Mini Review

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Reproducibility in biological models of the blood-brain barrier

Abstract: In the past decade, the importance of quality and reproducibility within research has been re-emphasized, consequently becoming a crucial part of scientific experiments. Their implementation into in vitro and in vivo biological experiments is challenging due to various parameters that can influence the final scientific outcome. In parallel to these activities, there is a huge scientific effort to improve today's medicines to make them safer and more efficient, and to cure untreatable diseases, such as many neurodegenerative diseases. Nanosized materials have been recognized as potential drug delivery systems in this arena due to their small size and surface properties, which enable the design and synthesis of safe and efficient delivery vehicles that might be able to cross the blood-brain barrier. However, the fundamental understanding behind their uptake mechanism and intracellular trafficking remains unknown. Simple and cost-effective in vitro blood-brain barrier models are widely used to address these questions. This paper aims to critically evaluate the current in vitro models using Transwell™ systems and to discuss alternative approaches towards more reproducible in vivo features.

Keywords: blood-brain barrier; in vitro; live cell imaging; nanoparticle; reproducibility; transcytosis.

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Introduction

The importance of reproducibility of scientific experiments has recently been highlighted (1). Assessing the reproducibility of even the most basic techniques and methods, including in vitro biological experiments, has become an essential part of scientific research. Biological experiments can be influenced by various parameters, such as passage-to-passage variation of cell behaviour, receptor expression, batch-to-batch variation of chemical substances, or the interaction between model particle and biological environment (2, 3). Hence, quality and reproducibility must be built into each individual part of the process, as they might have a significant effect on the final outcome and the reproducibility of the final results. Development of detailed protocols and implementation of interlaboratory comparison studies can help to ensure these issues (4).

There is a huge scientific and public interest to find new therapeutic agents for the treatment of neurodegenerative and other diseases localized in the central nervous system (CNS), such as Alzheimer's, Parkinson's or Huntington's diseases, which require the successful crossing of the blood-brain barrier (BBB) of the candidate agents. Due to their size and excellent surface properties, nanoparticles have been recognized as potential drug delivery systems (5), and they can be modified in versatile ways to target the CNS (6, 7).

The BBB strictly controls the molecular trafficking in and out of the brain using its physical, transport, enzymatic, and immune regulatory barrier functions (8, 9) (see Figure 1).

Physical barriers (formed by tight junctions and adherens junctions between the endothelial cells), transport barriers (composed of adenosine triphosphate binding cassette efflux transporters), and metabolic barriers (formed by enzymes) are responsible for keeping "unwanted objects", such as toxins, but also most drug candidates, out of the CNS (8–10). Conversely, transport of

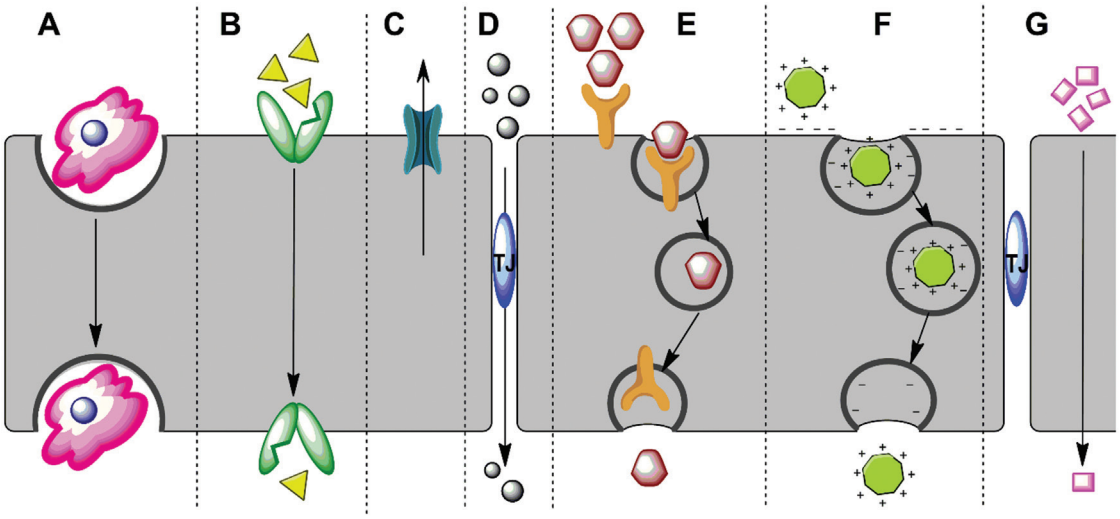

Figure 1 Pathways across the BBB. Grey rectangles represent the brain endothelial cells, which are connected through tight junctions (TJs). Lipophilic compounds are able to path through the barrier through transcellular lipophilic pathway (G), however, most of these compounds are pumped out via efflux transporters (e.g. P-glycoprotein) (C). Although tight junctions acts as a physical barrier and prevent the entrance of many compounds, small water-soluble compounds are able to path them through via the paracellular aqueous pathway (D). Many essential compounds, such as amino acids, peptides, or glucose, enter via transport proteins (solute carriers) (B). Other endogenous macromolecules, such as transferrin and albumin, path through the barrier via receptor-mediated transcytosis (E) and non-specific adsorptive-mediated transcytosis (F), respectively. Furthermore, mononuclear cells, such as monocytes and lymphocytes, can migrate through the barrier (A). Redrawn by D. Hudecz, based on refs (8, 10) with permission of Elsevier and Nature Publishing Group.

essential compounds into the brain is possible via paracellular and transcellular pathways, by influx transporters (specific solute carriers), or via receptor- and adsorptive-mediated transcytosis (8, 9).

The most investigated targeting route of the BBB delivery is receptor-mediated transcytosis and it is believed that nanoparticles are transported via this route (7). This targeting strategy is based on the coupling or grafting of receptor substrates that are known to cross the BBB via transcytosis, such as transferrin (11), insulin (12, 13), or apolipoprotein E (ApoE) (14), to the surface of the nanomaterial which trigger receptor-mediated transcytosis and thereby enable them to cross the BBB (6, 7). The success of such modifications using ApoE has been reported (15–17), but in many cases the nanoparticles follow and subsequently accumulate along the endo-lysosomal pathway (18), as is commonly observed with uptake of nanoparticles in other in vitro models (19–22), and never reach the brain. Hence, there is a need for a more specific nanoparticle targeting approach which requires the understanding of the fundamentals behind the transcytosis pathway. This could open a new era of CNS targeting, based on rational, specifically designed carriers.

In vitro BBB models are widely used to study such interactions as they provide a better insight into cellular mechanisms in comparison with in vivo models (23). In the past few decades, various in vitro BBB models have been developed, including cell based, non-cell based (24), and stem cell based models (25–27) [for the most recent reviews about different in vitro models of the BBB see refs (23, 28, 29)]. The cell-based in vitro models include primary, immortalized, and conditionally immortalized models from brain endothelial cells of different species, including rat (30–33), mouse (34, 35), bovine (36), porcine (37, 38), and human (39–41) [the reader can find more examples in a recent review by Bicker et al. (28)]. The endothelial cells in these models can also be co-cultured with astrocytes and/or pericytes (31, 33), which play important roles in the induction, maintenance, and function of the BBB (10, 42).

Investigation of transcytosis and other mechanisms through the BBB using in vitro models

In vitro permeability assays and uptake studies are most often performed in Transwell™ systems. These multi-well plates comprise an upper (apical- blood side) and a lower (basolateral- brain side) compartment which are separated by a porous membrane. The endothelial cells are seeded onto collagen, or collagen and fibronectin coated

membranes, and grown in culture medium until they reach a confluent monolayer. In order to obtain tighter barriers with better barrier function the endothelial cells can also be co-cultured with astrocytes and/or pericytes in mono-, co-, or triple culture models in contact and non-contact format (supporting cells are seeded on the basolateral side of the Transwell™ insert or to bottom of the basolateral holding plate, respectively) (28, 31) (see Figure 2).

Due to their simplicity, cost-effectiveness, high- to moderate throughput screening properties, and versatility, these Transwell™ systems are widely used both in academia and in the pharmaceutical industry. They can be applied for rapid measurement of the transendothelial permeability of substances through the BBB, and for the investigation of receptor expression and pathways of the BBB. These systems are also able to measure the transendothelial electrical resistance (TEER), which gives information about the tightness and paracellular permeability of the barrier/cell monolayer (higher TEER values indicate tighter barriers, hence, better mimicking of an in vivo model). The common drawbacks of these systems are the presence of a stagnant liquid layer as a result of their steady condition, and the paracellular leak pathway through the filter pore, which may influence the measured TEER values (23). However, it is possible to correct for these components and calculate the "real" transcellular endothelial permeability (43).

The combination of transmission electron microscopy (TEM) with traditional Transwell™ systems has revealed some crucial limitations of these models (18, 44, 45), which bring into question the reliability and reproducibility of these systems. These limitations include barrier imperfections, such as formation of multiple layers of endothelial cells on the filter and holes (see Figure 3); nanoparticle adhesion to the microporous filter pore; nanoparticle agglomeration in the culture medium and inside the cell; and leakage of the fluorescent dye. As a result of these limitations, the measured permeability might be an overestimation (due to holes, dye leakage) or underestimation (due to multilayer formation, nanoparticle adhesion and agglomeration) of the true value.

The observed limitations of Transwell™ systems confirmed that there is a need for more reliable and reproducible measurement techniques which could lead to an improved understanding of nanoparticle/drug candidate-cell behaviour, an insight of the detailed uptake and intracellular trafficking mechanisms, and reproducible quantification of nanoparticle crossing. Utilization of TEM gives better

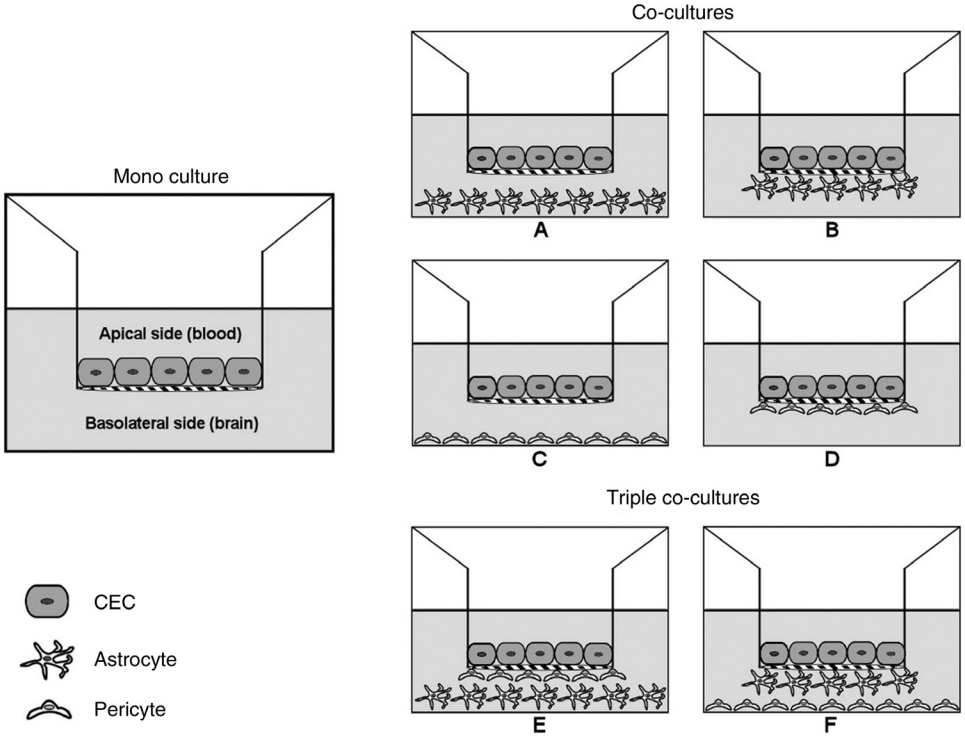

Figure 2 In vitro BBB models based on Transwell™ systems. The cerebral endothelial cells (CECs) can be cultured as mono-, co- (A-D), and triple co-cultures (E-F). In the co- and triple co-culture models, CECs are cultured with astrocytes and/or pericytes in contact (B, D) or non-contact (A, C) manner. Reprinted with permission of Bicker et al. (28). Copyright 2014 Elsevier.

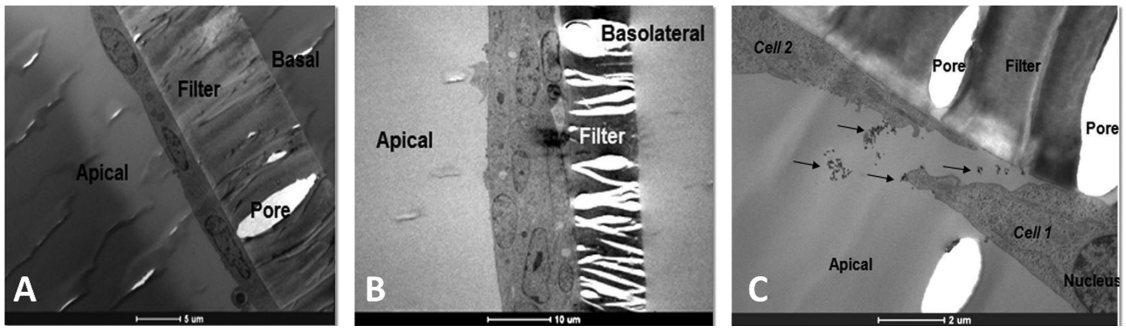

Figure 3 Transmission electron microscope images of the immortalized human brain capillary endothelial cells (hCMEC/D3) grown on collagen/fibronectin-coated 0.4-μm membrane. The endothelial cells can be grown as monolayer (A), multilayer (B), or as imperfect monolayer (C). In subfigure C, the arrows indicate 50 nm SiO_2 nanoparticles, which are approaching the filter in the area where cells are not in contact. Adapted from Ye et al. (18) with permission of The Royal Society of Chemistry.

insights into the localization and trafficking routes of nanoparticles (18, 46), however, due to the nature of these techniques (imaging fixed samples), it does not yield a dynamic picture. Immunohistochemistry in conjunction with confocal fluorescent microscopy has yielded further insights into these dynamics (16, 42, 47, 48). However, all of these techniques are based on fixed samples, thereby provide information as a snap shot and limited by the possibility of real time monitoring of the uptake mechanisms and intracellular fate of the nanoparticle.

Development of live-cell imaging techniques (19, 49) and fluorescent probes (50, 51) has enabled the study of dynamic events in a time resolved manner. These live cell imaging techniques include laser scanning- and spinning disc confocal microscopy, widefield fluorescence microscopy, total internal reflection fluorescence microscopy (TIRFM) (for monitoring events that take place on or near the basolateral side of plasma membrane) (52–54), Förster resonance energy transfer (for the measurement of intracellular molecular interactions) (55), fluorescence life-time imaging microscopy (for visualizing the life-time of a molecule's excitation state) (55). The reader can read more about these and other advanced live cell imaging techniques in refs (56, 57).

Most recently (45), nanoparticle translocation across the BBB was studied using live cell imaging techniques (spinning disc confocal microscopy and TIRFM) coupled with computational analysis. This study revealed the possibility to follow the spatiotemporal movement of nanoparticles quantifying transcytosis/translocation rate, lysosomal accumulation, or other localisation within the barrier. Barrier imperfections (barrier region with holes and/or multiple cell layers) were also observed with the applied live cell imaging techniques, and it was shown that significant amount of nanoparticles could rapidly traverse through the hole (see Figure 4). These events can only be seen using advanced live cell imaging technique, such as TIRFM.

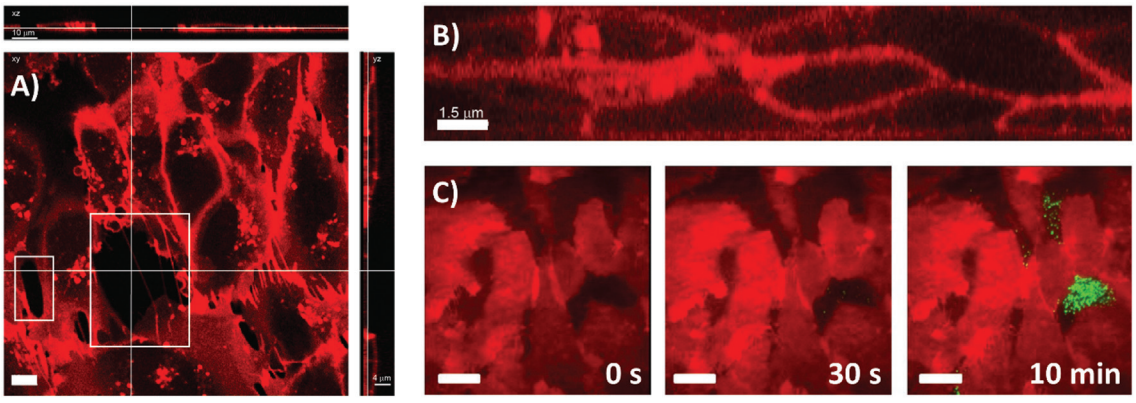

Figure 4 Imperfections of hCMEC/D3 cells grown on collagen-coated glass chamber. The images represent real time events, they were taken using spinning disc confocal microscopy (A and B) and TIRFM (C). Cell membrane was stained with CellMask™ Orange (red). Barrier cross-section with holes (A) (white rectangles) and multiple cell layers (B). Subfigure C) represents the transport of 100 nm carboxylated polystyrene nanoparticles (green) transport through barrier hole over time. Adapted with permission of Bramini et al. (45). Copyright 2014 American Chemical Society.

Steps towards a more realistic and reproducible in vitro model

Based on previous observations (18, 44, 45), imperfections exist in all in vitro barriers and monolayers models. Furthermore, the traditional Transwell™ systems suffer from numerous limitations, preventing their application for accurate and reproducible nanoparticle-BBB studies. Although, TEM imaging enables a more realistic study of such interactions, it does not provide time resolved information, for instance, an endocytotic event from the basolateral site might be mistaken with exocytosis, and events which might be identified as transcytosis might only be due to nanoparticle crossing through a hole located nearby the cell. Alternatively, live cell imaging techniques, including TIRFM, may overcome these issues. Furthermore, regions with a perfect cell monolayer should be investigated, rather than the whole cell population, which might contain holes or multiple cell layers.

Additionally, to have a more comprehensive picture towards the in vivo situations, it is important to apply several models, since every model has their limitations. For instance, different in vitro BBB models have slightly or radically different levels of protein, receptor and transporter expression depending on the type of the model (29, 58). It is also known that primary models have higher TEER values than immortalized models, which means that their junctional protein level expression is higher (23).

Moreover, before the actual nanoparticle-BBB studies, it is necessary to understand the transcytosis mechanism through the BBB. The assumed mechanism of transcytosis is: cell entry mainly via clathrin- or caveolea-mediated endocytosis, or via phagocytosis or macropinocytosis (59), followed by vesicular trafficking, by the avoidance of endo-lysosomal degradation pathway or recycling mechanism, and finally exocytosis on the basolateral side (60). Use of positive controls of transcytosis, such as insulin (12, 13), ApoE (14), transferrin (11), and in house verification of reproducible results are critical and essential part of the above studies.

In addition, application of shear stress in the in vitro BBB models and the use of co-cultures can further improve the models by mimicking the blood flow of in vivo situations, as demonstrated by dynamic in vitro models (61) and microfluidic platforms (62–65) which incorporate these concepts. Microfluidic systems provide the possibility to mimic the anatomical size and hemodynamic shear stress; they are cost effective, require fewer samples than traditional Transwell™ systems, and give the opportunity of high throughput screening (62–64). These devices are based on a similar concept as Transwell™ systems, i.e. they consist of an apical and a basolateral compartments, which are separated by a porous membrane (62, 63, 65) or small gaps (64). Most of these systems are based on a so-called sandwich concept; however, Prabhakarpandian et al. (64) have recently presented a different design, synthetic microvasculature model of the BBB (SyM-BBB), in which the position of apical compartment is side-by side with the basolateral compartment, enabling real-time optical monitoring. Figure 5 illustrates the concept behind microfluidic BBB (μBBB) (basolateral side is below the apical) and SyM-BBB (apical side is next to the basolateral side).

There is a fast development in the area of microfluidics, for example Booth et al. (65) have recently reported a multi-channel, multi-assay platform, which enables the study of wide range of wall shear stress conditions and multiple types of assays in a reproducible and controllable manner. A potential drawback to these models is that they are based on the same concept as Transwell™ systems, whose reliability and reproducibility have been

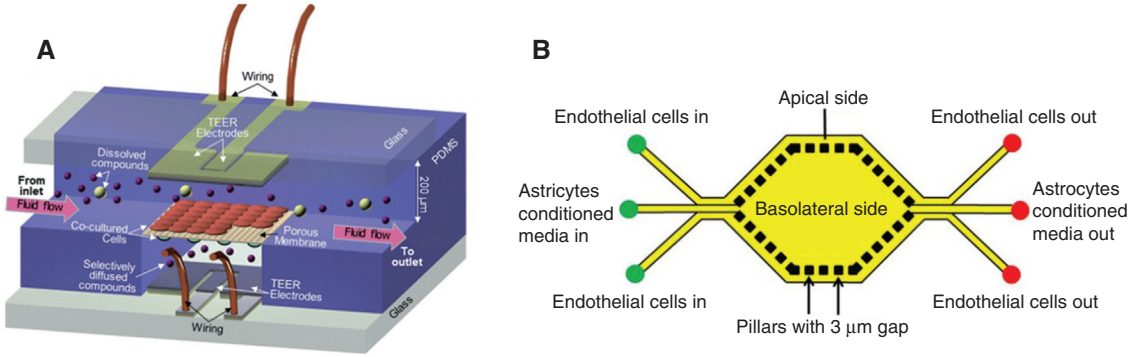

Figure 5 Design of μBBB (A) and SyM-BBB (B) models. Adapted from Booth and Kim (62) and Prabhakarpandian et al. (64) with permission of The Royal Society of Chemistry.

questioned within this current review. However, the design of SyM-BBB and its future derivatives provide the possibility of high throughput pharmaceutical screening and real-time imaging, which enables closer monitoring of holes, cell morphology, and cytotoxicity.

Conclusion and outlook

Although live cell imaging techniques and their combinations with microfluidic systems seems to provide a better understanding and real time monitoring of the intracellular fate of nanoparticles within the BBB, they are not ideal from an industrial point of view due to their current lack of high throughput screening capabilities. On the other hand, these techniques offer the possibility of more reproducible experiments compared to the traditional high throughput Transwell™ systems, as all processes can be followed visually in a time resolved manner. Regions with imperfect barrier properties can be deselected, and thereby the influence of uptake or retention of nanoparticles through these regions can be excluded from the final estimated nanoparticle uptake. Furthermore, as the accurate investigation of intracellular trafficking and other mechanism through the BBB is only possible using advanced live cell imaging techniques, these techniques will become an essential part of nanomedical and biological barrier research.

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Diána Hudecz started her PhD studies as a Marie Curie Early Stage Researcher within the PathChooser ITN Programme in January 2014. During her work, she is investigating the nanoparticle transport within and through the blood-brain barrier using in vitro models and different imaging techniques. A further important aim of her project is to gain quantitative and reproducible experimental data. Diána holds masters degrees from the Technical University of Denmark and from the University of Copenhagen in Chemical Engineering and Pharmaceutical Sciences, respectively. She completed her BSc studies in Chemical Engineering at the Budapest University of Technology and Economics in Hungary.

Louise Rocks joined the Centre for BioNano Interactions in November 2012 as a Postdoctoral Fellow. Her research is focused on the effectiveness of molecular targeting with nanoparticles, specifically, the complications that arise in complex biological fluids. Louise obtained an MSc in chemistry from the University of Strathclyde, Glasgow, prior to undertaking a PhD in the development of a sensitive approach to in vitro/ex vivo biomolecule imaging using surface enhanced Raman spectroscopy.

Laurence W. Fitzpatrick first joined the Centre for BioNano Interactions in February 2011. During which time he worked closely with Dr. Peter Sandin, which resulted in a publication in ACS Nano in January 2012. Laurence returned to the group as a PhD student in September 2012 and is funded by the Irish Research Council under the EMBARK Initiative. His main interests are bio-nanoparticle imaging in cells and tissues and his work focuses on what drives a sub-population of nanoparticles to “unexpected places” within cells, as well as different internalization rates for different sub-populations of particles. He completed his bachelors and graduated with a second class honours BSc in Science with Nanotechnology (Physics Major) in October 2012 from DIT.

Luciana-Maria Herda joined the Centre for BioNano Interactions in January 2014 as a Marie Curie Early Stage Researcher and full-time PhD student, part of the PathChooser ITN programme. Her work focuses on strategies to design functionalised nanoparticles for targeting and controlled negotiation of biological barriers (blood-brain barrier). Luciana graduated from “Carol Davila” University of Medicine and Pharmacy (Bucharest) with a degree in Pharmacy and in September 2013 she completed her MSc in Drug Discovery and Development at University College London School of Pharmacy.

Prof. Kenneth A. Dawson is Director of the Centre for BioNano Interactions (CBNI). He is also a lead investigator of the bionanoscience activities in University College Dublin, and Chair of Physical Chemistry. His professional roles include participation in the EMEA Nanomedicines Expert Group, membership of SCENIHR, participation in the OECD and ISO working groups on standards for Nanotechnology, Chairing the International Alliance for NanoEHS Harmonoisation and co-ordinating several large research projects in the area of nanosafety and nanomedicine. His work has focused on the biomolecule corona that surrounds nanoparticles in biological milieu and how this mediates nanoparticle interactions with living systems. The long-term goal of his research is the development of a rational framework to understand the interactions of nanoparticles with living systems.