New Oral Nanomedicines: Transporting Therapeutic Macromolecules across the Intestinal Barrier

Reporting

Project Information

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RESULTS PACK
Nanomedicine – innovative ways of treating challenging conditions

Final Report Summary - TRANS-INT (New Oral
Nanomedicines: Transporting Therapeutic Macromolecules across the Intestinal Barrier

Executive Summary:
Currently marketed injected peptide and protein therapeutics have a great impact on the treatment of important systemic diseases, however, so far, they cannot be administered in the fashion that has the highest patient compliance: the oral route.

The cohesive forces that stand behind TRANS-INT was a clear concept and a shared ambition. The concept being the rational design of oral nanomedicines based on safety, mechanistic, bioengineering and pharmaceutical technology criteria.

The overall objective of TRANS-INT was to develop new oral nanocarriers and new potential oral nanomedicines for the treatment of diseases with high economic and social impact and also to contribute to the generation of knowledge regarding the interaction of the nanomaterials with GI barriers.

To address these objectives, the TRANS-INT research activity has been distributed among different work packages (WPs). The achievements of these WPs are described below.

The activity of WP1-WP2 has led to the development and characterization of potential nanomedicines (13 prototype families), which were selected after continuous screening of hundreds of prototypes, based on a defined Target Product Profile (TPP). Of the 13 selected prototypes, 8 were optimized and explored for possible incorporation into a solid dosage form. Of these prototypes, 5 could be presented in the form of SmPill® minispheres or incorporated in an enteric capsule. And of these, 2 were also investigated for their quality properties after storage as a solid dosage form for up to 3 months, under ICH conditions.

WP3-WP4 covered the in-vitro/in vivo evaluation in terms of cytotoxicity and mechanistic issues. These studies were performed for 2-13 prototypes, either in the Caco-2 model, in isolated human jejunal mucosae or in mouse/rat models. The toxicity assays gave positive information, while the mechanistic issues behind the behaviour of each prototype were widely variable and depended on their composition. Some prototypes were highly distributed and were retained along the intestinal mucosa, reaching, in some cases, the intracellular space, whereas others were mainly retained in the mucus layer. Overall, a good correlation was found among the data obtained in rodents and in isolated human jejunal mucosae, however this was not always the case with data obtained when using the Caco-2 model. The lack of mucus in the Caco-2 model was thought to be a reason for major discrepancies between models.

Finally, the results obtained by WP5 and WP6 illustrate the efficacy of selected formulations following intraintestinal injection to anesthetized or fully awake normoglycaemic and hyperglycaemic rats. Overall, among the 10 prototypes investigated, 3 gave positive responses, especially in the diabetic rat model, however, there was a discrepancy among the data obtained from the different models. Only one prototype could be tested in large animal models (pig model), and the results from this experiment show a modest performance. Some of these studies are now being continued with partners’ specific resources.

WPs 7-8-9 cover training and education; exploitation and dissemination; and management. The results of
these transversal WPs have been very positive based on (i) the great number of training exchanges (20), and workshops (10) performed, and high number of PhD students and postdocs trained (30); the high dissemination of the consortium activities, with 155 documents and 3 patent applications already recorded and, (iii) the high involvement of senior and junior researchers in the management of the project.

Overall TRANS-INT provided a positive experience, with an important cohesive force, which led to the generation of knowledge that will greatly impact the oral peptide delivery field in terms of the potential of nanotechnology.

Project Context and Objectives:
The context and the rational

Currently marketed injected peptide and protein therapeutics have a great impact on the treatment of important systemic diseases, however, so far, they cannot be administered in the fashion that has the highest patient compliance: the oral route. This result is to be expected given that the gastrointestinal tract is specialised in degrading peptides and does not allow their transport across the intestinal mucosa. The availability of an oral form for the administration of peptides could lead to substantial improvement in the treatment of important chronic pathologies, such as diabetes. A successful oral delivery technology would need to fulfill the following key tasks: to protect the macromolecules from degradation, and to facilitate their transport across the intestinal epithelium in a safe, cost-effective and efficient fashion. To this end, we propose the use of nanocarriers specifically tailored to deal with the GI environment.

The concept behind TRANS-INT has been the rational design of these oral nanomedicines based on integrative knowledge, networking intelligence and creativity. This design has been driven by:

i) Biopharmaceutical mechanistic criteria (we have gained information about the interaction of nanomaterials with the GI mucosa)

ii) Safety criteria (we have mainly used biomaterials which are already accepted by regulatory agencies or are in clinical evaluation)

iii) Materials bioengineering criteria (we have formulated biomaterials in order to produce nanocarriers with the required functionalities: protection of the peptide, efficient drug transport across the intestinal mucosa, and adequate and controlled drug release)

iv) Pharmaceutical technology criteria (we defined a number of production technologies, which are easily scalable and allow an adequate payload).

In order to make significant advances, TRANS-INT understood that it was crucial to identify the mechanistic behaviour of nanocarriers in the biological system in a systematic and in-depth fashion. Thus, the TRANS-INT motto has been: “Understand the barrier; understand the carrier”.

The overall objective of TRANS-INT has been to develop new oral nanocarriers and new potential oral nanomedicines for the treatment of diseases with high economic and social impact, as well as to contribute to the generation of new knowledge regarding the interaction of nanomaterials with the gastrointestinal barrier.

The overall strategy of the work plan relied on the following major fundamentals:

1) THE DESIGN: The rational and specific design of nanostructures was based upon improved knowledge
of the mechanistic issues regarding how the nanostructure interacts with the GI barrier. A critical driver of our design was to study the ability of the nanocarriers to conveniently associate and deliver the selected macromolecules within the required timeframe. This design has led to the engineering of multifunctional nanocarriers by combining well-known biomaterials in new nanostructural compositions.

2) THE BIOMATERIALS: TRANS-INT has made use of properly characterized biomaterials, with a track record of biocompatibility and biodegradability. The majority of the GRAS materials or excipients were produced under GMP conditions.

3) THE TECHNOLOGIES: The majority of the technologies used in TRANS-INT were simple and mild processes, easily scalable without the need for high-energy sources, that could damage delicate peptides. These technologies were also selected based on their high peptide entrapment efficiency, with optimum association between the peptide and the biomaterials.

4) PREDICTIVE “IN VITRO” MODELS: The selection of the nanocarrier prototypes was done according to a continuous screening process based on a defined Target Product Profile (TPP). Therefore, using relevant and well-accepted in vitro tests and models was crucial for the success of the project. A relevant example is the use of human intestinal tissue mounted in an Ussing Chamber to screen epithelial uptake of prototypes and to attempt to relate the data to efficacy in rat models.

5) THE ASSESSMENT OF ADDED VALUE, EFFICACY AND SAFETY: This was guaranteed by the evaluation of lead prototypes according to a multi-reference centre validation scheme driven by the partners and end-user companies. The necessary evaluation in animal models was performed according to the 3R’s principle: replacement, reduction and refinement. In addition, working with the Advisory Board, both at the inception and throughout the project, we ensured that the technologies under development and the protocols devised for efficacy and safety evaluation were as far as possible within the EU regulatory framework.

The activities driven by these fundamentals are described in detail below. These activities are described in terms of WPs organization (similar/complementary activities are included in the same WP), the decision making process (the results of the activities provide decision-making tools) and the timing. These three ways of presenting the work provide a complete view of the work plan strategy.

WP1: NANOCARRIER DESIGN, DEVELOPMENT AND CHARACTERISATION
WP2: FORMULATION OF PEPTIDE DRUG CANDIDATES
The overall effort in this WP has provided information about hundreds of prototypes and the optimization of the 13 prototype families. The delivery platforms were classified into 3 categories; nanocapsules, nanoparticles and micelles, and most of their components were selected because of their acceptable regulatory profile. The delivery platforms were adapted to the formulation of 5 antidiabetic peptides (human insulin, insulin glulisine, modified insulin, and the GLP-1 analogues exenatide and liraglutide), as well as an anti-pain peptide. The formulations were screened according to the following ranking of properties: size and surface charge -> peptide association efficiency -> shelf stability -> stability in simulated intestinal fluids and in cell culture media -> in vitro release profile -> proteolysis -> mucodiffusion. Among the 13 prototypes selected, 8 of them were optimized and explored for their
incorporation into a solid dosage form. Specific prototypes were also investigated for their physicochemical and pharmaceutical properties after being stored as a solid dosage form for up to 3 months, under ICH conditions. Up to 5 prototypes were explored for their incorporation into the SmPill® minispheres technology with 3 having an adequate outcome. The rest of prototypes were presented as powders or incorporated in an enteric dosage form (Prototype 3 and Prototype 13).

The final result was a variety of rationally designed nanocarriers with some capacity to overcome the gastrointestinal barriers, and endowed with the ability to load and control the peptides’ release. Major impacts are the provision of: (i) nanotechnology approaches to combine therapeutic peptides with biomaterials and to engineer specific properties for the said combinations; (ii) robust assays with standard operating procedures (SOPs) to assess proteolysis, stability in complex simulated intestinal media, peptide release and proteolysis, and mucodiffusion of nanomedicines; and (iv) technology approaches to produce prototypes in a solid form (freeze-dried powders, enteric capsules and SmPill® minispheres) that help to preserve the stability of the prototype during storage.

WP3: IN VITRO MECHANISTIC AND TOXICITY STUDIES
WP4: PRELIMINARY IN VIVO MECHANISTIC AND TOXICITY STUDIES
The prototype families (13) underwent toxicity evaluation in the Caco-2 monolayers and 6 of them were evaluated for their toxicity in the human intestine. Nine prototype families were investigated for their capacity to modify the transepithelial electrical resistance (TEER) and to be internalized by the Caco-2 monolayers, and 6 prototype families were also evaluated for their capacity to cross-isolated human intestinal tissue. The results indicated that the interactions of the prototypes with the intestinal mucosa were dependent on the size and composition of the prototype. Prototypes 6 and 12 showed the greatest internalization by the intestinal tissue.

In vivo biodistribution/imaging studies were performed using fluorescent (6 prototypes) and radioactive markers (2 prototypes). The results showed a significant retention of specific prototypes along the intestinal tract, thereby evidencing the positive interaction of specific nanocarriers with the mucosa, however, no translocation of nanocarriers was observed. In vivo immunological studies highlighted the promising immunological profile of the selected prototypes in that there was no evidence of stimulation of local immune events in mice by the peptide-loaded nanocarriers tested.

Finally a comparative analysis was performed of different nanocarriers in terms of toxicity and mechanism of interaction with the intestinal barriers using model cell lines (Caco-2), isolated human intestinal tissue and in vivo mouse/rat models. Overall, the results of WP3-WP4, highlighted the low cytotoxicity and immunotoxicity of selected prototypes as well as their capacity to withstand the harmful intestinal environment and to facilitate the retention of the prototypes at the level of the intestinal mucosa. This information is key to understand whether a specific prototype would be more adequate for systemic peptide delivery or rather for local drug delivery to the GI tract itself.

WP5: IN VIVO PHARMACOKINETIC/PHARMACOLOGICAL EVALUATION OF NANOMEDICINES
WP6: PRECLINICAL EFFICACY, TOXICOLOGICAL, IMMUNOLOGICAL EVALUATION
Ten (10) prototype families were tested for their efficacy in normoglycaemic rats, and 6 of them were tested in hyperglycaemic rats, using the oral and intra-intestinal injection model without anaesthesia. In
addition, confirmatory studies were also performed with 6 selected prototypes using an anaesthetised normoglycaemic rat model (Sanofi R&D model). The results of these studies indicate that 3 prototypes (modified insulin -Loaded nanoemulsions, Glulisine-loaded Protamine nanocapsules and Zinc-Insulin nanocomplexes) exhibited significant responses in normoglycemic or diabetic rats. In addition, 4 insulin prototypes elicited responses that were only significantly higher at specific time points than those corresponding to the controls and 3 glulisine-loaded prototypes did not provide a significant response in any of the models investigated. Finally, exenatide and liraglutide-loaded prototypes (NLCs and Cyclodextrin-Liraglutide complexes) provided responses that had no pharmacological relevance. However, the evaluation of these prototypes in the Sanofi R&D model did not result in a positive outcome. In addition, an exploratory in vivo study performed in pigs (WP6, Seroscience), indicated that insulin was not absorbed following the administration of the selected prototype.

Overall, the final outcome from these WPs is a comparative analysis of different nanocarriers in terms of their capacity to enhance the systemic absorption of the associated peptides (3 types of insulin and 2 GLP1-analogues) and their subsequent hypoglycaemic response using different animal models. The conclusion was that the efficacy observed is model-dependent, a result that suggests that further studies in large animal models are necessary in order to validate the potential of the most promising technologies. A critical issue in future work will be the in vivo evaluation of prototypes at the final dosage form. Hopefully, this work will culminate with an understanding of the basis for the formulation of oral peptide drugs and the delivery of one preclinical candidate. In addition to the potential development of an oral dosage form for systemic peptide delivery, we noticed the fact that, based on the biodistribution data, some prototypes might be more adequate for the local delivery of peptide drugs to the intestinal tract.

WP7: TRAINING AND EDUCATION
The education and training programme included:
- Scientific sessions on peptide drugs and oral drug delivery, including debates on the current literature and a session on intellectual property and presentation skills.
- Twenty (20) researchers visited partner labs for up to 12 month periods, including a one-day open lab event.

The final result was a total number of 30 Ph.D. students/post-docs trained in a cross-disciplinary environment and acquiring relevant knowledge regarding the development of medicines and the translation challenges associated with it. The special skills these young researchers acquired through this program are already having an impact in their professional career, as supported by the fact that some of them are currently working in the academic or industrial fields, while others have become entrepreneurs.

WP8: EXPLOITATION AND DISSEMINATION
The project website has contributed successfully to the exchanging of information both within the consortium and with outside stakeholders. The implemented e-tools (e-brochure, e-poster, e-newsletter) are still now being disseminated via the website and by email.

TRANS-INT has had and will continue to have, an impact in the world-wide industry/academy research
activity in the field. The project has so far resulted in 3 patent applications, 25 publications in scientific journals and 130 presentations in conferences. A monographic issue including TRANS-INT partners has been published in the ADDR journal.

A joint conference between several FP7 consortia (ALEXANDER and TRANS-INT) and an IMI consortium (COMPACT) was held in Dresden, Germany, on 9-11 November 2015.

A number of workshops were organized in alliance with the NANOFAR Erasmus Mundus Ph.D. School and also with the Spanish-Portuguese Local Chapter of the Controlled Release Society (CRS). These workshops and conferences have greatly contributed to the PhD students’ and post-docs’ organization and communication skills.

WP9: MANAGEMENT
Partners and, notably young researchers, have been highly involved in the management of TRANS-INT based on a clearly defined decision making process. An active external scientific board and the executive committee are being critical in accelerating this process.

The management structure has involved all partners, including young researchers, who have participated actively in a well-defined decision making process. The management training is expected to have an impact in other consortia as well as in the organization of the partners’ laboratories.

Overall, the scientific and technical knowledge generated regarding the characterization of nanomaterials and their interaction with the gastro-intestinal barriers is expected to have a high impact in the development of peptide-based oral nanomedicines. Hopefully, some of the most promising candidates will soon reach the preclinical development phase. On the other hand, the training and skills acquired by the young researchers will definitely help them to further develop their careers in industry and academia.

Project Results:
The description of the main scientific results obtained in the TRANS-INT consortium is presented below in individual WPs.

WP1: Nanocarrier design, development and characterisation

The main objective of this work package was the development of a series of nanocarrier prototypes, with different structures, including solid nanoparticles, nanocapsules consisting of a liquid core (aqueous or oily) and micelles. The main tasks associated with this objective and the corresponding achievements are described below.

1.1. Nanocarrier selection and development nanotechnologies
1.2. Nanocarrier characterization
1.3. Nanocarrier optimization
1.4. Final processing of nanocarriers and pharmaceutical presentation

1. Nanocarrier development, characterization and optimization
The activities of this work package resulted in the development of a very high number of different nanocompositions (> 1000). A selection of them, which fulfilled the selection criteria defined by the consortium, progressed to WP2 for peptide loading and led to a total of 13 nanocarrier prototype families. Most of these prototypes are based on well-characterised and safe polymers (e.g. polysaccharides, polypeptides), lipids, oils and in some cases, inorganic materials, whereas others were made of novel materials, i.e. modified cyclodextrins or modified chitosan. The preparation methods were selected according to the criteria of simplicity, mildness and scalability of the formulation process and were adapted to the specific composition of each prototype. These methods included ionic gelation, complexation, self-assembly, solvent displacement, among others. These methodologies also allowed the stable incorporation of fluorescent markers into the nanocompositions in order to enable mechanistic evaluation and in vivo microscopic tracking in subsequent work packages. In this regard, maintaining the original physico-chemical properties of the carrier upon labelling and avoiding the leakage of the fluorescent dyes over time were identified as important challenges.

Figure 1. Schematic representation of the different nanostructures

In addition, a further output from this work package was the inclusion of epithelial cell penetrating compounds and permeation enhancers in construct formulations. These molecules included established permeation enhancers (e.g. medium and long chain fatty acids) as well as cell penetrating peptides (CPPs), all aimed at improving transport across the intestinal barrier. The following four CPPs were prepared by the IRB Barcelona, and made available to all the partners: r8 (octaarginine), D-Cys-r8 and the lipophilic derivatives C12-r8, and cholesterol-r8. These compounds were prepared by solid-phase peptide synthesis and were fully characterized. The lipophilic derivatives were also made with suitable fluorescent labels.

The systematic screening of these nanocompositions required the use of standardized methodologies in order to enable identification and optimisation of the most promising prototypes in terms of:

- Handling and storage stability.
- Colloidal stability in complex biological fluids, including cell culture media and simulated intestinal fluids (SIF, FaSSIF, FeSSIF).
- Mucointeraction (including mucodiffusion and mucoadhesion).

Regarding nanocarrier stability, the following standard operating procedures (SOPs) were developed and adopted by the WP1 partners:

Table 1. SOPs

In addition to the implementation of these SOPs, an important aspect of these systematic screening studies was the assignment of a Reference Centre partner for each specific technique. This allowed the assessment and selection of the candidates for peptide encapsulation in work package 2.

Among the above characterization studies, measuring colloidal stability in complex biological media was found to be the most challenging task. The presence of enzymes and lipids in fasted- and fed state
intestinal media were found to interfere with these analyses and this made it particularly difficult to draw conclusions regarding particle stability in such conditions. Therefore, several complementary techniques (TEM/SEM imaging, lipolysis studies etc.) were also performed to confirm the results obtained with the SOPs.

2. Mucointeraction studies

This activity involved the determination of the mucoadhesion and mucodiffusion properties of the TRANSINT prototype families using a series of techniques: dynamic light scattering (DLS), microfluidics, capillary-based methods, multiple particle tracking (MPT) and fluorescence recovery after photobleaching (FRAP).

2.1. Analysis of mucoadhesion by DLS (USC)

To analyse the mucoadhesion of nanoparticles we proposed the combination of two different approaches. On one hand, as a fast screening assay to evaluate the interaction of nanoparticulate systems with mucin, we proposed the analysis of size and z-potential of the particles in a porcine mucin solution by dynamic light scattering (DLS). Due to the intrinsic limitations of this technique it was not possible to carry out the assay under physiological conditions, i.e. a solution of 2-5% (w/v) of mucin. For this reason we suggested the analysis of these physicochemical properties using a range of mucin concentrations (from 1•10^-3 up to 1•10^-1 % (w/v) in simulated intestinal fluids) to follow the interaction of the particles with mucin over time. In spite of its limitations, this assay was found to be a useful tool for the screening of the affinity of nanoparticles to mucin in the early development phase of the prototypes.

2.2. Analysis of mucoadhesion by fluorescence microscopy (USC)

As a second assay for the selective in vitro screening of the interaction of nanoparticles with mucin, we proposed the semi-quantitative analysis of fluorescent nanoparticle retention on a mucin film by fluorescence microscopy. Briefly, a coverslip was coated with mucin and a solution of fluorescent nanoparticles was incubated with the coverslip for 2h at 37ºC. The excess nanoparticles were washed out thoroughly and the coverslip was analysed by fluorescence microscopy. Fluorescence images of each nanostructure were used for the semi-quantitative determination of the retention in the mucin film (a minimum of 15 images were analysed for each prototype).

2.3. Analysis of mucodiffusion by the capillary technique (USC)

As a selective in vitro screening method to analyse the mucodiffusion rate of nanoparticles we proposed the use of a mucin gel-filled capillary. The capacity of the nanoparticles to diffuse through the capillary was studied using fluorescent nanoparticles. Monitoring the fluorescence front displacement in a fluorescence microscope every 5 seconds allowed us to determine the time required by each prototype to cross 1 mm of the mucin gel. In a subsequent step, the assay was fine-tuned by the substitution of the mucin solution 5% (w/v) by porcine jejunum mucus. As porcine mucus is thicker than the mucin solution it was necessary to modify the approach to assess the mucodiffusion rate/penetration capacity of the nanostructures. Under this scenario the diffusion profile of the fluorescently labelled nanocarriers from the mucus/nanoparticles interface towards the mucus was then analysed in a fluorescence microscope after the incubation of the samples.
capillary in a vertical position at 37°C for 10 minutes. Representation of the normalized fluorescence from the mucus/nanoparticles interface towards the mucus allowed us to calculate the penetration capacity of the nanoparticles at a specific time point. In parallel to the determination of the penetration capacity of the nanostructures we are also working on the calculation of the diffusion coefficient, using the same raw data, by computational simulation.

Figure 2. Microcapillary setup for measuring the mucodiffusion of fluorescent nanocarriers

An alternative to this capillary technique was a microfluidic chip-based technology used by UCD. It has also been optimized for tracking the behaviour of the fluorescent charged and uncharged nanocarriers upon contact with freshly-obtained porcine duodenal mucus. This technology provides information through the direct monitoring of particle diffusion by fluorescence microscopy in order to compare the mucopenetration capacity of the different nanocompositions developed within the consortium.

2.5. Analysis of mucodiffusion using FRAP

FRAP is a microscopy method based on fluorescence recovery after photo-bleaching for the analysis of mucus-interactions. A FRAP experiment measures the rate at which fluorescent particles diffuse back into a volume that has been photo-bleached by a high intensity laser beam. The quicker the recovery of fluorescence in the bleached region, the faster the carrier diffuses in the mucus. The nanocarrier diffusion coefficient (D) can be determined through analysis of the recovery function. In addition to providing diffusion constants in mucus, the FRAP experiment gives a measure of nanocarrier mucus-adhesion properties, through the determination of a parameter called the trapping constant (K), which is the weight % of trapped nanocarriers in the mucus.

Figure 3. The FRAP experiment

2.6. Multiple particle tracking analysis (MPT)

The MPT analysis allows the determination of the diffusion coefficient and the diffusion mode of each individual nanoparticle, as well as the ensemble mean diffusion coefficient of the whole formulation in the specific biological matrix through the correlation of the mean square displacement (MSD) of the nanoparticles with time:

\[
D = \frac{\alpha}{\alpha - 1} \times \text{MSD}
\]

where D is the diffusion coefficient and \( \alpha \) indicates the diffusion mode of the particles. Free diffusion of the particles is defined by \( \alpha = 1 \), while \( 0.9 > \alpha > 0.2 \) reflects the hampering of the diffusion of the particles by the mucus. Finally, \( \alpha \leq 0.2 \) is a clear signal of the immobilization of the particles in the mucus mesh.

Figure 4. Measurement of nanoparticle diffusion by multiple particle tracking

The different prototypes developed by the TRANS-INT consortium could not be analysed using the same mucoadhesion/mucodiffusion techniques, a fact that makes it difficult to elaborate an overall comparative study of the TRANS-INT prototypes. Table 2 illustrates the prototype families analysed using the different techniques.
Table 2. The prototype families analysed using the different techniques

The overall conclusions that could be extracted from the different techniques are:

- The surface properties of the nanocarriers and, notably the presence of polyoxyethylene coatings as well as anionic surface charge, reduced interaction with the mucus and had a positive effect on the mucodiffusion of the nanocarriers.

- The size of the nanocarriers had a significant influence on their mucodiffusion properties, i.e. the smaller sized nanocarriers (less than 100 nm) were more mucodiffusive than the larger sized nanocarriers.

- The positive charge of the nanocarriers had a negative impact on their mucodiffusion behaviour.

All of the mucodiffusion techniques analysed have specific “pros” and “cons” and the data obtained in mucin or fresh porcine mucus should be interpreted cautiously. In our opinion, the results of these studies should be analysed jointly with those obtained in the in vitro cell culture studies and also in the in vivo studies. Only the integrated analysis of the in vitro-in vivo behaviour could provide insights on the effects of the mucodiffusion properties on the in vivo performance of the nanocarriers.

3. Final processing of nanocarriers and pharmaceutical presentation

In the last stage of the project, WP1 partners explored two main strategies for the processing of the nanocarriers and their pharmaceutical presentation. The first one consisted of the conversion of the prototypes into a dry powder formulation. In most cases, freeze-drying was identified as the simplest method, in combination with the use of different sugars or cryoprotectors. These cryoprotectors were generally sugars and the type (e.g. mannitol, sucrose, trehalose) and amount needed varied according to the prototype composition.

A simple and straightforward formulation strategy involved the incorporation of the freeze-dried powder into a capsule coated with a pH-sensitive coating. This was only performed for the drug-loaded nanocarriers and it is described in WP2.

The second strategy was based on the proprietary technology of Sigmoid Pharma, SmPill®, that allowed the formulation of the nanocarriers into oral minispheres/beads. Altogether, 5 prototypes were converted into these SmPill® beads as the final dosage form.

Figure 5. The SmPill technology

In conclusion, WP1 activities helped the consortium re-define a significant number of technologies from partner laboratories. Among the different properties analysed in this WP, three specific ones were particularly challenging, namely: (i) characterization of the stability in simulated intestinal media in the presence of pancreatin enzymes and bile salts, (ii) characterization of the mucodiffusion properties (different techniques had to be used for the characterization of different prototypes) and (iii) labelling with...
fluorescent tags without altering the physicochemical properties of the nanocarriers. In addition, it should be pointed out that, in the case of several technologies, specific properties, i.e. stability and mucodiffusion, could only be characterized for the peptide-loaded nanocarriers and not for nanocarrier controls with no drug. This was due to the fact that these nanostructures consisted of a complex of the peptide drug with a specific biomaterial. A result of this screening was the definition of 13 series of prototype families, which were loaded with different peptides, as indicated in WP2.

WP2: Formulation of peptide drug candidates

The aim of WP2 was to use the nanocarriers identified in WP1, to encapsulate candidate peptides. The different prototype families would then be used to test the formulation, characterisation and optimisation strategies that would be used for further development and evaluation in downstream work packages.

An outcome from the initial transition of the peptide-loaded prototype families from WP1 into WP2 related to the interaction of the nanocarrier with the peptide. As a consequence, the physicochemical properties of the resulting nanocarriers were affected by the nature of the peptide associated to it, and, consequently the resulting prototypes needed to be re-defined. The resulting peptide nanocarriers required additional characterisation independent of the data obtained in WP1. Accordingly, the partners decided to place comparatively more emphasis on the characterisation of the peptide-loaded carriers, compared to the 'empty' nanosystems, and to focus screening on the evaluation of the peptide loaded carriers in downstream work packages.

In order to develop optimal peptide loaded carriers the overall objectives were broken down into the following specific tasks:

1. Development of peptide-loaded nanoparticle formulations.
3. Optimization of the peptide-loaded nanocarriers.
5. Final processing of the nanocarriers, and their pharmaceutical presentation, and stability.

The formulations were based on the initial nanocarrier prototype families (PFs) introduced and evaluated in WP1, i.e. a total of 13 prototype families, as indicated in the table below. These included a range of different types of nanocarriers such as nanocapsules (Prototypes 1,2,3 from USC and Prototypes 4,10 from UA), nanoparticles, nanocomplexes and micelles (Prototype 5, 6 and 13 from USC and Prototype 11 from UCLouvain, and Prototypes 7 and 12 from UCD, Prototype 9 from UCLondon and prototype 8 from UCC).

1. Candidates peptides

The peptide selection was led by Pharma partner (Sanofi R&D), in dialogue with those groups with responsibilities for nanocarrier development. Initially, Sanofi R&D supplied the consortium with gram quantities of three different peptides, i.e. two types of insulin (Insuman® and Apidra®), and a pain-
relieving peptide, labyrinthopeptin. Driven by its wide use as a benchmark peptide and its availability of large amounts the initial focus for many of the prototype families was on evaluation of the loading capacity of nanocarriers with insulin.

Two different variants of insulin peptides were made available initially, human insulin, hexameric form (Insuman®), and glulisine insulin, monomeric form (Apidra®). The decision to work with the two variants was based on chemical and physicochemical differences between the two peptides.

In addition to the insulins two other diabetes related peptides were included into the peptide repertoire later in the project, i.e. the glucagon-like peptide-1 agonist (GLP-1) analogues exenatide and liraglutide, which again differ in their physicochemical properties and in their pharmacokinetic profiles. The analgesic, labyrinthopeptin peptide, initially provided by Sanofi R&D proved to be very interesting due to its high lipophilicity and stability leading to interesting in vivo data for several PFs but it could not be tested widely due to the discontinuation of the project for this peptide by Sanofi R&D.

Table 3. Peptides used in each Prototype Family

2. Know your peptide - peptide characterization and stability profiles

At the start of the project it was also envisaged that all peptides, but particularly less well-known peptides, would need to be characterised and suitable assays subsequently developed. Assay requirements for the initial preparation of nanoparticulate formulations typically involved validated HPLC or LCMS methods. These methods were provided by Sanofi R&D and validated by IRB-Barcelona and were shared with partners in the form of SOPs. IRB has also contributed to the project by synthesizing new CPPs, i.e. hydrophobized D-octaarginine (R8-C12 and R8-Cholesterol) that were included in some of the formulations. In addition, the stability and structural integrity of the peptides was monitored by circular dichroism.

3. Rational nanocarrier design- definition of the Target Product Profile

As the evaluation of the peptide loaded nanocarriers in the downstream WPs involved a broad range of complex in vitro techniques, as well as in vivo experiments, a funnelling strategy was developed to select the prototypes that were the most suitable for further development. The development of the selection criteria was driven by a number of considerations to allow quantitative measurement of the following properties:

a) potentially predictive of the performance, based on mechanistic hypotheses, e.g. the assumption that carriers would need to protect the peptide, to penetrate mucus, and to either release their cargo either at the epithelium or following epithelial uptake;
b) ensure the prototypes meet essential PD experimental requirements, e.g. dose volume based on peptide activity;
c) limit the selection to prototypes meeting the appropriate pharmaceutical requirements in a dry powder dosage form (i.e. exhibiting a peptide payload consistent with the amounts to be administered in preclinical safety studies to comply with the peptide dose range);
d) take into account the cost of goods by minimising peptide waste (as evaluated from encapsulation efficiency).

The selected assays needed to allow reasonably high throughput, and used the equipment and methodologies specified in the SOPs, in order to serve as screening assays.

The decisions on the properties that are likely to affect the overall suitability and in vivo performance were widely debated, which reflected the fact that it remains unclear how nanoparticles in general can facilitate oral peptide delivery or in fact whether any specific prototype would enhance epithelial peptide uptake at all.

The development of useful criteria was thus based on current knowledge of the physiology of the GI tract, on a large body of nano-enabled oral peptide delivery literature, as well as on pharmaceutical and commercial requirements.

Key assumptions flowing from this were that suitable carriers would have a reasonable peptide loading and a relatively high entrapment efficiency and could be delivered in standard dosing regimes as well as minimising peptide waste. Furthermore, we postulated that properties such as size and surface charge would influence the delivery by a carrier and that a detailed standardised characterisation of these colloidal properties would be important. The stability of any formulation in terms of peptide loading and colloidal properties was also considered to be important in order to allow analysis and testing by partners in other WPs. A further assumption made was that in addition to having a minimally-acceptable shelf stability, suitable carriers would both maintain colloidal integrity and would provide some protection to the peptides in cell culture media and simulated intestinal fluids. Assays were devised, or adapted, to monitor in vitro peptide release profile and protection from proteolysis.

Based on this analysis, the following criteria were adopted for the Target Product Profile (TPP):

- Nanocarriers should be reproducibly produced with a size smaller than 400 nm, preferably close to 100 nm and with a variable zeta potential, preferably close to neutral or negative. These parameters are supposed to influence their stability and mucodiffusion properties.
- Nanocarriers should be stable upon exposure to intestinal media, in the presence of enzymes.
- Nanocarriers should be stable upon storage at 4ºC for at least a week, in order to enable their adequate manipulation and characterization.
- Nanocarriers should be converted into a freeze-dried powder or alternative form of final administration (SmPill® minispheres provided by Sigmoid).
- Nanocarriers should exhibit moderate to high mucodiffusion properties.
- The final peptide loading of the nanocarriers should be at least 2%, preferably 20%.
- The nanocarriers should protect the associated peptide from degradation in the presence of pancreatin.
- The nanocarriers should provide a controlled release of the associated peptide. As a first requirement, and being conscious of the frequent lack of in vitro-in vivo correlation, it was requested that nanocarriers should not have a burst effect higher than 50% in 30-60 minutes.

The assays were validated and shared as SOPs in order to allow the characterisation of the prototypes.
according to common criteria and standardised assay procedures. Considerable effort was put into achieving this, not only to allow the ranking of prototypes and to prioritise further development, but also to facilitate direct comparisons between different prototypes with a view to confirm mechanistic hypotheses around specific nanoparticle properties being detrimental or beneficial for oral peptide delivery.

Table 4. SOPs

The consortium developed, adapted, and validated assays to quantitatively characterize each prototype, in some cases using a number of different conditions. For example, a number of different compositions have been developed for simulating intestinal media in order to represent the conditions in the GI tract. However, the ability of such simulated media to predict effects on nanoparticle systems in vivo remains unconfirmed. In fact, some of the established standardise assays are probably less useful for evaluating nanocarriers than they are for small molecules.

Other challenges emerged around applying the same assay technique based on the characteristics of the individual carrier systems; for example, due to different physicochemical properties, it was not possible to define a common method for the determination of peptide encapsulation and the final bench-mark values to be measured with an appropriate technique were agreed instead.

Combining the various prototype families and peptides, the consortium has developed and characterised hundreds of formulations which were ranked within each prototype family to allow the selection of the most suitable composition and processing conditions. The different prototype families were also compared, which led to the elimination of some of the less suitable prototype families.

The assay grid proved successful, in terms of the prioritisation of specific prototypes, and this allowed the funnelling of suitable nanocarriers to downstream WPs. As a consequence of the feedback from these evaluations prototypes in some cases underwent further optimisation.

The consortium proceeded with further development on the assumption that the most convenient dosage form would involve a dry formulation to be used in a tablet or capsule. Furthermore, for most peptides some protection in the stomach from a coated formulation will be beneficial even though nanocarriers in that overall construct should provide some degree of protection.

In addition to scaling up the preparation of the final dosage forms, dry peptide loaded nanoparticle powders were prepared and characterised. As a potential gastro-protective dosage form the consortium evaluated the combination and ‘encapsulation’ of the prototypes within the SmPill® mini pill provided by Sigmoid Pharma. This technology was capable of processing liquid suspensions as well as dried formulations, although the targeted peptide loading is typically more easily reached with dried formulations.

In conclusion, the experience from WP2, on the design, characterisation and manufacture of peptide loaded nanocarriers, has shown that in cases where there is a good match between the peptide’s physicochemical properties and the efficiency of the carrier nanoparticle formulations there was clear potential in terms of enhancing oral delivery. On the other hand, other peptides have proven more
challenging to encapsulate efficiently and their effect on oral peptide delivery was likely to be more
variable. While the selection criteria allowed effective funnelling of prototypes for development it appears
that in their current form such criteria are not necessarily predictive of in vivo performance and in fact
successful prototypes in some cases did not strictly meet the hypothesised selection criteria.

WP3: In vitro studies of toxicity and mechanism of action

The objective of this WP was to evaluate the cytotoxicity and mechanism of action of the different
nanocarrier prototypes developed in WP1-WP2. The nanocarriers were selected (13 prototype families
and several subclasses) according to the defined TPP, as specified in WP1-WP2. A number of sequential
assays described below allowed us to rank the prototypes according to their toxicity and capacity to
overcome the model Caco-2 barrier as well as the human jejunal mucosa barrier in vitro. These studies
were:

- Task 3.1 – Cellular toxicity and functionality (cell damage and immunological responses)
- Task 3.2. Evaluation of the interaction of nanocarriers with the intestinal epithelium and transport of the
  associated peptide

Regarding these in vitro assays, the following standard operating procedures (SOPs) were developed and
adopted by the WP3 partners:

Table 5. SOPs

The main data resulting from this screening are described below.

1. Cellular toxicity and functionality (cell damage and immunological responses)

Veneto Nanotech/ECAMRICERT was the Reference Centre responsible for evaluating the cytotoxic
effects of selected prototypes on the intestinal epithelial cell line, Caco-2, by measuring metabolic activity
and membrane integrity. A number of assays were performed for the cytotoxicity evaluation such as ATP
content, MTS (mitochondrial activity), Neutral Red assay (NRU) and cytosolic LDH release according to
the SOPs prepared by the consortium, lactate dehydrogenase (LDH) release according to the SOPs
prepared by the consortium. In some cases, prototypes could not be tested using one specific technique
because of analytical interference with assay components. UCLouvain also performed preliminary
cytotoxicity studies for several prototypes using the MTT assay (mitochondrial activity, a variation of
MTS).

The half maximal effective concentrations, EC50 (the concentration that reduces cell viability by half of the
maximum effect), and the minimal effective concentration (MEC) were calculated from the concentration-
response curves observed for the different prototypes. The results clearly indicated that, in undifferentiated
Caco-2 cells, the cytotoxicity was prototype-dependent. A large range of EC50 values from above 8 to less
than 0.1 mg/ml were observed. No relationship could be found between particle size and zeta potential
and toxicity. However, the components, and in particular some surfactants and agents affecting the
membrane properties greatly influenced cytotoxicity. This was to be expected, as these particles would be expected to have a greater internalization rate. These data on potential cytotoxicity were used for further in vitro studies (Task 3.2).

The most cytotoxic prototypes, according to the in vitro pre-screen assays described above, were further evaluated using the lipidomic assay by CEA. Specific lipidomic effects, a significant increase of signals corresponding to some specific ceramides and sphingomyelins, were observed for prototype 10 (Eudragit® nanocomplexes), at concentrations that did not lead to significant toxicity according to the standard cytotoxicity assays.

A subsequent cytotoxicity screening was also performed in human intestinal epithelial tissues by UU. Toxicity was assessed by monitoring the tissue electrophysiology and response to cAMP agonists in the Ussing chambers (viability), LDH release (membrane damage), tissue ATP content (viability), and induction of cytokine release from the jejunal tissue (potential inflammatory response). Cytokine release was measured using a Mesoscale instrument in multiplex mode allowing simultaneous quantitation of 30 cytokines. Overall, with the exception of one prototype, the nanoparticles tested by UU showed limited toxicity to the jejunal tissue. This was likely due to the generally good safety profile of the ingredients selected to produce the prototypes.

Prototype 3 in particular, Protamine nanocapsules, was also tested for potential immunological responses. The results of the in vitro read-outs indicated the absence of significant immunological responses in dendritic cells from mice. Efforts were, therefore, concentrated on the evaluation of the in vivo immunological responses in mice.

Overall, the conclusion from this toxicity screening was that the majority of the prototypes exhibited very promising toxicity profiles and, therefore, no prototypes were excluded as a result of this screening.

2. Evaluation of the interaction of nanocarriers with the intestinal epithelium and transport of the associated peptide

In the first step of the screening, intestinal cell lines, such as monolayers of enterocytes, including Caco-2 and also the mucus-producing HT29-MTX cells, were used either as monocultures or co-cultures. The interaction of the prototypes with the intestinal cell lines was studied qualitatively and quantitatively. For quantitative evaluation, flow cytometry was used to analyse the interaction of the prototypes with the Caco-2 cell line. For this, cells were seeded in 24 well plates for 48 hours, and then nanoparticles were incubated with the cell line for 2 h. For a qualitative evaluation, the nanoparticles in the Caco-2 cell were visualized under confocal microscopy. The transport of the fluorescent compound associated to the nanocarriers was also evaluated in the basolateral side by fluorimetry.

Sixteen different compositions belonging to 13 different prototype families were evaluated. Among them, only 4 prototypes exhibited evidence of internalization within the monolayer. The rest remained mainly associated with the monolayer epithelium. Overall, the results indicated that the interactions observed were highly dependent on the composition of the prototypes, with nanocomplexes with positive surface charge and/or with the presence of permeation enhancers, i.e. chitosan and arginine-rich polymers,
showing the highest degree of interaction. In some cases this internalization was accompanied by a transient decrease in the transepithelial resistance (TEER) of the Caco-2 cell monolayer, which might suggest the alteration of the tight junctions. Regarding the transport of the fluorescently-labelled peptides, in general, a small fraction was found to traverse the cell monolayer.

In a second step, a screening was performed with a reduced number of prototypes (9 nanocompositions belonging to 6 prototype families) in the ex-vivo human intestinal model mounted in an Ussing chamber. Visualization of nanoparticle interactions with tissue was achieved using laser scanning confocal microscopy (LSM), epifluorescence microscopy, and structured illumination super resolution microscopy (SIM). Additionally, a tissue CLARITY protocol was developed allowing the detection and 3D rendering of nanoparticles in intact, un-sectioned tissue.

The results showed different levels of interaction of the prototypes with the intestinal mucosa, which could be classified as (i) no permeation, (ii) weak permeation and (iii) active endocytosis. The first category included PFs 2 (Polyarginine nanocapsules), 10 (Eudragit® nanocomplexes), and 11 (Nanostructured Lipid Carriers, NCL) that showed pronounced mucus binding. No nanoparticles could be detected in close proximity with the tissue by microscopy, and, accordingly, these nanoparticles showed zero permeability (Figure 6). The second category consists of prototype family 3 (Protamine nanocapsules), which showed pronounced mucus binding but could also be detected at low levels in the tissue and exhibited some uptake. Nanoparticle PF 6 (PGA-PEG/r8-glulisine nanocomplexes, C12R8 np) constituted the third category. These nanoparticles were highly mucodiffusive and no binding to mucus could be detected (Figure 6). The nanoparticles were endocytosed to a high degree by the enterocytes of the intestinal epithelium and showed a small but measurable permeability. The permeability could, to a large extent, be abolished using chemical inhibitors of endocytosis, strongly suggesting it is due to active cellular processes.

The comparative analysis of these data with those obtained in the Caco-2 model cell line indicates important discrepancies. In fact, Prototypes 2, 10 and 11, showed a good internalization by the monolayers. This could be attributed to the absence or limited presence of mucus in the monolayers. The only two prototypes that behaved comparatively were PF 6 (PGA-PEG/r8-glulisine nanocomplexes, C12R8 np), which showed a high internalization in both models, as well as PF 12 (undisclosed for IP reasons). This could also be related to the good mucodiffusive properties of these prototypes.

Figure 6. Interactions of tested nanoparticle prototypes with human jejunal tissue. A: Prototype families 2a, 10, and 11 showed pronounced mucus binding. No nanoparticles could be detected in close proximity with the tissue. These nanoparticles showed zero permeability. (Nanoparticles from family 10 in green, nuclei in blue) B: Prototype family3 nanoparticles showed pronounced mucus binding but could also be detected at low levels in the tissue and exhibited some permeability. Nanoparticles in tissue were always found beneath defects in the epithelium (blue arrow). (Nanoparticles in green, actin stained in red to visualize an epithelial defect in vicinity of blue arrow) C: Prototype family 6a nanoparticles were highly mucodiffusive and no binding to mucus could be detected. The nanoparticles were endocytosed to a high degree by the enterocytes of the intestinal epithelium and showed a small but measurable permeability. (Nanoparticles from family 6a in green, nuclei in blue) D: Permeability of prototype family 6a nanoparticles could be inhibited by endocytosis inhibitors such as the dynamin inhibitor Dynasore indicating that active...
endocytosis is the permeation mechanism

The study of the peptide transport was also performed using the Caco-2 cell model and the human intestinal model. Peptide-loaded nanoparticles were incubated with the cell monolayer for 2 h before collecting the transported sample in the basolateral side. The transported peptides were expected to be quantified using LC/MS/MS at CEA, however, using this technique the peptide transported could not be detected. Similar results were obtained upon addition of the prototypes to the human intestinal tissue mounted in a chamber. Analysis of insulin permeability by LC/MS/MS at CEA could detect no insulin permeability in samples from prototypes 3, 6 and 12 that were, apparently, more permeable (the three nanoparticle prototypes showed measurable permeability).

In conclusion, taking these results together, the main conclusions from WP3 are:

- The cytotoxicity of the prototypes varied significantly depending on their composition. The maximum toxicity observed in the Caco-2 model cell line corresponded to an IC50 value of approximately 0.1 mg/mL. This value, together with the negligible toxicity observed upon contact of the prototypes with the intestinal epithelium, indicate that all the designed prototypes were considered to have a low toxicity in this preliminary screening.

- The capacity of the prototypes to interact and be internalized by both, Caco-2 monolayers and the human intestinal epithelial tissue mucosae, was dependent on nanoparticle composition. Those consisting of a R8-insulin nanocomplex surrounded by a PEGylated polymer were found to have the greatest internalization capacity.

- None of the prototypes tested led to a significant transport of the fluorescent tag or the peptide associated across the epithelium.

Therefore, the overall conclusion was that irrespective of their composition, the capacity of nanocarriers to facilitate the transport of the associated peptide across the epithelium was in all cases very limited. This is a relevant conclusion in two main senses:

1) interaction of a nanomaterial with the intestinal mucosa does not necessarily translate into the translocation of either, the nanomaterial or the associated peptide across the intestinal mucosa.

2) the accumulation of the nanocarriers and associated peptides within the intestinal mucosa might be of potential interest for the treatment of GI disease.

WP4: Preliminary in vivo mechanistic and toxicity studies

This WP involved the preliminary assessment of pharmacodynamics (PD) and pharmacokinetics (PK), and the biodistribution of selected prototypes in rodents. Taking into account the toxicity data from WP3 and the fact that the majority of the carriers were made from excipients and materials with Generally Regarded As Safe (GRAS) status, toxicology changes to the intestine were expected to be minimal.
The main studies associated to this WP were:

Task 4.1 – Particle interaction with epithelial barriers and biodistribution
Task 4.2. – Preliminary immunological evaluation

1. Particle interaction with epithelial barriers and biodistribution

The UCD “rat intestinal regional instillation model” was one of the experimental approaches taken for this study. This non-recovery model involves dissection and exteriorising of the rat gut loop, with an intact mesenteric blood supply, while the rat is maintained under anaesthesia for 4 hours wrapped in a warm blanket. Advantages of this model are: a relatively high throughput, consistency of the dissection, and the capacity to compare nanocarrier insulin formulations with datasets achieved in parallel with known epithelial permeation enhancers, such as sodium caprate and sucrose laurate esters. Its disadvantages are that the anaesthesia could impact intestinal absorption and alter levels of endogenous insulin. Figure 7 shows the positive behaviour observed for 2 of the prototypes investigated using this instillation model.

Figure 7. Instillations in rat jejunal loops by UCD. Upper left: Glucose levels achieved for Prototype 8 (PD, n=4). ● PBS; ■ insulin solution; ▲ insulin in nanocarrier. Upper right: Plasma levels achieved for Prototype 8 (PK, n=4): o insulin in nanocarrier; Δ insulin solution (s.c.). Lower left: Prototype 12 (PD, n=6). ● PBS; ■ insulin solution; ▲ insulin in nanocarrier. Lower right: Prototype 12 (PK, n=6): symbols as for lower left. Dose was 50 IU/kg (instillations), 1 s.c.

An important finding from these experiments was that at least some selected nanocarriers could, indeed, work in non-diabetic rats. The reason why other prototypes did not perform well might be related to the experimental model or to the fact that they do not perform according to expectations.

The fate of fluorescently-labelled nanocarriers with and/or without peptides was investigated following oral administration/in situ instillation to rats/mice. Fluorescence data from UCLondon with prototype 9 (Chitosan based GCPQ) showed evidence of particle uptake by the mouse intestine, as some particles were visualized behind the epithelium. ECAMRICERT, IOV, and UCD attempted similar studies in rats. Most of the studies they performed found signals suggesting that several nanocarriers were stuck in overlying mucus, or alongside the folds of the microvilli, despite their neutral zeta potential. However, other prototypes with similar physicochemical properties, showed the capacity for adherence to the epithelium and internalisation, presumably by endocytosis. As an example, Figure 8 shows fluorescent images from UCLondon of the labelled prototype 9 (Chitosan-based GCPQ), revealing a signal in and beyond the epithelium.

Figure 8. Labelled PF 9 adhering very closely to the brush border of the mouse small intestine following gavage appears to be taken up by the epithelial cells and into the lamina propria. Co-localisation with the Dylight 488 tomato lectin suggests some uptake into circulation

Figure 9. Texas Red (TR)-labelled PF 9 (GPCQ-TR) adheres to mucus and is abundant between the villi of mice
In another example, IOV/ECAMRICERT carried out fluorescence imaging of DID-labelled PF 2 (Polyarginine nanocapsules) in rats. Their results show evidence of DID-labelled prototype 2 interaction with the duodenal wall after 60 minutes, but not at later time points (Figure 10).

Figure 10. IOV/ECAMRICERT evidence of labelled PF 2 along the side of duodenal villi in rats following oral gavage

Overall, the fluorescence imaging studies show that the interaction of the different prototypes investigated with the intestinal mucosa depends on the nanocarrier. Polyarginine nanocapsules appeared to have measureable interaction with the epithelium, whereas such interaction could not be determined for protamine nanocapsules. On the other hand, the GCPQ prototype was able to penetrate the mucosal layer and reach the intestinal villi, and some particles were found to reach the layers underneath the epithelial barrier, i.e. the submucosa, although this is not a quantitative assay. Finally the NLCs prototype was found to have a fast interaction with the intestine, and remained widely distributed along the intestine for extended periods of time. The biodistribution pattern was not, apparently, modified upon incorporation of SLNs into the SmPill minispheres. Overall, despite the qualitative character of these results, the overall conclusions are in agreement with those observed in vitro using the human intestinal tissue model.

In vivo fluorescent biodistribution studies were complemented with studies using radiolabelled nanocarriers, as performed at USC. As an example, Technetium-99m (99mTc) labelling was applied to Prototype 6: PGA-PEG/R8-glulisine nanocomplexes (USC). The radiolabelled carriers were administered orally to Wistar rats at a dose of 50 IU insulin/kg, corresponding to 250 μCi. The imaging was performed with SPECT-CT acquisition. The biodistribution study concluded that the 99mTC-labelled PGA-PEG/R8-glulisine were retained in the small and large intestines up to 26 hrs, in contrast to the control that remained mainly in the stomach (Figure 11). However, no significant radioactivity could be observed in the rest of the body, a fact that suggests that the particle interact with the intestinal mucosa and remain at that level, without systemic absorption, even after extended periods of time.

Figure 11. In vivo tissue distribution of the orally administered 99mTc- PGA-PEG/R8-glulisine (ENCPs) (A) and the free 99mTc control (B)

IOV/ECAMRICERT performed the preliminary immunological evaluation of two selected prototypes after acute and chronic treatment to mice. Mice were dosed daily with different nanocarrier prototypes (Prototypes 6 and Prototype 13) for up to one month, and then examined local mucosal immunology and systemic immunology read-outs. As an example Figure 12 shows the histological analysis of the intestinal tissue after chronic administration of Prototype 6: PGA-PEG/R8-glulisine nanocomplexes (USC). No major histological differences between control and prototype-treated duodenum and large intestine were observed at selected time points. No differences were found between duodenal and colonic expression.

Figure 12. Immunohistochemistry of the small intestine after Octa NCPs administration with CD3 and CD68. In the figure co-localization with DAPI is reported (magnification 40X with dry objective). (Ctrl=untreated)
Overall, no visible morphological alteration was observed in the intestinal mucosa following administration of different prototypes. In the light of preliminary immunotoxicological results obtained during Task 4.2 we could consider the tested prototypes potentially safe according to these initial preclinical assays. However, further advanced toxicological studies are required in order to draw a final conclusion on the safety of these nanoformulations.

In conclusion, a summary of the results reads as follows:

- The results obtained in the rat loop gut instillation model indicated that only 2 prototypes (out of 7 tested) delivered peptide and altered pharmacodynamics according to the expectations. However, this model may have some limitations due to its use of anaesthesia and the fact that it is not an oral model.

- The biodistribution data obtained with fluorescent and radioactive nanocarriers indicated that there is a significant interaction between most of the nanocarriers and the intestinal mucosa, a fact that, in some cases, translates into a prolonged residence time as noted by the SPECT studies (in some cases of more than 12 hrs). In addition, confocal images suggest that the degree of interaction of the nanocarriers with the mucosa varies depending on their composition. While some nanocarriers, i.e. protamine nanocapsules, appear significantly stuck on the mucus, others, e.g. PGA-PEG/R8-glulisine nanocomplexes or GCPQ nanocomplexes, were shown deeply internalized. In general, no transport of the nanocarrier across the intestinal mucosa could be observed.

- The local intestinal immunological data indicate that the nanocarriers tested do not cause a relevant immunological response after chronic administration to mice.

Therefore, the overall conclusion of these studies is that the nanocarriers investigated in the TRANS-INT consortium have a deeper or more superficial interaction with the intestinal mucosa depending on their composition. However, no clear correlation with the physicochemical properties of the nanocarriers could be established. For example, some prototypes with similar sizes (200-300 nm) and zeta potential (close to neutrality) showed a very distinct interaction with the mucosal barriers. In some cases, the interaction was important enough to prolong the permanence of the nanocarriers in the mucosa for long periods of time and generate PK/PD responses. However, no translocation of the particles across the intestine of rodents could be observed. Despite their internalization, specific nanocarriers showed an adequate biocompatibility.

An additional conclusion was related to the agreement of these data with those observed with the human intestinal in vitro model.

WP5: In vivo pharmacokinetic/pharmacological evaluation of nanomedicines

The main activities associated to this WP were:

Task 5.1 - In vivo evaluation of the pharmacological profile
Task 5.2 - In vivo evaluation of the drug pharmacokinetic profile
The results of these two activities are jointly presented below.

The original aim of WP5 was to investigate the PK (plasma peptide concentration) and/or PD (plasma glucose levels) of orally delivered antidiabetic peptides (up to a maximum of 20 formulations) in small experimental animals (rodents). To help us choose the most appropriate type of rodent animal model for the study, several considerations should be made. Firstly, the aim of developing insulin-based therapies is to treat diabetes. However, there are two main types of this disease (type I and type II), each with different etiologic and pathological features. Type I diabetes is characterized by the destruction of endogenous beta-cells, hence, in Type I diabetes patients, endogenous insulin production is lacking from the outset. On the other hand, type II diabetes is, in most instances, related to obesity, and its hallmark is usually normal or elevated insulin levels (although after long term evolution of the disease, the insulin levels could be low) and insulin-resistance in the liver. Therefore, there is a general lack of consensus on which specific experimental animal model is most relevant to test new insulin-based therapies.

There are several models that can be used, each with different pros and cons. The use of normal animals (meaning non-obese and non-diabetic animals) is an obvious choice because, among other advantages, they do not require specific preparations/training or treatment before the experiments. Obviously they will exhibit normal insulin sensitivity and a counter-regulatory response to hypoglycaemia. An alternative would be the use of rodents with chemically induced diabetes.

To induce diabetes, the animals are administered chemicals that are toxic for beta-cells. By destroying the insulin producing cells, these chemicals stop endogenous insulin production, thus causing hyperglycaemia. Although the most widely used chemical in this context is streptozotocin, there is a wide variation on the specific protocol (dose and duration of treatment) used. In general, diabetes is fully developed 5-7 days after a single streptozotocin administration at an intraperitoneal dose of \( \sim 40\text{mg/mL} \).

One advantage of this model is that, because animals so treated have a low endogenous insulin production, they are more sensitive to exogenous insulin administration than normal animals. Two of the drawbacks of this model are: 1) that animals lose weight and 2) that there is a large variation on diabetes development among individuals because the destruction of beta cells is quite different in each animal and 3) the data may yield false positives for nanocarriers in terms of translation potential. Hence, it is appropriate to define inclusion criteria (e.g. glucose levels > 300 mg/dl) for the animals to be tested. In our studies, the testing was done mostly in normal animals (non-diabetic, food ad libitum), although, in some cases, we also used the other two models (animals with fixed food-regime or diabetes). The experimental details of our studies are as follows:

At the USC, experiments were performed using the two models indicated in Figures 13 and 14. In total, 10 formulations belonging to 8 different prototype families were tested in the non-diabetic model, and 5 selected formulations were tested in hyperglycaemic rats. In addition, the performance of specific peptide-loaded prototypes was assessed following different doses and administration conditions (sc., oral gavage and/or by direct jejunum and/or ileum instillation, using different volumes).

The different studies were chosen after taking into account the nature of the formulation to be tested (some formulations are more sensitive to degradation in the stomach and duodenum than others). In addition, the
comparison of these modalities of administration was expected to provide mechanistic insights for subsequent optimization of the prototypes. Subcutaneously injected insulin (either human insulin or glulisine) was used as a reference to estimate the relative oral PD effect vs. SC and the relative oral bioavailability. As indicated in Figures 13 and 14, sampling of blood was normally performed for up to 8 hrs and, in a few experiments, for up to 12 or even 24 hrs.

Prototype formulations tested at USC were selected taking into account the TPP defined in WP1-WP2 regarding the physicochemical and pharmaceutical properties associated with the rational design of the nanocarriers. In addition, these selected formulations were found to exhibit a low-medium cytotoxicity profile. Some of them were also found to exhibit an adequate interaction with the intestinal mucosa.

These protocols of administration were followed for different prototype families. Taking into account the data reported in the literature, emphasis was made in comparing the results obtained in experiments performed with normal versus diabetic rats.

At Sanofi R&D, 6 selected formulations were tested for their evaluation in normoglycaemic rats using a protocol that differs substantially from the one indicated in Figure 13. The main difference relates to the fact that animals were anesthetized during the whole experiment using pentobarbital. In addition, samples of blood were only taken for up to 4 hrs.

Figure 13: Protocol for the administration of formulation using normal animals (i.e. non-obese and non-diabetic animals)

Figure 14: Protocol for the administration of formulation using diabetic rodents (diabetes induced by streptozotocin administration during 1 week)

Overall, the results obtained at USC led to the following conclusions:

− Prototype 13 (coated Insulin nanocomplexes) led to significant and prolonged responses in both normal and diabetic rat models. This was the prototype eliciting the most promising profile as shown in Figures 15 and 16. It should be noted that the response was more noticeable when the formulation was tested in the diabetic model.

− Prototype 3 (Protamine nanocapsules) led to a modest reduction in the glucose levels in a normal rat model (20-30% decrease), however this response was not increased when prototype 3 was tested in diabetic models.

− Prototype 1 (Insulin nanoemulsion) did not lead to a reduction in the blood glucose levels in a normal rat model, however, it did elicit a significant response in a diabetic model.

− Prototypes such as polyarginine nanocapsules, chitosan nanoparticles, Eudragit® nanocomplexes and GCPQ nanocomplexes led to a minor but significant glucose response only at some specific time points (1-2 time points), following either intra-duodenal or intrajejunal administration. The response observed was similar in both normal and diabetic models.
Finally, PGA-PEG/R8-insulin nanocomplexes and Cyclodextrin-insulin nanocomplexes did not lead to a significant response following either intra-duodenal/intra-jejunal administration. The response observed was similar in both normal and diabetic models.

The GLP-1 agonist loaded nanoformulations failed to show a meaningful biological effect under our experimental conditions.

In conclusion, only 3 of the prototypes tested—protamine nanocapsules, insulin nanocomplexes and insulin nanoemulsions led to meaningful responses in either normal or diabetic animal models. These values are in agreement with those previously observed in the literature for other insulin-based nanoformulations.

In the non-anesthetized rat diabetic model (USC) provided the most significant responses, whereas the anesthetized rat model (Sanofi R&D) did not show any specific response nor any concentration of insulin in blood up to 4 hrs after inoculation. Whether one experimental protocol is more meaningful than the other remains to be elucidated. However, one could speculate that the transport of the nanoparticles and their consequent release of insulin could be reduced in an anesthetized model, which would explain the lack of a significant response in this case, in the time frame tested.

Irrespective of the influence of the animal model on the overall output of this WP, it should be noted that these results are somehow in contradiction with what was expected from the in vitro experiments performed in the Caco-2 model cell line and in human intestinal tissue and the in vivo biodistribution studies. In these models, for some specific formulations that did not give a significant response, a very important penetration of the nanoparticles and of the associated insulin was observed.
Two main hypotheses have been formulated to explain these data so far:

1- The positive in vitro data (stability, control release, caco-2 permeability...) obtained for some prototypes may have a limited predictive value of the performance of oral peptide formulations in vivo.

2- The high variability in the response to insulin, even after subcutaneous administration, and the animal model itself may be responsible for the highly variable responses observed for all formulations. In this sense, it is important to keep in mind that in order to draw a clear conclusion, it will be necessary to perform the experiments in large animal models. This evaluation will be performed for selected prototypes after the TRANS-INT consortium comes to an end.

It is also important to highlight that specific formulations that did not lead to significant peptide absorption, i.e. PGA-PEG/R8-insulin nanocomplexes and GCPQ formulations, among others, so this might be of interest in achieving a local release at the level of the intestinal tract. This situation might be of interest for the treatment of local intestinal diseases with new peptide drugs.

Finally, it is worth noticing that although, based on these data it seems that insulin might not benefit from most of the technologies described here, other peptide drugs with a lower molecular weight and a higher stability might be good candidates for such delivery technologies. In fact, one explanation for the fact that some nanoformulations accumulate at the mucosa level, could be that insulin is simply not released from the nanocarrier inside the cell, or that it is degraded inside the lysosomal compartments as it crosses the intestinal wall. Such a difficult barrier may be easier to overcome for smaller peptides than for large labile peptides, such as insulin.

There is no doubt that TRANS-INT has provided a unique opportunity for academia and industry institutions from all over Europe to address the problem of how to transport therapeutic macromolecules across the intestinal barrier. There was great, positive interaction within and between academic and industrial institutions, between established scientists and scientific newcomers. Overall, a greater understanding of the underlying mechanisms of oral delivery and intestinal absorption of peptides, such as insulin, was reached, in particular, relating to i) how to bring drugs to the best intestinal region site for the absorption, ii) how to protect the drugs from degradation, iii) how to get them through mucus and the various cell layers once they reach the tissue and iv) how to increase the final drug load and bring it to its target.

However, the fact that the data obtained at USC and Sanofi R&D (and indeed at UCD in WP4) are considerably different suggests that the decrease of glucose in the blood is highly dependent on the study conditions. Further research is needed to evaluate the nature of these differences and their impact on the process of taking peptides, such as insulin, across the intestinal wall and other natural barriers.

WP6: Preclinical efficacy, toxicological an immunological evaluation

This WP provided preliminary preclinical data regarding the efficacy of one selected prototype in the pig model. Experiments to fulfil the task associated with this WP are still under way and no conclusive data
Potential Impact:
This section describes the potential impact of the TRANS-INT project, including socio-economic impact and the wider societal implication of the project as well as the main dissemination activities and exploitation of results.

1 Potential impact
The potential impact of the TRANS-INT project was evaluated with regards to five different aspects; those aspects are briefly discussed in the sub-sections below:

- Impact on advancement of research;
- Impact on EU economic development;
- Impact on public health;
- Impact on legislation/technical standards/guidelines;
- Improved understanding by academics and research organizations of the pharmaceutical industry and regulators.

1.1 Impact on advancement of research
One of the main objectives of the TRANS-INT project was to generate comprehensive knowledge on nanocarriers and nanomedicines as well as on their behaviour under biological conditions. This knowledge was generated in the course of the project. The following results were achieved:

• Knowledge on how the nanocarriers can be efficiently loaded with peptides;
• Knowledge on how the composition of the nanocarriers and the drug loading can affect the release properties;
• Knowledge on how the composition of the nanocarriers influenced their stability in the biological environment, i.e. gastrointestinal tract;
• Knowledge on how the composition of the nanocarriers influences their interaction with the biological barriers, especially in terms of mucodiffusion through and capacity to be internalized and/or get across the intestinal mucosa;
• Knowledge about the toxicity and immunological responses generated by the nanocarriers.
• Knowledge about the in vitro stability of the nanocarriers as a suspension and as a freeze-dried form.
• Knowledge about the factors that affect the technological processing of the nanocarriers into a final dosage form.

These results were achieved thanks to the contribution of all partners involved in work packages 1 – 6 and are going to have a direct impact on their research projection. This will happen for both academic and industrial partners, as they will have the possibility to apply the acquired knowledge in future projects that they will run on their own or in collaboration with partners external to the TRANS-INT consortium. Especially in the latter case, it is very likely that the impact of TRANS-INT will go far beyond the consortium partners.

1.2 Impact on EU economic development
At the start of the TRANS-INT project, it was expected that it would impact the European pharmaceutical industry.
industry and result in an improved competitiveness of its biotech/health sector.

After project completion, it is possible to affirm that, even if TRANS-INT was not able to progress to the pre-clinical stage, it gave us an improved knowledge of the nanocarriers that can be converted into an oral dosage form to deliver biopharmaceuticals and peptides, as outlined in section 1.1 above.

Biopharmaceuticals and peptides represent an area of the pharmaceutical industry that is receiving an increasing interest and economic investment; however, their lack of oral bioavailability is a major difficulty that is hindering a complete exploitation of these market opportunities.

We believe that the knowledge generated by the TRANS-INT project in the last five years has shed light on the formulation requirements that need to be implemented in order to develop nanocarriers and nanomedicines with improved oral bioavailability.

The three companies involved in the project, Sigmoid, Seroscience and Sanofi R&D, recognized that the acquisition of critical knowledge will help them to improve the development of peptide delivery technologies. In addition, a wide range of European pharmaceutical companies will be able benefit from the project results not only from the acquisition of the knowledge achieved, but also as a result of licenses and co-development strategic plans. A boost to industrial alliances and collaborations can be generated as the knowledge has resulted from a multi-disciplinary project that involves biology, physical, organic and analytical chemistry, pharmaceutical technology, pharmacology and immunology. Usually these competences are not retained by a single pharmaceutical company, especially in the case of SMEs, but can be acquired and appropriately understood when specific alliances are established.

It must be clear that the economic benefits are not limited to the industry partners, but can be extended also to the academic ones; as discussed in more detail in section 2, three patent applications have been generated so far. Two of them were filed by academic partners (USC and UCD) of the TRANS-INT consortium, and one of them was the result of the collaborative alliance between USC and Sanofi R&D. In addition, a fourth patent is currently being prepared by the USC. This is an indication that there will be clear market and license opportunities for those partners that will boost both their research projects and also the possibility to create spin-off companies.

1.3 Impact on public health
It is known that the oral route is the most acceptable to patients and therefore it has the highest compliance; as explained in section 1.2 however, this route has proven to be highly limited for biopharmaceuticals and many of the synthetic peptides due to the poor oral bioavailability and their difficulties in overcoming the biological barriers.

The TRANS-INT project has provided several possible solutions to overcome these issues, therefore unlocking the full potential of this class of medicines.

Although the consortium has worked on an anti-pain peptide, the focus was more into the development of an oral anti-diabetic type 2 dosage form.
Global diabetes prevalence has been constantly increasing in the last 20 years, passing from 151 million in 2000 to an estimated figure of 438 million in 2030. This disease is known to be associated with poverty, as most patients are from low- and middle-income countries and belong to the lower socio-economic groups in the richer countries.

The results achieved by some of the formulations prepared and analysed in the course of the TRANS-INT project show that an oral dosage form can potentially be developed starting from those prototypes. This would have a significant impact on the current therapy not only by improving the patient compliance, but also, and more importantly, by decreasing the side effects and reducing the concomitant and subsequent diseases such as diabetic foot, neuropathic/chronic pain, eye diseases, etc.

An added benefit would consist in a significant improvement of the quality of life for those patients and their restored ability to actively participate in society and work.

Finally, the availability of oral nanomedicines for the treatment of type 2 diabetes would significantly reduce the public health care costs.

Firstly, oral administration is the most cost-effective administration route due to improved patient compliance and the avoidance of expensive injection devices with sterile material.

Secondly, the improved oral bioavailability will reduce side effects and lower the public care associated costs, reducing the requirement for trained personnel while concurrently increasing the number of patients that can actively participate in society and work.

TRANS-INT used a small size anti-pain peptide, with a very positive outcome, as well as 4 different types of insulin and two different GLP-1 analogues. Given the large array of physicochemical properties and the extraordinary complexity of the anti-diabetic drugs selected (very sensitive to degradation, very low permeability and high PK/PD variability), the knowledge generated will be extremely valuable and potentially transferred to other biopharmaceuticals and peptides, as already explained in sections 1.1 and 1.2. This means that the results achieved can be used for a more general objective, which consists of increasing the application of nanotechnology in medicine in a variety of diseases that would go far beyond type 2 diabetes.

1.4. Impact on legislation/technical standards/guidelines

As the nanomedicines represent a new class of pharmaceuticals within the larger group of innovative medicines, their definition and the terminology used at international level has not been fully agreed; despite this the number of nanomedicines that were introduced to the market has constantly increased over the last years, the protocols for the assessment of their safety and efficacy were not standardised and every product was assessed on a case-by-case basis.

The TRANS-INT project was executed keeping in mind its potential impact on the relevant regulatory aspects; the formulation development and in vitro and in vivo analyses were conducted after consultation with the project advisors, some of which have been Ad-Hoc project advisors for EMA and other regulatory
It is important to highlight that a very important aspect of TRANS-INT was “Quality”, and because of this, the operational work of the consortium relied on the premise of having “Reference Centres” that performed the comparative analysis of the formulations developed. This comparative analysis was essential for the continuous screening process and the selection of prototypes to be evaluated in advanced phases. These reference centres elaborated specific SOPs for all the methodologies used by the consortium. These specific guidelines have been made open to the public via the TRANS-INT web site. We believe that the meticulous work carried out in this regard will give a contribution to the standardization of manufacturing and analysis methods to facilitate the introduction of nanomedicines into the pharmaceutical market.

1.5 Improved understanding by academics and research organizations of the pharmaceutical industry and regulators

Directly in connection with section 1.4 described above, one of TRANS-INT objectives was to improve communication between academic and industrial partners and to increase their knowledge in the regulatory framework and the technology transfer process. In turn, this knowledge would have been transferred to patients, via the industry, the regulatory agencies and the clinicians.

This result was successfully achieved by the project through the organization of appropriate sessions as part of the six-monthly review meetings, where experts from the participating companies and external consultants were discussing with the academic partners explaining the regulatory requirements.

In addition, as part of work package 7, specific training sessions were organized where these requirements were communicated to young researchers.

It is also important to highlight that Sanofi R&D and SIGMOID were highly involved in the Executive Committee and also in the Industrial Liaison and Exploitation Committee, both committees met together on a monthly basis. Therefore, this continuous communication allowed the consortium to receive direct advice from industry in all steps of the project. In addition, as highlighted above, the members of the advisory board, Prof. Robert Langer, Prof. Ruth Duncan, Prof. Rogerio Gaspar, Prof. Randall Mrsny and Prof. Felipe Casanueva, are highly involved in the translation of technologies and provided the consortium with high level advice in regulatory aspects and translational research.

2. Dissemination activities and exploitation of results

This section describes the activities for the dissemination and the exploitation of results that were implemented during the TRANS-INT project. These activities were completed as part of WP8, which had three main objectives:

a. To define and implement an integrated strategy for TRANS-INT dissemination and exploitation; this was achieved through the development of a dissemination and exploitation plan (see section 2.1)

b. To regularly inform all stakeholders about TRANS-INT and the project results;

c. To promote the (use of) TRANS-INT’s results and advertise the benefits of the project;
These objectives were achieved through the completion of the following tasks:

- Development and maintenance of the TRANS-INT website, discussed in section 2.2;
- Dissemination to all key audiences, discussed in section 2.3;
- Exploitation of results, discussed in section 2.4.

2.1. Dissemination and exploitation plan

The aim of this plan was to describe how TRANS-INT would make optimal use of the tools available for dissemination and exploitation of the project’s achievements. The plan was prepared by WP8 leader (Sigmoid Pharma) in collaboration with the project coordinator (USC), the Industrial Liaison and Exploitation Committee (ILEC) and the Executive Committee (ExC).

The plan was regularly updated during the execution of the project and made available to the participants of the TRANS-INT consortium.

The dissemination plan described how the project intended to share the outcomes with stakeholders, relevant institutions, organizations and individuals, in order to contribute to the overall dissemination strategy for the programme.

The first step consisted of determining the subject for dissemination and establishing the anticipated outputs and outcomes of the project. In this regard, the main objective of the TRANS-INT project was to develop innovative nanocarriers specifically adapted to deal with the gastrointestinal system. These nanocarriers should not only be capable to protect the macromolecules from degradation, but also to facilitate their transport through the intestinal epithelium in a safe and efficient way.

The efforts of the TRANS-INT project concentrated on developing nanodrugs for diseases with high socioeconomic impact, and especially for diabetes. The TRANS-INT project’s results were expected to impact therapies related to these diseases. Oral delivery would improve the quality of life, while also improving patient compliance. Achieving oral delivery of these peptides will contribute also to a significant reduction in long term health care costs, as patients will move to these important therapies earlier in their disease.

TRANS-INT’s activities also included detailed studies evaluating toxicity and immunological reactions of the nanocarriers, as the hazards that may be introduced by using nanoparticles for drug delivery may be beyond that posed by conventional delivery systems.

The second step consisted of identifying target audiences.

The most obvious audience for the TRANS-INT research outputs is the scientific community. The technological advances developed within the TRANS-INT project would be applicable for other nanocarriers, nanomedicines and disease areas than those studied in this project. These results can help
to provide guidelines and to build strategies for future research projects in the area of nanodrug delivery.

However, the holistic approach followed in the TRANS-INT project led to information relevant for a wider audience; Among all potential TRANS-INT stakeholders, the following key audiences were prioritized:

- Pharmaceutical and healthcare industry involved with or associated to drug delivery, drug development and manufacturing that may be interested in further applications of the technologies developed within the project;
- Clinicians: TRANS-INT efforts concentrated on communicating with clinicians, so that the new nanomedicines are understood and quickly adopted by the clinical community;
- Policy makers and governmental bodies, as the development of oral nanomedicines for the treatment of diseases with a high socio-economic impact would significantly reduce the public health care costs, as discussed in section 1.3. It was therefore decided to closely interact with policy makers and governmental bodies that may design and implement actions to favour the adoption of the new nanomedicines, and may decide to deploy funds for further research development;
- Regulatory bodies: to ensure that the developed nanodrugs fulfil the existing regulations regarding their safety, quality and efficacy. TRANS-INT results may be of help to different bodies like the European Medicines Agency (EMA), the International Standards Organisation (ISO) and the European Group on Ethics in Science and New Technologies (EPE) to evaluate the adequacy of current health and safety regimes to effectively regulate current and anticipated applications;
- Health care and Patient’s organisations, to inform them about the project’s benefits. These organisations can be seen as information multipliers since they usually organise large public communication events;
- General public: communicating with the general public is very challenging due to its heterogeneity. Therefore TRANS-INT’s efforts to reach a general audience focused in contributing to the public’s understanding of the true potential of nanomedicines towards fighting the targeted diseases and this was achieved through media interviews, press releases and public lectures;
- Networking with other R&D projects: exchange of knowledge with other R&D projects in related research areas both at EU and international level would contribute to foster synergies with them.

The communication strategy was tailored to provide appropriate messages to the target audiences; four purposes were identified:

- Raise awareness – let others know about the TRANS-INT project and the research activities within it;
- Inform – educate the targeted audiences;
- Engage – get input/feedback from the various audiences;
- Promote – ‘sell’ the TRANS-INT outputs and results.

Depending on the target audience, an appropriate dissemination action/tool and purpose was selected, as presented in Table 3 below.

Table 6. Dissemination actions/tools vs. audiences and purposes

The exploitation plan was implemented to describe how TRANS-INT would screen all results to ensure optimal exploitation.
One of the first actions of this plan was to create an Industrial Liaison and Exploitation Committee in order to ensure the continuous monitoring of all project activities so that IP and/or know-how would be identified and protected. It was also decided that this committee would be informed of all initiatives related to IP protection and potential commercialization of results.

Each WP Leader was responsible for presenting the Industrial Liaison and Exploitation Committee, findings for evaluation and this committee was responsible to advise the Executive Committee on actions to be taken. The Industrial Liaison and Exploitation Committee attended and presented to the Executive Committee the outcomes for recommendation. The communication between both committees was enabled through their participation in monthly jointly meetings. In this way, the Industrial Liaison Committee was able to advise on how to better exploit results and to seek approval for recommendations.

The exploitation plan comprised the following activities:

a. An opportunity to analyse and identify the potential industrial or clinical applications as well as the customer target groups;
b. A technical and commercial assessment of the most effective routes for exploitation, e.g. licensing to third parties, development in-house or with external partnerships;
c. A feasibility and value assessment of how far a result should be developed by the consortium so as to optimise its commercial value within the resources and the strategic aims of the project;
d. The performance of a preliminary market analysis to assess the potential commercial value which can be generated;
e. The development of a marketing strategy if the output is to be licensed to a third party.

2.2 Development and maintenance of the TRANS-INT website

The TRANS-INT website (http://www.trans-int.eu) was created soon after the start of the project as the primary dissemination route through which the project would be presented.

The website aimed to provide access to general information on the project, the project main findings and other relevant information as for instance the training offered by the consortium. Special attention was paid to ensure high visibility to the potential industrial applications derived from the TRANS-INT research activity. The website was also used as the main vehicle to make available all public deliverables.

The website was structured in the following sections:

- Home page: the home page has a self-explicative menu to allow for an easy navigation through the different web sections. It shows research aims and activities/main findings of the TRANS-INT project. This information is displayed through the use of attractive visuals accompanied with a brief description and a link to access more detailed information. The home page also incorporates direct access to news and events of relevance to the TRANS-INT activity;

- News & events section: accessible from the home page, it includes news, media coverage and updates about the project. It also displays information on events related to TRANS-INT activities;
• Partner section: including detailed information on the different research groups and institutions taking part in the project and a map showing the geographical distribution of the participating institutions to show the European dimension of the TRANS-INT activities;

• Main findings section: this section provides the general public with basic non-confidential information on the TRANS-INT research activities in such a way that they can be understood by non-specialists, thereby improving the public’s understanding of TRANS-INT related science. Moreover, a reservoir of the TRANS-INT scientific publications is also available through this section.

It includes a brief description of the publications (e.g. title, authors, journal, abstract) and a link to the site where a full version of the article can be found. This includes also abstract and conference presentations.

Last, the public deliverables are freely available;

• Job offers section: The TRANS-INT web page was also used to advertise the job positions offered by the consortium. The job adverts included a broad description of knowledge and competencies required for the job, as well as information on the job provider, duration of the position and the contact details for job application;

• Training section: this section displays the different training opportunities offered within the TRANS-INT project including a brief description of the courses and workshops, the location and the time schedule. It also contains links to webinars (Web-based seminars) and other training material

• Contact us section: this section displays publicly the contact details of the coordinator acting as a single contact point for the TRANS-INT consortium;

• Intranet: this section consists of a password controlled private section to allow for internal communication, discussion and exchange of documentation (Standard Operating Procedures, draft papers, meeting minutes, private deliverables, etc);

• Logos: the web page header displays a distinctive logo of the TRANS-INT project together with the European emblem and the FP7 logo.

The web page was updated continuously and from September 2013 was linked to Google Analytics so that it was possible to determine the success of the website as a dissemination tool.

2.3. Dissemination to all key audiences
Intense dissemination activities were performed by all partners in the course of the TRANS-INT project. In fact, the outcome, as per May 2017, has been: 23 scientific publications, 6 books or book chapters, 26 conference abstracts and 5 PhD theses defended. However, most of the scientific data remain to be disclosed due to the TRANS-INT exploitation plan and the fact that results have been generated until the end of the project. Based on this, it is expected that the consortium will generate a minimum of 20 more papers and a 5 more PhD thesis. Due to this fact, the web page will remain open and the Industrial Liaison and Exploitation Committee will remain operative for one year after the end of the project.
Of particular importance was the themed issue published by the journal Advanced Drug Delivery Reviews in November 2016 under the title: Oral delivery of peptides: opportunities and issues for translation (The EU FP7 TRANS-INT Consortium). The issue contained 12 review articles from the TRANS-INT partners and was edited by Prof. Alonso (USC) and Prof. Brayden (UCD).

Important dissemination activities were also performed in collaboration with other European Consortia and Scientific societies. For example, in 2015 we celebrated a conference jointly organized by 3 European Consortia, the TRANS-INT consortium, the ALEXANDER consortium from the FP7 Program and the COMPACT consortium from the IMI Program. This conference entitled “Crossing Biological Barriers” was held in Dresden in 2015 and it had many participants.

It is particularly worth mentioning the plenary and invited talks delivered by the PIs which focused on TRANS-INT. These talks were delivered at the most prestigious conferences, i.e. the CRS Annual Conference and the AAPS Conference amongst others.

The TRANS-INT consortium collaborated as well with the NANOFAR Erasmus Mundus PhD Program consortium in the elaboration common workshops. These workshops, highly attended by PhD students from all over Europe, were of great success and represented a very good opportunity for the dissemination of the knowledge and training skills generated by the TRANS-INT consortium.

Finally, an additional important dissemination tool was represented by the TRANS-INT e-newsletters and e-brochures that were published. These publications provided an overview of project progress by each work package.

2.4. Exploitation of results

One of the first actions to be implemented, with regards to the exploitation of results, was the establishment of the rules to be observed when disseminating TRANS-INT results.

In order to facilitate document review by the Industrial Liaison and Exploitation Committee, a ‘Publication Request Form’ was introduced. All documents for dissemination were to be submitted to the ILEC in the presence of a completed ‘Publication Request Form’. Furthermore, to promote dissemination the review period for review articles (containing background IP only) was agreed to be 2 weeks. The review publication also needed to be accompanied with a ‘Publication Request Form’. These actions have been working successfully and have provided clarity to the review process and have facilitated dissemination activities greatly.

One of the most important achievements was the submission of patent applications from the consortium partners. In addition, one of the patent applications was jointly presented by academia (USC) and industry (Sanofi R&D). This shows the potential industrial relevance of the TRANS-INT results as well as the high level reached by the research work.

Three patent applications were submitted during the TRANS-INT project; the patents are summarized in Table 4 below and are described in detail in deliverable 8.9.
Finally, it should be highlighted that the Industrial Liaison and Exploitation Committee of the TRANS-INT Consortium will remain active and analysing the potential IP issues related to the publications that are and will be produced in the coming months.

It can be assumed that the owners of the patents indicated above will continue the work done in TRANS-INT and will search for paths to increase their technologies in value and, probably, the development of new oral peptide-based nanomedicine candidates.

List of Websites:

**PROJECT WEBSITE:** [www.trans-int.eu](http://www.trans-int.eu)

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http://www.farmfak.uu.se/farm/lmformul-web/index_en.shtml

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Related documents

final1-trans-int-final-publishable-report-figures-and-tables.pdf

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