocapsules. In a first step, a PEG spacer was linked on the capsule and modified to create an SH-reactive group. In a parallel reaction, SH-groups were introduced to the mAb. By combining the two activated species a mAb-HSA nanocapsule was prepared.

Enzymatic synthesis of mAb-HSA conjugates

In addition, TUG/BOKU developed an enzymatic coupling procedure as alternative method for cross-linking of mAbs and other proteins – in our case HSA. The conventional production of protein conjugates involves several reaction steps including chemical modification and activation of both proteins followed by cross-linking often involving toxic chemicals. Here we used the enzyme tyrosinase from *Agaricus bisporus* to prepare a mAb-HSA conjugate under mild reaction conditions and avoiding chemical cross linkers. To facilitate the enzymatic cross-linking reaction, natural low molecular weight phenolic molecules like e.g. caffeic acid, which are accepted as substrate by tyrosinase were used. The reaction mechanism of this enzyme is explained in Figure 5.

possible conjugates

300 kDa
216 kDa
132 kDa
366 kDa
282 kDa
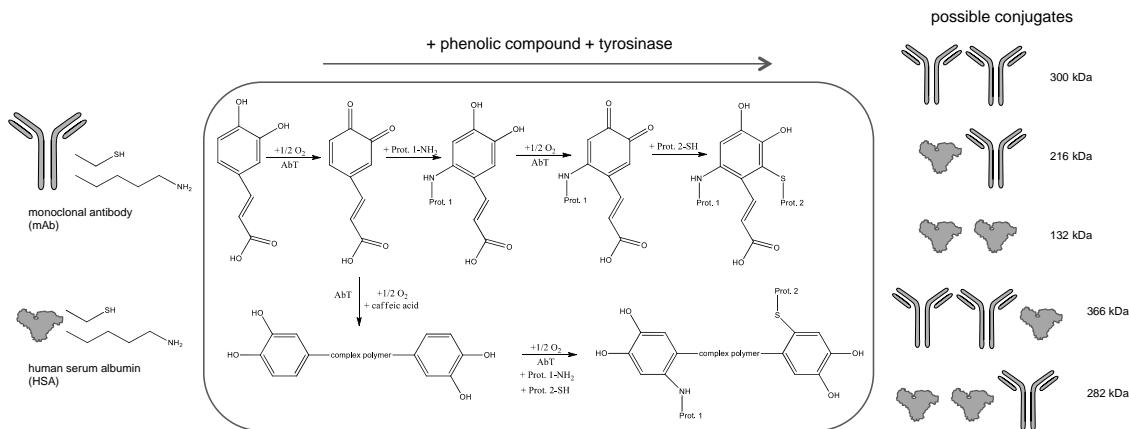
Figure 5: Reaction scheme of enzymatic cross-linking of mAb and HSA. Middle: Scheme describing the reaction mechanism of the enzyme tyrosinase: A natural small phenolic substrate (e.g. caffeic acid (CA)) is oxidized by tyrosinase to give reactive quinones. In the next step, these quinones couple to –NH$_2$ or –SH residues of mAb and/or HSA and cross-link the two proteins in this way (see upper panel). With the addition of further phenolic cross-linkers a natural spacer between the two proteins is formed by the polymerization of the phenolic substrate (see lower panel). Right: possible conjugates being generated during enzymatic cross-linking process of mAb and HSA, including predicted molecular weights.

The reaction progress was analysed by SDS-PAGE using fluorescence-labelled HAS and colloidal Coomassie brilliant blue staining. Using this method, TUG/BOKU found reaction products in the molecular weight range of 216 kDa, which is the predicted molecular weight for a 1:1 mAb-HSA conjugate (see Figure 6). To confirm the composition of this conjugate, LC-MS/MS was performed, which showed indeed the presence of mAb and HSA. For further analysis the conjugate was purified by size exclusion chromatography (see Figure 6).
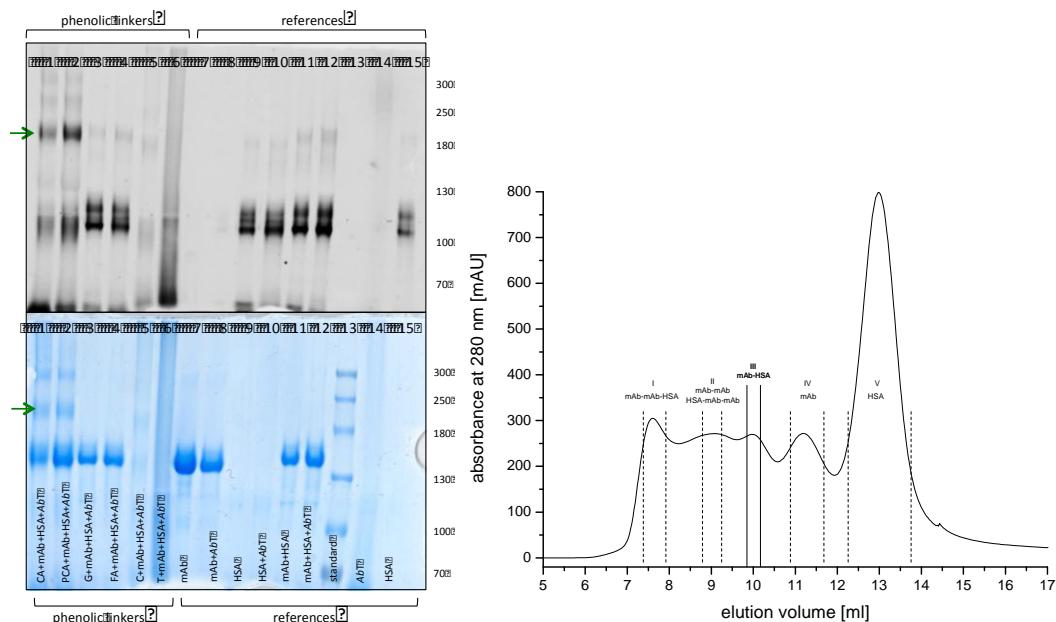
Figure 6: SDS-PAGE analysis of different tyrosinase-generated mAb-HSA conjugates and the starting reference proteins. Top picture: fluorescence scan of the SDS-PAGE gel. FITC-labelled HSA is giving a fluorescent signal, which was detected by fluorescent scanning of SDS gel. Using fluorescent scanning, fluorescent-labelled proteins can be detected in lower amounts than with Kang staining. **Bottom picture**: Kang staining of the same gel showing cross-linking of mAb and HSA by tyrosinase in combination with different phenolic molecules (lane 1-6, CA caffeic acid, pCA p-coumaric acid, G guiacol, FA ferulic acid, C catechol and T tyrosinase). The green arrows mark the mAb-HSA product band at 216 kDa, which is found only with CA and pCA (lane 1 and 2). Lanes 7-15 show

references where HSA, phenolic molecules, mAb and AbT were replaced with reaction buffer. **Right figure**: Size exclusion chromatography of the enzymatic mAb-HSA coupling reaction. The peak corresponding to the size of 216 kDa was purified for further experiments.

The binding capacity of the enzymatically-conjugated mAb-HAS nanocapsule was determined by ELISA and flow cytometry. Both methods confirmed that the mAb resisted the coupling procedure and recognised its antigen. Furthermore, no unspecific binding to MHC class II negative cells was seen. These results were published recently in the Journal RSC Advances [3].

Generation of nanodevices functionalised with hydrophobic-tailed mono- and bispecific antibodies

A further strategy involved the genetic fusion of a hydrophobic linker to antibodies to allow their efficient integration into the lipid bilayer of liposomal nanodevices without chemical modifications. Monospecific antibody-based liposomal nanodevices were created by incorporation of a single hydrophobic-tailed antibody fragment into liposomes (Figure 7A).

Bispecific nanodevices consisted of a liposome with incorporated hydrophobic-tailed antibody fragments directed to two different antigens (Figure 7B). As the most advanced approach, MUW and UMINHO generated a liposome loaded with a unique hydrophobic-tailed bispecific antibody (Figure 7C).
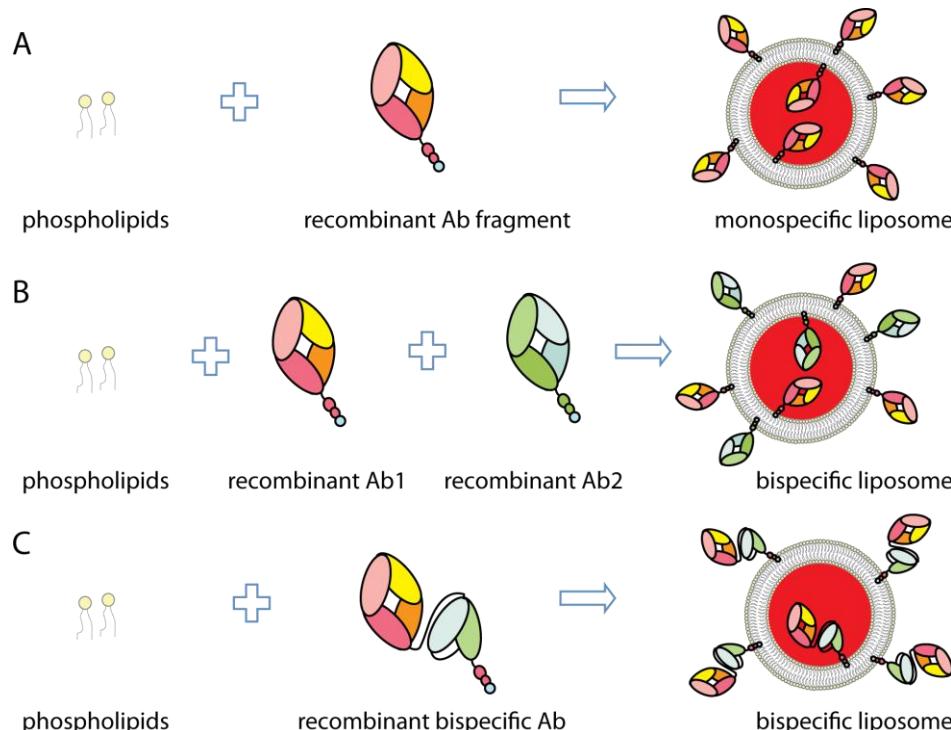
Figure 7: Various formats of recombinant hydrophobic-tailed antibody-based liposomal nanodevices. A. Monospecific liposomes were created by combining phospholipids and a recombinant hydrophobic-tailed (blue segment) antibody fragment directed against a highly expressed marker on the macrophage surface. For visualization, the liposomes were filled with the fluorescent dye Alexa Fluor 647. We designed the antibody fragment for deep incorporation into the lipid bilayer of the liposome with the hydrophobic linker (blue segment) and produced the fusion construct in yeast. **B.** The same approach as in B was used to create bispecific liposomes where two antibody fragments specific to two different macrophage surface markers were incorporated. **C.** As an alternative, the liposome was functionalized with a uniquely designed hydrophobic-tailed recombinant bispecific antibody directed against two different markers of macrophage subsets.

References:
[1] A. Rollett, T. Reiter, P. Nogueira, M. Cardinale, A. Loureiro, A. Gomes, A. Cavaco-Paulo, A. Moreira, A.M. Carmo, G.M. Guebitz. *International Journal of Pharmaceutics* 427 (2012) 460-466.
[2] A. Rollett, B. Thallinger, A. Ohradanova-Repic, C. Machachek, E. Walenta, A. Cavaco-Paulo, R. Birner-Gruenberger, J.G. Bogner-Strauss, H. Stockinger, G.M. Guebitz. *RSC Advances* 3 (2013) 1460-1467
[3] A. Rollett, T. Reiter, A. Ohradanova-Repic, C. Machachek, A. Cavaco-Paulo, H. Stockinger, G.M. Guebitz. *International Journal of Pharmaceutics* 458 (2013) 1-8

1.3.3. WP3 - Biological activity (Leader: IBMC)

Macrophages expressing folate receptor (FRβ) are abundant in the inflamed sites of Rheumatoid Arthritis (RA) and are known to play an important role in the pathophysiology of the disease. For this reason, the Nanofol project aimed to target these cells.

The main objective of WP3 was to assess the specificity and biological activity of the folate-based nanodevice (FBN) developed within the Consortium. Such task was performed initially using primary human and murine macrophages *in vitro* cellular models. In a later stage, animal models of RA were used to provide a final indication of the therapeutic efficacy of such devices.

Characterization of in vitro macrophages

The phenotype of macrophages located in the inflamed sites of RA patients is yet not fully characterized. This knowledge is of great importance if a successfully targeting of these cells is aimed. In order to get a better understanding of macrophage biology, different populations of macrophages (expressing or not FRβ developed *in vitro* from human peripheral blood monocytes were studied in detail in phenotypic and functional analyses. A transcriptomic study of such populations was also made to reveal genes that can discriminate the various functionally distinct FRβ-positive macrophage subsets in order to get tools capable of targeting these discrete populations.

Uptake specificity of nanodevices

The uptake specificity of many different fluorescent FBN (HSA particles, BSA particles and liposomes) by primary macrophages expressing FRβ was assessed in this task. The initial lack of specificity was overcome by successive improvements in the particles. A promising specificity was soon observed when macrophages were incubated with HSA particles containing folate (FA) at the surface. Nevertheless, these particles revealed to be toxic to the cells. A low cytotoxicity and a very specific uptake by FRβ-expressing macrophages were then finally achieved for an improved formulation of liposomes, which were better masked and contained an increased concentration of surface FA (Figure 8). These liposomes were the most successful FBN developed. The following step consisted in loading these particles with an anti-inflammatory drug or with RNAi molecules and assess whether, after internalization by macrophages, they would have the desired biological effect: inactivate or kill macrophages.

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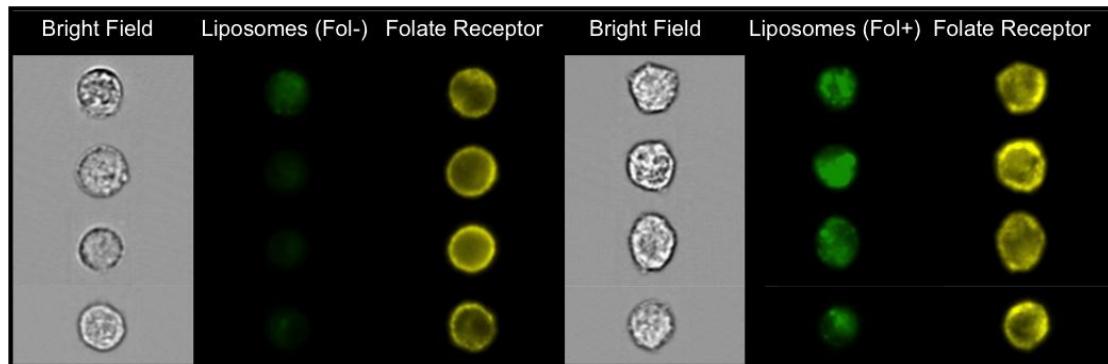
b
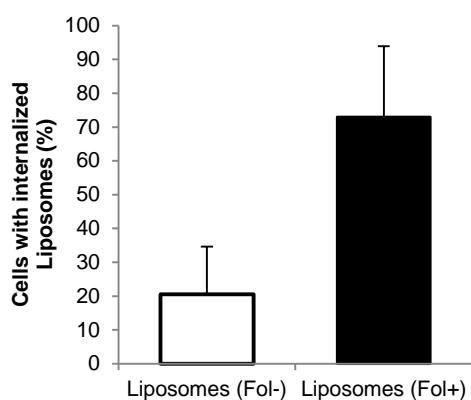
Figure 8: Internalization of fluorescent liposomes by *in vitro* differentiated primary macrophages. Human macrophages were differentiated from blood monocytes for 7 days with 50 ng/ml M-CSF and activated for 24 h with IL-4 (20 ng/ml). Activated macrophages were incubated at 37 °C for 1 h with 100 µg/ml liposomes containing or not folate. (a) Internalization of liposomes (green) was analyzed with an ImageStream multi-spectral imaging flow cytometer (Amnis) after labeling the folate receptor β with a fluorescent antibody (yellow). The panel shows representative sections of images. (b) The percentage of cells with internalized particles is shown in the graph as the mean+standard deviation of 3 different donors.

Pharmacological drug's release and effect on activated macrophages

Having shown the uptake specificity of liposomes, the effectiveness of liposomes loaded with anti-RA drugs commonly employed in the treament of arthritis in humans was tested. The choice was made to use in the following studies methotrexate (MTX), a classical anti-RA drug, also a folic acid analog.

Both soluble MTX and MTX-loaded FBN were tested *in vitro* in human and murine macrophages. An increase in the apoptosis and cell death levels was observed for soluble MTX in human FRβ-expressing macrophages, showing that the drug was effective in the type of cells that we were targeting. MTX-loaded FBN also led to increased cell death levels, although not to the extent of the soluble drug. Similar results were observed using RAW cells, a murine macrophage cell line model. These results were nonetheless promising, as they revealed that the drug was being released from the liposome and could induce a therapeutic effect. Taking together the specificity of the device and successful drug delivery, our results are rewarding and consistent, and fulfill the initial objectives of the project. The final test was to assess the specificity and biological effectiveness of MTX-loaded liposomes in an *in vivo* model.

RNAi delivery from FBN in activated macrophages

One of the aims of the Nanofol project was to test FBN loaded with RNAi molecules as an alternative to the drugs traditionally used for the treament of RA. To achieve this task, our transcriptomic study in macrophages expressing or not FRβ together with an exhaustive literature review were performed in order to select potential targeting genes. RNAi against some of the selected genes were tested and the most effective was incorporated into liposomes and tested in human and murine macrophages. A promising gene expression reduction was observed both in human as murine macrophages, showing that the RNAi molecules were being released from the liposome. Nevertheless, the biological effect of such gene repression is still under evaluation.

In vivo therapeutic efficiency

A main objective of the Nanofol project was to demonstrate the potential of the new FBN liposome formulations prepared within the Consortium in the treatment of RA in animal models. This work was essential in order to make projections on future therapeutic uses of these formulations in humans.

This subtask intended to demonstrate, using *in vivo* systems in the mouse, a specific uptake of FBN in macrophages expressing the FRβ and the effectiveness of FBN loaded with an anti-arthritis drug to treat animals suffering from experimental arthritis. Both objectives have been successfully achieved.

Firstly, we were able to show at the cellular level that liposomes with FA were strongly uptaken by murine peritoneal macrophages expressing FRβ. These experiments were performed using an experimental activation system allowing us to elicit FR□-positive macrophages and to check *in vivo* whether FBN were specifically uptaken by these cells. This result was important as it demonstrated the validity of the biological approach seeking at targeting this particular cell population of macrophages known to play a prominent role in the pathophysiology of arthritis.

Similarly, we were able to show that these devices accumulated in inflamed joints, where resident FRβpositive macrophages are very abundant. These results were obtained in a mouse "collagen-induced-arthritis" (CIA) model, which is the closest model from human RA, and is widely used for pre-clinical studies aiming at testing new drugs for future developments in humans (typical arthritic joints are shown in Figure 9, left). For these experiments we used fluorescent FBN and, whenever possible, *in vivo* imaging technologies were employed (Figure 9, right) in order to reduce the amount of mice used for the studies and thus fulfill the ethical recommendation of the three R rule (reduce, refine, replace).
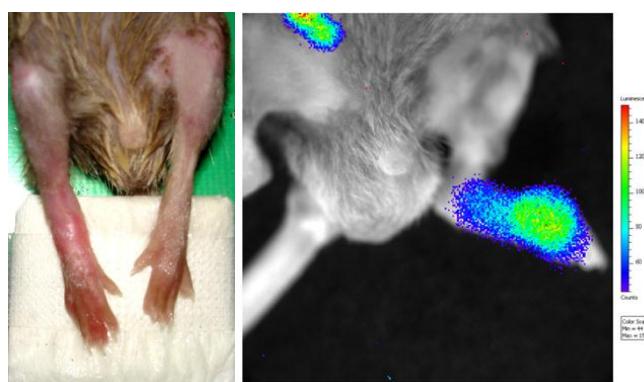
Figure 9: Left. Typical arthritic lesions after collagen injection to DBA1 male mice, associating joint swelling and strong local inflammation. Right. A bioluminescence IVIS Spectrum device (Caliper Life Sciences) was used to monitor myeloperoxidase activity in inflamed joints of anesthetized arthritic mice after injection of Luminol.

The second challenging issue of the program was to show that a clinical benefit could be obtained with these liposomes containing FA, as compared with treatments using the soluble anti-arthritic drug MTX. We first verified the efficiency of the MTX treatment in the CIA model in the mouse. Unquestionably, the drug strongly reduced arthritis both at the clinical and histological levels (Figure 10).

We were next able to demonstrate that a liposomal device containing MTX works much better than the drug alone. Moreover, this effect was further enhanced when FA was expressed on the device. We are still uncertain of the specific pathways impacted by MTX (i.e., the folate metabolic pathway) in the arthritis environment to reduce the inflammatory process. We nevertheless showed that the treatment can affect the phenotype of resident macrophages.
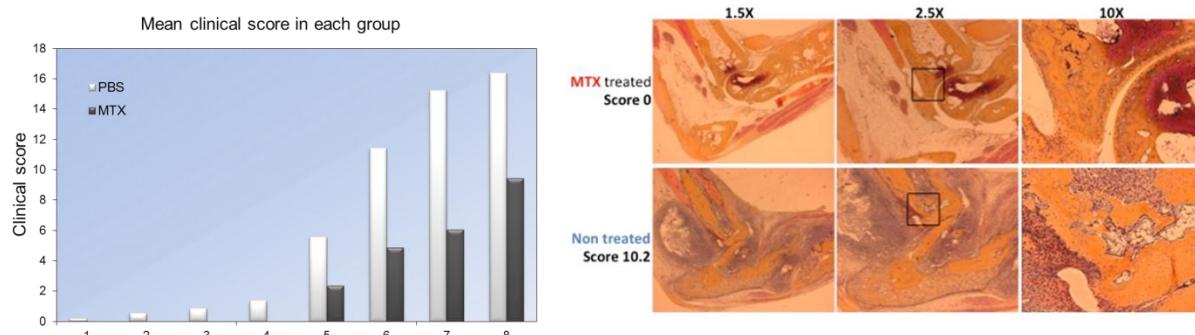
Figure 10: Left. Arthritic mice (12 mice in each group) were treated with serial injections of MTX intraperitoneally twice a week, during 3 weeks. Clinical scores were measured on each mouse twice a week with a total of 8 examinations (corresponding to the values on the horizontal axis). Shown are the mean clinical scores in each group measured for each examination. Right. Paraffin embedded sections from ankles of DBA1 mice treated or not with MTX. Global arthritis clinical scores are also indicated. Fixed and decalcified ankles were paraffin embedded at the end of the protocol and 7 μm sections were stained with Hemalun-Eosin-Saffron. Swelling of one arthritic untreated joint is shown in the lower panel, with huge mononuclear cell infiltration and bone destruction, as revealed by orange-saffron staining. In comparison, ankles of MTX treated mice (upper panel) exhibit a normal shape without swelling or cell infiltration. Bone structure is also conserved.

We can conclude from these results that FA improves liposome uptake in arthritic joints and the clinical benefit of anti-arthritis drug, with a remarkable reduction of the disease in a murine pre-clinical model. The new FBN developed during the Nanofol project are therefore very promising tools for the treatment of chronic inflammatory diseases in human, such as RA, but possibly also of cancer, since cancer cells usually express very high levels of folate receptors on their cell surface. As such, we believe that the results of the Nanofol project achieved within this specific WP have provided experimental data that supports the effectiveness of FBN in relevant models of disease and pave the way to further clinical designs.

1.3.4. WP4 - Bioavailability of FBN delivered orally (Leader: SYNOVO)

Objectives
WP4 was concerned with the issue of practical administration of the nano-devices to patients in a form that is stable, tolerable and effective. The ideal route for chronic medication in the oral route because the gut is a natural organ of absorbtion. However, liposomes are traditionally not sufficiently stable to withstand this route, and we have, therefore, sought to either strengthen the liposomes, or protect them against digestive enzymes, or administer them via other routes for comparison.
Alternative routes include:

- Intravenous (infusions are a common approach in cancer, or acute and life-threatening inflammation)
- Intra-synovial (suitable for steroidal depot therapy in severe arthritis)
- Sub-cutaneous (common for current biologicals)
- Inhaled (suitable normally only for high potency, high solubility drugs) or for anti-inflammatory therapies for lung diseases

The WP had three key tasks:
4.1   Establish in vitro assays for FBN uptake.
4.2   Establish Labelling for detection
4.3   Formulation for both parenteral and oral administration of FBNs..

In earlier work, we showed that the nanodevices being produced by Minho were remarkably stable. In work reported elsewhere (see WP1), data show that the quality of the materials has been improved both in terms of selectivity and stability. In the same process, additional fluophores were added to assist detection in vitro.

On assessing the original in vivo pharmacokinetics we showed that the basic vehicle, whether folate decorated or not, was an effective means to promote exposure in vivo to methotrexate.
Following improvements to the vehicle at minho, it was shown by IBMC that folate decoration lead to a receptor specific uptake to a greater degree than in earlier prototypes.
In this report, we describe the pharmacokinetics of methotrexate delivered with these particles via the intravenous, oral and inhaled routes in animals which are either stimulated to present an acute inflammation, or which are normal.

Methods
Nanodevices were prepared by Minho using protocols reported in WP1.
For all studies, MTX was prepared for administration at the same dose. For intranasal (i.n., inhaled) formulation, MTX was formulated by dilution in normal saline.
Methotrexate (MTX) was used as the primary indicator of liposome activity/presence. The reason is that the distribution of the active ingredient reflects the final pharmacological activity.
Analysis was via LC-MS-MS using a triple quadrupole mass spectrometer (Synovo) Agilent 1260 HPLC coupled to a Sciex API4000 device. Elution was via a gradient from 2 to 100% methanol over 4 minutes, total run time-12 minutes.

Results
Lipsomes prolong plasma MTX exposure.
When applied i.v., liposomal MTX is distributed from plasma much more slowly than unformulated MTX. These data suggest that the nanodevices are stable in plasma and contain the MTX against a concentration gradient.
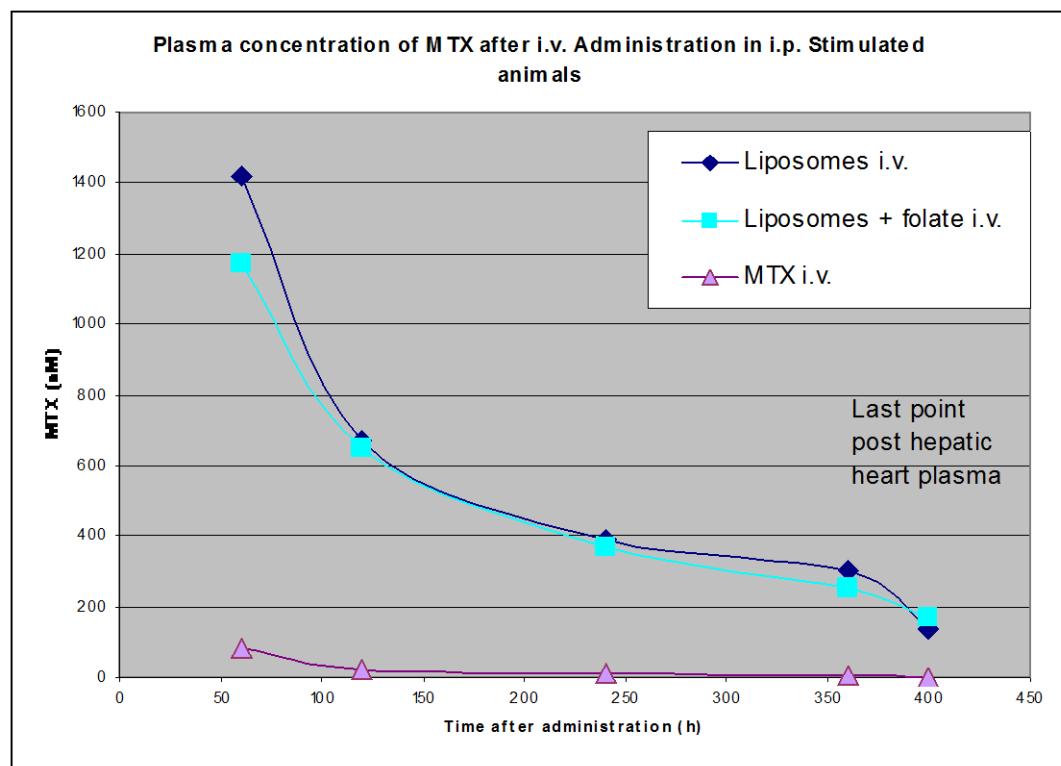
Figure. 11. Pharmacokinetics of MTX when provided as free drug dissolved in serum, vs. MTX in liposomes. All materials were injected i.v. at a dose of 0.6 mg/kg MTX.
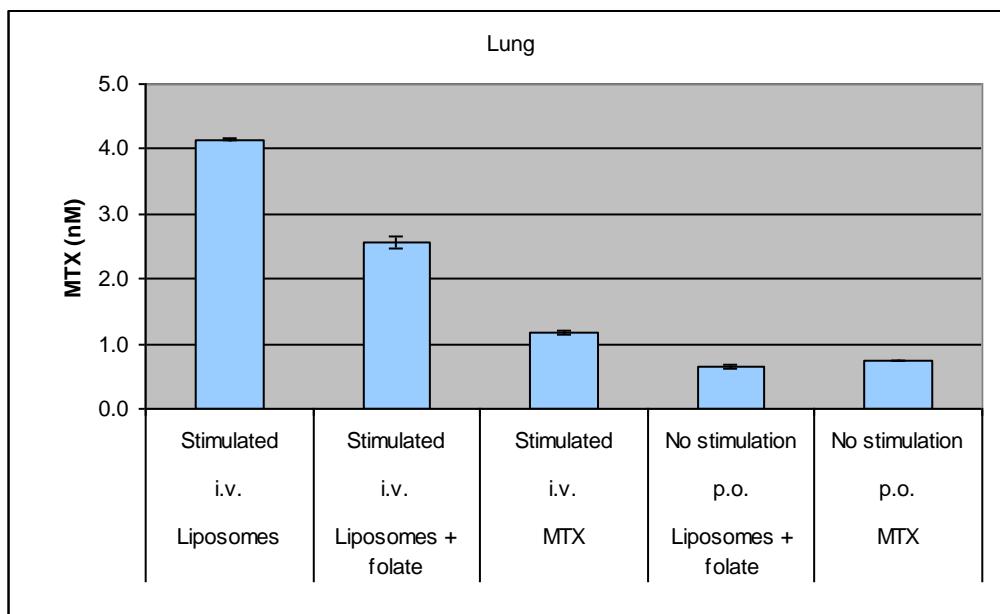
Figure. 12. Distribution of MTX when provided as either a free drug dissolved in serum, vs. MTX in liposomes. Data are for lung in nM on the basis of the assumption that tissue is 90% liquid and d=1. Materials were injected i.v. at a dose of 0.6 mg/kg MTX or p.o. at a dose of 1.2 mg/kg MTX.
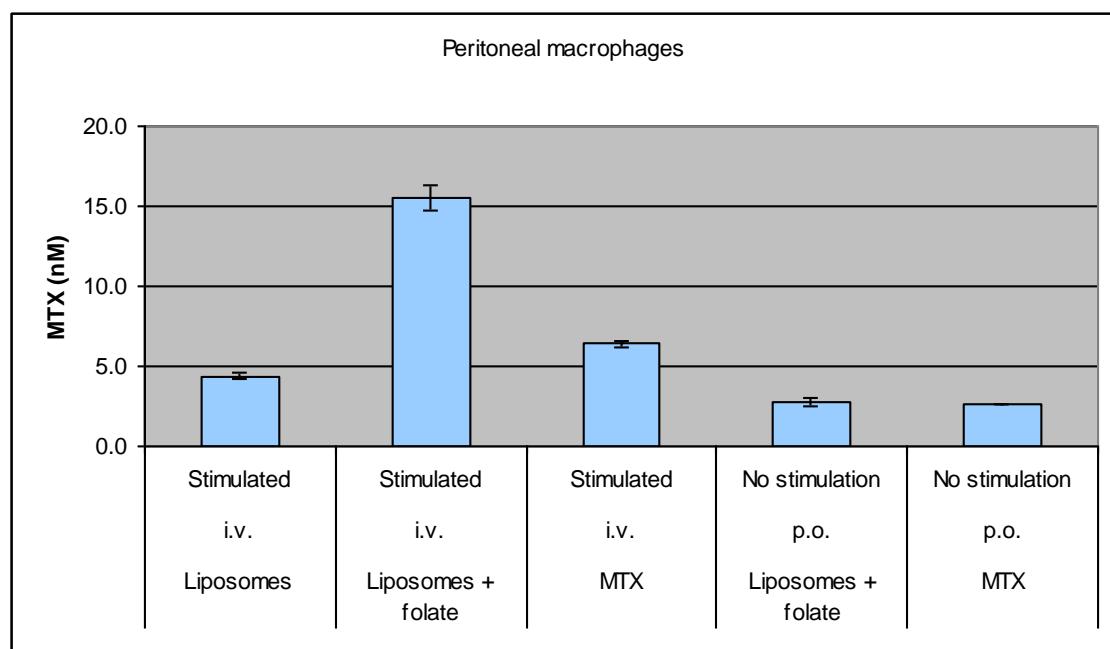
Figure. 13. Distribution of MTX when provided as either a free drug dissolved in serum, vs. MTX in liposomes. Data are for Peritoneal Macrophages in nM on the basis of the assumption that tissue is 90% liquid and d=1. Materials were injected i.v. at a dose of 0.6 mg/kg MTX or p.o. at a dose of 1.2 mg/kg MTX.
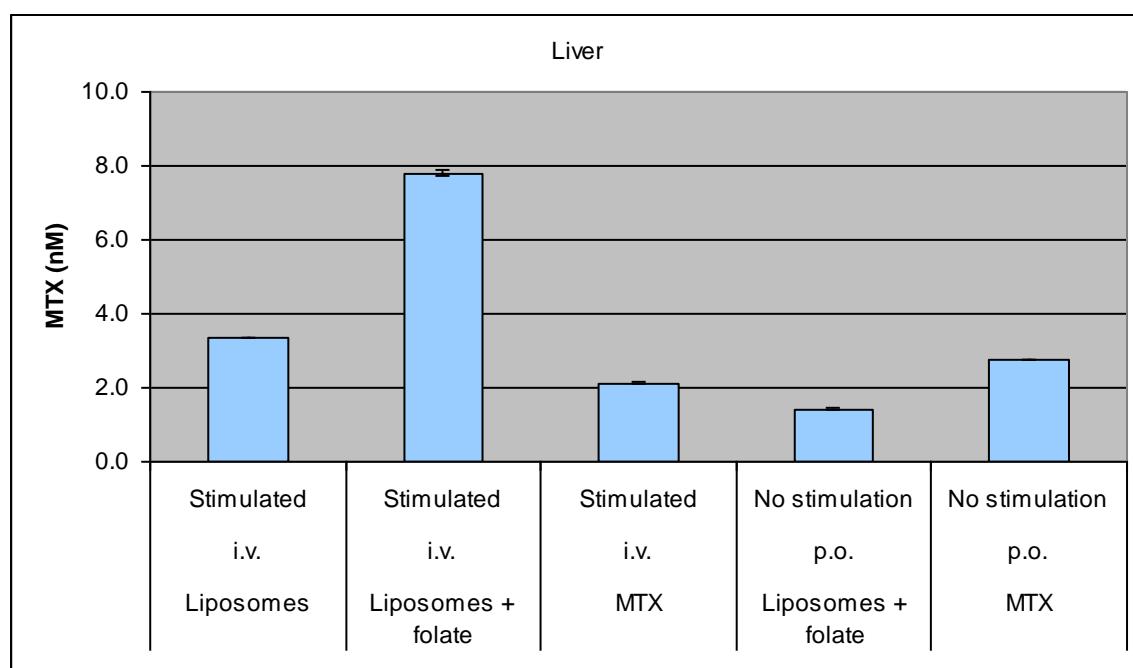
Figure. 14. Distribution of MTX when provided as either a free drug dissolved in serum, vs. MTX in liposomes. Data are for lung on the basis of the assumption All materials were injected i.v. at a dose of 0.6 mg/kg MTX.

These data indicate two key points:
The first is that in the presence of a sink or localised set of folate receptor expressing cells, there is a folate mediated increase in the apparent concentration of MTX in the compartment. The apparent increase is in the order of 3- to 4-fold over either free MTX or liposomal MTX without decoration.
The second is that the folate decorated liposomes have at least the bioavailability of the free folate, if anything, with a longer terminal half-life. The key challenge now is to determine

whether the liposomes are entering the gut lining intact, and whether they are "inflammo-tropic" thereafter. In this study, the system of high application volume was used to obtain a high by-pass of the stomach.

Clinical significance

In the clinic, MTX is administered both orally and via injection. The oral application is more prone to side effects, especially in central organs. For this reason, many patients prefer the injected form. However, this is also associated with issues. In current therapy, methotrexate is used once a week and is supplied in excess, however, through rapid elimination, it is largely removed from the body over 1 to 2 days with the first 24 h being associated with side-effects including lethargy. The initial high dose is considered necessary to fully bind target sites and obtain an effect. The bulk of the methotrexate applied normally is not delivered to the target site but instead taken into the main metabolic organs (liver spleen kidney) where it exerts a general metabolic suppression that is felt as lack of energy by patients. This particular side-effect is reported most frequently by women (ca. 75% of reports).

The presumed mode of action of these undesired effects are actions on the following proteins or enzymes (Weisman et al., Arthritis Rheum. 2006 Feb;54(2):607-12.

Risk genotypes in folate-dependent enzymes and their association with methotrexate-related side effects in rheumatoid arthritis):
- Methylenetetrahydrofolate reductase (MTHFR) 677TT, (brain/CNS)
- Thymidylate synthase (TSER) *2/*2 (variable number of tandem repeats), (hairloss)
- Amino imidazole ribonucleotide transformylase (ATIC) 347GG, (gastrointestinal) and,
- Serine hydroxymethyltransferase (SHMT1) 1420CC (brain/CNS/hairloss)

The dose used is designed to still provide sufficient MTX to the peripheral targets despite the large amounts taken into the organs directly.

In contrast to MTX, the FBN is selectively retained in plasma and through the larger size of the liposomes, is not subject to immediate absorbtion and filtering by the main organs. This means that the FBNs can circulate to their peripheral target and be bound there instead of being non-selectively absorbed by the intestine, liver, kidney and brain.

The results show that FBNs exhibit much longer exposure time in blood than does free methotrexate. Moreover, they suggest that the targeting vehicle actually reaches the target cells in good amounts, and brings the drug with it.

In an ideal pharmacological system the compound would be present at its target in low but stable amounts sufficient to exert an effect. Excess amounts would then be no longer available for the main metabolic organs, which are anyway not involved in the pathological response.

Given the advantages of the nanofol formulation have conducted a range of studies to show how these advantages can be used in a simulated clinical setting.

1.3.5. WP5 - Life Cycle & Risk Analysis (LCRA) and Ethical Issues (Leader: INERIS)

The overall aim of WP5 was to accompany innovation by providing risk analysis links to the development of nanobiodevices (NBD), from their conception to their elimination, including their use.

Task 5.1 was expected to provide recommendations / advice for producers and users of the NBD in the framework of nanosafety, first within the project, then by considering an industrial production.

Task 5.2. was devoted to *in vitro* and *in vivo* assessment of NBD toxicokinetics.

A life cycle approach (**Task 5.3**) was then considered in order to comprehend all the elements to take in consideration. The analysis covered all life stages, from the conception / development of the NBD, through the use to treat chronic inflammatory diseases in patients, up to its elimination. The first tasks provided some elements to help for the life-cycle analysis (LCA) related to the NBD, regarding the conception/production at first (Task 5.1), then regarding the use of the device on humans, by assessing the toxicity of both individual components and whole folate-based nanobiodevice for a further estimation of the risk assessment for treated patients (Task 5.2).

This approach was completed with a global socio-economic study regarding the use of the NBD that will be carried out with a cost/benefit assessment (**Task 5.4**).

Ethical issues were considered throughout the whole project especially related to animal experimentation. Issues were analysed in a way to find concrete solutions for reducing animal testing (**Task 5.5**).

Task 5.1: Industrial hygiene

The European research project Nanofol is part of a general initiative to develop new medications in the area of nanosciences.

The safety of nanostructured products – "nanosafety" – is an essential component of the nanosciences.

With this in mind, the Nanofol project includes a specific work package devoted to nanosafety (WP 5).

This deals specifically with the problem of nanosafety in the experimental work carried out at Nanofol (Task 5.1). This part of the project concerns the obligation of the employer to make every effort to protect workers, among others, from all types of risk, and in particular from chemical risk. In this framework, INERIS aims to help ensure that the participants in the Nanofol project take better account of the nanorisks in their laboratories.

Two delivrables are planned, on one hand to describe the exposure in laboratories and inform the users on the nanosafety recommendations (M12); on another hand to deal with nanorisks concerning the final product (M48).

For the case of the laboratory visited, the following recommendations can be formulated (Table 1).

The nano-waste management is a huge problem because of a less number of providers which are able to deal with it. With the aim to make the nano-waste management easier, we have proposed to find a way to destroy nanobiodevices, especially liposomes. It is necessary to dilute liposome suspensions in hydrogen peroxide and heat to 60 ° C, to achieve this destruction.

Themes	Recommendations
Layout of the premises	
	Replace the tiling and paint on walls as necessary in order to have walls that are easy to decontaminate
Confinement of the source of hazard and protection of operators	
	Use of hood checked annually for all handling, with a HEPA (≥ H14) or ULPA (≥ U14) filter. *In the present state of our investigation, double confinement during experiments is not strictly required (nanovesicles in aqueous dispersions)*
	Use of two pairs of nitrile gloves*, glasses*, suits*, safety shoes* during handling or local transport

Confinement with regard to the environment	
	Handle nanomaterials only under a hood (including cytometry)
Waste management	
	Separate nanowaste from other waste; adapt storage (double confinement) in order *to guarantee no emission of nanovesicles in pulverulent form;* select a disposal system suitable for nanowaste
Transport	
	Use suitable double confinement* systematically during movement of nanomaterials
Training	
	Provide training material specific to the risks related to the use of nanoparticles
Signage	
	Establish signage specific to nanomaterials
Accident	
	Develop a procedure suited to the activity of the laboratory to plan the approach to be adopted in case of accidents (spill, rupture of confinement, contact with the skin, etc.)

Table 1: Recommendations with regard to nanosafety.
*: measures concerning percutaneous absorption risk

One of our purposes is to identify a tracer of our nanobiodevice. Such a tracer has to:
- be present on the vector rather than inside
- not to be present in the environment,
- be easily identifiable.

Transmission Electron Microscopy (TEM) is one of the more efficient tools to characterised any nanomaterials. However, TEM requires the analysis under dry conditions which, in general, may destroy the liposomes. In such a case, only membranes are observable. In order to handle this, a droplet is deposited on a TEM grid to identify nano-objects. Fortunately liposomes provided by UMINHO are so stable that few of them are still observed after drying. This is an indication of a strong modification of the liposomes.

The phosphorus present in a phospholipidic membrane is detected by microanalysis. Consequently a spherical object with the phosphorus could be a tracer.

A visit at the producer (UMINHO) highlights some improvements in the management of nanomaterials. Key enhancements are:

Management of the work in fume hood when the production of nanomaterials is found,

Establishment of the signage specific to nanomaterials,

Use of suitable double confinement systematically during movement of nanomaterials.

No sampling carried out during the visit at UMINHO have given rise to a presence of a liposome or crashed membrane on the grid which could highlight a release of aerosolized liposomes.

Task 5.2: Toxicokinetics and toxicodynamics

Two kind of nanobiodevices (NBD) were produced and assessed in the project :

1. BSA[2]-based nanoparticles (BSA-NPs) with tween or poloxamer, containing or not folate-peptide at the surface and containing or not anti-inflammatory drug inside (celecoxib or methotrexate-MTX).

2. Liposomes made of EPC[3]/CH[4]/DSPE-MPEG[5] + Vit. E, containing or not folate-peptide at the surface and containing or not anti-inflammatory drug inside (celecoxib, CORM-2 or MTX). In the last available formulation, EPC was replaced by DOPE[6] in an attempt to make the liposomes sensitive to acidic pH

The NBD were provided suspended in phosphate-buffered saline (PBS). The size distribution of the suspensions were measured by dynamic light scattering with a Zeta-sizer (Malvern). **NBD suspensions were stable, with a unimodal distribution of size, centered around 100 nm for BSA nanoparticles and around 130 nm for EPC and DOPE-liposomes (Figure 15). Contrary to expectations, DOPE-liposomes appeared very stable in suspensions of various acidic pH (2 and 5) (Figure 16).**
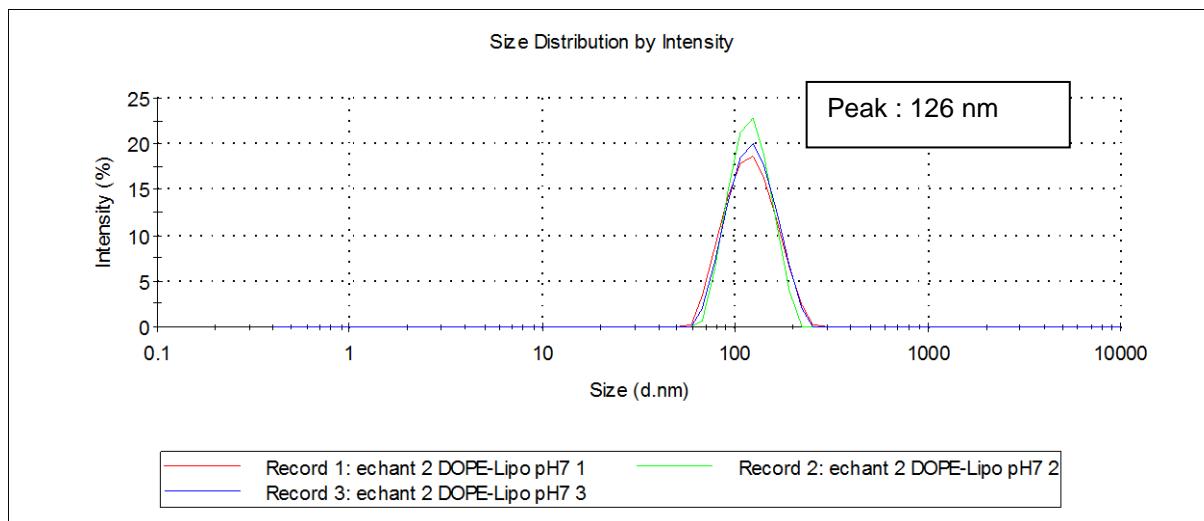

Figure 15: Size distribution of a DOPE-liposome suspension in PBS

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[2] BSA: Bovine serum albumin
[3] EPC: Egg phosphatidylcholine
[4] CH: Cholesterol
[5] DSPE-MPEG: [N-(carbonyl-methoxypolyethylene glycol-2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine]
[6] DOPE: 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
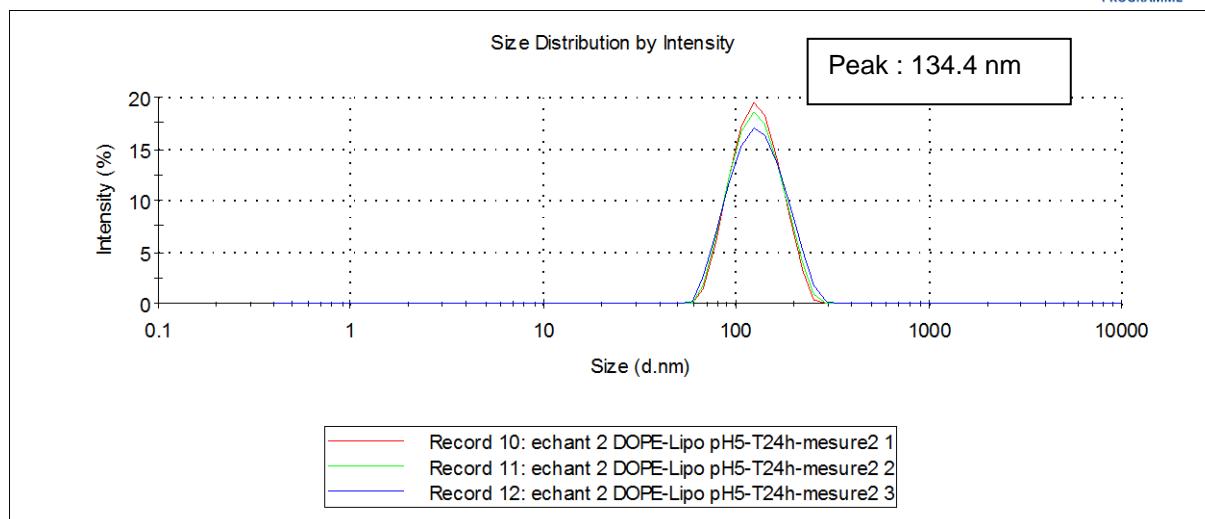
Figure 16: Size distribution of a DOPE-liposome suspension in PBS (pH 5) at T+24h

In vitro toxicity of NBD (cytotoxicity, inflammatory and oxydative properties) was assessed on non target human cell lines of various origin : pulmonary cells (A549), hepatic cells (HepG2), renal cells (Caki-1), monocytic cell line (THP-1), fibroblasts (BJ5ta) and also on a murine macrophage cell line (RAW. 264.7). Cytotoxicity was assessed using routine cytotoxicity tests (namely metabolic tests such as SRB, MTT assay and derivatives and Alamar blue™ and also LDH assay which reflects the cell membrane integrity), at concentrations up to 5 mg/mL for BSA-NPs and 0,8 mg/mL for liposomes.

The main finding was that, in our experimental conditions, these formulations presented a very low (BSA-NPs) or no (EPC-liposomes) cytotoxicity regardless of the presence or absence of folates and drug. Encapsulation of the anti-inflammatory drug methotrexate (MTX) in liposomes seems to protect the cells against MTX effects, meaning a great stability of liposomes in our cell culture conditions and thus a poor intracellular delivery of MTX by liposomes. One explanation could be the very high resistance of liposomes to phagosomal degradation. A corrective action to overcome this issue might be to incorporate pH-sensitive lipids into the device to make them sensitive to the very low pH of phagosomes (this point remains to be done as DOPE-liposomes appeared stable in acidic suspensions).

The pro-inflammatory potential of BSA-NPs 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 at doses of 0.156 or 0.625 mg/ml. **In these experimental conditions, NBD display no pro-inflammatory properties on non target cells**.

The induction of oxidative stress was studied with the DCFH-DA probe, which measures the presence of intracellular reactive oxygen species (ROS). Oxidation of the probe due to ROS, switches it to a highly fluorescent compound which is detectable. **Results showed no significant oxidative properties of BSA-NPs on human fibroblasts. Similar results were obtained with liposomes with and without encapsulated celecoxib.**

As nanobiodevices (NBD) were expected to be injected intraveinously, their stability in the presence of serum and their haemolytic properties were assessed. **Suspensions of BSA-NPs or liposomes in 80% serum remain stable for at least 24h.** The mean sizes of the main peaks were slightly enhanced, with a value of 126 nm (96 nm in PBS) for BSA-NPs and 136 nm (126 nm in PBS) for liposomes. This might be due to an adsorption of seric proteins at the surface of the NBD, thus enhancing the size of the droplets.

The capacity of the NBD to destroy red blood cells was assessed in vitro on rat blood according to the standardised protocol ATSM E 2524 " Standard Test Method for Analysis of

Hemolytic Properties of Nanoparticles". Briefly, BSA-NPs or EPC-liposomes were mixed with blood and incubated for 3h at 37°C. A centrifugation was then performed and Hb was measured in supernatant.

As shown in Figure 17, **no significant haemolytic property was noticed for all the formulations tested.**
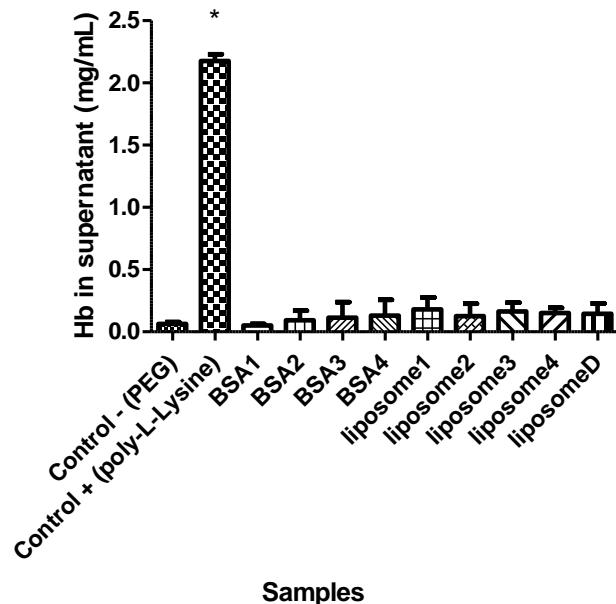
Figure 17: Haemolytic properties of NBD towards rat blood. Values are means ± SD for 3 independent experiments. (* significantly different from negative control)

It was decided, within the consortium, to focus on one particular kind of NBD, i.e. folate-liposomes containing the anti-inflammatory drug methotrexate (MTX) inside. Therefore, in vivo studies performed in mice were done on free or liposome-encapsulated MTX.

Mice were subcutaneously injected with free or EPC-liposome-encapsulated MTX at a unique dose of 0.5 mg MTX /kg (which is close to the weekly dose received by patients suffering from chronic inflammatory diseases). At various intervals of time, ranging from 15 min to 360 min for free MTX and from 15 min to 72 h for encapsulated MTX, mice (3 per groups) were sacrificed and various organs removed for further MTX analysis, in order to determine the biodistribution of free or encapsulated MTX.

The encapsulation of MTX in liposomes modified the fate and behavior of MTX in the body. When injected as free molecule, MTX concentrations in the body fell within a few hours. On the contrary, encapsulated MTX seemed to be slowly released by the liposomes, resulting in low concentrations of free MTX in the body but a higher persistence. However, analysis of kinetics data for encapsulated MTX using the PBPK (Physiologically-Based PharmacoKinetics) model by Bischoff et al. (1971) for which we showed a good agreement with our kinetics data for mice exposed to free MTX could not explain the time course of liposome-released MTX.

Mice of the biodistribution study were also assessed for free and encapsulated-MTX general toxicity. Blood analysis (i.e. numeration, formula, biochemistry and cytokine dosage) was performed 60, 180 and 360 min after injection of free MTX and 60, 180, 360 min and 24h after injection of liposome-encapsulated MTX. In order to assess the toxicity of the NBD by themselves, additional mice were subcutaneously injected with EPC or DOPE-liposomes without MTX inside (at a concentration of about 2 mg/kg).

With a single subcutaneous injection of either 0.5 mg/kg of free or encapsulated-MTX or of 2 mg/kg liposomes, the general toxicity appeared limited in healthy mice: no clinical signs and no modification of blood numeration/formula and pro-inflammatory

cytokines were observed up to 24h after the injection. A transient increase in hepatic enzymes was noticed with free MTX but not with encapsulated MTX. Liposomes without MTX inside seemed to slightly altered some blood markers. This finding, together with the expected longer lifespan of encapsulated MTX in the body, justify further toxicological studies on liposomes with or witout MTX inside, at higher doses and/or at repeated doses. This will require larger quantities of NBD than those available in the project.

As a general conclusion:
- In our experimental settings, NBD display low or no toxicity on non target cells, on blood and on healthy animals,
- NBD induce a higher persistence of encapsulated drug in the body suggesting the use of lower amount of drug to treat patients
- These findings, together with those of task 5.1. and other results obtained within the consortium (efficiency, bioavailability…), were taken into account in the life cycle analysis

Task 5.3: LCA

The Life Cycle Analysis (LCA) conducted for NANOFOL follows in most aspects the methodology of the ISO 14040 standard. It applies a cradle to grave approach, covering material and energy flows and related emissions and waste from the production of raw materials through the manufacturing and use steps up to the treatment of residues. Transport activities between and within the different life cycle aspects are equally included in the analysis.

LCA input data originates from various sources, including a literature review and information provided by industry and NANOFOL partners. No information was found about a possible ecotoxicity of nano vectors (BSA and liposomes). Therefore the LCA, and further on the SEA (Socio-Economic Analysis), assume that no impact is expected from the release of BSA or liposomes in the environment. Both LCA and SEA also make the assumption that the currently commercialised and the prospective nano based drugs have identical therapeutic benefits.

In the LCA impacts were calculated with respect to a common functional unit, the 'treatment of a rheumatoid arthritis (RA) patient during one week'. The LCA analysed and compared four scenarios. Two reference scenarios for existing commercialized treatments, one assuming an oral administration (REF ORAL), the other assuming subcutaneous injection (REF INJ). And two prospective, nano medicines based on liposomes (NBD INJ LIP) and BSA (NBD INJ BSA), respectively, both assuming subcutaneous injections as administration mode. As an important aspect, the NBD scenarios are calculated for a lower dose of the active agent MTX, in line with results from WP 3 that an encapsulation of the active agent allows increasing the effect of this agent.

The major conclusions of the LCA are:
- The scenarios with nano vectorised solutions show distinctly lower impacts in terms of human toxicity. This is mainly determined by the lower dose of MTX administered per functional unit, which considerably reduces human toxicity for the patient.
- For most of the other LCA impact categories, the NBD scenarios, especially the liposome scenario, show higher impacts than the REF scenarios, as illustrated in Figure 18.[7] The higher impacts are primarily influenced by raw material manufacturing, i.e. the use of trichloromethane for liposome nanovesicles and bovine albumin serum nanovesicles.

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[7] In
Figure results are presented in a relative way, setting the scenario contributing most to a given environmental effect at 100%, and expressing the results for the other scenarios relative to this highest contributing scenario.
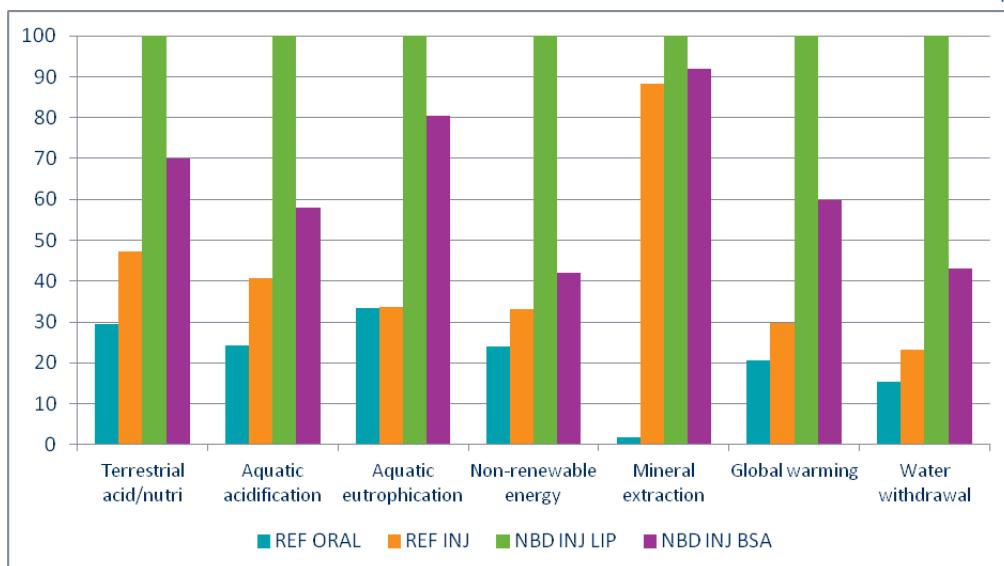
Figure 18: Life cycle comparison (excluding human toxicity and ecotoxicity impacts) without nurse transportation; Source: Catalan & Hamon (2013)[8]

- With respect to ecotoxicity, the results of the LCA are less clear cut. Whereas the liposome scenario implies lower ecotoxicity impacts than both reference scenarios, the BSA scenario leads to higher impacts than REF ORAL.
- When taking into account nurse transportation in the analysis - the three treatment alternatives relying on subcutaneous injection imply administration by a nurse - the first conclusion on human toxicity is no longer valid according to the LCA. Indeed, nurse transportation becomes the primary cause of impacts in terms of LCA categories.

In order to reduce all environmental and health impacts analysed, the LCA recommends developing a prospective drug that can be auto-administered. Furthermore, recycling of chloroform would reduce toxicity impacts.

Task 5.4: SEA

The LCA was complemented by a socio-economic analysis (SEA), consisting in a partial cost-benefit comparison between the existing, commercialised and the prospective, nano based treatment. This analysis aimed at capturing in a common, monetary unit the overall sustainability of the nano based medical treatments from a societal perspective.

A bibliographic study demonstrated the feasibility of monetising the major LCA impact indicators for which impacts differ significantly amongst the four scenarios studied. These comprise human toxicity (valued based on the Value Of a Life Year), ecotoxicity (valued via monetary values available for the indicator Potentially Disappeared Fraction of species) and global warming (valued based on estimates of Social Costs of Carbon and Marginal Abatement Costs). Internet research and exchange with project partners allowed selecting proxies for costs of the alternative treatment options.

The SEA thus takes account of the lower secondary effects for patients, the environmental impacts, and the additional cost of the new medicine for the public health system. Benefits were calculated as the monetised equivalent of avoided health and environment impacts when replacing the existing by the nano medicine and additional costs as the price difference between the traditional and new medicines. For the prospective drugs price proxies were suggested by an expert from the project partner SYNOVO. Both for impact and cost indicators ranges of monetary values were used in the assessment to capture the sensivity

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[8] Catalan, C. & Hamon, L. (2013): Comparative Life Cycle Assessment of Rheumatoid Arthritis Treatments – Final Report, Report prepared for INERIS by QUANTIS.

of results to economic assumptions. All economic values are expressed in the € 2012 price base.

Major results of the SEA, calculated excluding the impacts related to nurse transportation, are as follows:

- The monetary values of the aggregated impact on health and the environment are higher for the existing than for the prospective, nano based drugs. Replacing the existing by the prospective drugs thus leads to benefits. This result holds for high and low monetary estimates for the impact indicators and can thus be considered as robust.

- These monetised additional benefits for human health and the environment range from € cents 10 to 70 per functional unit for the different combinations of high and low monetary estimates per LCA indicator (cf. Figure 19). The benefits were calculated for a replacement of REF ORAL by NBD INJ LIP but are representative of the replacement of either REF by either NBD scenario.

- This result is essentially driven by the monetized human toxicity impacts to the patient which were calculated as an addition to the LCA for the specific purpose of the Nanofol SEA. In fact, for the reference scenarios the total human health impact (human toxicity to the patient and to the general population) accounts for more than 99% in the total monetized benefit, for the prospective scenarios it accounts for 90% to 94%. In other words, lower secondary effects to the patient of the nano based drugs are more significant than some environmental drawbacks.
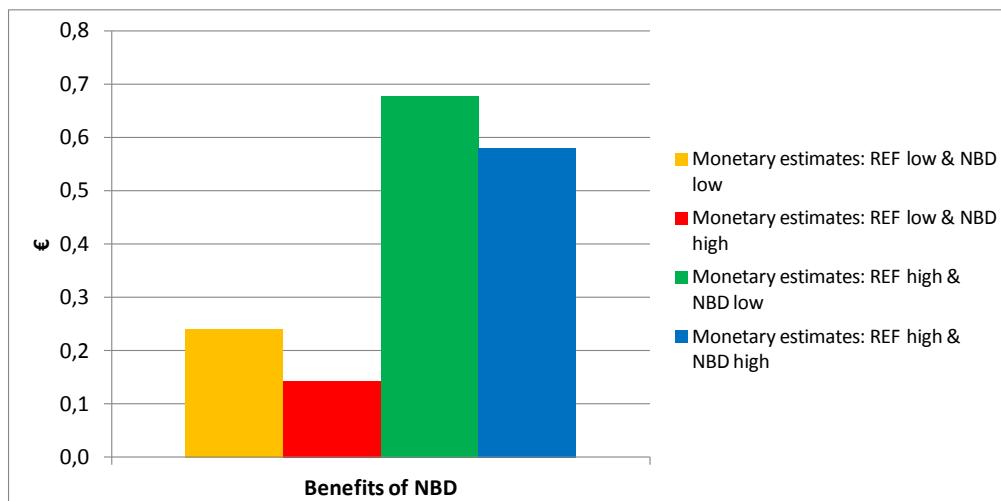
Figure 19: Benefit range when replacing existing RA medicine (REF) by nano vesicle medicine (NBD)

- The assessment of net benefits (calculated as benefit minus additional cost) is limited to a comparison between the different subcutaneous injection scenarios, as oral treatments are generally less expensive than injections and therefore the two are not really comparable. Having excluded the impacts related to nurse transportation in our calculation, we do not take into account the costs for the nurse intervention either.

- The net benefit for replacing REF INJ by NBD INJ LIP or by NBD INJ BSA results in values approximately ranging from € -3.6 to € +8 for the different combinations of low and high value estimates for LCA impact indicators and costs. Benefits exceed additional costs only under low cost assumptions for the nano based drugs. The future price of these drugs, therefore, appears to be key in ensuring the overall sustainability of the innovative treatment.

- It should be noted that uncertainty about costs, especially for the prospective drugs, is high. The absence of precise cost information clearly constitutes a limitation to the analysis.

- All results were calculated under the assumption of the drug alternatives having identical therapeutic benefits. If this were not the case, the difference between current and future drugs would need to be included in the SEA. This might affect overall conclusions.

Task 5.5: Ethical aspects and animal testing reduction

The objectives of The European project Nanofol were to develop new diagnosis and therapeutic agents for rheumatoid arthritis (RA), using nanodevices targeting specifically activated macrophages. Several phases of the project required the use of animals (only mice), especially to validate *in vivo* the targeting of inflammatory site and therapeutic efficacy of the nanoparticles. Indeed, animal models of human pathologies, and particularly rodents, are increasingly used in pre-clinical research, allowing the description and understanding of human diseases and the evaluation of potential treatments. **Thus, the central ethical question that needed to be tackled during the Nanofol project was the use of animals.**

This was done at two levels, as required by the EU standards:

1) **We first followed the 3-R's rule, *i.e.* the aim of the experiment cannot be satisfied by use of another method not involving animal experimentation ('replacement'), as few animals as is possible are used ('reduction'), and the animals are not exposed for more suffering than absolutely necessary ('refinement').** Our experimental design has fulfilled these three imperatives thanks to several action plans:

- Use of *in vitro* assays was preferred wherever possible to replace animal experimentation. This held true with the majority of the experiments that have been performed within the various tasks in the different WP of the project to check the nanobiodevices. But replacement was not always possible for some *in vivo* experiments, for which no alternative to relevant models of RA in mice exists to evaluate the targeting and the clinical effect of nanodevices.

- In this case, and to further reduce to a minimum the number of animals used, only a few nanobiodevices were tested at relevant doses. These rules have been followed for experiments checking the therapeutic activity of the nanobiodevices and their pharmacology and toxicology properties.

- The well being of the animals (refinement) was also of importance during the project and the degree of suffering by animals has been always minimized. Direct pain to the animals was restricted as much as possible, with examination performed on anesthetized animals. Important efforts were also made to make preferential use of small animal imaging methods since they are non-traumatic and non-invasive tools for result evaluation.

2) In addition, as the Nanofol proposal takes place in European countries in which clinical research and experimentation are regulated by both national and international legal and ethical rules, **the research program was also conducted in full agreement with relevant local or national rules and regulations of the country where the research was carried out and subjected, as appropriate, to prior authorisation of the project by competent research ethics (s) of that country.** Accordingly, all the activities carried out with mice during the project were respectful of the different standards stemming from our "quality-insurance" policy, promoting awareness of best practice in the care of laboratory animals, as recommended by national and/or EU legislations **(2010/63/EU directive)**, including: i) a prior authorization for all projects using animal models; ii) the creation of a local structure responsible for the welfare of animals; iii) a level of initial training for project leaders, and, for everyone, a specific training in animal experimentation; iv) the daily animal monitoring obligation. We also followed the general guidelines underlying the conception and use of pathogen free animal facility that have been drawn up so as to cope with emerging and forthcoming needs and challenges enacted by the scientific community throughout the world, both to practical and ethical ends.

1.4. Potential impact and dissemination and exploitation of results

1.4.1. Potential impact

NANOFOL nanobiodevices targeting activated macrophages may become an interesting therapy solution. The results indicate that better clinical scores can be FBN rather than tradicional drug for RA. Some other potential alternatives base on target therapy. Such new therapies could reduce secundary effects and reduce time of hospitalization of patients.

Folate-based nanodevices can be applied to other situations other than chronic inflammatory diseases such as cancer. The oncology market is the third largest pharmaceutical market, and is currently experiencing strong growth. Worth an estimated $77 billion in 2013, GMR Data forecast that the global cancer drugs and treatments market will reach $143.7bn by 2023.

(http://finance.yahoo.com/news/cancer-drugs-treatments-market-data-160400335.html).

A recent report foresees a critical expansion in the nano-based drug delivery market from its current $3.4B (about 10% of the total drug delivery market) to about $26B by 2012, being this only a promising beginning for the $220B forecasted by 2015 [The Nanoparticle drug delivery market, Científica, 2006].

The nanobiodevices developed in the NANOFOL, namely Liposomal FBN with MTX has been shown to be a better therapeutic apporach than MTX alone. Those results are a major breakthrought in RA therapy, because the new liposomal fomrulations:
-   present less secondary effects
-   are more efective than the drug alone

Despite the economic evaluation has been made, major benefits are expects from the NANOFOL developed tecnology. It is an optimal directed therapy which will ensure minimal drawback for the patient and hopefully optimal recovery circumstances.

Commercialising more effective medical systems with no or substantially reduced side-effects will have positive consequences on market share for European industry.

The development through the NANOFOL project of a new cost-effective alternative treatment for inflammatory diseases such as RA will help EU market increase its market share in this highly competitive sector.

NANOFOL contributed to improve European biotech industry through the development of novel treatments for chronic inflammatory diseases. The NANOFOL project when aplied in the clinics will:

-   significantly increase economic growth by improving European citizens quality of life. Indeed, as recommended in the Healthy Ageing European project, NANOFOL will promote economic growth and competitiveness by ensuring that older people with chronic conditions and disabilities will remain active and independent thanks to the development of a cost effective alternative for diagnosis and treatment of many chronic inflammatory diseases.
-   dramatically increased competitiveness of European healthcare industries: NANOFOL will provide favourable conditions for industrial innovation ensuring that R&D is translated into affordable and safe wealth-generating products. Within this project, affordable Folate Based Nanobiodevices for simultaneous therapy and diagnostics will be developed.

The project will thereby improve the European Union's competitiveness in the activity area of knowledge nanotechnology-based delivery systems by building a platform on which spin-offs can be generated.

NANOFOL took up the European Challenge presented in the European Science Foundation Policy Briefing on rheumatic disease (2006), by ensuring the:

- Integration of successful national research efforts into a pan-European research strategy with a consortium bringing 8 European countries together;
- Integration of basic, clinical and health care research for a fast translation of new concepts thanks to the active involvement of high-tech SMEs ;
- Integration of competence in molecular biology, genetics and immunology with a multidisciplinary consortium.

1.4.2. Dissemination and exploitation of results

Since the very beginning of the NANOFOL project, the members of the consortium have been very active disseminating the work that was being carried out in the project, the results achieves and the existance of the project itself. As a result, 69 dissemination actions of any kind have been reported to far. Most of them consisted of the participation of the partners in conferences/congresses were the research done in the project was presented either in posters, oral communications, or abstracts.

Most of the dissemination actions were targeted towards the scientific community, due to the nature of the research conducted. Nevertheless, a significant share of the dissemination actions targeted the industry, in order to raise their interest and try to reply to unmet market needs. Apart from the scientific community and the industry, general society was also targeted with the objective of disseminating to taxpayers that EU funding is being invested in R&D and the objectives pursued by the NANOFOL project, which respond to the demands of the European society.

Since the beginning of the project, results have not been extensively published in peer-reviewed journals in order to protect the scientific data and results produced. Four papers have been published in peer-reviewed journals, two of them in the last year. However, several papers are planned to be submitted to journals in the following months. In fact, eight papers are currently being writen by members of the consortium and will be submitted in the next weeks.

Regarding the exploitation of the results achieved in the project, valuable foreground has been produced and identified as susceptible of industrial application. The main exploitable results are identified below:

- Result number 1: Fusion proteins rational design peptides linked to folate for targeting diseases with an over expressing of folate
- Result number 2: Characterised nano-vehicles, with folate at its surface, incorporating pharmacological drug
- Result number 3: Process for the production of nano-vehicles with folate and/or Mab at its surface, incorporating a either pharmacological drug
- Result number 4: New monoclonal antibodies with desired specificity and affinity to targets
- Result number 5: Genetically engineered monoespecific antibodies coupled to the surfactant neck domain construct and/or HSA

Since the begining of the project, The NANOFOL partners envisaged three main exploitation routes for the foreground produced, depending on the results, their maturity, the investment needed to take them to the market, and their fit with the strategy of the different members of the consortium:

- Direct exploitation by project partners: It is considered the best direct exploitation route for that foreground that fits with the strategy, and competences of one of the members of the consortium. This is the case of an especific Folate Receptor Beta Antibody (result 4) that is ready to be exploited and will be manufactured and distributed by the partner EXBIO.
- Spin offs: Several partners have experience in the creation of successful spin off companies. This would be the ideal route for foreground produced by several partners, with no direct fit with the business strategy of any of them but being sound enough to establish a new venture on it.
- Technology transfer: For other results of the project that do not fit directly with the interests of any of the members of the consortium and are not sound enough to establish a new venture, transferring the technology to other companies might be of interest.
- Given that there is good evidence that a clinical benefit may be obtained from liposomal methotrexate, two of the partners have indicated their willingness to generate a spinout to clinically develop this technology as an infusion system.
- One of the SMEs plans to patent and clinically develop its antibody technology derived from the project.

Now the project has come to its end, some of the results obtained seem to be promising but need further development before they can effectively be exploited. This is the case of the innovative nanobiodevices developed and their use to formulate active ingredients to target activated macrophages. In this regard, some members of the consortium are considering the possibility of submitting a new project for Horizon 2020 that will develop this technology further and will take them closer to market.

1.5. Consortium and contact information

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Partners
- Suanfarma SA – Spain
- Technische Universitaet Graz – Austria – until November 2012
- Nederlandse Organisatie Voor Toegepast Natuurwetenschappelijk Onderzoek TNO – Netherlands
- Instituto de Biologia Molecular e Celular IBMC – Portugal
- Institut National de la Santé et de la Recherche Médicale INSERM – France
- Medizinische Universitaet Wien – Austria
- "Aurel Vlaicu" University of Arad – Romania
- Synovo GmbH – Germany
- Institut National de l'Environnement et des risques INERIS – France
- Exbio Praha AS – Czech Republic
- ALMA Consulting Group SAS – France
- Universität für Bodenkultur Wien – Austria – from December 2012

1.5. Acknowledgements

Section 2 - Use and dissemination of foreground

2.1. Section A: Dissemination (Public)

2.1.1. List of scientific (peer reviewed) publications (A1) - Public

Template A1: List of scientific (peer reviewed) publications, starting with the most important ones											
No	WP***	Title	Main authors	Title of the periodical or the series	Number, date or frequency	Publisher	Place of publication	Date of publication	Relevant pages	Permanent identifiers** (if available)	Is/Will open access*** provided to this publication?
1	WP1	Liposome and protein based stealth nanoparticles	Artur Manuel Cavaco-Paulo, Eugénia Nogueira, Ana Loureiro, Patrícia Nogueira, Jaime Freitas, Catarina R. Almeida, Johan Härmark, Hans Hebert, Alexandra Moreira, Alexandre M. Carmo, Ana Preto and Andreia Gomes	Faraday Discussions	14/06/2013	Royal Society of Chemistry	online	14/06/2013	1		No
2	WP1	Folic acid-functionalized human serum albumin nanocapsules for targeted drug delivery to chronically activated macrophages.	AlexandraRollett, Tamara Reiter, Patrícia Nogueira, Massimiliano Cardinale, Ana Loureiro, Andreia-Gomes, Artur Cavaco-Paulo,	International Journal of Pharmaceutics	Vol. 427, Issue 2	Elsevier		10/05/2012	460–466		No

No	WP	Title	Main authors	Title of the periodical or the series	Number, date or frequency	Publisher	Place of publication	Date of publication	Relevant pages	Permanent identifiers** (if available)	Is/Will open access*** provided to this publication?
3	WP2	HSA nanocapsules functionalized with monoclonal antibodies for targeted drug delivery	Alexandra Moreira, Alexandre Carmo, Georg M. Guebitz Alexandra Rollett, Tamara Reiter, Anna Ohradanova-Repic, Christian Machacek, Artur Cavaco-Paulo, Hannes Stockinger, Georg M. Guebitz	International Journal of Pharmaceutics	Vol. 458, Issue 2	Elsevier	Online	2013	1-8		No
4	WP2	Enzymatic synthesis of antibody-human serum albumin conjugate for targeted drug delivery using tyrosinase from Agaricus bisporus	Alexandra Rollett, Barbara Thallinger, Anna Ohradanova-Repic, Christian Machacek, Evelyn Walenta, Artur Cavaco-Paulo, Ruth Birner-Gruenberger, Juliane G. Bogner-Strauss, Hannes Stockinger and Georg M. Guebitz	RSC Advances	Issue 3	Royal Society of Chemistry	Online	2013	1460-1467	DOI: 10.1039/C2RA 22560C, Paper	No

Currently, members of the consortium are working on several scientific (peer reviewed) joint publications. Regardless of project completition, the consortium expects that the dissemination of results will continue and joint papers are expected to be published in the following months. Below, a preliminary list of some of the manuscripts currently under preparation is described (not included on the EC portal):

No	WP	Title	Main authors	Title of the periodical or the series	Number, date or frequency	Publisher	Place of publication	Date of publication	Relevant pages	Permanent identifiers** (if available)	Is/Will open access*** provided to this publication?
1	WP1	Chemically modified BSA for specialty functions of BSA particles	Ana Loreiro, Ana S. Abreu, Marisa Passos Sárria, Gonçalo J. L. Bernardes, Andreia C. Gomes, Artur Cavaco-Paulo	---	---	---	---	---	---	---	---

2	WP2	Targeting macrophages in a arthritis mice model with stealth folate liposomes	Eugénia Nogueira, Ana Loureiro, Nuno G. Azoia, Marisa P. Sárria, Ulyana Shimanovich, Celine Charvet, Gilles Renault, Franck Lager, Delphine Le Roux, Irene C. Mangialavori, Patrícia Nogueira, Jaime Freitas, Catarina R. Almeida, Anna Ohradanova-Repic, Christian Machacek, Alexandra Rollett, Michael Burnet, Johan Härmark, Gonçalo J. L. Bernardes, Hans Hebert, Georg Guebitz, Hannes Stockinger, Alexandra Moreira, Alexandre M. Carmo, Juan Pablo F. C. Rossi, Andreia C. Gomes, Ana Preto, Georges Bismuth, Artur Cavaco-Paulo	---	---	---	---	---	---	---	---
3	WP3	Difference from "nothing" - minor changes of exposure conditions to a chemotherapeutic drug conducts to different cellular responses	Marisa P. Sárria, Teresa Matamá, Artur Cavaco-Paulo, Andreia C. Gomes	---	---	---	---	---	---	---	---
4	WP2	Functional characterisation of folate receptor β-expressing macrophages	Anna Ohradanova-Repic, Christian Machacek, Vladimir Leksa, Gerhard J. Zlabinger, Goran	Blood							

			Mitulovic, Celine Charvet, Gilles Renault, Martin Bilban, Thomas Burkard, Michael B. Fischer, Eva Petrova, Miloslav Suchanek, Takami Matsuyama, Artur Cavaco-Paulo, Georges Bismuth, Hannes Stockinger								
5	WP2	Novel function of FRβ in myeloid cells	Anna Ohradanova-Repic	J Immunol							
6	WP2	Mono- and bispecific antibody fragments for specific targeting of activated macrophages	Anna Ohradanova-Repic, Christian Machacek, Srdjan Janjusic, Gordana Wozniak-Knopp, Miloslav Suchanek, Vaclav Horejsi and Hannes Stockinger								
7	WP2	Expression profiling of macrophage-associated folate receptor beta revealed by novel monoclonal antibodies	Christian Machacek, Eva Petrova, Hana Tichovska, Karel Drbal, Miloslav Suchanek, Andrew Rees, Anna Ohradanova-Repic and Hannes Stockinger								

2.1.2. List of dissemination actions (A2) - Public

No	WP and (if applicable) result number	Type of activities*	Main leader	Title/ Subject/ Reference	Actual/ planned date or status	Place	Type of audience **	Size of audience	Countries addressed	Draft received (Yes/No)	Final version received (Yes/No - anticipated date DDMMYY)
								Template A2: List of dissemination activities			
1	WP1	Posters	TUG	Microcapsules for targeted drug delivery	02/03/2011	7th International Conference on Polymer and Textile Biotechnology. Milan (Italy)	Scientific community (higher education, Research) - Industry	100	mainly Europe + international	Yes	Yes
2	WP2/WP3	Posters	MUW	GPI-anchored folate receptor β is a marker of M2-type macrophages	03/12/2010	Annual Meeting of the Austrian Society for Allergology and Immunology. Vienna (Austria)	Scientific community (higher education, Research) - Industry	500	European countries (specially: Austria, Croatis, Czech Repuplic, Hungary, Slovakia and Slovenia)	Yes	Yes (16/11/2010)
3	WP1	Organisation of Conference	TNO	On-chip microreactor system for the production of nano-emulsion loaded liposomes: towards targeted devices	04/05/2011	6th International Conference on Microtechnologies in Medicine and Biology. Lucerne (Switzerland)	Scientific community (higher education, Research) - Industry	500	International	Yes	Yes (09/02/2011)

4	WP2	Posters	MUW	Characterization of the folate receptor beta microenvironment	15/06/2011	7th YSA PhD Symposium at the Medical University of Vienna. Vienna (Austria)	Scientific community (higher education, Research)	100	European	Yes	Yes (13/06/2011)
5	WP1	Organisation of Conference	UMINHO	NANOFOL Folate-based nanobiodevices for integrated diagnosis/therapy targeting chronic inflammatory diseases	30/05/2011	Workshop: The Targeted Drug Delivery Cluster. Euronanoforum 2011. Budapest (Hungary)	Scientific community (higher education, Research) - Industry - Policy makers	1000	European	n.a.	n.a.
6	WP2	Posters	MUW	Characterization of the folate receptor beta microenvironment	01/09/2011	GenAu Summer School. Litschau (Austria)	Scientific community (higher education, Research)	40	European Countries	Yes	Yes (13/06/2011)
7	WP2	Posters	MUW	The role of folate receptor β-positive macrophages in chronic inflammatory diseases	27/09/2011	Retreat of the MUW. Vienna (Austria)	Scientific community (higher education, Research)	100	Austria	Yes	Yes (13/09/2011)
8	WP1	Oral presentation to a scientific event	TUG	Visualization of Drug Delivery Microparticles using Confocal Laser Scanning Microscopy	29/09/2011	ICPE 5th International Congress on Pharmaceutical Engineering. Graz (Austria)	Scientific community (higher education, Research) – Industry	100	International	Yes	Yes (15/06/2011)
9	WP2	Organisation of Workshops	EXBIO	Analysis of complex macrophage phenotype in diverse human pathologies	08/10/2011	Conference of Analytical cytometry VI. Prague (Czech Republic)	Scientific community (higher education, Research) - Industry	100	Czech Republic	n.a.	n.a.
10	WP1	Posters	TUG	Preparation and characterization of surface functionalized biopolymer-	04/03/2012	3rd International Congress BioNanoMed 2012.	Scientific community (higher	100	international	Yes	Yes (20/02/2012)

				nanocapsules for drug delivery		Krems (Austria)	education, Research) - Industry				
11	WP5	Posters	INERIS	LCA of Nanodrugs: Adding Constraints	24/06/2012	Goldschmidt 2012. Montreal (Canada)	Scientific community (higher education, Research) - Industry - Policy makers	1000	international	Yes	Yes (05/06/2012)
12	WP2	Posters	EXBIO	Macrophage population continuum in chronic inflammatory diseases detected by novel 8-color phenotypic	23/06/2012	CYTO 2012. Leiptzig (Germany)	Scientific community (higher education, Research) - Industry	1000	international	Yes	Yes (1/03/2012)
13	WP2	Posters	UMINHO	Preparation and Characterization of BSA nanospheres as drug delivery systems	25/03/2012	ACS National Meeting - 2012. San Diego (USA)	Scientific community (higher education, Research) – Industry	1000	international	Yes	Yes (08/06/2012)
14	WP2	Posters	MUW	Production of recombinant Fab antibody fragments in the methylotrophic yeast Pichia pastoris	27/09/2011	Retreat of the MUW. Vienna (Austria)	Scientific community (higher education, Research)	100	Austria	Yes	Yes (12/09/2011)
15	WP2	Oral presentation to a scientific event	MUW	Characterization of the protein microenvironment of the folate receptor beta	06/06/2012	EFIS-EJI Immunology Conference. Tatra mountains (Slovakia)	Scientific community (higher education, Research) - Industry	200	Europe	Yes	Yes (08/06/2012)
16	WP2	Posters	MUW	The role of folate receptor β-positive macrophages in diseased tissue	05/09/2012	European Congress of Immunology. Glasgow, (UK)	Scientific community (higher education, Research) -	1000	European	Yes	Yes (12/06/2012)

							Industry			
17	WP1	Oral presentation to a scientific event	UMINHO	Preparation and Characterization of nanospheres as drug delivery systems	03/12/2012	The 2nd International Conference on Nanotek and Expo. Philadelphia (USA)	Scientific community (higher education, Research) - Industry	1000	International	Yes	Yes (12/06/2012)
18	WP5	Posters	INERIS	Comparative Life Cycle Assessment of rheumatoid arthritis treatments.	12/05/2013	SETAC Conference. Glasgow, UK	Scientific community (higher education, Research)	1000	international	Yes	Yes (11/12/2012)
19	WP2	Posters	TUG	Surface modified protein nanocapsules for targeted drug delivery	07/04/2013	ACS National Meeting and Exposition, New Orleans, Louisiana (USA)	Scientific community (higher education, Research) - Industry	1000	international	Yes	Yes (10/12/2012)
20	WP2	Posters	TUG	Synthesis of surface modified biomaterial nanoparticles for targeted drug delivery	08/09/2013	ESB 2013 - 25th European Conference on Biomaterials. Madrid (Spain)	Scientific community (higher education, Research)	500	Mainly european	Yes	Yes (21/01/2013)
21	WP6	Oral presentation to a wider public	SYNOVO	Nanoparticular formulation for targeted therapy and imaging of RA	11/01/2010	Advancing Biologics from the Lab to the Clinic. Brussels (Belgium)	Industry		Europe/USA	n.a.	n.a.
22	WP1	Oral presentation to a scientific event	UAV	Top-down glycolipidomics by fully automated chip-based nanoelectrospray collision-induced dissociation	15/02/2010	21st Annual Austrian Spectrometry Forum. Vienna (Austria)	Scientific community (higher education, Research)	500	European Countries.	n.a.	n.a.
23	WP1	Oral presentation to a scientific event	UAV	Glycomics of biglycan glycosaminoglycans by chip-nanoESI MS	07/03/2010	43rd Annual Conference of the German Society for Mass Spectrometry.	Scientific community (higher education, Research)	375	Europe	n.a.	n.a.

							Scientific community (higher education, Research)				
						Halle (Germany)					
24	WP6	Organisation of Workshops	TUG	COST868 action-meeting	08/03/2010	Porto (Portugal)	Scientific community (higher education, Research)	200	European countries	n.a.	n.a.
25	WP1	Posters	TUG	Microspheres for targeted drug delivery systems	04/03/2011	COST 868 Meeting. Milan (Italy)	Scientific community (higher education, Research)	200	European countries	Yes	Yes
26	WP1	Oral presentation to a scientific event	UMINHO	Preparation and Characterization of protein nanospheres	21/03/2010	American Chemical Society Meeting. San Francisco (USA)	Scientific community (higher education, Research)	1000	International , USA	Yes	Yes
27	WP1	Posters	UMINHO	Characterization of liposomal delivery for Methotrexate	21/03/2010	American Chemical Society Meeting. San Francisco (USA)	Scientific community (higher education, Research)	1000	International , USA	Yes	Yes
28	WP6	Interviews	IBMC	Multiple interviews with experts	29/03/2010	World Immune Regulation Meeting - IV "Innate and Adaptative Immunoregulatory Mechanisms. Davos (CH)	Scientific community (higher education, Research) - Industry	1000	international	n.a.	n.a.
29	WP6	Organisation of Conference	UAV	Lectures on high performance mass spectrometry	24/04/2010	1st International Conference of the Romanian Society for Mass Spectrometry. Sinaia (Romania)	Scientific community (higher education, Research)	100	International	n.a.	n.a.
30	WP6	Interviews	IBMC	Multiple interviews with experts	11/05/2010	SINAL 2010, 4th Portuguese National Meeting on Signal	Scientific community (higher education,	100	Portugal	n.a.	n.a.

						Transduction. Faro (Portugal)	Research)				
31	WP1	Oral presentation to a wider public	UMINHO	Protein based nano- and microspheres for medical applications	23/05/2010	Particles 2010. Lake Buena Vista (Florida)	Scientific community (higher education, Research) - Industry	1000	International	n.a.	n.a.
32	WP4	Exhibitions	SYNOVO	Nanoparticular formulation for targeted therapy and imaging of RA	09/06/2010	Drug Discovery Leaders Summit. Montreux (Switzerland)	Industry	500	International	Yes	Yes
33	WP6	Oral presentation to a wider public	ALFAMA	Functionalized QuantumDots for Celullar Imaging&Chemical Access to Prosttranslational Modified prot.	22/06/2010	Course Lecture: Nanotechnologies Applied to Diagnosis and Disease Therapy. Uminho (Portugal)	Scientific community (higher education, Research)	50	European countries	n.a.	n.a.
34	WP6	Organisation of Conference	UMINHO	Phd Course: Nanotechnologies Applied to Theragnostics: From Diagnosis to Disease Therapy	14/06/2010	Porto, (Portugal)	Scientific community (higher education, Research)	50	European countries	Yes	Yes (18/06/2010)
35	WP6	Posters	UAV	Nanoelectrospray MS applications in biomedicine	15/06/2010	European Nanobiotechnology Congress. Munster (Germany)	Scientific community (higher education, Research) - Industry	1000	European countries	Yes	Yes
36	WP1	Oral presentation to a scientific event	UMINHO	Folate-based nanobiodevices for integrated diagnosis/therapy targeting chronic inflammatory diseases	29/07/2010	4th internat. meeting on develop. in mat., proc. and applic. of emerging tech (MPA). Braga (Portugal)	Scientific community (higher education, Research)	500	Portugal	n.a.	n.a.
37	WPX	Oral presentation	SYNOVO	TBC	22/08/2010	14th International Congress of	Scientific community	1000	International	n.a.	n.a.

		to a wider public				Immunology. Kobe (japan)	(higher education, Research) - Industry				
38	WP6	Organisation of Workshops	TUG	COST 868 - Meeting	02/09/2010	Analipsi (Greece)	Scientific community (higher education, Research)	100	European countries	n.a.	n.a.
39	WP2/WP3	Posters	MUW	GPI-anchored folate receptor B is a marker of activated macrophages	04/09/2010	9th EFIS - EJI Tatra Immunology Conference. Strbske Pleso (Slovakia)	Scientific community (higher education, Research)	100	European countries	Yes	Yes (16/11/20 10)
40	WP2/WP3	Posters	MUW	Folate receptor B is a marker of activated macrophages	28/09/2010	EBS&EFIS Workshop "Inflamm. Dis. and Imm. Resp: Basic Aspects, Novel Appr& Exp. Mod. Vienna (Austria)	Scientific community (higher education, Research)	100	European countries	Yes	Yes (16/11/20 10)
41	WP2/WP3	Posters	MUW	GPI-anchored folate receptor B is a marker of activated macrophages	21/09/2010	Retreat od the Center for Pathophysiology, Infectiology and Immunology. Vienna (Austria)	Scientific community (higher education, Research)	100	Austria	Yes	Yes (16/11/20 10)
42	WP6	Exhibitions	SUAN	Dissemination of the Brochure	29/09/2010	Biospain 2010: Spanish Biotechnology Congress. Pamplona (Spain)	Scientific community (higher education, Research) - Industry	1000	Spain & European Countries	n.a.	n.a.
43	WP6	Exhibitions	SUAN	Dissemination of the Brochure	05/10/2010	CPHI Worldwide: Pharmaceutical Ingredients International Event. Paris (France)	Industry	1000	International	n.a.	n.a.

44	WP1	Oral presentation to a scientific event	UAV	Chip-based electrospray mass spectrometry for glycolipidomics in biomedical research	19/10/2010	1st Middle Eastern and Mediter. Sea Reg. Mass Spectrom Workshop. Weizmann Institute. Rehovot (Israel)	Scientific community (higher education, Research)	500	European countries	n.a.	n.a.
45	WP6	Organisation of Conference	UAV	Courses and lectures on mass spectrometry applications in biomedicine	10/11/2010	international Symposium on Research and Education in Innovation Era. 3rd edition. Arad (Romania)	Scientific community (higher education, Research)	100	European countries	n.a.	n.a.
46	WP6	Articles published in the popular press	TUG	Novel Approaches towards bioresponsible biopolymers and functional materials	01/12/2010	Research TU-Graz	Scientific community (higher education, Research) - Industry - Civil society - Policy makers - Medias	1000	Austria	Yes/No	No
47	WP6	Press releases	UAV	Various press releases informing about UAV's participation in Nanofol	01/12/2010	Regional and national newspapers	Civil society - Policy makers - Medias	1000	Romania	Yes	Yes
48	WP6	Organisation of Conference	UAV	Courses lectures on Biological Mass Spectrometry	01/05/2011	2nd International Conference of the Romanian Society for Mass Spectrometry. Timisoara (Romania)	Scientific community (higher education, Research) - Industry	100	international	n.a.	n.a.
49	WP6	Exhibitions	SUAN	Dissemination of the Brochure	29/06/2011	Bio 2011. The Global Event for Biotechnology.	Industry - Policy makers	15000	international	n.a.	n.a.

						Washington DC (USA)				
50	WP6	Exhibitions	SUAN	Dissemination of the Brochure	25/10/2011	CPHI Worldwide. Pharmaceutical Ingredients International Event. Frankfurt (Germany)	Industry	28500	International	n.a.	n.a.
51	WP1	Posters	UMINHO	Liposomal formulations for specific drug delivery	25/03/2012	ACS National Meeting - 2012. San Diego (USA)	Scientific community (higher education, Research) - Industry	1000	Intenational	Yes	Yes (19/03/2012)
52	WP2	Posters	INSERM	Folate Receptor 2 expression levels by macrophages does not correlate with the stage of inflammation	05/06/2012	European Congress of Immunology. Glasgow (Scotland, UK)	Scientific community (higher education, Research)	1000	european	Yes	Yes (14/06/2012)
53	WP6	Web sites/Applications	ALMA	http://www.nanofol.eu/	01/07/2010	Online	Scientific community (higher education, Research) - Industry - Civil society - Policy makers - Medias		World	n.a.	n.a.
54	WP6	Articles published in the popular press	UMINHO	6M Public Executive Summary	28/07/2010	On the Nanofol Website	Scientific community (higher education, Research) - Industry - Civil society - Policy makers - Medias		World	Yes	Yes
55	WP6	Articles	UMINHO	12M Public Executive	28/01/2011	On the Nanofol	Scientific		World	Yes	Yes

		published in the popular press		Summary		Website	community (higher education, Research) - Industry - Civil society - Medias			
56	WP6	Articles published in the popular press	UMINHO	18M Public Executive Summary	28/07/2011	On the Nanofol Website	Scientific community (higher education, Research) - Industry - Civil society - Policy makers – Medias		World	Yes	Yes
57	WP6	Articles published in the popular press	UMINHO	24M Public Executive Summary	27/01/2012	On the Nanofol Website	Scientific community (higher education, Research) - Industry - Civil society - Policy makers - Medias		World	Yes	Yes
58	WP6	Articles published in the popular press	UMINHO	30M Public Executive Summary	26/07/2012	On the Nanofol Website	Scientific community (higher education, Research) - Industry - Civil society - Policy makers - Medias		World	Yes	Yes
59	WP6	Articles published in the popular press	UMINHO	36M Public Exectuive Summary	23/01/2013	On the Nanofol Website	Scientific community (higher education, Research) -		World	Yes	Yes

							Industry - Civil society - Policy makers - Medias				
60	WP6		Articles published in the popular press	UMINHO	42M Public Executive Summray	17/07/2013	On the Nanofol Website	Scientific community (higher education, Research) - Industry - Civil society - Policy makers - Medias		World	Yes	Yes
61	WP1		Oral presentation to a scientific event	UMINHO	New Peptide-linker of Folate-target liposomes for specific drug delivery.	12/09/2013	ACS meeting. Indianapolis (USA)	Scientific community (higher education, Research) - Industry	1000	internationa	yes	yes (29/05/20 13)
62		WP2	Posters	MUW	Deciphering the function of folate receptor beta	19/6/ 2013	9th YSA PhD Symposium (Vienna, Austria)	Scientific community (higher education, Research)	300	Austria	Yes =EFIS-EJI Conferen ce, 2012	Yes (08/06/20 12)
63		WP2	Posters	MUW	Deciphering the function of folate receptor beta	17/09/2013	4th Retreat of the Centre for Pathophysiology, Infectiology and Immunology (Vienna, Austria)	Scientific community (higher education, Research)	100	Austria	Yes =EFIS-EJI Conferen ce, 2012	Yes (08/06/20 12)
64		WP2	Oral presentation to a scientific event	MUW	Deciphering the function of folate receptor beta	10/10/2013	2nd Meeting of Middle European Societies for Immunology and Allergology (Opatija, Croatia)	Scientific community (higher education, Research)	200.,	European countries	Yes =EFIS-EJI Conferen ce, 2012	Yes (08/06/20 12)
65		WP2	Posters	MUW	The role of folate receptor ß-positive macrophages in diseased tissue.	30/11/2012	ÖGAI Symposium 2012 (Klosterneuburg, Austria)	Scientific community (higher education,	100	Austria	Yes= Europ. Congr. Imm.	Yes (12/06/20 12)

#	WP	Type of activity*	Partner	Title	Date	Place	Type of audience**	Size	Countries		
						Research)				2012	
66		WP2	Posters	MUW	Phenotypical and functional diversity of folate receptor β-positive macrophages in diseased tissue	17/09/2013	4th Retreat of the Centre for Pathophysiology, Infectiology and Immunology (Vienna, Austria)	Scientific community (higher education, Research)	100	Austria	Yes= Europ. Congr. Imm. 2012	Yes (12/06/2012)
67		WP2	Posters	MUW	Phenotypical and functional diversity of folate receptor β-positive macrophages in diseased tissue	10/10/2013	2nd Meeting of Middle European Societies for Immunology and Allergology(Opatija, Croatia)	Scientific community (higher education, Research)	200	European countries	Yes= Europ. Congr. Imm. 2012	Yes (12/06/2012)
68		WP2	Oral presentation to a scientific event	MUW	Novel macrophage subsets with potential implication in inflammatory diseases	20/11/2013	NANOFOL/NanoDiaRA conference (Lisbon, Portugal)	Scientific community (higher education, Research)	80	International	No	No
69	WP4		Oral presentation to a wider public	SYNOVO	TBC	09/06/2010	Drug Discovery Leaders Summit. Montreux (Switzerland)	Industry	200	International	Yes	Yes
70	WP6		Articles published in the popular press	UMINHO	48M Public Exectuive Summary	31/01/2014	On the Nanofol Website	Scientific community (higher education, Research) - Industry - Civil society - Policy makers - Medias		World	Yes	Yes

* A drop down list allows choosing the dissemination activity: publications, conferences, workshops, web, press releases, flyers, articles published in the popular press, videos, media briefings, presentations, exhibitions, thesis, interviews, films, TV clips, posters, other.

** A drop down list allows choosing the type of public: Scientific Community (higher education, Research), Industry, Civil Society, Policy makers, Medias ('multiple choices' is possible.