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SERENADE: safer and ecodesign research and education applied to nanomaterial development, the new generation of materials safer by design

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SERENADE is a French project which aims to develop and apply the “safer by design” process to create safer nano-products. It achieves this goal by combining knowledge and scientific approaches from a range of disciplines towards this common goal. This tutorial review presents the conceptual approach to “Safer by Design” and provides several examples of case studies primarily for TiO$_2$ (anatase) present in paints and cements to demonstrate how the approach can inform design decisions. Particular attention is paid to chronic low dose exposure scenarios.

Environmental significance

This article is devoted to some new data concerning the life cycle of nano-products in relation to the synthesis of safer by design products as paints containing TiO$_2$. The end of life and exposure of cements and paints containing anatase and also the impact of nanoparticles on the WWTP have been studied. The effects on plants have also been evaluated for CeO$_2$ at low concentrations. The data have been obtained with low concentrations of nanoparticles and after a relatively long time. Some new data concerning health risks (autophagy, ingestion of TiO$_2$ as a food additive, reprotoxicity) have been developed. This paper is a review of the research studies on the first three years of an 8 year project. The conclusion shows the evolution of the research studies which are developed in the frame of case studies.

Introduction to “safer by design”

Safer by design is not really defined but a generic concept based on the integration of the life cycle assessment into managing the environmental health security (EHS) in order to optimize the benefit/risk ratio in terms of societal and economic risks. This approach, which embraces economic and societal challenges when developing nano-enabled products by considering risk assessment across the product life cycle, is accepted by authorities in the USA and EUROPE. A recent European project NanoREG 2 is organized around three pillars of the safer by design (SBD) concept: i) safe products by...
design based on the chemical and physical properties of the nanomaterials, such as shape, aspect ratio, crystallographic faces, and chemical additives in nano-enabled products which can maximize benefits and minimize risks, ii) safe use of products containing nanomaterials, including minimizing waste production and safe handling and recycling, and iii) safe industrial production because workers and production sites are the most likely place in the life cycle for exposure. This implies the necessity to develop knowledge for industry through training, and qualification of workers and health and safety departments. The production of SBD guidelines on the best available techniques for workplace safety, including equipment qualification and certification schemes and protocols, is also addressed as relevant tools for promoting safety at the workplace.

The safer by design concept is relatively new for industry or scientists as the development of nanotechnology considers EHS for the first time, compared to previous technologies, during its development. The NSF in the US (Strategy for Nanotechnology-Related Environmental Health and Safety)\(^1\) and the European Commission in Europe have granted various projects in support of this aim. Research focusing on Safer by Design concepts is implemented by projects like SUN (http://www.sun-fp7.eu), NanoREG I (www.nanoreg.eu), Nanoreg II (http://www.nanoreg2.eu) and ProSAFE (www.h2020-prosafe.eu) establishing a close collaboration between authorities, industries and scientists leading to efficient risk management approaches. SERENADE is an ongoing effort to implement the safer by design approach at the research and development stages, with the aim of putting new products on the market that have a good balance between safety, functionality and costs. This requires a broad interdisciplinary approach including chemistry, physics, biology, chemical engineering, entrepreneurship, ecology and modeling to assess both performance and risks of a new nano-enabled product.

I. The “safer by design” concept and objectives

Fig. 1 shows the different stages of SBD research, which includes the regulation and education of engineers and young scientists.

The objectives of the safer by design approach are the following:

- design of nanomaterials safer for both human health and the environment in order to promote the sustainable and responsible development and competitiveness of companies involved in nanotechnologies;
- implementation of metrological tools for occupational workers, population and environmental media;
- implementation of technologies for the end of life of the products: recycling, waste management, water treatment;
- development of a new approach of entrepreneurship by integrating findings on marketing, communication or ethics, which are today at the core of many nanotechnology debates, in order to shape a sustainable market infrastructure for their innovations.

These objectives can be met through five Priority Research Actions (PRA) (Fig. 2A) jointly implemented with Priority Education (and outreach activities) Initiatives (PEI) (Fig. 2B).

A. PRA 1: ecodesign concept

The ecodesign concept aims to create nano-enabled products that are effective, but also do not pose unnecessary risks. This entails materials design choices that consider both the material’s functions and its lifecycle risks. The ecodesign concept is jointly developed with small and medium enterprises (SMEs) and laboratories, which are experts in characterization and development of new materials. This PRA at the core of the SERENADE project is also developed...
with an entrepreneurship approach because the nanotechnology industry is entering new markets—involving an entirely new market dynamic—that contain largely unknown risks:

- concerning the value chain of existing sectors (e.g., whether or not there are existing sales and distribution channels or sales forces),
- concerning the differentiating factors of the new products they are offering with respect to competing technologies (e.g., perceived better performance)
- and concerning their acceptance by the market (e.g., identification and understanding of customer expectations and the market dynamic).

B. PRA 2: exposure reduction

PRA 2 aims at reducing the exposure to nanoparticles, nanomaterials and nano-by-products across the entire life cycle from synthesis (occupational exposure), the use phase of the nano-enabled product (release of nano-by-products

Fig. 3 Exposure of humans and the environment to various ENMs.
under the conditions of normal use and/or accidents), to end of life. In particular, PRA 2 also aims at analyzing and modeling the releases of ENMs from the nano-enabled products developed by the building industry (self-cleaning paints, glasses, cements, wood, etc. with TiO$_2$, CeO$_2$, Ag$_0$) and agroindustry (gas barriers, packaging, soft drinks, food, cosmetics, etc.). Understanding of the kinetics and mechanisms of nanomaterials released along the chain value is important. It is also necessary to evaluate the relationship between the chemical composition and structure of the products, the kinetics of release, and the properties of the particles released into the environment along the value chain. Fig. 3 illustrates the flow of ENMs at different levels of the value chain.

C. PRA 3: biocompliance criteria
This research area aims at analyzing the human and environmental impacts by using pristine ENMs (accidental release or manipulation by workers) as well as ENMs released from nano-enabled products that are complex particles generated during product fabrication or in the release process. These materials have different properties compared to the pristine materials. The interactions of these materials with other materials in the environment are also studied to evaluate real exposure routes and the differences in reactivity due to the chemical complexity of the released particles from various products. The transport within the nano-enabled products such as cements, paints, and plastics and also the transfer to and transport in surface water, soils, sludges, and finally agricultural soils are very important combined with possible transformations (Fig. 3).

D. PRA 4: end of life
This PRA is devoted to the end of life impacts, including the importance of modeling releases and the chemical complexity of particles released from the pure ENMs at the primary NM manufacturing site, into intermediate product fabrication locations, and from nano-enabled products during use. These studies concerning the environment’s role (humidity, rain, temperature, etc.) in the release and transformation of ENMs coming from nano-enabled products need an interdisciplinary approach$^2$ (Fig. 4).

E. PRA 5: risk forecasting
PRA 5 is devoted to risk forecasting. It is developed through a strong relationship with CEINT (Center for the Environmental Implications of NanoTechnology) to propose a process that should include the following six key features: (1) the ability to generate forecasts and associated levels of uncertainty for questions of immediate concern; (2) a consideration of all pertinent sources of nanomaterials; (3) an inclusive consideration of the impacts of activities stemming from nanomaterial use and production that extend beyond the boundaries of toxicology and include full life cycle impacts; (4) the ability to adapt and update risk forecasts as new information becomes available; (5) feedback to improve information gathering; (6) feedback to improve nanomaterial design. In contrast with work addressing hazards, there have been very few studies evaluating the factors controlling environmental exposure despite the fact that risk management strategies for nanomaterials, where needed, are likely to depend on exposure management. A risk management strategy rooted in a fundamental understanding of the possible pathways of exposure to nanomaterials leads to a broad array of
options for managing risks, which spans protective devices for workers in nanomaterial fabrication industries, standards for product disposal or recycling, the use of pollution reduction equipment, changes in human behavior and, in extreme cases, an outright ban on the production of a given nanomaterial. An evaluation of exposure would therefore appear to be an excellent starting point in predicting the potential for risks posed by a given nanomaterial. However, considerable amounts of information are required to estimate environmental releases and exposure, information that is only partially available. Monte Carlo methods and Bayesian network methods can be used to formally represent uncertainty in models that link production estimates to environmental releases, transformations, and persistence (Fig. 5).

II. The current safer by design approach

The safer by design approach is emerging. The current aspects of the SBD approach are illustrated using several case studies from SERENADE research: i) the synthesis of nanocellulose particles for future applications, ii) the exposure of TiO$_2$ nanoparticles from real nano-enabled products including paints as well as formation of unintentional volatile organic compound (VOC) by-products, iii) the affinity of NMs in waste water treatment plant biomass and with compartments of natural media (free water, sediments, soils, etc.) as it is an important parameter for forecasting environmental risks, and iv) evaluation of some new biological effects: autophagy, reprotoxicity and also genotoxicity from exposure to nanomaterials in food, as well as the effects of some nanomaterials on bacteria, aquatic organisms and plants. A large part of these data was associated with the best technologies to characterize the location of ENMs in complex media such as organs, cells, soil and plant bacteria, and plants on different scales from $\mu$m to nm and analyze the chemical evolution of ENMs using NMR, XANES, and EXAFS. These approaches have been used over the last 5 years to evaluate not only the impacts of nano-enabled products including paints, cosmetics, electronic devices, and agro-food plastic coatings, but also the end of life of these products and consequences on the efficacy of WWTP in terms of CH$_4$ production, treatment of nitrate and organic pollutants and also the application of biosludges to agricultural lands. These data are then used to identify new nanomaterials or to modify existing nanomaterials to lower the risks of these products without losing the benefits that they provide.

II.A. Crystalline nanocellulose (CNC)

In the last decade, there has been increasing interest in the development and characterization of emulsions stabilized by colloidal bio-based particles, i.e. Pickering emulsions. Notably, nanocrystals of biomolecules are of particular interest since they are more likely to be benign and more sustainable to produce. It is possible to obtain highly stable oil-in-water Pickering emulsions stabilized only by unmodified cellulose nanocrystals (CNC). These particles are 200 nm long and about 10 nm thick. The amazing properties of these systems in terms of stability and mechanical behaviour for encapsulation of any oil are due both to their anisotropic shape and to the irreversible nature of their adsorption. Furthermore, the

Fig. 5 Simplified flow of nanomaterial through the production process and to the environment (WWTP = waste water treatment plant) (from ref. 2).
CNC preparation by enzymatic or acid hydrolysis, or oxidation processes, may introduce charged groups at the surface. This provides good colloidal stability thanks to charge repulsions but can prevent their stabilizing ability. The low toxicity of CNC has been demonstrated. However, new methods of processing CNC and demonstrating their ability to replace petroleum-based materials with bio-based CNC were needed to stimulate new markets.

The SBD work provided the understanding of the parameters responsible for the stabilization of cellulose nanocrystals (CNC) at the water/oil interface under various conditions. Therefore, three CNC batches with variable surface charge densities were prepared and characterized. AFM, TEM and small angle neutron scattering (SANS) were used to visualize the different morphologies and determine the dimensions of isolated and aggregated CNC. A fractal aggregation process with fractal dimensions of 2.1 to 2.3 was determined. Pickering emulsions were produced with various amounts of CNC. The evolution of drop diameters of the emulsions showed two distinct areas depending on the concentration and surface charge density of the CNC. One, at the lower concentrations, was governed by the limited coalescence process at constant drop coverage. At the higher concentrations, densification of the interface was observed at a constant drop diameter. The structural study by SANS defined thicknesses from 6 nm to 22 nm and various roughnesses of the cellulose layer according to the conditions. Finally, adding an internal phase led to high internal phase emulsions (HIPE). These emulsions were characterized by confocal laser scanning microscopy and drop diameter distribution. It was observed that by adding oil to the starting emulsion, a second population of larger drop diameter arises. This has been attributed to the modulating surface charge density of the CNC. One, at the lower concentrations, was governed by the limited coalescence process at constant drop coverage. At the higher concentrations, densification of the interface was observed at a constant drop diameter. The structural study by SANS defined thicknesses from 6 nm to 22 nm and various roughnesses of the cellulose layer according to the conditions. Finally, adding an internal phase led to high internal phase emulsions (HIPE). These emulsions were characterized by confocal laser scanning microscopy and drop diameter distribution. It was observed that by adding oil to the starting emulsion, a second population of larger drop diameter arises. This has been attributed to the modulating coverage of the surface that led to coalescence beyond a critical value of concentration. As a result, this work demonstrated the ability to modulate emulsion properties such as internal phase fraction, drop diameter and texture based on the process, concentration of CNC, ionic strength and surface charge density of the cellulose nanocrystals.

This work has been a decisive step to provide new opportunities where these sustainable nanoparticles can replace petroleum-based polymers in formulations. It allowed better control of the CNC synthesis and processing steps, enabling them to become a platform for forming hybrid CNC-nanoparticle complexes. Associating more toxic nanoparticles with CNC could decrease their risk–benefit ratio, for example, in applications such as paints in order to decrease the formation of HNO$_2$.\textsuperscript{5–7}

II.B. Exposure from nano-enabled products: self-cleaning paints and cements

The use of TiO$_2$-NMs in construction materials is growing. Indeed, TiO$_2$-NMs are incorporated into various materials like paint and cement due to their photocatalytic properties to obtain self-cleaning and indoor and outdoor air treatment properties. Photo-catalytic paints are an excellent example of a remediation technology using sustainable energy, as they only require UV-VIS light to work. This passive technology can also greatly reduce energy inputs for improving indoor air quality such as mechanical ventilation and air purifiers, which require considerable amounts of electrical energy. While the influence of photocatalytic paints has great potential to lower energy inputs for treating indoor air, data suggest that these paints produce a remarkably high indoor HONO (nitrous acid) flux of \textit{ca.} $2.5 \times 10^{10}$ molecules cm$^{-2}$ s$^{-1}$ based on the heterogeneous NO$_2$ reactions with photo-catalytic paints containing 7% TiO$_2$ (w/w). This may be an undesirable outcome from a human health perspective.\textsuperscript{8–11} The SBD approach will be used to optimize the photo-catalytic nanomaterials in order to reduce the concentrations of indoor pollutants such as VOCs without the generation of secondary contaminants such as HONO. The morphology of TiO$_2$ anatase\textsuperscript{12} and the formulation of the paint are areas of future study to optimize the design of photocatalytic paints.

In the case of photo-catalytic cements, processes that lead to leaching of TiO$_2$ from the matrix will decrease the material’s efficacy and lead to unwanted environmental release of NMs. Tests were conducted to understand the factors that influenced NM leaching to make those materials more efficacious and safer. Leaching tests were adapted to specifically simulate cement alteration and aging. Three photocatalytic cements with increasing initial porosity were leached at lab-scale and each exhibited different degradation rates. Only particulate, pristine TiO$_2$-NMs were present into aquatic media (mimicking surface water). Complete pore network morphology characterisation was performed from analysis of nanometer-scale 3D data (nano-tomography X-ray data at the cement surface). This detailed characterization on the nanometer scale revealed a release-predicting parameter. The bottleneck size of $762 \pm 10\%$ nm (size of the smallest channel between 2 pores connected to the cement surface) was identified as the main morphological parameter controlling the diffusion of TiO$_2$-NMs from the interior to the surface. The predicted TiO$_2$-NM release rates based on the measurement of total pore volume connected to the cement surface by the bottleneck size of $>762 \pm 10\%$ nm explained between 15 and 104% of the experimentally observed release rate. The use of nano-CT was essential to identify and quantify this release-controlling parameter.\textsuperscript{13} This understanding can be used to design the pore network of a cement to minimize release of the embedded NMs (Fig. 6).

Durability i.e. determining whether NPs that are added to a paint can be released after aging and/or during abrasion is an important aspect in the life cycle of a paint product. A neat aging protocol combining mechanical abrasion and atmospheric weathering was developed to assess the parameters affecting release. For instance, the application of mechanical stresses alone was found to generate emitted ENM aerosols in which the ENM is always embedded inside the product (paint) matrix. But if the mechanical stresses are coupled with the environmental weathering then the eventual deterioration of the product after a certain weathering...
duration may lead to the emission of free ENM aerosols too. All these experimental findings pertaining to the effect of the mechanical stresses alone, have also been put into the perspective with classical material and mechanics state laws using a predictive analytical model. Close agreement of the modeled results with the experimental values validated the model. This validated model was then used to perform a sensitivity analysis on the model parameters to rank the influence of a 25% variation of each of their values on the modelled particle emission for the given conditions. The study confirms the ability to alter the chemistry of the nano-enabled product in order to limit the loss of embedded NMs along the life cycle.

II.C. TiO\(_2\) and CeO\(_2\) exposure evaluations in aquatic and terrestrial mesocosms and resulting environmental impacts

The effects of NMs were assessed on a plant and model bacteria, and finally on a simplified ecosystem based on a plant-soil-bacteria system using global (transcriptomics, metabolomics) and targeted (molecular and cellular response, enzymatic activities, etc.) approaches. Various TiO\(_2\) particles (anatase cubes and sticks, and rutile sticks) were tested. Designing the properties of TiO\(_2\) had a major impact on the interaction with \(Escherichia\) coli as a bacterial model. The shape and the nature of crystalline faces modulated the impact of TiO\(_2\) NMs on the viability of cells. Fibers of mono-crystalline sticks (200 nm length), exhibiting [101] reactive faces through stacking defects, were more deleterious to cells than compact shapes (30 nm cubes or smaller sticks) or rutile sticks (100 nm length) with no [101] faces. The impact of the NMs on cell viability was related to cell exposure (CytoViva® microscopy). Using mutants affected by oxidative or membrane stress and qRT-PCR, we showed that the shape and the nature of the crystalline faces exposed determined the mechanism of interactions. At a concentration inducing no cell death (10 mg L\(^{-1}\)), anatase fibers generated membrane stress requiring the sigma factor rpoE, while rutile sticks and anatase cubes triggered an oxidative network of enzymes involved in superoxide and hydrogen peroxide dismutation and iron sequestration. For nanoTiO\(_2\) in interaction with \(E.\) coli cells, the best compromise to retain the photo-catalytic activity and to minimize the impact was to design small compact cubes of anatase TiO\(_2\), with no [101] faces.

The impact of NMs and of their design was also assessed on a plant–soil–bacteria system. Canola was selected, as it is an important oil-producing cultivar in Europe. A clay-loam calcareous soil (pH 8.19) from near Aix-en-Provence was used. The NMs were tested at 1 mg kg\(^{-1}\) exposure, which is a concentration close to the PEC (predicted environmental concentration). The parameters studied covered plant growth and antioxidative response, and impact on microbial community structure and their enzymatic activities after one month of plant growth. The TiO\(_2\) NMs impacted neither the germination of canola nor the plant growth (root and shoot biomasses). However, a morphology-specific response was observed: anatase cubes significantly affected enzyme activities related to N recycling (protease).\(^{19,20}\) These works show that pure anatase or rutile TiO\(_2\) which could be released or used in agriculture exhibit reactivity which seems to depend on the aspect ratio \(i.e.\) on the extension on some faces. Avoiding these morphologies could help to minimize the long-term risks from increasing concentrations of these NMs in agricultural soils.

Similar studies were conducted with CeO\(_2\) NMs. Canola plants were grown for one month in soil spiked with nanoceria (1 mg kg\(^{-1}\)). To define the role of nanodesign in environmental impacts, nanoceria with different sizes (3.5 or 31 nm) and with and without a coating (citrate) were studied. We measured microbial activities involved in C, N and P...
recycling in the rhizosphere and unplanted soil. The bacterial community structure was analyzed in unplanted soil, the rhizosphere and plant roots by 454 pyrosequencing of the *rrs* gene. This revealed size-dependent impacts, ranging from decreased microbial enzymatic activities in planted soil to alterations in bacterial community structure in roots. Particle size/aggregation was a key parameter in modulating these nanoceria effects on root communities. The citrate coating lowered the impact on microbial enzymatic activities but triggered variability near the plant roots, which called into question the lack of effects on microbial activities and bacterial community structure. Some nanoceria favored bacteria resistant to heavy metals, antibiotics and disadvantaged taxa associated with soil suppressiveness toward plant pathogens. This work provides a basis to determine outcomes of nanoceria in soil, at a dose close to predicted environmental concentrations, and to design them so as to minimize these impacts.\(^{19}\)

**CeO\(_2\) exposure evaluation in aquatic mesocosms.** Nanomaterials are likely to be introduced into the environment in a chronic low-dose manner. However, very few studies are assessing the potential impacts from such long-term low-dose exposure. Chronic dosing of CeO\(_2\) NPs over 1 month, to achieve a final concentration of CeO\(_2\) of 1.1 mg L\(^{-1}\), was carried out to evaluate the exposure versus time for benthic grazers and planktonic filter feeders.\(^{21}\) The addition of industrial uncoated and citrate-coated CeO\(_2\) with different sizes of 4 nm (pristine and coated) and 31 nm (pristine) was analyzed in terms of homo- and heteroaggregation with clays (kaolinite), dissolution rates and sedimentation rates from the water column to the sediment (Fig. 7).

These studies provide several important behaviors of these NPs. For example, the size of NPs is an important determinant of whether the particles undergo homo- or heteroaggregation at the same applied mass. This is due to the difference in number of NPs; the NPs with a size of 4 nm contain many more CeO\(_2\) particles than the larger particles, and thus primarily undergo homoaggregation. This, in turn, affects the environmental distribution and the possible effects on the benthonic or pelagic species. The particles with the smallest size present the highest reactivity in terms of reduction rate\(^{22}\) and also the chemical coating species modify the reduction rate and biological effects.

**Nanosilver materials for novel agrochemicals and from WWTP sludge for agriculture.** There is great potential for nano-enabled fertilizers and pesticides that are applied...
f oliarly. However, the foliar uptake pathways are poorly understood. The uptake of Ag-NPs in the crop species *Lactuca sativa* after foliar exposure and their possible biotransformation and phytotoxic effects were studied. In addition to chemical analyses and ecotoxicological tests, micro X-ray fluorescence, micro X-ray absorption spectroscopy, time of flight secondary ion mass spectrometry and electron microscopy were used to localize and determine the speciation of Ag at sub-micrometer resolution. No sign of phytotoxicity was observed, Ag was effectively trapped on lettuce leaves and thorough washing did not decrease the Ag content significantly. We provide the first evidence for the entrapment of Ag-NPs by the cuticle and penetration into the leaf tissue through stomata, for the diffusion of Ag in the leaf tissues, and oxidation of the Ag-NPs and complexation of Ag⁺ by thiol-containing molecules. Such type of information is crucial for better assessing the risks associated with Ag-NP-containing products for producing more effective agrochemicals.

**Analysis and modelling of interaction of NPs in WWTP.** The fate of many NMs in consumer products is the wastewater treatment plant. The presence of NMs in industrial and urban effluents could modify the efficacy of the treatments in terms of elimination of organic pollutants, N and P treatments and also the use of biosludges for CH₄ production and agricultural application. The first studies used pristine NMs and coated (CeO₂) NMs in order to understand the fate and potential impacts on WWTP operations and understanding the ultimate fate of NMs in the WWTP is essential for building confidence that NMs will not cause unwanted consequences. A model for nanoparticle (NP) distribution and transformation in a wastewater treatment plant (WWTP) was developed and parameterized for four metal and metal-oxide NPs (ZnO, Ag⁰, TiO₂ and CeO₂). ZnO, CeO₂ and Ag⁰ were tested with different surface functionalization compared with bare NPs. All four NPs were predicted to associate with the solid phase indicating significant accumulation in the biosolids. The association with bacteria is ~90% for CeO₂, ZnO and TiO₂ and ≥60% for Ag⁰ nanoparticles. High rates of transformation are calculated in the aerobic compartment. Due to high insolubility and negligible redox transformation, the only process predicted to impact TiO₂ NP fate and transport in WWTPs was distribution between the solid and liquid phases. The transformations of NPs are due to oxidation (Ag⁰) and precipitation in Ag₂S nanoparticles, reduction for CeO₂ in CePO₄ and transformation of ZnO in ZnS. These transformed NPs are more thermodynamically stable.²⁷⁻³⁴ The influence of the coating was found to be very important in terms of the affinity of NPs for bacteria present in the sludges. Using a laboratory reactor with activated sludge in aerobic mode, the affinity of bare and coated industrial CeO₂ NPs for bacteria was measured. Fig. 8 shows that the citrate-coated CeO₂ has much lower affinity for bacteria in biosolids than uncoated CeO₂. This has implications for the intimate fate of NMs in WWTPs and should be considered in the design.

The reduction kinetics of CeO₂ NPs was also different for coated and bare CeO₂. The reaction worked faster in the case of bare CeO₂, reaching 30% within the bacterial aggregates and ~12% in the case of coated CeO₂ after 24 h. This shows that direct contact with the bacterial membranes plays an important role with regard to physicochemical transformations of metal oxide nanoparticles.²⁷

The presence of surface functionalisation with organic or mineral molecules reduces the transformation kinetics and toxicity. Surface-functionalised nanoparticles can slow down transformation kinetics (e.g. oxidation, reduction) and could reduce toxicity, which would be a positive effect.

**Modeling the affinity of NPs for biomass.** The affinity of the NPs for secondary sludge (anaerobic reactor) provides information on the fate of NMs during anaerobic digestion. The attachment behaviour of TiO₂ and Ag⁰ coated with gum arabic has been evaluated by measuring the adsorbed mass of particles at different contact times (Fig. 9).

Here, $γ$ is the time variable distribution coefficient and $C₀$ is the concentration of colloids (bacteria). The slope of the line during the log-linear phase of adsorption, $m$, is defined from the equation shown in Fig. 9, where $Cₙ$ is the mass concentration of background particles and $n₀ − n$ is the number of NPs that have heteroaggregated. The parameter $α$ is the affinity (or sticking) coefficient between NPs and background colloids, $β$ is the rate constant of breakup between NPs and background colloids, $kₙ$ is the rate constant of breakup of aggregates and $M₀$ is the mass of background colloids removed by settling. This approach has been applied to a variety of different NPs including GA Ag, PVP Ag, pristine CeO₂ and citrate-coated CeO₂, TiO₂ and ZnO. The calculated $α$ values were all quite low, between 0.0003 and 0.012. Despite these relatively low values, the data suggests that removals in a typical anaerobic digestor will range from 70 to 97%. This means that particles will be largely in the solids, but in some cases a significant fraction (30%) may reside in the effluent water from the process. This understanding can be used to model the fate of NMs in these systems, and to provide better estimates of release points and release concentrations of NMs into the environment.
III. Study of the toxicity (autophagy, genotoxicity and reprotoxicity) of TiO$_2$ and CeO$_2$

Better understanding of the factors influencing NM hazards (toxicity) is needed to be able to more safely design nanomaterials. Significant efforts are therefore aimed at examining the biological effects of TiO$_2$, CeO$_2$ and Ag$^0$, and linking the observed effects with the NM and system properties. In the SBD approach, only relevant cell lines are used. For example, toxicity resulting from inhalation (a predominant exposure pathway) was assessed using lung cells. Caco-2 enterocytes and HT29-MTX mucus-secreting cells are used to study the role of TiO$_2$ in foods, and its reproduction toxicity (reprotoxicity) is studied using in vitro fertilization of mice. The unique characteristics of NMs relative to molecular toxicants give rise to new potential toxicity mechanisms. One such novel mechanism, autophagy, explained, at least in part, nanoparticle effects. Nanoparticles with different shapes, faces and coatings are tested in both acute and chronic exposures. This approach provides information on how the properties of a NM affect its toxicity, and therefore some guidance on design choices that can mitigate these important toxicity mechanisms.

III.A. Autophagy

Autophagy is a physiological process that allows the autodigestion of subcellular components in lysosomes. This process helps to inhibit inflammation and oxidative stress in cells. NPs often end up taken up by lysosomes, and it is unclear if the presence of NPs interferes with autophagy. We determined if the presence of TiO$_2$ in lysosomes interfered with autophagy using 7 different titanium dioxide (TiO$_2$) NPs varying in size, crystal phase, and/or surface properties. Thor-ough characterization of all particles was performed and their effects on the autophagy pathway were analyzed in a murine macrophage cell line (RAW264.7) in terms of initiation/elongation (activation of mTORC, expression of Atg mRNA/proteins), autophagosome accumulation (LC3-II expression), lysosomal activity/fusion with autophagosomes (SNARE, LAMP, Cathepsin expression and/or activity), and autophagy flux (p62 expression). The consequences of such exposure to NPs in terms of inflammation and oxidative stress were also quantified (inflammatory cytokine, pro- and anti-oxidant gene/protein expression). We demonstrate here that exposure of RAW macrophages to the TiO$_2$ particles led to the accumulation of autophagosomes, together with partial (TiO$_2$ NP) blockage of the autophagy flux (accumulation of p62). No apparent alteration of the autophagosome/lysosome fusion process was observed for any of the TiO$_2$ NPs used. In order to further understand the origin of the alteration of the autophagy flux, we evaluated the early steps of autophagy, particularly at the initiation and elongation levels. No alteration in the elongation of the phagophore could be observed for any of the NPs. The TiO$_2$ NPs induced a pro-inflammatory response (secretion of TNF, MIP-2, and IL-1$\beta$) as well as an oxidative stress (increased expression of Heme oxygenase and superoxide dismutase). No major difference was observed considering the different physico-chemical properties of the various types of TiO$_2$ particles. Taken together, these results demonstrate the alteration of the autophagy process by TiO$_2$ NPs. These studies may potentially provide an in vitro evaluation model for the detection of early toxic effects of nanoparticles.$^{31-33}$

III.B. Effects of TiO$_2$ as a food additive

Among the different routes of exposure, the oral route remains the least documented, although nanomaterials are...
commonly used as food additives, or incorporated into pack-
aging in contact with food or water, to provide their texturing
and anti-microbial properties, or as simple colorant agents.
The oral and gastrointestinal mucosa are the first regions
that comes into contact with the ingested nanoparticles.

TiO₂ is an important case study material because it has
been widely used by the food industry for decades in confection-
ery, sauces, and pastries as white food coloring (E171 ad-
ditive). TiO₂ is also present in toothpaste. The nano-
particulate nature of TiO₂ powder used as a food additive is a
question of intense debate within the SERENADE consortium
as well as at the European Commission level. Indeed, even if
it is not intentionally synthesised as nanoparticles, the size
distribution clearly shows a significant fraction of particles
smaller than 100 nm, but the question is how many.

Compared to other types of (nano)particles, ingestion of
TiO₂ is high, with children (<10 years) being the most ex-
posed (up to 3 mg kg⁻¹ per day). Ultimately, given its high
consumption and the current lack of understanding of its
long-term health effects, three SERENADE partners combined
efforts to focus on (nano)TiO₂ provided by food (E171), its
fate and effects on the digestive tract and associated immune
system in comparison with the reference P25 nanosized TiO₂
provided by ANSES (NM105 OECD). The primary size distrib-
ution of the E171 TiO₂ powder (anatase) is centered at 100
nm, with 44.7% of the particles having a diameter lower than
100 nm (transmission electron microscopy measurements).
The toxic effects of E171 were evaluated on two cell models,
representative of the gastrointestinal tract. First, we used the
Caco-2 enterocyte cell line, differentiated into mature
enterocytes by culture 21 days post-confluence. Second, we
used a co-culture of Caco-2 enterocytes and HT29-MTX mu-
cus-secreting cells; this model is representative of the epithe-
lium lining the ileum. These two cell models were exposed to
E171, as well as to two reference TiO₂ nanoparticle hatches
(Evonik P25, because this TiO₂-NP is largely used in the liter-
ature, and anatase 12 nm nanoparticles, termed A12, as a
model of the nanoscale fraction of E171). Two exposure
modes were used: either acute exposure (6 h, 24 h or 48 h to
20, 50 or 100 μg mL⁻¹ E171 or NPs) or chronic exposure (10,
50 or 100 μg mL⁻¹ E171 or NPs for 21 days). Our results show
that cytotoxicity was never observed, regardless of the particle
or NP, exposure conditions, and cell line. However, both P25
and E171 induced a concentration-dependent increase of re-
active oxygen species (ROS) content in Caco-2 and Caco-2/
HT29-MTX cultures. The highest ROS contents were observed
in cells exposed to P25, then to E171, and then to A12. More-
over, the Caco-2/HT29-MTX co-culture was more sensitive to
these particles/NPs than the Caco-2 monoculture. We then
evaluated the expression of genes encoding anti-oxidant en-
zymes, in exposed cells. P25 induced decreased expression of
superoxide dismutase 1 (SOD1) and glutathione reductase
(GR), but not of superoxide dismutase 2 (SOD2) and catalase
(CAT) in both cell models after short time exposure (6 h but
not 48 h). Conversely, E171 induced decreased expression of
superoxide dismutase only, also after 6 h of exposure. Finally,
we investigated the modulation of expression of some efflux
pumps from the ABC transporter family. These transporters
are involved in the efflux of xenobiotics from gut cells in case
of contamination. We demonstrated upregulation of the genes
encoding these pumps in Caco-2 cells exposed to
A12. The approaches used here, i.e. assessing both
chronic and acute effects, and using cell lines that are
aligned with the expected exposure routes, give a better as-
essment of potential effects than using less relevant cell
lines. These results also indicate that the impact of ENMs in
food products on human health warrants further exploration.

III.C. CeO₂ and reprotoxicity (PRA 3)
The effects of NMs on reproduction, if present, would pose a
significant risk. Studies were conducted to better understand
how NP properties may lead to reprotoxicity. During in vitro
fertilization of mouse 1272 oocytes, we showed a significant
decrease of fertilization rate, at a very low concentration (0.01
mg L⁻¹) of CeO₂ NPs. We also showed significant DNA dam-
age induced in vitro by CeO₂ NPs on mouse spermatozoa
and oocytes at the same low concentration using the Comet
assay. As an orthogonal method, we used transmission
electron microscopy to determine the location of accumu-
lation of the particles within or outside the cells. These images
showed that, at a high concentration (100 mg L⁻¹), is also
required for visualizing the particles, the particles were taken up
by endooytosis by the cumulus cells surrounding the oocytes,
and indicated accumulation along the spermatozoa plasma
membranes and oocytes’ zona pellucidae. There were no
nanoparticles in the cytoplasm of the spermatozoa, oocytes
or embryos. This study demonstrates for the first time the
impact of CeO₂ NPs on in vitro fertilization, as well as their
genotoxicity on mouse spermatozoa and oocytes, at low nano-
particle concentration exposure. Decreased fertilization rates
may result from: (1) CeO₂ NP’s genotoxicity on gametes; (2) a
mechanical effect, disrupting gamete interaction; (3) oxida-
tive stress induced by CeO₂ NPs. These results add new and
important insights with regard to the reprotoxicity of
nanomaterials, and suggest the need for additional in vivo
studies after low-dose exposure. This also indicates that addi-
tional work is needed to understand the NP properties that
lead to this behavior so that new designs may be proposed.

Conclusion and future
This tutorial review provided several examples of how inter-
disciplinary research can begin to develop the understanding
of how NM properties affect both the desirable and undesir-
able behaviors. This understanding then provides a roadmap
for designing materials with maximum benefits and minimal
risks. For example, characterizing the processes of the aging
of paints and cements containing TiO₂ at the nanoscale led
to an understanding of how appropriately designing the ma-
trix properties can prevent release of the embedded NPs. In
addition to better understanding of the properties controlling
fate, well-conceived toxicity testing can reveal the properties
of NPs that result in their biological effects. Chronic low-dosing scenarios can also identify new modes of particle-specific toxicity such as autophagy and reprotoxicity, which will have to be addressed to allay safety concerns. Nanoparticle properties affecting the end of life fate have been measured and modelled, e.g. the affinity of NPs for other colloids (mineral or organic) and NP distribution in WWTPs. Importantly, these examples show that the complex problem of safer by design approaches to nanotechnology requires highly interdisciplinary teams, careful and thorough characterization of the particles and system, and experiments that need to be designed specifically to study the relationship between exposure, chemistry evolution and biological effects for a range of nanomaterial types embedded in various product matrices, e.g. cosmetics, plastics, paints, and cements. Consideration of all aspects of the product life cycle must also be considered, from the production process to the end of life. Ultimately, this body of evidence across a range of material and product types will lead to design rules that maximize product benefits while minimizing the risks to human health and the environment.

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