NANOINTERACT: A rational approach to the interaction between nanoscale materials and living matter?

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Abstract. The importance of understanding the interactions between nanoscale materials and living matter has now begun to be appreciated by an extraordinarily large range of stakeholders, including researchers, industry, governments and society, all of whom appreciate both the opportunities presented by and challenges raised by this arena of research. Not only does it open up new directions in nanomedicine and nanodiagnostics, but it also offers the chance to implement nanotechnology across all industry in a safe and responsible manner. The underlying reasons for this arena as a new scientific paradigm are real and durable. Less than 100 nm nanoparticles can enter cells, less that 40 nm they can enter cell nucleus, and less that 35 nm they can pass through the blood brain barrier. These are fundamental length scales of biological relevance that will ensure that engineered nanoscience will impinge on biology and medicine for many decades to come. One important issue is the current lack of reproducibility of the outcomes of many experiments in this arena. Differences are likely a consequence of such things as uncontrolled nanoparticle aggregation leading to unpredictable doses being presented to cells, interference of the nanoparticles themselves with many of the tests being applied, differences in the degree of confluency of the cells used, and a host of other factors. NanoInteract has shown how careful control of all aspects of the test system, combined with round robin type approaches, can help resolve these issues and begin to ensure that the field can become a quantitative science. The basic principle of NanoInteract is that given identical nanomaterials, cells and biological materials, and using a common protocol, experiments must yield identical answers. Thus, any deviations result from errors in (applying) the protocol which can be tracked and eliminated, until quantitatively reproducible results are obtained by any researcher in any location. This paper outlines the NanoInteract programme, illustrates key advances, and highlights early successes. (www.nanointeract.net)

1. Introduction

It is clear that as applications and uses of nanotechnology increase, so too will our exposure to nanoparticles and nanomaterials. Our ancestors were exposed to only very limited types of particles throughout human evolution [1] and there were few particles smaller than 70 nanometers in diameter in the air throughout prehistory, until we learned to harness fire for use. This may mean that we have not built up any defences to nanoparticles [1]. Certainly where certain sizes and types of particle have been released into the environment, profound and unpredictable consequences for human health emerged, for example in the case of asbestos-induced mesothelioma. In this instance, the impact on many sectors of the developed worlds’ economy, from manufacturing to insurance, has been highly damaging.

The problem facing nanotechnology is that there exists no clear understanding of the nature and origin of the risks involved in the manufacture, processing, and use of nanoparticles, both research and regulatory frameworks being at early stages of development.[1] Juxtaposed with this concern is the growing opinion that it will be increasingly difficult to commercialise, indeed even to seek venture funding for commercialisation of nano-systems, without having addressed the issues associated with the (perceived) risk. The potential socio-economic benefits in addressing these issues in a deep, structural (and timely) fashion can hardly be overstated.

Thus it is becoming increasingly clear that unless the issues regarding the safety of nanoparticles and nanotechnology are resolved, the technology may never reach it’s full economic potential, and fulfil its promise to revolutionize medicine, computers and other arenas.
The NanoInteract programme has been designed to address the issues related to uncertainty and irreproducibility in the assessment of nanotoxicity, and to develop a complete understanding of the mechanisms of nanoparticle interaction with living systems. It brings together approaches from physical chemistry, biophysics and protein chemistry, classical toxicology and ecotoxicology and modern biological technologies to develop a new fundamental framework, and to begin to validate the new approaches which offer potential for higher throughput screening of the estimated 30,000 nanoparticles that are currently being developed in laboratories and industries around the world.

A key concept that has emerged in NanoInteract is the idea that nanoparticles in a biologically relevant environment (cell media, plasma, etc.) draw to themselves a number of proteins and lipids that form a sort of dynamic ‘corona’ in slow exchange with the environment [2, 3]. It is this nanoparticle-protein complex that represents the “biological identity” of the nanoparticles, and is this that interacts with the cellular machinery. Interestingly, natural organic matter also appears to function in a similar manner [4]. Such interactions with proteins can have dramatic impacts on the dispersion properties of the nanoparticles, as well as on the protein aggregation behaviour [5, 6].

An overview of the NanoInteract work-programme and the conceptual framework is shown in Figure 1. The project combines the classical toxicology and ecotoxicology approaches in order to assess their applicability to the questions, within a programme where attention to systems parameters is applied at all stages of the experimental design. Thus, as illustrated in Figure 1, key stages in the research process including: (a) ensuring that the nanoparticles used are of the highest purity and monodispersity possible, with any eluting species removed where possible, and characterised where they cannot be removed; (b) dispersion in the relevant biological media for the experiment, such as cell culture medium containing 10% foetal calf serum from a common source, with the dispersion properties characterised over timescales relevant to the experiments using a range of complementary techniques and taking into account measurement artefacts such as the weight-bias which can result in a high number of small particles being hidden by a small number of large particles; (c) characterisation of the protein and biomolecule corona that associates to the nanoparticles in the test media, specifically identification of the proteins and their residence times, as only those proteins that are associated with the nanoparticles for a timescale of several minutes can be considered biologically relevant; (d) connection of the protein corona with the mechanism of uptake utilised by the nanoparticles, including identification of the receptors and transport proteins involved in the uptake and transport processes, and elucidation of the final sub-cellular localisation and the kinetics of nanoparticle uptake; and (e) assessment of the biological impact of nanoparticles using a range of advanced biological techniques such as proteomics and transcriptomics, and connection of the impacts to the nature of the nanoparticle corona, and the final sub-cellular localisation.

As so much is unknown, unproven and irreproducible in the field at present, one of the most important issues to resolve is to establish a rational, quantitative basis for nanotoxicology, and within NanoInteract the approach taken to date has been the application of round-robin type approaches, where results are confirmed independently in up to three different locations (partner or collaborator laboratories). The basic principle of a round-robin (and of the NanoInteract project) is that given identical nanomaterials, cells and biological materials, and using a common protocol, experiments must yield quantitatively identical answers. Thus, any deviations result from errors in (applying) the protocol which can be tracked and eliminated, until quantitatively reproducible results are obtained by any researcher in any location. This ensures that the results are unbiased, scientifically sound, and not open to alternative interpretation. Within NanoInteract we have successfully applied this approach to identification of the protein corona surrounding polymeric nanoparticles [3], nanoparticle-induced fibrillation studies [5] and to genotoxicity studies using silica nanoparticles [7]. Currently, the round-robin approach is being applied to the development of a standard nanoparticle protein corona, in conjunction with NIST (National Institute of Standards and Technology in the United States).
2. Materials and Methods

2.1. Round-robin
A round-robin defines a useful protocol in challenging cases where the science is not yet understood. Everybody gets same answer using the same protocol proving that the test is robust. Anything leaving room for interpretation in a protocol is identified by the round robin process via a total process of evaluation and comparison of results obtained by the different partners. Given identical materials, and a well developed protocol carefully applied, experimental results must converge. Widespread differences that cannot be resolved by a round robin processes suggest that either the test is unsuitable for nanomaterials or that the protocol is incomplete. Options to proceed include finding a new test or evolving the protocol.

2.2. Nanoparticle protein corona
Nanoparticles that enter the blood stream become coated with proteins. Recovery of the adsorbed proteins by centrifugation with controlled washing steps and times, in conjunction with consideration of the affinities and association / dissociation rates of the proteins, ensures that only truly particle-associated proteins are recovered and identified [3]. Copolymer nanoparticles, 0.5 or 2 mg in 0.05 or 0.2 ml of 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 ml EDTA were incubated with increasing amounts of plasma on ice. The final volume was constant within each experiment. After 1h, the samples were moved to 23°C to promote aggregation. The particles were pelleted by centrifugation (13 kRPM, 2 minutes), washed three times with 0.5 ml 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 ml EDTA and the vials changed after each washing step. Bound proteins were removed from the particles by adding SDS-PAGE loading buffer and separated by 12% or 15% SDS-PAGE. Bands were cut out from SDS PAGE gels, digested by trypsin, and studied by mass spectroscopy (nanoscale liquid chromatography quadrupole time-of-flight MS/MS). Spectra were analyzed by Mascot software to identify tryptic peptide sequences matched to the International Protein Index (IPI) database (www.ebi.ac.uk/IPI). A second independent identification experiment was performed using nano-electrospray liquid chromatography mass spectrometry (Nano-LC MS/MS). Spectra were searched using the SEQUEST algorithm [8] against the International Protein Index (IPI) database.
2.3. Nanoparticle-induced Fibrillation
Dialysis-related amyloidosis involves the fibrillation of protein β2-microglobulin (β2m) which occurs at increased serum concentration in patients with renal failure. The molecular events leading from the native folded monomer to the fibre are not resolved, but nucleation is the key step, after which fibrillation proceeds rapidly. The effect of nanoparticles on β2m fibrillation was studied at 37°C in the absence and presence of nanoparticles under a set of solution conditions. The protein concentration was 40, 80 or 125 µM (as determined by absorbance at 277 nm), in 6, 10 or 20 mM sodium phosphate buffer, pH 2.5, with 0.02% NaN₃, and 50 mM NaCl. Buffer stock was added to the monomer solution, pH checked on a thoroughly rinsed pH electrode, and the solution was then filtered (0.2 µM filter) before it was incubated at 37°C with or without 0.01 mg/ml nanoparticles (copolymeric nanoparticles, cerium dioxide nanoparticles or quantum dots). Experiments were carried out in three different locations, using an identical protocol. To improve the statistics and eliminate irreproducibility due to small differences in solution conditions, experiments were set up in groups from the same solution, with 48 to 120 samples in each group. Each sample of 0.5 ml was shaken in a 1.5 ml Eppendorff tube at 250 rpm close to horizontal orientation. To monitor the appearance and growth of fibrils, aliquots from the tubes were pipetted at different time points to a black 96 well plate and the thioflavin T fluorescence (20 µM thioflavin T added) was measured at 475 nm with excitation at 435 nm in a plate reader [5].

2.4. Silica genotoxicity (Comet assay)
The aim of this study was to perform the most complete, thorough and well-controlled investigation of the genotoxicity of commercially available and laboratory synthesized SiO₂ nanoparticles performed to date using the Comet assay test at the same doses used by Pacheo et al. [9], and validating the findings from the study at two independent laboratories. Its purpose is primarily to show that careful application of protocols, standards and methodologies renders the Comet test of these materials quantitatively reproducible.

Silica nanoparticles from Sigma (Ludox, alumina and sodium counter-ion stabilised, both 30nm) and non-stabilised amorphous silica nanoparticles from Glantreo (nominally 30, 80 and 400 nm) were assessed at physiologically relevant concentrations of 4 µg/ml and 40 µg/ml with 3, 6 and 24 hours incubation times using cultured 3T3-L1 fibroblasts supplied to all partners from a central stock, and using a common protocol carefully applied. Nanoparticles were sonicated in an ultrasonic bath for 15 minutes prior to dilution in ultrapure water and subsequent preparation of working solutions in cell culture media. Cells were dosed immediately and incubated under normal cell culture conditions (37°C, CO₂, etc.).

A detailed protocol for the Comet assay was described and rigorously applied at the two test locations. Each particle type was tested in detail with multiple repetitions and multiple time points at the first site (UU) and selected particles were independently chosen at a separate laboratory at the 6h time point (NIOM) and the results compared. Additionally, slides from each location were scored at the other location, for complete validation of the results. Comets were analysed using a Nikon Eclipse E400 fluorescence microscope using a x 40 dry lens (NA 0.75). Comet analysis was carried out using Komet 5.0 (Andor™ Technology), % tail DNA and olive tail moment were selected for statistical analysis. For each individual slide 50 cells were analysed at random locations across the slide.

3. Results and Discussion

3.1. Protein Corona
The exchange times (of the ‘hard corona’) can be so slow that many early biological responses are already defined by these associated biomolecules [10]. It is therefore these that define the biological identity of the nanoparticle, and it is important to learn their identity and more broadly to develop methods to assess them. Significant progress is being made in this arena.
Following incubation of nanoparticles with plasma, the proteins bound to the nanoparticles are recovered by centrifugation and washing. The washing and centrifugation protocol was investigated in detail, to ensure that only proteins genuinely bound to the nanoparticles are recovered, rather than those that are present simply due to their abundance in plasma. After one wash, the amount of albumin is still significant but not after three washes. A total washing time of 20 minutes was implemented to retain proteins with relatively high affinity and slow exchange. Five proteins were consistently associated with the particles.

In two independent experiments this led to identification of human serum albumin (69 kDa; p<10^{-6}), apolipoprotein AIV (43 kDa; p<10^{-6}), apolipoprotein E (34 kDa; p<10^{-6}), apolipoprotein AI (28 kDa; p<10^{-6}), and apolipoprotein AII (8.7 kDa; p<5 \times 10^{-5}). The molecular weights of these proteins match those estimated from SDS-PAGE in the present experiments (shown in Figure 2). Judging from the band identities, the most abundant protein on the particles is apolipoprotein AI, and the amount of HSA is low in comparison. The concentration of apolipoprotein A1 in human plasma is only about 1-2 mg/ml, compared to 30 mg/mL for HSA.

**Figure 2.** Left: Illustration of the dynamic nanoparticle protein corona where rapid exchange occurs with the surrounding proteins, and proteins with high affinity replace lower affinity higher abundance proteins.

Right: 1D-gel showing the major proteins bound to copolymer particles of increasing surface hydrophobicity. Note the very low binding of HSA (human serum albumin) to the particles despite it being the major component of human plasma [3]. The fact that identical materials with different size and surface charge bind different proteins into their coronas has deep implications for regulation of nanomaterials, and suggests that in the future nanoparticles may be able to be classified according to the proteins that they bind, and potential impacts from nanoparticles may be predicted based on protein coronas.

**Table 1.** Selective binding of different apolipoproteins to polystyrene nanoparticles of different sizes and different surface charges.
Interestingly, apolipoprotein A1 is the major component of HDL (high density lipoproteins), and is found on chylomicrons, large lipoprotein particles created by the absorptive cells of the small intestine, which are used in cholesterol transport in vivo [11, 12]. This has potentially significant implications for the uptake and transport of nanoparticles, as, for example, apolipoprotein E has been shown to enhance transport to the brain [13]. More recent results from the NanoInteract project have shown that changing the size and/or surface charge of nanoparticles influences the proteins that bind to the nanoparticles in a biological media, including resulting in specific binding of Apolipoprotein E or Apolipoprotein F which is known to promote transport across the placenta, as shown in Table 1 [10].

3.2. Fibrillation Assays
β2m-fibril growth appears to follow a (seed-independent) homogeneous nucleation-and-growth mechanism, requiring high concentration of the monomer only, at pH values less than 4.8, where unfolding of the protein is rather extensive. Nucleation is a stochastic event, so fibrillation by this mechanism is characterised by a lag phase that increases as we approach the heterogeneous nucleation regime. However, once the process is started it quickly goes to completion.

Experiments (in total 432 tubes) were set up at 50 mM NaCl to enable the collection of statistical amounts of data in a reasonable time. Under these conditions the presence of 0.01 mg/ml nanoparticles was observed to enhance in the rate of fibrillation significantly. The ‘lag phase’ (likely a reflection of the time for a critical nucleus to form) for two groups of experiments in tubes are presented in the curves shown in Figure 3 (left), and in the form of histograms in Figure 3 (right). From the time dependence of fibril formation, it appears that the enhanced rate of β2m fibrillation in the presence of the nanoparticles is due to a significantly shortened lag phase, or time to form a critical nucleus. Indeed, in the absence of nanoparticles, the lag phase is so long that fibrillation has not even begun during the timeframe in which the process has gone to completion in the solutions with nanoparticles. More recent experiments using amyloid-β (one of the proteins associated with Alzheimer’s disease) have shown the opposite trend, with selected nanoparticles slowing down or even reversing the fibrillation process.[6]

![Figure 3. Left: ThT fibrillation assays in the absence (black) and presence (blue and red) of copolymer nanoparticles of different hydrophobicity. Blue curves show the reduction in the lag time in the presence of hydrophilic copolymer nanoparticles, and red curves show the effect of more hydrophobic copolymer nanoparticles. The concentration of β2m is 0.5 mg/mL in buffer containing 20 mM phosphate and 50 mM NaCl at pH 2.5. The particle concentration in all cases was 0.01 mg/mL. Right: Distribution of lag times showing the statistical distribution of the lag times.](image)

3.3. Comet Assay Alignment
Application of the round-robin process to the Comet assay was quiet a challenge, and involved several iterations, including alignment of the protocol between the two laboratories, use of same Comet assay reagents (including cells, serum, etc.), inter-lab visits and sample exchange, comparison of analysis
software and hardware and elimination of variation due to different analysis software and hardware, and application of strict random sampling rules. Table 2 shows a set of data from the two laboratories, showing that quantitative agreement can be obtained.

Once the protocol had been refined, and quantitatively similar results were obtained at both test sites, this careful, systematic and multi-site study demonstrated that amorphous silica nanoparticles from different sources (Sigma and Glantreo) do not cause genotoxicity detectable by the Comet assay in the 3T3 cell line under the conditions described, as shown in Figure 4. The results were found to be reproducible in separate tests in two independent laboratories and with different personnel analyzing the Comet results. This study does not detect genotoxicity from amorphous silica nanoparticles in the size range from 20 nm to below 400 nm, with both positive and negative surface characteristics.

![Figure 4](image-url)

**Figure 4.** Examples of Comet assay images, recorded with Nikon Eclipse E400 fluorescence microscope using a x40 dry lens (NA 0.75), a) 3T3-fibroblasts incubated for 24 h with 40 µg/ml 30 nm silica nanoparticles from Glantreo, b) incubated for 24 h with the dialysate, c) negative control, d) positive control. All images are from the experiments at UU.

**Table 2.** Comparison of Comet assay results of Sigma Ludox CL mean and standard deviation of Tail%DNA, conducted at two different NanoInteract partner locations.

|                      | Partner X |                  | Partner Y |                  |
|----------------------|-----------|------------------|-----------|------------------|
|                      | Tail%DNA  | Mean             | Tail%DNA  | Mean             |
|                      | 6h        | SD               | 6h        | SD               |
| Sigma Ludox CL 420883| 6.24      | 5.12             | 7.44      | 7.50             |
| 40 µg/ml silica NP   | 8.46      | 8.86             | 6.42      | 8.56             |
| Dialysate            | 7.26      | 5.99             | 6.12      | 7.31             |
| Negative control (DMEM)| 34.30    | 18.44            | 52.34     | 17.99            |

4. Conclusions

NanoInteract is a European research project investigating the interactions between nanoparticles with living systems, using a range of approaches from classical toxicology and ecotoxicology, through physical chemistry and biophysics, and advanced biological techniques such as transcriptomics and proteomics. The key elements of NanoInteract are control of the system at all stages, from nanoparticle purity, dispersion in biological media, control of cell confluency and the exposure dose, etc., and application of round robin approaches to ensure that quantitatively reproducible results leave no room for doubt or uncertainty regarding the results, and ensures that the results can be trusted by all of the relevant stakeholders, from governments to NGOs to the general public.

An important aspect of the work is the application of a combination of existing biophysical and physical characterisation techniques to the issue of protein conformation upon binding. We have found that size and surface charge affect the nature of the proteins that bind to the nanoparticles in physiological solutions, thereby conferring different biological identities onto the nanoparticles. This may have significant impact in terms of regulation of nanoparticles in the future, and potentially for development of quantitative structure-activity relationships (QSARs). We also note the surprising feature that nanoparticles are sometimes able to induce dramatic effects on protein interactions, such as the case for protein fibrillation, and of course this observation, when combined with the potential for nanoparticles to transport to the brain implies the need to study this whole arena in more depth.
Having understood the nature of the expressed biomolecules the challenge now is to study where the particles go (using imaging techniques) and what effects they have on cells / organisms when they reach there. Significant progress has been made here within NanoInteract, and will be the subject of another article. A key achievement of the field would then be to connect this biological identity (corona) to the observed biological impact. This has not been achieved yet, but progress is being made within NanoInteract.

Perhaps the most significant outcome of the NanoInteract project to date is the suggestion that, contrary to often stated beliefs, in vitro toxicity test may evolve to become entirely quantitatively reproducible across multiple laboratories. This is a hopeful message as nano-EHS studies are cited broadly, not just in the scientific community, but also in the public media.

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References

[1] European Commission, Nanotechnologies: A Preliminary Risk Analysis on the Basis of a Workshop Organized in Brussels on 1-2 March 2004 by the Health and Consumer Directorate General of the European Commission. 2005.
[2] Cedervall T, Lynch I, Lindman S, Nilsson H, Thulin E, Linse S and Dawson K A 2007 PNAS 104 2050
[3] Cedervall T, Lynch I, Foy M, Berggård T, Donnelly S, Cagney G, Linse S and Dawson K A 2007 Angewandte Chemie Int. Ed. 46 5754
[4] Brant J A, Labille J, Bottero J Y and Wiesner M 2007 Environmental Nanotechnology: Applications and Impacts of Nanomaterials McGraw Hill NY ed. 231
[5] Linse S, Cabaleiro-Lago C, Xue W-F, Lynch I, Lindman S, Thulin E, Radford S, Dawson K A 2007 PNAS 104 8691
[6] Cabaleiro-Lago C, Quinlan-Pluck F, Lynch I, Lindman S, Minogue A M, Thulin E, Walsh D W, Dawson K A and Linse S 2008 JACS ASAP published
[7] Barnes C A, Elsaesser A, Arkusz J, Smok A, Palus J, Lesniak A, Salvati A, Hanrahan J P, de Jong W H, Dziubaltowska E, Stępnik M, Rydzyński K, McKerr G, Lynch I, Dawson K A and Howard C V 2008 Nano Letters, ASAP Article DOI 10.1021/nl801661w
[8] Yates J R, Eng J K, McCormack A L and Schieltz D 1995 Anal. Chem. 67 1426
[9] Pacheco S E, Mashayekhi H, Jiang W, Xing B and Arcaro K F Abstracts of the 2007 Annual Meeting of the American Association for Cancer Research
[10] Lundqvist M, Stigler J, Cedervall T, Elia G, Lynch I and Dawson K 2008 PNAS 105 14265
[11] Berggard T, Arrigoni G, Olsson O, Fex M, Linse S and James P 2006 J Proteome Res. 5 669
[12] Mahley R W, Innerarity T L, Rall Jr S C and Weisgraber K H 1984 J. Lipid Res. 25 1277
[13] Michaelis K, Hoffmann M M, Dreis S, Herbert E, Alyautdin R N, Michaelis M, Kreuter J and Langer K 2006 J Pharmacol Exp Ther. 317 1246