Development and evaluation of a novel tool for physiologically accurate data generation

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Scientific coordinator: Dr Martha LILEY
CSEM - CENTRE SUISSE D'ELECTRONIQUE ET DE MICROTECHNIQUE SA - RECHERCHE ET DEVELOPPEMENT
Tel: +41 32 720 5184
Fax: +41 32 720 5740
Email: martha.liley@csem.ch
www.inlivetox.eu

1. Executive summary

Increasing concerns about the safety of new active chemical ingredients used in pharmaceuticals, cosmetics and the food industry have been further highlighted by the emergence of materials with dramatic new properties based on nanoparticles (NPs). NPs are particles with at least one dimension on the nano-scale of 100nm or less. Ethical concerns about the use of animals to test the safety or efficacy of new compounds are growing. *In vitro* testing offers a potential solution to the challenge of how to ensure that as NPs are developed and used, any unintended consequences of exposure to humans are minimised. The InLiveTox project, funded by EU over the past three years, has significantly advanced the capability of *in vitro* testing in particular of NPs.

The InLiveTox project focused on the impact of NP exposure via ingestion, on the vascular endothelium, liver and gastrointestinal tract (GI). Exposure via ingestion is particularly relevant due to the inclusion of NPs in food, food packaging and in oral medicines. The key questions to be addressed were: (i) How do these tissues individually respond to NPs? (ii) How do the interactions between the different tissues modulate their responses? (iii) How does inflammation affect the toxicity of NPs and their ability cross the intestinal barrier?

The partners in InLiveTox were an interdisciplinary consortium including European leaders in nanotoxicology, pharmacy and engineering. The consortium developed a novel modular fluidics-based *in vitro* test system and demonstrated its use to model the response of selected tissues to the ingestion of NPs. The results from the *in vitro* system were validated by an *in vivo* study of NP biokinetics and toxicity by ingestion in rats carried out in parallel. Comparison of the data obtained in vivo on exposure by injection and ingestion with data obtained from standard (static, single cell type) assays and the InLiveTox system showed a remarkable pattern of differences and similarities particularly when studying inflammation. There are clear differences in the physiological relevance of the different approaches.

The InLiveTox system developed in this project has wider application than just the testing of response to NPs. It can be used as a testing and research tool in toxicology and pharmacology for any new chemical entity. Throughout the project there has been extensive dialogue with members of an Industrial Advisory Group whose from the pharmaceutical, cosmetics, food and household products manufacturing industries.

Technical outputs from the project include tested prototypes of a cell culture system that will be commercialized by partners from the consortium. Cell culture protocols and assay protocols for toxicity studies and screening of ingested NP toxicity will support the adoption of this new system by industry and the academic research community.

2. Context and objectives

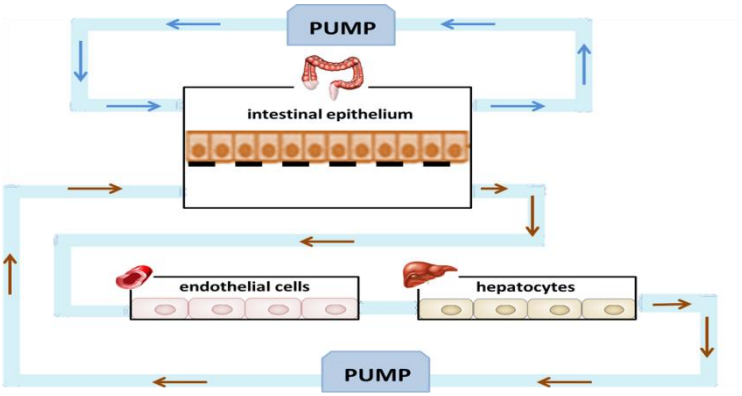
Context

Engineered nanoparticles (also called manufactured nanomaterials) can be considered a new class of substance, since chemical and physical properties at the nano-scale can differ hugely from those of bulk materials. Typical examples include silver particles used as an antibacterial and antifungal agent in water bottles, zinc oxide used to block UVA and UVB sun rays in sunscreen, and the various materials used as pigments in paints and cosmetics. The novel properties of NPs – that make them so attractive for many applications - means

that they can also interact with the body in different and unexpected ways, and their small size allows them to potentially cross barriers in the body that hold back larger materials. For this reason a rapid and thorough evaluation of NP toxicity is essential.

Given the large numbers of NPs that exist in the lab and sometimes in commercial products, researchers and industry are faced with the almost impossible task of analysing the safety of thousands of new materials in a very short time frame. It has become clear that, from both ethical and financial viewpoints, new animal-free testing methods are necessary. Unfortunately, the animal-free cell culture or "in vitro" models currently in use are often not representative of the human body. They simplify enough to allow the production of data, but often so much that the data is not representative and cannot replace most testing on animals. Most in vitro tests work with one type of cell in culture, but this is, of course, not the way cells work in our body, where the surrounding environment, including other cell types, has a strong influence on how cells behave. The interactions between different organs, or the transport of a material through barriers, for example from the intestine into the bloodstream, cannot yet be modelled in a realistic, or "physiological", way.

The EU-sponsored project InLiveTox (www.inlivetox.eu) has brought together engineers and biologists from universities and research institutes across Europe in order to develop an improved cell culture system. This model will help us gain insight into how NPs taken up in food or otherwise ingested can cross the intestinal wall and how they can affect the different tissue in the body.



Objectives

The global objectives of InLiveTox were:

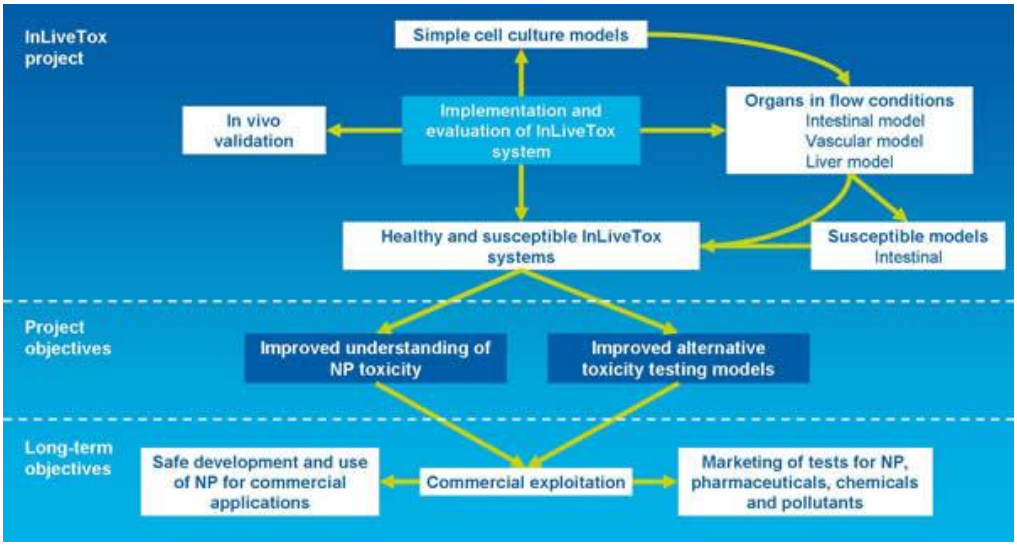
- To improve understanding of NP toxicity by ingestion
- To develop improved *in vitro* toxicity testing methods

In order to achieve these objectives the project aimed to develop a modular microfluidics-based *in vitro* test system, the ILT system (WP1); a system in which different cell types – each modelling a target tissue - could be maintained interact via cytokines. The target tissues chosen for this project were the GI tract, the liver and the vascular endothelium so that NP toxicity by ingestion could be modelled with the system.

Subsidiary objectives in the project were the establishment of common culture conditions for the cells and test protocols for the analyses to be carried out with the ILT system (WP2). Once established, these were then to be implemented in the ILT system (WP3).

Validation data were to be obtained via an *in vivo* study of NP toxicity by ingestion in rats carried out in parallel. Data on both biokinetics and toxic response from the target tissues were the goal of WP4.

The final validation, bringing together in vivo data with new data generated by the ILT system, was the objective of WP5.



The long-term objectives of the project are:

- to ensure the safe development and use of NMs for commercial applications
- to commercialise a test system to screen NMs for their toxicity.

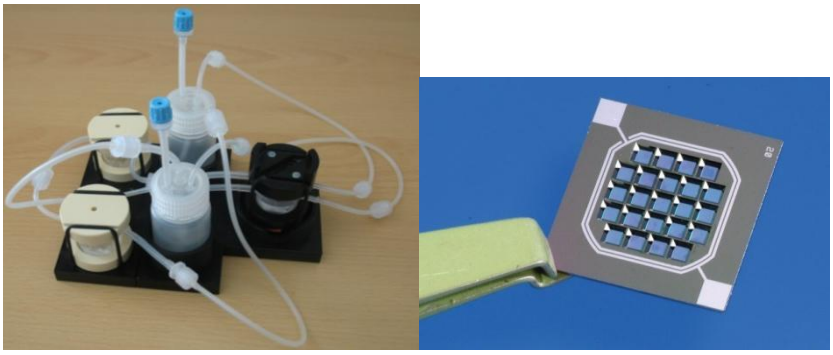
These two objectives are related to the foreseen impacts of the project, with future commercialisation of the InLiveTox system making it available to the whole toxicology community.

3. Description of the main S&T results/foreground

WP1: Development of microfluidic systems

WP1 was the engineering and design workpackage of the project, and also involved mathematical modelling. The underlying concept of the project is that of connected cultures in which 2 or more tissues are connected together by a fluid flowing between them, much as in the human body in which distant tissues and organs are connected by the bloodstream. Our aim was to engineer a connected culture fluidic system in which nanoparticles could pass through an intestinal barrier and then influence downstream tissues to simulate the route of ingested nanoparticles in the body. It was decided early on in the project to build a modular fluidic system so that different tissues could be added or removed from the fluidic

circuit as required. We also choose to use cell culture systems which were easily translatable to biological experimental standards.
The first part of the WP was dedicated to the scaling of the InLiveTox system using a method known as allometry. This method is based on the well-known fact that the different features of animals are correlated to their body mass. Using allometry we calculated the liver, vascular tissue and intestinal cell numbers necessary to properly represent the human body in the reduced scale of our system. At the same time we also considered other parameters such as flow, oxygen and fluid forces. Having identified the cell numbers and other parameters we proceeded with the design of the system. During this period the engineering partners introduced the others in the consortium to fluidic culture methods using a bioreactor chamber known as ILT0. ILT0 is a interconnectable commercial cell culture chamber designed and commercialized respectively by 2 partners in the team.
The main challenge in the workpackage was the design of the intestinal chamber which requires a semi-permeable membrane to separate the intestinal side from the blood side and 2 different fluid flows on either side. As we had anticipated this, two versions were developed, ILT1 and the much improved version ILT2.
Challenges identified were i) the need to continuously monitor the function of the intestinal cells using a technique known as TEER (transepithelial electrical resistance). TEER is a standard and sensitive method of analysing the integrity of epithelial barriers like the intestinal wall.
ii) the need to use ultrathin membranes to allow nanoparticles to get from the intestinal side to the blood side. Most membranes available commercially get clogged up with nanoparticles
To meet these challenges an ultrathin silicon nitride membrane was fabricated with built in electrodes for TEER measurements (see figure). The first bioreactor chamber ILT1 was designed to hold the membrane and had electrical contacts for TEER. After the first few prototypes were fabricated and tested, a training course was held to introduce partners to the new system. The cell culture partners then started using the ILT1 to perform baseline measurements on intestinal epithelia. A few months later enough feedback had been collected to begin the redeisgn of ILT2. Basically biological partners had difficulty in seeding cells on the microfabricated membranes and then placing the membranes in the ILT1. Other problems concerned leakage, air bubbles and difficulty with stable TEER measurements. ILT2 was designed using innovative solutions (some were patented) to overcome these problems. It was also more robust and the TEER measurements more stable due to new watertight contacts and more robust electronics. The final part of the workpackage was dedicated to training of other partners, modelling of nanoparticle passage through the membrane, modelling of nanoparticle sedimentation in the fluidic system and troubleshooting when technical hitches arose during cell culture.



WP2: In vitro biological models

Laying the ground work for all further in vitro studies in the InLiveTox project, WP2 established protocols to ensure reproducible and stable size distributions in the NP suspensions as well as defining assays and endpoints to quantify cell viability and toxic responses in the InLiveTox system. Baseline data under static conditions was generated for comparison in later stages of the project. Furthermore the in vitro models used in the project were optimized with regards to culture conditions, selection of functional markers and adaptation to higher throughput applications (modified 3D model of the inflamed intestinal mucosa).

As the InLiveTox system is based on studying the cross talk between three different tissue models connected to each other via a fluidics systems, common culture conditions had to be identified for the three cell lines Caco-2 (enterocytes), C3A (hepatocytes), and HUVEC (endothelial cells). A common medium was developed based on EMEM. This medium, referred to as EMEM-GF, affected neither cell adhesion nor morphology either in static or dynamic conditions. Under the chosen conditions, all cells maintained their viability as well as their tissue-specific phenotype and were able to withstand the shear stress of dynamic conditions. Albumin expression was used as the phenotypic endpoint for C3A hepatocytes while expression of von Willebrand factor was the marker for endothelial cells and barrier function quantified via transepithelial electrical resistance characterised functionality of Caco-2 cells.

In order to interpret the toxicity of nanoparticles and understand the underlying mechanism of potential toxic effects, the physicochemical characteristics of the nanoparticles need to be known. Previous studies have correlated toxicity of various nanomaterials to parameters such as surface are, surface reactivity or phase composition. Furthermore, it was shown that the dispersion medium used can greatly influence the physiochemical properties of nanoparticles. Therefore, we characterized nanoparticles to be used in the InLiveTox system with regards to their size, polydispersity, aggregation behaviour and surface charge in various media to be used for in vivo and in vitro investigations. Most nanomaterials were found to form stable dispersions in EMEM-GF with particle sizes close to the nominal size. Only NM101 TiO_2 NP showed significant aggregation behaviour to 700 nm sized clusters (primary particle size 7-11 nm). Serum proteins sterically enhanced stability of the nanoparticle dispersions as aggregation behaviour was more pronounced in serum free conditions. Dispersions were stable for at least 48 h, allowing their application in the InLiveTox system.

Parameter	Assay
Functionality	Albumin (C3A), vWF (HUVEC), TEER (Caco-2)
Membrane integrity	LDH, Propidium iodide
Metabolic activity	Alamar blue
Oxidative stress	Glutathione depletion (GSH)
Cell apoptosis	Caspase activity, Fas-Ligand
Inflammation	Cytokine expression at mRNA level (realtime PCR) Cytokine expression at protein level (Bioplex)

Table 1: Panel of assays applied in the InLiveTox project

Different assays evaluating various aspects of NP toxicity such as membrane damage, cell death, apoptosis, inflammation and oxidative stress were established in the partner labs and adapted for use in the interconnected fluidic ILT setup. Furthermore baseline values for toxicity for the different ILT NP and individual cell lines under static conditions were determined.

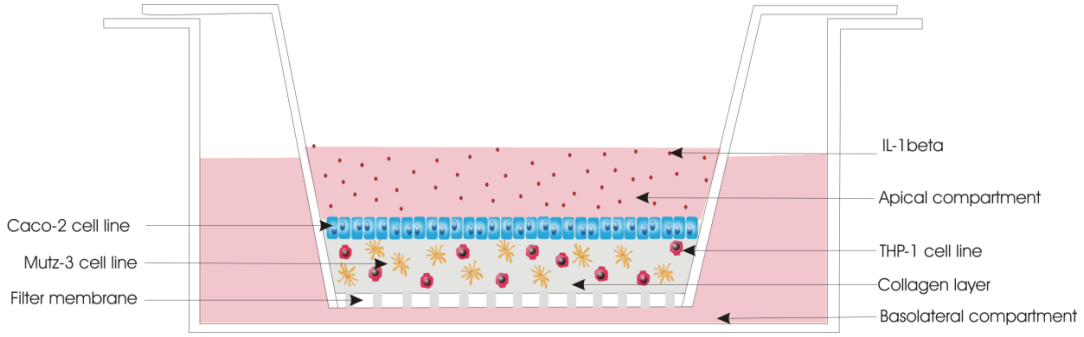
		HUVEC	C3A	Caco-2
Alamar blue	55 nm fluoresbrite			
	211 nm fluoresbrite			
	Au 15 nm			
	Au 80 nm			
	NM 101 TiO2			
	NM300 Ag			
LDH	55 nm fluoresbrite			
	211 nm fluoresbrite			
	Au 15 nm			
	Au 80 nm			
	NM 101 TiO2			
	NM300 Ag			
GSH	55 nm fluoresbrite			
	211 nm fluoresbrite	NA		
	Au 15 nm	NA		
	Au 80 nm	NA		
	NM 101 TiO2	NA		
	NM300 Ag	NA		
FAS ligand	55 nm fluoresbrite		ND	ND
	211 nm fluoresbrite		ND	ND
	Au 15 nm		ND	ND
	Au 80 nm	ND	ND	ND
	NM 101 TiO2		ND	ND
	NM300 Ag		ND	
Cytokine expresssion	55 nm fluoresbrite	ND	ND	
	211 nm fluoresbrite	ND	ND	
	Au 15 nm	ND	ND	
	Au 80 nm	ND	ND	
	NM 101 TiO2	ND		
	NM300 Ag	ND		
Cytokine release	55 nm fluoresbrite		ND	
	211 nm fluoresbrite		ND	
	Au 15 nm		ND	
	Au 80 nm		ND	
	NM 101 TiO2		ND	
	NM300 Ag		ND	

Table 2: Summary of determined toxicity levels of ILT nanoparticles in different indicator assays and cell lines; ND = not determined; NA = not applicable; green = not toxic over the determined concentration range; yellow = slight toxicity at high concentrations; red = high toxicity

For all three cell lines, NM300 Ag NP were found to be highly toxic at very low concentrations. NM101 TiO2 NP demonstrated strong pro-inflammatory activity in HUVEC cells and to a limited degree also in C3A cells. There is some indication that very high

concentrations of 55 nm and 211 nm fluoresbrite polystyrene NP induce toxicity in C3A and HUVEC cells as shown in the Alamar blue and LDH assay. For Caco-2 cells there seems to be slight oxidative stress reaction induced by Au 15 nm NP.
In general, C3A cells and HUVEC were more sensitive for potential toxic effects of NP than Caco-2 cells, as they showed lower LC50 values and gave slightly positive readouts for the polystyrene NP at high concentrations and for inflammation induced by TiO2.
The LDH assay and measurement of apoptosis via FAS ligand and of inflammation via IL-8 release seems most suitable for routine application in the ILT setup as all three biomarkers can be measured from the cell supernatant without need of removing the cells thus allowing online monitoring. Furthermore, the measured LC50 values were about 5-10 fold lower in LDH compared to Alamar blue assay, indicating higher sensitivity. IL-8 release mirrored the cytokine expression at mRNA level.

For the healthy intestine there are several more or less complex multi-cellular in vitro models of the intestinal mucosa described in literature and patents, in most cases based on permanent intestinal epithelial cell lines such as Caco-2 or HT-29 However, only one of these models (combining Caco-2 cells with M-cell like Raji cells) has been used to study NP interactions focusing on uptake over healthy intestinal mucosa without assessment of local inflammatory or cytotoxic reactions. As InLiveTox also wanted to address engineered nanomaterial interaction with the susceptible barrier, we introduced toxicity testing an inflamed intestinal model. Previously, a 3D co-culture model based on Caco-2 enterocytes and primary immune cells had been established. Allowing easier lab to lab transfer and adapting the model to higher throughput screening, primary blood derived macrophages and dendritic cells were replaced by permanent cell lines in the InLiveTox project.



THP-1 and U937 cells were evaluated to replace the primary blood derived macrophages while MUTZ-3 cells were targeted to replace the dendritic cells. Studies on activation of THP-1 and U937 cells identified THP-1 cells as the only suitable candidate. As a first step a Caco-2/THP-1 double co-culture was established. In a second step, MUTZ-3 cells were integrated to form a triple culture of enterocytes and dendritic and macrophage like cells. The modified setup performed comparable to the original triple culture both in the non-inflamed state and upon stimulation with pro-inflammatory cytokine IL-1β. Barrier properties were reduced and an increased release of pro-inflammatory marker IL-8 was observed

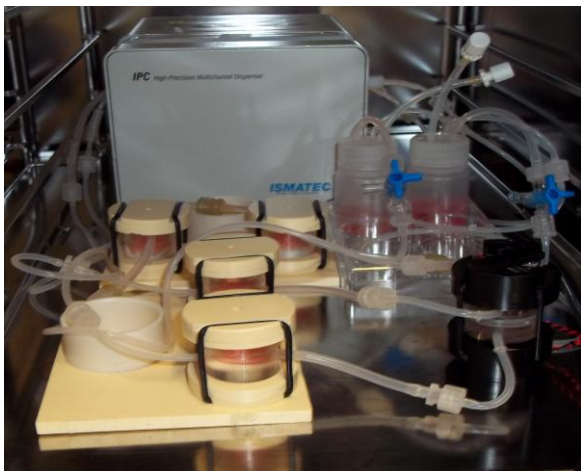
WP3: Implementation of biological models in the ILT system

This work package focussed on taking the cell culture models from WP2 and establishing them in the fluidics system(s) of WP1 to provide the starting point for the validation work of WP5.

The first task in the work package was to establish the ILT1 system at the different partners' institutes. After the training course for ILT1 was carried out, USAAR and UNIPI were provided with complete ILT1 systems and started working with them in their own laboratories. After some weeks of work by the consortium using ILT1, feedback from the different users was analysed and was used to define the requirements for the second version bioreactor, ILT2 that was developed in WP1.

It was originally planned to establish the 3-tissue culture model – with all three model tissue maintained in the same fluidics system – using ILT1 and ILT0 at this point. However, this was not feasible. Instead, the individual tissue models were set up in ILT0 (for the C3A and HUVECs) or ILT1 bioreactors and the viability and function of the models under the common culture conditions was tested.

The 3-tissue culture model, was, however, established using the ILT2 bioreactor. The complete 3-tissue model is shown set-up in an incubator below. As described in previous WPs, the models established were: for the intestinal epithelium CaCo-2; for the vascular endothelium, HUVEC, for the liver, C3A.



One tissue model that was not established in the ILT system was the triple culture model of the inflamed intestine. This model (described in WP2) was based on a collagen layer deposed on the surface of the microporous cell culture support. Unfortunately, the collagen layer effectively blocked the transport of NPs from apical to basolateral compartments, making the model unsuitable for study of NP transport. In addition, the collagen layer adhered poorly to the silicon nitride microporous cell culture support used in the ILT system. This did not pose a problem under static culture conditions. However, under flow conditions, the model intestinal barrier peeled off the support so that the separation of the two (apical and basolateral) flow circuits was not maintained. For these reasons, it was decided to abandon the establishment of this model and to focus efforts on the remaining tasks in the project.

Finally, at the end of WP3 a system baseline was established in the absence of NPs. The baseline provided a negative and a positive (0.1%Triton-X 100) control for the subsequent experiments in WP5. The baseline included assays for viability, inflammation, apoptosis and function.

WP4: in Vivo assays

The animal studies used rodent models to determine the quantitative biokinetics of intravenously injected and orally administered radio-labelled NM (TiO2, Au and Ag) with a special emphasis on cardiovascular and liver uptake since these two targets relate to the targets included in the InLiveTox model. Tissues from these animals were used to investigate toxicological responses, again focusing on the gut, cardiovascular system and liver. This data was then compared with other biokinetic studies using similar particles, but other routes of exposure (e.g. respiratory route).

Oral gavage biokinetic studies

- After oral administration to rats, only a rather small fraction of all of the NM administered were absorbed through the gastro-intestinal-tract (GIT) wall into blood circulation for subsequent accumulation in organs and tissues. The major fraction of the ingested dose for all NM administered were eliminated from GIT the into faeces.
- The 24-hours biokinetics study of the orally delivered monodisperse, different sized (1.4, 2.8, 5, 18, 80, 200nm) gold nanoparticles (AuNP) showed a clear, inverse size-dependent absorption through the gut wall into blood circulation (0.4% of administered 1.4nm AuNP to 0.02% of 80nm AuNP). This led to low but detectable accumulations in organs of the following declining order: kidneys, liver, spleen, lungs, heart, brain. Interestingly AuNP accumulated also in the skeleton. This pattern differed strongly from the pattern after intravenous injection of the same AuNP indicating different surface modifications according to the route of entry which may relate to the formation of the protein corona.
- Similar to AuNP most of the orally administered, agglomerated titanium dioxide NP (TiO_2 NP) of 70nm size were also excreted in faeces. Compared to 80nm AuNP the GIT absorption of TiO_2 NP was five times higher indicating NP material differences. Accordingly TiO_2 NP in organs and skeleton were higher but ranked similar as for 80nm AuNP. Absorption through the gut into blood circulation increased during the first 24 hours but decreased during the next six days indicating NP clearance from the body.
- Silver nanoparticles (AgNP) were not used for biokinetics studies due to enhanced physic-chemicalreactions during the process of radiolabelling.

Intravenous injection biokinetic studies

- All NM administered intravenously were almost completely retained in the body and accumulated in organs and tissues,.
- Intravenous injection of NP suspensions predominantly resulted in accumulation in the liver, justifying the choice of hepatocytes in the InLiveTox model.
- These studies allowed for kinetic measurements of the hepato-biliary clearance of NP into the small intestine and further into faeces. The monodisperse, different sized (1.4, 2.8, 5, 18, 80, 200nm) AuNP showed a clear inverse size dependency of this hepatic clearance pathway. Clearance of the 70nm TiO_2 NP was similar to that of 80nm AuNP but increased linearly with time up to 2% of the initial NP dose over one month indicating a persistent but moderate NP clearance pathway out of the liver.
- Due to the physic-chemical reaction during the process of rdiolabeling leading to the destruction of the silver NP, instead intravenous injected silver ions were used due to the

potential for AgNP to dissolve. Silver ions were found to accumulate less than 5% in organs and the skeleton but were not excreted in urine as expected but predominantly in faeces indicating effective translocation from blood into the gut lumen. This was not seen for the metal ions of the other NP.

Oral gavage toxicology studies

- A depletion of reduced glutathione (GSH) levels in the liver were used as a marker of oxidative stress. No significant effects were observed for intestinal or hepatic oxidative stress for any NP administered by gavage.
- Similarly, no significant changes in GSH levels were observed in Peyer's Patches after gavage of Au NPs of 15 and 80 nm.
- However, there was a significant increase in GSH in Peyer's Patches after gavage of 250 µg TiO_2 and Ag per animal indicating oxidative stress in this target tissue for these two particle types.

Intravenous injection toxicology studies

- Of the injected particles (Ag, 15 nm Au and TiO_2), only the TiO_2 particles caused a decrease in reduced GSH content of livers. No significant changes in GSH levels were observed in Peyer's Patches and intestines after injections of any of the particles used.

Inflammation

- Exposure of rats to NPs by both injection and ingestion resulted in changes in the expression of genes involved in inflammation, oxidative stress and apoptosis in the target organs examined (liver, Peyer's Patches, intestine and aorta). Some of the most interesting results were:
    - Changes in gene expression were not limited to the primary target organs (i.e. livers/aorta for injection and intestine/Peyer's Patches for ingestion), but systemic changes were also observed
    - Changes in gene expression were dose-dependent, and a particularly interesting difference was observed in Peyer's Patches, where small doses of 15 nm Au particles (50 µg per animal) caused an increase in the expression of a number of genes, but higher doses (250 µg per animal) caused a strong decrease in gene expression.
- Overall, 80 nm Au particles caused the fewest changes in gene expression, and when compared to 15 nm Au particles, there was a pronounced size effect for the Au NPs. When examining H&E-stained sections of livers, intestine, Peyer's Patches and aorta, no obvious effects such as strong influx of inflammatory cells or tissue damage could be observed.
- No NPs were detected in the Peyer's Patches of rats exposed to the range of NPs used in the *in vivo* exposures (gavages) using TEM.

Ag, TiO_2 and 15 nm Au particles were detected by TEM in livers of animals exposed via the tail vein. Ag and TiO_2 NPs were detected in both hepatocytes and Kupffer cells, and some had reached the nuclei of hepatocytes. The particles appeared in small clusters and free within the cytoplasm/nuclei. In contrast, 15 nm Au particles were only detected in the cytoplasm of Kupffer cells, and were without exception enclosed within membrane-bound vesicles.

WP5: Validation of the system and analysis of NP toxicity by ingestion

The first deliverable in WP 5 was to produce and distribute a protocol handbook for assessing NP toxicity using the InLiveTox system. This handbook which was produced has been shared among the ILT partners and includes:

- an introduction to the system
- instructions for setup, use, cleaning and membrane regeneration
- cell culture under standard conditions, common medium conditions and transfer of cell culture membranes or cover slips into the ILT2 system
- particle suspension preparation and exposure of cells
- protocols for a number of toxicological endpoints such as cytotoxicity and viability, medium analysis for soluble mediators
- protocols for cell-specific functional tests such as stains for tight junctions and TEER measurements for Caco-2 cells, von Willebrand factor staining for Huvecs, and albumin measurement in the cell culture medium for C3A cells

D5.2 was concerned with characterising fate and behaviour of NPs within the completed InLiveTox model and the impact of the particles on the cells in the system.
This deliverable was delayed because of the additional time needed to finalise the ILT2 system and deal with some problems as highlighted in WP3. Successful experiments were carried out using Ag NPs, which were selected because they had the highest toxicity to all three cell types used (see WP2).
A range of concentrations of NM300 NPs was applied to the ILT2 system, including a dose which was non-toxic in static experiments, a dose with low toxicity and one with high toxicity above the LC_{50}. Smples were taken over the course of 24 h. Effects of the particles on cell-specific markers such as TEER and albumin release could be measured, as well as cytotoxicity and inflammatory mediators in the medium.
It was also apparent that the barrier function of the Caco-2 cells prevented damage by Ag particles to the downstream Huvec cells, but that when the Caco-2 cells were damaged, downstream cells were affected by Caco-2 signalling and/or Ag NPs passing through the compromised barrier to the downstream cells.

D5.3
Data from the single cell studies, inflamed cultures and 3-tissue experiments under flow conditions were analysed by 3-way Principle Components Analysis assessing a combination of variables (subjects), objects (response variables) and conditions. Although the technique has been used for complex chemometric data, and has also been recently employed for classifying neurons, this study is the first application of 3-way PCA to toxicity data. 3-way techniques are designed for descriptive analysis of complex data sets for example when morphological data on cells are collected at different time points .Such techniques summarize all information in a three-way data set by means of a few components, and describing the relations between these components. In order to perform the 3-way PCA, the data need to be to re-organized. The toxicity data were assembled into an nxmxp matrix with cells (C3A, HUVEC, CACO-2, CACO-2 inflammed model) as m, nanoparticles (Ag, Au 18 nm and 80 nm, PS-FITC, TIO2) as n and the concentrations (from 0 to the maxmium exposure) as p.
To obtain principal components the covariance matrix of data is decomposed in a eigenvectors matrix and a eigenvalues matrix. Eigenvectors are the columns of the rotational matrix that is transposed and multiplied for the data matrix. The multiplication result is the principal components matrix. (Mathematically it corresponds to performing a series of orthogonal rotations on a cubic core array, G, expressing the correlations between the data from the “datamatrix” denoted by objects, variables and conditions planes. The orthogonal rotations are iterated until a body-diagonal common orientation is reached). The minimum

number of components is selected on the basis of an optimized data fitting and stability and interpretability criteria such that data variance is maximized . In fact, only a small number of principal components are chosen and they constitute the axes of the plots in which data are reported in D5.3.

In summary, regarding viability assays (Alamar Blue and LDH), it was concluded that C3A cells are the most sensitive to NP exposure, followed closely by Huvec and then Caco-2 and the inflamed model.
Inflammation as analysed by IL-8 release was higher for TiO_2 than for Ag NPs, related to cell death caused at relatively low concentrations of Ag. The inflamed model gave the highest response, and C3A were the most sensitive cells, followed by Huvec and Caco-2.
Viability of cells in the static and dynamic 3 tissue model was also examined. Under static conditions, there was a clear difference in susceptibility of the different cell types, with Huvec slightly more susceptible than C3A, and Caco-2 the least susceptible. However, under flow conditions with crosstalk between cells, there was overlap between all three cell types, pointing out that the 3 tissue model under flow conditions is more than just the sum of the three single cell types, and represents interactions between the cells which cannot be modelled in a single cell system or without medium flow.
Overall, using silver data, cells in the system are more susceptible to NP exposure when under flow than as single cells in static conditions. This includes data from Huvec and C3A cells, which are not directly exposed under flow conditions, and indicates once more the importance of a co-culture system which enables communication between the different tissue models.

D5.4 was a report about the toxicity of nanoparticle toxicity in the inflamed intestine model. The results were compared to the non-inflamed triple culture model and the Caco-2 monoculture.
Testing in the non-inflamed and inflamed co-culture model of the intestinal mucosa changed the toxicity profile of the engineered nanoparticles investigated in the InLiveTox project. TiO_2 nanoparticles showed no toxic effect in any of the three cell models evaluated nor did they induce any inflammation. In contrast, two different-sized Au NPs had no significant effect in the different toxicity endpoints evaluated but induced strong inflammation in both triple culture setups. This inflammation couldn´t be observed in the Caco-2 monoculture.
Differences between the three cell culture test systems were most pronounced for Ag NP. Although the particles showed clear toxicity in both mono- and co-cultures the LC 50 values were markedly lower in the triple culture setups compared to Caco-2 monoculture. Furthermore significant inflammation was observed in the inflamed and non-inflamed co-culture model while being weakly pronounced in the monoculture.
In general the co-culture model due to the presence of professional antigen presenting cells more easily picked up pro-inflammatory potential of NP. Furthermore, a preferential uptake of NP into the immune cells can be assumed, as epithelial damage was significantly reduced in the co-culture setups. The higher LC50 values and NOEL observed in the triple cultures cannot be related to a change in cell number. In the mature triple culture a ratio of ~$1*10^6$ enterocytes to $1*10^4$ dendritic cells and macrophages, respectively is reached, thus the contribution of the immune cells to metabolism and LDH leakage is neglectable.
The innate immune cells have a protective effect reducing epithelial exposure and inducing an adequate immune response in answer to a perceived threat. In the inflamed model, this protective function is impaired. The already weakened epithelial barrier breaks down earlier upon NP exposure as seen in TEER measurements. Furthermore, epithelial cell death is more pronounced. In low doses toxic NP had an immunosuppresive effect on the innate immune cells and at higher doses the immune answer induced fell short to the reaction in the healthy, non-inflamed epithelium.

In summary, testing in the triple culture setups provided valuable data for interpretation and correlation of in vivo results and mechanistic understanding of NP interaction with the gut mucosa.

In conclusion, the developmental and experimental work in WP5 has resulted in a working system for NP exposures with a set of instructions which ensures reproducibility of experimental procedures among ILT users. The baseline created in WP3 has been expanded upon with further experiments including a range of doses of high-toxicity Ag NPs, which show effects on all three types of cells. These effects vary between cell types and are dose-dependent, and differ from those observed in single cell or static cultures. Finally, another refined cell culture model, the 3D inflamed and non-inflamed co-culture model of the intestinal mucosa, also showed variations between the single cell models and this more physiological setup, and clear differences between the normal and inflamed model were apparent, which is an important step to *in vitro* modelling of not only the healthy, but also the diseased gastrointestinal tract.

4. Potential impact

Socioeconomic impact and wider societal implications

Socio-economic impact can occur at the micro level (companies or partners using the project results) at the meso level (specific industry sectors or geographic regions benefitting) or at the macro level, where benefits are experienced across countries or the whole EU. The InLiveTox project will have impact at all these levels.

At a micro level, the project has delivered an exciting and innovative technology that has the potential to underpin new product developments in the field of *in vitro* testing. This has created (and will continue to create) commercial opportunities for some project partners and for those organisations that licence the technology from the partners – as demonstrated by the very high level of interest observed from potential end users in both industry and research organisations. The very high quality of the project work and the results obtained will further enhance the reputations of the research organisations that participated and strengthen their capability to take a leading role in future internationally leading research in the field of *in vitro* testing and toxicity.

At a meso level, the results of the InLiveTox project have the potential to change the way that the pharmaceutical, chemical, cosmetic and food sectors of industry are testing the safety and efficiency of new materials. The improved methods could deliver significant economic benefits both through reduction of testing costs compared to the use of animals, but also through the opportunity to bring safer products to market faster than existing methods. The technology developed in the project could provide a significant competitive advantage to the early adopters.

At a macro level, the project confirms the internationally competitive position that Europe's research organisations hold in the fast developing field of *in vitro* testing. A report by the US National Research Council in 2007 entitled "Toxicity Testing in the 21st Century" outlined the scale of the challenges and suggested a 15 year time scale for the replacement of many animal testing methods by in-vitro techniques.

The WYSS Institute in Boston, USA, has recently been granted $26 million for a project to develop a 'lab-on-a-chip' solution to toxicity testing. The capability demonstrated by the InLiveTox project shows that EU researchers could be leaders in this field, if they are able to secure similar levels of funding.

The motivation for such an investment is the size of the existing (£1.8 billion) market for drug toxicity testing and also the emerging (£2 billion) market for the testing of chemicals to comply with REACH legislation.

Any change in methodology for the testing of new drugs will require regulatory approval. The regulatory bodies (ECVAM, FDA etc) are justifiably cautious in approving new methods. However the cornerstone to any change is sound science and the work of the InLiveTox team has demonstrated the capability of European research organisations to make significant progress.

To summarise the potential impact of the InLiveTox project:

- Direct commercial benefit to the partners or their licensees, opening up new commercial product opportunities that could reach revenues of (€7 million) within 5 years.
- Potential annual savings for the pharmaceutical and chemical sector industries using the technology measured in €10's of millions in the short-term (5 years) and €100's of millions in the longer term (10 years).
- Potential safer drugs and reduced risk in chemical trials through the reduction in false positives or false negatives in toxicity testing.
- Significant reductions in the number of animals used for testing of new drugs and chemicals.

Main dissemination activities

A project identity set consisting of project logo, leaflet and all associated templates was created at the start of the project and distributed at many conferences and exhibitions throughout the duration of the project.
InLiveTox website (http://www.inlivetox.eu/ was created by ALMA and updated with data from all the partners, and will be maintained by Alma Consulting for a period of 5 years following the completion of the project.

Three Press Releases were made between June 2010 and the end of the project and articles or interviews appeared in Edinburgh Napier News, Radio Suisse Romande, l'Express/l'Impartial, Le Temps, La Gruyère, Migros magazine, The Scotsman, BBC news and Lepoint.fr .

Two workshops and Seminars were delivered in Ispra and Saarbrücken in 2010 and 2011.

Specific Training Courses are planned by Pisa University and Kirkstall to disseminate the methods developed during the project. The first of these was held on 17&18 September 2012 in Rome.
It is expected that these courses can be self financing and so can continue at regular intervals during 2012 and 2013.

Papers were presented by the project partners at 13 conferences in Rome, London, Lausanne (2), Saarbrücken (2), Montpellier, Edinburgh, Grenoble, Krakov, York, Essen and Utrecht.

The project results were exhibited at 3 major trade shows in Paris, Manchester and Munich as well as several smaller events.
The following scientific papers have been published already or will be published in the near future:

Title	Main Author	Citation	Publisher	DOI
Quality control in in vitro nanoparticle testing: Collagen coating can optimise particle distribution on adherent cell lines and improve data quality	B. Gaiser	Modern Polymeric Materials for Environmental Applications", Vol. 4, Issue 1, 2010	Ed. K. Pielichowski	ISBN is 978-83-930641-1-3
A complementary definition of nanomaterial	W. Kreyling	Nanotoday, Volume 5, Issue 3, June 2010, Pages 165–168	Elsevier	10.1016/j.nantod.2010.03.004
Generation and characterization of stable, highly concentrated titanium dioxide nanoparticle aerosols for rodent inhalation studies	W. Kreyling	JOURNAL OF NANOPARTICLE RESEARCH, 2011, vol. 13 no. 2 p. 511-524	Springer	10.1007/s11051-010-0081-5
Engineering quasi-vivo® in vitro organ models	A. Ahluwalia	Adv Exp Med Biol. 2012;745:138-53.	Springer	10.1007/978-1-4614-3055-1_9
Size and surface charge of gold nanoparticles determine absorption across intestinal barriers and accumulation in secondary target organs after oral administration		Nanotoxicology, February 2012, Vol. 6, No. 1 , Pages 36-46		doi:10.3109/17435390.2011.552811) http://informahealthcare.com/doi/abs/10.3109/17435390.2011.552811
Particle size-dependent and surface charge-dependent biodistribution of gold nanoparticles after intravenous administration	Stephanie Hirn	Eur J Pharm Biopharm. 2011 Apr;77(3):407-16	Elsevier	http://www.sciencedirect.com/science/article/pii/S093964111000370X

Title	Main Author	
Using in vitro models to assess the toxicity of nanomaterials	**Vicki Stone**	In Vitro Toxicology Society

Title	Main Author	

Three chapters in a book entitled New developments in Cell-Based In-Vitro Testing - Methods & Protocols have been written by InLiveTox partners. The Book is available on-line in June 2012 by Pan Stanford Publishing. www.panstanford.com

Direct mailshots of a Newsletter were made to 24 industrial companies who were potential users and 2 organisations involved in regulatory matters (ECVAM and LGC)

Exploitation of results and commercialisation strategy

The overall objective of the strategy is to maximize the uptake of the project results. The plan accommodates both those beneficiaries who wish to be involved directly in commercial activities and those who wish to licence others to do this on their behalf. The plan takes account of regulatory aspects and ethical issues.

There are two main sectors that are being targeted in the commercialisation plan: the academic research community working on advanced in-vitro models of cell culture and the commercial companies involved in the testing of safety or efficacy of compounds for the pharmaceutical, cosmetic household products or food industry.

In the commercialisation plan careful consideration was given as to whether to publish, licence to others or develop commercially ourselves. The latter two options are viable because steps have been taken to protect the IP by patent, copyright designs or secrecy.

Products and Services

The following products and services that build on the IPR created during the project will be commercialized by the partners.

- Protocols to set up cell cultures to create viable biological tissue models of the intestine and other barriers within the body (SERVICE)
- Protocols to determine Cytotox and inflammatory responses (SERVICE)
- Data on biokinetics from in vivo studies (SERVICE)
- Modular microfluidics based in vitro test system “InLiveTox System” including necessary protocols of how to use it in various applications (PRODUCT)
- Improved microfabricated membranes for cell culture (PRODUCT)

Routes to Market

The service offers described above will be marketed direct by the organisations involved.
For the products a different approach is being taken by Kirkstall. i.e. initial market creation in the local market (UK) by direct sales
This is followed by selection of distributors to serve regional markets.

Competition

The market for products and services in the field of in-vitro testing of safety and efficacy for drugs, cosmetics and chemicals is very large and hence there are many competitors already and many more emerging. The leading players have invested heavily in development of their

offers and then followed by perhaps even greater investment in getting their tests validated and approved by the regulatory bodies such as ECVAM.

The InLiveTox consortium members will be seeking further investment and partnership with major global companies to ensure the maximum take up of the technologies developed during the project.

The service offers could be available before the end of 2012. The Product offers are expected to take one to two years further development subsequent to the end of the project.

Web site: www.inlivetox.eu

Partners:

Centre Suisse d'Electronique et de Microtechnique SA (CSEM) (CH)
Dr. Martha LILEY - Martha.LILEY@csem.ch

University of Pisa (I)
Dr. Arti AHLUWALIA - arti.ahluwalia@centropiaggio.unipi.it

University of Saarbrücken (DE)
Claus-Michael LEHR - lehr@mx.uni-saarland.de

Helmoltz Centre in Munich (DE)
Wolfgang KREYLING - kreyling@helmholtz-muenchen.de

Kirkstall Ltd (UK)
Malcolm WILKINSON - jmw@kirkstall.org

Heriot-Watt University (UK)
Dr. Vicky STONE - V.Stone@hw.ac.uk

Alma Consulting group (F)
Anne-Cécile de Giacomoni - acdegiacomoni@almacg.com

Inlivetox consortium