NeuroNano

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Part A

The EU FP7-funded project *NeuroNano* conducted an investigation into how nanoparticles interact with the human brain. The overall aim was to establish a risk assessment framework to categorise nanomaterials in terms of their potential for access to the brain, and potential for harm once there.

Two potential sources of impact from nanoparticles were investigated: the potential or the nanoparticles to generate so-called reactive oxygen species (ROS), and the potential of the nanoparticles to induce or promote protein mis-folding and/or fibrillation. Note that ROS form as a natural byproduct of the normal cellular metabolism, but that during times of environmental stress (e.g., UV or heat exposure), ROS levels can increase dramatically.[1] Deposition of insoluble fibrillar protein aggregates in the brain and neurons has been observed as a major pathological change in many neurodegenerative diseases, although it still remains controversial whether such aggregates are a cause or a symptom of the diseases.[2] Thus, the key question being addressed within the *NeuroNano* project was whether the presence of nanoparticles in the brain would exacerbate existing toxicity mechanisms, and if so, if we could identify key physico-chemical properties that contributed to these mechanisms, in order to design safer nanomaterials.

Neurodegenerative diseases affect 1.6% of the European population and the number of people contracting these diseases is increasing dramatically as the population of Europe ages. It has been suggested that engineered nanoparticles that appear in environmental pollutants could pose a threat to human health and may be linked to neurodegenerative diseases, such as Parkinson's and Alzheimer's. For example, there is some evidence that there is an increased incidence of these types of diseases in heavily polluted cities.[3]

The *NeuroNano* project resulted in a number of key findings:

- The existing body of research into the impacts of nanomaterials was not sufficient for the project partners' purposes. As a result, they had to produce their own evidence base from scratch, using both cellular systems and carefully selected animal studies.

- Despite the brain being a "sacrosanct" organ which most particles cannot enter, very small quantities of nanoparticles can pass through the blood-brain barrier (BBB). However, there is no evidence that the amounts of engineered nanomaterials that pass the barrier are sufficient to affect human health.

- Several new modes of interactions of nanoparticles with the BBB were observed, using the most advanced microscopy every applied to such systems. For example, a propensity for nanoparticles to accumulate in the lysosomes of the BBB endothelial monolayer was observed. Additional work is required to understand if similar effects are also observed in animals, as current approaches to quantify uptake *in vivo* do not distinguish between the nanoparticle load in the brain versus in the endothelium separating the brain from the blood (i.e. within the BBB).

The *NeuroNano* project's findings are being fed into future standardisation and regulatory measures regarding the production and application of nanoparticles, as part of on-going efforts to promote the sustainable and responsible development of nanotechnology and to improve the life quality of Europe's ageing population.

Key outputs include a range of radiolabelled and fluorescently labelled nanoparticles (which are being further developed in the FP7 Infrastructure project QualityNano, several new approaches for assessment of nanoparticle-protein and nanoparticle-cell and barrier interactions, a large set of data that is feeding in the EU FP7 modelling project NanoTransKinetics.

References:
[1] Devasagayam, TPA et al., *J. Association of Physicians of India (JAPI)* 2004, 52: 796.
[2] Walsh DM, Teplow DB. *Prog Mol Biol Transl Sci.* 2012, 107:101-24.
[3] Calderón-Garciadueñas L, et al., *Toxicol Pathol.* 2007, 35:154-62.

Summary description of the project context and the main objectives.

The overall science and technology objective of the *NeuroNano* project was to determine if engineered nanoparticles could constitute a significant neuro-toxicological risk to humans for two diseases endpoints, namely Alzheimer's and Parkinson's diseases. Importantly, the *NeuroNano* consortium did not presume a neurotoxic hazard from engineered nanoparticles / nanomaterials. Instead, the project sought to understand if exposure to nanoparticles resulted in them reaching the brain, and if so under what circumstances. Additionally, the project aimed to understand whether the presence of nanoparticles in the brain could exacerbate or accelerate existing cellular mechanisms such as the rate of generation of Reactive Oxygen Species (ROS) or the rate of fibrillation of proteins involved in the neurodegerative diseases of interest, amyloid-beta and alpha-synuclein, respectively.

The project was designed to be compliant with the European Commission's policy on reduction, replacement and refinement (the 3Rs) of the use of animals in scientific testing and experimentation. As such, much of the work was performed using *in vitro* methods, many developed specifically within the project, and which are now being further refined for onward development towards standardised alternative tests, both within the QualityNano research infrastructure project (www.qnano-ri.eu), and in other EU projects such as MARINA (www. www.marina-fp7.eu/). Where animal studies were necessary (such as for assessment of the distribution of nanoparticles following exposure via different routes, and to assess the impacts of nanomaterials in the brain on animal behaviour and amlyloid plaque load), there were designed to correlate and potentially confirm *in vitro* findings, and were designed to minimise the numbers of animals needed whilst still being scientifically robust. The tiered approach to the three stands of work relating to the assessment of neurodegenerative disease (solution studies, cell studies, and finally animal studies) represents a scientifically and ethically balanced approach to the work, balancing the necessary level of scientific excellence with the need to reduce the numbers of animal experiments.

The *NeuroNano* project built on important findings from a previous EU FP6 project, NanoInteract[1], which had identified the nanoparticle protein (biomolecule) corona as a basis for characterising what cells (organisms / animals) actually "see" and interact with. It is now well accepted that in typical biological environments, nanoparticle surfaces are covered by ambient proteins, resulting in a well-defined long lived 'hard corona', which obscures the bare surface in early nanoparticle.[1, 2] Within *NeuroNano*, the hypothesis was that the *nanoparticle corona could form the basis of a new structure-activity relationship for neurotoxic cellular impacts of nanomaterials*. It is thus believed that one should correlate the properties of the corona to the biological activity, rather than the material properties themselves. Within the NeuroNano project efforts were made to establish such corona-activity relationships (intra and extracellular) for a range of cell types, as well as tissue samples. This is important because, if the logic chain holds – the biomolecule corona around a nanoparticle determines the biological identity, which determines the degree of oxidative stress and amyloid load, and the mapping onto disease. Ultimately the corona and its re-arrangement is the key factor to determine hazard. It is hard to avoid this conclusion as all the evidence points towards materials themselves never coming into direct contact with living matter in a biological milieu.

Another important finding from the NanoInteract project was that nanoparticles could influence the rate and mechanism of protein fibrillation, as investigated using a combination of the thioflavin T (ThT) assay and Electron microscopy imaging. Several particle-protein combinations had previously been investigated, and both enhancement and impairment of the rate of protein fibrillation have were observed in the presence of nanoparticles.[3-5] The mechanism of nanoparticle impact of protein fibrillation is interesting; the surface of the nanoparticle acts as a sort of 'catalyst' in which it sheds small clusters of pre-fibril clusters, oligomers of different sizes, and some of these constitute critical nuclei for further growth of the clusters. This mechanism is itself of some concern, for many specialists now consider that it is the protein oligomers that are neurotoxic species, not the fibrils, in Alzheimer's

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[1]http://www.nanosafetycluster.eu/eu-nanosafety-cluster-projects/sixth-framework-programme-projects/nanointeract.html

disease[6, 7] and this has long been one of the prominent views in Parkinson's disease also.[8-10] The findings evoked a strong response in the research and regulatory community, likely because they highlighted a novel mechanism (completely unexplored) that could present a new form of hazard for which there is currently no conventional toxicological test, and no research reported.[11] Within the NeuroNano project the connection between nanoparticle physic-chemical characteristics and protein fibrillation was investigated as part of a set a graded studies to determine the ease with which fibrillation occurs for the relevant particles, producing a 'fibrillation score', termed Amyloid Fibril Generation Potential (AFGP), by analogy to the Free Radical Generation Potential score for oxidative stress (see below).

At the time of development of the *NeuroNano* project, much of the knowledge of nanoparticle-induced oxidative stress has been derived (or deduced) from environmental or occupational exposure to small particulates including asbestos, coalmine dust, quartz, and PM10.[12-14] Oxidative stress caused by particles and other sources is capable of graded (dose-response-related), effects in cells.[15, 16] At low level there may be only very mild effects that are dealt with by the cells constitutive anti-oxidants and are not 'sensed'. At higher levels there can be activation of anti-oxidant defences, like catalase, to deal with mild oxidative stress. As the extent of oxidative stress increases, there is activation of stress-responsive signalling that leads to changes in gene expression for inflammation in epithelial cells, for example. At very high doses there can be cell death by necrosis or apoptosis. Thus, there were several pathways by which nanoparticles might generate (and respond to) harmful oxidative stress in the brain:

1) Direct effects of nanoparticle-derived free radicals on neurones after contact or entry into them;

2) Contact or entry into neurones causing proteasomal or mitochondrial dysfunction;

3) Uptake by microglial cells causing activation of NADPH oxidase and an oxidative burst.

If indeed oxidative stress is an important mechanism in the brain, then it was anticipated that those particles most active in the generation of free radicals production would be more harmful than others.[17] However, given the complexity even in cell systems, it was possible that oxidative stress might be correlated not just with the degree of necrosis/apoptosis, but also with mitochondrial dysfunction, proteasomal dysfunction, induction of antioxidant defence, respiratory burst and inflammatory gene expression by microglial cells. To form a coherent picture the *NeuroNano* team proposed the application of gene arrays, proteomics and redox proteomics to determine the nano-particle-induced perturbation of cellular metabolism and oxidative stress pathways.

Whilst the precise mechanisms leading to neurodegenerative diseases are still not fully clarified (and were even less so at the time of initiation of the NeuroNano project), it is broadly agreed that the key effects involve the presence of early pre-fibrillar structures, neuroinflammation and ROS-related processes. All of the links in the causal chain are now present for a credible expectation that nanoparticles could have impacts on the onset and progression of neurodegenerative diseases, and thus present a neurotoxicity risk of greater or lesser significance depending on their physico-chemical composition and potential to access the brain.

Alzheimer's disease is characterized by loss of short-term memory and visual-spatial skills. It is diagnosed clinically based on characteristic neurological and neuropsychological phenomena. There is as yet no commonly accepted marker for the disease, and certain diagnosis is made only post-mortem. Recent research has generally considered the disease is caused by misfolding of either amyloid β or tau proteins.[18] Evidence that amyloid β is the key is provided by the fact that the Down syndrome patients (who almost invariably progress to Alzheimer's at an early age[19]) have an additional copy of the gene for amyloid β precursor. There is also growing support for the hypothesis which suggests that the cytotoxic species is an intermediate oligomeric misfolded species of the protein, rather than the mature fibrils. It may be that Alzheimer's is primarily a disease driven by extracellular events where by Amyloid Precursor Protein (APP) is cleaved leading to release of the amyloid β fragment containing residues 1-40/42, and that the resulting extracellular amyloid β then forms the toxic oligomers (pre-fibrils) leading to the disease. Indeed the delivery of biological extracts containing these oligomers to mice leads directly to Alzheimer's-like symptoms.[6] Exposure to environmental toxins is believed to

increase the likelihood of developing Alzheimer's disease, and there have been persistent claims that the ROS mechanism is also involved, but the manner is ill-understood as yet. Studies in cities with high pollution loads, where there are significant loads of nanoparticles of the size that can access the brain have suggested that Alzheimer's could be associated to this form of pollution, and it is now known that nanoparticles can dramatically affect the rate of protein fibrillation (including amyloid β).[3]

Parkinson's disease is characterized by muscle rigidity, tremor, a slowing of physical movement, followed by (in extreme cases) a loss of physical movement. Neurons in diseased tissue generally contain Lewy bodies (dense aggregates involving proteins α-synuclein and Parkin). Parkin, a protein responsible for targeting damaged proteins for destruction in the proteasome, is believed to involved in the onset of the disease, leading to protein accumulation, ER stress and production of ROS, resulting in an increased misfolding of α-synuclein. There is sufficiently broad consensus that protein fibrillation is at the root of the disease that it is a major target for drug therapy. In general Parkinson's disease is believed not to have been common until the beginning of the Industrial Revolution. There has thus long been a suspicion that toxic substances might lead to generation of ROS and possibly represent some sort of co-factor for the disease.[20, 21] In summary then, besides the physical presence of any foreign body in the neurons, the additional elements of α-synuclein fibrillation and ROS are quite clearly issues of central concern.

The key conclusion was that oligomeric protein fibril clusters and oxidative stress are both believed to be the cause or (in the cases of ROS) significant contributing factors in neurodegenerative diseases.[22, 23] Thus, according to our understanding, all of the key warnings signals for neurodegenerative hazard were present at the time of formulating the project: nanoparticles can reach the brain, and when they get there, evidence (*in vitro* and in some cases *in vivo*) suggests that they are able to trigger the two key processes (oxidative stress and accelerated fibrillation) associated with neurodegenerative diseases.

To this end, the overall objective of the *NeuroNano* project was divided into eight scientific objectives, as follows:

1. Develop nanoparticles that have appropriate labels of sufficient activity and duration for neurotoxicological studies. In particular, NeuroNano aimed to develop nanoparticles that retain their label activity (radio, fluorescent etc.) over time periods of up to two or more years.(WP1)

2. Identify classes of nanoparticles that lead to either (a) oxidative stress in relevant cell models (WP2); (b) increased rates of fibrillation (WP3); (c) up-regulation of the relevant pathway proteins involved in the diseases (at the cellular level) (WP4). These explicit measurables of oxidative stress and amyloid load (in solution, in cells), validated by animal models (WP4) will provide the basis for identification of hazard for those particles (WP5).

3. Identify the physiochemical properties, and surface expression properties that lead to given levels of oxidative stress and amyloid load. *NeuroNano* sought to correlate the physiochemical properties with (a) increased rates of fibrillation; (b) up-regulation of the relevant pathway proteins involved in the diseases (at the cellular level); or (c) oxidative stress in relevant cell models. *NeuroNano* also sought to test the hypothesis that the composition, organization, and reorganization time scales of the biomolecule corona (the proteins that adsorb to nanoparticles under physiological conditions) can be more naturally correlated to these key biological impacts, rather than any specific set of material properties. (WPs 1- 4)

4. Understand what constitutes a lead nanoparticle candidate for passing the Blood Brain Barrier (BBB). The original supposition was that not all nanoparticles that could be toxic will in fact pass through the BBB. Indeed, preliminary data suggest that there is a very great dependence on particle type and size, and these effects must be investigated and understood. Part of our strategy here was to carry out early screening of the particles using the *in vitro* BBB model in parallel with limited animal trials. *NeuroNano* also sought to clarify if the nanoparticle corona is more naturally correlated with the ease of crossing the BBB that the underlying nanoparticle physico-chemical properties. (WP4)

5. Quantify transport efficiency to the brain. *NeuroNano* sought to quantify the amount of nanoparticles that arrive in the brain (of small animals) from each of the potential exposure routes (inhalation, instillation, ingestion, intravenous). Furthermore, *NeuroNano* sought to show how this depends on the material, again both in the conventional sense (size, zeta potential etc.), and on the surface expression, or evolving protein corona. (WPs 1, 4)

6. Understand the detailed pathways that nanoparticles take to reach the brain. The animal studies were also intended to provide as much information as possible about how the nanoparticles reached their destination, including the detailed nature of what is expressed on their surface as they pass through different intermediate tissue types on going to the brain. Thus, even if no neurotoxicity was observed, the investment of resources, and animal experimentation would result in significant new data and a deeper understanding of the *in vivo* biodistribution of nanoparticles and the role of the protein corona. (WPs 1, 4)

7. Determine if there are indeed neurodegenerative disease endpoints for a range of animal models, and relate the extent of those endpoints to the amounts and properties of nanoparticles that have reached the brain. If, according to the usual measures of diagnosis (cognitive, behavioural and pathological), nanoparticles were found to affect the time of onset, or extent of the disease, then several examples of arrival dose versus response (by these conventional methods of diagnosis) would subsequently be constructed. The *NeuroNano* project thus sought to test the hypothesis that the more natural correlation is between the elevation of oxidative stress/amyloid mass and the disease outcome, i.e. to test the concept of the FIBROS property hazard map. (WPs 1, 2, 3, 4)

8. Provide input for a risk assessment framework (and screening protocol) for engineered nanoparticles for use by regulatory authorities and industry. The ultimate aim of the NeuroNano project was to begin development of a simple rational screening methodology for neuronanotoxic hazard, the so-called FIBROS map. The basis of the FIBROS map was the hypothesis to be tested within NeuroNano that screening for hazard in the future could be based on the three (presumed) contributing factors of engineered nanoparticles to neurotoxicity: access to the brain, oxidative stress generation potential, and amyloid fibril generation potential, possibly encapsulated in the arrival efficiency of the nanoparticles to the brain, and the location on the FIBROS hazard map. (WPs 1, 2, 3, 4, 5)

The *NeuroNano* project was thus a very focussed and detailed study of the potential for engineered nanoparticles to pose a neurotoxicity risk, and the development of a framework within which to predict the level of nerutoxic risk posed by specific nanoparticles. The main project elements and logic flow are summarised in Figure 1 below.
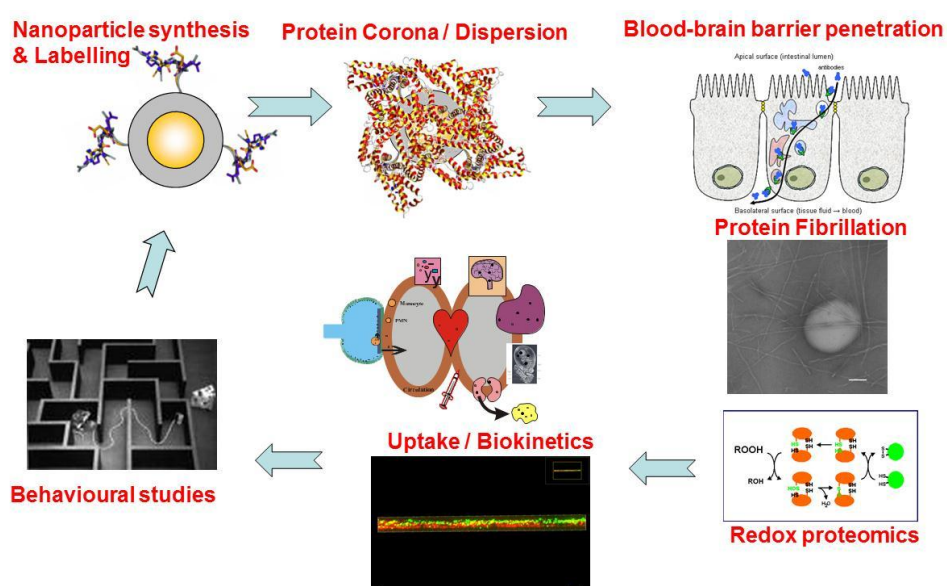

Figure 1: Flow chart of the research elements contained within the FP7 *NeuroNano* project.

S & T results from NeuroNano.

The *NeuroNano* project was organised into 5 scientific and 2 management Work Packages, as illustrated graphically in Figure 2. The inter-dependencies of the Work Packages are also illustrated. The flow of work in the 3 central experimental Work-Packages was based on a tiered approach, where experiments were conducted in order of increasing system complexity – experiments in solution, experiments *in vitro*, and finally, experiments *in vivo*. The purpose of this approach was to establish (where possible) *in vitro* methodologies to assess the potential neurotoxicity of engineered nanoparticles, and to reduce the numbers of animals needed in the course of the project, in line with the European Commission 3Rs policy regarding animal studies (reduction, replacement and refinement - Directive 86/609/EEC).
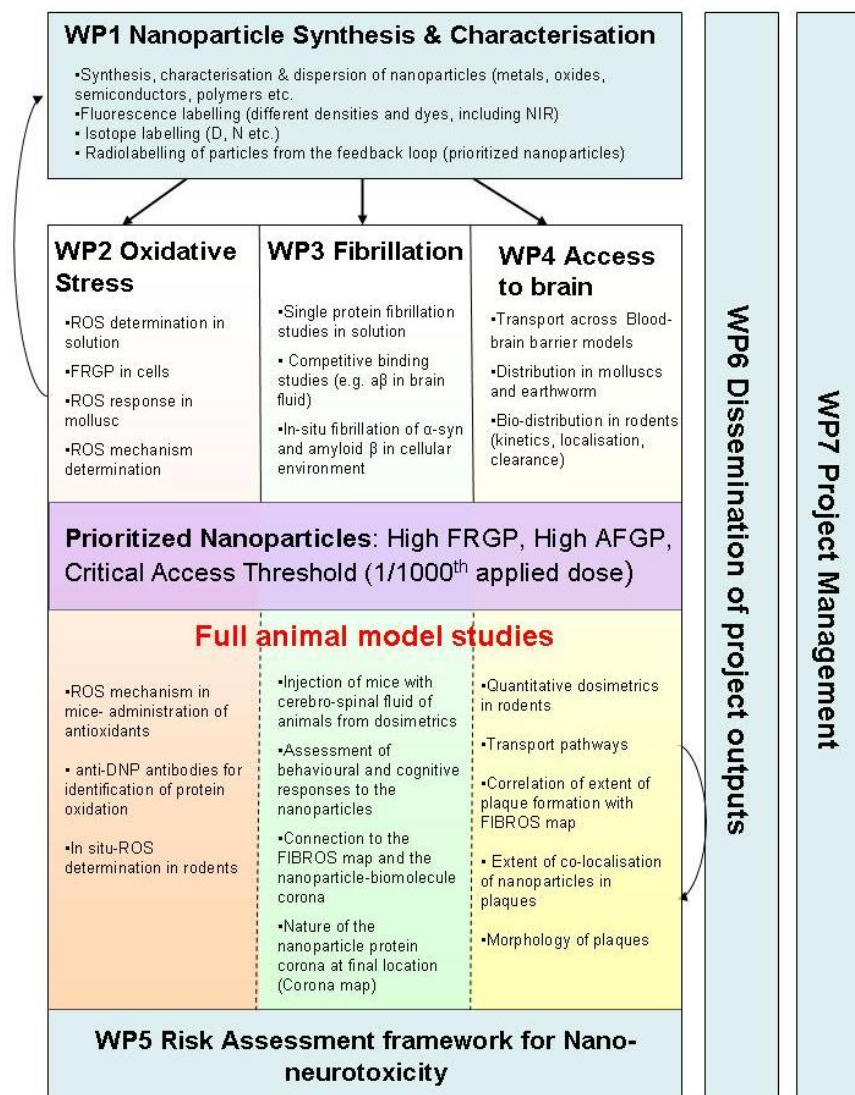

Figure 2: The structure and organisation of the *NeuroNano* Workplan and work packages.

Progress towards the objectives and beyond the State of the Art

NeuroNano was an extremely ambitious project that set itself objectives far beyond the state of the art in the field. *NeuroNano* set itself eight broad objectives against which its success could be measured, spanning the activities of the five research work packages, and the dissemination workpackage. A summary of the *NeuroNano* consortium's progress towards achieving each of its objectives, during the three years of research activity, is given in the paragraphs below:

Objective 1: Develop nanoparticles that have appropriate labels of sufficient activity and duration for neurotoxicological studies

Radiolabelled nanoparticles: The main task of the JRC in the project was to develop cyclotron-based methods for radiolabelling of nanoparticles. Initially, extensive effort went into investigating the radiolabelling of TiO_2 nanoparticles (selected as a priority nanoparticle at the project kick-off meeting for *in vivo* biokinetics studies at HELMUC). The result of theoretical considerations and many experimental tests was a successful protocol for ion-beam activation and subsequent dispersion and size selection of ST-01 TiO_2 NPs, achieving samples suitable for the *in vivo* studies, whilst also ensuring that the structure of the nanoparticles was undamaged by the selected activation procedure. Several test irradiations on different types of TiO_2 were carried out, and XRD structural damage assessment was performed on P25 and ST-01 TiO_2 nanoparticles after proton irradiation. The XRD results indicated that no significant damage was caused, even at activity levels as high as 1MBq/mg, achieved after 20 hours irradiation. Much time was spent in developing a dispersion and size-selection protocol in order to achieve a "bioavailable" suspension of activated ST-01. This included several steps of dispersion, leaching, centrifugation, "washing" and filtration. The results are described elsewhere,[24] but it can be noted that the suspension consisted of nanoparticles of approximately 100nm hydrodynamic size (DLS performed at HELMUC), that were stably labelled to an activity level of more than 1MBq/mg. The *in vivo* studies indicated only minimal release of 48V from the activated ST-01 TiO_2 nanoparticles.

Throughout the project further experiments have been carried out on TiO_2, as it proved to be an extremely useful material (see below) for validation of calculations of the thermal effects of ion-beam irradiation on nanoparticle samples in general. Following this, experiments were also carried out on Fe_3O_4, CeO_2, nanodiamonds, carbon black, and carbon nanotubes. Gold nanoparticles were also successfully activated and were used for *in vitro* work at IHCP/JRC.

In addition to direct ion-beam activation, a highly novel method for nanoparticle radiolabelling, based on recoil of [7]Be from a Li containing powder, was developed. This method was shown to also be suitable for radiolabelling of SiO_2 and Al_2O_3 nanoparticles and nanotubes.

In addition, the JRC activated Au and Ag electrodes for use in the HELMUC spark ignition aerosol inhalation facility, an additional task not originally foreseen in the NeuroNano workplan that is highly relevant for the project results. Finally, radiochemical synthesis of [56]Co labelled SiO_2 was achieved, and progress was made regarding synthesis of [89]Zr labelled Al_2O_3.

Another approach of synthesis of labelled nanomaterials from activated precursors was also investigated, and radiochemical synthesis of SiO_2 labelled with [56]Co was achieved. A foil of iron was irradiated to produce the radiotracer, and then dissolved and the [56]Co separated. This was added to nanoparticle synthesis precursor solutions and SiO_2 nanoparticles were then created. Practically all the [56]Co was incorporated in a stable way in the SiO_2 nanoparticle matrix. Preliminary experiments showed that [89]Zr Al_2O_3 could be synthesised in a similar fashion and radiochemical synthesis of several other NP types is now being pursued.

Fluorescently labelled nanoparticles: UCD were responsible for development of fluorescently-labelled particles that fulfilled the dual goals of being suitable for use in a biological context, such that they did not elute the fluorescent label inappropriately, and being sufficiently bright and stable that they can be tracked over time in complex milieu. Indeed, within the previous NanoInteract project, UCD had observed that many laboratory synthesized and commercially available fluorescent nanoparticles eluted free dye which confounded the uptake experiments, and had developed approaches to assess the elution of free dye from nanoparticles.[25]

Within the NeuroNano project, UCD focussed on development of optimised fluorescent silica nanoparticles that were resistant to dissolution *in vitro* over 48 hours**.** During the course of the NeuroNano project, the UCD team observed that the commonly employed Stöber type silica nanoparticles degrade by hydrolytic dissolution, which is accelerated in the presence of fluorescent moieties, which increase the porous nature of the particles, and under biological media conditions as

compared to water alone. UCD have thus developed a method to render such nanoparticles' biologically stable for at least 48hrs, using an arginine-based approach to construct nanoparticles with a core-shell architecture, in which the highly fluorescent silica core is made by the Stöber method and a silica shell is added using the arginine approach in a two-step process. In making such particles, no effort need be made to limit the fluorescence intensity of the core, as this is no longer a significant element of the structural integrity of the nanoparticle.[26]

Three shell sizes were created (by the arginine method) on washed 40nm Stöber-FITC silica NPs as shown in Figure 3. These particles showed good dispersibility in relevant media (as is usual for silica nanoparticles). They were incubated under biological type conditions with resultant dye leakage/dissolution monitored by time-resolved 1-D SDS-Page Gel and molybdate assay. The modified particles show vastly improved stability against dye leakage (Figure 3a). The absence of any visible dye release over 48 hours (see Figure 3b) is quite striking. When combined with the freedom to load a large amount of dye (or other internal) label these structures represent an ideal combination for biological application. Figure 3c, the epifluorescence microscopy imaging after 22 hours incubation, in agreement with the gel result, show the increased background fluorescence for the Stöber and Stöber Shell particles as compared the Arg-Sil Shell ones (rightmost panel). Indeed, under different biological conditions, the contrast can be more striking.
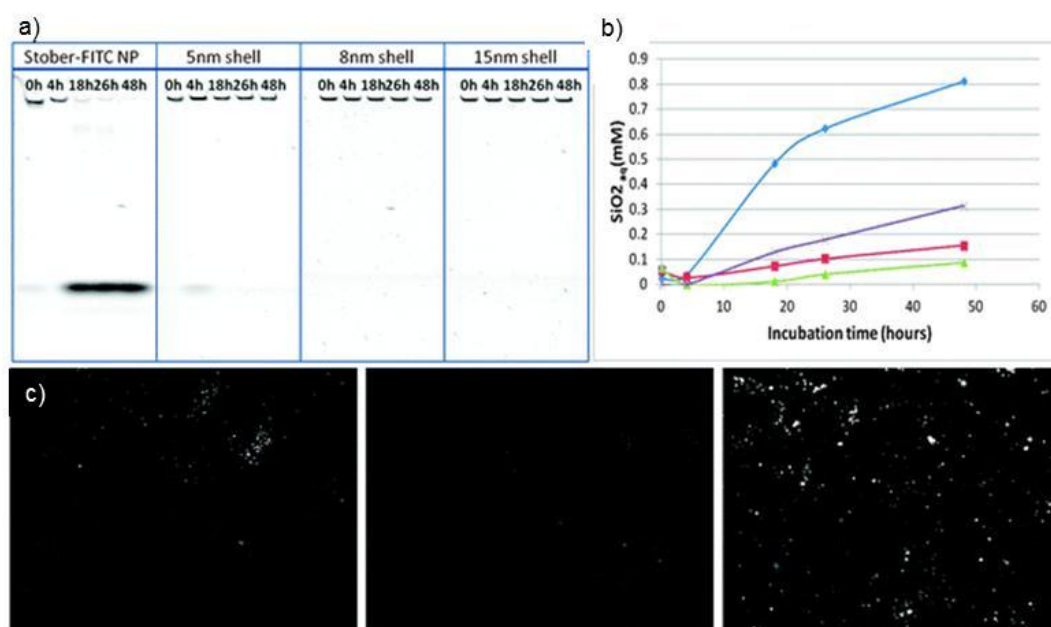

Figure 3. (a) 1-D SDS Page Gel showing time resolved dye release form the relevant particles following incubation in biological type conditions (HEPES pH 7.4, NaCl 148mM, CaCl$_2$ 1mM). (b) Corresponding dissolution 10 monitored by molybdate assay Graph showing dissolution profile for particle core (blue), shell I (green), shell II (red), shell III (purple). (c) Epifluorescence microscopy images (FITC channel, exp. time 400 ms) of nanoparticles in A549 cells following 22 hours incubation, from left to right Stöber FITC, Stöber-FITC-StöbShell, Stöb-FITC-ArgShell1. From [26].

Note: These particles are being further developed within QNano (the Research Infrastructure for nanosafety assessment) in WP6 on the optimisation of labelled nanoparticles for biological assays, and are being considered as for onward development as a QNano quality assured test nanomaterial.

Objective 2a: Identify classes of nanoparticles that lead to oxidative stress in relevant cell models

The original hypothesis was that the potential of the nanoparticles to cause oxidative stress (the Free Radical Generation Potential, FRGP) would differ for different particles depending on their chemical

composition and potentially surface curvature and other properties, and that this could be connected to the physic-chemical properties of the nanoparticles as a hazard classification approach.

Thus, UEdin determined the intrinsic free radical activity of the NeuroNano panel of using the using DMPO spin trap approach to measure hydroxyl radicals. This well established approach uses Electron paramagnetic resonance (EPR) or electron spin resonance (ESR) spectroscopy, which is a technique for studying chemical species that have one or more unpaired electrons, such as organic and inorganic free radicals or inorganic complexes possessing a transition metal ion. Spin traps, such as Tempone H (superoxide anions) or DMPO (hydroxyl radicals) serve to 'trap' the fugitive free radicals so that their concentration can be assessed. Figure 3 shows the free radical activity determined by Tempone H for the various titania nanoparticles and the vehicle controls in four diverse cell lines (A549 adenocarcinomic human alveolar basal epithelial cells, 1321N1 a human brain astrocytoma, RAW a mouse macrophage cell line and Sh-SY5Y a human neuroblastoma cell line).

From the *NeuroNano* panel of nanoparticles, only one particle, TiV+ was cytotoxic and haemolytic. However, this particle (TiV+) did not have an excess of free radical generation as measured by EPR compared to the non-toxic NPs in the panel, as shown in Figure 3. Thus, the source of its toxicity is not related to ROS production. Similarly, amine-modified polystyrene were found to induce platelet aggregation, but not via the classical up-regulation of adhesion receptors. Rather the amine-PS nanoparticles appeared to act by perturbation of the platelet membrane, revealing anionic phospholipids. Neither oxidative stress generation by particles nor metal contamination was responsible for these effects, which were a result of differential surface derivatization.[27] The study reveals that NP composed of insoluble low-toxicity material are significantly altered in their potency in causing platelet aggregation by altering the surface chemistry.
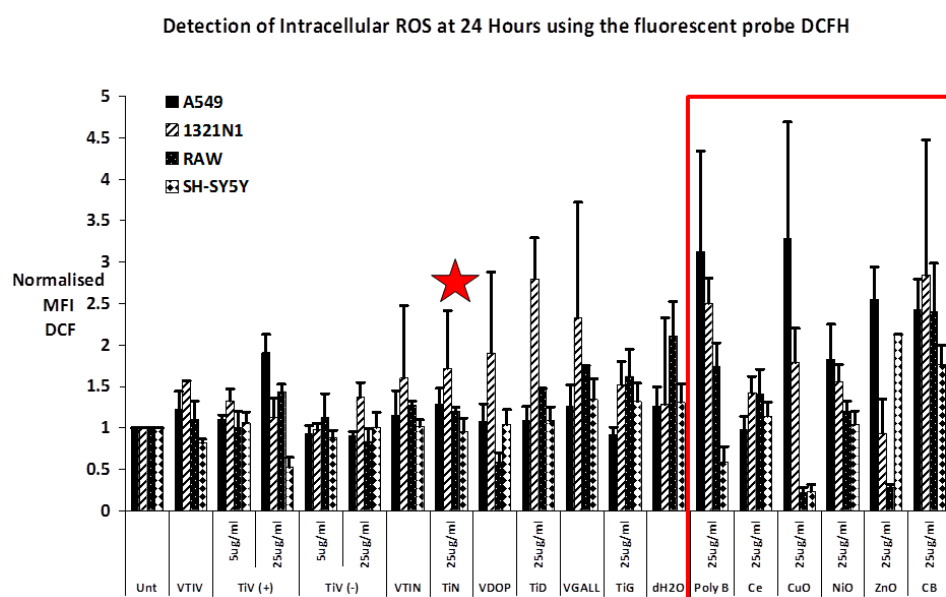

Figure 12. No nanoparticle treatment caused a significant increase in intracellular ROS in any of the four cell lines tested. TiV+ nanoparticles (red star) did not cause increased oxidative stress, but the trend for oxidative stress with the positive control nanoparticles (Poly beads, CeO, CuO, NiO, ZnO, and CB) is clear (highlighted in red box).

In a previous study with a large panel of metal oxide nanoparticles, UEdin found a clear relationship between high positive zeta potential and haemolytic activity and between high positive zeta potential and ability to cause inflammation in rat lungs.[28] Thus, the zeta potential of the full *NeuroNano* panel of particles was determined as a possible factor in toxicity in the light of research in other projects. The TiV+ were found, along with the aminated polystyrene beads (one of the positive control nanoparticles), to have a high positive zeta potential under mild acid conditions, as shown in Figure 4.
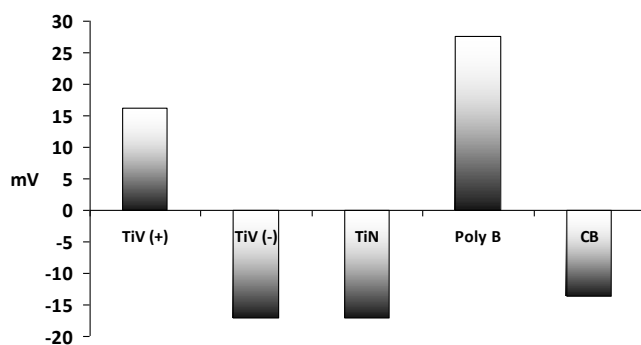
Figure 4. Zeta potential as an alternative to oxidative stress as a structure/toxicity model.

Thus, oxidative stress is not as direct a correlator with potential impact as had been expected *a priori*. Additionally, evidence emerging (beyond NeuroNano) of an inflammatory impact from nanoparticles that do not show ROS potential[29], suggesting that there are other mechanisms of inflammation that need to be investigated.

Objective 2b: Identify classes of nanoparticles that lead to increased rates of fibrillation

At its conception, *NeuroNano* envisaged that, in analogy to the Reactive Oxygen Generation Potential (FRGP) concept described under objective 2a above, a set of solution studies to determine the ease with which fibrillation occurs for the relevant proteins (i.e. amyloid-β and α-synuclein for Alzheimer's and Parkinson's diseases, respectively) in the presence of different nanoparticles, producing a 'fibrillation score', termed Amyloid Fibril Generation Potential (AFGP).

However, as per the case with oxidative stress, this proved to be somewhat overly simplistic, and indeed since induction of fibrillation is a surface area effect, all nanoparticles induce or modulate the rate of protein fibrillation. Thus, nanoparticles can affect protein fibrillation in two distinct ways – either enhancing the rate of fibrillation (increasing the likelihood and occurrence of nucleation) or inhibiting the rate of fibrillation (reducing the likelihood and occurrence of nucleation). In both cases, the observed effects can be a result of changes in local protein concentration and/or changes in protein confirmation. Indeed, dual effects have also been observed, whereby the effects observed were dependent on the ratio of particle surface area to protein concentration, showing the subtle inter-play of local concentration and protein confirmation on formation of the critical nuclei. These are stochastic events, and are the first step in the fibrillation processes. Thus, at different ratios of particle surface area to protein the same particle can accelerate or delay protein fibrillation.[30]

Similar effects were observed with superparamagnetic iron oxide nanoparticles (SPIONs): depending on their surface coating charge, a surface area dependent "dual" effect was observed; with lower concentrations of SPIONs inhibiting fibrillation, while higher concentrations enhanced the rate of Aβ fibrillation. Coating charge influenced the concentration at which the acceleratory effects were observed, with the positively charged SPIONs promoting fibrillation at significantly lower particle concentrations than either negatively charged or essentially uncharged (plain) SPIONs. This suggested that in addition to the presence of SPIONs affecting the concentration of monomeric protein in solution (and thereby the nucleation time), there were also effects of binding on the Aβ conformation, which was mainly detected with the positively charged SPIONs.

A further complicating factor arises from the fact that *in vivo* the nanoparticles are surrounded by a corona of proteins and other biomolecules which reduce the availability of the nanoparticle surface for interaction with amyloidogenic proteins and likely also mediates the impacts of nanoparticles *in situ*. Thus an important step forward in developing our understanding of Aβ-nanomaterials interactions under more biologically relevant conditions was to consider the case where the NM surface is already

pre-covered with proteins and thus potentially less accessible to the Aβ, i.e. to study the effect of protein corona coated NMs on Aβ fibrillation.

The effects of protein coated nanomaterials on the fibrillation of Aβ were investigated via simple ThT assays and TEM imaging, in order to investigate whether the protein corona shell on the surface of NMs has an effect on Aβ fibrillation. Several different NMs (i.e. silica (100 nm), silica (200 nm), polystyrene with carboxyl surface modification (100 nm), and multi-walled carbon nanotubes (CNT; diameter: 10-40 nm and length: 0.1-10 µm prepared by Partner 13 UFC) were employed in this study and their effects on the Aβ fibrillation process were probed in the absence and presence of a protein corona, as well as well as under conditions where the protein corona was denatured. Figure 5 shows the results of ThT assays for Aβ in the presence of 100 and 200 nm silica nanoparticles without and without protein coronas (formed at 10% or 100% plasma, representing *in vitro* and *in vivo* exposure conditions, respectively) under normal and denaturing conditions.

The measurements show that the "bare" nanomaterials used accelerated the rate of Aβ fibril formation, in agreement with literature. However when the NMs were pre-coated with a plasma protein corona it resulted in a considerable increase in the lag times for the fibrillation process, compared with that in the presence of the pristine nanoparticles. For 100 nm silica particles, fibrillation in the presence of the corona-coated NMs actually occurred more slowly than for free Aβ. The extent to which the lag time for Aβ fibrillation increased depended on the nanoparticle-protein corona composition. Particles incubated in 100% plasma showed greater inhibitory effects than particles coated in 10% plasma, in for all NMs used in this study. NMs bearing a protein corona slowed the fibrillation of Aβ, regardless of size, surface chemistry, charge or aspect ratio when compared with the same pristine (bare) nanomaterial.
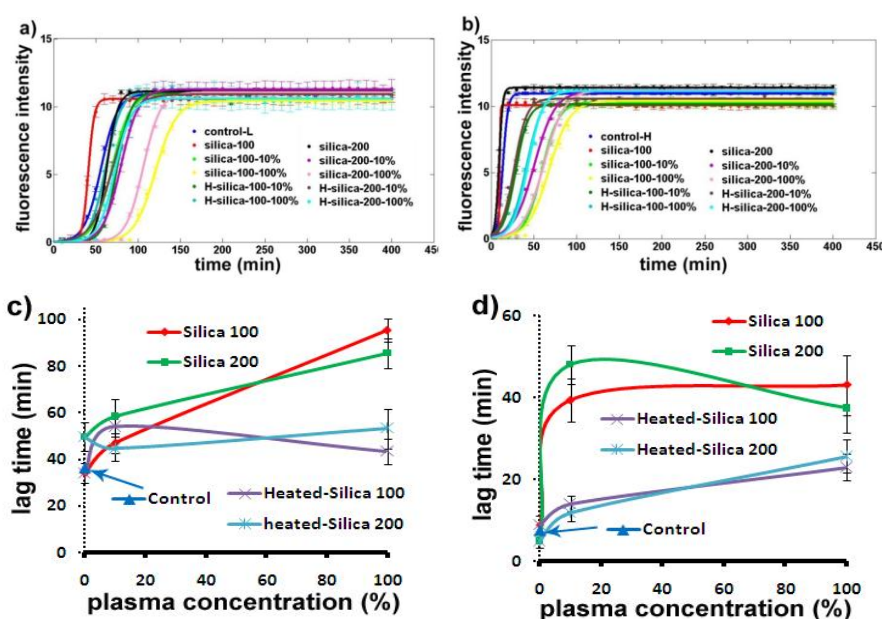

Figure 5: Time-dependent ThT fluorescence was probed at 480 nm (excitation wavelength of 440 nm) for (a) 20 µM, (b) 40 µM Aβ fibrillation in the presence of corona- and disrupted corona-NMs, and (c) and (d) calculated lag times for Aβ fibrillation from (a) and (b) above for 20 and 40 µM Aβ, respectively).

Importantly, in order to be effective as an inhibitor of Aβ fibril formation the proteins in the nanoparticle-protein corona must be in their native form: corona proteins that have been denatured by heat were found to actually accelerate the formation of protein fibrils, mostly likely due to the exposure of hydrophobic amino acid residues from proteins that do not refold after heating.

Objective 2c: Identify classes of nanoparticles that lead to up-regulation of the relevant pathway proteins involved in neurodegenerative diseases (at the cellular level)

The control of cell number plays a critical role in organ development and in maintaining the integrity of structural elements. Balance between cell proliferation and cell death represents the central aspect of this regulation, as well as in maintaining homeostasis.[31] Failure to regulate apoptosis is a common feature in several diseases, including autoimmune disorders, neurodegenerative diseases, cancer and AIDS.[31] In contrast to rapid turnover of cells in proliferative tissues, neurons commonly survive for the entire life of the organism. This enduring nature of neurons is necessary for maintaining the function of those cells within neuronal circuits. Excessive death of one or more populations of neurons results in disease or injury. For example, death of hippocampal and cortical neurons results in Alzheimer's disease (AD), death of mid brain neurons results in Parkinson's disease (PD), death of neurons in the stratium results in Huntington's disease (HD) and finally, death of lower motor neurons results in amyotrophic lateral sclerosis (ALS).[31]
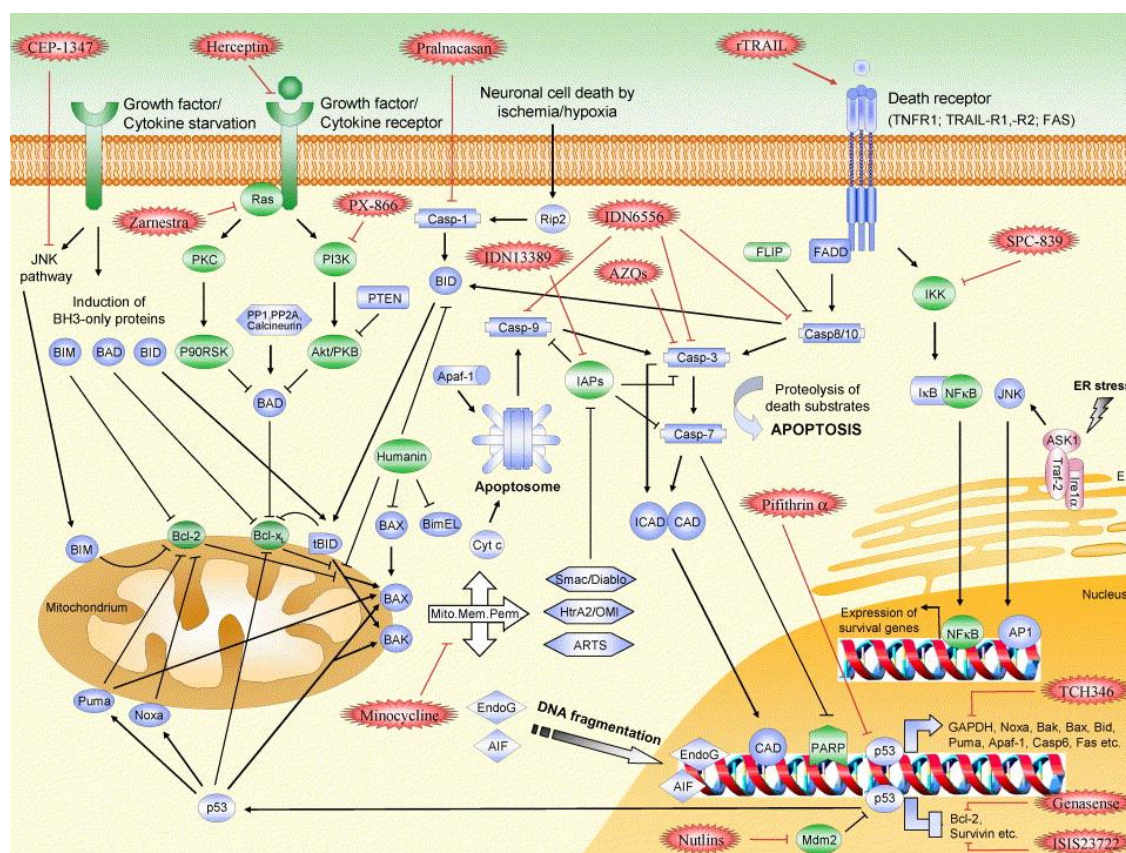

Figure 6: Apoptosis pathways and drug targets. Deregulation of apoptotic pathways can lead to different types of diseases such as neurodegenerative diseases and autoimmunity. Molecules with pro- or anti-apoptotic function are highlighted in blue or green, respectively. Potential drugs are represented in red. (From [31]. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Using the human brain astrocytoma cell line (1321N1) and the human lung carcinoma cell line (A549) extensive work on the mechanism of apoptosis induced by 50nm amine-modified polystyrene nanoparticles has been performed, and was correlated with the redox proteomics and gene expression changes. A clear role for oxidative stress has been observed in the apoptosis induced by 50 nm amine-modified nanoparticles in human astrocytes. The sequence of events for these nanoparticles is localisation of the nanoparticles in the lysosomes, leading to lysosomal swelling and rupture, which triggers loss of mitochondrial membrane potential resulting in depletion of cellular ATP level, which

causes cellular ROS generation and in turn leads to the oxidative DNA lesions followed by DNA fragmentation,[32] as shown schematically in Figure 8. Besides acting on mitochondria, ROS have been reported to induce lysosomal permeabilization and lysosomal enzymes have been found to act on mitochondria and promote mitochondrial ROS generation. Therefore, a feedback loop is formed for more lysosomal permeabilization.[33] In this study ROS generation was measured to examine the extent of oxidative stress induced by NPs inside 1321N1 cells.
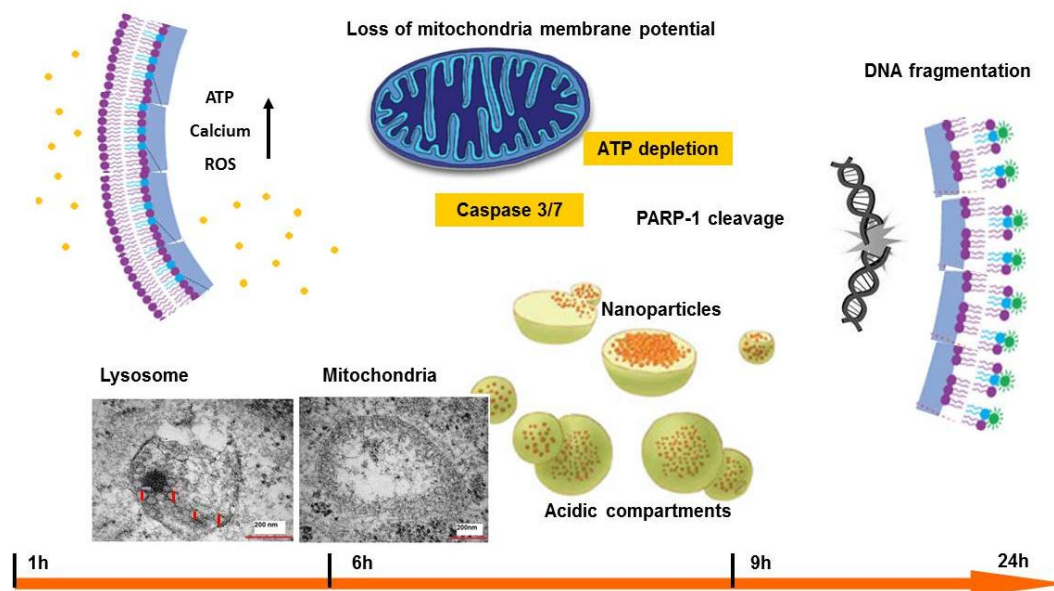

Figure 7: Schematic illustration of the time-resolved onset of the different steps in the onset of apoptosis in A549 cells following incubation with 25μg/mL 50nm amine-modified polystyrene nanoparticles.[32]

From the studies utilizing the amine-modified fluorescently-labeled polystyrene nanoparticles, there is evidence of some early stage apoptosis, ROS, and intracellular calcium even in the first few hours of exposure of the cells to the nanoparticles. These may be attributed (although unclear to what degree) to residual positive surface charge, still expressed due to incomplete coverage of proteins (for which there is certainly some evidence at only 10% FCS), or earlier signals arising from the transition through the endosomal pathway. In any case, after 8 hours, the system evolves as one would expect, with PARP activation, following caspase 3/7 activation, and decrease in ATP levels (as shown in Figure 7), membrane permeabilization, accompanied by high calcium levels and ROS generation (as shown in Figure 7),[34] and finally DNA fragmentation.

A key question in terms of understanding the impact of nanoparticles is to understand the fate of the nanoparticles, and their corona of proteins acquired externally to the cell from the cell culture medium, as the particles are taken up by cells and transported to a final subcellular location, typically the lysosomes. UCD have developed an approach to determine the fate of the nanoparticle corona, and specifically to determine if it is sufficiently strongly bound to the nanoparticles to be retained during nanoparticle uptake and to understand how the presence of this passifying layer of proteins affects the impact of nanoparticles on cells.[35]

The full bovine serum used for tissue culture was fluorescently labelled by reaction with an Alexa 488 dye which forms covalent linkages with the amino groups of the serum proteins. Extensive optimisation was necessary to identify the best reaction conditions and, more importantly, to clean the labelled protein from the residual unreacted fluorescent dye molecules. Cleaning was achieved by passing the labelled serum through HPLC through G25 Sephadex columns. By labelling the full serum, rather than using single labelled proteins, we could form a labelled corona of the same complexity of that formed on nanoparticles exposed to cells under standard culture conditions and we could track its evolution during nanoparticle uptake and intracellular trafficking.

Use of labelled serum in this way has allowed us to determine that in the case studied here (50nm amino-modified polystyrene (NH$_2$-PS) nanoparticles) the corona proteins are retained, at least in part, during uptake on the nanoparticles, and thus to generate the first evidence that the corona can enter with the nanoparticles inside cells.[35] Following uptake, time resolved fluorescence imaging indicated that the corona-nanoparticle complexes are also trafficked together until final nanoparticle destination into the lysosomes, thus the original labelled corona is still retained, at least partially, even when the nanoparticles arrive to their final destination of lysosomes. Once there, a progressive decrease of the corona fluorescence could be monitored, probably due to enzymatic degradation at lysosomal level.[36] This progressive corona degradation was clearly accompanied by lysosomal swelling and thus lysosomal rupture, release of lysosomal content into the cytosol and consequent apoptosis followed, as previously reported for these nanoparticles in these cells.[32]

The programmed cell death induced by NH$_2$-PS NPs can also be followed by the regulation of gene expression. In order to monitor the dynamic genetic response to NH$_2$-PS NPs on the gene expression level, we performed microarray in a time resolved way. The first clear outcome was that the cellular responses to NH$_2$-PS NPs were strongly regulated at the gene expression level. The expression levels of some genes were changed up to 40 times more than that of control cells (data not shown). Moreover a clustered hierarchically of the change of gene expression levels relative to that of untreated control was performed. The result indicates four large clusters of genes with very similar transcriptional dynamics (monotonic up-regulated, early up-regulated, monotonic down-regulated and early down regulated). To find the biological significance of these 4 clusters of genes, we looked for biological functions and process overrepresented in each cluster using gene set enrichment analysis (GSEA).[37] The trends observed in the time resolved analysis ensure the confidence of the data.[38] The up-regulated cluster includes genes involved in cell proliferation, pro-apoptosis and DNA damage; the down-regulated cluster contains genes involved in mitochondrial functions and cell division cycle, etc. Many genes are up or down regulated after only a few hours of exposure. Overall this analysis confirmed the programmed nature of the impact of NH$_2$-PS NPs and the clusters of genes with the strongest changes in expression are in agreement with the observed apoptosis. More detailed results are presented in the following sections.

Objective 3: Identify the physiochemical properties, and surface expression properties that lead to given levels of oxidative stress and amyloid load.

NeuroNano sought to test the hypothesis that the composition, organization, and reorganization time scales of the biomolecule corona (the proteins that adsorb to nanoparticles under physiological conditions) can be more naturally correlated to the key biological impacts associated with neurodegenerative diseases (i.e. oxidative stress and protein fibrillation), rather than any specific set of material properties. Thus, for the test nanomaterials, the nature and impact of the protein corona was assessed as the basis for such a correlation.

The corona is a very selective layer of proteins and other biomolecules, which strongly adsorbs on the nanoparticle surface for time scales longer than nanoparticle uptake in cells, thus constituting a new complex unit interacting with the cellular machinery. The formation of a corona affects the material properties also, by simply lowering the surface free energy of the bare material.[2]

UCD have recently shown that identical particles and cells, under otherwise identical conditions, undergo very different interactions with cells, resulting in very different biological outcomes, depending on whether the particles are presented to the cells in the presence or absence of a preformed corona in serum.[1] Although the details of the uptake mechanisms are still unresolved (indeed, details are still missing even for nanoparticles exposed to cells in the presence of serum),[39] it has been observed that nanoparticle uptake under serum-free conditions is, in most cases, higher than that measured for the same nanoparticle in the presence of serum.(Guarnieri, 2011 #506) Figure 8 summarises the very different uptake behaviour observed under serum free and serum conditions, with uptake dramatically higher in the absence of serum, with resultant higher impact on the cells.
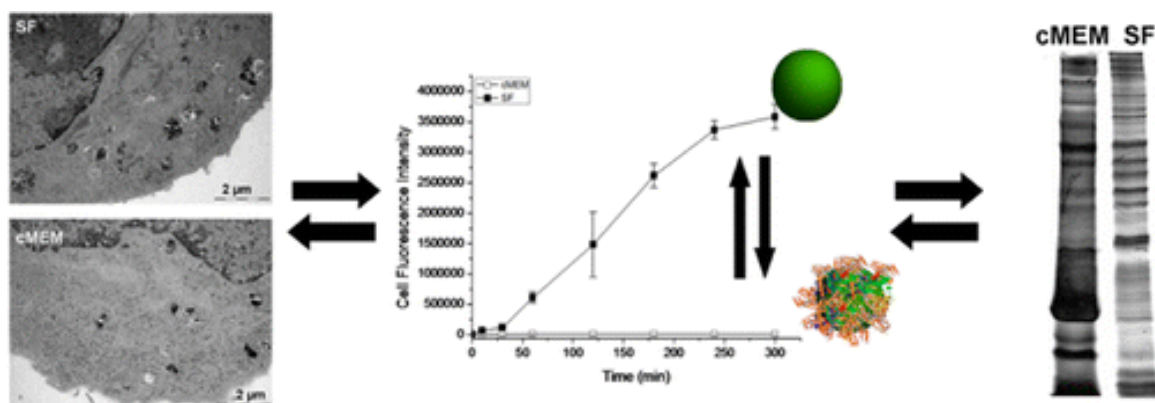
Figure 8. Fluorescently labelled silica nanoparticles (50 nm) were dispersed in serum-free MEM medium (SF) and complete medium supplemented with 10% serum (cMEM) and their uptake, localisation were determined. The different exposure conditions not only affect the uptake levels (centre) but also result in differences in the intracellular nanoparticle location and impact on cells (left). Interestingly, after only one hour of exposure, a corona of very different nature forms on the nanoparticles exposed to cells in the absence of serum (right). From [1].

Using confocal microscopy we could confirm by immunostaining that the major final localization of the nanoparticles exposed under serum free conditions was in the lysosomes, as was also the case for the nanoparticles exposed in complete medium (with 10% serum). This was particularly clear after long exposure time (24 h), where a high level of co-localization could be seen with lysosomal marker (LAMP1) positive structures, even though not all of the nanoparticles were (yet) found there under the serum free conditions. This could also be related to the presence of nanoparticles that seemed free in the cytosol by Electron Microscopy (EM) analysis. Moreover, confocal imaging confirmed the presence of residual clusters of nanoparticles outside of the cells (and also on the glass slide) under serum-free conditions, as was noted also by EM.

Nanoparticle protein corona studies

HMGU performed an *in vitro* incubation of triphenylphosphine surface-modified gold nanoparticle (5nm, 15nm and 80nm, identical to those used in the biodistribution studies) with diluted serum of mice. The nanoparticles were incubated for 24h at 37°C. After incubation the nanoparticles were washed three times and an SDS gel electrophoresis was made. After Coomassie blue staining the gel spots were cut and prepared for subsequent digestion with trypsin. The digestion was performed according to an established standard protocol of the core facility Proteomics of the Helmholtz Center Munich. After digestion the Mass Spec analysis was made with the help of the Proteomics Analyzer 4700, which is a tandem time of flight matrix assisted laser desorption and ionization apparatus (MALDI-TOF-MS). From this analysis, a number of different proteins which conjugated significantly to the gold nanoparticles. Table 1 shows that the 80nm NP did not bind to as many serum proteins as the smaller sized NPs, likely a result of their reduced surface area compared to the smaller NP. Interesting is that there are some proteins which adsorb only to a specific size of particles, like ApoA2 or ApoA4 to 15nm particles and Krt78 protein to 5nm particles or alpha-1-antitrypsin to 80 nm particles.

UFC developed a series of porous silica nanoparticles (~ 60 nm, with three different microchemical surface chemistries [Si-OH, Si-NH$_2$ and Si-P(CH$_3$)O$_3$H]) and assessed their interactions with proteins. After dispersion in the serum (10 or 55% representing *in vitro* and *in vivo* conditions, respectively), the presence of the hard corona not only increased the average particle size but also increased the polydisersity of the dispersions. After several washing and centrifugation processes to ensure the elimination of weakly bound proteins, the presence of the "hard corona" of strongly adsorbed proteins, on the porous silica nanoparticles surface was clear from the increased particle diameters for the protein-coated porous nanoparticles as determined by dynamic light scattering analyses (DLS).

kDa	Protein			5nm	15nm	80nm
11	Apo A2			x		
15	Hämoglobin subunit alpha (beta)			x	x	x
26	Outer dense fiber protein 3b			x	x	
30	Apo A1			x	x	
36	Apo E			x	x	
45	Apo A4			x		
46	Alpha-1-antitrypsin					x
46	Fibrinogen gamma			x	x	x
52	Clusterin			x	x	
55	Krt78 protein				x	
55	Fibrinogen beta			x	x	x
55	Fibrinopetide B			x	x	x
56	Vitronectin			x	x	x
59	Histidin-rich glycoprotein				x	x
69	Serum Albumin			x	x	x
86	Gelsolin			x	x	
129	Alpha-1-globin			x	x	x
166	Alpha 2 Macroglobulin			x	x	
166	Murinoglobulin 1			x	x	
186	Complement C3 (2x)			x	x	

Table 1: Qualitative results of serum protein binding to 5 nm, 15 nm and 80 nm gold NP.

No significant change in protein corona composition was evident for the Si-OH and Si-P(CH$_3$) samples. However, a tendency towards decreasing intensity of the protein bands for Si-NH$_2$ sample, especially to proteins bands with lower molecular weight, was observed. Quantitative analyses of hard protein corona (% of pellet weight) by thermogravimetric analysis confirmed this also: the Si-NH$_2$ sample has a lower content of protein in the hard corona after incubation with 55% of plasma, at the same mass (500µg/particle), as compared to the Si-OH and Si-P(CH$_3$) samples. A similar trend was also observed at a normalized surface area (~ 800 g/m^2). This work is being written up for publication at present.

Objective 4: Understand what constitutes a lead nanoparticle candidate for passing the Blood Brain Barrier (BBB)

In the conception of the *NeuroNano* framework for assessing the neurodegenerative potential of nanomaterials, it was recognised that not all particles that could be toxic will in fact pass the Blood Brain Barrier. Indeed, preliminary data suggested that there is a very great dependence on particle type and size, and these effects must be investigated and understood. Part of our strategy was to carry out early screening of the particles using the *in vitro* BBB model in parallel with limited animal trials. We will also seek to clarify if the particle corona is more naturally correlated with the ease of crossing the BBB. The idea was that if these models proved effective, then all candidate nanoparticles would also be screened for likelihood to pass the BBB, and this new approach would reduce the reliance on animal models, in line with European Commission policies on reducing animal usage.

Again, this initial conception while valuable in framing the project, was somewhat limited by our collective lack of knowledge at the time, as it missed two key factors that have since emerged as being of very considerable importance, namely:

(1) that nanoparticles can (potentially) signal across barriers via paracrine signalling, as has been demonstrated for the wear-induced particles across the foetal barrier,[40] and with the NeuroNano project for non-toxic carboxyl-modified polystyrene nanoparticles.[41]

(2) The low amount of particles reaching the brain could be a result of significant accumulation of nanoparticles in the brain endothelium itself. Indeed, using the *in vitro* barrier model, we observed significant accumulation of nanoparticles in lysosomes of the barrier cells (see Figure

9), from as early as 4 hours after exposure, and reaching a plateau level at account 24 hours, suggesting that the barrier itself could be a target for nanoparticle accumulation.

To study particle internalization in cellular lysosomes, LAMP1 antibody (a specific lysosomal antibody) was used. In figure 30, co-localization can be observed after 4 hours, indicating nanoparticle entry into lysosomes.
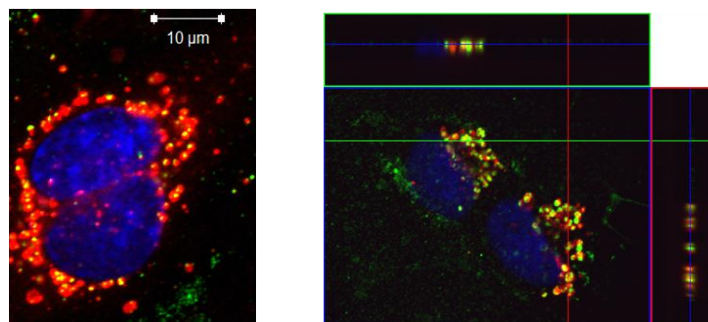

Figure 9: Confocal images after 4 hours of exposure of A549 cells to 100nm PS-COOH NPs shows nanoparticles accumulated in the lysosomes. Blue, nuclei (DAPI); red, lysosomes (LAMP1 antibody); green, nanoparticles.

While such significant accumulation of non-toxic nanoparticles, such as the PS-COOH shown here, and also silica nanoparticles or gold nanoparticles which we have also studied using this model, do not seem to perturb the cells, there are indications that signalling molecules are secreted by the endothelium in response to the presence of the PS COOH NPs, which are subsequently amplified by the astrocytes when using a co-culture model via paracrine signalling.[41] This suggests that accumulation of large numbers of PS COOH NPs in lysosomes of the *in vitro* BBB monolayer impedes the signals that would lead to an inflammatory reaction and possible cell death. Growth of human astrocytes below the BBB monolayer allows the communication of supporting signals *in vitro* which aids in mimicking a more realistic *in vivo* situation, allowing these signalling pathways to occur.

Clearly, assessing impacts of somewhat less benign particles accumulating in endothelium is necessary, although as the particles themselves begin to induce effects on the endothelium barrier separating out the sequences of signalling events will become more difficult, and isolating paracrine signalling from inter-cellular signalling will require detailed molecular biology studies.

Other work performed by UCD was to assess the role of specific transport proteins in determining the date of nanoparticles, and specifically their propensity for transcytosis across the *in vitro* BBB model.
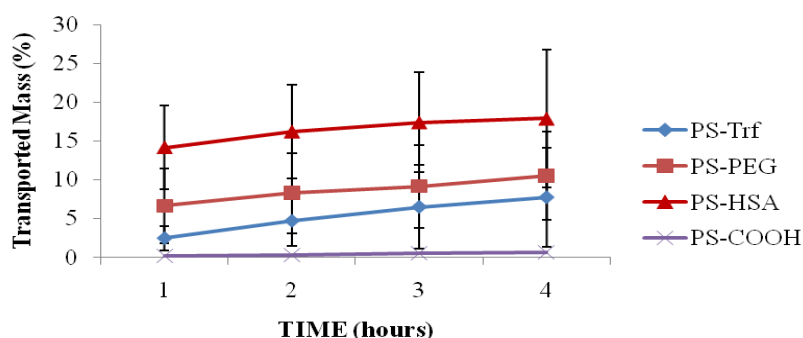

Figure 10: Effect of grafting with known transport molecules on the transcytosis efficiency of 100 nm PS-COOH nanoparticles, namely Transferrin (Trf) and human serum albumin (HSA) or with polyethylene glycol (PEG) which is generally considered to reduce protein binding to particles. Cells were grown on transwell filters for 24 hours, and were then exposed to 100 µm NPs on the apical side over 4 hours. The fluorescence in the basal chamber was determined, and converted to the transported mass of particles over time.

On-going work has suggested that escaping from the endo-lysosomal pathway is a key step, and that HSA may be more effective at achieving this than transferrin. Additionally, the orientation of the grafted proteins matters greatly in ensuring effective functioning and receptor engagement, and thus strategies to address this are being developed within UCD.[42]

Another important finding from the screening efforts using the *in vitro* BBB model was that approaches that are standard in other arenas (such as drug screening) are in many cases not sufficiently characterised for work with nanomaterials which engage receptors actively and undergo energy-dependent pathways. For example, while standard processes to confirm the formation of barriers and tight junctions, such as staining with claudin-5 and even TEM imaging applied in a routine manner appear to show barrier formation and good barrier integrity as shown in Figure 11, more detailed studies in UCD have shown that in very many cases such *in vitro* barriers, unless carefully optimised, may have many holes through which the particles can passively diffuse, thereby confounding the determination of transcytosis.[43] An example of this is shown in Figure 12, and similar data has been generated using confocal imaging.[44]
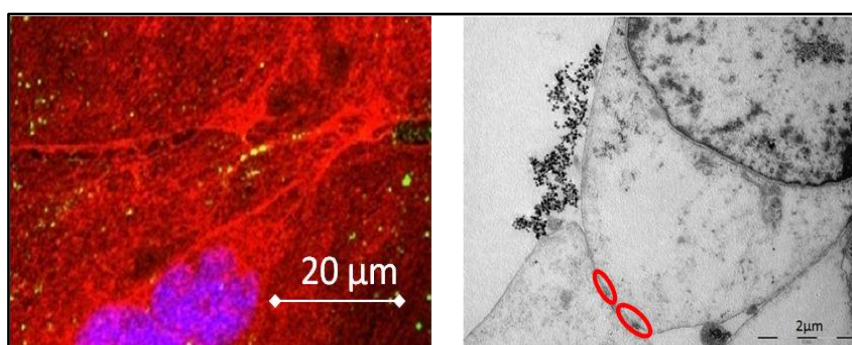

Figure 11: TJs integrity and stability was also checked after NPs exposure. The image on the left shows claudin-5 (red) expression is still well identified even in the presence of 100-nm PS-COOH NPs (yellow-green); on the right, an EM image shows 50-nm SiO$_2$ NPs in contact with two cells forming the barrier, without any TJs disruption. From [44].
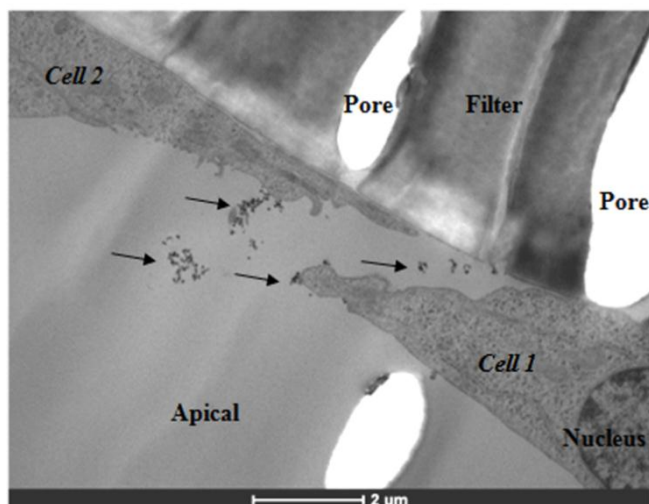

Figure 12. Indications of monolayer interruptions and imperfections in an *in vitro* human Blood Brain barrier model. TEM imaging indicated that the BBB monolayer did not cover the whole surface of the transwell filter: in this image, a small interruption to the barrier integrity could be observed between two neighbouring cells (Cells 1 and 2). This could potentially allow nanoparticles to travel through the filter. Without knowing that there were imperfections in the barrier (i.e. without TEM imaging), this could be misinterpreted as transcytosis. The arrows indicate 50 nm SiO$_2$ nanoparticles approaching the filter in the area where cells are not in contact. From [43].

Objective 5: *Quantify transport efficiency to the brain. NeuroNano sought to quantify the amount of nanoparticles that arrive in the brain (of small animals) from each of the potential exposure routes (inhalation, instillation, ingestion, intravenous)*

In vivo Exposure by Peripheral Injection to nanoparticles in rats

UU investigated the amount of nanoparticles reaching the brain of rats using a combination of TEM imaging and ICP-MS quantification of particle load.[45, 46] Peripheral injections of ceria (64 nm), titania

(3-9 nm), silica (50 nm), gold (BBI 30 nm), gold-ApoE-PEG 2000 (30nm), gold-ApoE-PEG 5000 (30nm) and gold-ApoE-PEG 10000 (30nm) were carried out using intravenous and intra-tracheal methods. Particle suspensions were intratracheally instilled synchronous with the animal's inspiration followed by 300 μl of air. Animals were culled after 24 hours and blood was collected immediately by cardiac puncture, aliquoted into EDTA Vacutainer tubes and chilled. An aliquot of CSF was removed from the cisterna magna and frozen immediately. Animals were then perfused through the heart with PBS solution and all major organs (brain, heart, lungs, liver, kidneys, spleen, pancreas, stomach, intestines and muscle) removed for ICP-MS analysis. Ultrathin sections (100nm) were microtomed and mounted on copper TEM grids. Samples were imaged with a FEI Titan transmission electron microscope equipped with an energy dispersive X-ray (EDX) elemental analysis system. Figure 13 shows an example of TEM/EDX images of Titania particles found in liver and lung. No particles were found in brain tissues.
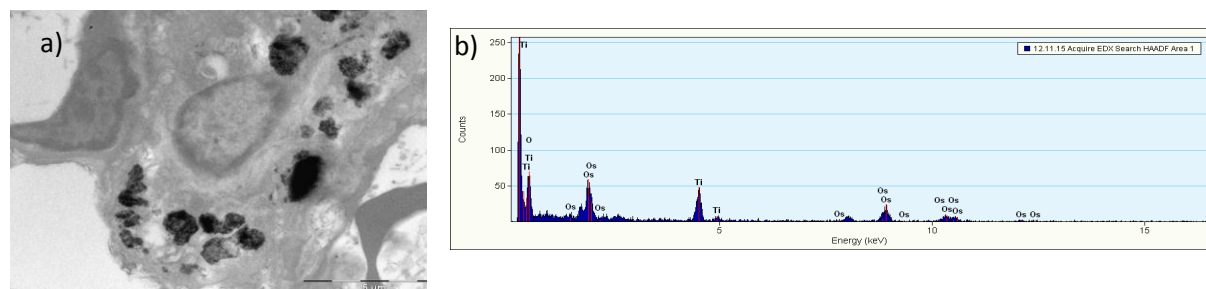

Figure 13: TEM/EDX image (a) of Titania in lung of IT dosed animals and corresponding spectra (b).

Sample uptake was then measured by ICP-MS and calculated as ng of cerium or titanium per gram of wet tissue measured; or per ml of cerebro-spinal fluid (CSF). No significant increase or decrease in nanoparticle elemental concentration was found in brain tissue or CSF from animals injected with titania by either route, when compared to control by t-test as shown in Figure 14. However, a significant increase in cerium content against control (6-fold; $p<0.05$) was observed in brain tissue of rats injected IV with ceria. A 1.45-fold increase in cerium content in brain from IT injected animals was also found, however this was not deemed statistically significant (data not shown). This data may suggest that ceria passes the blood brain barrier readily.
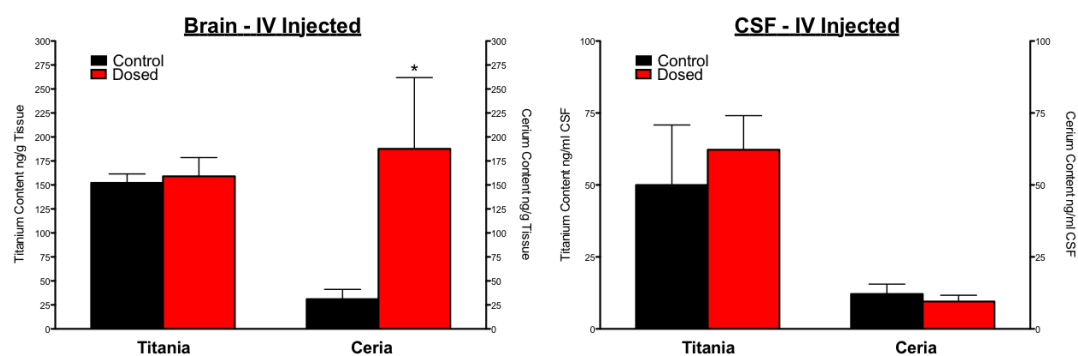

Figure 14: Uptake of ceria and titania after either IT or IV administration to brain and CSF of rats. Values are means±S.E.M. of 5 observations. *$p<0.05$ compared with control tissue.

In vivo biodistribution studies – comparison of different exposure routes - intratracheal instillation to lungs (IT), intravenous injection to blood (IV) or by intraoesophageal instillation (gavage, IO)

HMGU studied the biodistribution of radiolabelled (with [198]Au) monodisperse gold nanoparticles, coated with triphenylphosphine, of 1.4 nm, 5 nm, 18 nm 80 nm, 200 nm size) in rats following exposure to the nanoparticles via three different exposure routes. Additionally, the effect of surface charge was assessed using 2.8 nm gold nanoparticles that were either amino- or carboxyl-surface-modified.

Twenty-four hour gold nanoparticle accumulation in the brain of rats is shown for each administration route in the three panels of Figure 15. While brain accumulation of the gold NP decreases significantly from 1.4 nm to 5 nm after IV injection this is much less observed after IT or IO instillation.

Note that 1.4 nm and 2.8 nm gold NP fractions administered by IO instillation are an order of magnitude lower than those after the other two administration routes. Furthermore, it is remarkable that similar fractions of 5 nm – 200 nm gold NP were found after either IV instillation or IV injection. Taking into account that gold NP retention in the lungs is between about 0.9 and 0.99 with increasing NP size and hence 1-2 orders of magnitude lower fractions translocating from lungs to blood compared to direct IV injection, this indicates a much more efficient accumulation of gold NP having crossed the air-blood-barrier (ABB) than IV injected NP. One reason for the observed difference is the different dynamics of the bolus IV injection versus a more prolonged translocation across the ABB after IT instillation. However, this phenomenon is likely to be mediated by different protein coronas on the surface of the gold NP.

The brain accumulation of 5 nm – 200 nm gold NP is rather similar following administration by two of the routes (IV, IT).[47] However, IO led to the highest accumulation in the brain for the 18 nm gold NP, indicating that something happens with these nanoparticles in the gastro-intestinal tract which alters their biokinetic fate.[48] The most obvious explanation is a different specific protein coating, related to the exposure route. In the stomach, it is conceivable that the acidic environment, as well as the presence of gastric enzymes, is likely to degrade the triphenylphosphine surface coating of the applied NP. Thereby, protein coating of the blank NP surface may occur. The enhanced size selective accumulation of 18 nm gold NP indicates that the size and the curvature of the NP surface seems to modulate protein binding leading to a gold NP-protein conjugate with a preferred affinity to structures in the brain.

There was no significant difference between the brain accumulation of the 2.8nm gold NP either amino or carboxyl-coated for IT and IV administrations. However, after IO no negatively charged 2.8 nm NP could be detected in the brain whereas a low but measurable amount of positively charged 2.8 NP was detected.[49]
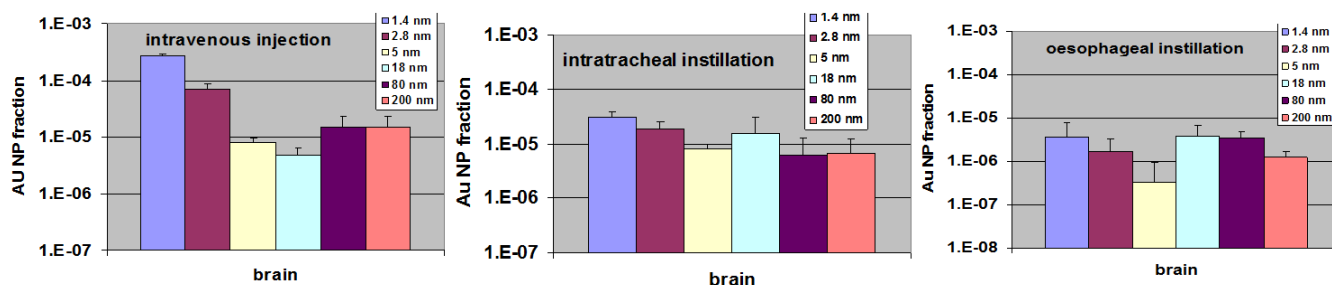

Figure 15. Twenty four hour gold NP accumulation in the brain of rats are shown for each administration route in the three panels (mean and SD, n=4).

Thus, route of exposure matters in terms of how much of the applied particle dose reaches the brain, although even with the most efficient transfer to the brain, less than 1% of the applied dose reaches the brain. Nanoparticle size also plays a role in determining the amount of access to the brain, although to a much smaller degree that with only the very tiny 1.4 nm particles showing a significantly enhance uptake into the brain via these routes. Surface charge also plays a role, as does surface chemistry, since we observed almost no titania particles in the brain, but some evidence of ceria nanoparticles following exposure via IV injection.

Objective 6: Understand the detailed pathways that nanoparticles take to reach the brain

The animal studies described under *Objective 5* above were also intended to provide as much information as possible about how the nanoparticles reached their destination, including the detailed nature of what is expressed on their surface as they pass through different intermediate tissue types on going to the brain. Thus, even if no neurotoxicity was observed, the investment of resources, and

animal experimentation would result in significant new data and a deeper understanding of the *in vivo* biodistribution of nanoparticles and the role of the protein corona.

This approach also complies with the EC directive on the reduction of animal experimentation, whereby the guiding philosophy of *NeuroNano* was to minimise the use of animals in this research by adhering to the 3 Rs rule (**reduce** the number of animals overall by **reusing** them when possible and **refining** the study design so that animals are used only for specific purposes where *in vitro* models are inappropriate). Thus, *NeuroNano* planned all animal experiments in a manner consistent with **reducing** the number of animals required to a minimum and the **reuse** of tissue from control and nanoparticle exposed animals, as appropriate so that all issues are addressed by the same animals, rather than multiple parallel usage.

Thus, in addition to assessing the biodistribution of nanomaterials and quantifying the amount reaching the brain from the different exposure routes, *NeuroNano* was also interested to show how this depends on the material, again both in the conventional sense (size, zeta potential etc.), and on the surface expression, that is, on the evolving protein corona. Additionally, the majority of the mechanistic studies were performed using *in vitro* cell and barrier models.

Mechanisms of uptake and intracellular transport of nanoparticles

To date, most studies have focused on identifying the point of entry and final destination of nanoparticles. However, to truly understand and predict the fate and impacts of nanoparticles, and specifically to identify rare events, a deeper knowledge and understanding of the entire intracellular itinerary and event sequence will be essential. It is, in principle, possible that the original corona, combined with new molecules picked up inside the cell, could, even if rarely, provide the nanoparticles with signals to allow them to access different subcellular destinations rather always following the endo-lysosomal pathway.

The transfer of cargo between membrane organelles of the endocytic pathway is a complex process involving multiple intermediates and a wide variety of trafficking machinery molecules.[50, 51] One of the major classes of proteins regulating all intracellular traffic is the Rab family of small GTPases.[52] All membrane structures participating in trafficking events are likely to contain at least one Rab family member, and therefore, UCD hypothesized that, by studying the co-movement of NPs with various Rabs, we would be able to precisely establish the complete intracellular pathway taken by NPs. In order to explore this approach, we transfected cells with various constructs encoding fluorescently tagged Rab proteins associated with endosomal–lysosomal trafficking. For our initial studies, we transfected cells with a construct encoding mCherry-Rab7, a small GTPase essential for the maturation of late endosomes, and fusion with lysosomes, and with a number of other relatively well-characterized Rab GTPases, namely, Rab5A, Rab9A, and Rab11A, all associated with various endocytic compartments.

UCD have utilised rapid time-lapse confocal imaging of nanoparticles in living cells, in which we label various intracellular membranes with fluorescent reporters. Rather than relying on the use of potentially biased and limited fixed-cell co-localization coefficients, our approach takes advantage of both the spatial and the temporal information that can be gained from using living cells.[53] Specifically, we consider the correlated co-movement of colocalized structures and thereby provide enhanced confidence in the colocalization metrics obtained.

These studies have revealed that 40 nm NPs rapidly transit through Rab5-positive membranes, before being passed into acidic compartments decorated with Rab9 and Rab7 (Figure 16). Only a minor population of the internalized NPs was detected in Rab11-positive membranes, indicating that recycling of these NPs back to the cell surface was not a preferred trafficking pathway. Finally by acquiring three-dimensional information in living cells, we provide the greatest possibility of identifying all of the particles within cells which allow us to detect relatively rare events in NP trafficking.

Rare events are important observations if we are to definitively understand how NPs interact with the intracellular environment. These phenomena may have important practical consequences for

identifying new routes out of the endo-lysosomal pathway, the appreciation of which will be essential if we are to further develop nanomaterials for efficient drug delivery in a safe manner.
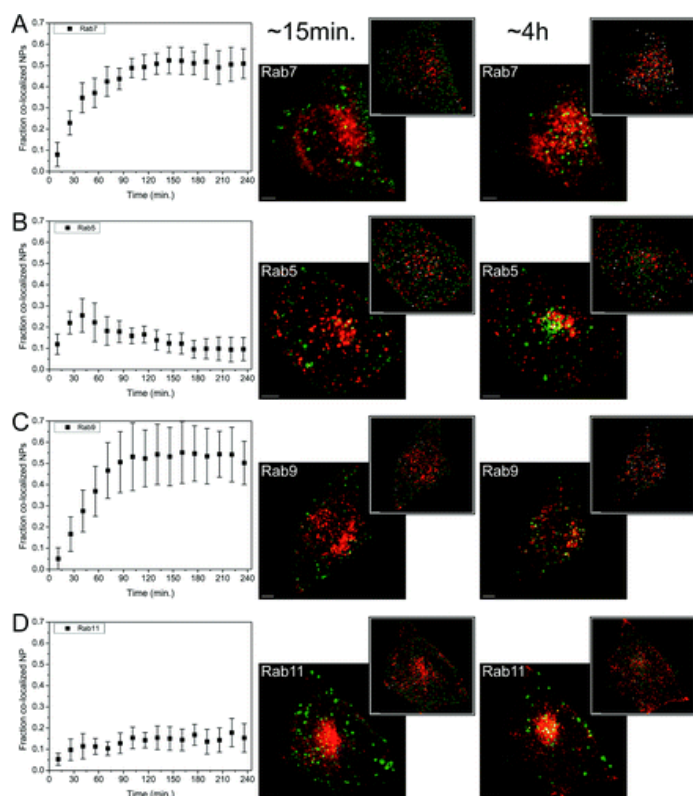

Figure 16: Time profiles of colocalization between 40 nm NPs and Rab7 (A), Rab5 (B), Rab9 (C), and Rab11 (D) together with representative images of the HeLa cells at the first (15 min) and final time points (4 h). The colocalization values are given as the fraction of cell-associated NPs colocalizing with each Rab. Each time point is an average of three consecutive frames from five different cells, and the error bars indicate the standard deviation between cells. The images show both the masked out raw and the corresponding segmented, digitized image. NPs are shown in green, Rab structures are shown in red, and in the segmented image, the colocalizing NP objects are shown in white. All scale bars represent 5 μm. From [53].

Role of cell cycle in nanoparticle uptake (including in polarised / barrier cells)

UCD have recently demonstrated the strong relation between nanoparticle uptake and cell cycle in proliferating cells and developed methods to measure if nanoparticle export is present[54]. In proliferating cell populations, during cell division, nanoparticles are split among daughter cells, thus the internalized nanoparticle dose is diluted. These findings can have important implications for slow- or non-dividing cells, such as for instance the blood barrier itself, where such diluting effect may be missing or less active, and if export is not present, bioaccumulation could arise over longer time scales. Thus, together with the question of transcytosis, it is important to study how nanoparticle uptake and cell cycle relates in such specialized barriers and if nanoparticles can impact the barrier itself.

For this purpose, we have used the *in vitro* BBB model developed within the project and we have investigated whether these cells are indeed cycling. This was done by exposure of the base analogue EdU to the BBB model to see if incorporation was present, as a sign of DNA synthesis, thus indicating active cell cycle. EdU is incorporated in cells synthetizing DNA, thus the percentage of EdU positive cells can be used to obtain the percentage of S phase cells and the evolution of the labelled subpopulation in the following hours can be used to monitor the cell cycle and estimate the cell cycle length[54].

A first result was that indeed some cells in the barrier synthetise DNA and divide (Figure 17). The percentage of these cells (S phase cells) in the differentiated BBB layers (7 days after seeding) was lower than in the case of isolated younger cells, not yet differentiatied, and decreased over the next days, as the percentage of cells in G0/G1 increased (this is a rather common observation when nutrients are depleted in confluent cell cultures[54]). By following the evolution of the labelled 'cycling' population of cells in the following hours and days (Figure 17), we could also estimate the length of the different cell cycle phases and found that first divided cells appeared after 8 h, and all labelled cells were divided after ~24 h. We estimated the cell cycle length to be around 40 -44 h.

We then used 40 nm PS-COOH polystyrene nanoparticles (100 ug/ml) in order to study kinetics of nanoparticle uptake in the BBB model. We observed that nanoparticle uptake in the barrier seems to be lower than in younger (1 and 4 days old) isolated cells. Uptake levels were rather similar between 1 day old (sub-confluent) and 4 days old cells (confluent cells, but with no tight-junction expression yet), so we concluded that this effect on nanoparticle uptake was connected to the development of tight junctions and the differentiation into a BBB layer. This can have important implications for *in vivo* studies, as it would suggest that many experiments performed in vitro could have much higher nanoparticle uptake levels than what more differentiated cells *in vivo* may show. Moreover the cell fluorescence distributions obtained on the BBB layer were very broad in comparison to 1 and 4 days old cells, thus indicating that nanoparticle uptake in the BBB layer was more heterogeneous within the population and subpopulations behaving differently seemed to be present. No obvious connections could be found between these subpopulations with different nanoparticle uptake and cell cycle phases.
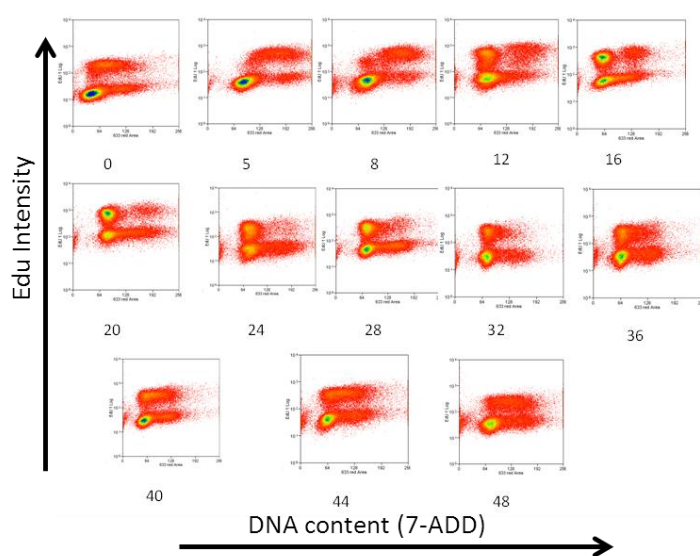

Figure 17: Cell cycle in the hCMEC/d3 BBB model. After formation of a BBB monolayer (7 days after seeding), the differentiated BBB cells were exposed for 1h to the base analogue EdU and then grown for further hours (as indicated below each plot), prior to detection of EdU intensity and of the total DNA content (by 7-ADD staining) by flow cytometry. Results showed that several cells were synthetizing DNA (EdU positive) and that 8 h after the EdU pulse fist positive cells divided (thus diluting their EdU content). All EdU positive cells were divided after roughly 24 h.

In vitro transport studies in the model BBB – evidence of nanoparticle transcytosis

In recent years, significant effort has been directed towards mimicking the *in vivo* BBB using immortalised cells. The hCMEC/D3 cell line was chosen as it is the closest available correlate to the human *in vivo* situation, and does not require co-culture with primary astrocyte cells, making it easier to work with for quantitative studies. In addition, the porous transwell system upon which the hCMEC/D3 monolayer was grown has previously been utilised in the presence of monolayers from alternate cell lines by other groups (19, 20).[55] As this cell line can differentiate into a monolayer in culture after 7-10 days, seeding the cells onto a permeable filter, the so called "transwell" system (Figure 2) enables an *in vitro* BBB model to be established. The transwell is composed of an insert filter on which the cell monolayer is grown, and an acceptor well, with the apical chamber mimicking the blood and the basolateral chamber mimicking the brain. Thus, molecules loaded into the apical chamber are presented to the cellular barrier formed on the filter, which can then to be taken up by the cells by a receptor-mediated (transcytosis) process.

Figure 18 shows a series of EM images of hCMEC/D3 cells grown on collagen/fibronectin coated transwell filters (0.4 μm pore size) exposed to 100 μg/ml 50nm SiO_2 NPs for 1 hour. In Figure 10A uptake can be observed as the plasma membrane envelopes the 50nm SiO_2. In Figure 10B the SiO_2 NPs can be observed in endosomes and lysosomes, and a nanoparticle can be observed on the basolateral side of the cell which is suggestive of an exocytosis process, whereby the particles are released from the basolateral side of the cell. In Figure 12, a small number of 0nm SiO_2 NPs can be seen adhering to the pore of the blank PET membrane 4 hours post NP exposure. Various other filter membranes are currently under investigation to circumvent this issue.
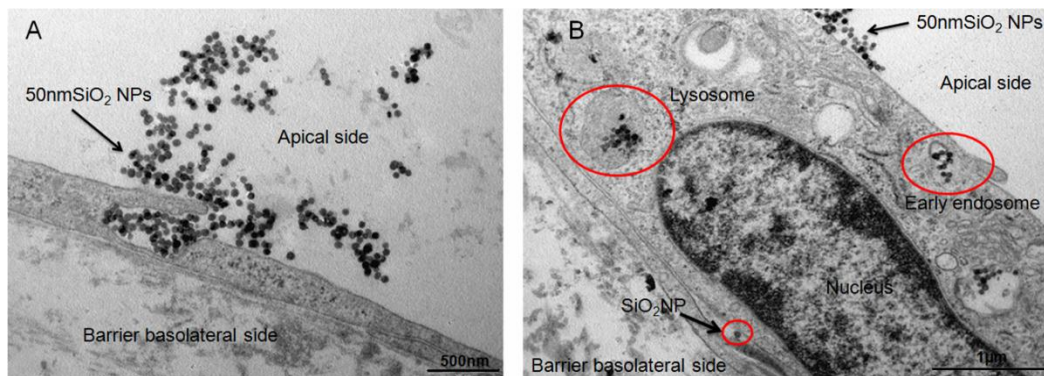
Figure 18: EM images of transport of 50nm SiO$_2$ NPs through the hCMEC/D3 monolayer four hours post-exposure. Scale bars are 500nm and 1μm respectively. Uptake of 50nm SiO$_2$ NPs by the plasma membrane was visualised in the hCMEC/D3 monolayer. The image on the right depicts localisation of 50nm SiO$_2$ NPs within a polarised hCMEC/D3 cell. From [56].

Extensive screening using a wide range of nanoparticles, and imaging with both electron and confocal microscopy has demonstrated that filter membranes exhibited some incompatibility/difficulties during nanoparticle transportation across the hCMEC/D3 model, e.g., nanoparticle adherence to filter pores, poor nanoparticle equilibration through filters and potential dye leakages of nanoparticles. Moreover, the formation of a human endothelial cell multilayer was also a concern in relation to growth on the filters, as demonstrated via the TEM images in Figure 9 above. After considering the above factors, UCD decided to focus on high-resolution TEM imaging to qualitatively analyze the possibility of nanoparticles penetrating the *in vitro* BBB. Strikingly, all tested nanomaterials in our studies entered the endothelial cell monolayer by endocytosis mechanisms. In relatively rare case a few nanoparticles were found to reach the basal side of the BBB by trancytosis, indicating clear evidence of nanoparticle penetration through brain endothelium.[43] It is also clearly evident that such penetration still relied on a high dose of nanoparticle exposure to the *in vitro* BBB. The concentration of which may greatly exceed what would arrive into the brain capillary system *in vivo*, considering nanoparticle initial passage through the circulatory/excretory system post animal exposure.

Role of proteins in corona in vivo on nanoparticle distribution

The pulmonary route is considered to be one of the primary exposure routes for inadvertent exposure to nanomaterials, as well as being very promising for drug delivery by inhalation. In this regard, understanding the role of lunch surfactant proteins on the biodistribution of nanoparticles is vital. Directly after their deposition, inhaled Au NP come into contact with pulmonary surfactant protein D (SP-D). SP-D can agglomerate Au NP *in vitro*, and this may influence the clearance as well as the systemic translocation *in vivo*.

HMGU undertook a study to investigate the clearance and translocation of Au NP at a very early time point after inhalation, as well as the influence of SP-D on this. Aerosolized 20-nm radioactively labelled Au NP were inhaled by healthy adult female mice. One group of mice received dissolved 10 μg of SP-D by intratracheal instillation prior to the Au NP inhalation. After a 2-hr Au NP inhalation period, the mice were killed immediately, and the clearance and translocation to the blood stream were investigated.[57] The highest amount of Au NP was associated with the lung tissue. In the bronchoalveolar lavage fluid (BALF), more Au NP remained free compared with the amount associated with the BALF cells. The amount of Au NP cleared by the mucociliary escalator was low, probably because of this very early time point. Instillation of SP-D prior to Au NP inhalation had no statistically significant effect on the biodistribution of the Au NP, and that SP-D has only a minor effect on Au NP translocation and clearance at a very early time point.[57]

Objective 7: Determine if there are indeed neurodegenerative disease endpoints for a range of animal models, and relate the extent of those endpoints to the amounts and properties of nanoparticles that have reached the brain

NeuroNano aimed to determine whether, according to the usual measures of diagnosis (cognitive, behavioural and pathological), nanoparticles were found to affect the time of onset, or extent of the selected neurodegenerative diseases (i.e. Alzheimer's and Parkinson's disease). Thus, from the animal studies described above, histopathological analysis of plaque load was performed, and behavioural studies on animals exposed to nanoparticles were also performed. Again, in line with the drive to reduce animal testing, a number of other species were also assessed, which are considered to be important as environmental sentinel species and models for oxidative stress (mussels) and or neurodegeneration (earthworms).

Mytilus edulis (mussel)

The mussel genus *Mytilus* is one of the most widely-used groups of sentinel species in aquatic toxicology, having been used for many years in environmental monitoring programs such as Musselwatch.[58] Advantages of this species include the mussels' wide geographic range, abundance, ease of identification, filter-feeding habit and sedentary nature. The animal is also robust enough to withstand moderate levels of pollution. This allows a broad range of loadings, with a resultant range oxidative stresses. It is also believed that the overall range of oxidative stress processes in *Mytilus* is comparable to that of animals, and therefore its whole-organism proteome is an attractive analytical tool to screen for tissue level oxidative stress. Indeed, in recent years, proteins expressed in *Mytilus* have come to be widely used as biomarkers of environmental stress, which is now considered a form of validation of the species for environmental (including oxidative) stress.[59]

Within *NeuroNano*, *Mytilus edulis* was used to probe uptake, distribution and physiological / biochemical effects resulting from nanoparticle exposure. A comprehensive set of exposure experiments has been completed for *M. edulis* exposed to three doses of CuO_2 and TiO_2 nanoparticles. Neutral red retention time (NRRT) assays were performed on haemolymph, a physiological index of lysosomal membrane instability in response to environmental stress.[60] The three main organs of the animal (gill, mantle and digestive gland) were excised for further analysis. Metal analysis indicated that Cu accumulated predominantly in the gill in a dose-response manner, with a smaller amount accumulating in digestive gland (Figure 19). In addition, haemolymph from control mussels were exposed on microscope slides to increasing doses of nanomaterials [61] and it was found that neutral red retention time was significantly reduced in mussels exposed to CuO_2 nanoparticles compared to controls. Sections of fresh tissue were chosen for histological staining. These reveal gradual deposition of brown cells / granules along the mantle margin illustrating the animal's response to exposure.[60] These granules have been found to affect the turnover of intercellular proteins [62] and are often found in *M. edulis* and other molluscs when exposed to environmental stressors such as contaminants (organic and metallic) [63].
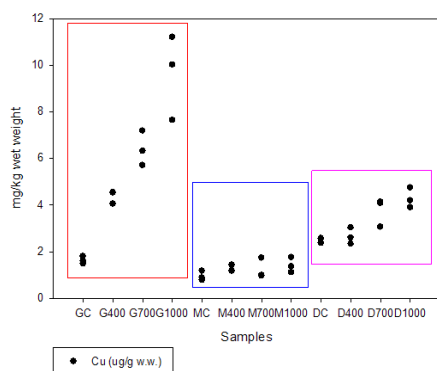

Figure 19: Copper levels in triplicate extracts of gill [64], mantle (M) and digestive gland (D) of *M. edulis* exposed to 400, 700 and 1,000 ppb CuO nanoparticles (C indicates control). From [60].

Nereis virens (ragworm)

The marine polychaete, *Nereis virens,* a common species in estuarine sediment and infaunal deposit feeder, was used as a further animal model. This species is known to select and ingest large amounts of fine sediment particles that tend to be enriched in organic material and metals. The Worms used in this exposure trial were bought at Halfway angling centre, Cork. Clean sediment was bought in from a local supplier (artificial play sand).

Worms were acclimatised for 5 days to clear the guts by placing in a large 100 litre tank with sediment up to 3 cm in height and containing artificial seawater between 16-20 psu at a constant temperature of 12ºC. Air was constantly supplied to the container, a lid was placed on top to prevent evaporation and water, salinity and food supply was checked every day. After 5 days the worms were removed from the, starved for 24 hours to ensure complete clearing of guts and divided between 12 separate containers (triplicate for each nanoparticle exposure and a control), 20 individuals per container, at the same salinity levels, depth of sediment and a constant air supply. Sediment had been placed in each container 24 hours previous to placement of *N. virens* to allow for settlement of particles. The nanoparticles were placed directly into the water column and the exposure concentrations are as follows; TiO_2 – 2 ppm, CrO_2 – 1000 ppb, CuO_2 – 1000 ppb & control. Samples were taken at three time points (24 hours, 4 days and 10 days), with 5 individuals taken from each container/sample point, a total of 15 individuals/nanoparticle and a total of 60 individual's /sample time.

Each worm was dissected and some gut, head and tail tissue of each individual was taken for analysis. All tissue samples were fixed in Davidson's fixative at 4ºC for 48 hours and then transferred to 70% ethanol. Tissue was processed and sectioned at 5µm prior to staining with haematoxylin and eosin (H/E) and then mounted using DPX. Slides were screened using a Nikon YS2-146972 light microscope, using a 10X, 20X and 40X magnifications. Screening of tissue samples from these animals is currently on-going and will be reported in due course.

Neuropathological and histological studies of rat brains

The effect of titania nanoparticles (ST01 titania anatase) on plaque formation was analysed in the 5XFAD Alzheimer's mouse model. These transgenic mice express 5 forms of human familial Alzheimer's Disease mutations (Co-overexpression of the human APP695 with the Swedish (K670N, M671L), Florida (I716V), and London (V717I) FAD mutations in the same APP molecule and human PS1 harbouring two FAD mutations, (M146L and L286V). These mice develop amyloid plaques, show impairments in neuronal transmission and synaptic plasticity as they age, and develop memory impairments similar to patients of Alzheimer's disease. Amyloid deposition and gliosis is accelerated in these mice compared to other models, with significant levels observed at 2 months of age.

Animals aged 6 weeks were injected at UU with ST01 titania anatase, 3-9nm (55nm by DLS) by intra-tracheal instillation. Animals were dosed 4 times intra-tracheally with 25 µl of particle, allowing a 48 period between injections. The study design and sacrifice dates were as follows (n=6/group):

1.   Non-transgenic control – 2, 4 and 6 months old

2.   Transgenic control – 2, 4 and 6 months old

3.   Non-transgenic titania dosed – 2, 4 and 6 months old

4.   Transgenic titania dosed – 2, 4 and 6 months old

Animals were perfused and brains collected for subsequent plaque load analysis (IHC: amyloid beta (1-42) and dense core plaques: Congo red). 6 brains were paraffin embedded per block (72 animals in 12 blocks) and sectioned at 5 µm exhaustively (~350 sections collected on APES-coated slides/6 animals of ~1400 sections in total). Every 40th section was sampled and sections (~35/animal) were de-paraffinised and stained with Congo Red and Haematoxylin as a counterstain. IHC staining for ß-Amyloid of the paraffin sections on slide was very labour- and cost- intensive: efforts to improve the method (dilution of reagents and use of staining dishes) are still being worked on. The main approach was neuropathological review of the histological material by a pathologist.

No significant differences in plaque loading were detected by this qualitative but sensitive approach. The neuropathological review conclusion is that intra-tracheal exposure to ST01 titania anatase (3-9nm primary particles, 55 nm in dispersion) did not cause any detectable neuropathology effects.

To test whether there was any subtle alteration to mouse brain microstructure that was undetectable to pathological review, quantitative stereological analysis of the retro-splenial coxtex was performed. The rationale for this investigation was to examine the brain region which has one of the highest levels of peri-natal apoptosis during development. The 6 month exposure time point was selected to maximise the possibility of detection of chronic low dose neuropathological change. The endpoints studied were the volume density of plaque, total plaque volume and total activated astrocyte number.

No significant differences in any of the three end-points (the volume density of plaque, total plaque volume and total activated astrocyte number) were demonstrated between control and treatment groups following 6 months exposure to titania nanoparticles administered intra-tracheally. This correlates well with the fact that no titania was determined in animal brain samples following IV or IT exposure to the same titania nanoparticles, as determined by TEM and ICP-MS (Figures 13 and 14).

Behavioural studies in rats and mice

Behavioural and memory changes are the first subtle changes of brain functionality that are observed well before any anatomical changes occur in neurodegenerative diseases. Thus, in addition to looking for physiological markers of neurodegeneration in response to exposure to nanoparticles, *NeuroNano* partners also performed some behavioural studies on nanoparticle-exposed and control animals.

Different doses of ST01 titania anatase (3-9nm primary particles, 55 nm in dispersion) with different stabilisers (e.g. gallic acid, citrate or a combination) were tested with these animals, and analyses made at different time points after exposure. Cognitive performance in behavioural tasks was analysed using a battery of spatial and nonspatial memory tasks, motor tasks, and anxiety / emotional tasks. The development of potential cognitive impairments will be tracked over time.

Animals were trained in an operant conditioning task to respond under an alternating-lever cyclic-ratio schedule (ALCR). Subjects capable of carrying out a complete schedule were subsequently implanted with a permanent ICV cannula, allowed to recover for 7 days and dosed with ST-01 titania (3-9 nm) nanoparticles stabilised with either citric acid or gallic acid. Animals were then subject to further ALCR schedule and the number of lever switching errors, incorrect lever perseverations and response rate recorded.

The conclusion from these studies is that very little behavioural effects were observed in the rats or mice. Further data analysis is still underway and will be published over the coming months. These findings are also consistent with very small amounts of the ST-01 titania particles reaching the animal brains following IV or IT exposure.

Objective 8: **Provide input for a risk assessment framework (and screening protocol) for engineered nanoparticles for use by regulatory authorities and industry**

The ultimate aim of the *NeuroNano* project was to begin development of a simple rational screening methodology for neuronanotoxic hazard, the so-called FIBROS map. The basis of the FIBROS map was the hypothesis to be tested within *NeuroNano* that screening for hazard in the future could be based on the three (presumed) contributing factors of engineered nanoparticles to neurotoxicity: access to the brain, oxidative stress generation potential, and amyloid fibril generation potential, possibly encapsulated in the arrival efficiency of the nanoparticles to the brain, and the location on the FIBROS hazard map.

As described in the sections above, a number of challenges to the model arose from the research performed within the *NeuroNano* project, and from emerging data external to the project, as is usual for such a speculative project in an emerging scientific field. Some of the most significant findings that

need to be included in an adapted form of the risk model include the fact that neither oxidative stress could nor fibrillation potential could be simply correlated with nanoparticle physic-chemical parameters, the fact that access to the brain may not be necessary for nanoparticles to exert an effect on cells on the brain side of the blood brain barrier, and the fact that nanoparticle accumulation in the endothelial barrier might result in the barrier itself being a target for nanoparticles with unexpected consequences. Thus, toxicity to the endothelium itself needs to be considered, as well as long tern accumulation effects even for particles that are inherently non-toxic, such as the COOH-PS particles, but where subtle cytokine effects can be amplified by the presence of other cell types, including those not directly exposed to the nanoparticles.

Whether the large accumulation of nanoparticles in lysosomes observed in the *in vitro* model is representative of the situation *in vivo* has yet to be determined, and was the subject of much lively debate at the NeuroNano – ESF EpitopeMap co-sponsored workshop held in Dublin on 6th and 7th December 2011 in conjunction with the NeuroNano final meeting. While such large numbers of lysosomes had not been observed *in vivo*, this may be because they had not been looked for. Additionally, *in vivo* quantitative studies have an inherent limitation that it is difficult to separate the brain tissue from the barrier endothelium, so exact localisation is hard to determine (in barrier endothelium or actually in brain tissue itself). A number of recommendations around the need for elucidation of these issues were included in the report from the workshop (available from the NeuroNano project website and the NanoSafety Cluster website).

Thus, a ranking of the nanoparticles in the FIBROS map was not really possible, however, we proposed an advanced model for further development by the community, as shown in Figure 20.
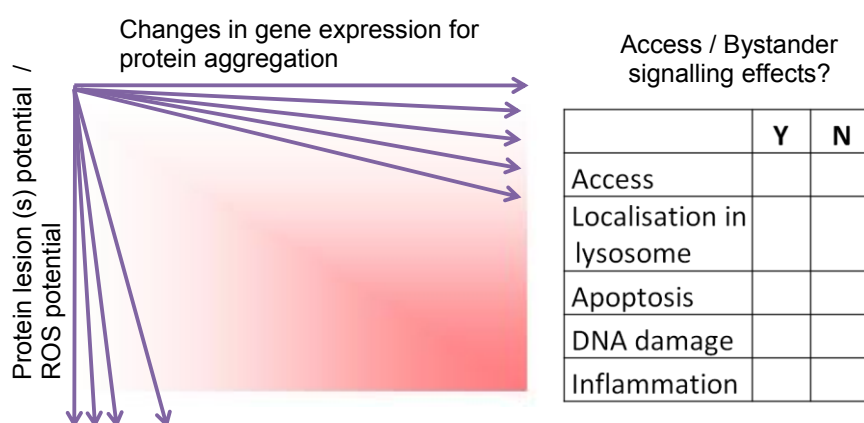

Figure 20: Expanded FIBROS map, which includes a spectrum of parameters in each category as well as taking account of the fact that direct contact with nanoparticles may not be required, and that there may be a complex interplay with other endpoints such as inflammation and apoptosis.

We can summarise the findings from the *NeuroNano* project as they relate to the concepts of the FIBROS map as follows:

Fibrillation potential

- Induction of fibrillation is a surface area effect – all nanoparticles induce or modulate the rate of protein fibrillation, and there appears to be a detailed interplay between monomer, oligomer and fibril for residence at the nanoparticle surface.

- The presence of a protein corona on nanoparticles, such as would inevitable occur during nanoparticle uptake *in vivo*, slowed the fibrillation of Aβ, regardless of size, surface chemistry, charge or aspect ratio when compared with the same pristine (bare) nanomaterial.

- In order to be effective as an inhibitor of Aβ fibril formation the proteins in the nanoparticle-protein corona must be in their native form: corona proteins that have been denatured by heat were found to actually accelerate the formation of protein fibrils, mostly likely due to the exposure of hydrophobic amino acid residues from proteins that do not refold after heating.

- Further work is required to understand more fully the specific mechanism by which fibril formation is inhibited by the presence of a protein corona around nanomaterials, and also to determine whether slow protein degradation *in vivo* in the presence of nanoparticles could contribute to protein fibrillation and neurodegeneration over the long term.

- The fact that particles bring their corona with them all the way to their final location in cells suggests that there may also be subtle effects from the presence of serum proteins in cells when they are normally excluded from cells. Whether this would also have a role in protein fibrillation in cells, or indeed induce other impacts requires further investigation.

- The fact that the positively charged polystyrene particles, which induced apoptosis in astrocytes, were also found to up-regulate signatures related to protein aggregation and fibrillation is significant, and suggests that this could be an alternative measure against which to assess fibrillation potential of nanoparticles. A wider panel of particles would need to be assessed, along with an in-depth biomarker validation process in order to demonstrate this link robustly.

Reactive oxygen stress generation potential

- No link between cellular oxidative stress and nanoparticle effects could be confirmed. Indeed, the particle that was found to induce the most effects in cellular assays, the TiV+ which was cytotoxic, haemolytic and induced mitochondrial damage, did not have an excess of free radical generation as measured by EPR compared to the non-toxic nanoparticles in the panel.

- A link between particles with a positive surface charge and toxicity was also investigated, and while the connection held in some assays, the TiV+ particles were found to damage mitochondrial function, whereas the positively charged control nanoparticle, amine-modified polystyrene nanoparticles were not, suggesting that more than simple charge was involved in the induction of this particular effect.

- Oxidation of protein thiols and carbonyls is a generally-applicable, sensitive marker of oxidative stress induced by nanoparticles in both cells and more complex organisms such as mussels and worms (much more robust than measuring cellular oxidative stress in the presence of nanoparticles). However, as with the biomarkers for protein fibrillation, significant additional screening, plus validation work is required.

Access to the brain

- Route of exposure matters in terms of how much of the applied particle dose reaches the brain, although even with the most efficient transfer to the brain, less than 1% of the applied dose reaches the brain.

- Using ST-01 titania nanoparticles, brain accumulation was found to peak at around day 7, but particles could still be detected by day 28 following a two-hour inhalation (mean and SD, n=4) in rats. On the other hand, following peripheral injection of the same nanoparticles in rats, titania particles were found only in liver and lung and no particles were found in brain tissues.

- A significant increase in cerium content against control (6-fold; $p<0.05$) was observed in brain tissue of rats injected intravenously with ceria nanoparticles. This data may suggest that ceria passes the blood brain barrier readily.

- Nanoparticle size plays a role in determining the amount of access to the brain, although to a smaller degree that expected for exposure via intertrachael instillation and intravenous administration: brain accumulation of 5 nm – 200 nm gold NPs is similar for all particle sizes tested. Only the 1.8 nm particles showed significantly enhance brain uptake via these routes.

- The exposure route dependence is related to the nature of the biofluid milieu that the particles initially encounter and thus to the nature and composition of the protein corona that forms: exposure via the oesophagus led to the highest accumulation in the brain for the 18 nm gold NPs, indicating that something happens with these NP in the GIT which alters their biokinetic fate. The most obvious explanation is a different specific protein coating, related to the exposure route. In the stomach, it is conceivable that the acidic environment, as well as the presence of gastric enzymes, are likely to degrade the triphenylphosphine surface coating of the applied NP. Thereby, protein coating of the blank NP surface may occur.

- Although uptake is rather low compared to the initially injected dose, conjugation of gold nanoparticles with either ApoE or albumin significantly increased the amount of particles detected in all investigated brain compartments compared to the bare citrate stabilized gold NP. Retention after 24h was very similar in the total brain for both apoE conjugates.

- The distribution pattern for single brain compartments was very similar for both apoE conjugates. Therefore we conclude that both conjugation methods led to an increased translocation into the brain across the blood-brain barrier. Compared to the apoE conjugates the albumin conjugates seem to have an even better access to the brain.

Potential for indirect signalling

- The low amount of particles reaching the brain could be a result of significant accumulation of nanoparticles in the brain endothelium itself. Indeed, using the *in vitro* barrier model, we observed significant accumulation of nanoparticles in lysosomes of the barrier cells, from as early as 4 hours after exposure, and reaching a plateau level at account 24 hours.

- While such significant accumulation of non-toxic nanoparticles, such as the Ps-COOH shown here, and also silica nanoparticles or gold nanoparticles which we have also studied using this model, do not seem to perturb the cells, there are indications that signalling molecules are secreted by the endothelium in response to the presence of the PS COOH NPs, which are subsequently amplified by the astrocytes when using a co-culture model.

- Growth of human astrocytes below the BBB monolayer allows the communication of supporting signals *in vitro* which aids in mimicking a more realistic *in vivo* situation, allowing these signalling pathways to occur. Assessing impacts of less benign particles accumulating in endothelium is necessary, although as the particles themselves begin to induce effects on the endothelium barrier separating out the sequences of signalling events will become more difficult, and isolating paracrine signalling from inter-cellular signalling will require detailed molecular biology studies.

Need to connect access to the brain with impact

- The neuropathological review of animals exposed intra-tracheally to ST01 titania anatase (3-9nm primary particles, 55 nm in dispersion) indicated that these particles did not cause any detectable neuropathology effects: No significant differences in the volume density of plaque, the total plaque volume or the total activated astrocyte number were demonstrated between control and treatment groups following 6 months exposure to titania (ST-01) nanoparticles administered intra-tracheally.

- Thus, from a risk assessment viewpoint, simply observing that a small percentage of particles can reach the brain may not actually represent a risk, if there is no associated hazard. However, given that these studies were performed on limited numbers of animals, a very limited panel of particles, significantly more research is required in order to make a real connection between access and impact. Additionally, the particle tested were themselves very benign, and thus more inherently toxic particles should be tested in the future. However, from the viewpoint of the FIBROS model for risk assessment, it is clear that access and impact need to both be assessed, and dose-response elements included also.

All *NeuroNano* publications are available at www.ucd.ie/cbni.

Potential impact & main dissemination activities / exploitation of results.

Dissemination activities and the exploitation of results

The *NeuroNano* project has already resulted in over 50 publications[2], with several more in press or under review. The knowledge and experience gained is now forming the basis for other projects, both in the EU and across the world, including incorporation into several other EU projects, such as QualityNano, the EU infrastructure for nanosafety assessment which is disseminating many of the *NeuroNano* findings to the community via training schools and the development of positive and negative control nanomaterials; and NanoTransKinetics, a modelling project that aims to develop a predictive tool for determining uptake, localisation and impact of nanomaterials based on their physic-chemical characteristics and their subsequent biomolecular corona.

Additionally, *NeuroNano* protocols and methodologies are being incorporated into several other on-going international efforts, such as MARINA (http://www.marina-fp7.eu/), the Celtic Alliance for Nanohealth (http://celticnano.eu/) and the Helmholtz Virtual Institute for NanoTracking (http://www.hzdr.de/db/Cms?pOid=34014&pNid=2452). Members of the *NeuroNano* consortium have been actively involved in dissemination of the outputs from the project to regulatory bodies and policy makers, via representation on the OECD working party on manufactured nanomaterials, via ISO and CEN, via the DG Sanco Scientific Committee for Emerging and Newly Identified Health Risks (SCENIHR) and via the European Medicines Agency (EMEA) and the European Food Safety Authority (EFSA).

From the project conception stage, emphasis was placed on ensuring impact for, and contribution to standardisation efforts, including:

- ensuring that the *NeuroNano* partner's efforts to establish best practices within the group should lead to protocols of value to the international community of scientists working in the nanotoxicology arena. The project was designed so that members of the Advisory Board who liaise with bodies responsible for standards such as CEN WG 166, ISO newly created TC on nanotechnology will be aware of the best practices that we are developing.

- building on the technical and focussed nature of the project, which was heavily biased towards quantitative and verifiable methods, to ensure that the outputs are framed in such a way that they contribute key scientific data to the development of standards, test and measurement methods in the field of nanoparticle interactions with biological systems. Interfaces were established with the Research Framework program, COST, EUREKA and CEN-STAR.

NeuroNano has over the course of its 36 months provided essential scientific data which will assist in the planning and development of reference technical instruments needed for EU policies such as the revision of Environment Directives, REACH, protection of health, food safety, etc.

Enduring impact in a range of scientific and quality assurance areas

The *NeuroNano* project has already had, and will continue to have, impact far beyond its size and budget, in part due to the formative stage of the research field at the outset of the project, and in part as a result of the fact that it has facilitated the development of several insights that will have a durable impact on the research field. Among the key scientific developments resulting from the *NeuroNano* project were the fact that the cell cycle may play a significant role in determining the accumulation or otherwise of nanoparticles in key cells, the fact that the nature of the protein corona can be significantly different under *in vitro* versus *in vivo* conditions due to the significant differences in the amounts of proteins present relative to the available particle surface area.

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[2] Full list of NeuroNano publications is provided as part of the summary of dissemination activities, and is also available on http://www.neuronano.eu/sections/AboutNeuroNano/ProjectOutputs.

Additionally, *NeuroNano* has pioneered several new tools and approaches within the project, which are now being developed into a set of high level protocols and best practice papers for broader dissemination to the community. *NeuroNano* has also resulted in the development of several new particles that are now available more broadly to the research community, such as the various radiolabelled particles developed by the JRC and the background research on the potential of the amine-modified polystyrene nanoparticles for use as positive control nanoparticles for apoptosis. These are now being developed further within the QualityNano research infrastructure project, and are expected to be launched in early 2013.

Further details on some of the key impacts are given below.

Radiolabelled nanoparticles synthesised by JRC

A key output from *NeuroNano* is the availability of a range of radio-labelled nanoparticles for use in dosimetric / biokinetics or environmental fate and behaviour studies, or other studies where tracking of nanoparticles over specific time periods is required.

Pre-synthesised Au, CeO_2, Fe_3O_4, and TiO_2 nanoparticles can be radiolabelled using ion-beam methods, and Au and Ag electrodes can be activated for subsequent nanoparticle synthesis.

Significant progress towards activation of carbon-based nanoparticles has been made, with direct high-energy proton bombardment and a novel recoil method both achieving good levels of activity in MWCNTs. Some further work has to be performed to check for radiation damage effects. The recoil technique can be used to radiolabel nearly any type of nanoparticle, and successful initial experiments have been performed on SiO_2 and nanodiamonds.

Radiochemical synthesis methods can also be used to synthesise labelled nanoparticles starting from precursors with trace amounts of radioisotopes. In this way, SiO_2 nanoparticles labelled with a Co-56 radiotracer have been successfully created. This route can potentially be used to synthesise many different types of radiolabelled nanoparticle under carefully controlled laboratory conditions.

Access to radiolabelled nanomaterials is possible via the QualityNano Research Infrastructure (www.qnano-ri.eu), or by contacting the JRC (Neil.Gibson@jrc.ec.europa.eu), assuming that all necessary safety provisions and permissions for handling radioactive materials are in place, that the irradiation/labelling process can be appropriately included in the JRC cyclotron's schedule, and that the requesting institute provides the necessary input (knowledge, assistance, etc.) in the case of new experiments.

Positive and negative control Nanoparticles for apoptosis and cell cycle regulation

Having studied in detail in several cell lines and several species the impacts of amine-modified polystyrene nanoparticles, and found a consistent and dose-dependent onset of apoptosis (linked with autophagy under some circumstances), as well as a low-dose impact on cell cycle, these nanoparticles were determined to be an ideal candidate for onward development, and round-robin validation as positive control nanomaterials for apoptosis and cell cycle regulation. The fact that they are easily labelled for tracking and surface functionalised with a variety of functional groups adds to their attractiveness as a positive control candidate. Since the carboxyl-modified equivalent particles show almost no effects on tested cells or organisms at equivalent exposure doses and times they are an ideal candidate for onward development as negative control nanoparticles for the same end-points.

Since the development of such positive and negative control nanoparticles is outside the scope of NeuroNano, the body of data was handed over to the QualityNano project (formerly QNano) for onward development and pre-validation, with the final plan being that IRRM might develop them as certified reference materials.

Protocols covering a range of aspects of nanoparticle interactions with cells, barriers and organisms

Protocols covering a variety of aspects, from nanomaterials synthesis and characterisation, surface functionalisation and dispersion, to all aspects of nanoparticle uptake and localisation, apoptosis, assessment of changes in gene expression, oxidative stress and redox proteomics, dosimetrics and biokinetics and assessment of behavioural impacts of nanomaterials, have been developed within *NeuroNano*. Many have already been published as part of scientific manuscripts, and many others are currently in final stages of preparation for publication.

As part of the wrap-up of the *NeuroNano* project, these protocols are currently being re-formatted into the template designed by NanoImpactNet and will be made available to the community via the NanoImpactNet protocols database, which has been taken over by the NanoSafety Cluster.

New assays and/or experimental approaches developed within NeuroNano

Effect of ratio of available protein to nanoparticle surface area on resultant corona (in vitro versus in vivo coronas)

A key advance developed within NeuroNano has been the understanding that the corona is context dependent: i.e. the corona formed under low protein conditions (i.e. *in vitro* conditions) may not be identical to that formed under high protein conditions (i.e. *in vivo* conditions).[65] The protein adsorption for two compositionally different NPs, namely sulphonated-polystyrene (PSOSO$_3$) and silica (SiO$_2$) NPs was studied at different protein concentrations keeping the particle surface area constant. NP-protein complexes were characterized by Differential Centrifugal Sedimentation (DCS), Dynamic Light Scattering (DLS) and Z-potential both *in situ* and once isolated from plasma as a function of the protein/NP ratio. A semi-quantitative determination of their hard corona using one-dimensional sodium dodecyl sulphate poly-acrylamide gel electrophoresis (1D PAGE) and electrospray Liquid Chromatography Mass Spectrometry (LC MS/MS) was introduced, which allowed us to follow the total binding isotherms for the particles, identifying simultaneously the nature and amount of the most relevant proteins as a function of the plasma concentration. We find that the hard corona can evolve quite significantly as one passes from protein concentrations appropriate to *in vitro* cell studies to those present in *in vivo* studies, which has deep implications for in *vitro-in vivo* extrapolations and will require some consideration in future.

Thus, it is a quite general observation that binding leads to relatively complete surface coverage for even low plasma concentrations. The protein concentration study also suggests a progressive displacement of proteins with lower affinity in favor of those with higher.[65] However, there are significant differences compared to the more usual forms of adsorption, including the fact that, when formed, the protein layer is essentially irreversible on the time scales of the experiments carried out here. We interpret this to mean that the system seeks to lower its (initially high) surface energy by selecting and exchanging on shorter timescales from the whole set of proteins that diffuse to the surface.

Cell cycle effects

Work at UCD regarding the role of cell cycle in determining nanoparticle uptake rates and intracellular dose[66] have resulted in some important protocols and experimental design considerations. In summary, the nanoparticles studied here do not disrupt cell cycle progression and, once internalized, localize in lysosomes, where they remain. Nanoparticle export is negligible and dilution of the intracellular nanoparticle load occurs by cell division. Uptake rates during the different cell cycle phases are comparable. Nevertheless differential accumulation in the different phases, with characteristic ranking in the order G2/M > S > G0/G1, arises because for times shorter than cell cycle duration, G2/M-

phase cells have not yet divided, and therefore have accumulated nanoparticles for longer, whereas cells in the S phase have accumulated nanoparticles after cell division and G0/G1-phase cells have just divided, and have not had time to internalize many new nanoparticles. Nanoparticle uptake curves that deviate from linearity result from averaging over the uptake of cells that are in the different cell cycle phases.

It is now clear that future studies and models of nanoparticle uptake must accommodate the cell cycle, these being inseparable phenomena, even where the rates of uptake are independent of cell cycle phase. For example, biological or toxicological experiments using the same cells and nanoparticles, but prepared slightly differently, may have different proportions of cells in the different cell cycle phases. As many biological effects will depend on the internalized nanoparticle concentration, the role of the cell cycle is also implicit in such experiments.

From a broader perspective there are also important implications. The nonlinear uptake kinetics could be mistaken for export, masking a permanent intracellular load. Hypotheses of cell-level clearance should therefore be evaluated explicitly in the future. From a practical point of view, the techniques developed here are well-placed to address many aspects of the impact of cell cycle on the interactions of nanoparticles with cells.
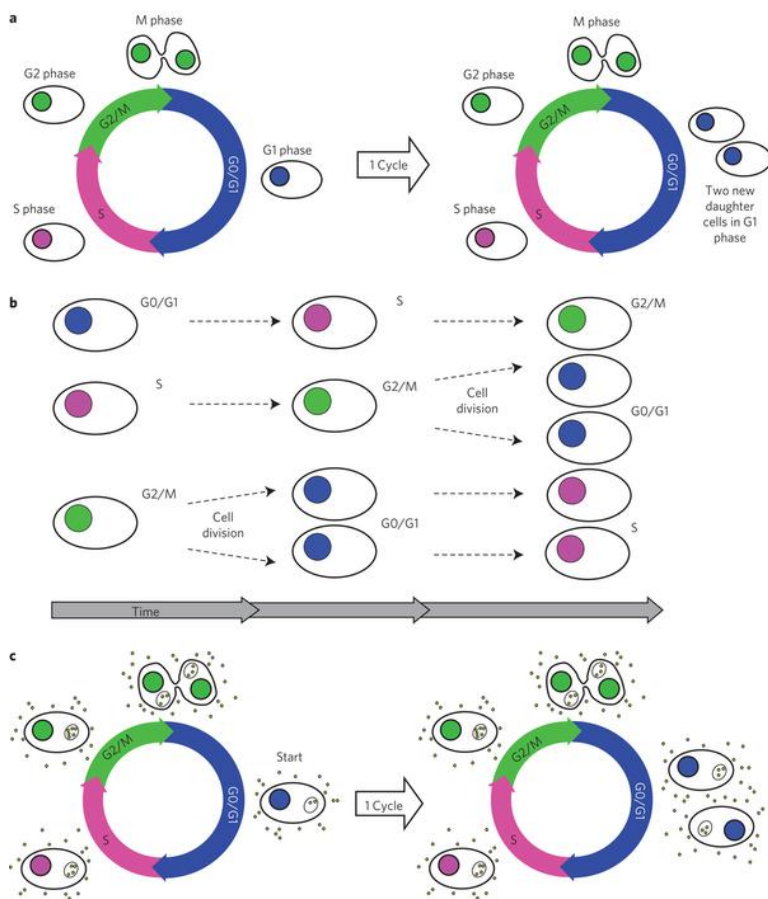

Figure 21: a, The cell cycle, which is a series of events that lead to cell division and replication, consists of four phases: G1, S, G2 and M. Cells in the different phases are distinguished by blue, pink and green nuclei for the G1, S and G2/M phases, respectively. The cell cycle commences with the G1 phase, during which the cell increases its size. During the S phase the cell synthesizes DNA, and in the G2 phase it prepares for cell division, which occurs during the M phase. The two daughter cells then enter the G1 phase. **b**, A cell culture contains a mixture of cells in different phases of their cell cycle, simultaneously undergoing progression and cell division. **c**, Nanoparticle uptake in a cycling cell. The yellow-green circles represent the nanoparticles, which, inside cells, accumulate in the lysosomes, represented by the oval compartment. When the cell divides, the internalized nanoparticles are split between the two daughter cells. Figure not to scale.

TEM protocol for assessing integrity of the in vitro BBB

In light of the increasing drive to reduce reliance on animal testing there is a concerted effort to develop and validate alternative *in vitro* methods for research, screening and regulatory assessment of the impacts of chemicals, including nanoparticles. However, to date there remain important concerns about the reliability and relevance of such *in vitro* methods, and whether they represent a realistic alternative to animal studies. Methods to ensure the quality and reproducibility of *in vitro* methods and the comparability of the data generated to the *in vivo* models they are intended to replace are required to begin to address these issues.

A significant outcome from *NeuroNano* is a protocol to characterize and monitor the quality of *in vitro* human cellular barrier models using high resolution Transmission Electron Microscopy (TEM), which can be applied for transport assays, mechanistic studies and screening of drug/compound (including nanoparticle) penetration across such biological barriers. Data from two examples of biological barriers are given, namely the hCMEC/D3 endothelial blood-brain barrier model, and the Caco-2 intestinal epithelial barrier model, are given to show the general applicability of the method.[67]

As described above and shown in Figures 11 and 12 key elements of the protocol include ensuring that barrier is intact, the tight junctions are formed and that there are no holes in the barrier which would allow particles to diffuse through the membrane thereby confounding measurements of active transport via transcytosis.

The neutral red retention time (NRRT) assay

The NRRT assay is useful for detecting decreased lysosomal membrane stability in haemocytes sampled from bivalves, a phenomenon often associated with exposure to environmental pollutants including nanomaterials. Bivalves are popular sentinel species in ecotoxicology and use of NRRT in study of species in the genus *Mytilus* is widespread in environmental monitoring. The *NeuroNano* partner UCC has shown that this assay can be applied to a panel of metal and metal oxide nanoparticles (2ppm) and distinguish between those that are toxic and those that are not, and connect this with markers of oxidative stress or other toxicity pathways, and they suggest that this could support use of NRRT as a generally applicable *in vitro* assay in nanosafety assessment.[68]

Impact for standardisation

Several of the approaches developed or optimised in *NeuroNano* are suitable for standardisation, and indeed NeuroNano was presented at two of the European Commission workshops on standardisation activities from EU NMP projects, as follows:

Dr. Iseult Lynch presented data specifically related to standardisation from *NeuroNano* at the NMP Seminar on Standards & Standardisation on December 14th 2010 in Brussels. Talk title: "NeuroNano (EU FP7 214547-2) Contribution to standardisation processes". Significant interest was expressed by CEN in the aspects of the protocols for Differential Centrifugal Sedimentation characterisation of nanoparticle dispersions *in situ* in biological fluids and in the validation of the *in vitro* model Blood brain barrier, and follow-up is underway.

As a follow-up event, the Industrial Directorate of DG RTD organised for NMP project partners a Seminar on "Standardisation in Research and Innovation, practical tools for the dissemination and implementation of research results" which was held on 10th November 2011, and at which Dr. Iseult Lynch gave an invited presentation session on the NeuroNano project's experience of the Standardisation process focussing on step 1 "*preparing for standardization*".

Following on from that meeting, *NeuroNano* initiated contacts with CEN - European Committee for Standardization / CENELEC - European Committee for Electrotechnical Standardization to identify next steps (including via the QualityNano project).

Among the items that have been extensively discussed as a candidate for standardisation is the combined use of Differential Centrifugal Sedimentation and Transmission Electron Microscopy to describe the "dose" of nanomaterials actually presented to cells/animals under *in-situ* conditions. Differential Centrifugal Sedimentation is one of the only methods to accurately describe (semi-quantify) the amount of monomeric particles, versus particle agglomerates of 2, 3 or many particles upon interaction with the exposure medium components (e.g. proteins, natural organic matter etc.), and Transmission Electron Microscopy is used to visualise the dispersion state of the particles *in situ* in the dispersion medium, as shown in Figure 22.

Why do we need to describe the dose of nanoparticles?

Dose – response is the basis of hazard assessment, bus assumes that entire mass of nanomaterials is in monodisperse form with no aggregates / agglomerates – typically not the case, especially when dispersed in "real" biofluids, such as cell culture media containing 10% Foetal Calf Serum.

Thus, there is a need to know the *in situ* dose in nanoform (< 100 nm). To achieve this, *NeuroNano* proposed:

⇒ Characterisation *in situ* in biofluids

⇒ Combination of DCS and TEM describes dose presented to cells

⇒ Time-resolved data (evolution of sample)

⇒ Applicable to any biofluid, including environmental (e.g. natural organic matter).
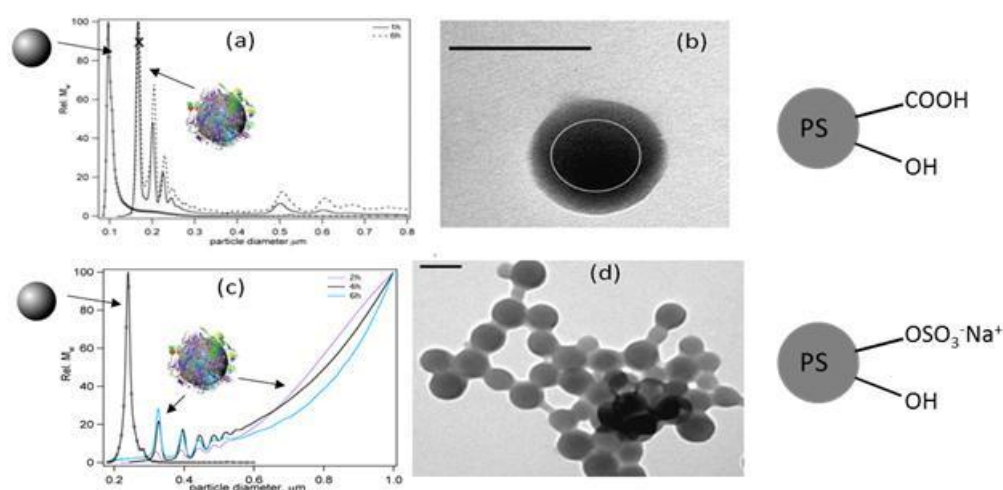

Figure 22: DCS data for two different nanoparticles dispersed in plasma. In the first case (top), particles remained well dispersed, with the majority of the particles as monomers, and some dimmers and trimers, and a very small amount of larger aggregates. In the second case (bottom) the particles agglomerated very quickly in the presence of proteins, with almost no particle monomers remaining due to agglomeration, as also confirmed by the TEM image.

Impact for regulatory agencies and policy groups

Neurodegenerative diseases currently affect over 1.6% of the European population, with dramatically rising incidence that is likely (in part) due to the increase in the average age of the population. There are persistent claims, based on the epidemiology, that pollution may be a cofactor in Alzheimer's disease, although the evidence is controversial. The risk that engineered nanoparticles could introduce unforeseen hazards to human health is now a matter of deep and growing concern in regulatory bodies, governments and industry. However, at present there is only circumstantial evidence that nanoparticles could impact on such diseases.

The 'blood–brain barrier' (BBB) is a protective mechanism that separates the bloodstream from brain tissue while allowing passage of essential nutrients to the brain. Although this neuroprotective function is vital, the BBB also impedes the passage of pharmacologically beneficial substances in instances of CNS diseases, such as Alzheimer's disease, Parkinson's disease, neuro-AIDS, stroke and dementia. Thus, despite the existence of these transport pathways, pharmaceutical companies have invested significant sums in the design of drugs that can cross the BBB, with very limited success. The limited penetration of drugs into the brain is the rule, not the exception, and in fact more than 98% of all small molecules do not cross the BBB.[69]

Due to their small size and large surface area that is rapidly coated with proteins, which thereby confer on them a biological identity, nanoparticles have unique access to the cellular machinery and can potentially cross biological barriers such as the BBB, offering extraordinary hope for treatment of diseases such as HIV and Alzheimer's disease, but also raising significant concerns regarding their safety. Thus, based on the information presented above, whereby nanoparticles of common materials such as silica and polystyrene appear to be able to utilise active transport mechanisms across the human BBB model, even at sizes up to 200 nm, albeit in low numbers compared to the doses presented, it is clear that nanoparticles cannot be considered as chemicals for risk assessment purposes. Thus it is recommended that regulators consider nanomaterials as biological entities in terms of the assessment of their environmental safety and exposure.

It is further recommended that significant additional research be performed as to the mechanism and kinetics of nanoparticle transport into and across the endothelial barrier and the consequences of nanoparticle accumulation in the endothelium be assessed. Additionally, a dedicated effort to assess the effects of nanoparticle signalling across and from biological barriers is required.

Another issue that emerged is the critical knowledge gap regarding the relevance of in vitro models for predicting *in vivo* impacts. Thus, the whether the large accumulation of nanoparticles observed in the *in vitro* Blood-brain barrier model translates into particle accumulation in the BBB *in vivo* is as yet unknown – approaches to separate the barrier endothelium from the rest of the brain tissue would be required to address this and to quantify the nanoparticle load in the barrier endothelium as distinct from within the brain tissue.

Given the emerging understanding that nanoparticle protein coronas formed under in vitro conditions may have no resemblance to those formed under *in vivo* exposure conditions, there is a critical need to evaluate the current practice of performing *in vitro* culture experiments with 10% serum proteins since this results in nanoparticle coronas that may not be biologically relevant and thus may lead to non-physiologically relevant impacts and unnecessary concerns. This is a debate and set of questions that needs to be undertaken sooner rather than later if there is to be a truly scientific underpinning for future regulatory decisions regarding nano-enabled products and processes.

Recommendations for further research (from *NeuroNano* Final meeting and workshop)

As part of the *NeuroNano* final meeting (5th December 2011), and in collaboration with the ESF EpitopeMap Research Networking Programme[3], a joint workshop was held in Dublin on 6th - 7th December 2011 that brought together leading European experts[4] in Engineered nanoparticle transport across biological barriers to identify the central questions that require scientific and policy attention as a matter of priority. Among the outcomes from the workshop of particular relevance for *NeuroNano* and its stakeholders were:

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[3] EpitopeMap is an ESF Research Networking Programme to understand the role of the protein corona in determining nanoparticle biocompatibility.
[4] Experts included David Begley, Jörg Kreuter, Patrick Case, Antonio Pietroiusti, Peter Wick, Wolfgang Kreyling, Christina Schulze, Andreani Odysseos, Marcello Cacace, Margaret Saunders, Luisa Campagnolo, Joseph Brain, Kenneth Dawson, Iseult Lynch, C. Vyvyan Howard and Neil Gibson.

Accidental 'targeting' of ENP to the brain – need for implementation of "protein corona" screening

Many of the "success" stories to date in terms of using engineered nanoparticles to deliver drugs to the brain have been the result of the ENP surface offering an appropriate scaffold for known blood-brain-barrier receptors, such as the LDL receptor. In fact, one key study shows that having a specific surfactant coating (polysorbate 80 - Tween® 80) that spontaneously binds apolipoprotein E and apolipoprotein A-I from the dispersion medium was *as effective* at delivering the drug to the brain as the specifically surface-engineered ENP where these apolipoproteins were chemically grafted to the ENP.[70-72] This raises a very important question as to whether other ENP, which were never intended to reach the brain (or indeed other key organs, or the foetus), could unintentionally bind such targeting protein coatings and then reach delicate organs with very high efficacy.

Regulatory policy recommendation: Interaction of ENP with transporter proteins under competitive conditions could potentially be an indicator or risk, and could become part of a "safety by design" strategy for design of ENP surfaces.

Need to greatly improve in vitro and ex vivo models to correlate outcomes with in vivo effects in order to reduce reliance on animal studies and address developmental toxicity questions

A surprising outcome of the workshop was how little we know about the extent to which current *in vitro* blood-brain barrier models fully represent the *in vivo* situation, and how much is just taken as common knowledge. Concerns raised include the fact that: barrier models never achieve the same levels of electrical resistance as *in vivo* barriers; imaging of cellular barriers often reveal holes due to incomplete cell coverage, and mono-cell barriers are missing many of the signalling inputs that would be present *in vivo*, such as interactions with co-located pericytes and glial cells (in the case of the blood brain barrier) that also may induce tighter junctions and greater barrier specificity.

This could suggest that *in vitro* barrier models are unlikely to reproduce transcytotic pathways, and may be under-reporting on transport across the barriers, and over-reporting on transport into the barrier and accumulation within lysosomes inside the barrier cells. It was interesting to note that *in vivo* brain endothelial cells appear to have far fewer lysosomes than analogous cells studied *in vitro*. Careful quantification of these differences and an understanding of their significance are needed.

There was uncertainty about whether, how and over what timescale, biological barriers are renewed *in vivo*, and the consequences of this for bioaccumulation of nanoparticles, especially in response to chronic low level exposure. Finally, the question of indirect signalling effects from ENPs localised in or near barriers that do not themselves cross needs to be explored as a matter of urgency, especially with regards to the brain and placental barriers.

Funding recommendation: Research is needed to improve existing and create new barrier models and develop clear *in vitro-in vivo* correlations, including appropriate culture conditions (serum free) to optimise barrier functioning and cell-cell (paracrine) signalling. Research should be directed towards the use of co-culture and 3D cell culture approaches, and towards comparative *in vitro* and *in vivo* studies under similar conditions, and where limitations are documented and understood.

Infrastructure needs for nanosafety and nanomedicine

A key concern relates to large scale facilities and specialised research centres for supporting the safety and efficacy assessment of ENP (for example facilities for radiolabelling ENP and assessment of the biodistribution and biokinetics of radiolabelled-ENP). Funding to support such facilities, and the mobility of EU researchers / post-docs for their wider exploitation, over the coming decade is vital, as many questions regarding safety and efficacy of ENPs can only be answered by the use of not yet available radioactive nanomaterials. In a parallel fashion, support to maintain the intellectual infrastructure (appropriately trained personnel) is required to respond adequately to EU needs in relation to ENP.

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