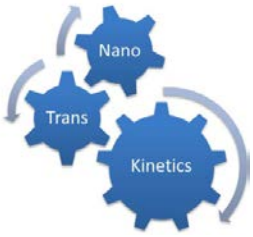
NanoTransKinetics

Modelling the basis and kinetics of nanoparticle cellular interaction and transport



Final report

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Contents

Executive Summary *(1 page max).*

The NanoTransKinetics project for modelling the toxicity of nanoparticles focuses on the interactions with biological systems on the hypothesis that prediction of the site of action will be pivotal to assessing potential hazards. The project has been developed in a four tiered approach: interaction with biological fluids, cellular membranes, in vitro cellular models and biological barriers (predominantly using in vitro models). The underlying science is that nanoparticles in contact with biological fluids are covered by a layer of molecules from the surrounding environment, constituting the biological identity of the nanoparticles. We believe that it is the adsorbed proteins and biomolecules that interact with the cell membrane receptors, suggesting that it may be possible to understand cell level interaction that are potentially relevant to humans.

NanoTransKinetics has at its foundation a reliance on an extensive collection of high quality data. Such a database of viable data can only be generated by intimate day-to-day links between experimentalists and theorists and, as such, is the cornerstone for the success of *NanoTransKinetics*. Therefore critical factors necessary for obtaining high quality data for input to nanoparticle modelling systems and databases have been identified. The project has also made significant strides towards developing phenomenological models and demonstrating their predicitve nature using the minimal set of physico-chemical data via epitope mapping of corona and in vitro liver models to determine interactions with cellular receptors. An open source suite of applications to simulate and predict the formation of nanoparticle/ protein complexes has been developed and disseminated throughout EU funded projects through interactions with WG4 and 6 of the NanoSafetyCluster, along with recommendations for all WG on critical factors for ensuring quantitatively reproducible experimental datasets.

Summary description of project context and objectives *(4 pages max)*.

Nanoparticles are enormously used in many sectors and are important both for their technology potentiality and the capability to enhance the performance of bulk materials. The very high surface area to volume ratio of many nanoparticles, though, allows them to adsorb particles from the environment. This is a crucial point for understanding the biological (toxicological) impact of nanomaterials. Many studies confirm that certain proteins and/or biomolecules can remain for a relevant time on the nanoparticle surface (protein corona), prevent the adsorption of other molecules, modify the nanoparticle chemical characteristic, and eventually determine the final localization and site of action of the nanoparticle in living organisms. The main object of *NanoTransKinetics* is to predict, mathematically, how nanoparticles behave in a biological environment, meaning interaction of the nanoparticles with biological fluids, membranes, and cells. To unveil the mechanism of interaction of nanomaterial with a biological environment is obviously a fundamental step that has a number of implications for instance in the nanoparticle regulation.

The implementation of the project relied on three main steps: firstly, collecting experimental data that were analysed to understand the behaviour of a nanoparticle in a biological environment. Secondly, to build up a phenomenological model from the experimental data, and finally to carry out experiments to test the validity of the model.
In addition, the project was structured in separate work packages that allowed modelling the nanoparticle interaction in different environments that eventually described how the nanoparticle behaves in a biological system. Thus, WP2 focused on the nanoparticle interaction with plasma and biological fluids. This task studied of the formation of the so-called *protein corona.* Particular efforts were also made in understanding how the biological entity or nanoparticle complex (nanoparticles plus adsorbed biomolecules) interacts with the environment. WP3 aimed at modelling the nanoparticle uptake in cells and organelles. WP4 describes the mechanism of interaction between a nanoparticle and a biological barrier. WP5 proposed the integration of the outputs from the previous WPs into a unitary vision and the development of user-friendly codes as predictive tools available to the wider research and regulatory communities. It also included a task of coordination of the efforts of the Work Group 4 (Databases) of the EU Nanosafety Cluster.

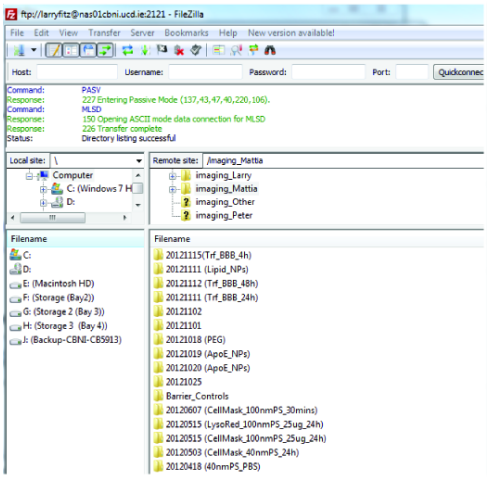
Description of main S & T results/foregrounds *(25 pages max).*

Experimental work and the main outcomes for each work package are described in detail below.

WP1: Data acquisition and quality assurance.

For a project such as NanoTransKinetics to be successful, there is a need for databases of high quality, reproducible data. Links to other key projects, both within the EU and the US, were developed and utilised with the explicit purpose of have accessing to high quality data. One of the early conclusions from analysing these initial datasets was the realization that additional data was required, however sources of validated datasets proved elusive. As such, more dedicated experiments began to produce detailed microscopic data on the interaction of nanoparticles with cells, upon which more detailed models were and are still being developed. Protocols developed in the initial period of the project for the assessment and annotation for incoming data was applied to ensure continuity within the Work Packages. This resulted in the generation of a database repository where the acquired, annotated data has been stored. This acts as a platform for data storage, integration and interpretation, where data generated by multiple individuals has been captured in an appropriate manner. This data is suitable for prospective modelling purposes where to-date, live-cell imaging and other data has been captured. A snapshot of the database repository, as well as a summary of the more dedicated experiments performed for NanoTransKinetics, is shown in Figure 1.

(a)



(b)

Number	Type of Data	Quantity of Recorded Data	Quality of Recorded Data
	Operator 1	ca. 0.7 TB Generated Data	
1	Nanoparticle - Blood-Brain-Barrier (BBB) Interactions.	ca. 350 GB.	Medium - High
2	Nanoparticle - Cell Interactions.	ca. 350 GB.	Medium - High
	Operator 2	ca. 0.4 TB Generated Data	
3	Nanoparticle - Cell Membrane. Dedicated to Membrane only Interactions.	ca. 200 GB	Medium - High
4	Nanoparticle - Surface Interactions (Investigated by TIRFM).	ca. 200 GB.	Medium - High

(c)

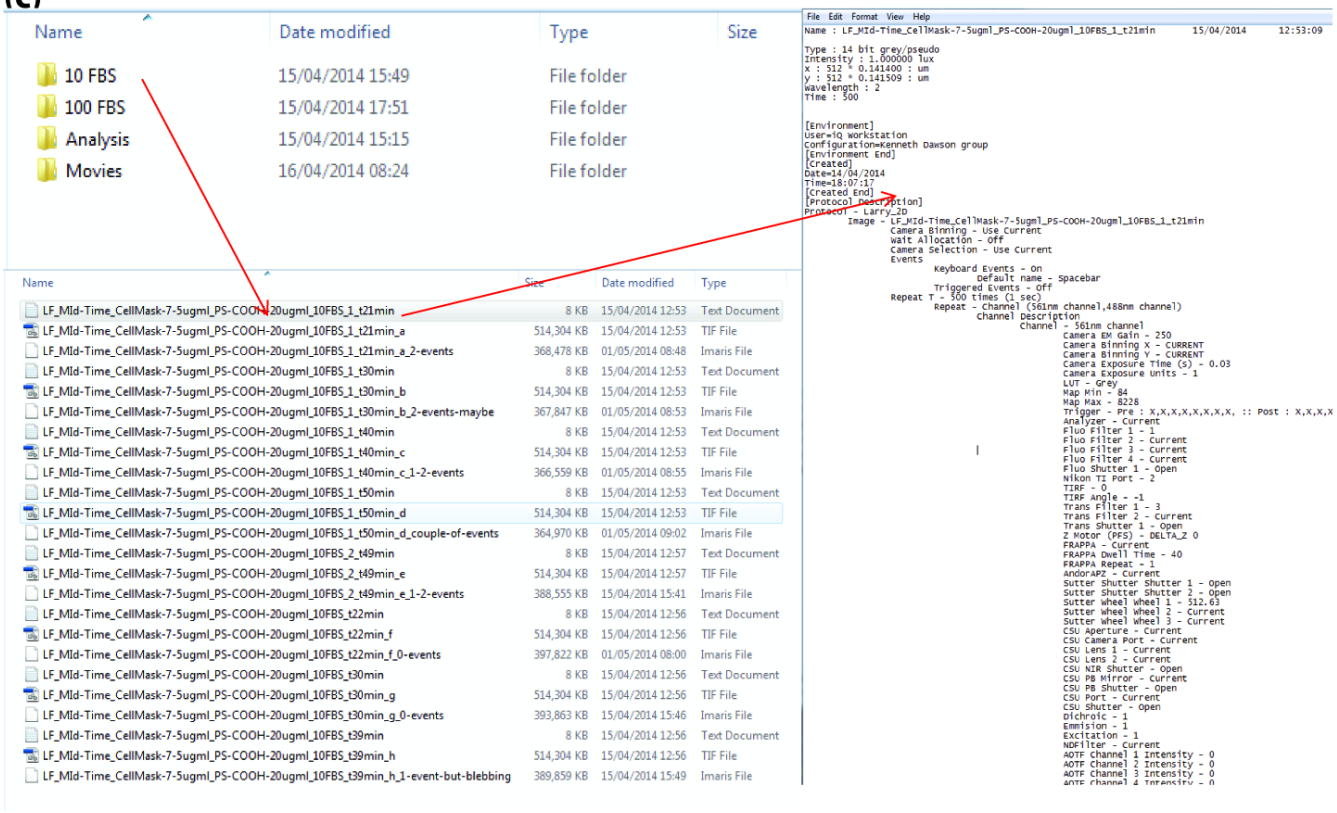


Figure 1: (a) Snapshot of the database repository on which the generated data is annotated and stored, showing a hierarchal structure. (b) Overview of the quantity of high quality data recorded over the duration of the project, grouped into different aspects of nanoparticle interactions with cells and barriers. (c) Example of the form of data generated for different conditions including sample descriptors for both the experimental conditions and also the acquisition conditions

WP2: Modelling nanoparticle interaction with plasma, extracellular matrix, and lipid membrane.

The concept of this WP was to set up phenomenological models that describe the behaviour of a nanoparticle in plasma, ECM and lipid membranes.

Literature data and data from previous European projects show that when nanoparticles (NPs) interact with biological media such as human plasma or serum, proteins and other biomolecules adsorb on the surface leading to the formation of the so-called *protein-corona.* However, these studies mostly enumerate the proteins or other biomolecules adsorbed and lack to scientifically indicate which the evolution of the protein adsorption. In addition, plasma and serum contain thousands of proteins and other molecules that make the representation of the interaction with nanoparticles difficult. To provide a model of such a complex interaction, the project was therefore developed in steps that aimed at simplifying the system:

1. The first step was to study the binding of a very simplified system: one protein and one nanoparticle. Here the novelty of the *NanoTransKinetics* project was to describe the binding by number of molecules adsorbed. This specific aspect, in spite of being the key to understanding the NP evolution in biological environment, was not well explored in the available literature.
2. Development of the model: the model was developed by analysing FCS (Fluorescence Correlation Spectroscopy) experiments on polystyrene NPs, using several variants of size and chemical surface composition. FCS was chosen because it is a potent technique that allows measuring kinetics of adsorption at molecular level.
3. The model was tested using nanoparticles having different composition and chemical characteristics. This is a key to verify the predictive capability of the model. Silica nanoparticles were chosen because of the high number of articles describing the interaction of those particles with proteins and complex fluids. Moreover, those particles are widely used in many sectors from the fabrication of biosensors to drug delivery, thus they have high impact for their toxicology potentiality.
4. Finally, the evolution of nanomaterial in the biological environment was also studied on the basis of the model. To achieve this result, the partners decided to study the dynamics of the proteins adsorbed on the nanoparticles (*protein corona*) in presence of competitive binding proteins.
5. A study of the interaction of the NP complex with the environment was also made. To this end, FCS was again used to describe the kinetics of adsorption. Also, partners decided to use vesicles made by a mixture of lipids, usually found in many cell membranes, in order to simplify the system.

The main achievement of this work package was to develop a model called “Strong Binding Model”. This model is able to measure the exact number of molecules adsorbed on the NP surface. Additionally, the proposed approach highlights the irreversibility of some types of adsorbed proteins binding nanoparticles. It allows distinguishing the hard and soft coronas.

However, this simplified model is only able to capture the behaviour of nanoparticles in presence of proteins having high affinity for the surface. A more realistic model was developed to take into account the competition among different proteins adsorbing onto the same NP. The coarse-grain model is based on a multi-scale approach and is described in the WP5.

Here, other techniques were also employed to study the dynamic of the corona in presence of competitive binding proteins. To achieve the goal the collaboration among the three European partners was very close and the access to high quality data from other European projects were a valuable support. The binding affinities K_D of the single proteins to silica were determined with differential centrifugation sedimentation (DCS) and microscale thermophoresis (MST) by UCD and LMU, respectively. The collected data were considered reliable data to develop and try the coarse-grain model out by UB.

Specifically, the coarse-grain model takes as phenomenological input the LMU and UCD measurements for mono-component protein solutions in contact with NPs and is calibrated using the data of LMU and UCD experiments. The calibration allows us to extract the binding affinity K_D of each protein to the NP surface. Once this parameter is estimated, the coarse-grain model is used to predict the protein corona formation of multi-component protein solutions in contact with NPs by means of the software suite BUBBLES and a Non-Langmuir Differential Rate Equation (NLDRE) theory, both developed by UB, as described in WP5. The model has been applied so far to the sequential adsorption of two and three proteins on silica NPs.

Finally, the study of interaction with the environment, namely lipid vesicles, shows that also vesicles have a corona. Therefore, the interaction between NPs and membranes might be driven by their respective protein coronas.

WP3: Modelling nanoparticle interaction with cells-uptake, distribution, sub cellular characterization.

The main goal of this WP is to address the uptake of NPs into cells. A significant amount of work was performed within this workpackage throughout the project.Naturally, as later experimental results reach maturity, the model was challenged for agreement and extensions undertaken, when needed. The resulting phenomenological model has been described in

Deliverable Report 3.1 (Phenomenological models for nanoparticle interaction with cells), and includes work that has been published in refs 1-3. The phenomenological model has been tested for several different particles and under different conditions, as described in more detail in Deliverable Report D3.3 (Confirmation of predictive capacity of models (time-dependent organelle concentrations). For example, the model predictions of a constant uptake rate fits well with the experimental observations for 20, 40 and 100 nm carboxylated polystyrene nanoparticles by the 1321N1 and A549 cell lines.[4] As another example, we may note a similar agreement for nanoparticles of a different material, namely silica,[5] (note, however, some issues at later times, likely due to dissolution of the silica nanoparticles[6]). The intracellular location was also invetsigated, with the nanoparticles following the endo-lysosomal pathway, fits the model. For other examples, see Deliverable Report D3.3.

A preliminary investigation for deepening the modelling approach has also been carried out using stochastic process algebras.[7] This approach, in contrast to the phenomenological approach described above, lends itself also to studying the variation among cells due to statistical fluctuations.

Furthermore, experimental methodology was developed based on direct observation and tracking of individual nanoparticles interacting with the outer cell membrane and inside cells using spinning-disk confocal fluorescence microscopy. This permitted visualisation of what *actually* happens when a nanoparticle interacts with the cell membrane, without any (obvious) inferential assumptions. Based on such data, one may extract quantities such as the residence times of nanoparticles at the cell membrane. Interestingly, the distribution of residence times appears to be exponentially decaying, with a characteristic time that scales with the nanoparticle radius. A model based on the diffusion equation, developed to describe the process, gives the same behaviour, thus laying the basis for understanding and describing the nanoparticle-cell membrane interaction.

The approach of the project by LMU was to create a multi-level computational model. The experimental data were collected performing transfection studies of mRNA lipoplexes. Those particles spontaneously enter into the cells and thus they can be considered a good model of nanoparticle.

The model was developed in steps. Firstly, the idea was to model the sequence of transfection phases as a typical mass-action chemical reaction (streamlined model). Then, the knowledge that endosomes can contain more particles prompted the need to develop a more complex model that took into account the fact that there exists endocytosis for multiple lipoplexes (or nanoparticles).

This kinetic model for mRNA delivery shows the ability to predict the transfection results. The model reproduces both the delay and dispersion of the onset time and also the dose-response relationship.

Another key point of *NanoTransKinetics* is to predict the concentration of nanoparticles in organelles and consequently to connect the outcomes with toxicological aspects such as apoptosis. On the basis of that our reasoning leads at developing a system for high throughput single cell experiments and analysis. An epi-fluorescence microscope at physiological conditions was used. In order to characterize different stages of apoptosis, adherent cells exposed to different dose of NPs were incubated with two florescent markers. The main advantage of this approach is to trace the time evolution of different markers that eventually allows for a better analysis of the NP uptake and toxicity effect due to high concentration of NP in particular sites.

WP4: Modelling the passage of nanoparticles across biological barriers such as the blood-brain barrier.

The phenomenological model for biological barriers built upon the model developed for single cells (Task 3.1), but includes one new feature, namely the existence of transport pathways across the barrier, as illustrated in Figure 15. Also note that in comparison with single cells, no account of cell division is included (though this process still occurs in the barrier to some degree).

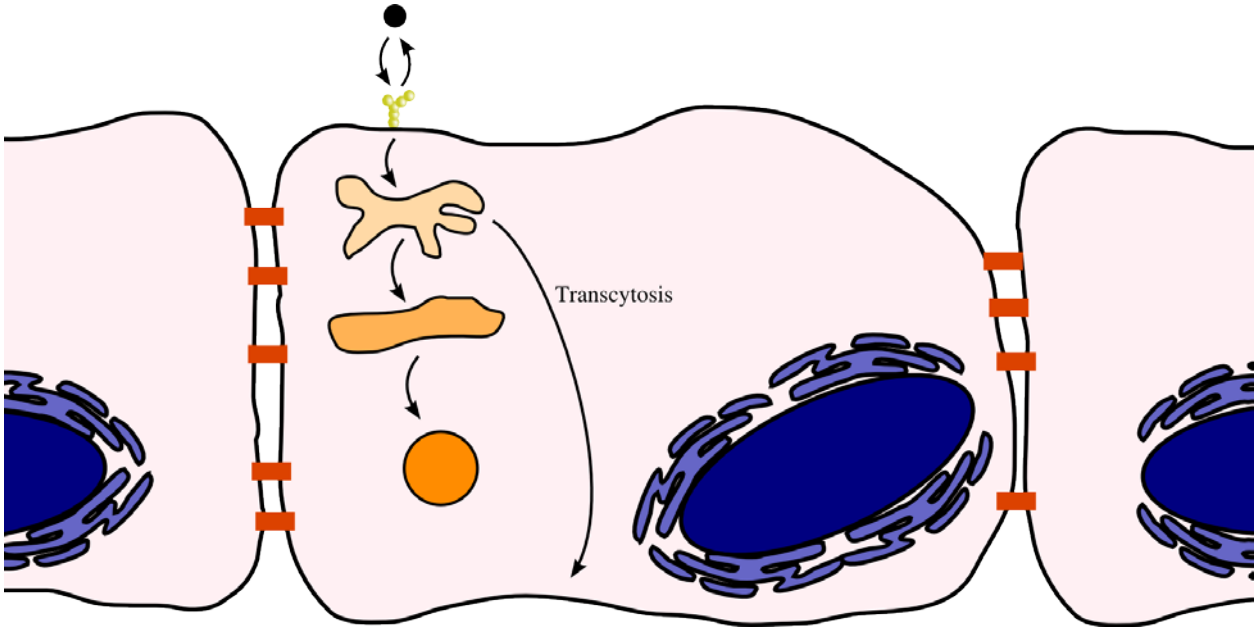


Figure 2. Phenomenological model for nanoparticle interactions with barriers, specifically the blood-brain barrier. The two boxes indicate two different 'modules' which can be taken from the phenomenological model for single cells as described in Deliverable Report 3.1. The new feature for barriers is the existence of transport pathways through the barrier ('transcytosis'). Of note are also the tight junctions that form an (for many substances) impermeable obstacle to transport between the cells.

The modelling effort has strongly liaised with the experimental effort within UCD (primarily on the basis of data generated within the FP7 project *NeuroNano* and the national project INSPIRE) to build the empirical basis upon which the models will be built. Experimentally, many problems have been found with existing barrier models and methodology when applied to nanoparticles, and this has hampered the development of the modelling in *NanoTransKinetics*. However, these problems have now been resolved – indeed, partly due to the involvement of *NanoTransKinetics* in the experimental effort. The development of a microscopic method for measuring nanoparticle interanalisation and transcytosis overcame many problems observed with routine methodologies such as transwell systems. A major conclusion from this work is that the there is very low rate of translocation through the barrier, most nanoparticle accumulating inside the barrier, often in lysosomes.

Further funding has been secured on the basis of this research, namely the MC ITN Pathchooser. UCD will continue their efforts to develop and experimentally validate models for studying the interactions of nanoparticles with biological barriers, using sophisticated microscopic techniques in a first attempt to rank nanoparticles based on their probability of

crossing biological barriers – towards prediction of brain accumulation and potential functional impacts

WP5: Data integration and development of predictive tools.

While the three modelling modules developed in WPs 2-4 (Nanoparticle-protein; nanoparticle-cell and nanoparticle-barrier) are all significant research advances in themselves, the truly predictive output arises from the integration of the modules, such that having experimental data for one module which can be fed into the models will enable prediction of the other end-points. It was the vision of this project to be able to start from any end-point and be able to predict any (all) other end-point(s) addressed by the model. The modular nature of the models will also enable new elements to be added as, and if, new toxicological end-points emerge for nanomaterials (note that at present very few exist, with apoptosis being one of the few confirmed as being induced by some nanoparticles).

This task is still in development, but is continuously being updated, particularly as the biological barrier models of WP4 continue to be investigated and developed in the MC ITN Pathchooser. Deliverable Report D5.2 (Data integrated across all aspects to enable mapping of all end-points based on any input parameter) will discuss the integration of the models as they stand at the end of the project, but it is envisioned that a full description will be soon published (with the inclusion of data from the follow up project Pathchooser).

The main result of WP5 is the development of a computer-simulation tool that we called "BUBBLES" which stands for "Bubbles is a User-friendly Bundle for Bio-nano Large-scale Efficient Simulations". *Bubbles* makes possible to predict the composition of the protein corona adsorbing onto a nanoparticle. The goal is to allow us to get this information for a large variety of experimental conditions without really repeating the experiments for all the different conditions.

The tool is organized in a software *bundle*, that is a set of informatics codes that take care of different aspects of the whole simulation. Because we want to make the tool accessible also to those that are not-experts in informatics, we made the bundle *user-friendly* by developing a self-explanatory web page that works as an interface of the user (Fig. 1).

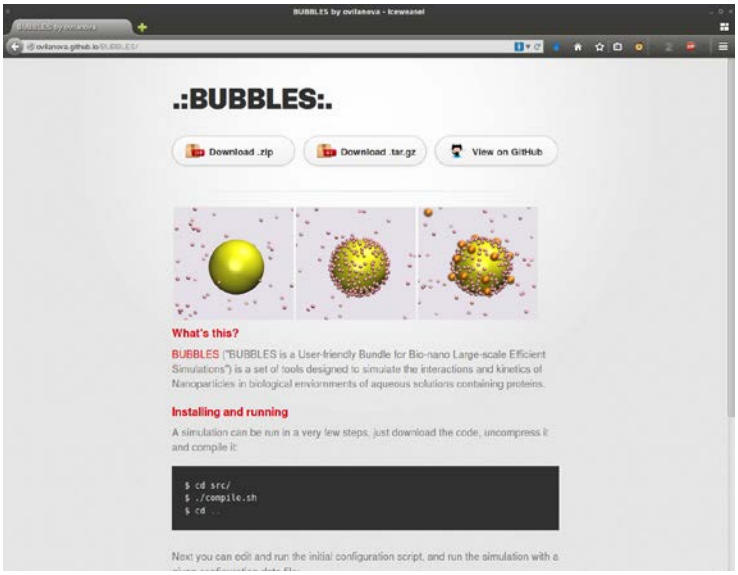


Figure 3: Snapshot of the BUBBLES main web page located at http://ovilanova.github.io/BUBBLES where we provide basic guidelines about the installation and the execution of the codes. Any user can freely access the application suite at https://github.com/ovilanova/BUBBLES.

Since our goal is to make this tool available for the entire scientific community, the stakeholders and whoever could be possibly interested in getting information about the composition of the protein-corona--nanoparticle complex, the entire bundle is *open-source*. This implies that it can also modified and improved by other researchers to make it more efficient or better adapted to different scientific contexts.

The simulations necessary for making the predictions need large calculation power, because the biological systems with which the nanoparticles interact are made of hundreds of millions of molecules. To make these *large-scale* simulations *efficient* we developed original codes that are able to take advantage of the standard Graphical Processing Units (GPUs) of any computer. GPUs are now considered to be the future of simulations because can perform calculations hundreds of times faster than those traditionally handled by the Central Processing Units (CPUs) of our fastest computers. The codes can run fast on any desktop or laptop computer, however are optimized for a cluster of GPUs to achieve better performances.

To be reliable the tool needs experimental inputs to define some parameters of the simulations. However, the required experimental inputs are such to reduce the complexity of the experiments.

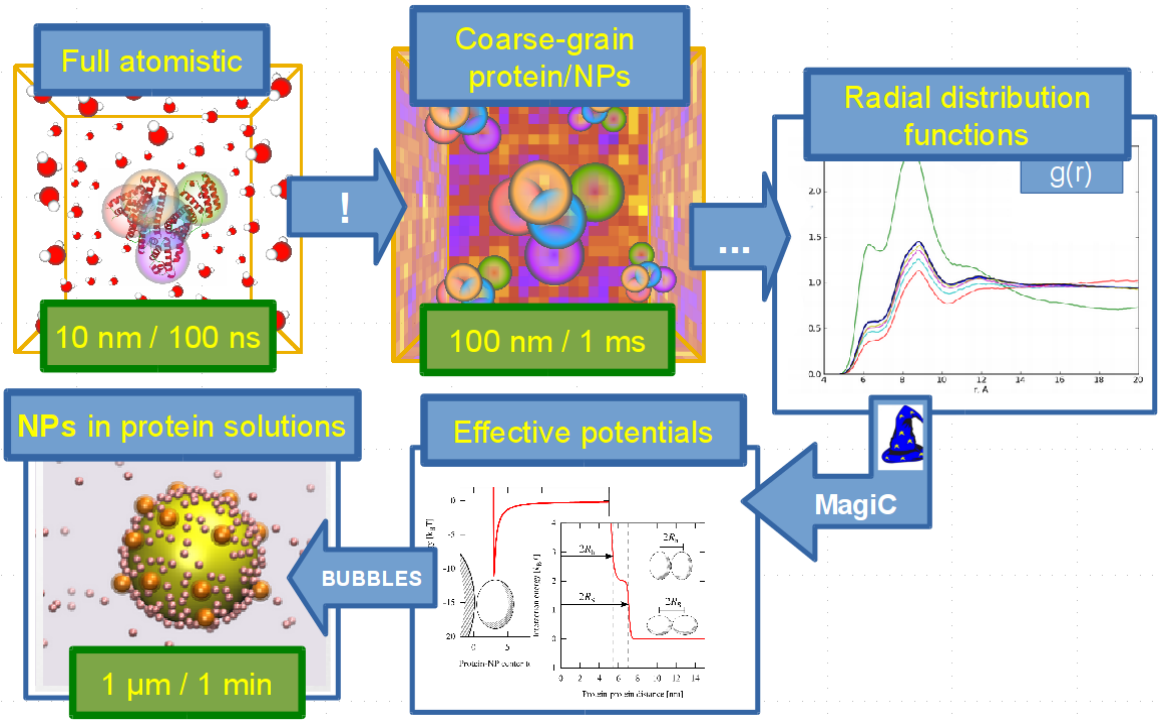


Figure 4: Schematic representation of our multi-scale approach: from a full-atomistic description of hydrated proteins and nanoparticles, to aqueous solutions with coarse-grained water and macromolecules that allows us to extract useful information about the relative structure (the radial distribution function) and, by using free-available software (such as "Magic") the effective interaction potentials that we implement in *Bubble* to describe the system at the largest length and times-scale.

We used our suite *Bubbles* to simulate the interactions of nanoparticles with proteins in aqueous solutions. We developed a multiscale approach, schematically described in Figure 2, to define the effective potentials that we adopt in *Bubbles*. The multiscale approach covers length-scales ranging from the 10 nm (atomistic scale) to microns (protein-corona—nanoparticle--complex scale) and times-scales going from hundreds of nanoseconds to minutes. We found an excellent agreement of the simulation results with the experimental results, validating the predictive power of our tool.

Potential impact and main dissemination activities and exploitation results *(10 pages max).*

One of the objectives of this project was to set-up standards, scientific approaches and tools that will allow the research community and the stakeholders to get relevant information about the fate of nanoparticles in leaving organisms for a large variety of different conditions without actually performing the experiments for all the different conditions. We pursued this goal by proposing standards for data acquisition and quality assurance of the experiments, developing models for nanoparticle in the extracellular medium, cell-uptake and biological-barriers crossing, and by making available to the public predictive tools. The knowledge gained in this project will be integrated into an upcoming QualityNano publication on common "pitfalls" in nanoparticle-cell interactions, a guide to ensuring high quality nanosafety science, with a specific section on data requirements for modelling and databases. Furthermore, an open source suite of applications to simulate and predict the formation of nanoparticle/ protein complexes have been developed and made available. In order to have widespread applicability of the modelling approaches developed within NanoTransKinetics, it was necessary to put a user-friendly interface for the tools to allow every potential user, even those with limited programming or computational backgrounds, to access it and work with it. To this goal we developed small software plug-ins to accompany existing imaging or proteomic software (e.g. Image J), such that data can be analysed and "correlated" with specific outcomes easily. This will also enable development of algorithms to link—for example, high content analysis assays—directly to the predictive models.

We will make maximum use of these results in the future by expanding our approach to a larger variety of systems. Based on the knowledge we acquired during the different stages of this project, we will build up a more general approach that could be applied to other biological-relevant problem. One that is at hand is the study of nanostructures that could be potentially relevant for drug delivery, nanodiagnostic of personal medicine.

This predictive ability of the tools we made available will allow the scientific community to reduce costs in terms of materials resources (the experiment are very expensive in many cases) and in terms of human resources (the experiments can require days of work of an entire experimental team). It will also allow different groups of scientist to make in a cost-effective and time-effective way systematic studies about how the nanoparticle fate changes by changing the environment to which they are exposed. For example, our tool to predict the

protein corona composition can be applied to a large range of protein-nanoparticle relative concentrations once a couple of cases are experimentally measured to set up the parameters of the model. Furthermore, the tool will allow studying the effect of changing the size of the nanoparticle, without performing the experiments.

The impact of these achievements is potentially relevant for NanoSafety, NanoMedicine and nanoDiagnostics and could lead to an optimal and safe exploitation of the nanoparticles in their everyday applications.

Dissemination

- **Publication of the web page with the tool "Bubble" (WP5) and its future expansion**
- **20+ published papers**
- **34 - conferences/meetings attended**
- **6 - workshops**
- **13 - other dissemination activities *(Departmental lecture, "Articles published in the popular press" / UB / "The strangeness of water" published in an on-line edition of EL PAIS (a leading Spanish Newspaper).***
- **In addition – 4 bilateral meetings**
    - ***UCD- LMU - NanoImpactNet - end of February 2012***
    - ***UCD- LMU - 9th International conference & workshop, Germany - March 2012***
    - ***UCD- UB - Statistical Mechanics in Barcelona, Spain - June 2012***
    - ***UCD- LMU – 26th conference of European Colloid and Interface Society, Sweden - September 2012***

NTK is associated with:

- ***<u>NanoSafety Cluster (NSC) – D7.2:</u>***
    - ***NSC WG4 Databases***
    - ***NSC WG6 Modelling***
    - ***Contribution to the NSC Website.***
    - ***Contribution to the NSC Compendium.***
- ***<u>Community of Research (CoR) – D7.2:</u>***
    - ***EU-US Communities of Research (CoR) - Databases & Ontologies.***

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