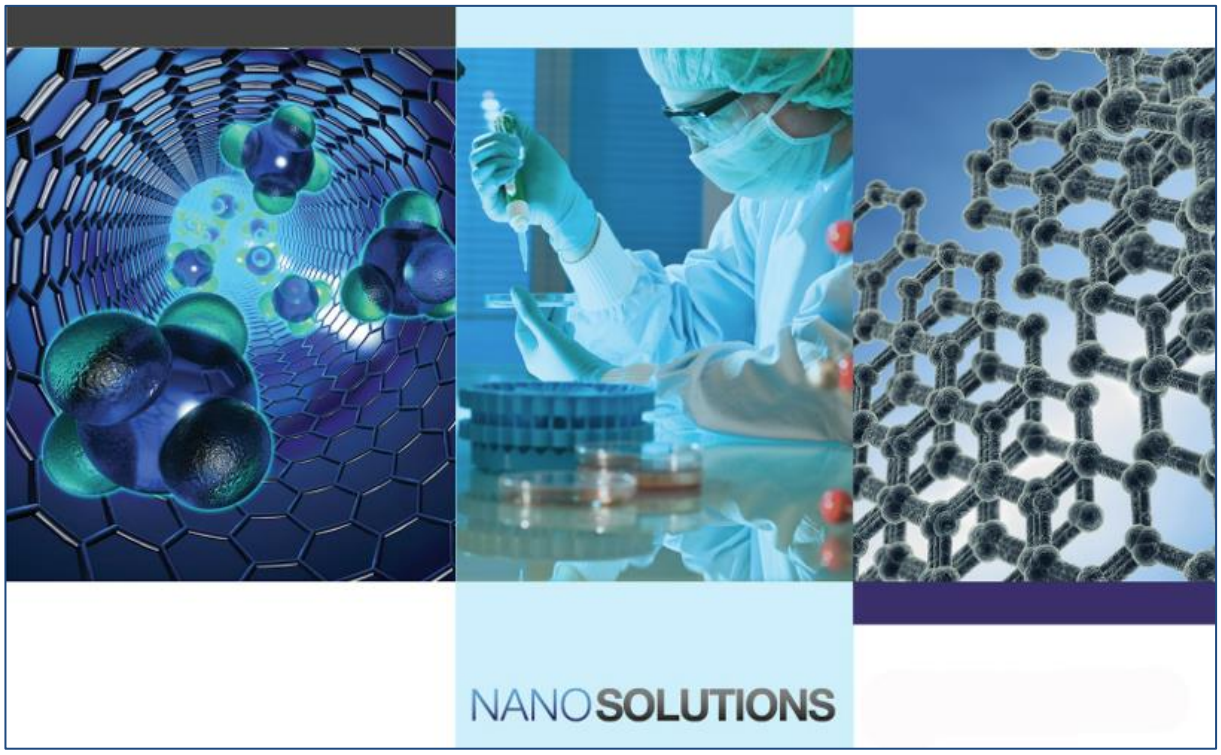
NANOSOLUTIONS



NANOSOLUTIONS Final Report:
Final publishable summary report

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1 Executive summary

Engineered nanomaterials (ENM) have attracted a remarkable interest in recent years, due to their technologically unique properties. These properties and their applications have built up immense technological and economic expectations for industries using ENM. However, some of these properties have given rise to concern that they may be harmful to humans. The NANOSOLUTIONS project has developed an approach for predicting the safety of ENM. This is based on an understanding of their interactions with living organisms at molecular, cellular and organism levels. The main objective of the NANOSOLUTIONS project has been to identify and elaborate on the characteristics of ENM that determine their biological hazard potential at various levels of biological organisation in a range of cells and organisms. The approach in NANOSOLUTIONS explores how interactions with cellular structures and molecular processes may lead to the impairment of key cellular or organismal functions. Using a panel of 31 distinct ENM with different core chemistries and surface modifications, the project has provided evidence that these adverse effects may be associated in complex and diverse ways with multiple properties of ENM. A key goal of the project has been to develop a set of biomarkers of ENM toxicity to assess and predict the safety and toxicity of ENM across species. ENM interactions with living organisms are complex and may depend both on the chemical composition of the material, and other physicochemical and surface properties, and on the acquired 'corona' of adsorbed biomolecules. The objective of this research has been to build a means to develop an "ENM Safety Classifier", based on the material characteristics of ENM, using the understanding of their interactions at different levels within living organisms. To this end, a wide range of in vitro and in vivo models relevant to human health and the environment were utilized. A considerable focus was devoted to omics-based approaches in order to capture the mechanisms of action of ENM. The project has identified a limited number of chemical and biological features which predict - with a high accuracy - whether a given ENM belongs into one of three hazard classes (low or no hazard, intermediate hazard, or high hazard). The nanosafety classifier is a computational tool and it is continuously developed as more data are entered.

The main innovation of the NANOSOLUTIONS project has thus been the development of the ENM Safety Classifier. This novel hazard profiling principle will help in understanding and defining the toxic potential of different types of ENM. It can be used by the ENM industry as well as the regulatory community to manage, reduce ENM-associated uncertainties, and bring clarity to the current debate, since it enables classifying ENM into different hazard categories. During the course of the project, high-throughput screening (HTS) platforms for rapid screening of ENMs, based on robust and validated in vitro assays, have also been developed and optimized for ENMs. These platforms can be used to implement new assays based on the biomarkers identified by the Safety Classifier. The data gathered in the project has also contributed to the life cycle impact evaluation of ENM-based products, and will ultimately clarify their global environmental impact. Testing and validation of the tool is in progress and has the potential to be used within a few years. NANOSOLUTIONS has utilized a huge array of technologies from materials science to system biology, and has had access to eminent, relevant expertise. Validation of the ENM Safety Classifier tool has been carried out with industrially relevant materials. NANOSOLUTIONS will strive to make all data available to other qualified parties. This open access to high-quality data on the material characteristics of various classes of ENM and on the relevant biological outcomes across several species, including healthy and susceptible individuals, will serve as a valuable resource for future ENM safety prediction and classification.

2 Summary description of the project context and objectives

Engineered nanomaterials (ENM), defined as having at least one dimension of ≤100nm, have attracted remarkable interest in recent years due to their technologically and economically interesting properties. The properties of ENM and their applications have built up immense expectations for industries using ENM, both in terms of technological and economical benefits. However, some of these properties have given rise to concern that they may be harmful to humans.

Scientists, regulators and industrial representatives have begun to investigate the features of ENM in order to assure their safe use in nanotechnologies. The European Commission has also explored in depth the characteristics of ENM and issued a document addressing ways through which to assure their safety.

The NANOSOLUTIONS consortium, launched in April 2013, has created means to develop a safety classification for ENM, based on an understanding of their interactions with living organisms at molecular, cellular and organism levels. Many important functions of living organisms take place at the nanoscale. The human body uses natural nanomaterials, such as proteins and other molecules, to control its many systems and processes. A typical protein such as haemoglobin is 5 nm in diameter.

The main objective of this project has been to identify and elaborate on characteristics of ENM that determine their biological hazard potential. This potential includes the ability of ENM to induce damage at different levels of the organism by interacting with cellular structures, which may lead to the impairment of key cellular functions. The project has provided clear evidence that these adverse effects may be especially mediated by ENM-induced alterations in gene expression and translation by using 31 distinct ENM.

An important goal has been to develop a set of chemical and biological features of ENM toxicity which are relevant to assess and predict the safety and toxicity of ENM across species. ENM-organism interaction is complex and depends not only on the composition of the ENM core, but more specifically on its physicochemical properties, and ENM's surface properties. The objective of this research has hence been to build a means to develop an "ENM Safety Classifier", based on the material characteristics of ENM, using the understanding of their interactions at different levels of living organisms. The Classifier can be used by the ENM industry as well as the regulatory community to manage, reduce ENM-associated uncertainties, and bring clarity to the current knowledge gaps, since it enables classifying ENM into different hazard categories. During the course of the project, high-throughput screening (HTS) platforms for rapid screening of ENMs, based on robust and validated in vitro assays, have also been developed and optimized for ENMs. These platforms can be used to implement new assays based on the biomarkers identified by the Safety Classifier. The project has been indeed able to provide a limited number of features which predict with a high accuracy whether a given ENM belongs into one of three (high, intermediate, low to no hazard) hazard classes in the NANOSOLUTIONS context. These platforms can be used to implement new assays based on the biomarkers identified by the Safety Classifier. The data gathered in the project has also contributed to the life cycle impact evaluation of ENM-based products, and will ultimately clarify their global environmental impact. Testing and validation of the tool is in progress and has the potential to be used within a few years. NANOSOLUTIONS has utilized a huge array of technologies from materials science to system biology, and has had access to

eminent, relevant expertise. Validation of the ENM Safety Classifier tool has been carried out with industrially relevant materials.

The NANOSOLUTIONS project has been divided into 13 work packages (WPs), with the collaboration of all of the 38 partners in different WPs, each of which had its own separate leader. Professor Kai Savolainen, Finnish Institute of Occupational Health, was the Coordinator and Professor Harri Alenius (FIOH/UH) the Vice-co-ordinator. The partners had an outstanding expertise in omics technologies. In addition to transcriptomic and proteomic assessments, the project also assessed the epigenetic effects of ENM. The task of collecting, processing and analysing the experimental data generated in WPs 5 to 10, using state-of-the-art and beyond-state-of-the-art systems biology approaches, has been the topic of WP11. This task has been carried out with a great success, as indicated above. WP12, in turn, has developed the “ENM Safety Classifier”. WP12 has also tested and validated the predictive potential of this novel model in close collaboration with the industrial project partners. Finally, WP13 has been elaborating on numerous dissemination activities in order to target and engage the relevant stakeholders; not only the scientific community and ENM manufacturing industries, but also, crucially, regulatory agencies.

2.1 Description of work performance of the project and main results

The material distribution and the Standard Operation Protocols (SOPs) have been finalized and completed. All 31 ENMs have been also fully characterized to provide the ENM Safety Classifier with information about the industrially relevant ENMs that have been tested in the project. The combined expertise by the project partners enabled a high quality-high accuracy characterization of the ENM used in the project. This was also a crucial component of WP3 (Materials) of the project, and highly important for assessing the biological and environmental impact of the ENM. The work on the behavior of the dispersed ENMs on biological fluids has also been carried out, and identified dissolution rates of the tested nanomaterials in different biological fluids, as well as nanoparticle tracking analysis (NTA) to determine changes of particle size distribution/settling in those fluids. The work highlighted the need for standardized methodologies for dispersion of the ENM in large-scale toxicity studies. This was crucial to allow the integration of results from several partner-laboratories into the ENM Safety Classifier.

The work on life-cycle analysis (LCA) has been brought to completion by WP4. Simulation of the ENMs release and release characterization at laboratory scale have been carried out and a variety of applications ranging from sportswear textiles to motor oil additives have been fully explored. Also, the basis for deriving the effect factors of NMs and their application of the LCA case study for quantum dots have been established.

One of the major objectives has been to carry out the experimental work to test the ENM and generate data, in a coordinated manner, on ENM interactions with living systems; this work has been performed in WP5-WP9. For the first time, the relevance of the glycosylation on the bio-nano interactions has been shown. The work has also highlighted the significance of a protein corona (biomolecules attached to the surface of a nanomaterial in a biological milieu) on nanoparticles in modulating particle properties and their biological interactions. The study showed that the post-translational modification of proteins can significantly impact nanoparticle–cell interactions by modulating the protein corona properties (WP5).

The results from WP6 (Cell models) highlight dose- and time-dependent effects of the tested ENMs, but also underscore that different surface functionalizations of ENMs have distinct effects on the toxicity in different cellular models including macrophages, lung cells, T cells, and mesenchymal stem cells. In WP7 (Cross-species models) a toxicity screen has been

attempted with all materials for two model organisms – microbes (*E. coli*) and the water flea (*D. magna*) to determine low, medium and high toxicity materials. In the test conditions, the cadmium telluride quantum dots with a cadmium core (CdTeQDs) have been the most toxic from all the tested nanomaterials to microbes. In WP8 (Disease models) the data has shown that the surface functionalization of quantum dots determines their association with atherosclerotic lesions in the carotid artery of ApoE-/- mice fed a high-cholesterol diet. Also, in asthmatic mice, core CuO exposure activated innate immunity reactions while it diminished T-cell mediated adaptive immunity response. In WP9, translocation studies have been conducted across different barriers at cellular, tissue, organ and organism level. The results provided evidence that the surface functionalization of ENM determine their behavior across endothelia barriers and cell membranes and influence nanoparticle uptake.

OMICS methods have been addressed in detail and the set-up for the omics experiments have been carried out as planned in WP10. In addition, the project has developed the computational framework for the ENM Safety Classifier and agreed on the data formats and data repository (WP11). The ENM Safety Classifier highlights the most relevant features that, across the data layers, will predict the safety of the nanomaterials and classify against the effect. A novel computational method has been developed for feature selection and prioritization from omics data based on fuzzy logic and random forests approaches. The method is able to retrieve sixteen very robust features which could predict with a high accuracy the hazard potential of a given ENM (r = 0.8–0.9). The WP12 (Safety classification) has carried out an option analysis of the Classifier. It has also conducted prototyping and testing of the tool, and has developed a specification for a high throughput system for future testing and analysis. Thus, the computational infrastructure of the Classifier is in place, and has been tested with ENM from outside the NANOSOLUTIONS Project.

The dissemination activities of the project have been completed by WP13. The *International Congress on the Safety of Engineered Nanoparticles and Nanotechnologies* (www.ttl.fi/senn2015), was organized in Helsinki, on 12-15th April 2015, attracting around 200 international experts and scientists around the world. The Congress provided a great forum for sharing the results of the project so far and discussing the latest knowledge on the safety of engineered nanomaterials and nanotechnologies. In addition, various articles and newsletters were disseminated to the target audiences, and social media was in active use. The NANOSOLUTIONS project in collaboration with the Working Group on Systems Biology of the EU NanoSafety Cluster organized the *System Biology in Nanosafety Research Nobel Forum Conference* on 9–10th November 2015 in Stockholm. Topics covered were omics technologies and computational approaches, and the application of these tools and approaches in nanosafety research. Several EU-funded nanosafety projects were represented among the speakers. At the end of the project a Joint Conference of five major EU nanosafety projects entitled *New Tools and Approaches for Nanomaterial Safety Assessment* was organized in Malaga on 7–9th February 2017, with around 230 participants. Furthermore, the NANOSOLUTIONS project is contributing to a nanosafety workshop in the *EuroNanoForum 2017* to be organized on 23rd June 2017 in Malta.

2.2 Description of the final results and their potential impact and use

The main innovation of the NANOSOLUTIONS project has been the development of the ENM Safety Classifier. This novel hazard profiling principle will provide a basis that enables us to understand and define the toxic potential of all types of ENM. It can, and will, be used by companies that manufacture ENM and by the regulatory community to manage hazard, reduce

uncertainty, and clarify the current debate; since it will provide the potential to effectively "de-classify" many types on ENM in many applications, in terms of safety risks.

The development and exploitation of this tool will support the innovation process by:

- Reducing the overall uncertainty concerning the safety of ENM, and identifying which ENM and which applications actually possess significant toxicity potential, and those that do not;
- Enabling industry to select, early in the innovation path, the appropriate ENM on the basis of their toxic potential as well as their functionality, and further to prioritise the use of those ENM with low toxic potential in their products;
- Reducing industry's testing and product registration costs, enabling companies to focus investment on materials that do not have a significant toxic potential;
- Providing a clear knowledge and common vocabulary by which information concerning the potential safety issues, and the need to control exposures, may be passed along the supply chain;
- Providing industry at all stages of the innovation chain with confidence that the materials that they are using do not represent future business risks (reputation, litigation) resulting from unforeseen toxicity;
- Allowing regulators to prioritise regulatory activity as regards the ENM with significant safety issues, and to minimise the regulatory requirements for other ENM;
- Reassuring the general public that their health and the environment will not be harmed;
- Creating the possibility of widening the range of ENM and applications, free from concerns about potential safety issues;
- Supporting the safe and confident exploitation of ENM in a huge range of products and processes for the benefit of Europe and its citizens.

The data gathered in the NANOSOLUTIONS project will also contribute to the life cycle impact evaluation of the life cycle analysis (LCA) of ENM-based products, and will ultimately clarify their global environmental impact. Testing and validation of the LCA tool will be carried out in close collaboration with ENM manufacturing companies, and the results will be disseminated to the relevant regulatory agencies in order to encourage the implementation of this novel tool. In order to identify the most relevant hazard-associated features as well as the most critical molecular signatures that predict the safety of ENM, NANOSOLUTIONS will utilize state-of-the-art and beyond-state-of-the-art systems biology and bioinformatics techniques. These novel approaches are being actively developed and successfully implemented by the bioinformatics partners of the Consortium for predicting the effects of pharmaceuticals.

NANOSOLUTIONS has utilized an impressive number of technologies from materials science to system biology, and has had access to eminent, relevant expertise. A screening of ENM properties and their relation with biology has been performed, focussing on representative materials and the different types of surface chemistry. NANOSOLUTIONS will strive to make all data available to other qualified parties, for example through the European NanoSafety Cluster. This open access to high-quality data on the material characteristics of various classes of ENM and on the relevant biological outcomes across several species, including healthy and susceptible individuals, will serve as a valuable resource for future ENM safety classification.

3 Description of the main S&T results/foregrounds

3.1 WP3 Materials

The overall objectives of WP3 (Materials) were to provide a set of stable and well-characterized engineered nanomaterials (ENM) and to ensure the stability of ENMs in aqueous stock suspension until the toxicity experiment. A total of 31 engineered nanomaterials (ENM) was acquired for performing the toxicity studies in the end-user labs and thereby generating a complete set of data to feed and develop the prototype NanoSafety Classifier. The selection of ENM types was based on a pre-established set of selection criteria, which were:

- the selected ENM should exhibit a wide range of toxicities;
- the ENM should be used by European industry with likelihood of human exposure (intentional and non-intentional), or be relevant in the future;
- the selection should support European legislation initiatives.

The selection of ENM covered both 3D (nanoparticles) and 1D (nanotubes or nanorods) ENM. The ENMs were provided by NANOCYL or synthesized for the project by the project partners PlasmaChem, University of Bordeaux (UB), University of Manchester (UNIMAN) and CiC BiomaGUNE. Nine types of ENM selected for the project cover a wide range of relevant industrial applications of ENM:

- TiO_2 particles 10-20 nm (Primary particle size) (pigment/colorant in paints, cosmetics, food);
- TiO_2 rods 1:5 aspect ratio (similar applications/industries as TiO_2 particles);
- CuO particles 10-20 nm[a] (antifouling agent in paints and biocide in textiles);
- Au particles 3-5 nm[a] (potential future use in nanomedicine for e.g. drug delivery, bioimaging);
- Au particles 10-20 nm[a] (potential future use in nanomedicine for e.g. drug delivery, bioimaging);
- Ag particles 10-20 nm[a] (antimicrobial agent in food packaging, biocide sprays);
- CdTe particles 3-5 nm[a] (used in LED/solar cells/lasers, inkjet printing applications);
- Nanodiamonds' particles 3-5 nm[a] (additive in engine oils, used in plastic reinforcements);
- Multiwall Carbon Nanotubes (MWCNTs) 1:100 aspect ratio (composite materials, sporting goods).

The synthesis of ENM with the same core material in different sizes (3-5 and 10-20 nm Au particles) and shapes (TiO_2 particles and rods) allowed investigating the role of size and shape of ENM on their toxicity. In order to study the influence of the surface properties, each ENM was available in 3 specific functionalized variants. The ENM were functionalized with carboxyl groups, ammonium groups or polyethylene glycol, thus yielding ENM with pH-dependent negative and positive charges, respectively, or improved hydrophilicity. Additionally, in the case of TiO_2 particles and rods, CuO particles and MWCNTs the non-functionalized variants (i.e., with no functional groups on the particle surface) were also available, which meant an additional 4 ENM to use for toxicological testing.

The 31 ENM were distributed in powder or liquid suspension form to the project partners and dispersion procedures were developed. A range of dispersion protocols were developed

optimized and standardized with respect to ultrasound power input and treatment time. They were tested as part of a quality assurance scheme under the leadership of the laboratories in charge of the work on dispersion-SOPs and basic characterisation of the ENM. Only by tightly complying with the developed protocols, it could be assured that the ENM suspensions produced in the different test laboratories of the ENM had the same characteristics. Such similar characteristics were crucial to allow integration of results from several partner-laboratories into the ENM Safety Classifier. The work conducted in the project highlights the need for standardized methodologies for dispersion of ENM in large scale toxicity studies involving several laboratories.

Physico-chemical characterization of the 31 ENMs was performed and the obtained metrics transferred to the ENM Safety Classifier. The physico-chemical characterisation of the ENM comprised basic characterisation using transmission electron microscopy (TEM), dynamic light scattering (DLS) and zeta-potential measurement for provision of particle primary size / particle shape, hydrodynamic size and surface charge, respectively.

The physico-chemical characterisation also included a more advanced characterisation stage, which was based on the combined expertise of WP3 partners. The metrics selected for advanced characterisation of the ENM comprised the study of impurities by inductively coupled plasma mass spectrometry (ICP-MS). The release of metal from the ENM was also determined by ICP-MS after dialysis. The ENM were largely non-soluble or did not release any metals within a 24 h test period, with the exception of release of Cu, Ag, Cd and Te from the CuO CdTe and Ag-PEG ENM variants, and a minute release of Fe from MWCNTs. The hydrodynamic diameters were determined by the combined size-separation/size-detection technique, differential centrifugal sedimentation (DCS), which demonstrated comparable sizes with those determined by DLS for especially the CuO ENM variants. The presence of the different functional groups was verified with different characterization techniques including X-ray Photoelectron Spectroscopy (XPS), Fourier Transform Infrared Spectroscopy (FTIR), Thermogravimetric Analysis (TGA) and C,H,N elemental analysis.TGA demonstrated the total mass of functional groups/impurities represented in a few cases more than 50 % of the total mass of the ENM. This information should be taken in consideration when interpreting the toxicity studies. The results from XPS and Raman spectroscopies largely verified the presence of the functional groups on the ENM according to the planned scheme. In particular, FTIR provided highly detailed information not only on which functional groups were present, but also the side chains upon which they were bonded to the ENM cores.

Besides the acquisition and characterisation of the 31 ENM, WP3 aimed at understanding the behaviour of the dispersed ENMs in selected biological fluids and cell media. Dialysis experiments for determination of dissolution rates and nanoparticle tracking analysis (NTA) for studying changes of particle size distribution/settling were performed. In terms of dissolution, TiO_2 ENM (both particles and rods) were found to be very stable in all tested media with very limited dissolution. CdTe ENM demonstrated the highest dissolution followed by the CuO nanoparticles. The role of the coatings on metal release from the test materials was difficult to determine as it was not always the same across the different media.

NTA data indicated that the nature of the coating may determine particle agglomeration in biological media. Despite this, it was difficult to describe a common agglomeration behaviour pattern for all tested ENM in the different media: the results suggested that there was a trend for the functionalized ENM to agglomerate more and form larger agglomerates compared to the core ENM. This trend was observed for most of the ENM in all media tested except for the M7 media, where the type of functionalization had no effect.

Although some patterns were observed (e.g., ENM dissolve more in the artificial saliva and artificial urine solutions; carboxylate-coated ENM form larger agglomerates in artificial urine), the occurring particle-to-particle and particle-to-media interactions are complex. From the current experiments it was concluded that the dissolution and agglomeration/aggregation of the particles is determined by the particle chemistry (both the core and the coatings), as well as the composition of the biological media. None of these factors is solely responsible for the behaviour of the ENM in biological media, and instead, both factors act together.

3.2 WP4 Life-cycle analysis

INKOA performed a selection of representative applications incorporating each ENM type to be studied in the execution of NANOSOLUTIONS, according to the information provided by industrial partners in the consortium. Additionally, a description of the life cycle stages of the ENM depending on the application or consumer product was carried out; special relevance has been given to the life cycle stages beyond the manufacturing stage.

Thereafter, the life cycle stages that are most likely to result in the transformation of the ENM and/or to result in the release of ENM were identified.Priority was given to the applications in conditions of normal use and tests were conducted under Task 4.2 and Task 4.3 on this basis.

The life cycle stages specifically described for each application, and estimations of the most relevant life cycle stages in terms of nano-release, were assessed by INKOA in collaboration with other partners. Finally, laboratory scaled release simulations were established by LEITAT on the basis of the former information.

According to the outcomes of the previous task, and the information provided by the industrial partners, ENM were selected. The NM-enabled products studied were grouped into four main application sectors: the textile industry (TiO_2, CuO, Ag NPs and MWCNT), the ink industry (CdTe quantum dots), nanomedicine (Au nanoparticles), and the automotive-oil additives industry (nanodiamonds). Experimental simulations, characterization of the materials before and after simulations, as well as the material released study were explored.Details can be found in deliverables D4.2 and D4.3. The simulations carried out were: washing for nano-enabled sportswear fabrics (TiO_2, CuO, Ag NPs), abrasion for polymeric nanocoatings applied on a car arm rest (MWCNT), printing for ink (CdTe QDs), oil recirculation for automotive-oil (nanodiamonds) and physiological fluids exposure for gold NPs. One real application for all nanomaterials was evaluated to enable the understanding of the possible transformations of these nanomaterials during common use conditions. An important goal was also to characterize possible ENM release from such products.

Different approaches were followed according to the nature of the experiment. For instance, waters were collected from fabrics washing experiments, CdTe emitted to air were collected during printing, or MWCNT embedded in polyurethane were collected in filters after being measured by particle counters. In other cases, such as the diesel oil containing nanodiamonds or gold dispersions, experimental work was focused on characterizing the transformations of ENM inside the products by means of different analytical techniques (e.g., ICP-MS, Raman Spectroscopy or TEM-EDX). According to the results obtained, the following general conclusions can be drawn (for further details see deliverable D4.3).

- Nanomaterials in the textiles (Ag, CuO and TiO_2) are weakly bound to the fabrics and hence easily released to the waste water treatment system.
- For antistatic fabrics containing MWCNT, abrasion experiment has revealed that low ENM releases from the matrix into air or soil occurred.

- In oils, nanodiamonds showed some chemical changes, especially metals transference from the engine to the ENM.
- In inks, CdTe particles were aerosolized during printing and emitted to air being especially of concern because Cd^{2+} is highly toxic.
- Au nanoparticles for biomedical applications may exhibit instability in both gastric and urine simulated fluids because of the formation of large aggregates that sediment over time.

In order to assess the release and transformation of the ink containing CdTe QDs, ENM in all the stages of its life cycle was explored. Abrasion of printed papers (rubbing) showed high cadmium transference from paper to the cotton abrader material. End-of life was simulated with leaching experiments indicating QDs were released. From the results it was concluded that tellurium is released much more readily than cadmium.

Additionally, the nanoparticle emissions during printing process in controlled conditions were characterized, and the assessment of their toxicological effect was carried out in routine cultures of pulmonary cell cultures and by using air-liquid interface with the same cells. These emissions were not large enough to elicit a toxicological cellular response.

The USEtox model was applied to derive human toxicity and freshwater effect factors for selected NANOSOLUTIONS ENM, namely MWCNT, TiO_2 and Ag. Work was carried out by TNO (on the human impact perspective) and LEITAT (for the freshwater ecotoxicity).

Subsequently, complete characterization factors (CFs) (effect factor, fate and exposure factor) for CdTe quantum dots (QDs) both for human and freshwater ecotoxicity have been derived.Finally, these values have been integrated into a Life Cycle Assessment (LCA) on the basis of the internationally recognized ISO framework for LCA (ISO 14040:2006) for PEG-CdTe in printing ink case study.The present action goes far beyond the state of the art in LCA studies for ENM in which these aspects generally remain uncovered.

3.3 WP5 ENM bio-nanointeraction in biological media

Nanotechnology has recently acquired new dimensions since advances in synthetic chemistry have made it possible to produce ENM of an extraordinary variety. For instance, it is now possible to synthesize them in a large range of sizes and shapes, and precisely control their physico-chemical aspects such as surface charge, hydrophobicity and surface modification. Regardless of the numerous applications that nanotechnology offers, their large scale production inevitably implies increased exposure to humans during the manufacture process but also throughout the material life cycle.

Nanomaterials behave significantly differently than other chemicals. Size and the material surface energy are considered to be key distinct properties conferring identity to particles at the nanoscale. Nanomaterial size is comparable to several biomolecules and it confers their ability to penetrate membranes, to be recognized by specific receptors and trigger potential adverse responses to cells or in an organism, and to accumulate in cellular compartments with unknown consequences. Therefore, an accurate study of the size and colloidal stability of ENM dispersions is a crucial step to ensure high quality dispersions and to identify the absence of different subpopulations in size or agglomeration/aggregation that will produce unreliable data.

The interactions between ENM and proteins are highly affected by the material physico-chemical properties, such as the chemical composition, the surface chemistry, the shape, the porosity, and the surface morphology which determine the nature of the protein corona

identities; both qualitatively (types of biomolecule forming the corona) and quantitatively (absolute amount). This means that for each ENM type that will be exposed in biological fluid, a distinct biomolecular corona is formed that will affect the biological outcome.

The protein corona is derived from proteins present in biological fluids, many of which are glycosylated. To date, the glycans on the proteins have been largely overlooked mainly because of the lack of dedicated infrastructure and workflow that could tackle the multidisciplinary aspects of the study. Within NANOSOLUTIONS, UCD has developed a running platform that has shown for the first time that the biomolecular corona is highly glycosylated and that changes in the glycan expression on the corona have a dramatic effect on the ENM bionano recognition. Quite remarkably, biomolecular corona are formed by proteins with high affinity with the ENM surface, regardless of their abundance in the media of origin as previously reported. Further studies have shown that glycosylation of the corona affects the cellular recognition and cellular response.

ENM physico-chemical properties govern the biomolecular corona formation and have a strong impact in the biological fate of ENM as approaching living organisms. Along with the protein component, the carbohydrate biomolecules should be taken into account as they are capable of controlling the ENM colloidal stability and the cellular response. The ENM dispersion complexity did not allow a straightforward correlation with the ENM properties and proteins, as by changing one parameter, such as charge or shape, the ENM distribution is also altered and the material surface area might be strongly affected. Lastly the initial corona composition study has revealed that the majority of the corona proteins are mostly dysopsonin proteins and thus these materials are likely not to be seen by the immunological system, however fluctuation in their abundance is specific to material and functional group.

A new platform has been set up for epitope mapping by UCD, where the protein orientation around ENM can be predicted by means of immuno-labels. This platform allows determining the statistical distribution of exposed protein epitopes presented across the nanoparticle corona surface on a particle-by-particle basis, identifying the epitopes expressed as well as their organization in relation to one another. The study has been published in Nature Nanotechnology and it has been shown to be a robust platform to map the detailed and exposed motifs on the ENM surface.

Physicochemical characterization of different ENMs in the presence of biological fluids, in particular human plasma, and quantitative composition analysis of the “hard corona” by a combination of proteomics and mass spectrometry with new methodologies based on immunolabelling with QDs have been carried out. The importance of profiling not only the protein corona composition as a whole, but also specific protein sequences that can potentially engage in nanoparticle-cell receptor interactions, has led to the development of a new platform for the molecular characterization of the protein corona. The platform has a focus on the organization and exposure of motifs or epitopes relevant for cell receptor interactions. A screening platform has been set up which enables the multi-detection of relevant specific protein sequences presented on the nanoparticle surface by monitoring qualitatively and quantitatively the interaction of QD immunoprobes with biomolecular corona protein domains on the NP surface. These interactions can be measured by using steady-state fluorescence spectroscopy or flow cytometry.

Mapping relevant epitopes on the biomolecular corona of nanoparticles (and their specific distribution both in relation to epitopes of the same class and epitopes of different classes) allows us to connect the real nature of the bionanointerface to cellular interactions, therefore obtaining a closer connection between a particle and its biological impact. Transfected cells

exhibited increased uptake of particles compared to non-transfected cells in various concentrations of IgG-depleted serum, suggesting specific receptor-protein interactions play a role in particle recognition. Results show that a higher number of particles are endocytosed into the transfected cells compared to the controls for all studied conditions.

A set of platforms to characterize the ENM biomolecular corona complexes in realistic environments and to study the interactions with specific cellular receptors has been developed in order to provide capacity for profiling the ENM corona to predict early interaction with selected biological targets (receptors). Highly monodisperse (in biological media), LPS free, particles have been used to established the methodology and other ENMs have been tested, showing the connection between specific motifs of the protein-corona composition and the ENM-corona binding partners.

3.4 WP6 Cell models

The overall aim of WP6 has been to apply a range of *in vitro* (cell-based) models and methods for the assessment of ENM toxicity, focusing on immunotoxicity/inflammogenic potential and genotoxicity/carcinogenic potential, and, moreover, to adapt selected *in vitro* assays for high-throughput screening. Additionally, WP6 has delivered samples for further assessment using so-called omics technologies in WP10. WP6 is unique in the sense that the 10 partner institutes were from 5 different geographic regions (Europe, North America, South America, Africa, and Asia). Moreover, in addition to academic partner institutes, two small companies (in Finland and Germany) were actively involved, not least in the high-throughput screening task. Overall, WP6 successfully fulfilled all of the expected objectives and the partners have thus generated a comprehensive set of results on a panel of 31 ENMs using a range of cell-based models and methods. These results have been transferred to WP11 to support the development of the Nanosafety Classifier. The work performed in WP6 has been presented at several international conferences and the WP leader also organized a conference on systems biology approaches in nanosafety research at the Nobel Forum at Karolinska Institutet in Stockholm in November 2015. The meeting provided an opportunity for scientists from different EU-funded projects as well as other international colleagues to present and discuss the latest findings in this emerging field.

The immune system is the first line of defense against foreign materials that enter our body, and this is why the work in WP6 was focused on possible adverse effects of ENM on immune-competent cells. The partners in WP6 have used transformed cell lines mimicking normal monocytes as well as primary human macrophages isolated from healthy donors. Using these models, we studied not only cell death by using conventional assays as well as label-free assays (using the xCELLigence Real-time Cell Analyser), but also the secretion of multiple soluble inflammatory mediators (cytokines and chemokines). In addition, exposure via inhalation represents the most likely route of exposure to ENMs in the occupational setting and there is a concern that certain ENMs may cause lung disease. For this reason, the lung cell line, BEAS-2B was employed both for conventional *in vitro* testing and for the high-throughput screening approaches. Another central question is whether ENMs may have carcinogenic properties, especially upon inhalation. For this reason, the work in WP6 also included genotoxicity assessment (i.e., assessment of DNA damaging effects) as a precursor to possible cancer development. To this end, lung cells as well as immune cells were utilized and the well-known comet and micronucleus assays were applied. In addition, we used mesenchymal stem cells to assess the impact of ENMs on cell differentiation and genotoxic effects. These cells possess a high capacity for self-replication and have the potential to differentiate into various different cell types when placed in an appropriate environment.

Cancer is increasingly viewed as a stem cell disease, due to the misappropriation of homeostatic mechanisms that govern tissue repair and stem cell self-renewal, and the study of ENM effects on stem cells is therefore of considerable relevance. The work in WP6 has taken as its starting point methods and protocols established in several other previous EU-funded projects including FP7-NANOMMUNE, FP7 NANODEVICE, and FP7-MARINA. Because WP6 was tasked with the delivery of samples to WP10 to enable global proteomic, transcriptomic, and epigenomic assessment of ENM effects, we opted for well-established cell models to ensure that the models would yield reliable and reproducible results. To this end, two cell lines were selected: the human monocyte-like cell line, THP.1 and the human lung epithelial cell line, BEAS-2B, and samples exposed to the full panel of 31 ENMs were delivered to WP10.

In addition to the use of conventional testing approaches, the partners in WP6 also developed novel high-throughput screening platforms for rapid screening of large numbers of ENMs. Indeed, the enormous diversity of ENMs in terms of their different sizes, shapes, compositions and coatings necessitates high-throughput screening protocols to test for potential hazards. HTS methods are automated methods based on robust *in vitro* assays capable of simultaneously assessing large numbers of chemicals or particles. In WP6, more than 100.000 data-points were generated using HTS assays for cell viability and other critical end-points. WP6 partners also adapted an HTS assay for genotoxicity assessment of ENMs. With such HTS platforms, large numbers of ENMs can be tested; this may not only speed up the hazard assessment of ENMs, but could also provide a valuable source of data for computer modelling to predict the hazard potential of ENMs. These assays are thus a tangible output of the work in WP6.

With respect to the results, one of the key findings in WP6 is that surface modification of ENMs plays a role for cytotoxicity, with amino-functionalized materials generally displaying more cytotoxicity. This was evident when the immune cell models were employed and a similar pattern, albeit not as straightforward, was seen for the lung cell models. In addition, the core chemistry (i.e., whether the particles tested were made of gold, or copper, or of other composition) also played a role. The work has also shown that the impact on stem cell differentiation of different ENMs is dependent on their surface chemistry; however, not all ENMs affect stem cell differentiation. Overall, the results generated in WP6 represent one of the largest data sets on cytotoxicity-immunotoxicity and genotoxicity of ENMs to date. These results were shared with WP11 in order to promote the development of the Nanosafety Classifier and the results are also important for the interpretation of the omics results produced in WP10.

3.5 WP7 Cross species models

The key role of WP7 in the NANOSOLUTIONS project was to provide *in vivo* data on the exposure of a wide range of organisms to ENMs. In addition, the work package also aimed to generate tissue samples that would be utilized for molecular biology (global RNA sequencing and proteomics) by other WPs in the project. All of the data has subsequently been used to build and calibrate the Nanosafety Classifier tool. The specific tasks in WP7 included; (i) *In vivo* exposures of test organisms to ENMs (task 7.1), (ii) mechanistic investigations on *ex-vivo* samples to determine mode of action and details of the biological effects (task 7.2), and (iii) accumulation and uptake kinetics studies to understand the bioaccumulation risk from ENMs (task 7.3). All the work has been achieved and the *in vivo* data has been made available to the EU Nanosafety data hub. Experiments were conducted on several model organisms. These were the soil bacterium *Escherichia coli K-12* strain, earthworms (*Eisenia fetida*), a nematode

worm that lives in the soil (*Caenorhabditis elegans*), the freshwater invertebrate (*Daphnia magna*), the edible marine mussel (*Mytilus edulis*), freshwater-adapted rainbow trout (*Oncorhynchus mykiss*), zebrafishes (*Danio rerio*), and mice.

One of the central scientific hypothesis in NANOSOLUTIONS was that the surface coating rather than the core chemistry should impart the biological effect. The experiments demonstrated some differential effects of the coatings. However, the effects were not consistent across materials or by model organisms. Furthermore, traditional toxicological end points (survival, growth, reproduction) and biochemical assays did not reveal a consistent ranking of the materials by coatings. In the zebrafish studies, it was possible to cluster data by material-type and possibly by coating using proteomics, provided the data were carefully sorted for the life stage. Regardless of the coating, overall the CuO, Ag NPs, and CdTe QD materials were the most toxic, while nanodiamonds and the TiO_2 materials had limited toxicity across all the model organisms tested. The toxic effects of MWCNTs were generally moderate, between these extremes. The observations were generally consistent with ranking toxicity by the chemistry of the core. The toxic mechanisms observed in the experiments included known modes of toxicity including oxidative stress, ionoregulatory toxicity, genotoxicity, inflammation, immune effects, and bioenergetics effects on growth and/or reproduction. There was no evidence of a single nano-specific or novel toxic mechanism.

Bioaccumulation and uptake kinetic studies were also conducted. The effort focused on the organisms that were large enough to dissect in order to obtain tissues for internal uptake measurements, or where a whole body burden could be measured. The studies especially focused on the CuO materials in earthworms, *Daphnia magna*, marine mussels and in zebrafish, as these materials were previously shown to be toxic to these organisms and the total Cu could be readily detected. Some data on TiO_2 in marine mussels was also obtained. Overall, the net Cu accumulation (uptake) from the CuO material exposures was broadly the same magnitude as that of the metal salt, $CuSO_4$, although the latter was a little faster and achieved at lower doses in the fish and earthworm studies.

In earthworms the CuO materials were cleared, as measured by decreasing body burdens over time in clean media. The apparent nano bioaccumulation factors (nBAF) were <1 indicating that the materials are not bioaccumulative in earthworms. This may be expected for an essential metal with a well-documented homeostatic mechanism in animal cells. In earthworms, there were no clear differences in the accumulation rates for different coatings; except for the CuO-PEG, which was slower. However, the accumulated Cu from the CuO-PEG exposures also had slow clearance from the body, and so the bioaccumulation risk was heading towards the hazard threshold with a nBAF of 0.8. In the zebrafish studies, concentration-dependent accumulation was seen in all treatments for the ENMs, but there was an important life-stage effect; with greater apparent uptake in the embryos compared to larvae. Critically, this was the opposite to the life stage effect of $CuSO_4$. The marine mussels showed some limited accumulation of either Cu or Ti from the relevant ENM exposures, and it is likely that the ionic strength of the seawater as well as the ability of the animals to secrete mucous materials leading to sloughing of biodeposits containing the ENMs prevented larger accumulation. The *D. magna* studies examined apparent body burdens over 24 h uptake or clearance experiments. Some apparent increases and losses were observed, but may be best explained by rapid adsorption/desorption phenomena rather than in true internalisation of the materials in the tissues. Nonetheless, the apparent uptake plots for most organisms fitted a rectangular hyperbola and therefore showed the expected features of uptake kinetics curves. Overall, the bioaccumulation experiments demonstrated that modified OECD methods provide data that can be fitted to uptake and excretion curves, and identify parameters that are

analogous to the bioaccumulation factors for soluble chemicals, although the basis of the observations here are from colloid chemistry. In conclusion, the WP7 *in vivo* experiments on a range of model organism was achieved, with the analysis of data from proteomics and RNA sequencing for the Nanosafety Classifier in other WPs. It was possible to identify ENMs of toxicological concern, the modes of action and the bioaccumulation potential; but there were no clear coating-dependent effect across all the organisms or all materials.

3.6 WP8 Disease models

The great majority of studies exploring potential pathogenic effects of ENM have been conducted using study settings mimicking effects in healthy individuals. However, individuals suffering from chronic illnesses, such as cardiovascular or pulmonary diseases, are thought to be more susceptible to develop health problems from particulate exposure. Therefore, the objectives of WP8 were to explore the effects of ENM exposure in diseased cells, tissues, or organisms either *in vitro* by using cultured human endothelial cells as well as a 3D *in vitro* airway model with cells from healthy and diseased human donors and or *in vivo* in murine cardiovascular disease and asthma models.

Findings from the first part of this WP demonstrate that the endothelial glycocalyx, a structure that constitutes a glycoprotein-polysaccharide meshwork coating the luminal surface of blood vessels, effectively controls ENM-endothelium interactions in the microvasculature. Constituents of the endothelial glycocalyx were found to physically cover endothelial adhesion and signalling molecules thereby preventing the endothelial attachment and uptake of ENM as well as their translocation through the vessel wall. Conversely, a degraded endothelial glycocalyx, e.g., under the pathological condition of ischemia-reperfusion, enables interactions of ENM with the microvascular endothelium, underlining the relevance of the endothelial glycocalyx for the protection of the tissue from blood-borne ENM *in vivo*. The results of two other studies strongly suggest that the surface functionalization of ENM determines their association with as well as their distribution pattern within atherosclerotic lesion *in vivo*, most likely by regulating the molecular composition of the biomolecule corona. The individual association behaviour of differently functionalized ENM appears to be based on a different affinity to molecular and cellular structures present in the atherosclerotic lesion environment.

The goal of the second part of this WP was to investigate the health effects of CuO ENM in both healthy and asthmatic individuals, and to elucidate the relevant molecular pathways involved in these effects. Using a murine asthma model, it was found that CuO ENM induced an acute inflammatory response when entering the lungs. Core CuO and its functionalized versions caused a significant influx of leukocytes, cells that protect our body against foreign invaders, into the airways of asthmatic subjects. Furthermore, relevant signalling molecules, that promote and mediate inflammatory reactions, were found in elevated levels in lung tissue of asthmatic mice as compared to their respective controls, especially in response to core CuO. Although the results obtained were qualitatively similar across the tested materials, it was found that surface modifications changed the immunomodulatory potential of the core CuO material. Based on the analysis of cells and solutes from the lower respiratory tract as well as on histological evaluation, the severity of pulmonary inflammation decreased in the order CuO-NH_3 > core CuO > CuO-COOH > CuO-PEG. Work using the MucilAir™ *in vitro* 3D human airway model focussed on the secretion of proinflammatory cytokines as a marker for the development of an inflammatory response in the airways upon exposure to core CuO and CuO-COOH. The co-culturing of fully-differentiated human airway cells at an air-liquid interface in a 3D configuration that harbour a variety of physiologically relevant cellular mechanisms makes it a relevant *in vitro* model that resembles the *in vivo* situation as close as possible. The

results show that a 1h-exposure through air to high concentrations of CuO and CuO-COOH NPs seemed to provoke an inflammatory response, hallmarked by a strong increase in the secretion of relevant proinflammatory mediators. Interestingly, exposure to CuO-COOH exposure was less toxic to the cells and also led to a less proinflammatory response. Despite the difference in the proinflammatory response between exposure to core CuO and CuO-COOH ENM, the analysis of gene expression profiles revealed a large overlap in affected pathways. Generally, stress-activated pathways and pathways involved in a proinflammatory response were upregulated. Another important, yet unexpected outcome of this study was the finding that cells that originated from healthy donors appeared to be more prone to develop an inflammatory response through CuO ENM exposure than cells from asthmatic individuals, while the latter showed a higher sensitivity for cytotoxicity. Taken together, the experimental setup used allows relevant exposure through air and is anticipated to enable relevant predictions of the bioavailability and adverse effects of inhaled compounds because the results do not need a translation step from dispersed, and thus altered, nanomaterials to real-life exposure. In conclusion, this WP demonstrated that combining the MucilAir™ model with an air exposure system is a practical and relevant approach to assess ENM inhalation toxicity *in vitro*, thus enabling the translation from *in vivo* animal data towards potential hazards of ENM exposure through air in humans.

In summary, WP8 has identified the endothelial glycocalyx as an important barrier against interactions of blood-borne ENM with the vessel wall and their translocation to extravascular tissue, verified the importance of the surface chemistry of ENM for their microdistribution in tissue and their association with cellular targets in atherosclerotic lesions, improved the understanding of how selected materials affect individuals with a pre-existing health condition, such as asthma, and identified similarities and dissimilarities between *in vitro* and *in vivo* models.

3.7 WP9 Translocation

The study of the uptake mechanism, biological fate and biodistribution of ENMs is crucial to understand toxicological endpoints of ENMs. The objective of WP9 was to study the translocation of ENMs across biological barriers at cellular, organs and organism levels in relation to the physical-chemical properties of the ENMs, in particular with their chemical functionalization.

The fate of ENMs was studied at cellular level. Intracellular uptake studies were conducted employing different techniques: Ion beam microscopy, flow cytometry, transmission electron microscopy, the CytoViva hyperspectral imaging system, confocal Raman microspectroscopy. The surface functionalization and more specifically PEGylation of ENM was confirmed to be a reasonable strategy to reduce the dissolution of ENM in acidic environments (e.g. CuO NPs), also reducing cytotoxicity, and the generation of reactive oxygen species (ROS). Cell homeostasis was affected by ENMs, as in the case of intrinsic concentration of iron and zinc considerably changed in cells exposed to CdTe and CuO. Surface functionalization affected the interaction of ENMs with cellular compartments. For example, while carboxylated CdTe NPs were mainly found also co-localized with the endoplasmic reticulum, PEGylated NPs were found associated to mitochondria and amine NPs with lipid bodies (Raman studies). For some ENMs was observed increased aggregation during cellular incubation (e.g. TiO_2-PEG, TiO_2-COOH and Ag-PEG) and the colloidal destabilization of the ENMs was correlated to a lower cellular uptake.

Translocation at higher complexity level was investigated across endothelial and placental barriers. In the blood-tissue barrier the integrity of the endothelial glycocalyx modulated translocation/uptake of ENMs *in vitro.* Furthermore, Au and quantum dots translocation from the blood stream into atherosclerotic plaques was characterized and the interaction between ENMs and the plaques showed to be dependent on the ENMs-protein corona formation.

Ex vivo placenta perfusion studies were performed for several NPs, which during the experiments generally underwent physico-chemical changes as agglomeration, dissolution and precipitation. The role of the surface functionalization showed to be again of primary importance as for Au NPs, which crossed the trophoblast barrier into the placental tissue in a size-dependent manner but can be effectively blocked by grafting PEG-moieties to the Au NPs. From a toxicological point of view, the exposure of placenta to CuO and CdTe NPs resulted in an interference with the release of placental hormones (e.g., beta-hCG).

Finally, translocation was studied at organism level. Single Photon Emission Computed Tomography and Positron Emission Tomography were used to directly follow the uptake, distribution and release of radiolabeled ENMs in animal models. Also in this case the surface functionalization was fundamental in determining the biological fate of the ENMs. Some ENMs (e. g. Ag NPs) could always be efficiently radiolabeled while other could not (MWCN), other could be labeled only when functionalized by a certain surface group (Au-NH_2 and Au-PEG). The different surface chemistry for the ENMs resulted in distinct biodistribution and release patterns.

The outcomes from WP9 confirmed the role of core nature, size, surface coating as determinant for ENMs translocation across biological barriers from the cellular level to tissue, organ and organism level. Moreover, some of the developed models (ex vivo placental perfusion models and cultured endothelial cells expressing glycocalyx) showed to give relevant toxicological indication as the importance of avoiding exposure to CuO and CdTe NPs during pregnancy and the importance of endothelial glycocalyx integrity in *in vivo* in microvessels.

3.8 WP10 Omics methodologies

WP10 was focused on the large-scale analysis of mRNA, microRNA and protein molecules, and in particular in the changes in the transcripts or proteins in response to treatment with different ENMs. WP10 had three specific tasks: 1) to study the potential binding of nucleic acids to ENM, 2) to determine the effect of different ENMs on the transcriptomes and proteomes of selected cell types and different organisms, and 3) to determine the changes in microRNA profiles in response to different ENMs. To do this, WP10 worked closely with WP6 and WP7 that supplied the treated biological material (the human cell lines, THP.1 and BEAS-2B, mouse lung RNA, and *E. coli*) and WP11, who bioinformatically analysed the data to produce a classifier for nanotoxicity.

The binding of nucleic acids to ENMs was tested experimentally by incubating DNA or RNA together with gold ENMs with different surface modifications. It was shown that negatively charged DNA and RNA bind more readily to positively charged gold ENMs coated with amino groups than to negatively charged gold ENMs coated with carboxyl groups. The interactions do not seem to be sequence specific. Unfortunately, there exist no methods available to retrieve ENMs from cells or tissues without disrupting the nucleic acid or protein core bound to ENMs and therefore it was not possible to study the bindings in *in vivo* situations. However, the protein coronas generated *in vitro* were determined and reported by WP5.

Monocytic THP.1 and lung epithelial BEAS-2B cells were exposed for 24 hours to 31 to different ENMs by WP6. RNA was extracted and used both to determine the transcriptome

(i.e., the total mRNA profiles) and microRNA in Task 3. The transcriptome was determined by STRT-RNA sequencing and DNA microarrays. In parallel experiments using cells treated in the same way, proteins were extracted to determine the proteome changes (i.e. the changes in the amounts of the different proteins). Further, C57BL/6 female mice were exposed to all 31 ENM and total RNA extracted from the lungs by WP7. The transcriptomes of mice samples were analysed by STRT-RNA sequencing and by DNA microarrays. *E.coli* was treated with a set of ENMs by WP7 and sent to Karolinska Institutet/b for proteomics analysis. The transcriptome of *E.coli* was analysed by global RNA sequencing at the University of Plymouth. In total, Karolinska Institutet/c has sequenced 510 samples and Karolinska Institutet/b determined the proteomes of 354 samples. The normalised data from both sets of analyses have been submitted to WP11 to be integrated in the safety classifier and to data repository.

The main goal of the University of Turku (U. TURKU) partner was to study the expression profile of microRNA in monocytic THP.1 and lung epithelial BEAS-2B cells exposed for 24 hours to 31 different ENM. microRNA are small non-coding RNA molecules (RNA that does not encode for a protein) which are known to regulate more than 60% of all protein coding genes. They are involved in several important biological mechanisms including early development, cell proliferation and cell death, fat metabolism and stem cell pluripotency. Dysregulation of microRNA has been associated with diseases such as cancer, heart diseases, kidney diseases, diseases related to development and function of the nervous system and obesity. A total of 210 RNA samples isolated from the exposed cells were delivered to U.TURKU partner who studied the expression profile of the genome-wide microRNA molecules in each exposed sample using next-generation sequencing system. In order to generate good quality microRNAseq data with higher number of sequencing reads, all samples were sequenced twice. Using different bioinformatics approaches, U.TURKU performed basic analysis which included data processing and normalisation of the pooled data from both sequencing process on each sample. The normalised data were forwarded to WP11 to be integrated in the Nanosafety Classifier (WP11). Moreover, all the raw data have been made accessible and been submitted to the NANOSOLUTIONS data repository server system supervised by WP11.

3.9 WP11 Systems biology analysis

The activities of WP11 rotated around three tasks:

- Data management;
- Development of the ENM Safety Classifier;
- Systems biology analysis of ENM mode of action (MoA).

Data management was mainly carried out by the Institute of Occupational Medicine, Edinburgh (IOM), BioByte, and University of Helsinki (UH). First, the data repository was established as a central data warehouse harbouring any protocols, data, and results originated from the activities of the whole consortium. Second, templates for storing the data were established and disseminated to the other partners. This ensured standardization and compatibility with other repositories outside the project. Attention was given to homogenize the data templates with those proposed by the FP7 project eNanoMapper and special agreement was established to ensure data and protocols transfers.

Development of the ENM Safety Classifier was mainly carried out by UH. Novel algorithms and computational methods were developed. Particularly, a novel multi-view adaptive genetic algorithm (MAGA) was established as the main method for carrying out the task of feature

selection and evaluation of the predictive models. Rich multi-view data retrieved from multiple partners (mainly in WP3, 6, 7, and 10) were preprocessed and further analysed in search of the minimal set of features able to predict the toxicity levels of the 31 ENM, tested both *in vitro* and *in vivo* in multiple exposure scenarios. Several models were established being able to predict cytotoxicity, genotoxicity and overall toxicity potential of ENM with high accuracy (> 90%). In collaboration with WP12, a set of computational validations were carried out on data retrieved from the FP7 project MARINA as well as on collaborative data from Health Canada.

Systems biology analysis of ENM Mode of Action (MoA) was mainly carried out by UH, UniSa, and TIGEM. Several computational methods were established to carry out systematic analysis of ENM MoA, as measured with omics technologies. Attention was given to the possibility to contextualize ENM MoA with respect of the MoA of drug treatments, chemical exposure and human disease. Further detailed analysis has been planned in future collaborative publications with several NANOSOLUTIONS partners (Karolinska Institutet, University of Plymouth, TNO).

3.10 WP12 Safety Classification

This work package has developed the ENM Safety Classifier by expanding further the concept and design and implementing it as a usable computational tool; through the prototyping and testing of the tool; and, based upon the resulting design and data requirements of the tool, it has developed a specification for a high throughput system for future testing and analysis of further and new ENMs. The further development of a formal conceptual model and design for the tool by the partners in this WP depended upon access to large volumes of data and information being readily retrieved and abstracted from the data repository in WP11, as well as to other knowledge from preceding WPs. It related particularly to the data management and analysis carried out in WP11, and the involvement in WP11 of IOM (as WP12 leader) – and similarly the Finnish Institute of Occupational Health (FIOH) as the tool developers – has greatly assisted the data availability and its flow.

The ENM Safety Classifier is a computational based predictive tool for the assessment of ENM safety. The tool will facilitate the prediction of hazard classification based on a minimal core set of input data, and is intended to be widely applicable by industry, (in particular to SMEs) regulators and academics involved in the development of materials. This task is linked with Task 11.1 (WP11). Development of the underlying data algorithms was done in WP11, based on analysis of extensive data collected in the project. These algorithms were linked to the minimal core dataset (identified in WP11) with the classification outputs. Key subtasks achieved within this task include (i) the development a specification of the optimum output classes which were achieved with an options analysis in consultation with the stakeholder groups. Options range was from a simple yes/no to consideration of dose for different endpoints, boundaries, uncertainties and the level of information which should be supplied by users. A stakeholder consultation/workshop was arranged to refine this specification. (ii) The development of a specification of the optimum inputs based on interaction with WP11. In this we identified the kinds of input data were necessary, such as, physical/chemical/morphological data, toxicological and ecotoxicological endpoints. Based on this specification, a data interface was developed to link the output data of WP11 and the input data for the Classifier. Based on these decisions, the algorithm which translated these inputs into safety classifications was implemented. This process has identified the optimal set of data which need to be collected and the format of the data. (iii) The Classifier was calibrated with existing complete data sets containing all of the required data inputs in addition to definitive measures of toxicity.

The Classifier was validated using data from FP7 project MARINA, using the silver nanoparticle, after consultation with industrial partners holding relevant, existing or under-development (industrially focused data sets). The validation of the Classifier consisted of collecting sorting and cleaning the data, running the evaluations, assessment and calibration of the validity of outputs. The Classifier's classification for silver nanoparticle as a material of 'medium' toxicity was accurate as this is well understood in nanosafety research. The Classifier functioning and the process of model validation were demonstrated in a workshop to the expert team from within the project stakeholder group.

A two-phased high-throughput testing platform was suggested to couple diverse technologies and assays with the NANOSOLUTIONS Classifier to group ENMs into low, medium or high toxicity materials. Data produced from cell culture model assays enabled initial and rapid generation of diverse dose-response and condition-dependent measures of the cytotoxicity and genotoxicity of ENMs. Scoring related to effective dose and detectable effects in the cell models enables a first level of "toxic potency" ranking of the ENMs. At selected exposure levels based on this ranking, quantitative PCR-based assessment of seven mRNA species predictive of cytotoxic and genotoxic potency thereafter enables completion of the toxicity classification via a second set of experiments. The selected mRNA species were chosen from the minimal feature set identified by the Classifier on the basis of providing better algorithm-based grouping of the ENMs than sequencing-derived mRNAs, miRNAs, proteins, corona proteins, and intrinsic ENM physiochemical data, and finally, from the ease of rapidly and reproducibly analyzing mRNA species quantitatively. The overall grouping assessment strategy of the platform thereby includes three high-throughput angles to the toxicity classification: initial dose-response cytotoxicity and genotoxicity analyses are coupled thereafter with expression analysis of mRNAs demonstrated by bioinformatics methodology to capture outcome of the cytotoxicity and genotoxicity effects, including separately and in combination. Applying more of the vast data generated in the project under machine learning principles enables further development of the Classifier as it becomes applied to independent EMN safety testing data in the future. The suggested platform would serve to effectively classify a corresponding number of ENMs as those assessed over four years in the NANOSOLUTIONS project within a time frame of 2-3 months, based overall on the generation of potentially 10^4-10^5 data points at high throughput.

4 The potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and exploitation of results

Introduction

In its 2020 strategy, the EU highlights nanotechnology as one of the key enabling technologies to promote smart, sustainable and inclusive growth throughout the EU and to ensure that the EU becomes the most competitive global knowledge-based society, providing prosperity and social stability for its citizens.

ENM and the technologies which utilise them are one of the critical pathways towards the creation of strong, competitive, and diversified manufacturing value chains. However, well-documented issues regarding the safety of ENM have given rise to increasing concerns, not

only for regulators and the general public, but also for the industries who are or may develop and use these materials.

Uncertainties related to the safety of ENM and associated technologies represent a major barrier to innovation based on these technologies, restricting the extent to which these materials have been exploited through the value chain. Hence major potential economic benefits resulting from materials, processes and products based on ENM have been lost or at least significantly delayed. Therefore, it is vitally important that these barriers should be overcome to open up these value chains and unleash the full economic potential of ENM.

A sound science-based foundation from which one can build a reliable and affordable safety classification of ENM and nanotechnologies is needed. This means gaining a clear understanding of the relationship between nanomaterial characteristics and their properties, such as their surface chemistry, as well as being able to identify the effects they can evoke in living organisms. The life cycle of the ENM used in different products must be considered.

Reaching these goals would remove a major obstacle to the exploitation of the full potential of these materials and the related technologies. In this way, the NANOSOLUTIONS project can help to achieve the goals of the EU 2020 Strategy in terms of promotion of industrial growth and the wellbeing of EU citizens.

Main achievements

In the Exploitation Strategy Seminar and Dissemination Workshop in February 2016 three key-exploitable results (KER) were identified. They have been described below.

Nanosafety Classifier (KER1)

NANOSOLUTIONS has created a state-of-the-art tool for safety assessment of nanomaterials – the Nanosafety Classifier. It represents a significant advancement from the currently available predictive models, with over 90 per cent accuracy.

The Nanosafety Classifier is a computational tool that can predict the environmental and health impact of nanomaterials based on their characteristics and behaviour. It is the first predictive tool able to identify the relevant information needed to regulate the safety of nanomaterials. The tool is continuously learning and the predictions keep improving as new data is fed into it.

This tool will reduce the cost of risk assessment for industry in using ENMs for their innovative new products and systems, at the same time facilitating the use of new materials. NANOSOLUTIONS will allow industry to change the way it carries out activities related to nanosafety, enabling it to incorporate safety as a part of its core business philosophy and working culture when dealing with ENMs. The classification tool will also help reduce the bureaucratic bottleneck that exists for ENM products by speeding up the safety approval process.

This novel, hazard-profiling model can be used by manufacturing industries and the regulatory community to reduce uncertainty and bring clarity to the current debate about the safety of nanomaterials. It does this by providing the potential to effectively “de-classify” (in terms of safety risks) many types on ENM in many applications.

Advances in safety assessment help regulatory bodies to develop robust and scientifically valid regulatory requirements that can be used at EU and national levels. The Nanosafety Classifier can be integrated into product development processes, which will help to improve the efficiency

and speed of the innovation process by letting manufacturers harness the concept of “safe by design”.

More than 30 previous EC-funded projects have examined the toxic effects of ENM in mammals and other species, provided technology solutions for delineating exposure to ENM and provided direct support to regulate ENM. NANOSOLUTIONS is, however, the first EC-funded project to have developed a general safety classification of ENM, based on an in-depth understanding of the characteristics of these materials and the mode of interactions of ENM across species, examining fundamental biological effects at the cellular, organ, and organism levels.

The chemical trading market globally is worth $ 5 trillion annually. Toxicology assessment and testing are less than 5% of this number with the share of nanomaterial hazard assessment being about 200-350 million annually. The classifier provides huge commercial potential for reducing costs and time in this arena, by offering industry safety classification services cheaper and quicker than what currently exists. Algorithms that enable the classifier are currently being considered for patenting, and a spin-off company is in advanced stages of discussion (with industry involvement).

Nanoparticles that kill cells have huge potential in drug development. Being able to predict the toxicology of particles can be used in “drug likeness” programmes. A NANOSOLUTIONS partner is exploring this area.

High-throughput screening platforms for engineered nanomaterials (KER2)

One of the tasks of the project was to establish high-throughput screening, HTS, for ENMs. Selected in vitro assays, using selected cell lines, have been optimised for HTS. Both companies involved in this work – Misvik and BioTeSyS – have added customer value from the incorporation of systems biology approaches to the safety evaluation of nanomaterials.

Huge numbers of ENMs will need to undergo safety testing in the future as more and more of them are used in everyday products. The estimated size of the nanotechnology market is at several tens of billion euros in the EU alone. ENMs are applied in a variety of business areas central to European business and society at large, so far without consistent implementation of a “safe-by-design synthesis” concept that integrates early safety evaluation. “Safe-by-design” production of ENMs through a reliable systems toxicology approach has the potential for enormous societal (health, life quality, environment, etc.) and economic impact.

Misvik Biology and BioTeSyS can now provide high throughput screening and systems toxicology-based services to the rapidly growing nanotechnology market. This will allow companies to test new ENM for their hazard potential much quicker and cheaper than ever before, lowering the cost of development and ensuring their products are safer by design.

Software for life cycle assessment (LCA) (KER3)

NANOSOLUTIONS has generated data that could contribute to improvements on methodologies used in the standard software used for Life Cycle Assessment (LCA). Data generated on release materials (nanomaterials quantification and identification) will contribute to future updates of methodologies used in the current life cycle analysis to be able to better assess and understand the global environmental impact that these materials have on the environment. After full implementation and improvements on the models and methodologies included in the standard life cycle analysis procedures, there will be an immediate impact on different users, such as industries developing nano-enabled materials (e.g. medical, construction, food, textile, ceramics, automotive, paints) or industries in charge of nanomaterial production that will get products into the market following circular economy principles. And as

until now these LCA software-based tools will also be used and run by environmental consultancy companies.

Existing LCA software (e.g. SimaPro) does not consider nano-specific characteristics. This is extremely important because nanomaterials cannot be treated the same as their macro counterparts or common chemical molecules. Therefore, all the efforts to generate data on the impact of the nanomaterial releases into the different environmental compartments and in human health will be of high importance in the area. With the modified version of the LCA software tool we will be able to assess the impact of using nanoadditives in conventional products and compare them with conventional materials.

Data

NANOSOLUTIONS has produced the largest dataset of cytotoxicity-immunotoxicity-genotoxicity to date. This huge amount of data is valuable to ongoing work and research in this field. Work is ongoing with eNanoMapper to ensure this data is included in the project's collaborative data sharing ecosystem currently being developed. The collaboration within the NSC will further extend the possibilities to utilize the data as well as other project results.

Dissemination activities

The dissemination and communications work carried out during the NANOSOLUTIONS project was designed to provide impact amongst the interested target audience as well as to raise visibility of the project at a regional, national, European and global scale. Several specific target groups were identified:

- Private sector actors: potential and current producers, suppliers, investors, stakeholders etc. and a range of industrial groups that will have distinct perspectives of the nanoparticle toxicologists.
- Public sector: decision makers, regional, national and European authorities linked to nanotechnology and potential uses for nano solutions.
- Mass media: will serve as a dissemination instrument to spread project milestones, results and impact.
- End users: Industry, manufacturers, general public and consumers
- Scientific community: has an interest in the project's findings for future developments

The dissemination process aimed to spread information among all potentially concerned stakeholders and to all levels of policymakers, as well as to certification and standardisation bodies and engineering/industrial organisations.

This approach was helped by the research and academic organisations involved in the project and the organisation of user and stakeholder interest groups. Insight Publishers Ltd (IPL) also made use of its existing extensive database that cuts across all relevant thematic sectors.

A database of target user groups was developed to enable strategic communications that were targeted for each identified group, using specific relevant information.

Scientific publications, printed and online press, and participation in conferences

Project partners were involved in the dissemination of research project results through publications in peer-reviewed journals, magazines, e-journals, and also by means of presentations and demonstrations in conferences, workshops, and exhibitions.

Publications describing the overall project aim, the consortium and the on-going activity were inserted in professional magazines on nanotechnology and safety classification.

Project activities were reported in three releases of "Projects Magazine", a leading European research and innovation publication. Delivery in print and digital format to 45,000 subscribers included targeted email alerts and newsletters highlighting NANOSOLUTIONS.

By the end of the project the partners reported 65 scientific publications (see http://nanosolutionsfp7.com/publications/published-work/); 139 oral presentations and 43 posters in various meetings and conferences, and 28 other dissemination activities, such as organization of a workshop, newsletters and press releases. Many publications are still on the preparation phase.

Conferences organized in the frame of the Project

The NANOSOLUTIONS project organized, or contributed in the organization of several conferences. The *International Congress on the Safety of Engineered Nanoparticles and Nanotechnologies* (www.ttl.fi/senn2015), was organized in Helsinki, on 12-15th April 2015, attracting around 200 international experts and scientists around the world. The Congress provided a great forum for sharing the results of the project so far and discussing the latest knowledge on the safety of engineered nanomaterials and nanotechnologies. In addition, various articles and newsletters were disseminated to the target audiences, and social media was in active use.

The NANOSOLUTIONS project in collaboration with the Working Group on Systems Biology of the EU NanoSafety Cluster organized the *System Biology in Nanosafety Research Nobel Forum Conference* on 9–10th November 2015 in Stockholm. Topics covered were omics technologies and computational approaches, and the application of these tools and approaches in nanosafety research. Several EU-funded nanosafety projects were represented among the speakers.

At the end of the project a Joint Conference of five major EU nanosafety projects entitled *New Tools and Approaches for Nanomaterial Safety Assessment* was organized in Malaga on 7–9th February 2017, with around 230 participants. In addition to the high-quality scientific part of the Conference a great effort was made in organizing a high-level panel discussion with representatives from regulatory bodies, decision makers and end-users. The participation of 14 panel members at the Conference for its whole duration was of vital importance in disseminating information to the various stakeholders.

The NANOSOLUTIONS project in collaboration with the Academy of Finland organized a workshop on nanosafety in the EuroScience Open Forum, ESOF on 22–27th July 2016.

Furthermore, the project is contributing to a nanosafety workshop in the EuroNanoForum 2017 to be organized on 23rd June 2017 in Malta. NANOSOLUTIONS WP leader Bengt Fadeel, Karolinska Institutet, is one of the main organizers of the workshop and will represent NANOSOLUTIONS in the event. The workshop entitled *Delivering safe nanotechnology to market* is dedicated to presentation, discussion and interaction with actives nano-sector players on the challenges and industry needs of bringing nanotechnology to market. Delegates are given an insight into the state of the art, while examining the changing regulatory framework, policy perspectives and societal expectations.

Targeted visits to promote NANOSOLUTIONS and NanoSafety Cluster (NSC) visibility

The NANOSOLUTIONS coordinator Kai Savolainen (also coordinator of the NanoSafety Cluster) has carried out targeted visits to key partners and global players in global nanosafety.

In 2013 spring he visited Australia and met civil servants responsible for nanosafety in Australia in CSIRO in Adelaide and Sydney. During a two months visit in Brazil in 2014 he had a meeting at the Brazilian Ministry of Research, Technology and Innovation in Brazil, the capital with Dr. Anna Gabriella Tempesta to promote collaboration between the project and the NSC. In autumn 2016 he attended the ProSafe Meeting in Paris representing FIOH, NSC but also NANOSOLUTIONS. He has also visited frequently the US and met there the Deputy Director General of National Science Foundation in Washington, D.C. (2013 Dr. Sally Tinkle; NANOSOLUTIONS IAG member, 2015 and 2016 Dr. Lisa Friedersdorf). The meeting in 2015 took place after the SOT Annual meeting, and the visit on 2016 after the Nanotoxicology meeting held in Boston on 1–4th June. He also met Dr. Tinkle in March 2017 after the SOT Annual Meeting in Baltimore. Dr. Tinkle currently works at the Science and Technology Institute in Washington, D.C. The US Federal Government currently outsources analytical work related to nanosafety and industrial collaboration as well as innovation and risk management of nanosafety. The Coordinator also visited the *Nanotechnologies: Occupational and Environmental Health Conference* held in Limpopo, close to Johannesburg in South-Africa in October 2015 to make contacts with, among others, South African scientists and regulators and those from around the world. All these dicussions have been highly useful and promoted the awareness of the project as well as of the NSC to various decision makers and regulators.

IAG collaboration

International Advisory Group collaboration was an important means to advance contacts to regulatory bodies and other main stakeholders of the project. The participation in the project meetings of DG GROW representative Ms. Maila Puolamaa was extremely active and the project received highly relevant comments from her and was mutually kept informed about what was going on in the Commission. When joining the IAG, Dr. Sally Tinkle represented US National Science Foundation. NSF has a remarkable influence on regulatory issues in the US even though it is a scientific body. The project also had a regulatory input from Japan (Dr. Jun Kanno, Director General of the Japanese Regulatory Research Institute) and Thailand (Dr. Sirirung Songsivilai, Director General of THAI NANOTECH, an organization of 1500 nanotech experts close to Bangkok). Dr. Jean-Marc Aublant represented CEN, and brought the European/global standardization view into the project. Also ECHA was approached and they accepted to be a member of the IAG but unfortunately they decided not to participate in the project work. The IAG further had representatives from BioNanoNet, ETPIS and NIA. Through the composition of the IAG a lot of thought was given to stakeholder and regulatory collaboration which was global in nature.

Graphic design and web development

A visual identity for the project was developed to reflect the project vision and key concepts, as well as to create an easily recognisable “image” to improve the project visibility. The project logo has been used prominently in all dissemination tools and printed materials. All communications materials from the project had consistent use of colours, shapes and messaging. All partners received branding guidelines from dissemination partner IPL as part of this exercise to ensure the brand consistency was maintained throughout the project and with every communication.

The NANOSOLUTIONS website was developed, and was the main promotional tool for publishing research results and documents. Use has been made of social media and news items within the NANOSOLUTIONS website to keep a regular flow of project news moving out to various online communities. There was an established process in place for all partners to

publish their latest information in order to ensure that up-to-date information was shared between the project and the external actors.

Newsletters

Seven digital, interactive newsletters were designed to keep the targeted audience fully up to date with the progress of the project. These were prepared and distributed electronically over the course of the project in order to support the dissemination strategy and secure an effective information exchange between the members of the project consortium, policy makers, stakeholders and the scientific community. They provided information on milestones and publications achieved in the project, as well as relevant developments in respective policy areas. They were directed at consortium members and their networks, industry, policy makers at European and national levels, stakeholders, participants in related research projects and the research community.

It is estimated that the eNewsletters reached around 43,000 subscribers.

Press releases

Thirteen press releases were used to diffuse information about NANOSOLUTIONS to a wide range of stakeholders including the general public. A series of general press communications were planned, while other opportunities for press releases were identified in conjunction with major milestones.

IPL identified specific multimedia contacts throughout Europe from its own database. These contacts had specific interest in and influence in this area and the database cuts across all mainstream European press, TV and radio as well as specialist publications and programming.

- Total number of delivered press releases: 14,495

IPL recorded 15 online coverage items following the press releases, and these brought:

- Online readership: 2.64 Milloin
- Estimated coverage views: 17,000
- Social shares: 309

Videos

Six videos produced in the project aimed to reach a wide range of target groups. Stakeholders and policy makers were targeted through the development of an institutional film for use at fairs and conferences. Civil society and the wider public were targeted through a video news release loaded on to major internet video channels such as YouTube and Vimeo. Various animations were also developed during the lifetime of the project.

Videos included:

- Introduction to the NANOSOLUTIONS project
- Interviews with key partners
- Safer by design video
- Recording of the ‘Dragons’ Den’ style session held between various partners
    - Nanosafety Classifier
    - Life Cycle Analysis
    - High Throughput Screening

Social media activity

- Twitter

The NANOSOLUTIONS Twitter profile has been used for four years and is currently followed by over 1,300 users. On average four posts a week have been completed on the Twitter account.

Some of the most successful tweets involved the infographics prepared by IPL in January 2017. All infographics described the key issues and important tools in the latest nano safety research and were branded with NANOSOLUTIONS logo. The infographics were widely shared, liked and viewed both on Twitter and LinkedIn.

- LinkedIn

The internal NANOSOLUTIONS group was active for two years, and involved 238 members. On average 5 posts and discussions were initiated each month, and it covered the latest information on the newsletters, press releases, latest articles and news stories as well as any ongoing issues.The information was also shared through public (external) groups and reached large numbers of people directly involved in European research.

Infographics

A series of five infographics were produced about some of the key concepts of the project. These explained in simple, accessible ways the context in which the project was taking place (the need for nanosafety assessment), as well as its three main results, the classifier, high-throughput screening and life-cycle analysis. These infographics were delivered successfully to a large, general public audience through social media and press and gained around 10,000 impressions on all platforms.

SEED

The SEED Research Library is a web-based hosting site developed by IPL where “SEEDs” of projects are displayed in thematic context. Users are able to follow those SEEDs in which they have an interest.

The NANOSOLUTIONS SEED was first hosted in the Library in month 16 and it is an ongoing activity. It has grown dynamically throughout the project’s lifespan and will be active for two years after the project. It will then be hosted in an archive section of the library, creating a permanent record of the project’s work and contact information of the partners.

Printed materials

Flyers that described the project’s main objectives, steps and expected outcomes were produced and printed as well as being made available for download. The flyers were distributed among partners to facilitate the promotion of NANOSOLUTIONS within the general scientific community and industry during workshops, conferences, seminars and personal communication. Flyers were also adapted for individual partners or events.

Posters were produced for events, workshops and conferences at which NANOSOLUTIONS had a presence.

A roll-up banner explaining project’s objectives was also designed and printed to use at events and conferences. To ensure dissemination continuity after the project’s end, the banner will be used in IPL’s booth at EuroNanoForum2017 in Malta in June 2017.

5 Project public website and contact details

http://nanosolutionsfp7.com/

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