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Physicochemical, colloidal and transport properties of nanoparticles

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Introduction

Following the definition from the European commission, all engineered particles below 100 nm in diameter are nanoparticles (<http://europa.eu> (2011)). By this definition, many “nanoproducts” exist on the market, in which nanoscaled particles are the main building blocks. From a more scientific viewpoint, nanomaterials are defined by the change of important physicochemical properties with decreasing crystal or particle size (Brune et al. 2006). Such significant changes do not normally occur in particles larger than 30 nm but mostly in particles smaller than 10 nm. The reasons for the changes in the material properties are the high surface/volume ratio and the changes in the electronic structure (quantum confinement). The surface/volume ratio not only is a geometrical parameter that increases the reactivity per mass of a material but it also affects thermodynamic properties such as phase stability and equilibrium, including the melting temperature, homogeneity of the particles and shape. Simultaneously, quantum confinement occurs, and the electrical and optical properties including the chemical reactivity are changed. A third origin of changes at the nanosize are properties such as ferromagnetism or ferroelectricity; they both depend on the interaction potential between the atoms (exchange energy), which decreases with a decreasing number of atoms per particle. If the thermal energy is larger than the interaction energy, the material loses its ordered arrangement of magnetic moments, and the

material becomes paramagnetic. Such properties depend not only on the temperature but also on the observation time. This short list demonstrates that nanoparticles are indeed very complex materials and that a large number of properties should differ from the bulk behavior if the size of the particles decreases. It is important to be able to predict the behavior of nanoparticles in a biological environment such as cell cultures or *in vivo*. In this chapter, only aspects that are directly relevant for their behavior in a biological environment are discussed.

Physicochemical properties of nanoparticles

The size, morphology, crystalline phase and crystal orientation of the crystal plane expressed at the surface determine the chemical and physical properties of nanoparticles. Properties such as melting point, surface tension, crystalline phase and catalytic activity are important characteristics for the application of the particles as well as for all aspects regarding interaction with living systems. In addition to the aforementioned physicochemical properties of nanoparticulate materials, Rivera-Gil listed colloidal stability, purity, inertness, size, shape, charge, and their ability to adsorb environmental compounds such as proteins (Rivera-Gil, 2013). These properties are again related to other basic physicochemical properties mostly in a complex manner, and the correlation of these properties with toxicity is not straightforward. The critical size, at which a significant deviation from the bulk properties occurs, depends strongly on the material but also on the property of interest (Barnard 2008). For example, a significant change (increase) in the band gap energy, an important parameter for optical and electrical properties, and thus also for chemical properties, can be observed mostly for particles smaller than 10 nm. In contrast, melting temperatures decrease if the size of the particle is less than 5 nm. Another interesting example is the behavior of ferromagnetic materials. The change from permanent magnetism (ferromagnetism for example) to paramagnetic behavior occurs for oxides in the

range of 20 to 30 nm and for metals below 5 nm. These few examples clearly illustrate that the properties of nanoparticles are not a constant value as for bulk materials; for each size, shape and environment, we observe different properties. Titanium dioxide, a well-investigated nanomaterial that is also a bulk product used in various industrial applications, is a highly complex nanomaterial. Different crystalline phases are known (rutile, anatase and brookite), but the thermodynamic stability of the phases is strongly dependent on the size of the crystals. Below 5 nm, anatase is the thermodynamically stable phase, whereas for particles larger 33 nm, rutile is the stable phase. Regarding reactivity, not only are small titanium dioxide particles more photocatalytic because the surface/volume ratio is larger; in addition, a strong increase occurs because the amount of the more reactive anatase is also increasing with decreasing size, and the band gap energy is increasing, which could have a significant effect on specific reactions. However, in reality, titanium dioxide nanoparticles exhibit a size distribution, and therefore, the crystalline phase cannot be simply predicted from the mean sizes of the powder. Additionally, the thermodynamically preferred crystal phases also depend on the shape and the thermal and chemical environment, especially from H₂O adsorbed at anatase or OH⁻ adsorbed at rutile, as both stabilize the corresponding crystal structure. The effect of surface chemistry on phase stability leads to the co-existence of both phases over a large size range from 5 to 33 nm, and an abrupt phase change such as Gibb's phase-rule is demanding is not observed (Barnard 2011). It is evident that the prediction of the properties of nanoparticles, for example, their capacity for the generation of reactive oxygen species, is very difficult and only possible with detailed modeling of the crystal and surface structure, the arrangement of atoms at the surface and their interaction with the surrounding environment.

Particle –particle interactions

The interaction between two particles is generally determined by three main factors: attractive van der Waals forces, repulsive electrostatic forces and steric hindrances. In addition to these forces, hydrophobic/hydrophilic interactions, depletion forces and/or osmotic pressure induced by dissolved macromolecules may be observed. In a biologically relevant environment, we must address all these forces simultaneously. In water, the standard theory for interactions between small particles in colloids is the Derjaguin, Landau, Verwey and Overbeek (DLVO) theory (Verwey 1948). The DLVO interaction potential is the sum of an effective repulsive electrostatic term and a van der Waals term, which is mostly attractive in nature. The van der Waals term is calculated as the integral of the interatomic dispersion interactions over the volume of both particles, while considering the dielectric properties of the medium between the particles (French 2000). Dispersion forces are the dipole interactions between molecules caused by the rapid change of the electron-nucleus arrangement, leading to changing dipoles that at any instant induce electrical interactions with another molecule/particle, resulting in interactive forces. Van der Waals potentials are calculated using a constant (e.g., Hamaker constant) times a geometrical term including the distance, shape and size of particles. Later works (Lifshitz 1956; Parsegian 1969; Hough 1980; Faure 2011) have demonstrated that the Hamaker constant can be correctly calculated and predicted based on spectroscopic measurements of the dielectric response of the material as a function of distance between particles and the media (water, cell media, air). Hamaker's van der Waals force description is widely accepted and valid for short-range interactions. For long-range interactions, other models exist (Vincent 1973), which also account for retardation effects because electromagnetic waves only have a limited velocity in the suspending media. Only limited data regarding the dielectric function of nanosized materials

exists, and therefore, bulk values must be used for the calculations. Electrostatic interactions result mainly from the overlapping of the ionic double layer of two neighboring particles. An exact analytical solution for the interaction energy of double layers can only be observed for non-correlated point charges at charged infinite plates. The Debye-Hückel theory uses the linearized Poisson-Boltzmann distribution to describe the concentration of charges (cations and anions) as a function of the distance from the plate surfaces. Hogg et al. and Bell et al. modified the result for charged infinite plates for the application to curved surfaces (e.g., Hogg 1966; Bell 1970). Figure 1a shows typical values for the interaction potential of van der Waals and electrostatic forces as well the sum of these two forces according to the DLVO theory for 100-nm diameter TiO_2 of in water with a zeta-potential of 30 mV. A barrier of 12 $k_B T$ (k_B is the Boltzmann constant) could clearly be observed at a distance of 4 nm from the particle surface. This value is just enough for the stabilization of a dilute suspension. When adding a polymer on the particle surfaces (also called a surfactant), the steric hindrance prevents attraction by van der Waals forces, and the suspension is colloidal stable.

Although the DLVO theory is well accepted and generally yields good predictions of the colloidal stability of suspensions of particles with diameters in the micrometer range, when the calculation is applied to nanosized objects (diameter <100 nm), the results do not always correlate well with experimental results. If we consider a suspension of 10-nm-sized maghemite nanoparticles ($\gamma\text{Fe}_2\text{O}_3$) in an aqueous solution of nitric acid at 0.01 M and a zeta potential of 20 eV, using DLVO theory, we can calculate an energy barrier against coagulation of 1-3 $k_B T$. This value would correspond to an aggregation time smaller than a second. However, this type of suspension can be maintained for more than 2 years at room temperature with no measurable aggregation. Fig 1b shows the decreasing DLVO potential for a TiO_2 in aqueous suspension

(zeta potential of 30 mV) with decreasing size. Only particles with a diameter > 50 nm are colloidal stable in pure water. To compute the effective electrostatic component, the ions are described by point charges, and two approximations are made: the Poisson–Boltzmann (PB) approximation (i.e., a mean-field treatment of ions) and an expansion of the charge density to linear order in the electrostatic potential. The interaction between highly charged particles in water is determined by the screening of the counterions and additional salt ions; the latter are highly present in biofluids. For strongly interacting concentrated particle suspensions, the effective interparticle forces exhibit a many-body character, which is induced by the nonlinear particle configuration dependent on counterion screening. The DLVO potential and its generic Yukawa expression for the potential of the mean force between particles developed to describe interactions in highly diluted and weakly interacting colloidal suspensions (Dahirel 2007) have been extensively used to describe concentrated suspensions with nanosized particles and high salt concentrations up to 0.15 M. Even recently, many experimental works on the suspensions of nanoparticles such as proteins (Zhang 2007) or iron oxides (Mériguet, 2004) could be interpreted assuming a Yukawa potential. These results suggest that these solutions of nanoparticles maintain the dominant features of colloidal suspensions, even if the size of the solute particles corresponds to the lowest limit of the colloidal domain. It is important to note that nanosized particles can exhibit quantum confinement and, therefore, different optical properties such as the dielectric function. Faure et al. (Faure 2011) recalculated the Hamaker constant of superparamagnetic iron oxide in various media, taking advantage of the recent publication of the optical properties of iron oxides published by Tepper (Tepper 2004). It was demonstrated that the Hamaker constant for 100-nm particles remains very similar to the bulk value; however, a more precise value is given. Knowing that iron oxide nanoparticles for medical applications are in the

size range of 7 nm, the nanosize will exhibit a non-negligible attractive forces. The Velegol group observed that van der Waals forces calculated using the classical approach are quite inaccurate for nearly all situations with nanocolloids (Kim 2007). The assumptions of a uniform dielectric function (even up to the surface), the absence of discrete atom effects or the assumption of interacting semi-infinite bodies all fail for the nanocolloid case. Another possible explanation for the discrepancy between the experimental and theoretical results is the breakdown of the assumption of a Poisson-Boltzmann-like ionic double layer. Two distinct nanosize effects can be identified that might break this assumption for sufficiently small particle diameters:

- In the DLVO theory, the ionic distribution around a particle is considered to be similar to the ionic distribution close to an infinite plate with equal surface charge. However, when the size ratio between the double layer thickness ($1/\kappa$) and the particle diameter is no longer negligible ($\kappa a < 10$), this assumption is no longer valid.
- In the DLVO theory, ions are approximated by point charges. However, when the size ratio between the solvated ionic species and the particle diameter becomes non-negligible, ions can no longer be regarded as point charges but as finite size particles that cannot overlap. In other words, the maximal volume or surface charge density in the media is limited by the ionic volume.

For particles suspended in blood, lung surfactant or cell media with serum for in vitro tests, the particles are not dispersed in pure water with a low salt content and a constant pH value. Biologically relevant fluids have high ionic strengths (0.15 molar), have a pH of 7.4 or less, such as 4.5 in endosomes, and contain macromolecules such as proteins with similar sizes as the particles, for example, albumin with a diameter of 5.5 nm. Fig 1.c shows the effect of the media

on the interaction potential of TiO₂ with a 100 nm diameter. It is clearly observed that the highest stability exists in pure water at low pH (high zeta potential), whereas in cell media at pH 7.4 and high ionic strengths, the particles agglomerate. After adding serum proteins that adsorb at the particle surface (Lundquist 2008), theoretically, steric stabilization should occur. An experimental investigation indicated that such conclusions are too simplified; the same particle can exhibit very fast agglomeration or very high stability depending on the type of coating, charge and the presence of serum (see Fig 2) (Fink-Petri 2007). In contrast, polymer-coated superparamagnetic iron oxide nanoparticles (SPIONs) were tested for colloidal stability and cell uptake. Although all the examined particles were colloidal stable in water and PBS buffer, these colloidal particles exhibited different stabilities in DMEM or RPMI media with and without fetal calf serum (FCS). Furthermore, the cell uptake (over various time intervals) depended on the cell media and the presence/absence of serum. Systematic variations of any of the parameters, including the change of polymer (PVA, amino-PVA) or cell medium (DMEM or RPMI) and the presence/absence of serum, had an observable effect on agglomeration and uptake (Fig. 3). It is clearly observed that particles in cell media with 10% serum agglomerate less; however, the uptake is also lower compared with particles in cell media without serum. The correlation between the agglomeration rate and uptake was 0.73 ($P = 0.02$). Replacement of PVA by amino-PVA, which means increasing the positive charge from 5 to 25 mV, led to significantly faster agglomeration and slightly higher uptake. The reason for this observation must be the interaction of the particles with proteins in the serum. Additionally, the high stability of PVA-SPIONs in FCS-supplemented media could be explained by depletion stabilization caused by the serum proteins, which have a similar size as the nanoparticles. Additionally, for particles >100 nm, sedimentation would be the dominant transport process, and therefore, the amount of particles in

contact with the cells could be larger (details are given in chapter “Particle transport”).

Protein adsorption

Pioneering work regarding protein adsorption on oxide nanoparticles was performed by the Dawson group (Lundquist 2008). These researchers demonstrated that even though many of the major highly abundant proteins could be detected in the corona independently of their size and surface charge, an entire range of other proteins form an important part of the corona. For a fixed material type, these researchers observed that the size of the particle and its surface modification are able to entirely change the nature of the biologically active proteins in the corona and thereby possibly also affect their biological function. Furthermore, Dawson stated that the fundamental question is the format of presentation of these proteins and to what degree they are still able to present native epitopes when embedded in the corona that are still open. Additionally, he claimed that particles studied *in vitro* (at low serum dilutions) may bear little relation to those that exist *in vivo* (high protein concentration as, for example, in the blood), suggesting the need for a re-evaluation of how such studies should be planned in the future and how *in vitro* - *in vivo* extrapolations could be performed. The nanoscale surface curvature strongly affects protein adsorption, such that the protein-binding affinities for nanoparticle surfaces are different from their analogous bulk material (Xin-Rui 2010), and the coronas associated with NPs of the same material but of different size can vary in composition (Shang 2009). Recent investigations suggest that the interaction between nanoparticles and plasma proteins and other blood components is a determining factor for the cellular up-take of the particles. Certainly, *in vivo*, the adsorbed protein layer may affect trafficking (Oberdörster 2010) and particle biodistribution (Ehrenberg 2009; Dobrovolskaia 2008).

Dawson and collaborators have shown in a large number of experimental investigations that the

surfaces of nanoparticles in contact with a biological fluid are modified by the adsorption of biomolecules such as proteins or lipids, leading to an interface layer that will finally determine the interaction of the “core/shell” system with cells and organs (Monopoli 2011). The so-called “protein corona” is not stable in composition during the first hours; it appears that first, the most abundant proteins adsorb fast but in the long term, proteins at low concentration but high adsorption energy are observed preferentially at the surface (Vroman effect). Dawson’s group called the first corona the soft corona and the latter the hard corona. Recently, it was demonstrated that for superparamagnetic iron oxide nanoparticles, this concept failed, and no change of the protein corona over time was observed (Jansch 2012). However, in all cases, it is increasingly accepted that the colloidal stability and overall scale of even nonspecific cell-particle interactions is determined by the degree of “screening” of the NP surface by the protein corona. In nanoparticles, for example, the superparamagnetic iron oxide of 7 nm (SPION) coated with poly(vinyl-alcohol) (neutral) or modified PVA with amino groups (positive charge) or carboxylic groups (negative charge) in contact with serum, the net charge of each particle has a negative value (-5 to -15 mV). A comparison of zeta potential values between SPIONs in PBS without and with serum reveals that the protein adsorptions to the surface of nanoparticles could cause a significant change to negative charges. By increasing the protein content, the zeta values would be further increased in the negatively charge direction (Wongsagonsup 2005). After washing with PBS (30 times), the obtained charge differed strongly due to the protein movement from the surface of the SPIONs. The protein-nanoparticle interaction caused a significant increase in the hydrodynamic size of the nanoparticles, which could be interpreted by the protein adsorption itself but additionally by the agglomeration. Severe washing of fixed nanoparticles reduced these hydrodynamic sizes; however, tightly bound proteins (such as attached proteins in

a stern layer) did not allow the particles to attain their primary size. Interestingly, at least 35 different proteins were detected in a corona of PVA-coated nanoparticles. The composition of the corona must therefore be very different from particle to particle because for steric reasons, only 5 to 10 proteins could be adsorbed per particle. Additionally, it is obvious from the detection of proteins at the surface of different charged PVA.SPION that the surface coating of the particles determines the type of proteins adsorbed (Fig. 4). PVA-coated particles partially exhibit a very similar adsorption; however, some proteins could be observed only on one type of particle or on two. Silica-coated particles (values acquired from Jansch (Jansch2012) exhibit a very different adsorption profile. This different protein adsorption led to different agglomeration behavior, as illustrated in Figure 3, which because of the unpredictable complex adsorption behavior, is not yet predictable. It is evident that such a different protein coating would also have a large effect on the interaction with cells, such as particle adhesion at the cell membrane, the uptake mechanism and biodistribution and toxicity. For example, it has been demonstrated that positively charged PVA-SPIONs interact more strongly with HELA cells than neutral or negatively PVA-SPIONs, which is consistent with the preferential uptake of cationic agents that has been widely described in the literature (Neuberger 2005). However, as discussed above, all the particles exhibited a negatively charged surface produced normally from the overall negative charge of albumin, such that direct electrostatic interaction of the original positively charged particle and the negatively charged membrane could be excluded as the mechanism for the attraction of particles by cells. In addition, the isoelectric point (iep) of the proteins, or in other words, the overall charge of the protein, has no or only a non-significant effect on the adsorption. Most of the adsorbed proteins have an iep < pH 7, which means that the proteins have a negative charge; however, we have also detected most of these proteins on negatively charged particles. It

appears that local charges at the protein surface (Sugio 1999) are more important for the protein-particle interaction than the overall charge.

Particle agglomeration

Nel et al. (Nel 2009) have provided a complete overview regarding the possible interaction of nanoparticles with the biological environment, especially regarding the reactions and interactions at the particle surface. These authors clearly demonstrated that a large number of reactions could take place but that only a few are known in detail. These authors also claimed that “It is well-known that nanoparticles agglomerate immediately upon addition to cell culture media.” In the paper, they mentioned that in addition to DLVO interactions, hydrophilic and hydrophobic interactions are responsible for this agglomeration; however, a quantitative prediction of the colloidal behavior is very difficult and not possible even today. Nel and coworkers developed a system for characterizing the agglomeration of nanoparticles in cell media and their effect on cell toxicity. The approach is based on fast through-put size measurement using the dynamic light scattering of samples in well plates (HT-DLS, Dynapro Plate Reader, Wyatt Technology). These researchers measured mostly the fast agglomeration of nanoparticles with agglomerate sizes up to several microns. Because sedimentation of agglomerated particles leads to incorrect measurements, the results produced by this method must be interpreted with care. Another method to detect agglomerates is the measurement of turbidity or light adsorbents in the UV/vis (Soos 2009). However, using this method, the effects from sedimentation, agglomeration of proteins, adsorption of proteins, etc. also affects the estimated particle/agglomerate size. An interesting study regarding the effect of cell media composition on the agglomeration behavior of titanium dioxide (P25, Degussa) was published by Ji et al. (Ji 2010). Six different cell culture media, including bronchial epithelial growth medium (BEGM), Dulbecco’s modified Eagle’s

medium (DMEM), Luria-Bertani broth (LB), tryptic soy broth (TSB), synthetic defined medium (SD), and yeast extract peptone dextrose medium (YPD), were selected. From the published results, it is evident that the cell medium alone leads to a strong agglomeration (high ionic strengths), whereas the addition of bovine serum albumin leads to some stable suspension and fetal bovine serum to stable suspension in all the investigated combinations, which is explained by protein adsorption (steric stabilization). These results partially confirm the results presented in Fig 2 but are contradictory to the statement of Nel et al. cited above. It appears that conclusions that are too general, such as all nanoparticles agglomerate in suspensions, insufficiently describe the reality. In addition, the results presented by Ji are not applicable to all inorganic particles. The results might be applicable for uncoated nanoparticles; however, nanoparticles with organic molecules as a shell would exhibit different behavior.

A well-established and accepted method and theory exist for the characterization of the agglomeration process of nanoparticles in well-defined solvent such as pure water with low ionic strength. Observations qualitatively agree with the trend from the DLVO predictions, although the predictions indicated that 32- and 12-nm particles were unstable in 10 mM NaCl. Experimental observations indicated that these particles were stable for extensive periods of time (He 2008).

On the theoretical side, the agglomeration of particles in aqueous media is well investigated, and models for the simulation and interpretation of the experimental results are available in the literature. Considering the significant developments that have occurred since the publication of the original paper of Smoluchowski (1917), agglomeration and flocculation modeling could be described as an established field of research (Thomas 1999). However, the successful application of these models is largely limited to idealized, artificial systems. In biological systems, which are

physically and chemically heterogeneous, the correlation between simulated and experimental data is still poor.

Particle transport

Particle transport processes to cells in cell culture during in-vitro assays have an important effect on the cell doses. As expressed by K. Wittmaak (Wittmaak 2011), most in vitro studies with nanomaterials would benefit from direct measures of cellular dose. However, the experimental measurement of cellular dose is often difficult or time consuming and, thus, is a considerable limitation of in vitro studies. Thus, measurements of the target cell dose will often not be available in published papers, which makes the comparison of different investigations difficult, and only limited results could be gained from metastudies. The dynamics of particles in liquids are well studied, and mathematical approaches for describing both transports by diffusion and gravitational settling have been developed by Mason (Mason 1924). Based on these approaches, Hinderliter and Teeguarden (Hinderliter 2010) have developed a computational model of nanoparticle transport, i.e., particokinetics by sedimentation and diffusion. This model can lead to a quantitative dosimetry for non-interacting (meaning no change in the size distribution during the in-vivo experiment) spherical particles and their agglomerates in a common cell culture system.

The model clearly shows that for small particles (< 50 nm), diffusion is the dominant transport mechanism, whereas for a particle diameter > 150 nm, sedimentation is important. In between, a minimum for particle deposition on cell surfaces is observed because transport by diffusion decreases with increasing particle size, whereas particle deposition increases with increasing particle size due to sedimentation. Therefore, it is incorrect to develop conclusions from experimental observations regarding the effect of size, including this critical range on toxicity,

without considering the very different amounts of particles in contact with cells. Additionally, weaknesses of the model are that only non-interacting particles are simulated and that the particles size is constant. As discussed in the paragraphs above, interaction occurs between the particles, the size could change (increase) and for most of the particles, the size range will move from the diffusion-controlled size area to the sedimentation-controlled regime. An additional difficulty is the density of the particles, which could decrease because the particles are not further primary particles but agglomerates with a maximum of random loose-packed configuration (54 vol% solid). The change in density will have an effect on the sedimentation velocity but not on the amount of particles transported by diffusion. Additionally, the boundary conditions of Masons solution were selected such that all particles touching the cell membrane are taken up directly, meaning that the cell acts as an infinite sink. It is well known that this phenomenon does not occur in cell culture systems. After touching the cell membranes, the particles must activate a uptake mechanism; often, a complex signaling process is therefore necessary. If the uptake rate is lower than the transportation rate, particle layers are formed at the surface of the cell. Because the particles exhibit fast protein adsorption and therefore a charge of -10 to -15 mV in cell culture, repulsion between the particles could theoretically occur, and only a monolayer will be formed. In cell culture, high ionic strengths of 0.15 mol will reduce this electrostatic repulsion and could permit the formation of thick particles layers, which can lead to a strongly reduced cell viability. To observe cytotoxicity depends therefore not only on the particle-cell interaction; it could also be caused by the type of particle-particle interaction and transport to the uptake ratio.

Various experimental approaches are available to monitor the transport rates of nanoparticles to surfaces (adsorption). Optical waveguide light mode spectroscopy (OWLS) and a quartz crystal

microbalance (QCM) can be effective tools for in-situ monitoring of the kinetics of the deposition of nanoparticles onto planar surfaces. The QCM-D method has been used to determine the mass of nanoparticles deposited on surfaces using the measurement of the energy dissipation factor or the decrease in the resonance frequency of a QCM-D crystal. For example, Chen et al. (Chen 2006) studied the aggregation and deposition kinetics of fullerene nanoparticles to silica surfaces. Their data revealed an increased rate of deposition with increasing salt concentration. Fatisson et al. (Fatisson 2009) studied the deposition of TiO₂ nanoparticles onto silica surfaces over a broad range of solution conditions, e.g., pH and ionic strength, illustrating the effect of electrostatic forces, interaction or repulsion as a function of pH. Hinderliter et al. (Cho 2011) measured the transport rates of fluorescent polystyrene nanoparticles by counting the number of particles reaching the bottom of cell culture dishes using total-internal-reflection fluorescence microscopy (TIRFM), where individual fluorescent nanoparticles near the surface are selectively illuminated while the background fluorescence from the particle in suspension is efficiently suppressed. This method allows the amount of newly arrived particles to be determined as a function of time (manual counting). The rate of transport of nanoparticles can also be indirectly determined from the cellular uptake rates. For example, Hinderliter et al. (Hinderliter 2010) measured the rate of transport of iron oxide nanoparticles of SPION taken up by RAW 264.7 macrophages as a function of time using a magnetic particle detector and quantified the results against standard curves using serial dilutions of SPION suspensions. Cho et al. (Cho 2007) calculated the number of gold particles taken up by cells using a UV-vis spectroscopic method based on extinction spectra versus calibration curves and compared the results with those of other conventional quantification methods such as inductive coupled plasma mass spectrometry (ICP-MS). Cho and co-workers have also

developed experiments to assess the effects of sedimentation on cellular uptake during *in vitro* tests by comparing inverted cell culture configurations to usual upright configurations of cells cultured at the bottom of wells. Table 1 provides a brief overview regarding the existing methods for the quantitative estimation of the transport of nanoparticles to cell membranes in cell cultures.

The amount of particles transported to cells in cell cultures with fixed cells can also be detected by chemical analysis. Fig. 5 shows the amount of PVA-coated iron oxide nanoparticles (SPION) observed after 1 and 3 h in close contact or inside the cells. Different cell culture media were used: DMEM and RPMI with or without 10% fetal bovine serum. Details about the experiment and the results are given in Fink-Petri et al. (Fink-Petri 2007). Because the size of the particles was also measured during the same period under similar conditions (lower concentration), it was possible to calculate the transport of nanoparticles by diffusion and sedimentation using a simplified method that calculated the diffusion and sedimentation separately. This calculation was possible because under the given conditions (the density of the loosely packed PVA-SPION agglomerates was 1.05 g/cm^3), the transport by sedimentation was only 2 to 5% of the overall transport. Figure 5 demonstrates that a relatively good correlation exists between the measured and calculated amount of particles in close contact with cells, which means that the transport of particles under the given conditions occurs by diffusion. It is also clearly observed that the amount of particles sticking to or up taken by the HELA cells in cell culture media without serum is only slightly lower than predicted, whereas the amount was clearly lower for particles in cell media with 10% serum. These results indicate that if the hydrodynamic particle size and the density of the particles as a function of incubation time are known, prediction of the transported amount in cell culture with a fixed cell should be possible. It also appears that for protein

adsorption, the adhesion of nanoparticles and their uptake is much lower, or in other words, the sticking coefficient for particles at the cell membrane must be much smaller than for particles without adsorbed proteins. Interestingly, the difference between the expected amount calculated with the particle size measured before adding the particles to the cell culture media, which is the typical procedure for toxicity tests, is 10 to 20% of the expected value for cell culture media with serum and 30 to 40% for serum-free cell culture media. This result implies that in typical in-vitro toxicity studies, the real amount of nanoparticles that is in contact with cells is difficult to predict and will be overestimated if the particles are mainly in the diffusion regime or underestimated if the particles are in the sedimentation regime. The reason for this over/underestimation is that with protein adsorption and agglomeration, the size is increasing, which leads to a decrease in the transported amount for diffusion-controlled particle transport and vice versa in the sedimentation regime.

Conclusions

The majority of the large number of product applications using particles with a diameter < 100 nm are mainly based on well-known materials such as silica, titanium dioxide or silver. Products that use nanoparticles because their properties differ from those of bulk materials are rare and are mostly still in the development phase. One possible reason for this rarity is that the processing, including the synthesis, remains a very challenging task for research and development as well as for translation to the industrial production scale. The particle–particle interaction, controlled by the forces between the particles, can be calculated based on the well-established DLVO theory if the correct material properties and ion distribution around the nanosized particles are known. However, these properties are mostly not known, and bulk values are used instead. This

substitution leads to the calculation of incorrect interaction potentials and eventually to false colloidal stability predictions. Fortunately, it appears that nanoparticles are colloiddally much more stable than predicted using standard DLVO theory. For applications in medicine or for investigations of toxicity, the coating of the particles is much more important than the core material in terms of the behavior of the particles. The well-known corona approach, adsorption of proteins at the nanoparticle surface, is very helpful in developing a framework for understanding nanoparticles in biological environments. Adsorption is a dynamic process, which means it is very difficult to link the actual protein composition and conformation with the behavior of the particles in the body. Additionally, the large number of different proteins observed on the surface of the particles indicates that an inhomogeneous coating exists. Therefore, each particle could have a different composition and thus exhibit different behavior in vivo. A positive effect is that in 1 pg of nanoparticles, the typical number of particles per cell is 1 million particles, which means we normally observe the average behavior of the particles. The combination of protein corona, modified attraction and repulsion forces and the high number of species in suspension defines the agglomeration behavior. In complex media, such as cell culture media with serum or in blood, the interaction of platelets and other blood cells with the nanoparticles also affects the colloidal stability. The complex behavior remains unpredictable; however, it is evident that in addition to van der Waals, electrostatic forces and the surface charge (zeta potential) affect the protein corona composition and therefore the colloidal stability. The difficulty is even greater because as mentioned earlier, protein adsorption is a dynamic process and the loosely bound highly abounded proteins could be exchanged over time with strongly binding low abounded proteins (the Vroman effect). It is evident that all these parameters have an effect on the transport behavior. However, it is astonishing that most of the in

vitro experiments that have quantitatively investigated the effect of nanoparticles on cells have not considered the transport behavior of nanoparticles in complex suspensions. As discussed, while the theoretical background as well as mathematical solutions for ideal situations are available, only in the last 2 to 3 years has the discussion started on how to apply this knowledge to real in vitro experiments. The first results indicate that the transport could be even slower than predicted by the theory, which suggests that more detailed research is necessary to understand the diffusion, sedimentation and, most importantly, the adhesion of nanoparticles at cell membrane surfaces.

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Table 1: Techniques available for in-situ nanoparticle adsorption kinetic studies on planar surfaces

Technique	Description	Model particles	Ref.
Optical Waveguide Lightmode Spectroscopy (OWLS)	Total internally reflected light within a high refractive index transparent layer.	SiO ₂	Huwiler 2007)
Quartz Crystal Microbalance (QCM)	An alternating field is applied between two metal electrodes attached to the surface of a piezoelectric crystal of a certain frequency. Adsorption of particle shift frequency.	SiO ₂ , TiO ₂ , fullerene	(Chen 2006) (Fatisson 2009) (Huwiler 2007)
Total Internal Reflection Fluorescence Microscopy (TIRFM)	Requires fluorescently labeled nanoparticles. Fluorescent molecules on adsorbed nanoparticles can be excited and monitored by a microscope focused on the surface.	Polystyrene	(Hinderliter 2010)

Figure captions

Figure 1: Interaction potential (DLVO) for titania particles of different size and in different media. A) Attractive van der Waals, repulsive electrostatic potential and total potential for 100-nm particles. The dotted line corresponds to particles with a strong bonded polymer or protein layer of 3 nm thickness. b) DLVO potential of titania particles of different sizes. c) DLVO potential of titania particles of 100 nm in different media. All values were calculated using Hamaker 2 (free access: <http://ltp.epfl.ch/page-35617-en.html>).

Figure 2: Agglomeration behavior of iron oxide nanoparticles of 7 nm coated with poly(vinylalcohol) in different cell media with and without serum (10%).

Figure 3: Cell-uptake (HELA cells) and agglomeration behavior of iron oxide nanoparticles of 7 nm coated with poly(vinylalcohol) in different cell media with and without serum (10%). Full symbol: incubation time 1 h; empty symbol: incubation time 3 h; circles: with serum; squares: without serum.

Figure 4: Relative amount of adsorbed proteins on nanoparticles with different charges (positive, neutral and negative) and coating materials (PVA and silica). The values for silica were obtained from Jansch 2012, and the values for iron oxide nanoparticles were obtained from Petri-Fink 2007.

Figure 5: Comparison of measured and calculated (diffusion as main transport mechanism) amount of iron oxide nanoparticles. Circles: cell media without serum; squares: cell media with serum; filled symbol: 1 h incubation time; empty symbol: 3 h incubation time. The values were obtained from Petri-Fink 2007.

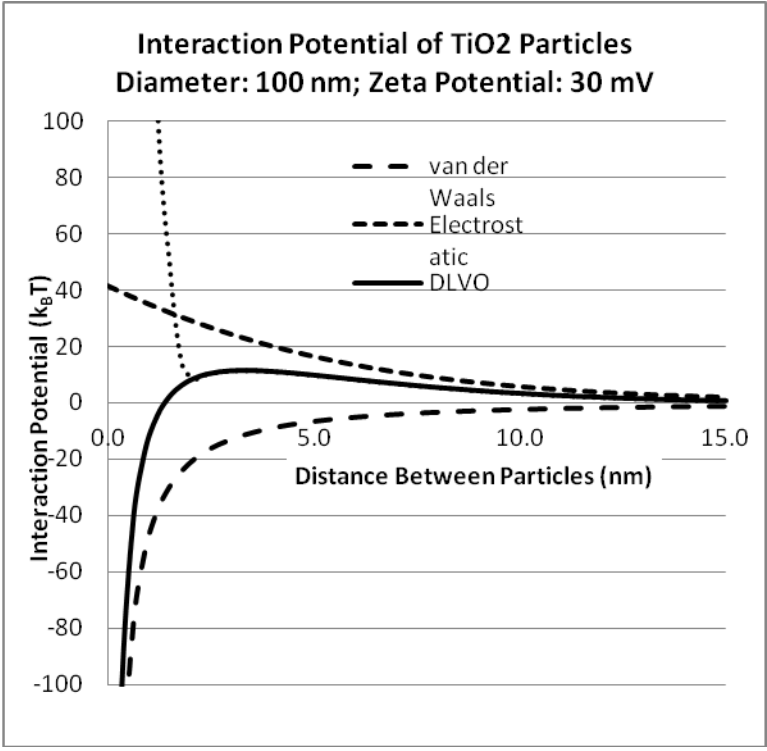


Figure 1a

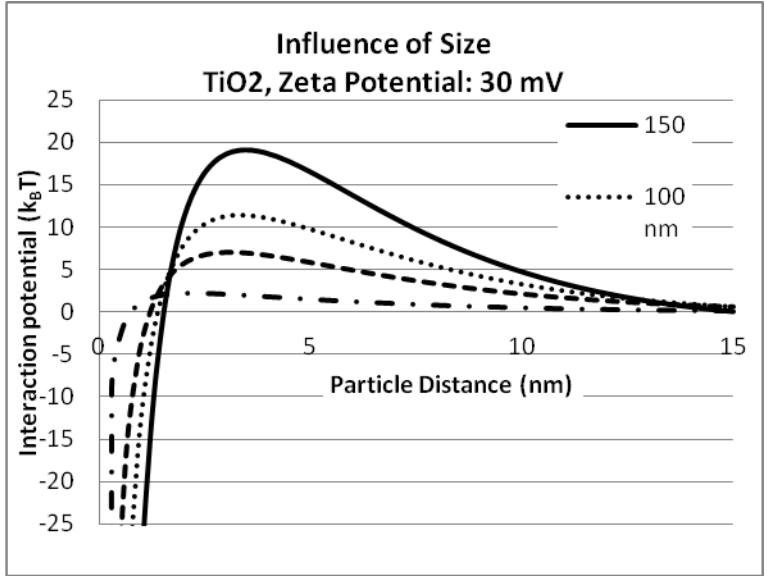


Figure 1b

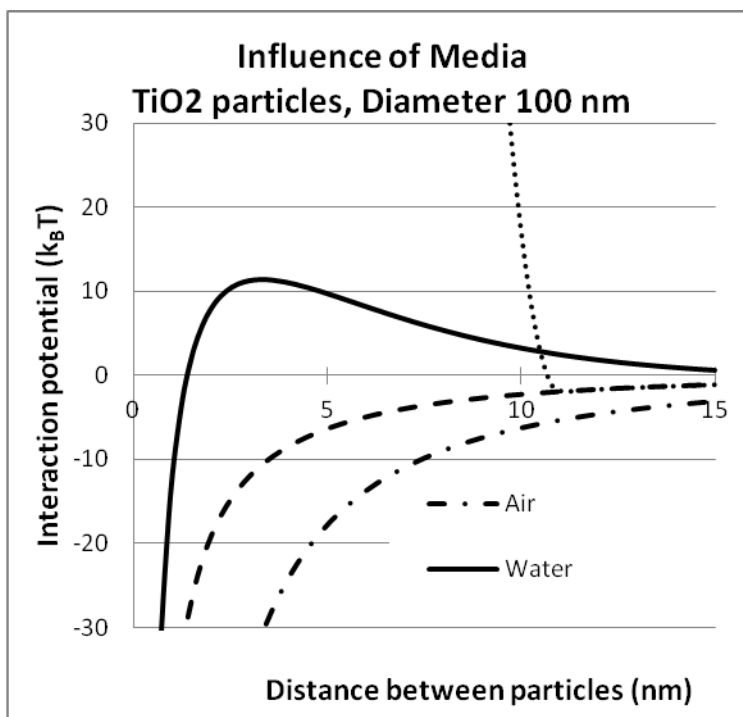


Figure 1c

Fig 1

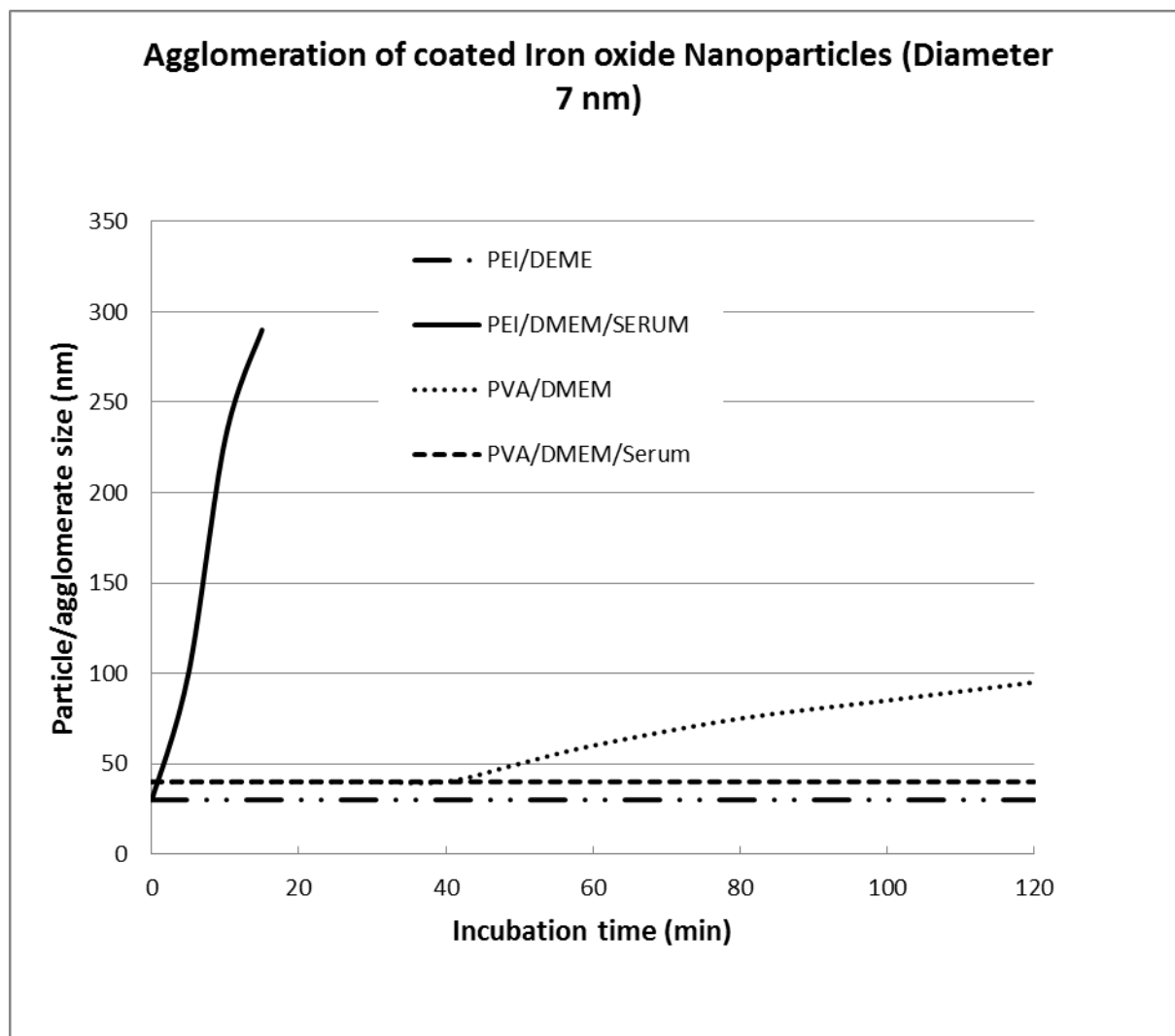


Fig 2

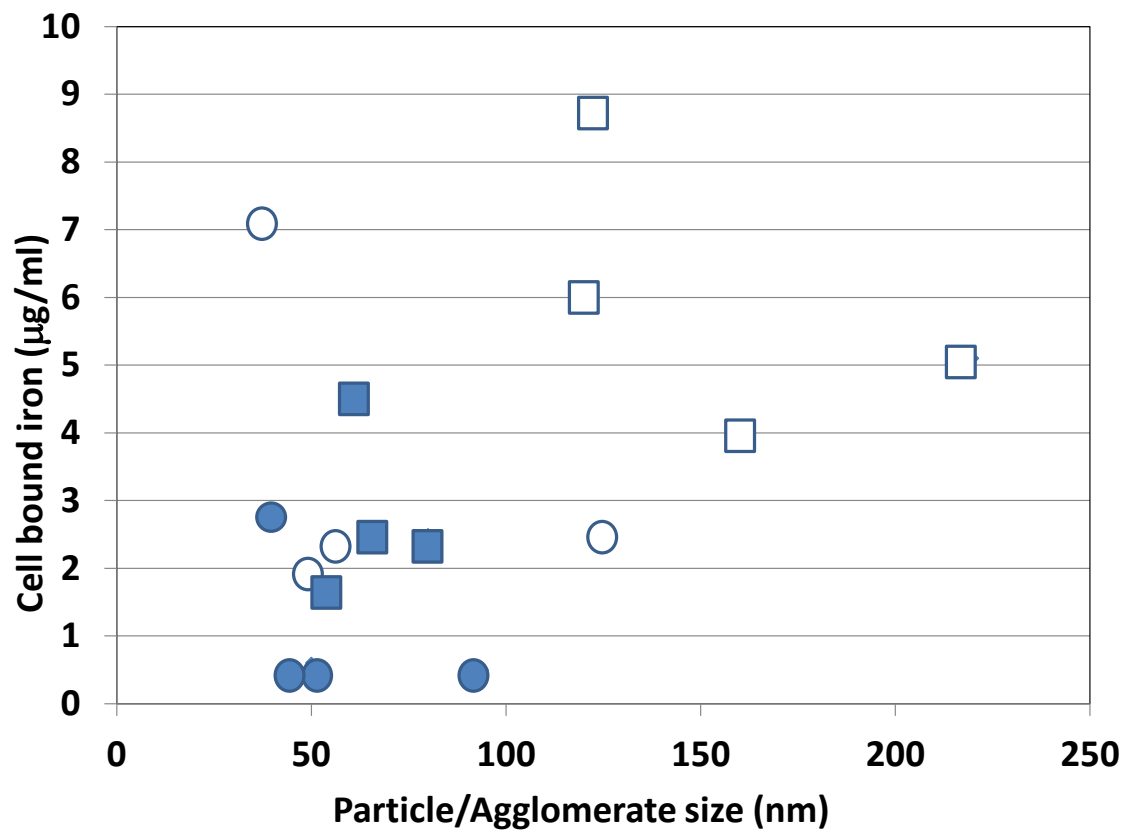


Fig 3

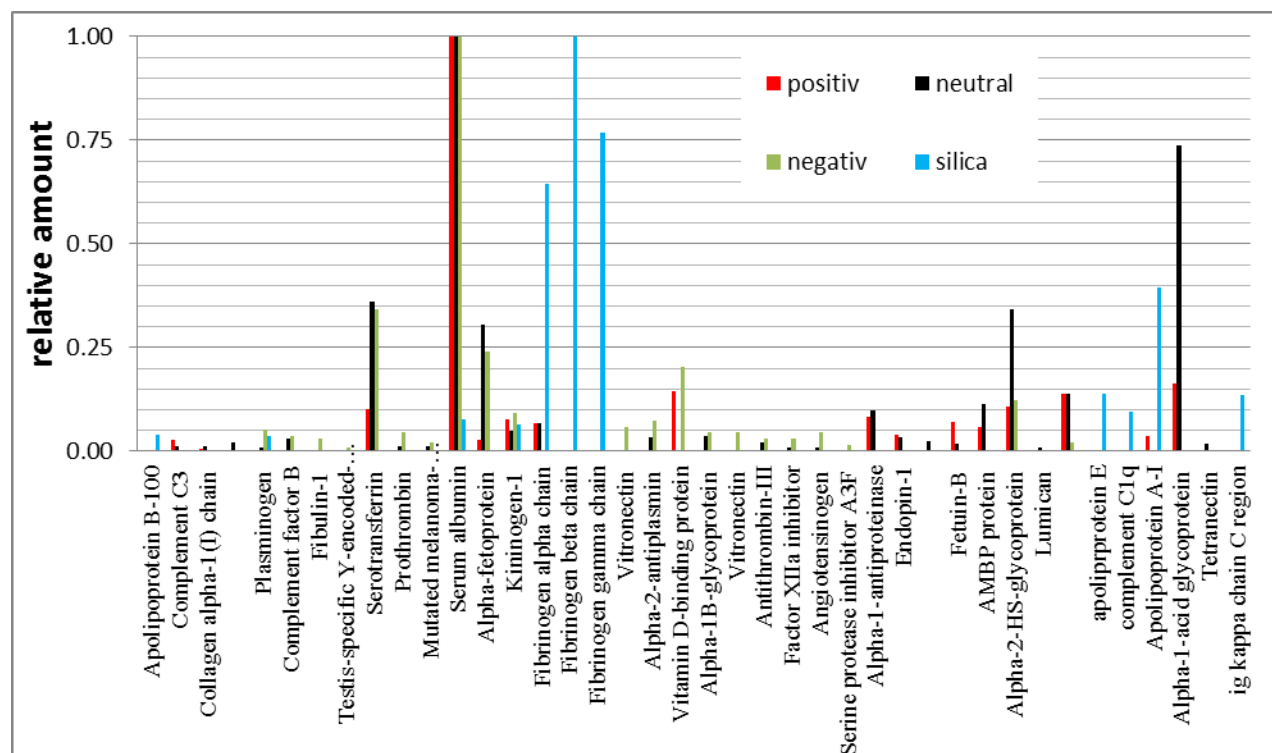


Fig 4

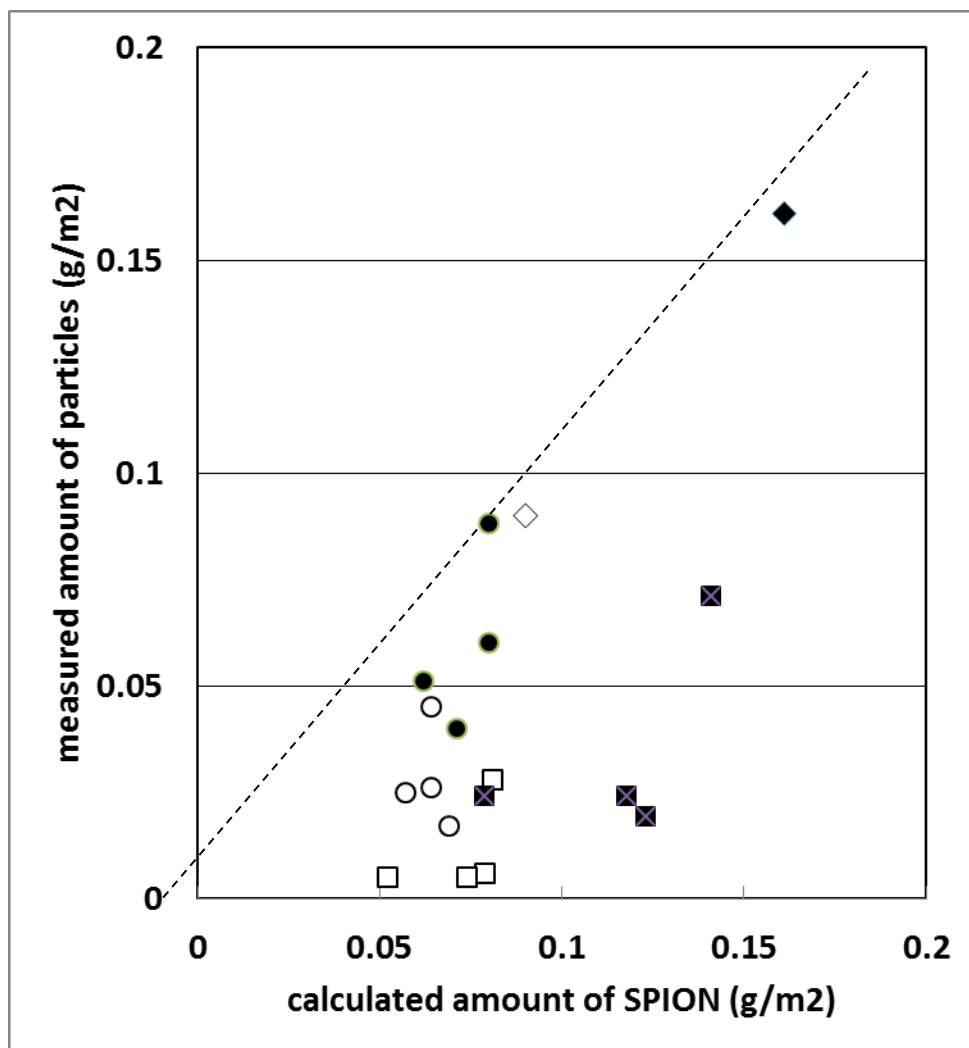


Fig 5