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Third National Conference

Nanotechnologies and nanomaterials in the food sector and their safety assessment

Istituto Superiore di Sanità
Rome, February 18, 2019

ABSTRACT BOOK

Edited by
F. Aureli, F. Ferraris, F. Iacoponi,
A. Raggi, S. Savini and F. Cubadda

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Department of Food Safety, Nutrition and Veterinary Public Health

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This volume gathers the abstracts of the contributions presented at the "Third National Conference on nanotechnologies and nanomaterials in the food sector and their safety assessment". The conference was co-organized by the Department of Food Safety, Nutrition and Veterinary Public Health of the Italian National Institute of Health and by the Directorate General for Food Hygiene and Safety and Nutrition of Italian Ministry of Health. The volume provides an overview of the applications, regulation, analytical determination, toxicology and risk assessment of nanomaterials in food products.

Key words: Nanomaterials; Nanoparticles; Food safety; Risk assessment

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Questo volume raccoglie i riassunti dei contributi presentati durante il "Terzo Convegno Nazionale nanotecnologie e nanomateriali nel settore alimentare e loro valutazione di sicurezza". Il convegno è stato co-organizzato dal Dipartimento di Sicurezza Alimentare, Nutrizione e Sanità Pubblica Veterinaria dell'Istituto Superiore di Sanità e dalla Direzione Generale per l'Igiene e la Sicurezza degli Alimenti e la Nutrizione del Ministero della Salute. Il volume offre una panoramica sulle applicazioni, la normativa, la determinazione analitica, la tossicologia e la valutazione del rischio dei nanomateriali nei prodotti alimentari.

Key words: Nanomateriali; Nanoparticelle; Sicurezza alimentare; Valutazione del rischio

Conference Chair: Francesco Cubadda

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Directorate General for Food Hygiene
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Ministero della Salute

Daniela Rodorigo
Director
Directorate General of the Collegial Bodies
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Ministero della Salute

Umberto Agrimi
Director
Department of Food Safety, Nutrition
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Istituto Superiore di Sanità

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NOTE FOR THE READER

This volume gathers all the contributions presented at the conference. Abstracts are divided into oral and poster presentations. For easy consultation, oral presentations are listed in the order of the programme.

Posters are listed after the oral presentations. The poster abstracts are numbered with a code including the letter “P” followed by a progressive number.

At the end of the volume, the authors’ index is provided for the reader’s convenience.

PREFACE

Nanotechnologies deal with the application of scientific knowledge to manipulate and control matter in the nanoscale – *i.e.* approximately 1 to 100 nm – in order to make use of size- and structure-dependent properties and phenomena distinct from those associated with larger sizes of the same material. Nanotechnologies are introducing dramatic changes in virtually all industry sectors by enabling management of characteristics such as material size, shape, morphology, chemical composition and molecular configuration for the improvement or development of new process and product properties. In the food sector, three main categories of products/applications of nanotechnologies and nanomaterials can be identified, namely agricultural production (*e.g.* nano-formulated agrochemicals and animal feeds), food processing (nano-sized ingredients and food additives), and food contact materials (*e.g.* nano-additives in plastics). Although making materials smaller can generate novel and useful properties, concerns have been raised on potential risks related to the interactions of nano-sized materials at the molecular or cellular levels, which may ultimately harm human health and the environment. Reducing the size of particulate materials to the nanoscale can impart certain changes in properties and biokinetics behaviour, which may also lead to altered toxicological effects compared with the corresponding conventional (non-nanomaterial) substance. However, approaching the safety assessment of products of nanotechnology is a challenge, since new concepts and tools for safety assessment of nanomaterials are needed. Current toxicity testing approaches used for conventional substances are still suitable starting points for risk assessment of engineered nanomaterials but they may need methodological modifications since they appear to be inadequate to detect all aspects of potential toxicity of nano-sized materials. A thorough physicochemical characterization is prerequisite for hazard identification and characterization both for the unambiguous identification of the nanomaterial and for providing important pointers for potential toxicity, and thus help in deciding an appropriate testing strategy. However, such a characterization may be challenging and requires the use of state-of-the-art techniques and appropriate multi-technique analytical methods. Moreover, the applications of nanotechnology to the food sector pose the issue of the real consumer exposure to nanoparticles through consumption of food and beverages. A critical issue is whether the nanomaterial undergoes degradation (*e.g.* dissolution) during gastrointestinal digestion, thus losing the nanoparticulate nature. Only if nanoparticles persist as such after gastrointestinal digestion they may be absorbed in the gut and there is a potential for internal systemic exposure. Then, once in the body, nanoparticles may cross biological barriers, including placenta, and the issue of potential toxicity arises especially for materials which may bioaccumulate in tissues. All these aspects lead to the need of developing a nano-specific risk assessment of the applications of nanoscience and nanotechnologies in the food and feed chain. This is the subject of the Guidance published in July 2018 by the European Food Safety Authority (EFSA), dealing with risk assessment of nano-applications in novel foods, food contact materials, food/feed additives and pesticides as related to their possible impact on human and animal health. The Guidance details data and information that need to be provided on certain nano-specific properties in addition to the data and information generally required by each application according to the relevant conventional regulation as stipulated in the relevant EFSA Guidance documents for the specific intended use. The EFSA

Guidance provides a structured pathway to assess potential risks not only of engineered nanomaterials as per legal definition, but also of any other type of substance falling under the food law that might present hazards related to the nanoscale, independently from regulatory definitions. In fact, it has to be appreciated that size-dependent properties and biological effects that are of potential concern for human health, specifically toxicokinetic behaviour and particle–cell interactions, are not rigidly related to specific legally defined size thresholds and may continue to occur even when the particles constituting the material have a size well above 100 nm. This may be the case for substances resulting from production processes that are aimed at reducing the average diameter of materials' particles (*e.g.* micronisation). The publication of the EFSA Guidance and of the revised European Commission's definition of nanomaterial are expected to improve the clarity of the regulatory framework concerning nanomaterials in food. Presently, the definition of engineered nanomaterial in the food legislation, as it appears in the Novel Food Regulation (EU) No 2015/22837 and in Regulation (EU) No 1169/2011 on the Provision of Food Information to Consumers, is not aligned with the general European Commission's definition of nanomaterial. The appearance of the revised version of the latter will produce such an alignment and, according to the Novel Food regulation, will lead to a single definition of engineered nanomaterial in the area of food law, required for consistency and coherence purposes. This will also support enforcement activities, *e.g.*, official control of engineered nanomaterials for food labelling purposes as established by Regulation (EU) No 1169/2011. This volume gathers the abstracts of the contributions presented at the 'Third national conference on nanotechnologies and nanomaterials in the food sector and their safety assessment' organized by the Department of Food Safety, Nutrition and Veterinary Public Health of the National Institute of Health on 18 February 2019. Since several years now the Department is committed to the assessment of the potential impact of the use of engineered nanomaterials on food safety and consumers' health. Following the first and second conference organized on the same topic in 2013 and 2016, respectively, the meeting is an occasion for the experts of the Department, along with experts from the European Commission (DG Joint Research Centre, Ispra), EFSA, and other research centres, to offer an up-to-date overview of this rapidly growing area from the standpoint of food safety. The conference is co-organized by the Ministry of Health, Directorate General for Food Hygiene and Safety and Nutrition, under the supervision of Alessandra Di Sandro as reference person, in the frame of the ongoing project 'Application of nanotechnologies in the agri-food sector: analysis and assessment of nanomaterials in food and preparedness of the food safety system', carried out by the National Institute of Health and funded by the Ministry of Health. The meeting stimulated interest from researchers, stakeholders and the public and promoted discussion on cutting-edge topics for science and risk assessment related to the applications of nanoscience and nanotechnologies in the agri-food system.

Francesco Cubadda

Conference chairman
Member of the Cross-cutting Working Group Nanotechnologies
of the EFSA Scientific Committee
National scientific expert in the EFSA Network for Risk Assessment
of Nanotechnologies in Food and Feed

Oral session 1

Chairpersons

Alberto Mantovani, Marco Silano

EFSA GUIDANCE FOR RISK ASSESSMENT OF NANOSCIENCE AND NANOTECHNOLOGIES IN THE FOOD AND FEED CHAIN – PART I

Reinhilde Schoonjans
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Nanoscience and nanotechnology are used in the food/feed chain as novel foods, food contact materials, food/feed additives and pesticides. In July 2018, EFSA published its Guidance for the risk assessment of such uses, and their possible impact on human and animal health. This Guidance offers to Applicants who seek market approval for their products, state-of -the art risk assessment approaches and test methods to follow in safety dossiers submitted under the respective food laws. With the new Novel Food legislation (EU) No. 2015/2283 in force since January 2018, this is a timely publication to meet such responsibility.

The origins of this Guidance go back to 2009, when EFSA wrote the first scientific opinion on potential risks and to 2011 when a first Guidance for risk assessment was published upon the request of the European Commission and independently of a regulatory definition. The updated Guidance of 2018, is based on the same scientific principles and is applicable to:

1. Engineered nanomaterial (1-100 nm) as defined in the Novel Food or FIC Regulation;
2. Materials >100 nm which could retain properties of the nanoscale;
3. Material not engineered as nanomaterial but containing a fraction of particles (<50%) in the size range <100 nm;
4. Different variants of nanomaterial;
5. Nanoscale entity made of natural materials deliberately produced to have nano-enabled properties or for encapsulating (bioactive) compounds;
6. Nanomaterial per EC Recommendation on definition.

The overall structure of the Guidance comprises detailed descriptions and rationales for selecting test methods for risk assessment, which are then shortlisted in summarising boxes under each section. The Guidance is nor a text book nor a check-list for dossier submission. It is up to the Applicants to select the best test set-up during a tiered approach for safety testing and to provide a rationale. Specific considerations related to the use as feed additive or pesticide (*e.g.* inhalation, dermal route of exposure, formulations versus active ingredients *etc.*.) are provided in an Appendix. Other Appendices deepen out further core issues with additional technical information. The main risk assessment scheme covers oral exposure from food/feed related uses and foresees direct and indirect routes of exposure. The tiered approach to hazard characterisation, starts with physico-chemical characterisation mandatorily including electron microscopy for size and shape determination.

This Guidance is in force since publication and workshops are organised to facilitate its implementation with EFSA staff, Member State representatives and Stakeholders intending to submit a dossier to EFSA. Furthermore, EFSA established a cross-cutting Working Group to support the implementation of this Guidance. In the coming year, activities will be starting to develop Part 2 of the Guidance for Environmental aspects.

EFSA GUIDANCE FOR RISK ASSESSMENT OF NANOSCIENCE AND NANOTECHNOLOGIES IN THE FOOD AND FEED CHAIN – PART II

Francesco Cubadda
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The EFSA "NanoGuidance" provides applicants and risk assessors with a structured pathway to assess potential risks of *(i)* engineered nanomaterials (as per legal definition) as well as *(ii)* any other type of substance falling under the food law that might present hazards related to the nanoscale, independently from regulatory definitions. Size-dependent properties and biological effects that are of potential concern for human health, specifically toxicokinetic behaviour and particle–cell interactions, are not rigidly related to specific (legally defined) size thresholds. They may continue to occur even when the particles constituting the nanomaterial have a size well above 100 nm. Furthermore, whereas physical, chemical and biological properties of materials may change with size, there is no scientific justification for a single size limit associated with these changes that can be applied to all nanomaterials. Therefore, potential risks arising from specific properties related to the nanoscale have to be assessed focusing on such properties and potentially related hazards, which may be independent of the proportion of particles constituting the material with a size below 100 nm.

A stepwise framework for nano-related hazard identification and characterisation is outlined in the Guidance to avoid any unnecessary testing. In the first step, it is investigated *in vitro* whether the nanomaterial degrades to non-nanomaterial forms (*e.g.* dissolves) under conditions representative of the gastrointestinal tract. Quickly and fully dissolving nanomaterials can be expected not to show nano-related behaviours and thus a standard risk assessment approach may be applied instead of further nanospecific testing. If the nanomaterial does not quickly and fully degrade, the next step entails gathering any available information from existing literature that meets quality criteria (*i.e.* that has adequate characterisation data on the nanomaterial tested) and identifying potential hazards through a set of *in vitro* studies, namely *(i)* degradation test under simulated lysosomal conditions, *(ii)* genotoxicity tests, *(iii)* relevant toxicity tests addressing endpoints such as impaired cell viability/cytotoxicity, oxidative stress responses, (pro-)inflammatory responses, and integrity of the gastrointestinal barrier. If *(i)* the nanomaterial is non-persistent and *(ii)* there is no indication of potential toxicity, an argument may be made to waive further nanospecific testing. If this is not the case, a pilot *in vivo* dose finding and ADME study followed by a modified 90-day oral toxicity test (OECD TG 408 with extended parameters from OECD TG 407) should be carried out for identification of the nanomaterials with potential to cause immunological, proliferative, neurotoxic, reproductive or endocrine-mediated adverse effects as appropriate. The results of the modified 90-day toxicity test should indicate whether further in-depth investigations

would be needed, *e.g.* human kinetic data from volunteer studies or additional studies on toxicokinetic, reproductive and developmental toxicity, immunotoxicity, neurotoxicity, carcinogenicity/mutagenicity, endocrine effects, gut microbiome.

Oral session 2

Chairpersons

Alberto Mantovani, Marco Silano

THE EC'S DEFINITION OF NANOMATERIAL: REGULATORY IMPLEMENTATION AND CHALLENGES

Hubert Rauscher
European Commission, DG Joint Research Centre, Ispra, Varese, Italy

The European Commission's Recommendation on a definition of the term "nanomaterial" (2011/696/EU) (EC NM definition) provides a general basis for regulatory instruments across all areas of European Union policy. The definition, or core parts of it, has been utilised in sector specific EU legislation, (*e.g.* Biocidal Products Regulation, Medical Devices Regulation, annexes of REACH) and is also used in several EU national schemes. It is also referred to in the EFSA Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. If a material is categorised as nanomaterial, regulatory consequences can include specific information requirements, safety assessment, explicit authorisation or product labelling, depending on the application. Nanomaterials therefore need to be reliably identified. This requires first of all a common understanding of the concepts and terms used in the EC NM definition. Furthermore, experimental methods need to be available which allow a reliable assessment of a material against the criteria of the nanomaterial definition, *i.e.*, whether more than half of the particles of a material have one or more external dimensions between 1 nm and 100 nm. The presentation analyses these conceptual and technical challenges and discusses the current state of efforts to implement the nanomaterial definition for regulatory purposes.

IN VITRO ASSESSMENT OF GASTROINTESTINAL AND LYSOSOMAL DEGRADATION/DISSOLUTION: THE NANOPERSIST PROJECT

Francesca Ferraris (a), Sara Savini (a), Federica Aureli (a), Jessica Ponti (b), Dora Mehn (b), Andrea Raggi (a), Francesca Iacoponi (a), Douglas Gilliland (b), Luigi Calzolai (b), Francesco Cubadda (a)

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Nanoparticles entering the human body via the oral route are subjected to conditions that are very different from those encountered via other exposure routes. Physicochemical properties of any ingested particulate material will be affected by extreme pH and ionic strength shifts encountered during gut transit, by the co-ingested food material, and by the enzymes and bile acids secreted within the gut. The extremely low pH of the stomach may promote partial oxidation/dissolution of nanoparticles constituted by soluble metals/metal oxides with release of constituent ions. The high ionic strength in the stomach and intestine critically affect nanomaterial properties, in many cases leading to agglomeration in the gastric phase and, due to concurrent changes in pH and other factors, to deagglomeration in the intestinal phase. Changes in the size-related properties, shape or surface characteristics affect the intestinal uptake of the particles and their kinetic behaviour once absorbed. Overall, interactions of nanoparticles with the gastrointestinal environment critically affect their biological and toxicological properties.

Within the NANOPERSIST project, the time-dependent transformations of real world particulate materials under conditions representative of the gastrointestinal tract have been studied by robust *in vitro* digestion methods with standardised protocols complying with the EFSA Guidance for nano-specific risk assessment. According to the Guidance, nanomaterials that quickly dissolve/degrade in the gastrointestinal tract do not give rise to nano-specific concerns and standard risk assessment can be followed. Only if nanoparticles persist as such after gastrointestinal digestion they may be absorbed in the gut. In this case, it is necessary to investigate their stability of under lysosomal conditions to assess the potential for intracellular accumulation. Biopersistence studies in a simulated lysosomal environment were also performed according to the EFSA Guidance and the critical time points identified therein (8-96 hours, as compared to 5-60 minutes of the intestinal phase of the digestion).

Ten different food-grade particulate materials belonging to four chemical classes (synthetic amorphous silica, titanium dioxide, iron oxides/hydroxides, and zinc oxide) were studied. A state-of-the-art multi-technique approach (transmission electron microscopy, single particle ICP-MS, AF4-UV-MALS-ICP-MS/MS, centrifugal liquid sedimentation, dynamic light scattering) was used for the physicochemical characterization of the particles in pristine conditions, after ingestion in fed (with food) and fasted (without food) conditions, and after lysosomal processing. TEM was essential to study particle

morphology and primary size; DLS and CLS gave an insight about the agglomeration behaviour of the pristine material at different pH conditions; spICP-MS with the use of µs dwell times combined with ion–molecule chemistry for resolution of spectral interferences enabled to obtain quantitative data on the mass concentration and number-based distributions in biological simulated fluids for most material types.

ORAL UPTAKE AND TOXICOLOGY OF FOOD-RELEVANT NANOMATERIALS: EXPERIENCES FROM BfR

Mario Goetz, Linda Boehmert, Holger Sieg, Albert Braeuning, Alfonso Lampen
Department Food Safety, German Federal Institute for Risk Assessment, BfR, Berlin, Germany

Nanotechnology is widely used in food and consumer products. Since more than ten years the risk assessment, research on oral uptake and toxicity of nanoparticles is done at the Department Food Safety of the BfR. Main questions that were addressed so far deal with the physico-chemical characterization, cellular uptake and transport via the intestinal barrier, as well as bioavailability after oral uptake *in vivo*, cellular effects, influence of ion release, protein corona and de novo particle formation and behaviour during the digestion process. As nanoparticles express a variety of different properties, like material, size, shape, coating, dispersion stability, ion release or protein corona that also depend and change with their environment, a classification strategy is still in progress. Therefore, we focussed first on metal based nanomaterials like silver, and continued with aluminium, iron oxide and zinc oxide.

Cellular uptake and transport via the intestinal barrier was investigated on different Caco-2 based *in vitro* models of the intestinal epithelial barrier. For this purpose, different coculture models were established to mimic the enterozytes as well as the mucus secreting goblet cells and M-cells. These experiments revealed an overall small uptake rate that nevertheless depended on the cell type involved in the model, the surrounding digestive fluids and the particle coating. Moreover, the transport of the particles though the intestinal barrier is also driven by the particle material itself. The identification of the corona proteins involved showed the interaction partner of the nanoparticle during uptake.

The digestion process and especially the intestinal fluids saliva, gastic juice and intestinal juice were simulated by an *in vitro* digestion system. In combination with powerful analytics like Small Angel X-ray Scattering (SAXS) and ion release experiments, this system helped to understand the fate of the nanoparticles after oral ingestion and their property differences compared to particles cell culture medium. It could be shown that the digestive process changes the ion release rate, agglomeration rate and intestinal uptake. Surprisingly, soluble metal ions gave rise to *de novo* formed particles during the digestion process as well as in some cell culture media. This complicates the approach to differentiate between particles effect and effect of released ions.

Poster Contributions

P01 USING ADVERSE OUTCOME PATHWAYS IN NANOTOXICOLOGY

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Department of Food Safety, Nutrition and Veterinary Public Health, Istituto Superiore di Sanità, Rome, Italy

Adverse Outcome Pathways (AOP) are a recent and promising approach in toxicology that connects, in a formalized and transparent way, mechanistic information to apical endpoints, by linking a molecular initiating event to an adverse outcome via key events. In Europe the development of AOP in toxicology is a major target of the ongoing project EU-TOXRISK (Horizon 2020 programme, grant agreement No 681002). AOPs may strengthen the predictive value of mechanistic *in vitro* assays or may link an apical finding in animals or humans to a putative mechanism; most important, AOPs exploit the added value of integrating multiple, different information, from molecular biology through to human medicine. AOP are "chemically agnostic", *i.e.*, describe process and events relevant to the understanding of toxicity, not mechanisms of single chemicals. However toxicological data on chemicals are critical for providing empirical support, increasing robustness and reducing uncertainties.

AOPs involving early alterations of cell-cell signalling may be highly relevant to the characterization of toxicological hazards by nanomaterials. Also, the developing field of nanotoxicology may support new AOPs. For instance, *in vivo* rodent assays carried out in our Institute with nano-sized titanium dioxide pointed out elements of a putative new AOP: increased testosterone biosynthesis interacts with insulin growth factor and promotes cell proliferation and increased height of villi in intestinal epithelium. The resulting final adverse outcome might concern either altered absorption of nutrients and/or enhanced proneness to tumour development. Our findings also indicated a role for steroid hormones in pathways leading to intestinal diseases.

Several studies, including from our Institute, showed that nanomaterials, such as titanium dioxide, may induce in rodents subtle histological alterations of the spleen (red and/or white pulp), kidney and endocrine tissues (ovarian granulosa, thyroid, adrenal cortex), often with sex-related patterns. The use of AOP may put such findings in context with both upstream mechanisms and downstream outcomes related to impaired body functions, hence supporting the assessment of the potential significance for humans. If consistent toxicity pathways are identified, using AOPs may identify endpoints or *in vitro* assays of particular relevance in nanotoxicology.

Physicochemical and toxicokinetics features are pivotal to predict and characterize potential hazards posed by nanomaterials. Nevertheless, specific aspects of nanomaterial-induced effects should not be overlooked, such as interactions with cell organelles (*e.g.*, lysosomes, mitochondria). In the field of food safety, special attention should be given to nanomaterial-cell interactions in the intestinal tissue, because intestine is the first and main site of exposure. The development of AOPs will, therefore, support the interpretation of nanotoxicology findings.

P02 FOOD-GRADE AMORPHOUS SILICA NANOPARTICLES INTERFERE WITH LPS-DEPENDENT TRANSDUCTION OF INFLAMMATORY SIGNALS

Massimiliano G. Bianchi (a), Roberta Ruotolo (b), Giuseppe De Giorgio (b), Giuseppe Taurino (a), Martina Chiu (a), Enrico Bergamaschi (c), Nelson Marmiroli (b) Ovidio Bussolati (a)

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Along with epithelial barriers, macrophages represent the first cells that interact with Engineered Nanomaterials (ENM) upon exposure. Many studies report that several ENM are able to trigger macrophage activation. However, possible interference of ENM with the effects of typical macrophage activators, such as bacterial endotoxin (LPS), has been much less investigated thus far. In this study, we have used two well characterized, food-grade Syntethic Amorphous Silica (SAS) Nanoparticles (NPs), the precipitated NM-200 and the pyrogenic NM-203 (obtained from the JRC Nanomaterials Repository, Ispra, Varese, Italy), to assess their effects on LPS-dependent activation of human macrophage-like cells.

THP-1 cells were treated with low doses of SAS NPs (2.5-10 $\mu g/cm^2$) and, after 24h, activated with 100 ng/ml of LPS. Cytokine secretion was assessed, along with the expression of pro-inflammatory genes, autophagy markers and transduction pathway components. Metabolic effects of LPS, such as the phosphorylation of the α-subunit of the initiation factor 2 (eIF2α), mTORC1 activation and Glutamine Synthetase (GS) induction, were also monitored.

At the doses used, SAS NPs had modest effects on macrophage viability but differentially modulated LPS-induced changes. NF-κB- and p38-MAPK-dependent induction of pro-inflammatory genes, such as PTGS2, ILB and CXCL8, were not affected by SAS NPs. In contrast, eIF2α phosphorylation and GS induction were inhibited by both nanosilica preparations, with a larger effect of NM-203. Exposure to SAS NPs also led to caspase-1 suppression and to a decrease in the LPS-dependent stimulation fold of IL-1β secretion. Conversely, SAS NPs did not inhibit LPS-stimulated mTORC1 activity. Moreover, SAS NPs produced a block of autophagy with a concomitant increase of the autophagy markers LC3II and p62, an effect partially inhibited by LPS. Effects on autophagy and GS expression were reproduced also in primary macrophages derived from human peripheral blood.

It is concluded that SAS NPs interfere with LPS-dependent changes associated with macrophage activation. Confirming previous data from our laboratory, obtained in different cell models, these results demonstrate that pyrogenic SAS NPs have larger biological effects than precipitated nanosilica. Given the use of amorphous silica nanoparticles as food additives, possible changes in the inflammatory response of exposed tissues deserve further investigations.

P03 FOOD-GRADE AMORPHOUS SILICA NANOPARTICLES INTERACT WITH INTRINSICALLY DISORDERED PROTEINS IN HUMAN IMMUNE CELLS

Roberta Ruotolo (a), Giuseppe De Giorgio (a), Massimiliano G. Bianchi (b), Giuseppe Taurino (b), Martina Chiu (b), Ovidio Bussolati (b), Nelson Marmiroli (a)
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Synthetic Amorphous Silica (SAS) is used in a wide variety of industrial applications including food products. According to the EU specifications, the forms of SAS used as food additive (E551) include pyrogenic or hydrated silica depending on the process (thermal or wet) used for their manufacture. These processes lead to the production of Nanoparticles (NPs) of SAS that interact to form larger aggregates and agglomerates. In the last few years, there has been increased debate regarding the health and safety concerns related to the use of consumer products containing NPs.

In this work we have characterized SAS NPs produced by wet route (precipitated silica, NM-200) or thermal route (pyrogenic silica, NM-203) and we have observed that NM-203 exhibits greater cytotoxicity than the precipitated form on murine and human macrophage cell lines.

To study the molecular interactions of NM-200 and NM-203 in a human monocytic cell line differentiated into macrophages (THP-1), we have isolated and identified the set of proteins from cell lysates which are adsorbed with a high level of affinity to the SAS NP surface. These proteins form a so-called "hard corona", the structure of which defines the biological identity of NPs. SAS NPs and protein extracts were incubated for 24 h with gentle agitation, and multiple centrifugation steps and extensive washes with buffers of different ionic strengths were used to release almost all non-bound and soft corona proteins. Hard corona proteins were recovered by centrifugation and identified (after tryptic digestion) using liquid chromatography–high-resolution mass spectrometry.

We have observed that NM-203 NPs adsorbs on their surface more proteins than NM-200. SDS-PAGE analysis shows similar protein profiles, but a different abundance of specific proteins that form the corona of NM-200 and NM-203. The hard corona of these SAS NPs was composed of a number of distinct proteins involved in crucial metabolic pathways: pre- and post-transcriptional modifications, translation, cell motility, molecular chaperoning. These proteins show large unstructured regions that provide high flexibility that promotes their adsorption to SAS NPs.

This study supports an increased cytotoxicity and protein binding of pyrogenic SAS NPs compared to precipitated form that could be attributed to the higher surface reactivity of NM-203. The identification of structural determinants of NP toxicity appears to be essential for a "safety-by-design" synthesis of NPs used as food additives or therapeutics.

P04 CHRONIC ORAL EXPOSURE TO SYNTHETIC AMORPHOUS SILICA (NM-200) RESULTS IN RENAL AND LIVER LESIONS IN MICE

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Synthetic Amorphous Silica (SAS) is a material composed of SiO_2 primary Nanoparticles (NPs), either present as such or as agglomerates and aggregates, and is widely used in many types of food processes and products as additive (E551). According to the EU specifications for E 551, the forms of SAS used as the food additive include fumed (pyrogenic) silica and hydrated silica (precipitated silica, silica gel and hydrous silica) depending on the process (thermal or wet) used for their manufacture. The primary NPs present in the food additive E551 may partly bind to form agglomerates in the food matrix, but after gastrointestinal digestion the gut epithelium appears to be predominantly exposed to nano-sized silica.

The impact of oral exposure to SAS on human health is not well characterized. Several *in vitro* studies showed a number of adverse effects of nanosized silica, dependent on particle size and type. Less information is available *in vivo* and specifically on the oral route of exposure. Biodistribution of SAS NPs is mainly directed to liver, which appears to be the target organ for toxicity, but there is a lack of long-term studies relevant to human dietary exposure.

To assess whether repeated, long-term exposure to SAS may result in adverse effects, mice were exposed for 18 months via drinking water to NM-200 - one of the reference precipitated silica used for applications related to food and included in the OECD program on ENM safety - at 4.8 mg NM-200/kg body weight/day, a dose relevant to the estimated dietary exposure to SAS in humans. The experiment focused on kidney and liver as target organs and was carried out in parallel using two different wild type mouse lines, namely C57BL/6 and C57BL/6S, and for a shorter duration the TgHuA53T transgenic mouse line. The three mouse lines differed for the expression of α-synuclein, *i.e.*, murine and human mutated (A53T).

The particle size distribution of the suspension administered to mice was characterized by AF4-ICP-MS/MS. Sensitive ICP-MS/MS determination of silicon revealed higher

contents in liver and kidneys of NM-200-exposed mice compared to unexposed aged-matched controls. Histological abnormalities, the most common being vacuolization of tubular epithelial cells, were detected in the kidney and inflammatory responses in kidneys and livers of exposed animals. The histological findings, in conjunction with the observation of detectable deposition of silica, highlight that chronic oral intake of SAS may pose a health risk to humans and need to be examined further.

P05 REPEATED ORAL ADMINISTRATION OF THE FOOD ADDITIVE E171 LEADS TO GUT AND LIVER ACCUMULATION ASSOCIATED WITH INFLAMMATORY RESPONSES IN MICE

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Titanium dioxide (TiO_2) is widely used in pharmaceuticals, cosmetics and as a food additive (E171). It contains a fraction of Nanoparticles (NPs) that can be absorbed systemically by humans after ingestion. Increasing concern has been raised about the impact on human health of the oral exposure of TiO_2 NPs from dietary and other sources. Notwithstanding several toxicological studies provided fresh evidence in recent years, a solid risk assessment of oral exposure to E171 has not been satisfactorily achieved.

We investigated whether repeated oral administration of E171 to mice, at a dose level comparable to estimated human dietary exposure, resulted in TiO_2 deposition in the digestive system, particularly gastrointestinal tract, and in molecular and cellular alterations associated with an the inflammatory response. To reproduce the first phase of digestion, a new administration approach involving dripping E171 into the mouth of mice was applied. A significant titanium accumulation was observed in the liver and intestine of E171-fed mice compared to controls; in the intestine the titanium was demonstrated to be present as TiO_2 particles via single particle ICP-MS analysis. Alterations of morphological hepatic parameters were still visible some days after the last dosing accompanied by an increase in superoxide production in the gastrointestinal tract and the induction of an anti-inflammatory response. Overall, these findings indicate that the risk for human health associated with the ingestion of products containing E171 needs to be carefully investigated.

P06 BIO-INTERACTIONS AND EFFECTS OF IRON- AND ZINC-BASED NPs IN XENOPUS EMBRYOS: A MODEL FOR FOOD NANOSAFETY

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Iron deficiency affects 1–2 billion people worldwide, in particular risk groups like pregnant and lactating women and young children. Iron sources approved for food fortification have disadvantages such as organoleptic changes and poor absorbability. Decreasing particle size to the Nanoscale (NP) could be a strategy to improve Fe bioavailability.

Zinc is an abundant trace element in the animal body and its importance on health is well documented. Since Zn cannot be stored, a regular dietary intake is required to satisfy the physiological needs. In fact, Zn has been traditionally used as food and feed supplement.

However, the benefits and risks associated with the use of NPs in the food industry need to be studied, especially during the critical window of development.

We approached this issue by using *Xenopus laevis* embryos to study the effects of iron- and zinc-based NPs at the level of the developing intestine. At an early developmental stage in fact *Xenopus* embryos start to swallow NPs in water suspension until the end of the primary organogenis, allowing the study of possible gut dismorphogenesis and disruption of the intestinal barrier.

ZnO, ZVFe, Fe_3O_4 NPs and the corresponding metal salts were administered to Xenopus embryos from blastula to larva stage at increasing concentrations. At the end of the exposure, embryos were screened for mortality and malformations, in particular for gut abnormalities, and for ultrastructural lesions at the level of the intestinal mucosa. The modality of NP translocation was investigated by confocal and electron microscopy techniques.

The results evidenced the ability of the ZnO NPs to significantly induce abnormal coiling and oxidative injuries to the intestinal epithelium, with many NPs taken up by the enterocytes or crossing the epithelium through the paracellular space. Smaller the NPs, higher the toxic effect.

Fe NPs resulted more biocompatible. No significant gut abnormalities were observed and only slight histological changes were induced at very high concentration. NPs were mapped in the intestinal mucosa, as well as in distal organs (i.e. liver), confirming their efficient absorption. The Fe intake resulted higher than that measured after incubation in $FeSO_4$ and $FeCl_3$.

In conclusion, ZnO and Fe NPs should be carefully evaluated as food ingredients for possible adverse effects in developing intestine. Gut morphogenesis and intestinal barrier differentiation during *Xenopus* development appear to be good models.

P07 HYBRID NANOEMULSIONS CONTAINING POLYPHENON-60 FROM GREEN TEA: BIOCOMPATIBILITY ON CACO-2 CELLS

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A Hybrid Nanoemulsion (HyNE) formulation was prepared to study its potential to improve the oral delivery of catechins - polyphenon-60 from Green Tea - an extract containing a mixture of polyphenolic compounds. The lipid-catechins solubility was first investigated and an appropriate lipid was selected for HyNE production. The long-term stability of the formulations was assessed after one year of storage (at 25 ºC and 4 ºC) by means of particle size, Polydispersity Index (PI), zeta potential (ZP) and encapsulation efficiency (EE) measurements. Biocompatibility of HyNE with Caco-2 cells was studied applying the (4,5-dimethylthiazol-2-yl)2,5-dyphenyl-tetrazolium bromide (MTT) and Oxidative Potential (OP) assay. *In vitro* drug release and transport studies were performed to predict the catechins *in vivo* release profile and to evaluate the delivery potential of the HyNE formulations, respectively. Stable non-loaded and cathecins-loaded HyNE systems with high EE and similar unsymmetrical/circular shape were obtained after storage. A classical Fickian diffusion is expected *in vivo* for catechins release from HyNE. Biocompatibility and increased catechins transport across Caco-2 cells were observed for the prepared HyNE formulations. The potential of HyNE formulations to deliver poorly water-soluble drugs such as catechins was confirmed.

P08 ANTIOXIDANT AND ANTITUMOR ACTIVITY OF *CROTON ARGYROPHYLLUS* KUNTH LOADED IN LIPID NANOPARTICLES

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The essential oil from *Croton argyrophyllus* Kunth has demonstrated antiproliferative, anti-inflammatory, antinociceptive and anticancer activity, and can be considered a source of phytochemicals for potential use in pharmaceutic and food sectors. Solid Lipid Nanoparticles (SLN) have been produced to load this essential oil and its antioxidant and antitumor properties evaluated *in vitro*. Lipid nanoparticles have been produced by phase inversion procedure (PIT method) followed by fast cooling in ice bath. Briefly, the water phase and the oily phase were separately heated at 80 °C. The aqueous phase was added dropwise to the oily phase, at constant temperature and under stirring (650 rpm). The mixture was cooled down to 60 ºC, required for the phase inversion (i.e. from w/o to o/w emulsion), followed by three thermal cycles (80-60 °C). The obtained mixture was immediately cooled-down in ice bath under continuous stirring for 2 h. Lipid peroxidation was measured by applying the production of thiobarbituric acid-reactive substances (TBARS) through the standard assay. The antioxidant activity was determined by the capacity of the antioxidants existing in the oil to scavenge the stable radical DPPH•. The cytotoxicity of Essential Oil-Loaded SLN (EO-SLN) was evaluated using human cell lines, namely, in HCT-8 (colon), OVCAR-8 (ovarian adenocarcinoma), and HL-60 (leukemia) by determining the reduction of the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). Both free Essential Oil (fEO) and loaded essential oil (EO-SLN) demonstrated to inhibit the Fenton reaction. Both samples demonstrated scavenging activity exhibiting fEO higher DPPH• scavenging capacity. The cytotoxic activity of fEO against OVCAR-8 was shown to be higher than against HCT-8 and HL-60, while could be improved when loaded in SLN.

P09 TOWARDS THE DESIGN OF NANOFERTILIZERS: EARLY EXPERIMENTAL EVIDENCES ON NANO-HYDROXYAPATITE

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Nanotechnology has been used in many fields of science like chemistry, physics, energy, material science and medicine. The urgent need of innovation in Agriculture has to meet both the future demand of food and the environment protection. For these reasons a responsible and safe application of nanotechnologies can play an important role, in particular in plant nutrition and plant protection. However, the impact of agriculture oriented nanotechnology papers has been negligible, so far. A recent survey shows that much less than 1% of scientific nano-papers published are dedicated to applications of nanotechnology in agriculture. This very low value depends on the fact that studies on nano applications in the primary sector started with a delay of about 15 years, if compared to other research areas.

Best management practices for crop fertilization are those that support the achievement of the main objectives of sustainable agriculture: productivity, profitability, and environmental health. Unfortunately, conventional fertilizers have low nutrient uptake efficiencies and are often associated with high losses and environmental negative consequences. Through the application of nanotechnologies in agriculture the nutrient elements will be possibly administered using "smart fertilizers". In this new scenario the nutrient will be supplied to plants as follows: *(i)* delivered as particles or emulsions of nanoscale dimensions; *(ii)* encapsulated inside nanostructures designed to allow the controlled release of nutrients; *(iii)* delivered in a complex formed by nanocapsules incorporated in a matrix of organic polymers. As far as the expected effectiveness of nanofertilizers, the potential of nanofertilizer application have not been extensively studied, yet.

The possible use of nano Hydroxyapatite (*n*HA) as P fertilizer or as a carrier of other plant nutrients is the subject of a scientific collaboration initiated between DI4A-University of Udine and CNR-ISTEC, Faenza. The poster describes some preliminary results regarding *(i)* the *n*HA synthesis and characterization carried out by analytical and advanced microscopy techniques, and *(ii)* germination tests of *Solanum lycopersicum* L. exposed to different concentration of *n*HA.

P10 SAFETY ASSESSMENT OF NANO-FERTILISERS: TIME TO TAKE ACTION?

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Fertilisers are chemical compounds intended to enrich agricultural soils and provide nutrients to plants. So-called 'EC fertilisers' are regulated by Regulation EC 2003/2003 on mineral fertilisers and may circulate freely within the EU market. The rules for other fertilisers ("national fertilisers") are currently not harmonised at EU level and are governed by national laws, although mutual recognition applies.

In March 2016 the Commission put forward a legislative proposal on fertilising products, with two main objectives: *(1)* sustainable fertiliser production from domestic sources, transforming waste into nutrients for crops; and *(2)* harmonised cadmium limits for phosphate fertilisers. However, a consistent assessment framework on fertilizers is still unavailable. Some European Food Safety Authority (EFSA) opinions on contaminants (e.g., nitrates, perchlorate) considered also the presence in fertilizers. The DG SANTE Scientific Committee on Health and Environmental Risks (SCHER) has provided scientific advice on specific fertilizers issues (presence of cadmium, use of calcium cyanamide), but paid limited attention to the potential human exposure via the soil-food chain.

Nano-agrochemicals are an important application area of nanotechnologies in the agri-food sector with nano-fertilizers featuring among the key innovative products. The extensive body of evidence on the fate and effects of nanoparticles in (edible) plants raises potential concerns in relation to:

- food safety (deposition of particles in plant tissues and soil-forage-animal carry-over)
- plant health (phytotoxicity)
- environmental impact (build-up in soil and adverse effects on soil organisms).

Nanomaterials may pose hazards and risks which are considerably different from conventional chemicals: the new conceptual framework of nano safety assessment should support a robust scientific approach to nano-fertilizers. Nano-fertilizers may lead to considerable agricultural benefits (*e.g.*, enhanced nutrient bioavailability), but also to potential risks. However, these issues are currently not addressed in the EFSA Guidance on Nano Risk Assessment since fertilizers do not fall within the remit of the Authority.

P11 BIOACCUMULATION STUDY OF TITANIUM DIOXIDE NANOPARTICLES IN EDIBLE MUSSELS

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Despite of the widespread production and use of engineered Nanoparticles (NPs) and their foreseen increased release into the aquatic environment, relatively little is known about NPs interaction with, bioaccumulation in, or transfer to the aquatic food web.

Due to the high diffraction index and strong light scattering and incident-light reflection capability, Titanium dioxide (TiO_2) NPs are widely used, mostly as white pigment in cosmetics, paints, dyes and varnishes, textiles, paper and plastics, food and drugs etc. Evidence has been collected of urban runoff water contamination by TiO_2 Nanomaterials (NM). Thus, aquatic ecosystems, such as the marine environment, are likely to be potential sinks for these NPs.

Edible bivalve molluscs are known to be strong natural filtrating systems: by accumulating NPs, they might represent a potential vector of such contaminants and a consequent route of exposure of humans to NPs. Moreover, the marine environment seems to facilitate ingestion of NPs by suspension-feeding bivalves due to the formation of aggregate that are better absorbed compared to the smaller primary particles that form the aggregates.

An *in vivo* study was carried out by exposing mussels (*Mytilus galloprovincialis*) to TiO_2 NPs in a controlled artificial marine environment, to understand NPs behaviour in the aquatic environment in terms of bioaccumulation of NPs in edible marine animals.

The detection and characterisation of NPs in marine environment and in marine species are quite complicated due to NPs unpredictable behaviour, fate and low concentrations. This makes the determination of the bioavailability and bioaccumulation of NPs in marine species a challenge. However, thanks to a very sensitive and powerful analytical technique known as single particle inductively coupled plasma mass spectrometry (spICP-MS), the levels of contamination of mussels exposed to NPs were determined. These preliminary analytical results hints at the potential exposure to NPS for consumers if mussels harvested in polluted environment are consumed.

From an analytical perspective, results confirm the potential of spICP-MS to detect inorganic NPs in complex matrices and its applicability in routine analysis.

P12 ESEM-EDS-BASED APPROACH TO ASSESS NANOPARTICLES FOR FOOD SAFETY AND ENVIRONMENTAL CONTROL

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Environmental contamination by metal Nanoparticles (NPs) have a close link to anthropogenic emission sources, such as combustion engine and power plants. NPs can negatively impact also food safety since environmental pollution, together with industrial and production processes, represents one of the main cause of possible unintentional inclusion of these compounds along the food chain. In recent years, potential environmental and health hazards of NPs have been extensively debated, proving that NPs toxicity depends significantly on their morphology, shape and size. In this study, Environmental Scanning Electron Microscopy (ESEM) equipped with Energy Dispersive X-Ray Spectroscopy (EDS) was exploited to directly visualize NPs to perform both qualitative and counting analyses without the need of standard material. ESEM-EDS technique was applied both to air samples and along the production chain of pasta (wheat ear, wheat, semolina and pasta). Sampling and sample handling are critical analytical steps for NPs analysis: to assess environmental contamination on raw materials and food samples, attention was paid to the development of sample treatment protocol in order to avoid potential artifacts and to the selection of proper filters in terms of materials and pore dimensions. For this purpose, food samples were immersed in milli-Q water and NPs were collected on polycarbonate 0.1 μm filters. The microscope was set to automatically acquire images over proper representative filter areas in filter point casually chosen, working at magnification and resolution convenient to nanoparticle visualization. Blank filter samples resulting from milli-Q filtration were used as zero background. Iron and titanium were the major constituents of the collected particles: Fe particles were mostly present in ear and wheat samples, whereas Ti particles in pasta samples deliberately produced exploiting a Ti-based alloy (hardness, lightness and ability to withstand extreme temperatures and to constitute machineries parts destined to friction) for prototypal extrusion inserts, typically not used in normal production processes. Finally, air monitoring near pilot plants was performed by using an eight-stage Andersen cascade impactor mounting polycarbonate 0.4 μm filters from I to VII stages and 0.1 μm filter for the final (VIII) stage. Preliminary experiments allowed to select 72 h as optimal exposure time for ESEM-EDS analysis in terms of number of particles collected and proper particle distribution on filter, whereas longer exposure times were used to perform gravimetric analysis: these analysis showed an increase in the concentration levels of total particles during winter, particularly in the case of fine Particulate Matter (PM2.5). Particles containing mainly Fe, Mn and Pb were identified. These results were confirmed by performing Inductively Coupled Plasma-Mass

Spectrometry analysis on the same filters. In conclusion, it is very important the development of production processes able to reduce the NPs contamination level from raw materials to final products, but also production line materials have to be strictly controlled, due to the possible occurrence of wear particles from plant components.

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