Tesi di Laurea

Colloidal characterisations for environmental exposure assessment in support of the risk assessment of nanobiomaterials for biomedical applications

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Summary

Engineered nanomaterials hold great promise in the medical field, as novel physico-chemical characteristics that emerge at the nanoscale may be utilised to overcome current medical challenges. Titanium dioxide nanoparticles are particularly noted for their biocompatibility, and photocatalytic activity. Such promise must, however, be weighed up against the potential human health and environmental impacts of widespread product deployment.

Despite the perception that the field of nanotechnology has developed in a relatively holistic manner, with innumerable journal entries concerned with toxicological testing of diverse ENMs, there has not been sufficient consistency with regard to standards, endpoints, etc. Thus, it could be summarised that a great deal of information has been gathered without great strides in knowledge.

This thesis endeavours to contribute to the current understanding of risk of nanotechnology-enabled health products. The regulatory landscape of ENMs in the medical field is explored, considering the legislation in place across the occupational, biomedical use and environmental life cycle stage.

In support of the elucidation of significant physico-chemical descriptors that may be utilised to predict the environmental behaviour of ENMs, colloidal characterisaitons of the clay mineral kaolinite were undertaken, complimenting the investigation of typical ENM TiO2 as well as a coated PVP form . Characterisition of the dispersions in various environmental matrices as developed by the H2020 nanoFASE project was undertaken by means of DLS, ELS and CSA techniques.

Motivation and Objectives
Between 2016 and 2024, the international market of nanotechnology is predicted to grow some 18%, and is rapidly expanding in the medical industry. Thus, nanotechnology-enabled health products are expected to significantly improve prevention, diagnosis and treatment of diseases in the decades to come. Nevertheless, along with benefits, the development of an ever-larger number of complex materials will result in unintentional release of materials in the workplace and the environment. Until recently, environmental risks of nanomedicines have not garnered the same attention as human health risks. Yet, investigations into the distribution of pharmaceuticals, which may display analogous environmental behaviour to NBMs, have revealed notable potential risks.

At a nascent stage of nanomedicine development, there are significant data, knowledge and regulatory gaps, for which expert judgement is the favoured method of risk evaluation to overcome significant uncertainties. However, understandably, a great variety of opinions are posed by experts.

A shift in the approach to risk evaluation of nanotechnology-enabled health products, and nanomaterials more widely, is required, which progresses from the incremental approach taken by European authorities thus far, towards a more holistic integrated risk assessment framework, perceptive of the subtleties of nanoscale phenomena. In order to cultivate such a framework, environmental and human health risks must be considered simultaneously, throughout the lifecycle of a given product.

The objectives of this thesis were to:
- conduct a review of EU legislation relevant to the employment of nanomaterials in the medical field;
- evaluate the physico-chemical properties relevant to titanium dioxide for medical applications;
- perform colloidal characterisations of TiO2 ENMs in relevant aquatic conditions

Chapter 1. Nanobiomaterials
1.1 Introduction

The trajectory of humanity’s understanding of itself and its place in the world is arguably reaching an inflection point, in which our collective innovation may contribute to our flourishing or our extinction. In such times, the potency of interdisciplinary collaboration is crucial to affront emergent complexity, and such a convergence of scientific thinking has been key to opening the nanoscale realm.

In his 1959 lecture ‘There’s plenty of room at the bottom’, Richard Feynman arguably anticipated the field of nanotechnology and biomedicine simultaneously. He laid down the conceptual foundations for the nanotechnological field by envisaging a world with miniaturised features – where vast tracts of information could be encoded into ever smaller spaces; where machinery could be modified and compacted. During the talk, he shared a peculiar thought: ‘although it is a very wild idea, it would be interesting in surgery if you could swallow the surgeon.’

It is somehow symbolic that physicists have been fundamental in shaping the evolution of contemporary biology, just as the revelation of DNA structure by Watson & Crick was indebted to the preceding work of Erwin Schrödinger. Now, thanks to technological advances allowing both the visualisation (e.g. Scanning electron microscopes, SEM) and engineering (e.g. electro-spinning devices) of structures at the nanoscale, the infinitesimal realm where molecular biology and physics collide is rendered finitesimal. Nanotechnology has conferred the potential to manipulate matter at the molecular level, opening a dialogue with elegant biological nano-machinery 3.8 billion years in the making. Thus, the internal surgeon may be awoken.

![Figure 1: A demonstration of the relative scale of biological entities](image)

The field of engineered nanomaterials (ENMs) has recently undergone exponential growth, particularly since the turn of the 21st century, and nanotechnology has been identified as part of the European Union (EU) 2020 Strategy as a Key Enabling Technology (KET).
Biomaterials are the subject of numerous proposed Horizon 2020 research projects and may be engineered at the nanoscale, thus categorised as nanobiomaterials (NBMs). The profound complexity of biology has led to the convergence of diverse disciplines, which gravitate towards the study of life. Medicine, as the economic driver of biological research, synchronises these fields towards the common goal of bettering human health.

Nanomaterials have had a long history in healthcare applications. Silver was the third metal known to be used by the ancients, after gold and copper, and was used empirically before the realisation that microbes were the agents of infection. Now, with cognisant knowledge of the mechanics of nanoscale systems, novel physicochemical properties may be exploited, and it seems that essentially every area of medicine may benefit from advances in nanotechnology. Among applications in diagnosis, drug delivery and regenerative medicine, the high surface to volume ratio of nanomaterials is vital to enhance surface energy and thus biological activity.

Alongside the rapid diffusion and expectations for these technologies, concerns have been raised about their possible impact upon human health and the environment. The same physicochemical properties responsible for technological efficacy can prove hazardous with potential toxic effects. Enhanced reactivity due to high surface area may correspond to greater biological harm. Additionally, a nanoparticle may access biological targets not reachable by larger, similar chemical entities. While advantageous in a therapeutic context, such deep material permeation may be detrimental upon unintentional exposure.

1.2 Definitions

Within an interdisciplinary framework, precise definitions are essential to provide stakeholders from different backgrounds with common points of reference. However, the innovative nature of the field results in various terms such as engineered nanoparticles (ENPs) or engineered nanomaterials (ENMs) being used in a distinctly variable way. Nano-biomaterials (NBMs), meanwhile, exemplify the complexity of the field, with the potential to blur the distinction between material and biological entities. These muddy waters plainly merit some clearing:

‘Nano’ (from the Greek, nannos, meaning dwarf) refers to one billionth, or $10^{-9}$ of an entity.

‘Nanotechnology’ is ‘the design, characterization, production and application of structures, devices and systems by controlling shape and size at the nanoscale.’

‘Nanomaterial’, as specified by the European Commission (EC) Recommendation (2011/696/EU), signifies: ‘a natural, incidental or manufactured material containing particles in an unbound state, as an aggregate or an agglomerate and where, for 50 % or more of the
particles in the number size distribution, one or more external dimensions is in the size range 1-100 nm.’

Within this definition are highlighted three subcategories of nanomaterial. For the purposes of this thesis the category of most interest is manufactured, or engineered nanomaterials; those purposely designed to exploit phenomena that occur at the nanoscale.

‘Nanomedicine’ may be defined as ‘the use of nanomaterials for diagnosis, monitoring, control, prevention and treatment of diseases’.

A standardized definition of ‘nanobiomaterials’ is currently lacking, though may be concisely described as ‘nanostructured materials for biomedical applications’.

Regardless, a notion central to the concept of a nanobiomaterial is found within its name: that such a material is designed to interact with specific biological systems. Whether derived from nature or synthesised in a laboratory utilising a variety of chemical approaches, NBMs may be developed to befit a whole or part of a living structure or device with the intention of performing, enhancing or replacing a natural function. They may be non-interactive with the surrounding biological system, such as a heart valve, or they may have an interactive functionality such as impregnated stents that release pharmaceutical agents. Explicitly, NBMs may facilitate the efficacy of medical devices and medical products, including advanced therapy medicinal products (ATMP).

Based upon the EC definition, various regulatory bodies release their interpretations of the nanomaterial designation. However, the use of distinct definitions across jurisdictions may act as an obstacle to applying universal regulations to identical ENMs. In European circles, consensus on medical related terms have converged on ‘nanomedicines’, describing products regulated as medicinal products, ‘nanomedical devices’, describing products regulated as medical devices, and ‘nanotechnology-enabled health products’ as a broader term that encompasses both product classes.

1.3 Risk Assessment and Management of ENMs

Engineering in the nanoscale dimension induces fervent promises of revolutionary consequences among academic and industry figures, across fields from clean energy to electronics. Still, these expectations must be tempered by the safety challenges that such radical development brings.
The safety of materials or processes in general may be evaluated using the concept of risk, which itself may have a different definition depending on the discipline in which it is considered. In psychology, risk is viewed as a cognitive experience, whereas to medicine and applied science it represents objective reality\textsuperscript{v}. The basis of risk assessment may differ also depending on the focus of the investigation, be it human health or environmental impacts. Nevertheless, the definition of human health and environmental risk may acquiesce simply to be hazard combined with exposure. Hazard is the inherent capacity of an agent to cause harm, while exposure is the opportunity for hazard to be expressed. Essentially, risk assessment (RA) utilises scientific principles to estimate the probability that adverse effects on human health or the environment could transpire from exposure to a given material. The resultant risk characterisation allows policymakers and industry figures to integrate evidence-based scientific information with socioeconomic information to intelligently manage the material at hand.

The chemical risk assessment process begins with problem formulation, outlining the goals and scope of the RA, before methodically proceeding with hazard and exposure assessment, dose-response approximation, risk characterisation and a consideration of uncertainty for the final assessment\textsuperscript{vi}.

At an early stage, the course of a given risk assessment will diverge depending on if the assessment is human or ecologically based\textsuperscript{vii}. However, the concept of integrated risk assessment (IRA) is important, recognising that mechanisms of toxicity are often similar across species, despite varying observed endpoints, and integration may enable collaboration between human health and ecological risk assessors\textsuperscript{vii}. Furthermore, the same processes may cause exposure to workers and the environment, and if the same ‘determinants of exposure’ link human and environmental exposure, integrative thinking may help to mitigate ‘problem shifting’. Nonetheless, despite the incorporation of integrated testing strategies into EU-wide regulation with the implementation of REACH in 2007, the impact of IRA-development has been limited\textsuperscript{ix}. Thus, slightly different means of quantification generally pertain human risk assessment (HRA) and environmental risk assessment (ERA).

In the case of HRA, an evaluation may be reduced to the risk characterisation ratio (RCR):

\[
RCR = \frac{\text{exposure estimate}}{\text{DNEL}}
\]

whereby the derived no-effect level (DNEL) signifies the “level of exposure above which humans should not be exposed”\textsuperscript{xx}.

Regarding ERA, the risk may be defined as follows:
\[ \text{RCR} = \frac{\text{PEC}}{\text{PNEC}} \]

whereby the predicted no-effect concentration (PNEC) is the concentration of a substance below which adverse effects are unlikely to occur during long- or short-term exposure; and the predicted environmental concentration (PEC) is the concentration of a substance expected to occur in a given environmental compartment. A risk quotient at or above one indicates a risk of adverse effects on organisms, while a value below one indicates that the risks by convention are acceptably low.

Now, the applicability of the traditional chemical risk assessment framework to nanomaterials is subject to strong debate. Over 15 years ago, a Royal Society report on nanoscience envisaged the necessity of legislative adaptation on a precautionary basis, and to a certain extent, the advice was heeded - from the outset, the interdisciplinary nature of the field fostered a collaborative approach that has ensured its conscientious development. As such, research into the potential health risks of ENMs was carried out in parallel with early technological innovation. A large body of work has subsequently demonstrated that nano-sized materials do not seem to elicit novel effects or nano-specific toxicities. This is the basis of controversy regarding of the classification of NMs itself; below the 100 nm cut-off, no distinct change in hazard has been observed. Thus, the paradigm of RA of chemicals is generally considered applicable to that of ENMs, if it is sufficiently adapted to address added complexities concerning identity, biological and environmental comportment.

At present, however, deficits in the common understanding of nano-bio interactions, distribution and accumulation mechanisms lead to largely qualitative assessment of risk reliant predominantly on expert opinion. Furthermore, the rapid evolution of the nanotechnological field poses great challenges to regulators' abilities to amend legislation. Thus, huge resources have been invested in the development of tools, protocols and guidelines to enable the risk assessment and management of NMs, with efforts carried out by a range of stakeholders. A plethora of RA frameworks for nanomaterials have been developed, and whilst each framework will have its own scope and advantages, the principle challenge they face is shared. Namely, constant development of ENMs leads to an ever-increased variety in the structure, size, shape etc. A multitude of slightly different ENMs accumulates, for which it would be both economically and ethically unviable to fully evaluate every variation. Consequently, a trade-off emerges between thorough accounting of data and the framework efficiency; critical information is prioritised and different frameworks aim to evaluate the limited nano-database uniquely and efficiently. Hristozov and colleagues conducted a
comprehensive review of frameworks and tools for the RA of ENMs, concluding that none of the reviewed frameworks satisfied all their evaluation criteria.

An important concept to have emerged may be that of ‘Regulatory Preparedness’ (RP), whereby regulators’ role shifts from being reactive to proactive, engaging actively with industry and innovators, whilst industry retains the legal liability for their products’ safety. Such an open dialogue may foster knowledge-sharing on how ENMs influence exposure and effects, improving the translation of scientific innovation into concrete action.

The current EU regulatory framework of nanomaterials is comprised of numerous pieces of legislation, sector-specific or otherwise, as summarised in Table 1. The European Community Regulation on chemicals and their safe use (Registration, Evaluation, Authorization and Restriction of Chemicals (REACH)) is the most comprehensive legal provision for substances in the EU and applies to chemicals in whatever size, shape or physical state. In principle, nanomaterials and their conceivable risks are covered by existing legislation, be it explicitly or implicitly. Still, when REACH regulation was being constructed, the field of nanotechnology was in its infancy, so provisions specific to nanoforms were not addressed. The ECHA gradually increased its activities around ENMs from 2011, including the establishment of the Nanomaterials Expert Group (ECHA-NMEG) in 2012. Moreover, the ECHA contributes to ongoing international regulatory activities such as the OECD Working Party on Manufactured Nanomaterials (WPMN). It was recognised by the EC that ‘Regulation (EC) No 1907/2006 sets the best possible framework for the risk management of nanomaterials … but more specific requirements within the framework are necessary’. Recently, amendments to REACH regarding information requirements for nanomaterials were formally adopted, and will come into force from 2020.

Table 2 Exemplary fundamental legislation by the EC that may apply implicitly to ENMs

<table>
<thead>
<tr>
<th>Legislation</th>
<th>Conclusions on ENMs</th>
</tr>
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<tbody>
<tr>
<td>Regulation (EC) No 1907/2006 (REACH)</td>
<td>There is no explicit definition or reference to nanomaterials in the REACH documentation, which by default renders identification and characterization difficult. Comprehensive nano-specific guidelines have, however, been developed and published to assist in the fulfilment to REACH criteria, from in silico methods to exposure assessment.</td>
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</table>
Distinctions between ENMs and materials are reflected by updated guidelines for mutagenicity, acute toxicity and procedures for toxicological waiving. The minimal set of nano-specific physicochemical characterisations are outlined, as is the necessity to measure relevant endpoints in relevant environmental and biological media.

A limited number of nanomaterials have a specific hazard entry under the CLP Regulation. The generation of new information on environmental hazards of chemical substances is not compulsory, thus manufacturers would be unlikely to pursue such affairs.

Airborne nanomaterials come under the objectives concerning particulate matter (PM$_{10}$ and PM$_{2.5}$), though specific control measures are not described, attributable to the fact that safe levels of ENM exposure and biological mechanisms of action are until now uncertain.

### 1.3.1 Life Cycle Approach to risk assessment and management of ENMs

With the development of emerging technologies in the past, the negative effects and impacts tended to emerge several years after the point of introduction. Vitally, issues arising from the application of nanotechnologies should be treated in a holistic manner. As a result, all life cycle stages should be considered and adverse side effects should not be overlooked, but confronted as early as possible. Although knowledge accessible at early developmental stages is usually restricted, the capacity for shaping developmental paths is significant. The term ‘life cycle’ is widely understood as the entire lifespan of a material or product, from the manufacturing stage through the use/application and ultimate end-of-life disposal.

ENM releases may occur at any stage of the life cycle; thus, a complete appreciation of the release potential is fundamental to ensure sustainable development of the technologies. A conceptual approach that has developed in recent years is *release as a prerequisite of exposure to ENMs*. Summarised as the so-called Framework of Release, the concept describes how a possible risk is only present if exposure is possible. Following release, the material emission and transport may lead to exposure of workers, consumers or the environment.
Since it became recognised that risk-relevant physicochemical (PC) properties of ENMs could change during the life cycle, the concept of life cycle thinking has become an integral part of RA framework development. Such an approach is crucial to achieve the reversal of the burden of proof from regulators to industry, a key objective of REACH regulation, and in enacting the polluter pays principle. However, as in the wider RA context, the adoption of nanotechnology into LCA frameworks is not a simple process, as summarised in Table 1.

**Table 1 The application of lifecycle assessment (LCA) to ENMs, from Nowack et al.**

<table>
<thead>
<tr>
<th>Aspect of Lifecycle Assessment</th>
<th>Present Challenge</th>
<th>Feasible Solution</th>
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<tbody>
<tr>
<td>Life cycle inventory</td>
<td>Lack of representative data, with high uncertainties in model outputs</td>
<td>Research should focus on use and end-of-life phases to fill knowledge gaps</td>
</tr>
<tr>
<td>Exposure modelling</td>
<td>Multimedia fate models classically utilise partition coefficients, which may not be appropriate for ENMs; modelling of ENM exposure may be restricted to dissolution, producing poor estimations of bioavailability; analytical measurements struggle to distinguish between ENM-derived nanoparticles and those natural occurring.</td>
<td>Models must account for the complex transformation processes of ENMs, including aggregation, agglomeration and dissolution</td>
</tr>
<tr>
<td>Effect modelling</td>
<td>There is a paucity of toxicological data across trophic levels for ENMs; toxicity testing is often undertaken with pristine material, which disregards transformations.</td>
<td>Toxicity values should only be extrapolated from transparent studies that report PC properties along with experimental conditions.</td>
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</table>

Across the life cycle, the assessment of the potential for human and environmental exposure is concomitant with the release potential of ENMs, as well as the different forms they may occur, be it as free ENMs, in an aggregated form or integrated within composites. There are two distinct, though related, exposure areas that require close investigation: occupational/consumer exposure and environmental exposure. In the occupational and consumer cases, exposure may be facilitated by the handling of industrial products and the use of consumer products, respectively. Still, eventually these ENMs are destined to enter the
environment, and so the occupational and consumer exposure assessment forms the basis for environmental assessment\textsuperscript{\textit{xiii}}.

When considering the different regulatory areas targeting ENMs, it is clear from the literature that information and measurement data for ENMs is most complete for worker exposure. Much less is known about consumer exposure, and the exposure of biota through the environment is the most challenging section\textsuperscript{\textit{xlv}}. The regulatory measures outlined below represent the standardised approaches by which the risk conveyed by a given material at a given life cycle stage should be assessed and managed.

1.3.2 Risk assessment and management of nanotechnology-enabled health products

Although the themes discussed do apply to nanobiomaterials for medical applications, there are aspects of the framing of NBM risk that are distinct.

Firstly, the desired impact of NBM in medicine is evidently to interact with the human biological system, with complex consequences just beginning to be revealed. Monopoli and colleagues have shown that nanoparticles adsorb many biomolecules (mainly proteins) upon exposure to biological milieu, resulting in the formation of a new interface termed the “protein corona”. Evidence has also been amassed to show that the protein corona regulates nanoparticle cell recognition, and hence plays important roles in modulating nanoparticle mobility and toxicity\textsuperscript{\textit{xlvi}}. Furthermore, given that 40% of nanomedicines submitted for approval between 2010 and 2015 were for cancer treatment\textsuperscript{\textit{xlvii}}, the positive functionality is a form of targeted toxicity. Indeed, the evaluation of Ag nanoparticles for antibacterial properties involved assessing candidate drugs for selective toxicity, i.e. antibacterial at specified concentration yet non-toxic to human, determined by the surface functionalisation and exposure time\textsuperscript{\textit{xlviii}}.

Secondly, although the patient efficacy and safety of NBMs in medical products are addressed by the appropriate agency, e.g. the European Medicines Agency (EMA), the potential environmental risks post-use are rarely discussed. This could be due to the high potential benefits from NBMs deemed to outweigh concerns of non-human stakeholders. This is despite the certitude that pharmaceutical products are detectable in surface waters across continents, and chronic low-level exposure may lead to adverse effects in non-target species\textsuperscript{\textit{xlix}}.

Thirdly, given the data gaps and uncertainties surrounding NBMs, the evaluation of risk is largely determined by expert judgment. Still, a variety of viewpoints are posed by different experts. Human health risk may be high considering that intravenous administration of NBMs may cause unexpected effects related to bioaccumulation\textsuperscript{i}. On the other hand, experts showed
that the risks of ENMs in medicine were perceived as low in comparison to risks presented by ENMs in other areas, such as cosmetics and pesticides. As such, Mahapatra and colleagues concluded that regarding the handling of uncertainties, ERA of nanomedicine is flawed to the point of irrationality, and an alternative approach to NBM risk governance is necessary.

1.4 Regulatory Overview of nanotechnology-enabled health products

A risk assessment of products of nanotechnologies undertaken by the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) laid the foundations for the evolution of European nanomaterial legislation. It was asserted that health and environmental hazards have been demonstrated across a range of manufactured nanomaterials, which indicated potential toxic consequences. According to Regulation (EU) 2018/1881, nanoforms ‘may have specific toxicological profiles and exposure patterns and may therefore require specific risk assessment and adequate sets of risk management measures’.

Regarding biomedical applications, the evaluation of medicines has been overseen by the European Medical Agency (EMA) since its inception in 1993. In general, products making medical claims will be regulated either by medical device legislation or under medical product regulation. The primary mechanism of action is key to define which category is applicable to a given material (Directive 2001/83/EC, Article 1.2(b)), although functionalized nanosystems may blur the borders between the two categories. Indeed, the current legislation remains stratified, plagued by a technological kaleidoscope that creates confusion between manufacturers and consumers. Though both regulated under European Union (EU) legislation, the regulatory processes for the two medical fields evolved separately. It has been argued that features of the pharmaceutical process should have applied to medical devices, and following recent controversies over Poly Implant Prothesese (PIP) breast implants and metal-on-metal hip prostheses, concerns over medical device regulation came under particular scrutiny. These separate incidents acted as catalysts for regulatory reform, resulting in a more harmonised overall paradigm established in the newly adopted Regulations. EU legislation regarding medical devices (Regulation (EU) 2017/745) was updated in 2017, and will come into force after a transition period of 3 years.

Life cycle concepts are ever more recognised as being integral to effective legislation. As previously discussed, within each stage of the life cycle, different populations of human or non-human organisms will be at risk, and a given nanomaterial may be found in various transformed states. Regulation (EU) 2017/745 states risk management as a ‘continuous iterative process throughout the lifecycle of a device’, while risk assessment outlined in Regulation (EU) 2018/1881 ‘shall consider all stages of the life-cycle of the substance resulting from the manufacture and identified uses’.
1.4.1 Biomedical use phase

As alluded to, NBMs in the biomedical field may be utilised in different technological structures for different uses, and the legal differences incur different regulatory pathways that may in turn call for different approaches to risk assessment. Still, the incorporation of nanomaterials into EU medical regulation has, comparable to REACH, proceeded slowly and is still incomplete. Furthermore, in addition to the categorical definitions outlined in Table 3, there are ‘borderline products’ whereby a medical device incorporates or administers a medicinal product.

<table>
<thead>
<tr>
<th>Biomedical category</th>
<th>Definition</th>
<th>Use</th>
<th>Legislation</th>
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<tr>
<td>Medicinal product</td>
<td>Any substance or combination of substances presented as having properties for the treatment or prevention of disease in human beings. The definition also includes any substance that aims to restore, correct or modify physiological functions by exerting a pharmacological, immunological or metabolic action, or to make a medical diagnosis</td>
<td>Diagnostics, drug delivery and regenerative medicine</td>
<td>Directive 2001/83/EC, Regulation (EC) No 726/2004</td>
</tr>
<tr>
<td>Advanced therapy</td>
<td>Gene therapy medicinal products (GTMPs) are products of biological origin containing recombinant nucleic acids, aiming to deliver genetic material to the target area. Somatic</td>
<td>Oncological cell therapy, gene therapy for protein</td>
<td>Regulation (EC) No 1394/2007/EC, 2009/120/EC</td>
</tr>
</tbody>
</table>
cell therapy medicinal products (SMCTs) encompass several types of cell therapies that display high heterogeneity owing to different origin, cell type, development stage and differentiation. A tissue engineered product (TEP) is made up of engineered cells or tissues administered with a view to regenerate, repair or replace human tissue. It may consist of tissues of human or animal origin, or both, which may be viable or non-viable.

Medical device

Any instrument, apparatus, appliance, software, material or other article intended to be used on humans for the diagnosis, prevention, monitoring, treatment or conception control by mechanical or physical means.


Although there are vast differences between the different categories of medical products, their regulatory route to market is comparable. With the intention of safeguarding public health, the market authorisation (MA), classification and labelling of medicines has been regulated in the EU since 1965. Authorisation of medicines is based upon three key criteria: quality, efficacy and safety, to ensure that products distributed across the EU provide a positive benefit-risk balance.

For new medicines, developers must demonstrate the safety and efficacy of their products by conducting clinical trials. They represent an essential component of evidence based medical research, to prove and identify benefits and risks in humans, and to compare whether a new treatment is better than existing alternatives. In this context, a better treatment does not necessarily mean greater efficacy; it may signify fewer adverse drug reactions (ADR).

Once medicines are placed on the market, they are monitored throughout their lifespan by the EMA Pharmacovigilance Risk Assessment Committee (PRAC). Pharmacovigilance is supported by Eudravigilance, a web-based information system that manages suspected side effects of medicines, and public hearings during safety reviews. Ultimately, safety monitoring ensures that the benefits of medicines outweigh their risks.

1.4.1.1 Biomedical product exposure
In accordance with Annex 8 of Regulation (EU) 2017/745, medical devices are subdivided into classes based upon how the product function may play a critical role in human health and safety. Depending on the medical device class, the requirements for receiving a CE mark varies from a simple self-certification for class I to a complete risk assessment of the product and manufacturing process by the Notified Body for a class III product. The medical device class is assigned based upon the intended purpose of the devices and their inherent risks. At present, medical devices incorporating or consisting of nanomaterials are classified simply according to Rule 19 of Annex VIII within Regulation (EU) 2017/745. All devices are classified as: class III if they present a high or medium potential for internal exposure; class IIb if they present a low potential for internal exposure; and class IIa if they present a negligible potential for internal exposure\textsuperscript{lxii}. The challenge is thus to arrive at an accurate assessment of internal exposure of medical devices. The expert advice most relevant to consider comes from the SCENIHR, in the form of the 2015 ‘Guidance on The Determination of Potential Health Effects of Nanomaterials Used in Medical Devices’\textsuperscript{lxiii}.

Comprehensibly, with a variety of uses of nanomaterials in medical devices, different categories may be recognized, such as non-invasive, invasive and implantable devices. Furthermore, the location of contact and contact time should be reviewed in order to formulate the appropriate biological testing regime (e.g. genotoxicity, cytotoxicity)\textsuperscript{lxiv}.

In all cases, the potential routes of exposure ought to be deliberated. In a nutshell, the risk of medical devices containing nanomaterials is mainly associated with the possibility of the release of free nanomaterials from the device, and their subsequent toxic effects. Still, the toxic effects of fixed nanomaterials should also be incorporated, as their unassuming chemical composition may influence reactivity. The factors affecting potential ENM release during use are manifold, including the given ENM, the product lifetime, the actual usage of the product\textsuperscript{lxv}. Experimental data on ENM release may still be scarce, though reported to be influenced by degradation by UV-light\textsuperscript{lxvi} and physical abrasion\textsuperscript{lxvii}. Free nanoparticles may reduce cellular viability, induce DNA damage and lead to both local and systemic effects\textsuperscript{lxviii}. This is an issue also discussed in the recent harmonized European standard ISO 10993-22 ‘Biological evaluation of medical devices Part 22: Guidance on nanomaterials’.

A phased approach is recommended by SCENIHR to evaluate the risk of nanomaterial use in medical devices, based on potential release and NM characteristics. The exposure is the outcome of potential release from the MD in actual conditions (exposure scenario) and the toxico-kinetics of the nanomaterial (internal exposure scenario). This is cross-referenced with outcomes of ISO safety testing. Table 4 illustrates the qualitative SCENIHR framework that determines the detail of risk assessment required according to the characteristics of a given medical device. By considering the expected release of nanoparticles in combination with the
invasive nature of the device subcategorised by the expected exposure duration, the level of risk assessment deemed necessary is computed.

**Table 4** Framework for risk assessment of nanomaterials in medical devices, (From SCENIHR 2015). \( F = \) full assessment, \( L = \) limited assessment, \( VL = \) very limited assessment, \( N = \) no further assessment

<table>
<thead>
<tr>
<th>Release of nanoparticles</th>
<th>Non-invasive</th>
<th>Invasive lung</th>
<th>Invasive other</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Short exposure</td>
<td>Long exposure</td>
<td>Short exposure</td>
</tr>
<tr>
<td>Low</td>
<td>N/VL</td>
<td>L/F</td>
<td>L</td>
</tr>
<tr>
<td>Medium</td>
<td>L/F</td>
<td>L/F</td>
<td>L/F</td>
</tr>
<tr>
<td>High</td>
<td>L/F</td>
<td>L/F</td>
<td>F</td>
</tr>
</tbody>
</table>

The phased approach itself entails an exposure assessment, which utilizes the likelihood of nanoparticle release to estimate potential exposure, considering the potential for particle distribution, and particle persistence. A hazard assessment is utilised to define the appropriate testing strategy, among relevant endpoints include irritation, immunotoxicity, cytotoxicity, genotoxicity. Systemic effects should be assessed on a case-by-case basis, though particular consideration should be given to the ability of particles to concentrate in draining lymph nodes and other organs within the mononuclear phagocyte system. Phase IV is the risk characterization, whereby the estimated risk is compared to the risk of comparable device that do not incorporate nanomaterials.

Methods exist to reduce the adverse effects such as nanoparticle surface optimization, adjusted binding of the nanoparticles within the ENM and safety by design (SbD) approaches. In the case of NBMs, the products may be assumed to be administered in a controlled fashion and thus the initial dose should be calculated. However, the active dose, that which reaches the target tissue/organ, is considerably more difficult to compute, and a rather complex issue. The action of medical devices upon untargeted tissues/organs is undesirable given the uncertainty of effect, and detailed information on the adsorption, distribution, metabolism and excretion (ADME) is required by Regulation (EU) 2017/745. Biocompatible polymers, meanwhile, are utilised to encapsulate or coat nanomedicines (e.g. polyethylene glycol, PEG), inferring stealth properties that allow the nanoform to circulate until reaching the target ix.

### 1.4.2 Occupational exposure

Although NBMs in the medical sector may provide great benefits to patients, they may represent new risks for workers. Indeed, it is suggested that occupational settings are where
the risks of hazardous materials are initially recognised, as workers may be the first to face exposure to novel materials for long intervals of time.

General EU regulation on worker protection does apply to nanomaterials, as summarised in Table 5, though nanomaterials are not explicitly referred to. The EC published a guidance on the protection of workers’ health and safety from the potential risks of nanomaterials, in which it claims that inhalation exposure represents the area of greatest concern in the occupational context, and special consideration is given to impacts on the respiratory and cardiovascular system. However, in a more recent review, Basinas and colleagues showed that the inhalation, dermal and ingestion routes of exposure may all be relevant.

**Table 5 Exemplary legislation in the occupational sector that may apply implicitly to ENMs**

<table>
<thead>
<tr>
<th>Legislation</th>
<th>Relevance to ENMs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical Agent Directive 98/24/EC</td>
<td>Employers must measure workers’ chemical agent exposure, unless it may be demonstrated that adequate protection is in place.</td>
</tr>
<tr>
<td>Carcinogen and Mutagen Directive (EU) 2017/2398</td>
<td>Employers must take measures to limit or eliminate the utilization of carcinogenic or mutagenic materials.</td>
</tr>
</tbody>
</table>

In international surveys undertaken to evaluate industry practices with relation to nanomaterials, it was reported that most companies dealing with nanomaterials applied safety practices based on conventional chemicals, with no adaptation for the distinct hazard potential of ENMs. Given the general uncertainties regarding the potential impact of ENMs to human health, it would be astute to take a more precautionary approach, which would also enable the fulfilment of the legislative requirements above.

The potential risk to healthcare workers through administration and involuntary contact with nanomedical products is largely unknown, and the workers may be unaware of the potential toxicity. The highest risk of exposure applies to those healthcare workers who prepare and administer the products.
Similarly, workers at the manufacturing stage may also be exposed to significant levels of ENMs, though through different exposure routes. It is largely recognised that the respiratory system is the prevalent route of exposure for ENMs, whereby the particle size determines the deposition, accumulation and systemic distribution in the body. Still, most of the research up to now has been focussed on determining airborne ENM concentrations, neglecting the potential for other exposure routes. An accurate identification of the probable exposure routes is necessary, as it informs the exposure assessment and subsequent risk management measures (RMM).

In occupational settings, ENM exposure may be quantified using a hand-held device, e.g. an optical particle counter (OPC), providing direct information on the number of particles. Several measurement and modelling techniques recommended for an occupational exposure assessment of ENMs are currently available, from the EC, ISO and the OECD; somewhat summarised in Table 6.

<table>
<thead>
<tr>
<th>Legislation</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISO TR 12885: 2008</td>
<td>Health and safety practices in occupational settings relevant to nanotechnologies</td>
</tr>
<tr>
<td>ISO 28439:2011</td>
<td>Determination of the number concentration and size distribution of ultrafine aerosols and nanoaerosols by use of mobility particle sizers</td>
</tr>
<tr>
<td>ENV/JM/MONO(2015)19</td>
<td>Harmonised tiered approach to measure and assess the potential exposure to airborne emissions of engineered nano-objects and their agglomerates and aggregates at workplaces</td>
</tr>
<tr>
<td>ISO/TR 18637:2016</td>
<td>Overview of available frameworks for the development of occupational exposure limits and bands for nano-objects and their aggregates and agglomerates (NOAAs)</td>
</tr>
<tr>
<td>ENV/JM/MONO(2017)30</td>
<td>Strategies, techniques and sampling protocols for determining the concentrations of manufactured nanomaterials in air at the workplace</td>
</tr>
</tbody>
</table>
A major challenge still facing exposure assessment in the regulatory framework is the differentiation between naturally occurring nanoscale particles and ENMs. Monitors detecting solely mass concentrations may not discriminate between particle types, thus neither possible sources. One solution may develop from aerosol mass spectrometer technologies, able to detect higher elements and metals. Furthermore, there is not a legally binding regulation defining how and with which instruments exposure measurements of ENMs should be conducted. Consequently, data quality and data interpretation are often variable and unsuitable to compare to each other. Nonetheless, measurement strategies have been shown to fulfil the requirements of reliability and relevance for human exposure assessment.

Currently, there are no internationally recognised standards regarding occupational exposure limits (OELs) of ENMs in the workplace, though unofficial values are proposed by different institutions. The British Standard Institute (BSI) suggest exposure limit values for various nanomaterial types, defined by mass. For instance, the exposure limit for nanomaterials based on carcinogenic substances is suggested to be 10 times inferior than for the micro-form. However, as will be explored in the successive chapters, the toxicity of nanomaterials is poorly described by mass; instead parameters such as shape, size and surface charge are superior determinants of toxicity.

An alternative means of exposure assessment is to utilise the concept of a biologically effective dose (BED). In particle toxicology, the BED is defined as “the entity within any dose of particles in tissue that drives a critical pathophysiologically relevant form of toxicity (e.g., oxidative stress, inflammation, genotoxicity, or proliferation) or a process that leads to it.” Fundamental to the BM concept are biomarkers: measureable parameters that divulge designated events in a biological system. Relevant exposure biomarkers include pulmonary cytokines, which may be detected as exhaled particles or elemental analysis in biological fluids. Still, these developments are in their early stages and a greater knowledge of ADME processes for ENM are required to identify nano-specific biomarkers.

Meticulously analysing the prospect of exposure measurements, strategies and assessments it becomes evident that a comprehensive occupational exposure assessment is not possible. Therefore, the European Agency for Safety and Health at Work advises a hierarchy of preventative measures to mitigate the exposure of workers to ENMs, known as STOP, outlined in CAD. The initial option should be to substitute the given nanomaterial for a less hazardous material. Of course, NBMs are utilised for the advantages brought by properties that emerge at the nanoscale. Where substitution is not possible, a NBM may be supplied as an aqueous dispersion, thus decreasing the dustiness and limiting inhalation exposure. Next in priority are technological measures, such as local ventilation, followed by organisational measures, such as minimising the number and/or duration of workers being
exposed. The final option is to use personal protective equipment (PPE) such as respirators and dermal protection.

1.4.3 Environmental exposure

The manufacture, utilisation and release of NBMs may lead to environmental exposure. Globally, environmental release of ENMs was estimated to exceed 340,000 tonnes by 2016, with most emissions ending up in landfills, followed by releases into soils, water bodies and the atmosphere. The nanomedical sector will, as it expands, contribute an ever-greater proportion of this total. In order to evaluate the environmental risk of NBMs, it is essential to know not only the potential hazard of the materials, but also the point of release in the material life cycle, their fate and behaviour in the environment, and consequent exposure. One must also appreciate the physicochemical properties of the material as well as possible transformations once in contact with environmental media. Indeed, the significance of transformations has been better understood only in the past decade. Transformations can reduce toxicity, but not in every case.

At the synthesis and manufacturing stage, the predominant channel for environmental exposure of ENMs is through the waste stream, with the possibility of direct air emissions. At the product use stage, ENMs may enter by means of wastewater, while the exposure pathway from disposal depends on the method of disposal. The level of release will depend on the potential mobility of ENM, be it incorporated within a solid matrix or liquid/gaseous form.

Nano-biomaterials used in medical applications will have multiple points of environmental entry, which may be intentional or unintentional releases, manufacturing emissions and the weathering of nano-enabled medical products during use.

The main exposure pathway for NBMs in therapeutic products is expected to be during patient use, through renal excretion into sewage and waste water treatment plants (WWTPs). Such WWTPs provide entry points for ENMs into freshwater and soil. Models of water treatment plants show that 90% of ENMs are taken up in sewage sludge, with under 10% reaching surface water bodies, making exposure of farmland likely, if agricultural utilisation of sludge is assumed. Considering this pathway, it is pertinent to deliberate biodistribution and biotransformation processes due to enzymatic action. Biodistribution studies have demonstrated that the clearance pathway of gold nanoparticles may depend on surface coating as well as size, and that the volume of an unaltered drug excreted may increase if the administered drug is encapsulated in a nanocarrier.

In the case of medical devices, quantitative information is limited, but improper disposal is estimated to provide the predominant pathway of exposure, with the proliferation of single-use, disposable diagnostic devices.
Another significant environmental exposure pathway occurs at the end-of-life stage, whereby NBMs may be disposed of in landfills or combustion at waste incineration facilities. In the case of combustion, the concern over airborne release of ENMs may be mostly disregarded, as technology applied to incineration facilities may effectively remove ENMs from flue gas. However, the little experimental evidence suggests that ENMs may increase the production of other pollutants, with waste containing ENMs generating 6 times the amount of PAH (polycyclic aromatic hydrocarbons) compared to waste containing their bulk equivalents. Moreover, the solid residues to which the nanomaterials bind persist are likely to end up with landfill. The possible release of ENMs from landfill is a poorly investigated field. Indeed, it is suggested that landfill sites are assigned as the final sinks in material flow models merely because the information on behaviour of ENMs is so scarce.

Unsurprisingly, the total containment of ENMs throughout the life cycle has been reported as improbable, given the generally low recyclability of products containing ENMs combined with tendencies in a globalized system for waste streams to terminate in developing countries with insufficient infrastructure to manage ENMs.

The significance of these possible NBM exposures depends upon various environmental fate process, including aggregation, dissolution and adsorption. Transformation products may be expected to emerge as a function of both the physicochemical properties of the NBM

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**Figure 2** A conceptual scheme showing probable sources, transport pathways and sinks of NBMs for medical applications (Mahaptra 2013)
as well as the environmental matrix. In turn, such transformations determine bioavailability, uptake, toxicity and overall environmental risk.

A common feature of nanomedical products is the application of polymeric coatings, used to increase stability in biological solutions. This indicates that they may persist for extended periods in the environment, migrating into the ecosphere and eventually into the food chain. Current evidence suggests the potential of ENMs to accumulate, although at low levels, in organisms such as earthworms. Furthermore, nano metal oxides have been demonstrated to be highly toxic to freshwater organisms, particularly crustaceans. Freshwater conditions may stabilise dispersions of nano metal oxides, increasing the bioavailability for fish and filter feeders. Moreover, cerium oxide nanoparticles, considered a promising nanomedicine for cancer, have been shown to adversely affect soya bean plants. Evidence shows that CeO₂ ENPs avoid biotransformation in the plant, supporting theories that nanomaterials may readily move across trophic levels. The interaction of nanomedicines with such a major food source must raise concerns of the benefit/risk ratio.

The evidence base of ecotoxicity studies dedicated to the effect of ENMs upon aquatic organisms has grown in recent years. Initially, studies have been 'proof-of-principle' experiments pertaining the concentrations of ENMs required to produce toxic effects. Effect concentrations reported are often high, in the range of mg L⁻¹, and calculated irrespective of bioavailability. A lack of sufficient monitoring technology and an absence of standardised methods renders conclusions of ecological implications difficult, and highlights the need of ecological perspective in nanotoxicology. Overall, a precautionary approach should be taken, based upon the more established evidence base of pharmaceutical products. Changes in behaviour patterns of marine amphipods were demonstrated to upon exposure to the selective serotonin reuptake inhibitor fluoxetine at just 10 ng L⁻¹.

As alluded to previously, given the problems encountered in clearly identifying specific ENMs in environmental matrices, there is effectively no exposure data available. Although such capabilities are expected to emerge, environmental models capable of predicting release and transformation of ENMs are crucial in the meantime. Nowack and colleagues pioneer the use of probabilistic material flow analysis (pMFA). To derive risk levels for the ecosystem, the approach is based upon probabilistic species sensitivity distribution (pSSD) for quantifying ecotoxicological risks and the calculation of risk quotient from the modelled PEC. A recent analysis of ten ENMs relevant to the Danish market found none of the selected ENMs constituted a general environmental risk.

1.4.3.1 Regulation of Environmental Exposure
Until now, with few exceptions, there are no specific provisions for nanomaterials within European environmental legislation (Table 5).

**Table 5 Exemplary EC, ISO and OECD environmental legislation that may apply implicitly to NBMs**

<table>
<thead>
<tr>
<th>EU Legislation</th>
<th>Conclusions on NBMs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Waste Framework Directive 2008/98/EC</strong></td>
<td>The categorisation of hazard waste relies upon the (inadequate) CLP regulation; no requirements regarding nanowaste are included; the state of the art waste treatment technologies remain inadequate to capture ENMs</td>
</tr>
<tr>
<td><strong>The Environmental Quality Standards Directive 2013/39/EC</strong></td>
<td>A 'watch list' of substances that may pose a significant risk, taking into account information including, where relevant, particle size, leads to the possible inclusion of nanomaterials in the list of priority substances, which would have a ripple effect on other water-related pieces of legislation.</td>
</tr>
<tr>
<td>Directive 2008/56/EC establishing a framework for community action in the field of marine environmental policy</td>
<td>All the limitations previously mentioned in relation to the lack of ecotoxicological data and difficulties with monitoring of nanomaterials in water are valid for the marine environment as well.</td>
</tr>
<tr>
<td>Regulation (EU) No 528/2012 concerning the making available on the market and use of biocidal products (BPR)</td>
<td>The BPR represents the most advanced EU legislation concerning nanomaterials, incorporating definitions and acknowledging that the nanoforms of active substances may exhibit different properties. Still, the implementation is hindered by lack of adequate methods to test the ecotoxicology, fate and behaviour of nanomaterials.</td>
</tr>
</tbody>
</table>

The regulatory framework for human medicines regarding environmental safeguard is also poorly defined, particularly for nano-enabled products. Conceivably, the perceived benefits of NBMs renders environmental concerns a low priority. Nonetheless, legislation is subject to constant evolution, as necessitated by the incremental approach adopted by the European Commission, directed to adapt existing laws in order to include nanomaterials.\textsuperscript{xlv}

In the case of medicinal products, the EMA demands an ERA within the marketing authorisation procedure, outlined in guidelines as a phased assessment approach.\textsuperscript{xlvii} In the
initial screening phase, the PEC of the active pharmaceutical ingredient (API) is calculated, as is the octanol-water partition coefficient. If threshold values are exceeded, phase II procedures are triggered, which incorporate a suite of acute ecotoxicity tests and persistence, bioaccumulation, toxicity (PBT) assessment, respectively.

Within medical device legislation, an ERA is not required. Nor is there reference to good practice regarding the device life-cycle or disposal methods. Thus, environmental risk assessment of medical devices may be legislated by REACH alone.

However, before considering the environmental safeguards of nanomaterials within REACH regulation, it is pertinent to note that the definition of a nanomaterial as adopted by the Commission may not be appropriate to deal with emerging nanomedical products. Crucially, products under development may in one or more dimension exceed the limit of 100 nm that defines a nanomaterial, yet still express the properties typical of nanomaterials that merit exceptional consideration. Secondly, REACH does not apply to materials manufactured in quantities of below 1 tonne/year, which may exclude a significant proportion of nanomaterials from being subject to the regulation.

The recently published REACH Regulation (EC) No 2018/1881 offers some guidance and may contribute to a more refined pathway to meeting the requirements of the other legislation. Minimum characterization information is outlined as necessary, whilst recognizing that relevant parameters depend on the individual case. Substances acknowledged as Persistent, Bioaccumulative and Toxic (PBT) or very Persistent and very Bioaccumulative (vPvB) are subject to an emission characterisation corresponding to all life-cycle stages.

Though welcome, the amendments do not provide a complete framework for the risk assessment of nanomaterials, and some test guidelines to fulfil the requirements may currently not be available. This is recognised, as is the necessity to ‘further develop guidance documents for the application of the test methods and waiving possibilities’.
2 Case Study: nano-TiO$_2$ in biomedical applications

Nanomaterials currently find purpose in the medical field by varied approaches: optimisation of existing drugs by nanoformulation; targeted drug delivery by encapsulation in vehicles such as nanoshells and magnetic nanoparticles; in vitro diagnostics that enhance analysis; regenerative medicine. As discussed, potential applications are effectively boundless, as the intrinsic size and surface area of ENMs allow such systems to access local targets with high specificity. However, technologies currently subject to research may take two decades before being available clinically, due to the rigorous testing regime required. Thus far, the most common application of nanomedicines is for cancer treatment$^{cix}$.

A positive biological outcome that predicates eventual product approval is contingent not only upon meticulous nanosystem design, but also on thorough knowledge of how nanomaterials and biological systems interact, for two principal reasons. Firstly, the physiopathological processes behind disease take place at the nanoscale, so a greater understanding of cellular processes informs the design of ENMs towards a given objective. Secondly, the interaction between the nanomaterial surface and biological medium, which is governed by a fluctuating layer of proteins and biomolecules that adsorb by a tendency to lower the free surface energy, is that which determines the cellular fate of nanomaterials$^{cii}$.

With advances in nanoscience, an important class of materials to emerge is that of composites that integrate inorganic nanoparticles with biologically active moieties, forming nanobiomaterials (NBMs). Such bioinorganic composites exhibit multifunctional properties with capacity to integrate with both inorganic supports via covalent bonding and biological entities via site-specific interactions with cell constituents.

Among the many uses of nano-TiO$_2$, biomedical applications have motivated strong interest owing to unique properties incorporating superb biocompatibility, high chemical stability and photocatalytic behaviour$^{ciii}$. These characteristics furnish potential for biomedical applications including drug delivery, bioimaging, photodynamic therapy for cancer treatment and biosensors. Utilising site-selective redox chemistry, a range of biological processes may be manipulated including cellular respiration and signalling, thus forming the basis for novel biotechnological tools controlled by redox processes$^{civ}$.

Since early times TiO$_2$ has been utilised as a white pigment$^{ciii}$, so that through the ages its low toxicity towards human and environmental ecosystems has been recognised. Yet, despite extensive studies of TiO$_2$ across various disciplines, the use of TiO$_2$ in medical applications is
relatively recent. The first examples emerged in the 1990s, before gaining momentum after evidence appeared of photoinduced cell death, after which point the publication count began to exponentially grow.

2.1 The relevant properties of TiO₂

Titanium dioxide occurs predominantly as three crystal structural forms: anatase, rutile and brookite. With distinctive crystalline organisation, different properties and photoactivities are observed due to diverse band structures; hence prospective biomedical applications may be determined by the crystalline form. In the nanocrystalline form, anatase plays the most important role in environmental applications and holds most promise for light-induced biomedical purposes. Anatase has a distorted octahedral coordination (O₆) with every titanium atom surrounded by six oxygen atoms in an elongated octahedral structure. Since first reportage of photocatalytic water splitting by a TiO₂ electrode upon UV light exposure, photoelectrochemical properties of TiO₂ systems have been widely probed.

TiO₂ is categorised as a semiconductor owing to the intermediate energy gap (E₉) between the highest occupied molecular orbital, the valence band (VB) and the lowest unoccupied molecular orbital, the conduction band (CB).

In the case of anatase, the large band gap of 3.2 eV corresponds to photons of wavelength 388 nm. Upon irradiation of light with a wavelength shorter than that of the band gap, electrons are promoted from the VB into the CB. This process leads to the generation of a positive ‘hole’ (h⁺) in the valence band and an electron (e⁻) in the conduction band. The photogenerated species may subsequently undergo recombination, or be translocated to a nanoparticle surface to influence aqueous redox processes. Positive holes may react with surface-adsorbed H₂O to produce hydroxyl radicals (OH⁻), whilst photogenerated electrons
are scavenged by molecular O$_2$ to generate superoxide radical anions (O$_2^{-}$). In solution, these radical species may react to form other reactive oxygen species (ROS) including peroxy radicals and hydrogen peroxide. With a multitude of possible ROS, the decomposition of cellular species may be expected both near the location of radical formation and at distance.

The ability to control the shape and size of TiO$_2$ results in systematic influence over particle crystallinity, as well as regulating redox potential and the exposure of crystalline planes that govern the adsorption and interaction of diverse molecular species. Anisotropic species relevant to biomedical applications include rods, bricks and tubes; each form may exhibit different surface areas, aspect ratios and surface atom organisation. For instance the high curvature of spherical and rod-like nanomaterials may distort significantly the Ti-O bond lengths, leading to distortion in the Ti symmetry from octahedral to square pyramidal. As such, nanoparticles with high curvature display better properties to accept selective binding of ligands that complete the coordination sphere.

2.2 Overview of medical applications of nano-TiO$_2$

2.2.1 Targeted drug delivery

The ideal medicine should maximise therapeutic action whilst minimising adverse side effects, with precise drug release at the right place and dose. Such concepts originate from Paul Ehrlich’s hypothetical ‘magic bullet’, which he later developed to share the Nobel Prize in 1908. Nanomaterials are auspicious tools for improved therapy, as manipulation at the nanoscale may improve drug delivery through the prospect of surface functionalisation leading to active targeting, with circumvention of obstacles such as the blood-brain barrier (BBB).

Targeted drug delivery systems consist of a device that transports the active ingredient. A great catalogue of nanomaterial forms has accumulated in recent years, consisting of various nanovehicles including dendrimers, nanoshells and polymer nanocomposites, which themselves may be fabricated into diverse shapes, from whiskers to capsules. In this way, the drug may be conjugated onto the nanosurface or contained in an internal reservoir. A prevalent methodology is to enclose hydrophilic drug products within the interior of amphiphilic hollow structures such as nanotubes.

Mesoporous materials are worthy contenders to achieve controlled drug release kinetics with site-selectivity. TiO$_2$ ENMs are particularly valuable due to their undercoordinated surface sites that enable surface functionalisation, as well as the facile control of chemical and electronic properties.

It is apparent that the system of drug-nanomaterial integration is an important determinant of its efficiency. Non-covalent complexation, for instance, may be expected to have different
consequences than covalent conjugation. TiO$_2$ NPs loaded with doxorubicin (DOX) by electrostatic complexation exhibited greater cytotoxicity, and were located in the nucleus, whilst the equivalent system covalently conjugated exhibited lower cytotoxicity, being located predominantly in the cytoplasm. Another method employs relatively weak physisorption of the oxygen-rich daunorubicin (DNR) in arrangement with one-dimensional nanowhiskers.

To evade nonspecific adsorption of abundant proteins that would disrupt efficacious drug release, surface modifications may be made with hydrophobic ligands. In this way, biocompatibility is enhanced, and established modifiers include polyethylene glycol (PEG).

ENMs may also be equipped to release drugs in response to pH. With a decrease in pH from 7.4 – 5.0, the accelerated release of both DNR and DOX has been documented, as such a shift in acidity causes a change in the surface charge of TiO$_2$ and discharge of a chemisorbed drug. Thus, the release of chemotherapeutic agents may be realised at pH levels typical of tumours.

Despite the irrefutable promise, clinical translation of ENMs for oncology has been until now limited. It is postulated that too much emphasis is placed upon the nanoformulation, with inadequate attention given to the underlying biology of disease. Most ENMs for tumour targeted delivery rely upon the enhanced permeability and retention (EPR) effect, without knowledge of the tumour pathophysiology. As a result, a median of just 0.7% of the nanoparticle dose administered is found to successfully reach a solid tumour. This low uptake raises the issue of subsequent environmental exposure to nanomedicines, a scenario subject to little research up to this point.

### 2.2.2 Photodynamic therapy

The photocatalytic activity of TiO$_2$ nanoparticles may be harnessed in the form of photodynamic therapy (PDT) for cancer treatment. PDT involves the local administration of a photosensitiser at a tumour, followed by agent activation by irradiation of light of an appropriate wavelength. Subsequently, photogenerated species may form powerful oxidants, predominantly singlet oxygen ($^1$O$_2$), a widely acknowledged facilitator of cell death. Compared with conventional therapies, PDT holds promise given its non-invasive nature.

The anticancer activity of photoexcited TiO$_2$ has been demonstrated both in vitro and in vivo, with UVA treatment clearly inhibiting tumour proliferation, and fabrication methods of water-soluble TiO$_2$ NPs have been developed. However, the use of TiO$_2$ NPs in PDT currently faces two major challenges. Firstly, pristine TiO$_2$ NPs may absorb only in the UV region, which restricts the feasible scope of the technique to surface cancers, since in the UV region, a significant fraction of the incoming radiation is absorbed by biological components such as proteins and haemoglobin. Above 1000 nm, water and fat tissues begin absorption,
which presents an optical window around the near-infrared (NIR) (700 – 1000 nm) as the feasible irradiation range for effective photoactivation. Secondly, ROS generation by UV irradiation is short-lived, insufficient to deliver a sustained deleterious effect to cancer cells. In this context, various methods have been adopted to extend the range of optical absorption of TiO₂ in the visible spectrum, including the conjugation of TiO₂ nanoparticles with enediol molecules, and the development of composite materials such as a graphene oxide/TiO₂ hybrid, which display good anticancer activity.

2.2.3 Combinational methods to overcome drug resistance

Considering challenges facing the therapeutic techniques outlined above, as well as the phenomenon of drug resistance that often frustrates chemotherapy, some imaginative approaches to cancer treatment are developed.

Acquired resistance of cancerous cells is often attributed to the epithelial-to-mesenchymal transition (ETM), a process of plasticity in which epithelial cell-cell linkages dissolve, and cells lose their polarity. A consequence of ETM of tumour cells is the downregulation of apoptotic signalling pathways as well as increased drug efflux, and the selective targeting of tumour cells to prevent this process could represent a method to overcome drug resistance. Mesoporous functionalised TiO₂ NPs that encapsulate DOX have been devised to target and block the ETM process, and through pH-responsive release and X-ray stimulation, the killing effect of tumour cells is enhanced. Furthermore, sonodynamic therapy (SDP) represents another possible avenue to overcome the shallow UV penetration of PDT. TiO₂ NPs are dynamic, loadable sono-sensitisers that by tumour-specific targeting may improve upon the efficiency of current nano-formulations.

2.3 Overview of (Eco)Toxicity of nano-TiO₂

With the applications and production volumes of nano-TiO₂ ever growing, potential for human and environmental exposure also increases. However, understanding of the toxicological characteristics that may present a threat to human and environmental health is still limited. The unique size, shape, surface area, surface chemistry etc. of nanomaterials may all be expected to affect their toxicity, rendering their precise evaluation difficult. Although it has been contended that size itself does not cause harmful effects, numerous studies have demonstrated that nanomaterials cause toxic effects not observed by chemically equivalent but larger particles. Indeed, it has been verified that the extent of TiO₂ induced cell death is inversely proportional to nanoparticle size.

Thus, ENMs may facilitate harmful effects in biological systems, either due to their intrinsic
properties or their small entity that permits access to targets not available to larger forms. At present, although data is not systematic, is scattered and contains large knowledge gaps\textsuperscript{clxxxiii}, the toxicology field has, unlike harmful substances of the past, advanced in parallel with the developments of nanotechnology\textsuperscript{clxxxiv}. Surveying the literature base concerning the physicochemical properties that determine toxicological outcomes, numerous mechanisms have been identified to cause NM toxicity; though the influence of reactive oxygen species (ROS) is particularly prevalent\textsuperscript{clxxxv}.

ROS is the general term for chemically reactive atoms or molecules that contain oxygen, and although reactivity is often due to the incidence of unpaired electrons, non-radical ROS also occur, such as hydrogen peroxide \(\text{H}_2\text{O}_2\). ROS play essential roles in modulating cellular events, and the superoxide radical \(\text{O}_2^-\) is a natural by-product of oxidative phosphorylation. Thus, cells have inherent mechanisms of defence to ameliorate potential harmful effects of ROS, such as the antioxidant enzyme superoxide dismutase (SOD)\textsuperscript{clxxxvi}. However, excessive ROS generation in response to external stressors may overwhelm the defensive cellular capacity, resulting in oxidative stress and cellular damage. The cellular targets of ROS include proteins, DNA and lipids. Therefore, the endpoints and locations of toxicity may be wide-ranging. For instance TiO\(_2\)-NPs applied to human peripheral blood mononuclear cells (PBMCs) for 24 h significantly reduced cell viability whilst also increasing production of ROS and inflammatory response cytokines such as interleukin-6\textsuperscript{clxxxvii}. Studies that indicate that nanoparticles are taken up by the reticuloendothelial system conjecture that the liver and spleen are the main target organs\textsuperscript{clxxxviii}. Meanwhile the high fraction of unsaturated fatty acids in the central nervous system (CNS) convey susceptibility to peroxidation by ROS. Evidence has accumulated that links TiO\(_2\) NP-induced oxidative stress with neuronal dysfunction. Elevated levels of glutamate in the extracellular region may accumulate in rat primary cultured hippocampal neurons, which may over-activate ionotrophic N-methyl-D-aspartate (NMDA) receptors, which can consequently open Ca\(^{2+}\) channels\textsuperscript{clxxxix}.

Toxicological paradigms are often extrapolated from data originating from animal studies. However the limitations of such an approach may be reflected by the fact that a mere 10% of drug developmental projects make it to market approval\textsuperscript{cxc}. Since the use of non-animal approaches is promoted by REACH, CLP and Biocidal Products regulations\textsuperscript{cxci}, underpinned by legislation on the protection of animals in scientific studies\textsuperscript{cxcii}, emerging \textit{in vitro} and \textit{in silico} approaches are more adept in the context of NBM risk assessment. The grouping of substances is accepted as a powerful tool to collect and rationalise data for hazard and risk assessment\textsuperscript{cxcii}. Moreover, quantitative structure-activity relationships ((Q)SARs), are crucial to quantitatively predict physicochemical, toxicological or environmental properties of materials based upon their structures. In this context, it is relevant to investigate the (eco)toxicology of TiO\(_2\) NPs based upon structural characteristics, as summarised by Table 2.
Table 3 The impact of various physicochemical properties upon mechanism of action, exposure, human toxicity and ecotoxicity potential of ENMs

<table>
<thead>
<tr>
<th>Property</th>
<th>Mechanism of Action</th>
<th>Exposure</th>
<th>Human toxicity</th>
<th>Ecotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size/Surface area</td>
<td>As particle size decreases, surface to volume ratio increases exponentially. The reactivity of a nano-form is dramatically enhanced with respect to the micro-form, as the lower coordination number of surface atoms impart higher reactivity. Moreover, most nano-bio interactions take place at the NP surface.</td>
<td>Biological circulation time that controls distribution will be determined by anatomical filters. ENMs &lt; 10 nm were preferentially found in the blood, kidney, lung and brain; larger particles were detected only in the liver, spleen and blood. Particles 200 nm and larger are more efficiently taken up by the reticuloendothelial system.</td>
<td>Ultrafine anatase TiO$_2$ NPs (25 nm) may trigger a stronger neutrophil inflammatory response than fine anatase (250 nm). Though an inflammatory response may only follow given aggregation sufficiently large to be recognisable by phagocytes.</td>
<td>Microbial toxicity has been reported as being most severe for TiO$_2$ NPs &lt; 40 nm. However across a nano-scale range (10$^{-1}$ – 10$^{-4}$ nm), the aggregation of TiO$_2$ NPs in aqueous medium leads to similar effective diameters, precluding any distinction between pristine particle toxicity.</td>
</tr>
<tr>
<td>Shape</td>
<td>High aspect ratio nanostructures (HARN) may share a structure-activity relationship with asbestos, interfering with the warping process of phagocytosis. Furthermore, epithelial barriers may be perturbed due to penetration of ENMs into the bronchial wall, ascribed to focal damage of the monolayer by NM aggregates.</td>
<td>Particle shape determines NP cellular uptake. Endocytosis of spherical NPs is easier and faster compared to fibre-like NPs. Frustrated phagocytosis ensures good biopersistence of TiO$_2$ nanofibres (NF) in vivo.</td>
<td>Haemolysis, a good in vitro indicator of pathogenicity, was more pronounced for TiO$_2$ NF than TiO$_2$ NP or crocidolite.</td>
<td>Rod shaped TiO$_2$ NPs have been demonstrably less toxic toward bacterial systems than spherical NPs.</td>
</tr>
<tr>
<td>Surface Charge</td>
<td>Surface charge plays a crucial role in nano-bio interactions. Cationic surfaces are generally supposed to be more active than anionic surfaces, possibly due to the attraction between cationic particles and the negatively charged phospholipid groups of membranes.</td>
<td>Colloidal behaviour of nanomaterials is significantly determined by surface charge, thus influencing organisms’ responses and fate and behaviour in the environment.</td>
<td>Positively charged TiO$_2$ may adsorb onto biological surfaces, and may promote lipid peroxidation, damaging the barrier function. Nanoparticle surface charge has been shown to modify blood-brain barrier integrity and permeability.</td>
<td>Positively charged CeO$_2$ NPs were toxic to microalgae due to the affinity of cationic particles to the negative protein domains in cell membranes.</td>
</tr>
<tr>
<td>Surface modification</td>
<td>Adverse impacts of TiO$_2$ NPs may be mitigated by the addition or modification of surface moieties. The relationship between the NP and the surrounding medium may be significantly controlled, to influence particle uptake, biological response and biodistribution.</td>
<td>Poly(ethylene-glycol) (PEG) is used to evade macrophage uptake, enhancing circulation. NP coatings may allow the material to oppose the protein corona. Without polymer coating, TiO$_2$ NPs aggregate rapidly with limited mobility; functionalised, mobility may be greater.</td>
<td>The conjugation of poly(amo)amine (PAMAM) dendrimers with PEG chains attenuates ROS production, decreasing cytotoxicity, representing applicability to TiO$_2$ NPs.</td>
<td>Citrate-capped Ag ENMs displayed biopersistence far greater than that of uncapped Au NMs.</td>
</tr>
</tbody>
</table>
2.4 NBM Fate and exposure in aquatic environments

TiO\(_2\) is reported as being the most produced ENM across the EU\(_{\text{ccxii}}\), which is likely to lead to a significant release of TiO\(_2\) ENMs to the environment\(_{\text{ccxii}}\), of which up to 30% is expected to enter surface water\(_{\text{ccxiv}}\). Surface water serves to distribute EMNs in the environment, establishing connections between different environmental compartments as well as to biota. Therefore, the aqueous environment embodies a fundamental starting point to expanding the knowledge base for ENMs environmental fate and behaviour\(_{\text{ccxv}}\).

It is recognised that in the environment EMNs tend to be transformed from their released form, and although some parallels may be usefully drawn between the behaviour of MNs to colloids, the novel physicochemical characteristics present a further challenge to determining their environmental fate and behaviour. A deeper understanding of underlying scientific processes is fundamental to rationalising various ENM transformations and distribution patterns. However, in a research landscape in which the efficacy and reliability of ecological ENM RAs are restricted by a lack of tools sensitive enough to discriminate them from background nanoparticles in the natural environment\(_{\text{ccxvi}}\), the more pressing needs are reliable data produced with validated methods\(_{\text{ccxvii}}\).

2.4.1 Environmental fate processes

Upon introduction to the aquatic environment, the fate and behaviour of ENMs will depend on the physicochemical properties of the given material, as well as medium-induced transformations. Though intrinsic properties such as size, surface energy and composition are significant, characteristics of transformation products are much more important than previously thought\(_{\text{ccxviii}}\).

![Figure 3 A heatmap showing the relative importance of distribution and transformation processes for the environmental fate of selected ENMs](image)

Environmental transformations of ENMs may be broadly categorised as chemical, physical or biological processes. Chemical processes include dissolution, redox and speciation; physical
processes include agglomeration and sedimentation; biological processes, often mediated by microorganisms, include biodegradation and biomodification. Furthermore, regardless of nanoecotoxicological conclusions, it is pertinent to consider the potential for surface-active ENMs to adsorb other pollutants, facilitating transport of otherwise poorly mobile substances. Ultimately, the multitude of environmental parameters that may characterise typical surface waters, such as pH, type of natural organic matter (NOM), concentration of suspended particle matter (SPM), poses a formidable challenge for systematic assessment.

2.4.1.1 Aggregation, sedimentation and deposition

It is widely acknowledged that the mobility of TiO$_2$ NPs in aqueous systems is strongly contingent on the aggregation process. The inclination of charged ENMs to interact and form stable suspensions may be described with the DLVO (Derjaguin, Landau, Verwey and Overbeek) theory of colloids, which comprises the combination of attractive interactions (Van der Waals forces) and repulsive interactions (electrical double layer interactions). Although the theory may be confounded by the nonsphericity and complexity of ENMs, it is generally acceptably applied, as the key factors in aqueous chemistry relevant to natural colloids are mostly applicable also to ENMs.

Literature has indicated that colloidal stability is determined by material surface properties as well as extrinsic properties determined by the medium composition. The crucial factors determining the aggregation of charged ENMs in environmental media include pH, ionic strength (IS), the incidence of divalent ions and the concentration of natural organic matter, in addition to the nanomaterial concentration.

The surface charge of nanomaterials originates from the contrasting nature of surface atoms to those of the bulk. In the case of inorganic metal oxides, surfaces are comprised of oxygen atoms with a lower coordination number. As a result, electro-neutrality of the mineral system is perturbed. The disassociation of surface hydroxyl groups is the main charging mechanism for metal oxide surfaces in an aqueous medium, and is pH dependent:

$$M - OH_2^+ \Leftrightarrow M + OH \Leftrightarrow M - O^-$$

The pH at which the net surface charge is neutral is known as the isoelectric point (IEP), and for TiO$_2$ ENPs is typically close to pH 6.5. Thus, below the IEP, oxygen atoms on the particle surface would accept protons and present a positive surface charge, whilst above the IEP protons would be disassociated, and display a negative surface charge. At pH close to the IEP, a substantial decrease in electrostatic repulsion results in a faster agglomeration rate.

A variety of studies have described the destabilising effect of electrolytes on ENM
aggregation in aqueous media. Naturally occurring electrolytes, both monovalent and polyvalent, were found to destabilize nanoparticles and thus enhance agglomeration and sedimentation. At a pH above the IEP, divalent cations may specifically adsorb to the ENM surface, screening the repulsive double layer interactions, consequently decreasing its range. The marine environment is characterized by greater ionic strength and lower NOM concentration, which could lead to screening of surface charge, agglomeration and settling. Indeed, chemical studies have reported that an increase in salinity just 2.5% above that of freshwater may affectively decrease colloidal concentrations in the water column. For this reason, coastal and marine sediments are regarded as likely sinks for ENMs.

The influence of natural organic matter (NOM) on the agglomeration of MN is complex, since it can both enhance and reduce agglomeration. The complexity of NM-NOM interactions is further highlighted by work showing how the mixing order between MN and NOM may influence agglomeration. Humic substances make up the major organic fraction, and tend to stabilize TiO$_2$ ENP dispersions at low IS conditions, due to a combination of increased electrostatic and steric repulsions. NOM are reported to adsorb onto the surface of ENMs, forming a surface layer that enhances ENM surface charge and stability, through electrostatic repulsions and steric effects.

In addition to the charge effects, surface modifications of TiO$_2$ ENPs may influence their fate and behaviour by steric effects. In biomedical applications, polymeric molecules such as polyvinylpyrrolidone (PVP) may be employed to convey stealth properties, or to improve biocompatibility; in an environmental context surfactants may enhance steric stabilization.

Agglomeration may be defined as the ‘Process of contact and adhesion whereby dispersed particles are held together by weak physical interactions ultimately leading to phase separation by the formation of precipitates of larger than colloidal size (agglomerates)’ in which weak interactions imply a reversible process. In contrast, aggregation is characterized by strong chemical or electrostatic bonds, and is thus an irreversible process. In practice, ENMs will not exist in a distinct form, rather occurring simultaneously in a combination of different states.

In natural aqueous conditions, heteroagglomeration is more likely to occur compared to homoagglomeration, as the anticipated ENM concentration is many orders of magnitude lower than natural colloids. The anticipated environmental concentration of ENMs in natural waters are typically under 20 µg L$^{-1}$, whereas natural colloid concentrations are usually between 1 and 20 mg L$^{-1}$ in fresh waters, higher in soil solutions, and moderately lower in marine solutions. Indeed, it has been demonstrated that heteroagglomeration is the most prominent process affecting the mobility of ENMs in river systems. However, heteroagglomeration measurement remains difficult due to the inability of instrumentation to detect the size ratio between ENMs and natural colloids.
Aluminosilicate minerals, such as clays of the kaolin group, abundant in aquatic systems, may be utilised as an analogue for SPM. Specifically, kaolinite is composed of alternating layers of tetrahedral silicate and octahedral alumina sheets. On tetrahedral faces, a negative charge dominates, resulting in an electric double layer subject to neutralisation by cations, whilst pH-dependent charge density arises on O faces and edges, and isomorphic cation substitution leads to irregular heterogeneity of surface charge. Thus, clay minerals possess diverse elements that contribute to transient and permanent surface charges, bestowing added complexity to the environmental matrix.

Agglomeration in the water column may lead to sedimentation. The modelling of sedimentation is a difficult task, as in natural waters the settling time is subject to numerous contributions including ENM interactions with SPM, size densities and fractal dimension of aggregates/agglomerates. Still, sedimentation is a major process that links the MN from the water phase into the soil phase. The clear association between agglomeration and sedimentation relates to the influence of gravity that leads larger masses to settle quicker.

2.4.1.2 Bioavailability, bioaccumulation

Concepts of bioavailability and organism uptake of ENMs are crucial to connect environmental chemistry theories with biological effects. Though quantitative literature on ENM uptake and accumulation is scarce, evidently organisms exposed to ENMs will incorporate them into their bodies, predominantly through gastrointestinal means. The significance of such uptake is subject to the capacity for NPs to enter cells, which may occur through different mechanisms. Crucial to the net absorption into an internal body is the ENM behaviour in the external media, where it may be subject to transformation analogous to those in the aqueous environment. Overall, bioaccumulation is, analogous to toxicity, dependent on numerous factors among physicochemical properties, particle composition, and environmental fate processes. Exposure to bioaccumulative ENMs is likely to cause adverse impacts more readily than exposure to other NMs.

2.4.2 Modelling NBM fate and exposure

To consider the fate and exposure of ENMs, the first stage of interest is environmental discharge. In principle they will be known, although the sources of NBM environmental release may be varied and difficult to quantify. The present modelling methods may be categorised as fate and behaviour models or mass flow analysis (MFA), where the former focuses more on likely environmental processes, while the latter tends to extrapolate from input data. A concern shared irrespective of the study approach is that few studies combine the behaviour of ENMs
across their pristine, weathered and transformed states, despite the tendency of the surrounding medium to determine transformative processes that alter ENM properties. An obvious limitation of MFA is a lack of satisfactory input data, which must be arduously gathered or predicted within wide bounds, thus constraining the model outputs as being rather speculative. Recently, models have incorporated stochastic aspects, whereby uncertainty and variability in the data is addressed by creating probability distributions for material concentrations across the life cycle. Fate and behaviour models comprise of detailed explanations of the key processes such as aggregation and dissolution. Deficiencies of these approaches include the assumption of steady state concentrations across compartments. Moreover, laboratory studies of ENMs fate and behaviour tend to be undertaken under simplified conditions, often at concentrations far greater than those predicted in the environment.

The goal of modelling methodology is to arrive at a reliable predicted environmental concentration (PEC) which may be compared to the predicted no adverse effect concentration (PNEC). This quotient forms the basis to derive the risk of a given ENM to the ecosystem. As mentioned, the analytical challenges of quantifying ENMs in the environment mean that there is precious few data on real-world concentrations beside which to authenticate modelling approaches. Consequently, modelling emerges as the only method to provide reasonable PECs. This may be a disturbing revelation given the wide range of values attained across the literature. The local median PECs for nano-TiO$_2$ across a number of Swiss river sections ranged from 11 to 1623 ng L$^{-1}$. Without actual concentrations to verify the results, it remains undetermined if the data ranges are due to actual differences in the environment, differences due to methodological approaches, or a combination.

2.4.3 NanoFASE Multidimensional parameter testing matrix

As described, environmental parameters are crucial in determining the behaviour of ENMs in surface waters. As such, to systematically evaluate agglomeration and transformation processes of ENMs in different surface water conditions, a multidimensional parameter matrix was established within the NanoFASE project which may be applied to assess various ENPs (Figure 4).
All chosen parameter values were based on chemical analyses of stream waters across Europe, compiled in the FOREGS database, and the matrix incorporates variations in pH, inorganic salts and natural organic matter (NOM), which in various configurations may represent freshwater and marine water scenarios. In addition, the influence of inorganic suspended particle matter (SPM) is considered.

Ultimately, comprehending how the colloidal stability of ENMs is subject to the surrounding medium may inform in silico modelling of properties and effects, refining the design of Intelligent Testing Strategies.

3. Physicochemical characterisation of nanobiomaterials

Novel physicochemical properties at the nanoscale convey innumerable possibilities, whilst simultaneously raising questions over potential health and environmental risks.
Within the Nano Environmental Health and Safety (NanoEHS) community, diverse opinions are held among stakeholders as to the adequacy of risk assessment frameworks and regulations for engineered nanomaterials (ENMs).

There is a basic consensus, however, on which nanomaterial properties should be analysed in order to assess exposure and hazard potential. This includes contributions from: the European Chemical Agency (ECHA), which suggest nanomaterial properties that should be evaluated in support of a chemical safety assessment (CSA); the organisation for economic cooperation and development (OECD), which through its series on the Safety of Manufactured Nanomaterials evaluated the methods applied in the OECD-WPMN testing programme; and International Organization for Standardization (ISO), that highlights the ISO standards most relevant to the biological evaluation of ENMs.

Nonetheless, size, the most fundamental property in nanoscience, remains a source of controversy in regulatory circles. By convention, the definitive range is between 1 and 100 nm. Yet, the maximum considerable size for a nanomaterial is arbitrary – an abrupt change in physicochemical or biological properties is not observed above this limit, and many nanomedical products may not be categorised neatly by this convention. In this context, a range of properties must be taken into account.

The success of any regulatory action is dependent on the ability to accurately identify a particular substance as a nanomaterial, and to develop a repertoire of robust methods that measure certain properties of the material or system to assess the risk. Such protocols must be accurate and reproducible, and there are two particular drivers for developing standard, reliable methods. Firstly, to determine if a material is in fact a nanomaterial, in accordance with the recommended EU definition, which requires accurate measurement of size, particle size distribution and/or measurements of the external specific surface area. Secondly, there is a need to facilitate read-across to anticipate possible fate and adverse effects from ENMs. The theory of read across is to correlate relevant physicochemical properties with toxicity and fate behavior such that the behaviors of new materials may be predicted from measuring specific physicochemical properties alone.

The specific ENM properties recently suggested by the Prosafe taskforce are many; exceeding the basic assembly required to characterize a nanomaterial, and they may be arranged into two categories: intrinsic particle properties, which are medium independent, and extrinsic particle properties, which are medium dependent. Different physical and chemical properties inevitably hold differing significance with respect to exposure and hazard potential. For instance, the intrinsic property of number average particle distribution is required to define a nanomaterial. An extrinsic property such as zeta potential may affect aggregation and
thus fate and persistence in the environment. A reactive property such as ROS generation will be an important determinant of toxicity.

At this point the association between intrinsic ENM properties and exposure or hazard characterization may not be fully illuminated. Intrinsic ENM properties do, nonetheless, directly influence extrinsic ENM properties that are strongly connected to exposure and hazard assessment. The dissolution rate of ENMs may be influenced by numerous intrinsic properties such as particle shape, size and specific surface area. ENMs with a higher dissolution rate may release toxic metals at a faster rate, leading to greater toxicity.

With the discernment between intrinsic and extrinsic properties, an awareness emerges that many ‘properties’ of a material may not be measured independently of the system in which they are found. Zeta potential only exists when particles are in contact with an ionic solution. Yet, the categorization of properties is not always absolute; the particle size distribution may change over time as particles dissolve.

3.1 An overview of conventional techniques for physicochemical characterisation of nanomaterials

3.1.1 Intrinsic Properties

3.1.1.1 Particle size distribution
A number of methods exist to determine the size distribution of primary particles in a material, each with its own optimal size range. These include methods that observe and count a small number of particles from a population (TEM, SEM SP-ICP MS), methods that track and average the behavior of a larger number of particles (DLS, NTA, X-Ray scattering), as well as methods that fractionate materials based on size or density prior to determining the size distribution (FFF, SEC). Although fractionation may reduce the complexity induced by polydispersity of samples, the influence of particle separation on NP dissolution or aggregation requires consideration. Size measurement of a particulate material should utilize different techniques depending on whether the NMs occur as a powder, dispersed in a liquid or are embedded in a solid material. Many of the different methods rely on different physical measurements or detection methods to infer particle sizes in the population. Consequently each result will provide a PSD based on a different property: Microscopy methods yield something close to the physical size of a NP (intrinsic); DLS, NTA yield a hydrodynamic diameter (system/time-dependent, thus extrinsic). The most generally applicable method used for nanomaterial characterization is electron microscopy, which readily quantifies size and morphology. Transmission Electron Microscopy (TEM), for
instance, shows interlab uncertainty of ~ 3% for simple near-spherical, near-monodisperse cases cclxi. DLS and NTA offer relatively fast measurements of hydrodynamic PSD based on NPs’ light scattering property and Brownian motion. The detection limit for NTA is generally higher but it can better handle polydisperse samples. However due to the use of assorted nanoparticle dispersion methods as well as different suspension media, reported PSD can vary significantly between laboratories.

In order to measure primary particle sizes and validate material against the current nanomaterial definition, light scattering or density separation methods will struggle as they cannot readily distinguish between a large particle and an aggregate of smaller particles. DLS is also unreliable for polydisperse samples or materials with an irregular shape cclxii. Moreover, the presence of larger particles confers a bias on the resulting spectra: larger particles tend to overshadow smaller ones, and number averaging of the scattering intensity is biased towards larger particles in a polydisperse system. Additionally, DLS provides information on the hydrodynamic size of nanomaterials in an environmental circumstance, which is an extrinsic property, and not necessarily the same as the primary PSD cclxiii.

![Scheme displaying how different PSD may result from the same polymer coated nanomaterial using different techniques](image)

Figure 5 Scheme displaying how different PSD may result from the same polymer coated nanomaterial using different techniques

In the search to achieve measurement reliability and reproducibility, the significance of dispersion protocols should not be downplayed. Through harmonization of the dispersing medium, sonification method and duration, consistent results across laboratories and across
techniques may be achieved. Therefore in the absence of protocols, measurements are less reproducible. Yet even with protocols in place, kinetic issues can considerably alter material behaviours. Investigating the degree of variability in TiO$_2$ nanomaterial dispersions by differential centrifugal sedimentation (DCS) methods, several steps in a protocol were highlighted as potential sources of variation, but final particle concentration was the most significant. The nanomaterial concentration influences the aggregation rate in the suspension, which manifests in the measured PSD. As is the case across the nanomaterial sphere, a ‘one-size-fits-all’ dispersion protocol is unlikely to be desirable. Rather, standardized protocols useful for common sample types should be adopted, and all associated metadata should be reported in addition to measurement data.

Generally EM methods are reliable and provide accurate measurements of the primary PSD. However, analyses are time-consuming, and in this regard DLS is a fine solution. However, methods are still required that relate measured values of the primary PSD needed to confirm the identity of a nanomaterial according to the current EU regulation. A tiered approach to measurement has been proposed, where the easy to apply methods such as DLS are applied to materials in order to screen for those that require additional confirmatory analyses by EM methods – this way resource requirements are held to a minimum.

Still, DLS is a generally accepted method of PSD for which an ISO standard exists (ISO 22412:2008). The ISO standard lists DLS as a method of estimation of average particle size and the measurement of the broadness of the size distribution. The applicability depends on several factors, related to the material and test conditions. Overall, its effective use is limited to stable particle suspensions of unimodal and relatively narrow size distributions.

3.1.1.2 Specific surface area

Nanomaterials are exceptional in part due to their high surface to volume ratio, and surface has become a decisive parameter from a toxicological perspective, since nano-bio interactions typically arise at the ENM surface. A number of methods are available to measure the specific surface area, prevalent among them the use of gas adsorption applied to powdered materials such as the BET method, which utilises the physisorption of N$_2$ upon powdered material. While the experimental methodology is robust and reproducibility generally good, there are currently no validated methods for nanoparticles in an aqueous suspension. Some studies have, however, utilised NMR to quantify the wettable surface area of suspended nanoparticles, from which the reactive surface area may give a better indication of the reactivity of the system.
3.1.3 Particle shape

The potential descriptors of nanomaterial shape are numerous, and in continual development to systematically capture the different ontologies, from conical, cyndrical to rod and ellipsoidal shapes. Electron microscopy is the most common tool to deliver qualitative measure of NP shape, though with well known limitations. TEM methods may only deliver dependable information on 2-D materials, and requires complementation by SEM or tomography to determine the 3-D shape.

3.1.2 Extrinsic properties

3.1.2.1 Zeta potential

The most widely reported extrinsic NM property is the zeta potential ($\zeta$). An abbreviation for electrokinetic potential in colloidal systems, the zeta potential represents the surface charge of particles, which is a key determinant of particle stability as well as cellular uptake and intracellular trafficking. A variety of reliable methods and models are available to determine the zeta potential, given its long history. Models evaluated in the literature include measurements of electrophoresis, electro-osmosis and electroacoustics; measurements of electrophoretic mobility of NPs is a prevalent method for aqueous dispersions. A detail of importance is that the zeta potential is modelled value rather than a direct measurement; issues are consequently possible with the elucidation and comparison of values between laboratories.

Common practice is the conversion of measurements of electrophoretic mobility into zeta potential using Henry’s equation and the Schmolukowski approximation. The modelled values of zeta potential for particles that act as ‘hard’ spheres, namely without a macromolecular coating, are generally consistent, within $\pm 2$ mV. Furthermore, the environmental parameters that may influence this value, including pH, ionic strength and ionic composition, are widely understood.

The determination of the zeta potential for ‘soft’ nanoparticles is more difficult. ‘Soft’ particles may be thought of as hard particles coated with a polymer or a protein. This nanomaterial class is indeed commonly encountered, as such coatings confer specific functionality to the particle. In biological solutions, protein ‘corona’ formation is also observed. The presence of a polymeric surface stratum on the nanoparticle perturbs the correlation between the electrophoretic mobility, surface charge and zeta potential. Thus, more complex numerical models are required, and the reported zeta potential for ‘soft’ particles
should be considered an apparent zeta potential; a degree of certainty further from the actual surface charge.

A number of issues may affect the integrity of the zeta potential, which may be of a practical nature, may involve interpretation, or the reporting and distribution of metadata to serve risk assessment purposes. Practically, the aggregation and settling of nanoparticles decrease a usable signal to noise. There may be issues involving the ionic strengths of solutions typical of environmental situations, which are too high, and electrodes are subject to blackening when organic coatings are present. Finally, a limitation of the method that concerns the formulation of results is the inadequate reporting of metadata. There is a range of variables crucial to determining zeta potential, including pH and ionic strength; without their recording, any efforts to utilize measure values of zeta potential for read across are impossible. To resolve this matter, some generalized protocols exist including ISO 13099-1.

Overall, a single reported value of zeta potential in a single well-defined medium is not useful. It represents a single measurement of a system-dependent variable. The pH of the isoelectric point (IEP) is more indicative of the materials property, as it is not dependent on pH or ionic strength. Still, the pH of the isoelectric point pH_{IEP} can be affected by specific sorption of ions, and thus a change in pH_{IEP} may indicate a modification in the particle surface constitution.

3.1.2.2 Solubility, dissolution rate

Solubility denotes the concentration of free ions in solution and in equilibrium with the nanomaterial phase. Although solubility may be attained in closed systems, a state of equilibrium is unlikely to be reached in an open system, or for slowly dissolving materials. Within sediment, molecules such as organic matter may complex the ions, whilst inside an organism cellular mechanisms may remove the ions as they are formed. In such situations, the rate of dissolution is the more appropriate parameter, as this may be compared to the rates of ion uptake, to discern if there is a potential for build-up of ions in the system.

The dissolution rate itself is entirely system-dependent, influenced by among others the pH, ligands existent, flow conditions. As such, functional assays may be designed that represent protocols for measuring dissolution rates in various media types; different assays should be created for measurement in water, soil, physiological fluids etc. Standard methods to measure nanoparticle dissolution are still being developed by the OECD.

3.1.2.3 Agglomeration, surface affinity
Agglomeration and aggregation of nanomaterials is commonly observed in fate and toxicity studies, and is somewhat inevitable given the inherent thermodynamic instability of colloidal NM suspensions. The agglomeration rate is a function of both particle and medium properties, and the phenomenon is essentially actuated by surface affinity: the fundamental property that governs the tendency of a NP to attach to another surface. The forces may exist between particles of the same material, homoaggregation, to another suspended particle type, heteroaggregation, or to a stationary surface, deposition.

A highly dynamic property, it is affected by anything that alters the nanoparticle surface, such as the adsorption of natural organic matter (NOM), or the solution ionic strength. The affinity coefficient, $\alpha$, describes the likelihood of attachment for each collision between two surfaces. The value is unity when there are no impediments to particle deposition, so attachment is favoured, and the value is below one when significant barriers exist. DLVO (Derjaguin, Landau, Verwey, Overbeek) theory is well-established and able to explain a number of trends observed for NP attachment to surfaces. However, its ability to predict behaviours in highly complex systems is limited. Instead a direct measurement of the affinity coefficient $\alpha$ is useful, and may efficaciously model homoaggregation and heteroaggregation.

At present there is an OECD draft guideline to measure NP agglomeration, which through a functional assay measures homoaggregation of NPs in an aqueous suspension; indicating the NP's stability against aggregation in a given medium. In such tests, nanomaterials are suspended in an aqueous medium, with defined composition, and the settling of particles over time is monitored by UV-Vis spectroscopy. Subsequently, particles that remain suspended at an explicit depth below the water-air interface are collected and quantified by ICP-MS to determine the suspended fraction after a given time.

An alternative method to monitor agglomeration behavior is to calculate surface affinity from an aggregation experiment. Currently, there is no standardized functional assay for surface affinity, but proposed methods involve time-resolved DLS. This is a well-established method for assessing the stability ratio, which is the inverse of affinity efficiency, of a colloidal dispersion. The protocol proceeds by measuring the aggregation rate, determined by increases in the hydrodynamic diameter with time early in the aggregation process, relative to the homoaggregation rate measured for the particle without a barrier to attachment. This method may be superior to that based on dispersion stability by UV-Vis, as it delivers a direct measure of attachment efficiency. Monitoring dispersion stability results only in an overall classification of stable, unstable or condition dependent.

All of these aggregation tests, proposed in aqueous solutions, are only applicable to particles with a density greater than that of water. Furthermore, particles with a strong affinity for the air-water interface will not settle consistently. An important limitation of the OECD
procedure is its inability to account for heteroaggregation behavior, a process significant for environmental fate.

For a small selection of materials (TiO$_2$ NPs and Ag NPs), inter-laboratory comparisons suggest that with well controlled experimental conditions, agglomeration of NPs may be measured effectively following the OECD guidelines.

3.2 Principles and techniques applied to TiO$_2$ case study

3.2.1 DLS

The evaluation of ENM particle size across various fields has mostly been carried out using dynamic light scattering (DLS), which is now considered the established technique. The DLS technique measures the time-dependent fluctuations in scattering intensity due to constructive and destructive interference that result from the Brownian motion of NPs within a sample.

Light scattering may be categorised into three domains based on a dimensionless size parameter $\alpha$, defined as:

$$\alpha = \frac{\pi D_p}{\lambda}$$

where $\pi D_p$ = particle circumference, $\lambda$ = wavelength of incident radiation. Based upon the value of $\alpha$, the domains are as follows: $\alpha \ll 1$ is Rayleigh scattering; $\alpha \approx 1$ Mie scattering; $\alpha \gg 1$ Geometric scattering. Hence, the particle dimensions determine which form of scattering occurs.

Particles suspended in a fluid are in constant motion, resulting from their collisions with other rapidly moving factions in the fluid. According to the Stokes-Einstein theory, particle motion is determined by, in addition to particle size, the suspending fluid viscosity, temperature, electrical charge and electrical mobility; as show in Equation 2:

$$d(h) = \frac{kt}{3\pi\eta D}$$

where $d(h)$ = hydrodynamic diameter, $k$ = boltzmann's constant, $T$ = temperature, $\eta$ = viscosity and $D$ = diffusion constant. As highlighted by the equation, the value produced by the DLS technique refers to how a particle diffuses within a fluid.

The suspension is illuminated with a monochromatic wave, which may be considered as a rapidly oscillating electric field. According to the semi-classical theory of light scattering, when
light enters the vicinity of matter, it induces those electrons to oscillate at the same frequency, consequently inducing a new oscillating electric field that radiates in all directions. The intensity of scattered light, which is the quality of interest, by an individual particle depends upon its size and shape as well as the disparity in refractive indices of the particle and the surrounding solvent molecules. In the case of nanoparticles, the dependence is straightforward, such that the scattering will be isotropic. According to the Rayleigh approximation, which in an aqueous system applies when \( d < \lambda/10 \), the Intensity of light scattered by NPs \( I \propto d^6 \).

The constantly mobile particles within the suspension cause constructive and destructive interferences which leads to fluctuations in intensity of scattered light over time. The analysis of the signal is facilitated by the intensity autocorrelation function (ACF), which is correlated as a function of delay time, \( \tau \). For a monodisperse system, the baseline subtracted ACF, \( C \), is an exponential decay of the following form:

\[
C = \exp(-2\Gamma\tau)
\]

with \( \Gamma \) = decay constant, itself readily derived from experimental data. The diffusion coefficient may be obtained from the relation

\[
\Gamma = D_t q^2
\]

where \( q = \) scattering factor, given by

\[
q = \left(\frac{4\pi n}{\lambda}\right)\sin(\theta/2)
\]

where \( n = \) refractive index of the liquid, \( \lambda = \) wavelength of incidence laser light, \( \theta = \) scattering angle. Inserting \( D_t \) into the Stokes-Einstein equation furnishes the particle size estimation.

### 3.2.2 ELS

The evaluation of ENM surface charge may be facilitated by electrophoretic light scattering (ELS). From the surface charge, the electrostatic interaction integral to the DLVO colloidal theory may be inferred, and thus aggregation behaviour may be monitored. Electric potential of a surface is the amount of work required to bring one unit of positive charge from infinity to the surface without acceleration. From a theoretical standpoint, \( \zeta \)-pot reflects the potential
difference between the electrical double layer (EDL) of electrophoretically mobile particles and the dispersant layer at the *slipping plane*.

When a charged particle is dispersed in solution, an adsorbed double layer forms on its surface. The inner layer is prevalently constituted of ions and/or molecules of the opposing charge to that of the nanoparticle (Stern layer) in order to compensate the central charge. Beyond this region the electrostatic potency of the particle surface charge decreases according to Debye's law, such that with every Debye length further from the central ion, the field decreases by a factor of $1/e_{\text{ccxviii}}$. This diffuse layer is composed of charged species of anionic and cationic form, a diffuse, dynamic layer that depends on a variety of factors including pH, ionic strength and concentration. When an electric field is applied to such a dispersion, the charged particles traverse towards the opposite electrode (electrophoresis). Within the EDL there is a hypothetical slipping plane which represents the interface between moving particles and the more distant dispersal layer, and from this particle fluid interface the zeta potential is measured.

Particles stimulated into movement during electrophoresis scatter an incident laser. Since they are in flux, the scattered light has a different frequency than the incident laser, and the frequency shift is proportionate to the particle velocity. The instrumentation to quantify this Doppler shift is shown in figure X. In brief the laser beam is split into two, of which one is directed to the sample while the other is the reference beam. The scattered light from the sample is combined with the reference beam to calculate the Doppler shift, from which the particle velocity may be deduced, and eventually the $\zeta$-pot calculated via a series of mathematical progressions.
The potential on the particle surface itself is defined as the Nerst potential \( \Psi \) and may not be directly measured. The potential at the surface \( \Psi_0 \) decreases linearly to a value \( \Psi_d \)

\[
\Psi = \Psi_d e^{-\kappa x}
\]

where \( \Psi = \) surface potential at a distance \( x \) from the stern layer, \( \Psi_0 = \) surface potential at the stern layer, \( \kappa = \) Debye-Hückel parameter, \( x = \) distance.

The extension of the double layer depends upon electrolyte concentration and valency of ions, described by the inverse of the Debye-Hückel parameter:

\[
\frac{1}{\kappa} = \left( \frac{\varepsilon_r \varepsilon_0 kT}{2n_0 Z^2 e^2} \right)^{1/2}
\]

where \( \varepsilon_r \) is the relative permittivity, \( \varepsilon_0 \) is the permittivity of free space, \( k \) is the Boltzmann constant, \( T \) is the absolute temperature, \( n_0 \) is the number of ions of each type present in the bulk phase, \( Z \) is the valency of the ions and \( e \) is the electronic charge.

Hence with increased ionic presence, the double layer is compressed and the \( \zeta \)-pot decreases. When two particles both with a double layer thickness of \( 1/\kappa \) approach such that double-layer overlap occurs, repulsion follows. The electrostatic energy of repulsion \( G_{el} \), given by

\[
G_{el} = \frac{4\pi \varepsilon_r \varepsilon_0 R^2 \Psi_d^2 \exp (-\kappa h)}{2R + H}
\]
Van der Waals (VdW) interactions may be calculated according

\[ G_A = -\frac{RA_{11}}{12h} \]

where \( R \) is the primary aggregate radius, \( A_{11} \) is the Hamaker constant, and \( h \) is the minimum inter-particle distance.

The total energy of interaction between two particles, \( G_T \), is given by the sum of repulsive and attractive forces:

\[ G_T = G_{el} + G_A \]

Colloidal stability is observed only when electrostatic forces dominate; particles are likely to aggregate and settle out if attractive forces predominate.

![Figure 7: Total energy of interaction between two colloids](image)

The \( G_T-h \) curve in Figure 3 shows a primary and a secondary minimum, with an energy maximum \( G_{\text{max}} \) at intermediate distance. The value of \( G_{\text{max}} \) is determined by the surface potential and electrolyte character. The condition for colloidal stability is to have an energy maximum much larger than the thermal energy of the particle (of the order of kT), in general corresponding to \( G_{\text{max}} \geq 25kT \). This may be achieved by having a high zeta potential (> 40 mV) and low electrolyte concentration (< 10^{-2} M), whereby the interaction is dominated by the double layer contribution. If the electrolyte concentration is increased, \( G_{\text{max}} \) decreases until it disappears at a certain electrolyte level.
3.2.3 CSA

Centrifugal Separation Analysis (CSA) has emerged as a method to assess dispersion stability of ENMs according to their sedimentation velocity, and has been efficaciously employed to calculate the sedimentation kinetics of TiO₂ NPs, MWCNs and CuO NPs. Usually the analytical centrifuge is employed to investigate the stability of a suspension over time, amid the prospect of re-agglomeration and flocculation processes.

The sedimentation behavior of a given particle may be influenced by the particle mass and hydrodynamic mobility, as well as fluid parameters such as viscosity and density. Stokes' law describes the motion of suspended particles for small Reynolds number, and the centrifuge method monitors the segregation of a homogeneous suspension due to acceleration forces.

Gravitational settling may be described by Stokes' law:

\[ \nu_s = \frac{d^2 g (\rho_d - \rho_c)}{18 \eta} \]

where \( d \) = sphere diameter, \( \rho_d \) = particle density, \( \rho_c \) = medium density, \( \eta \) = medium viscosity and \( g \) = gravitational force. The size of most particles renders the gravitational force insufficient to prevail over random molecular forces and influence separation. Centrifugation is a means to increase the magnitude of the gravitational field, and particles in suspension experience a centrifugal force in the radial direction moving away from the rotational axis, known as the relative centrifugal force (RCF). RCF is related to the speed of rotation in revolutions per minute (RPM) by Equation 1:

\[ RCF = 1.1179 \times 10^{-3} \times RPM^2 \times R \]

where \( R \) (m) is the radius calculated from the centre of the rotor to the point from which transmittance values were measured. During the centrifugal process, particle migration towards the base of the sample cuvette leads to variations in spatial particle concentration.

The principle follows that a sample is exposed to a continuous transmission of monochromatic light over the total length of the measurement vessel. The intensity of parallel light \( I_0 \) is perturbed by the sample, and the transmitted light intensity \( I \) is detected by thousands of sensors that span the sample length. The detected intensity is normalized to that measured for an empty cartridge, resulting in a sample transmission which takes values from 0 (total
obscurcation) to 1 (total transparency). The resulting transmission profile displays the intensity of light transmitted as a function of the radial coordinates, with the radius specifying the distance from the centre of the rotor. The variations of transmission profiles over space and time provide information on kinetic processes, enabling the calculation of sedimentation rates.

4 Materials and Methods

4.1 Materials

PROM TiO$_2$ uncoated nanoparticles (average particle size ca. 10 nm) were provided by NanoFASE partners as a water dispersion (ca. 1.2 wt%), as were PROM TiO$_2$ PVP coated nanoparticles as a water dispersion (ca. 1.9 wt%). The latter exhibited various shapes (square, spherical and rods), with the longest length of 11.9±3.3 nm and the shortest length of 5.1±3.3 nm. Kaolinite was purchased from The Clay Mineral Society (low-defect, Warren County, Georgia, USA). Before use, kaolinite was washed with 1M NaCl (10 times) followed by 1M NaOH (5 times). After each wash, solids were recovered by centrifugation (8000 rpm for 10 minutes, 8014 RCF) and after the final wash/centrifuge the solid was dried. Humic acid Suwannee River NOM (SR-NOM, International Humic Substances Society, MN) was employed as a surrogate total organic carbon (TOC) sample. All inorganic salts were of analytical reagent grade and purchased from Sigma-Aldrich (St. Louis, MI, USA). Ultrahigh-pure water (minimum resistivity: 18.2 MΩ/cm) used in the research was produced by a MilliQ water purifier system (Millipore, Bedford, MA, USA).

4.2 Methods

Three solutions at pH 5, 7 and 8.5 were prepared and adjusted by adding NaOH and HNO$_3$ to MilliQ water. The NanoFASE matrix was then prepared by adding to each solution firstly the salts CaCl$_2$:MgSO$_4$ in the ratio 4:1 followed by total organic matter (TOC = SR-NOM). The mixture was well shaken, and bath sonicated for 5 minutes. Meanwhile, a stock suspension of kaolinite in MilliQ water (10 gL$^{-1}$ and 1 gL$^{-1}$) was sonicated with an ultrasonic probe (UP-
200S Hielscher Ultrasonics GmbH, Germany) in an ice bath, delivering a power of 100 W for 15 min using a pulsed 80% mode. Different suspensions were then prepared by dispersing 1 gL⁻¹ and 500 mgL⁻¹ of kaolinite in 4 ml of NanoFASE matrix solution, then bath sonicated for 10 minutes. Aliquots of each dispersion were immediately analysed by Dynamic Light Scattering (DLS) and Centrifugal Separation Analysis (CSA), while Electrophoretic Light Scattering (ELS) measurements were taken afterward, ensuring the samples were bath sonicated for 5 minutes prior to measurement. Dispersions were obtained by adding to the matrix as outlined above 100 mg/L of given nanoparticles, and bath sonicating the suspension for 10 minutes. Hydrodynamic diameter, surface charge and sedimentation velocity were measured in duplicate and the data was expressed as an average for DLS/ELS and as a median for CSA. The experimental data obtained from the CSA technique was statistically analysed using a combination of statistical clustering and principle component analysis (PCA). Clustering methods facilitated the subdivision of the dataset into categories of samples with similar stability, whilst PCA allowed the obtained categories to be classified into high- or low-stability dispersions. Subsequently, the contribution of various extrinsic properties to the stability criteria was evaluated.

4.2.1 DLS and ELS

Hydrodynamic size and surface charge of the given sample was evaluated by DLS and ELS techniques, carried out by the multi-angle Nicomp ZLS Z3000 (Particle Sizing System, Port Richey, FL, USA). The hydrodynamic diameter was measured with an optical fiber set at 90° scattering angle (W = 25 mW and λ = 639 nm) over at least 6 min at room temperature. The surface charge of the electric double layer of each sample was acquired with ELS, by calculating the phase shift of ENMs with phase analysis light scattering (PALS). A 5V electric field was applied to obtain zeta-potential (ζ-pot) values from the mean phase shift with respect to time.

4.2.2 CSA

Centrifugal Separation Analysis (CSA) was employed to assess dispersion stability of ENMs and kaolinite in terms of sedimentation velocity, utilising the Multiwavelength Dispersion Analyzer LUMiSizer® 651 (L.U.M. GmbH, Berlin, Germany). The LUMiSizer facilitates the accelerated separation of different components in a dispersion by the application of a Relative Centrifugal Force (RCF), which at 2000 Rotations Per Minute (rpm) corresponds to a RCF of 537 at 120 mm from the centrifuge rotor, while at 1500 rpm corresponds to a RCF of 302. Sedimentation velocity data were calculated from the transmittance values which refer to
the amount of light passing through the sample cell over time at three different positions (115, 120 and 125 mm from the rotor). The wavelength of the transmitted light, through a polycarbonate cuvette with an optical path of 10 mm, was set at 470 nm. Particle migration due to centrifugal force results in a variation of local concentration, which corresponds to variation of transmission. The runtime of each analysis (41 min, 500 profiles) was chosen per the minimum time required to reach a plateaued state, ie. maximum transmittance that indicates complete ENM sedimentation. The linear dependency between sedimentation velocity and RCF allowed the extrapolation of sedimentation velocity at gravity by dividing the attained values by the RCF applied. All measurements were performed at 20°C.

4.2.3 Clustering, Principle Component Analysis (PCA)

The experimental data acquired from the CSA technique of samples dispersed at different pH, different levels of salts and different levels of NOM were analysed in order to categorise the dispersions into a class of high- or low-stability. Data standardization, in which for each element the mean was subtracted before the data divided by the standard deviation, preceded the clustering procedure. Three cluster algorithms were applied (Hierarchical clustering (HC), K-means (KM) and Fuzzy c-Means (FCM)), and agreed to arrange the data in such a way as to group samples with high CSA values together, and samples with low CSA values.
5 Results and discussion

5.1 Part One

In this section, experimentation was undertaken by colleagues, and the data is briefly interpreted to complement and contextualize subsequent scholarship.

To study the effect of different parameters upon the colloidal stability of kaolinite, the highest values of salts and TOC present in the NanoFASE matrix were applied, namely 10 mmol/L for salts and 10 ppm for TOC. The colloidal stability of kaolinite in terms of hydrodynamic diameter, zeta potential and sedimentation velocity was examined by initially considering the effect of pH, salts and TOC separately, then considering the effect of all three parameters together. The CSA experiments were undertaken at 2000 rpm (537 RCF).

As visualised by Figure 1, the sedimentation profiles of kaolinite measured by CSA were sensitive to different media conditions.

Furthermore, dispersions of a binary system of PROM TiO$_2$ NPs and kaolinite in environmental matrices was investigated; PROM TiO$_2$-PVP NPs were also examined by varying the
parameters using the same methodology.

5.2 Part 2

In order to explore more profoundly the NanoFASE matrix parameters, the amount of kaolinite employed was reduced from 1000 mg/L to 500 mg/L; salt concentrations were varied from 0.1 to 10 mmol/L; and SR-NOM concentrations from 0.1 to 10 ppm. The CSA experiments were undertaken at 1500 RPM, though since sedimentation velocity values are extrapolated to gravity force by dividing over the values obtained by the RCF, which depends on rpm, the results are effectively normalized and comparable to those reported above. An overview of the results for kaolinite is collected in Table 1, while dispersions with PROM TiO\textsubscript{2} and PROM TiO\textsubscript{2}-PVP NPs are displayed in Table 2 and Table 3 respectively.

5.2.1 Colloidal stability of kaolinite

<table>
<thead>
<tr>
<th>pH</th>
<th>CaCl\textsubscript{2}:MgSO\textsubscript{4} (4:1) mmol/L</th>
<th>SR-NOM (HA ppm)</th>
<th>Hydrodynamic diameter (nm)</th>
<th>ζ-potential (mV)</th>
<th>Sedimentation velocity (µm/s)</th>
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<td>1.50±0.04</td>
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<td>1.93±0.13</td>
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<td>-1±0.09</td>
<td>1.44±0.20</td>
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<td>0.1</td>
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<tr>
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<tr>
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<td>1</td>
<td>1318±282</td>
<td>-6.19±0.36</td>
<td>0.78±0.01</td>
</tr>
</tbody>
</table>
Influence of pH. Under neutral and alkaline conditions, the entire kaolinite surface may be negatively charged, and the system stabilized, whereas under acidic conditions, agglomeration may be more favoured. Indeed the mean sedimentation velocity was 1.03 \( \mu \text{m/s} \) at pH 7, 1.09 \( \mu \text{m/s} \) at pH 8 and increased to 1.45 \( \mu \text{m/s} \) at pH 5. The mean hydrodynamic size (\( d_z-\text{ave} \)) measured by DLS at pH 5 was 22\% greater than the mean \( d_z-\text{ave} \) observed at pH 7 and 8. Observing ELS measurements, positive values were noted at acidic conditions, whilst at the pH range 7-8.5 only negative zeta potential values were taken, as expected.

In detail, the stability of colloidal systems may be determined by DLVO theory as the sum of the particles’ VdW attraction and electrostatic repulsion between the surface electrical double layers (EDL). In this specific case, the net surface charge of kaolinite particles is a combination of two distinct types of surface charges: firstly, permanent negatively charged sites on the basal planes owing to the substitution of Si and Al ions for cations of a lower valency; secondly, pH-dependent polar sites, predominantly octahedral Al-OH and tetrahedral Si-OH groups, situated at the structural edges and exposed hydroxyl-terminated planes of clay lamellae. These amphoteric sites exhibit a positive or negative charge depending on the pH which may direct H\(^+\)/OH\(^-\) transfer from the aqueous phase. Previous investigations utilised a potentiometric acid-base titration and calculated the pH\(_{pzc, \text{edge}}\) of kaolinite as 5-6\(^{cclxxi}\). Thus, under low pH conditions, the edge surface charge becomes less negative or positive, electrostatic repulsion is lessened or inverted, and aggregation is enabled, often in an edge-to-face configuration\(^{cclxxxii}\). At pH 7 and 8.5, kaolinite edges, Al-O faces and Si-O faces all carry negative charges, and electrical repulsion may increase system stability\(^{cclxxxii}\).

Influence of salts. The destabilising effect of salts was evident at all pH values, as the suspensions with the lowest concentrations of salts and NOM displaying a mean sedimentation velocity two times that of the kaolinite suspension in pure water. As the salt concentration was incrementally increased from 0.1 mM to 10 mM, mean sedimentation velocity increased, rising 18\% at pH 5, 143\% at pH 7 and 47\% at pH 8. Similarly, the mean hydrodynamic diameter increased by 1\% at pH 5, 37\% at pH 7 and 50 \% at pH 8. In terms of zeta potential, the increased electrolyte concentration had a mixed impact. At pH 5 the mean \( \zeta \)-potential fell from -2.56 mV at 0.1 mM to -5.67 at 10 mM, at pH 7 it remained at around -3.8 mV, while at pH 8 the mean \( \zeta \)-potential increased from -5.61 mV to -3.5 mV.

These results are in accordance with previous laboratory studies of kaolinite in which the dispersion behavior of a pure clay suspension was found to be determined predominantly by
the ionic strength of the medium. By DLVO theory, electrolytes in the diffuse zone of the electrical double layer (EDL) may be classified as indifferent or specifically adsorbing ions. Indifferent counter-ions, e.g. Na\(^+\), compress the EDL without affecting the kaolinite surface charge. Multi-valent counter-ions, on the other hand, do reverse the surface charge by specific Stern layer adsorption. Increases in electrolyte concentration thus leads to a reduction in the EDL, allowing colloids to come closer, and enabling a greater contribution to the interaction energy from van der Waals forces, and leading to aggregation. With low ionic strength, the thickness of the Si-O face EDL may be thicker than that of the lamella, allowing the dominant EDL to spill over and effectively screen the positive edge charge. Only at high ionic strength may the EDL be sufficiently diminished such that edge surfaces are exposed, and face-to-edge agglomeration of kaolinite occurs.

**Influence of SR-NOM.** The effect of NOM upon kaolinite stability is to stabilise the suspension, as observed by the incremental changes in SR-NOM levels in the matrix. Between NOM levels of 0.1 ppm and 10 ppm, mean sedimentation velocity decreased by 61% at pH 5, 42% at pH 7 and 32% at pH 8. Mean diameter decreased by 45% at pH 5, and remained stable at pH 7-8. In all cases but one, an increased level of NOM enhanced the value of the zeta potential in the negative direction.

Previous studies of clay-NOM interactions focused on the role of NOM in modifying the clays’ surface electrostatic properties, showing that with the adsorption of NOM on the clay surface, predominantly through complexation between an edge aluminol with acidic functionality of NOM, the electronegativity of the clay surface increases, increasing electrostatic repulsion. The presence of sterics also play a role, and are suggested to be on the same magnitude as VdW attractive forces. In the acidic case, the kaolinite system is subject to attraction between the Al-O face/edge (+) and Si-O face (-), leading to a tendency toward agglomeration and instability. With high levels of NOM, adsorption reverses the cationic nature, eradicating potential aggregation through electrostatics, and thus colloidal stability increases. The adsorption of negatively charged moieties at pH 7 and 8.5 is not transformative for the colloidal surface, thus stability is not greatly altered.
5.2.2 Colloidal stability of PROM TiO$_2$ NPs
Table 10 Hydrodynamic diameters (nm), zeta potential (mV) and sedimentation velocity (µm/s) of a PROM TiO₂ uncoated (100 mg/L) suspension with different parametrical configurations of the NanoFASE matrix

<table>
<thead>
<tr>
<th>pH</th>
<th>CaCl₂:MgSO₄ (4:1) mmol/L</th>
<th>SR-NOM (HA ppm)</th>
<th>Hydrodynamic diameter (nm)</th>
<th>ζ-potential (mV)</th>
<th>Sedimentation velocity (µm/s)</th>
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**Influence of pH.** Under acidic conditions, uncoated TiO₂ NPs may be more stable compared to higher pH values. However, the mean sedimentation velocities across the pH range were similar, 0.27 µm/s at pH 5, 0.28 µm/s at pH 7, and 0.29 µm/s at pH 8. Mean hydrodynamic size was also comparable, with observed values of 1314 nm at pH 5, 1240 nm at pH 7 and 1569 nm at pH 8.5. Zeta potential results were as expected, demonstrating the tendency of metal oxides’ surface charge to reflect the pH of the media. Under acidic conditions, the mean ζ-potential was 2.66 mV, shifting to -1.84 mV at pH 7, and – 4.33 at pH 8. These values reflect the moderate nature of TiO₂ NPs within this matrix, and reasonably agree with predictions by DLVO theory. It has been calculated that an attractive Vtotal was observed around pH 6, around the pzc, whilst repulsive interaction energies prevail at pH 9. At pH 5, a sufficient positive charge may build up to prevent significant aggregation, whereas at pH 7 and 8.5,
repulsive forces may be present but not overwhelming. Thus, the values of zeta potential remained within the range of ± 30 mV, indicating a stable dispersion.

**Influence of salts.** The destabilising effect of salts was well described. With an increased electrolyte concentration from 0.1 mM to 10 mM, sedimentation velocity increased significantly. The mean velocity at high electrolyte concentration was 10 times greater at pH 5, 4.7 times greater at pH 7 and 3.5 times greater at pH 8. Similarly, the mean hydrodynamic diameter rose by 2.5 times at pH 5, over 3 times at pH 7 and 1.8 times at pH 8. Observed by ELS, ζ-potential at pH 5 fell from 4.9 mV at 0.1 mM to 0.72 mV at 10 mM, remained at around -2.6 mV at pH 7, while at pH 8 the mean ζ-potential mirrored the behavior of kaolinite, increasing from -6.39 mV to -0.34 mV. Above the PZC, divalent cations encourage aggregation due to specific adsorption which reduces the surface charge such that a potential barrier to aggregation is limited. The effect could be negligible below the PZC, although at any pH, increased electrolyte concentration provides specifically adsorbed ions, reducing the size of the electrical double layer surrounding the ENMs, increasing the rate of aggregation, aggregation size and sedimentation rate.

**Influence of SR-NOM.** The effect of NOM upon the TiO$_2$ NP suspension within the complex matrix was indeed complex, and difficult to extricate from the influence of pH. At pH 5 and pH 7, the mean sedimentation velocities were highest (~ 0.4 μm/s) with SR-NOM at 1 ppm, followed by 0.1 ppm, and the lowest mean velocities were observed at 10 ppm SR-NOM. At pH 8, a linear trend was observed, with mean sedimentation velocity decreasing as the NOM levels increased. The mean hydrodynamic diameter at 10 ppm SR-NOM decreased with respect to 0.1 ppm, independent of pH, although at pH 5 and 7 the hydrodynamic size at 1 ppm SR-NOM was comparable to at 0.1 ppm (± 50 nm). There was a clear trend for the mean ζ-potential to increase in the negative direction with increased levels of SR-NOM. Overall, it was clear that with higher levels of NOM in the solution, adsorption enhanced significantly the EDL repulsion, creating a net energy barrier and stabilizing the nanoparticles.

NOM would be expected to have a stabilising effect on TiO$_2$ NPs by adsorption of the negatively charged compounds, enhancing the negative charge and minimising aggregation. Under acidic conditions meanwhile, a degree of neutralisation occurs, causing destabilisation. It has been reported that the efficacy of NOM adsorption decreases with increased pH, as both the nanoparticle surface and the NOM may be negatively charged. Furthermore, NOM would be expected to be more soluble due to more dissociation of phenolic and carboxylic groups. Thus, the tendency for NOM to remain in solution would increase.

**Influence of salts and SR-NOM.** In the ternary systems of nanoparticles, salts and NOM,
synergistic effects may be difficult to predict. Humic acid, the predominant constituent of SR-NOM, are polycarboxylic acids, and as such with low pKₐ values may form complexes with cations such as Ca²⁺ and Mg²⁺. Precise intermolecular bridging between NOM and Ca²⁺ has been reported, that may increase nanoparticle aggregation in a medium containing the two species. Furthermore, the adsorption of NOM may increase with an increased ionic strength. This is attributed to changes in the shape of NOM from linear to spherical, due to the neutralisation of anionic phenolic and carboxylic groups by the electrolytes. Consequently, the NOM would be expected to assume a more compact coiled structure, enabling more NOM to adsorb onto the same surface area of TiO₂ NPs.

![Figure 11](image-url) PCA biplot analysing the sedimentation velocity of TiO₂ P25 (100 mg/L) dispersed in aqueous medium of various pH, ionic strength and organic matter content.

### 5.2.3 Colloidal stability of TiO₂ PVP NPs

<table>
<thead>
<tr>
<th>pH</th>
<th>CaCl₂:MgSO₄ (4:1) mmol/L</th>
<th>SR-NOM (HA ppm)</th>
<th>Hydrodynamic diameter (nm)</th>
<th>ζ-potential (mV)</th>
<th>Sedimentation velocity (µm/s)</th>
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Table 12 Hydrodynamic diameters (nm), zeta potential (mV) and sedimentation velocity (µm/s) of a TiO₂ PVP (100 mg/L) suspension with different parametrical configurations of the NanoFASE matrix.
Influence of pH. Analogous to uncoated equivalents, TiO$_2$ PVP NPs may be more stable under acidic conditions. Indeed the mean sedimentation velocity was observed to be 0.37 $\mu$m/s at pH 5, 0.49 $\mu$m/s at pH 7, and 0.51 $\mu$m/s at pH 8. Hydrodynamic diameter increased in a similar trend, rising from a mean of 2297 nm at pH 5 to 2796 nm at pH 8. The mean zeta potential also shifted in accordance to theory, with a value of 8.37 mV at pH 5, -0.18 at pH 7, close to the IEP, and -4.38 mV at pH 8, reflecting the tendency for protons to be lost from the nanoparticle surface, contributing to a more negative potential.

As nanoparticles are functionalized, their stability may differ to their uncoated counterparts. Of course, presence of the neutral PVP coating did not modify the $\zeta$-potential compared to the pristine sample. It would be expected, however, that modifying agents would improve the colloidal stability of the dispersions by steric means, preventing agglomeration of NPs with

<table>
<thead>
<tr>
<th>pH</th>
<th>Functionalization</th>
<th>Sedimentation Velocity</th>
<th>Hydrodynamic Diameter</th>
<th>Zeta Potential</th>
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respect to the uncoated form, thus reducing both the observed hydrodynamic diameter and sedimentation velocity.

**Influence of salts.** An increased electrolyte concentration generally coincided with greater aggregation. At pH 5, mean sedimentation velocity rose from 0.23 \( \mu \text{m/s} \) at 0.1 mM to 0.61 \( \mu \text{m/s} \) at 10 mM. At pH 7 the mean sedimentation velocity rose by 50\%, whilst at pH 8 it increased by 80\%. The mean hydrodynamic diameter also generally increased across the concentration divide from 0.1 mM to 10 mM. At pH 5, \( d_{\text{z-ave}} \) increased by 80\%, at pH 7 the rise was 14\%, whilst at pH 8 the mean \( d_{\text{z-ave}} \) increased by 65\%. Zeta potential results were difficult to rationalize, although it was demonstrated that independent of pH, the mean \( \zeta \)-potential was closest to zero, the point of maximum instability, at an electrolyte concentration of 10 mM. A high concentration of free ions, represented by IS, will screen the repulsive double layer interactions, consequently decreasing its range. Thus, the influence of VdW forces may become prominent, and aggregation increase.

**Influence of SR-NOM.** Comparable to behaviour of uncoated TiO\(_2\) PVP NPs, the influence of NOM was complex, and sensitive to the matrix pH. Under acidic conditions NOM tended to have a destabilizing effect, while at pH 7 and 8 seemed to stabilize the dispersions. At pH 5, an increased SR-NOM level from 0.1 to 10 ppm was accompanied by a rise in mean sedimentation velocity of 2.5 times. At pH 7 and 8, the accompanying change in mean velocity was a fall by 49\% and 37\%, respectively. These trends were also observed in the mean hydrodynamic size, which with an increased SR-NOM level from 0.1 to 10 ppm increased by 60\%, whilst at pH 7 and 8 decreasing by 48\% and 16\%, respectively. As in the case of uncoated equivalents, mean \( \zeta \)-potential of TiO\(_2\) PVP NPs tends to increase in the negative direction with increased levels of SR-NOM, shifting from 9.66 mV (0.1 ppm) to 3.55 mV (10 ppm) at pH 5, and from -3.58 mV (0.1 ppm) to –5.23 mV (10 ppm) at pH 8.
Figure 13 PCA biplot analysing the sedimentation velocity of TiO$_2$ PVP (100 mg/L) dispersed in aqueous medium of various pH, ionic strength and organic matter content.

6 Conclusions and consequences for environmental risk
The combined impact of pH, ionic composition, NOM and other components of aqueous media determine the tendency of nanoparticles to aggregate or stabilize. In turn, colloidal stability may affect the bioavailability of nanoparticles, and the precise environmental compartment in which they may reside. Ultimately, such fate and behavior may inform studies of toxicology and bioaccumulation that are relevant to environmental and human risk assessments, respectively.

With Z-pot values observed within ± 30 mV, TiO2 NPs, both uncoated and coated, may be expected to form unstable colloidal dispersions across all values conceived by the NanoFASE matrix. Nonetheless, ζ-potential only provides insight on electrostatic forces, and the vast range of values regarding sediment velocity and hydrodynamic diameter observed is testament to the dynamic behavior of NP dispersions within a narrow range of ζ-potential.

Clearly the most relevant conditions tested are those that correspond to real-world aqueous systems. Categories include seawater, estuarine water and freshwater. Seawater is characterized by high ionic strength (IS) and moderate dissolved organic content (DOC), freshwater by low IS and low DOC, whilst estuarine waters resemble freshwater conditions but for a marginally higher IS. The pH under all aqueous conditions is around 8ccxcviii.

In conditions of low NOM and high IS typical of marine water, aggregation was observed to be likely and rapid. For uncoated PROM TiO2 NPs at pH 8.5, NOM 0.1 ppm and electrolytes 10 mM, the observed sedimentation velocity was the highest of all conditions tested (0.6 μm/s), the hydrodynamic diameter the second largest (2306 nm), and the Z-pot observed to be close to zero. Under the same conditions, TiO2 PVP NPs did not display exceptional results with respect to other conditions, though the observed values were a similar magnitude to those of the uncoated case (dz-ave = 3077 nm, sedimentation velocity = 0.56 μm/s). With a high aggregation rate, TiO2 NPs may be expected to be swiftly removed from the water column. Consequently, benthic organisms could face a higher exposure risk, while a risk to aquatic organisms may only transpire if the loading were to be continuousccxcviii.

TiO2 NPs dispersed in a medium of moderate NOM and low IS more typical of freshwater displayed quite different behavior. Uncoated PROM TiO2 NPs at pH 8.5, NOM 10 ppm and electrolytes 0.1 mM displayed dz-ave of 462 nm and sedimentation velocity of 0.04 μm/s. Similarly TiO2 PVP NPs displayed the second lowest values of dz-ave and sedimentation velocity at 946 nm and 0.09 μm/s, respectively. Thus, TiO2 NPs may form stable dispersions in this media, with a low sedimentation rate. Hence, aquatic organisms active in the water column, such as fish and algae, may be exposed to small aggregates over a longer period.

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