Nanotechnology and Microbial Food Safety

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Nanotechnology has a multiple role in food industry, and as forecasted, its importance is increasing. Nanomaterials are used in food production or inspection, either in form of new materials with unique, nano-sized dispersity, providing new physico-chemical characteristics for these substances, or as novel methodologies utilizing nanostructures in analytical or technological processes. As food microbiology is affected, nanotechnology provides novel agents to inhibit growth of spoilage and pathogenic microbes, to prevent their attachment to surfaces, and even to eliminate them. Other applications provide tools for investigating microbes by detecting their attachment to food contact surfaces or specifying microbes and their growth. The new form of dispersity affects not only the activity of a given element or molecule against microorganisms, but also modifies metabolic and material transport systems of uni- and multicellular organisms. From this aspect, key questions in nanotoxicology are how nanoparticles enter cells and undergo within-cell or within-organism distribution; how cells or organisms are capable of dealing with these substances; how their biochemical mechanisms adapt or are evolved to process or metabolize nanoparticles. Regulation of the use of nanoparticles/nanotechnology has commenced at national and international levels: while allowing technological advancement in utilizing the microbiological/biochemical efficacy of nanoparticles, cautious measures have to be implemented to ensure food and environmental safety.

Keywords nanotechnology; antimicrobial effect; biofilm; packaging; AFM; biosensor

1. Introduction

A nanomaterial is defined as an “insoluble or biopersistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nanometres” as detailed in the recent EC Cosmetics Regulation [1]. Efforts are underway to establish a more comprehensive definition for nanomaterials. Hence, this is a provisional term until a uniform, European and international definition is made available [2]. Nanotechnology refers to a broad area of technological activity focused on the engineering and manipulation of these nanomaterials or nanoscale structures. As the physico-chemical properties of nanostructures are not governed by the same laws as larger structures, but by quantum mechanics, it is observed that these materials do not behave like their macroscale counterparts, and unique properties arise at the nanoscale. Most probably due to the higher surface/volume ratio (dispersity), colour, solubility, diffusivity, material strength, toxicity, also thermodynamic, magnetic, optical and other properties will be very different at the nanoscale as compared to the macroscale.

At present nanotechnology is being used in a range of applications, from the design of computer chip layouts and new polymers to commercial applications as in cosmetics and suntan lotions, drug delivery, surface coatings, and there is also the potential for food industry [3, 4]. The U.S. Department of Agriculture was the first to discuss at length the potentialities of nanotechnology in the agriculture and food sector in 2003. According to data from the Helmut Kaiser Consultancy (HKC) in 2007, there were more than 600 nanofood products already available on the global market, and approximately 250 firms were dealing with nanofood, primarily in the USA, Japan and China [5].

Nanofood is not by all means food modified at atomic level, or produced with nanoequipment. Nanofood is a product that was grown, processed or packaged with the help of nanotechnology or of equipment created with nanotechnology. The potential benefits for application of nanotechnologies in food production have been widely discussed and cover many aspects, such as efficient nutrient delivery, formulations with improved bioavailability, new tools for molecular and cellular detection of contaminants and food packaging materials [6-8]. There are a number of other emerging applications of nanotechnologies that could offer innovative solutions to the food sector and related industries or services. Examples include the use of nanoporous materials for water filtration and for removal of undesirable tastes, flavours or allergens from food products. Other developments nearing market include nano-coatings (e.g. of titanium dioxide) for photocatalytic sterilisation of surfaces and water, nano(bio)sensors for food safety, and nanobarcodes for food authenticity [9].

At the same time, the rapid emergence of nanotech applications in consumer products has raised a number of ethical and societal concerns ranging from possible health risks of using or consuming nanoenabled products, to their effects on the environment, intellectual property rights governing them, and the new privacy challenges they may raise [10]. The developments in food nanotechnology are comparable to those of genetically modified foods, another area that has been controversial, with many consumers being suspicious of the technology involved.

The Soil Association, the leading certifier of organic products in the UK, published the world’s first standards banning nanotechnology in 2008. They claimed that the risks of nanotechnology are still largely unknown, untested and unpredictable, and initial scientific studies show negative effects on living organisms. The organisation says that...
nanoparticles, believed by developers to offer advantages in formulation, especially in health foods, are made by “potentially toxic technology that poses a serious threat to human health”. Indeed, there are gaps in our knowledge regarding nanotechnology: A clear, fit-for-purpose, definition of nanomaterials and nanotechnologies is needed. Moreover, validated methods for detection and characterisation of nanomaterials in complex food matrices are not currently available.

Among others, Australia, Canada, the European Union, Japan or the United States have set up guidance documents and/or procedures for nanoenabled products based on existing regulations [11-14]. Nonetheless, most countries still have to decide if and how they want to regulate nanoenabled agro-food products. Options vary from self-regulation, to permits, standards and disclosure requirements, and by level of stringency, from a blanket ban to a simple data collection requirement. In the EU, the Directorate General of Health and Consumer Protection has set up the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR). This committee provides opinions on questions concerning emerging or newly identified health and environmental risks on issues which require a comprehensive assessment of risks to consumer safety or public health [15].

Nanomaterials are entering the food chain through well-known food products and their packaging, yet not only our knowledge about the possible effects of these substances on human and environmental health is rather limited, but food companies do not tend to acknowledge their intentional or often even unaware use: according to a recent survey, only 26 out of 2500 companies, including world-famous multinational food manufacturing corporations, responded to the request of information, and only 14 declared that they do not use nanomaterials. Nanomaterials are currently being used in the food industry [16] and in the agroeconomy sector in a broader range [17], mainly as agents for food packaging, substances to modify food characteristics and novel nanoencapsulation formulating agents for pesticides. The first part of our present overview summarizes various antimicrobial applications of nanomaterials in the food industry.

Nanotechnology is playing an increasingly important role in the development of biosensors. Sensitivity and other attributes of biosensors can be improved by using nanomaterials in their construction. Novel nanomaterials for use in bioassay applications represent a rapidly advancing field. In the last decade, various nanostructures have been investigated to determine their properties and possible applications in biosensors. These structures include nanotubes, nanofibres, nanorods, nanoparticles and thin films. In the second part of this overview biosensor techniques for microbial detection and microbial sensors are reviewed, in view of new optosensors, e.g. optical waveguide lightmode spectroscopy (OWLS) methods applying thin films, and of biosensors with enhanced sensitivity through nanoparticles and nanotubes.

The effect of nanoparticles used by different industries, including the food industry, on the target microbes and also on other living organisms in the environment (i.e. on the natural soil microbiota, the beneficial microbiota of the human and/or animal body, on the plants, and on the animal and human organism itself as well) is of great interest. The fate of nanoparticles applied in any form and step by the (food) industry after their useful life is over is also to be investigated. It is obvious that nanodispersity may affect not only molecular characteristics and biological effects we consider beneficial, but also those that have unfavourable impacts on human or environmental health [18-21]. Thus, nanodispersity not only provides novel physico-chemical characteristics, allowing outstanding microbial activities, but also modifies the cellular and ecotoxicological effects. Moreover, these effects may change by ageing of the nanodisperse system (through modification of particle size distribution by time), and various metabolic and material transport systems may also be affected in biological organisms, based on diffusional (passive), channel-based (active) or vesicular (endocytosis) incorporation of (bio)chemical substances and (bio)physical particles into cells, as well as macrophage-based removal (phagocytosis) from higher organisms, as summarised in the third part of this overview.

2. Application of antimicrobial nanodisperse systems in food industry

Nano-sized materials or nanotechnological steps can be applied at any stage of food production, i.e. during processing, packaging, labelling, transporting or even during tracing the food in question. Nanotechnology can be a part of the construction in the instruments used for processing nanomaterials, or the operation can be nanotechnology driven. Nanoparticles are to be used in foods as emulsions, composites or nanostructured materials. Built into the packaging materials they can play a role in ensuring microbial safety of food and in keeping the quality of food product.

2.1. Preservation

A key problem in food preservation is that the widespread production, use, and misuse of antibiotics resulted in the emergence of multiple drug resistant (MDR) infectious organisms. Recently, nanotechnology, specifically the use of nanomaterials with antimicrobial activity, has been presented as a new defence against MDR infectious organisms. They present a different approach to damaging microbial cell functions. Rather than focusing upon particular biochemical processes, as is the case with traditional antibiotics, they are likely to disrupt multiple cellular processes in a less specific fashion. This multifaceted approach may render it more difficult for microbes to develop resistance [22].

The commercially available nanosilver products make broad claims about the power of their nano-Ag ingredients, such as: “eliminates 99% of bacteria” renders material “permanently antimicrobial and antifungal”, “kills approximately
650 kinds of harmful germs and viruses”, and “kills bacteria in a short time as 30 minutes … 2-5 times faster than other forms of silver” [23]. Indeed, silver has a long history of being used as an antimicrobial agent in food and beverage storage applications. Numerous ancient societies stored wine and water in silver vessels. Anecdotal reports on the historic uses of silver include the examples of early American or New World settlers placing silver dollars or silver spoons at the bottom of milk and water bottles to prolong shelf-life, and of seafaring ships or airliners lining their water tanks with silver to keep water potable for long periods of time [24]. Silver was the sterilisation agent for water on the Russian MIR space station and on NASA space shuttles [25], and the broad-spectrum antimicrobial activity and relative low cost of silver have made it a candidate as the active disinfecting agent for water in developing countries [26]. In 2009, the U.S. Food and Drug Administration (FDA) modified the food additive regulations to permit the direct addition of silver nitrate as a disinfectant to commercially bottled water at concentrations not to exceed 17 µg/kg [27]. It is also being used in the medical sciences, i.e. against antimicrobial-resistant bacterial strains (e.g. multi-drug resistant varieties of methicillin-resistant Staphylococcus aureus), which have become a concern in hospitals. A definite advantage is that silver is capable to penetrate into biofilms. To date, several mechanisms have been postulated for the antimicrobial property of silver nanoparticles (AgNPs): adhesion of nanoparticles to the surface altering the membrane properties – AgNPs have been reported to degrade lipopolysaccharide molecules, accumulate inside the membrane by forming “pits”, and largely increasing membrane permeability [28]; AgNPs penetrating inside bacterial cell, resulting in DNA damage; dissolution of nano-Ag releases antimicrobial Ag+ ions [29]. The emergence of resistant bacterial strains is reported for silver as well, but is considered less probable than for synthetic antibiotics [30]. However, AgNPs used as drug disinfectant present certain risks by being toxic to mammalian cells [31], exerting toxicity on the liver and hepatocytes [32] and being capable of crossing the blood-brain barrier [33, 34] in rats; beside the long-evidenced health consequence that exposure to silver may cause argyrosis and argyria.

Copper nanoparticles have also been reported to be highly toxic against a wide variety of bacteria and fungi due to their high surface-to-volume-ratio, and generally kill cells by diverse mechanisms, such as membrane disruption, blocking biochemical pathways, complex formation with proteins, and DNA damage [35-37]. However, copper nanoparticles are known to be extremely sensitive to oxygen forming copper oxide nanoparticles, so it is desirable to use a matrix that will bind and protect the particles from an oxidizing environment [38]. On the contrary, other researchers claim that highly ionic nanoparticulate metal oxides, such as CuO, may be particularly valuable antimicrobial agents as they can be prepared with extremely high surface areas and unusual crystal morphologies [39].

Titanium dioxide is the most commonly used semiconductor photocatalyst. Activated by UV-A irradiation, TiO2 can kill both Gram-negative and Gram-positive bacteria, although some Gram-positive bacteria are less sensitive due to their ability to form spores [40]. More recently, nano-sized TiO2 was also reported to kill viruses including poliovirus 1 [41], hepatitis B virus [42] and Herpes simplex virus [43] through the formulation of hydroxyl free radicals and peroxide. It is of increasing concern, however, that TiO2 nanoparticles were demonstrated to induce in vivo side-effects in mice such as DNA damage and genetic instability [44], and therefore, their precautious and strict toxicity testing is urged [45].

Similar to TiO2, nano-sized zinc oxide has been used in sunscreens, coatings, and paints because of its high UV absorption efficiency and transparency to visible light. ZnO nanoparticles exhibit strong antibacterial activities on a broad spectrum of bacteria, though its antibacterial mechanism is still under investigation. The photocatalytic generation of hydroxyl peroxide was suggested to be one of the primary mechanisms. In addition, penetration of the cell envelope and disorganisation of bacterial membrane upon contact with ZnO nanoparticles were also indicated to inhibit bacterial growth. However, the role of Zn2+ ions released from dissolution of ZnO is not clear. It has been suggested that Zn2+ ions binding to the membranes of microorganisms can prolong the lag phase of the microbial growth cycle. Contradictory results have been reported about the impact of particle size on the antibacterial activity of ZnO. Some authors observed that smaller ZnO particles were more toxic than bigger particles, but no size related effect was found in another study. The related literature is summarised by Li et al. [46].

Other nanoscale materials with antimicrobial properties include nanoparticles based on magnesium oxide, cadmium selenide/telluride, gold, alginate, antimicrobial peptides, chitosan, fullerene, as well as carbon nanotubes. Several of these studies are targeted specifically at food or food packaging applications, and a recent publication reviewed the numerous classes of nanomaterial antimicrobials targeted for use in drinking water sterilisation [24]. The conventional bulk forms of silica and titanium dioxide are permitted food additives (SiO2 – E551 and TiO2 – E171), but there is a concern that the conventional forms may also contain a nano-sized fraction due to natural size range variation [47].

What is currently known about the mechanisms behind the antimicrobial activity of nanomaterials still indicates that the development of resistance is possible. Nanomaterials have the potential to induce horizontal gene transfer in environmental systems, which could lead to increased resistance. Nanoalumina (aluminium oxide, Al2O3) can promote the conjugal transfer of the RP4 plasmid from Escherichia coli to Salmonella spp. by up to 200-fold compared with untreated cells. The mechanisms behind this phenomenon has been investigated and it was demonstrated that nanoalumina is able to induce oxidative stress, damage bacterial cell membranes, enhance the expression of mating pair formation genes and DNA transfer and replication genes, and depress the expression of global regulatory genes that regulate the conjugal transfer of RP4. These findings are important in assessing the risk of nanomaterials to the
environment, particularly from water and wastewater treatment systems, and in the estimation of the effect of manufacture and the use of nanomaterials on the environment [48].

Beside the possible antimicrobial effect, nanomaterials also offer the possibility of a more efficient and targeted delivery of antibiotic agents. Better drug delivery can lower the likelihood of sublethal dosing of antibiotics as well as broad spectrum microbial exposures, which in turn could reduce the development of resistance to nanomaterials. Ceramic- or clay-based antimicrobial agents are a new breed of materials which act as a template on which an antimicrobial agent is absorbed or embedded due to high surface area. The advantage is the protection and slow release of the agents from the ceramic matrix increasing the overall efficacy of the material. Also naturally occurring clay minerals and their ceramic derivatives are chemically inert and nontoxic to the environment [15, 49].

2.2. Inhibition of biofilm formation

Formation of a biofilm begins with the attachment of free-floating microorganisms to a surface through weak, reversible adhesion via van der Waals forces. If the colonists are not immediately separated from the surface, they can anchor themselves more permanently. The first colonists facilitate the arrival of other cells by providing more diverse adhesion sites and beginning to build the matrix that holds the biofilm together. Some species are not able to attach to a surface on their own, but are often able to anchor themselves to the matrix or directly to earlier colonists. Once colonisation has begun, the biofilm grows through a combination of cell division and recruitment. The final stage of biofilm formation is known as dispersion, and is the stage in which the biofilm is established and may only change in shape and size.

Biofilm formation on surfaces has serious economic and environmental implications. Biofilm growth within a water distribution system, in industrial water cooling towers, in food processing areas, where water is permanently and abundantly used (i.e. fresh meat including poultry, cattle, sheep, goats, pigs, horses, as well as processing of vegetables including pre-cut fruit and vegetables, sprouting) can lead to problems, such as biocorrosion and biofouling accumulation. Biofouling readily occurs within a short time even on stainless steel and cannot be entirely removed by the cleaning processes. Fig. 1 shows an example from the poultry processing industry. The new, highly polished surface of stainless steel opening knife (a) becomes heavily fouled when used (b), and after the thorough cleaning at the end of the shift still shows a more or less continuous covering of the blade with fat and proteins. Milk processing systems are also prone to biofilm formation. Biofouling and biofilm formation is a problem not only to the food processing industry, but also to drinking water distribution systems. To prevent and control these occurrences, it is necessary to use suitable biocides to remove the biofilm and kill biofilm cells [50].

The primary colonisation strongly depends on the composition of materials, their chemical stability, as well as their surface properties. In most natural environments, association with a surface in a structure known as a biofilm is the prevailing form of microbial life. Microbial communities grow more stable when associated with surfaces or organised in aggregates [51]. Ponsonnet et al. [52] examined the pH variations in the local microenvironment created between the cell layer and the surface after bacterial adhesion. Van Houdt and Michiels [53] gave an overview of the occurrence, production and interaction of cell surface molecules and structures implicated in biofilm formation in E. coli, their influence at one or more developmental stages of biofilm formation. Therefore, the development of materials that inhibit the primary attachment of microbes and fouling materials to the surfaces are important. For non-food industrial purposes the application of Langmuir-Blodgett nanolayers of alkyl hydroxamic and phosphonic acids on iron and copper surfaces can be an effective way to prevent microbial corrosion of metals by reducing the attachment of corrosion associated microorganisms, and at the same time also showing bactericide or bacteriostatic activity [54].

Biofilms also harbour pathogenic microorganisms, among others Listeria monocytogenes, and are a possible continuous source of contamination. Chae et al. [55] found that epidemic strains of the bacterium had significantly
higher attachment ability than sporadic strains, but subsequent biofilm growth over 24 hours was not dependent on initial attachment. Studying the different microbial cell wall properties important in the attachment process, they found that neither surface charge, nor surface hydrophobicity were significantly correlated with the attachment to surfaces. The investigations proved that the ability of a *L. monocytogenes* strain to produce high levels of extracellular carbohydrates increases its ability to form biofilm. Atomic force microscopy (AFM), hydrophobicity and electron acceptor capability measurements were applied to study the surface properties of different materials used in kitchens to investigate the attachment of *L. monocytogenes* to surfaces and their viability [56]. It was found that the highest percentage (nearly 100%) of attached cells were viable on polypropylene surfaces, while significantly less (69.5 and 78.7%) cultivable cells were detected on marble and granite. The lowest percentage (18.5%) of culturable bacteria was found on a synthetic white silestone composite consisting of 94% quartz with antibacterial agent Triclosan incorporated. Cell viability is important, because only adhered cells that remain viable are responsible for post-process contamination. Recently enzyme-based listericidal nanocomposites have been developed [57]. Three facile routes have been reported for surface incorporation of the listerial bacteriophage endolysin Ply500: covalent attachment onto FDA approved silica nanoparticles (SNPs), incorporation of SNP-Ply500 conjugates into a thin polyhydroxyethyl methacrylate film; and affinity binding to edible cross-linked starch nanoparticles via construction of a maltose binding protein fusion. These Ply500 formulations were effective in killing *L. innocua* both in PBS and on lettuce.

Prevention of biofilm formation may be achieved via two routes (or their combination): either by inhibition of the attachment of bacteria to surfaces by the application of specific covering material, and/or by killing the bacteria (viruses, fungi) adhered to the surface. Main problems are the strength of adherence and the stability of the covering material, as well as its resistance against the food it gets in contact with. In case of microorganisms captured by water or air filters, the distribution of antimicrobial agents throughout the nanofibres of filter membranes would provide a cost-effective solution [58]. It is also a significant problem that microbes in the biofilm gain increased resistance to disinfectants and antibiotics due to better avoidance of exposure, than free floating cells [59, 60]. Living in the dense and protected environment of the biofilm allows bacteria to cooperate and interact in various ways. The extracellular matrix and the outer layer of cells protect the interior of the community. As described in the subchapter before, an alternative for antibiotics is the use of nanomaterials with antimicrobial properties, though it is highly probable that the protecting effect of the biofilm in that case would also prevail. Even the proven antimicrobial effect of some nanoparticles is dose dependent. An interesting phenomenon was observed by Shahrokhi and Emritzian, demonstrating that nanosilver at low concentration acts as a catalyst to increase bacterial metabolism. According to their results, 0.2 ppm of nanosilver promoted the formation of biofilm, while above this concentration it inhibited biofilm formation by different Gram-positive and -negative bacteria [61].

### 2.3. Packaging

Whilst most nanotechnology applications for food and beverages are currently at R&D or near-market stages, applications for food packaging are already becoming a commercial reality. Food packaging applications form the largest share of the current and short-term predicted market for nano-enabled products in the food sector [9]. When integrated into food packaging, nanosensors can detect specific indicators of pathogen metabolism or can alert or inform the consumer about a product’s temperature, light or O2 exposure (tampering) history. This could eliminate the need for expiry dates in some incidences and may even give the consumer a more accurate estimation of the state of spoilage of the food.

The combination of food packaging materials and active substances is a new way to control surface microbial contamination of foods. Some nanomaterials exhibit antimicrobial effects, as mentioned earlier. For such active packaging materials, sharing a common interface or physical contact with the food surface is essential [62]. These active food contact materials can extend the product shelf-life, enhancing food quality and safety and ultimately leading to less food waste.

Polymer nanocomposites are the latest materials to solve the multifaceted problem of food packaging (being most important the permeability to gases, water vapour or natural substances, and the protection against dirt, dust, light and pathogenic microorganisms). Filler materials can be wide varieties of nanostructured materials including clay and silicate nanoplatelets, silica nanoparticles, carbon nanotubes, graphene, starch nanocrystals, cellulose-based nanofibres, as well as chitin or chitosan nanoparticles or cyclodextrins. Next to their good techno-functional and other useful properties, they are environmental friendly materials, rendering the packaging material to degrade faster and better, and their cost effectiveness is also better [24].

Metal nanomaterials received substantial attention in this area, and products utilising the unique antimicrobial properties of metals, such as silver and gold, have been launched onto the market. Metal oxides have also been incorporated into commercialised products displaying light activated microbe inactivation. Ag nanoparticles absorb and decompose ethylene [63], thus food packaging films which incorporate Ag impart this effect on the associated food. This may contribute to its positive effects on the shelf-life of fruits and vegetables, by inhibiting senescence. Side-effects are reported to be rare, when AgNPs are applied at doses permitted by the FDA [64]. The antimicrobial efficacy of AgNPs has been studied against a number of bacteria, among them those of food safety concern, too. It was also
found, that AgNPs are effective against silver resistant strains of i.e. *E. coli* [65]. Inorganic nanoparticles, like AgNPs, can easily be incorporated into polymers while keeping their antimicrobial activity [66].

### 3. Application of nanotechnology-based microbial sensors in food analysis

Various nanostructures have been investigated to determine their properties and possible applications in biosensors to improve analytical characteristics such as sensitivity, limit of detection (LOD), miniaturisation or reuseability. These structures include nanotubes, nanofibres, nanorods, nanoparticles and thin films [67]. In addition, nanostructured sensor design also plays an increasingly important role in the improvement of analytical characteristics through quantum size effect, surface effect and macro-quantum tunnel effect.

There are numerous types of biosensors applying nanotechnology, also gaining different applications in microbial analysis for special aims, e.g. detection of specific bacteria, biofilm formation by bacteria, and development of microbial inhibition tests (Fig. 2). Due to the low infectious doses for most food-borne pathogens, rapid and sensitive detection methods are essential to ensure food safety. Advances in the development of nanomaterials have stimulated worldwide research in their applications for bioanalysis. Conjugation of biomolecules with nanomaterials serves as the foundation of nano-biorecognition. A variety of strategies including antibody–antigen, adhesin–receptor, antibiotic and complementary DNA sequence recognition have been explored for specific recognition between target bacterial cells and biofunctionalised nanomaterials. The incorporation of these biofunctionalised nanomaterials into current pathogen detection methods has led to rapid and nearly real-time pathogen detection, improved sensitivity and simultaneous detection of multiple microorganisms from either nutrient broth, liquid or solid food products, or biofilms [68].

#### 3.1. Biosensors for specific detection of bacteria

Selective discrimination of bacterial strain types and detection of actual cell concentrations is of great importance in all areas of microbial research including toxicity monitoring, clinical diagnostics, food safety and environmental science [73]. Novel sensitive sensing molecules (antibodies, lectins) and immobilisation methods, enhanced sensitivity and selectivity are needed for real-time detection of contaminating microbes present in different samples. Fig. 2a presents different strategies for immunosensing. Using immobilised antibodies as selective capture agents (*left*), bacterial substances or cells bind selectively to the immunoactivated sensor surface. Alternatively, when the surface of the immunosensor for organic microcontaminants, proteins or microbes is sensitised with an antigen or its protein conjugate (*right*), the microbial analyte content in the sample inhibits binding of the antibodies to the immunoactivated surface in a competitive manner. Another means of sensitivity enhancement is, when the sample and the antiserum are preincubated prior to being injected onto the sensor surface, and detection is carried out as a binding inhibition test, resulting in a further decrease in the LOD relative to the direct method. The signal measured by the immunosensor can further be amplified by nanoparticles coupled to the antibody used (Fig. 2b).

![Fig. 2 Thin film based optical biosensors for detection of microbial substances or cells.](image_url)

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The most studied and developed methods are for the selective determination of *E. coli* strains, especially *E. coli* O157:H7. This *E. coli* strain causes a severe food-borne disease through its secreted powerful poison, called verotoxin that binds to receptors in the human kidney, brain and gut cells, and leads to the death of the affected cells. *E. coli* O157:H7 is transmitted primarily through consumption of contaminated foods, such as raw or undercooked ground meat products, raw milk and contaminated raw vegetables and sprouts. Different types of immunosensors are reported for selective determination of *E. coli* cells. Subramanian [74] demonstrated a polyethylene glycol terminated alkanethiol mixed self-assembled monolayer on a surface plasmon resonance (SPR) immunosensor using purified monoclonal or polyclonal antibodies against *E. coli* O157:H7 immobilised on an activated sensor chip. Washa *et al.* [75] used SPR-based biosensor to detect *E. coli* in spiked skim milk using specific antibodies. Tan *et al.* [76] developed a poly(dimethylsiloxane) microfluidic immunosensor integrated with specific antibody immobilised on an alumina nanoporous membrane for rapid detection of food-borne pathogens *E. coli* O157:H7 and *S. aureus* with electrochemical impedance spectrum. Antibodies to the target bacteria were covalently immobilised on the nanoporous alumina membranes via self-assembled (3-glycidoxypropyl)trimethoxysilane. The impedance spectrum was recorded for detection of bacteria at frequencies ranging from 1 Hz to 100 kHz. Joung *et al.* [77] developed a nanoporous membrane-based impedimetric immunosensor for label-free detection of bacterial pathogens, such as *E. coli* O157:H7, in whole milk. The ionic impedance of electrolytes through alumina nanopores, due to antibody–pathogen interactions, was monitored by impedance spectra and analysed by normalised impedance change. An electrochemical-OWLS (EC-O WLS) technique was used in direct microbiological measurements applying polarisation potential in the measuring cell to facilitate the adsorption of *E. coli* bacteria. Significant difference was observed between the sensor signal of living and heat treated cells, so the mass of the immobilised cells and the ratio of living and immobilised cells were determined [78].

An SPR-based biosensor was developed for the rapid identification of *Campylobacter jejuni* in samples of broiler chicken. The specificity and sensitivity of commercial antibodies against *C. jejuni* was tested with *Campylobacter* strains and also with non-*Campylobacter* bacterial strains [79]. *S. typhimurium* as a common food-borne pathogen associated with the processing of poultry was determined by an SPR immunosensor that showed promising perspectives to detect the presence of *S. typhimurium* at 1×10³ CFU/ml [80].

Xi *et al.* [73] investigated a label-free impedimetric biosensor for discrimination and detection of bacteria based on the affinity of lectin with glycosyl complex on cellular surface. Two plant lectins, concanavalin A and *Ricinus communis* agglutinin, were deposited via electrostatic interaction on electrodes modified with polyelectrolyte film. The thus created lectin–polyelectrolyte film surface provided an appropriate biomimetic interface for specific adhesion of cells, e.g., Gram-negative (*E. coli* DH5a and *Enterobacter cloacae*) or Gram-positive (*Bacillus subtilis*) bacteria, yeast (*Saccharomyces cerevisiae*) and a mammalian cell line (HeLa). Wan *et al.* [81] reported an impedimetric immunosensor for rapid and non-labelled detection of sulphate-reducing bacteria, *Desulfovibrio caledoiensis* by immobilising lectin–concanavalin A using an agglutination assay. The immobilisation of lectin was conducted using amino coupling on the surface of a gold electrode derivatised with 11-mercaptoundecanoic acid.

DNA biosensors have gained increased attention over traditional diagnostic methods due to their fast and responsive operation and cost-effective design. The specificity of DNA biosensors relies on single-stranded oligonucleotide probe immobilised to a transduction platform. Mao *et al.* [82] investigated a QCM/DNA sensor based on the nanoparticle amplification method for the detection of *E. coli* O157:H7. A thiolated single-stranded DNA (ssDNA) probe specific to *E. coli* O157:H7 eaeA gene was immobilised onto the QCM sensor surface through self-assembly. The hybridisation was induced by exposing the ssDNA probe to the complementary target DNA, and subsequent mass and therefore frequency change of the QCM. Streptavidin conjugated Fe₃O₄ nanoparticles (average diameter = 145 nm) were used as “mass enhancers” to amplify the frequency change. Mo *et al.* [83] provided a QCM-probe biosensor based on thiol-derivatised *E. coli lacZ* gene probe. After the bacterial DNA was purified from the lysate, amplification by polymerase chain reaction (PCR) of a segment of the coding region of *E. coli lacZ* was carried out. The LOD of the combination of the PCR amplification and QCM detection was found to be < 10 fg of genomic *E. coli* DNA and as low as several viable *E. coli* cells in 100 ml of water. Thyu *et al.* [84] presented a cost-effective routine for the direct dispersion of multi-walled carbon nanotubes in DNA solution for detecting *E. coli* O157:H7.

Gnanaprakasa *et al.* [85] developed biosensor to detect the hippuricase gene (*hipO*) from *C. jejuni* using direct covalent coupling of thiol- and biotin-labeled ssDNA on both SPR and diffraction optics technology (DOT, dotLab) transduction platforms. A label-free detection system for DNA strands based on gold electrodes and impedance measurements was investigated by the immobilisation of 18-mer ssDNA via a thiol linker on gold film electrodes [86]. A new procedure based on photodeposition of AgNPs at TiO₂-coated piezoelectric quartz crystal electrode was developed to fabricate a highly sensitive PQC/DNA biosensor [87]. A rapid and reliable technique was developed for simultaneous detection of *S. typhimurium*, *E. coli* O157:H7 and *L. monocytogenes*. Magnetic nanobead-based immunomagnetic separation was used to separate the target bacterial cells while multiplex PCR was used to amplify the target genes in different food products [88]. Zhao *et al.* [89] constructed a rapid and sensitive DNA strip sensor, based on gold nanoparticle-labelled oligonucleotide probes for the detection of *Acidovorax avenae* subsp. *citrulli*. Both qualitative and semi-quantitative detection of the target DNA were proven, the qualitative LOD of the strip sensor was determined to be 4 nM.
The determination of the secondary metabolites of the different microbes is also an important task for immunosensor investigation. Sensitive detection of biogenic amines in raw, processed or fermented food by biosensors is of growing importance, due to the significant correlation of the occurrence of these substances with food contamination with microbes producing, among others, histamine, known to exert allergenicity [90]. Additional often studied mould secondary metabolites include mycotoxins, e.g. aflatoxins, ochratoxins [91-93], zearalenone [94, 95] and deoxynivalenal [96, 97].

3.2. Sensors to detect biofilm formation by bacteria on surfaces

In order to study the properties of formed biofilm or to develop anti-biofilm substances and surface treatments, it is of great importance to identify the physico-chemical parameters, which activate the sensor systems of colonist microbes, when they come into contact with a surface. Several methods are available to study biofilm formation. Investigating the effect of nanolayers on the microbial adhesion to and biofilm formation on different materials (glass, metals) next to the electrochemical measurements and traditional microbiological methods, AFM and quartz crystal microbalance (QCM, also termed quartz crystal nanobalance) were also used [54, 98, 99]. Over the traditional plating, surface microscopy, epifluorescence microscopy, scanning electron microscopy, as well as combination of biochemical methods and microscopy (fluorescence in situ hybridisation) the application of nanotechnology is becoming more common, such as AFM, QCM, total internal reflection microscopy, total internal reflection aqueous fluorescence microscopy and the application of biosensors. E. coli has been a useful model for the study of biofilm formation [100]. Ramsden et al. [101] applied the OWLS method for accurately parameterising the number and the shape of spreading cells. It has been observed for numerous types of cells that when a cell suspension is seeded onto a surface, cells will initially attach and subsequently undergo reorganisation that results in a flattening and spreading of the cell body. This implies that a small and subsequently growing flat region is being formed at the initial point of contact. It was concluded, that only the shape of cells changed, their number remained constant. The change in the measured signal resulted from the flattening and spreading of the cells on the sensor surface within a short time. The sensory method to detect microbial biofilm formation using biocompatible surface coating is depicted on Fig. 2c. QCM was also used as an extremely sensitive device for monitoring the biofilm formation and the inhibitory effect of biocides [99].

Komaromy et al. [102] successfully used e-beam lithography to produce arrays with gold/silicon oxide nanostructures in the form of dots and lines of different sizes and with different spacing. To assess the propensity of bacterial cells to adhere to these nanostructure arrays, these new materials as well as the planar reference surfaces were incubated with suspensions of E. coli and S. aureus. The results, as images of live cells or even cell damage or distortion on dot and line gold/silicon oxide nanostructured surfaces were evaluated with differential interference contrast microscopy. The use of nanostructured arrays for probing of bacteria–surface interaction may provide insight useful for testing special materials with pro- or antibacterial properties. While traditional light microscopy is capable of visualising structures of the size not smaller than cell organelles or viruses, smaller cellular structures (such as microfilaments, small membrane structures) down to globular proteins and amino acids and small bioactive molecules are visualised by microscopy techniques with resolution of atomic level, e.g. electron microscopy or scanning probe microscopy (such as AFM or scanning tunnelling microscopy). Other scanning surface investigation methods are also useful, i.e. confocal laser scanning microscopy that provides three-dimensional visualisation of thick biofilms of several cell layers [103]. For tapping surfaces and nanoscale analysis of microbial cells, AFM is rapidly evolving [54, 104]. AFM is also expected to be well-applicable for visualisation of binding/detachment of microbial antigens on/from immunosensor surfaces (as demonstrated previously for antigens of an organic microcontaminant trifluralin [105, 106], depicted in Fig. 3) or the effects of heat treatment or irradiation on the spores of B. cereus (as will be investigated in a current EU FP7 project SPICED “Securing the spices and herbs commodity chains in Europe against deliberate, accidental or natural biological and chemical contamination”).

Fig. 3 AFM image in two- (left) and three-dimensional (right) representation of anchoring a trifluralin-BSA conjugate on the aminossilanised SiO2-TiO2 surface of an OWLS sensor [105, 106].
3.3. Sensors based on inhibition of bacterial viability on surfaces

The chemical and mechanical stability of the applied microorganisms, as well as the techniques used for their immobilisation are often critical factors for the applicability of the microbial inhibition test technology [107-109]. Living bacterial cells could be immobilised by physical and chemical methods onto the measuring surface of the sensors. The method of immobilisation is extremely important, because it can influence cell viability. Horváth et al. [110] tested a grating-coupled planar optical waveguide sensor by monitoring the adhesion of E. coli K12 cells to the sensor surface. Label-free measurement techniques based on cysteine-terminated synthetic oligopeptides used for immobilisation of E. coli O157:H7 on gold substrate have been investigated. The attached living cells were exposed to toxic chemicals such as phenol, which induced a shift in the SPR angle [111]. An EC-OWLS technique without chemical modification during the immobilisation of bacteria was applied to study the stress effect of lactic acid bacteria [70, 71]. Different natural stress factors (e.g. acetic acid, lactic acid, hydrogent peroxide) were studied for their influence on the survival of lactic acid bacteria. Fig. 2d shows the sensing method of immobilised microbe-based sensor for cellular inhibition tests. Recently, a new group of enzymes, so-called silicateins, which form the axial filaments of the spicules of the siliceous sponges, have been identified and characterised. Silicateins catalyze the polycondensation and deposition of amorphous silica at mild conditions (low temperature and physiological pH). Thus, recombinant silicateins expressed in E. coli is applicable to form polysilica web around bacterial cells. Adányi et al. [72, 112] studied the immobilisation of silicatein modified E. coli BL21AI cells on SiO2-type chips and their biological properties, in particular their inhibitory effect of stressors/environmental pollutants on the novel bacterial sensor. The novel immobilisation strategy provided a simple technology for the development of microbial sensors, allowing real-time detection of the inhibitory effect of stressors/environmental pollutants.

4. Cellular and ecotoxicological effects of nanoparticles from the aspects of food safety

Due to their nanoscale fine structure and corresponding physicochemical characteristics, nanomaterials may possess unique biochemical and biological properties upon entering biological organisms, e.g. living cells or complex organisms, as compared to either true atomic/molecular solutions or larger particles of the same material. Toxicological research on nanomaterial safety is in its infancy. It is not known how the ingested nanoparticles will behave in the body; research in this area is at an early stage. The long term health consequences (if any) of ingestion of insoluble and biopersistent (‘hard’) nanoparticles via food are currently not known. There are considerable gaps in our understanding regarding the way nanoparticles interact with single cells (including bacteria) or multicellular organisms (including the human body). The limitations in knowledge are partly due to the lack of methodology for the detection and characterisation of engineered nanoparticles in complex matrices, e.g. water and food. The characteristics of these nanoparticles are likely to influence their absorption, distribution, metabolism and excretion. The metabolism (digestive) and material transport systems of uni- and multicellular organisms are based on diffusional (passive), channel-based (active) or vesicular (endocytosis) incorporation of biochemical substances and biophysical particles into cells, as well as macrophage-based removal (phagocytosis) from the living organisms. This is of essential importance in the incorporation of nanoparticles into living cells and multicellular organisms in two aspects: (a) how nanoparticles may enter cells and undergo within-cell or within-organism distribution; and (b) how cells or organisms are capable of dealing with these substances, i.e. how their biochemical mechanisms are capable or evolved to process/metabolize nanoparticles. These are key aspects that determine nanotoxicity at cellular or organism-wide level.

An essential defence line of organisms is composed of macrophages that engulf foreign substances and particles, and transport them to decomposition or removal from the organism. Macrophages typically recognize substances by biochemical (antigenic) features or particles by size (< 65 nm). The capacity of this defense mechanism is limited by the macrophage forming capacity of the organism, and is practically saturated or inactivated, when the number of the foreign substances entering the organisms surpasses the capacity of the macrophages. In addition, these processes not only may inactivate, but also possibly overactivate macrophage defence, causing dysfunction.

Mechanisms of cellular toxicity of nanoparticles include physical damage (accumulation on cell membranes or on organelle surfaces); membrane perturbation (lipid peroxidation, surfactant effects); effects on proteins and protein folding; and effects on biochemical processes through catalytic (e.g. oxidative damage) or inhibitory interaction. One of the first biological processes between nanoparticles and cells affected by them is interaction with the surface membrane of the target cell. This interaction may lead to membrane damage based on particle properties such as hydrophobicity, electric charge, penetrability, potential to disrupt membrane integrity. Upon entering the cell, nanomaterials as foreign substances may act causing cellular toxicity by key mechanisms including the generation of reactive oxygen species, oxidative stress, and to lesser extent, mitochondrial perturbation, protein denaturation and degradation, disruption of biological membranes, formation of foreign body granulomas, generation of neo-antigens, altered cell cycle regulation and DNA damage. These effects may lead to various symptoms in biological systems including inflammation, apoptosis, necrosis, fibrosis, hypertrophy, metaplasia and carcinogenesis [113].

A phenomenon that renders the assessment of toxic effects of nanomaterials difficult is that spontaneous physicochemical and metabolic processes may alter their toxicity. Size distribution of given nanoparticles varies with...
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Long term drawbacks, it is important to possess the means of evaluating any possible associated risks. The areas of database is required [126]. To avoid the situation in which the technological advantages may be counterbalanced by manufactured nanomaterials used in consumer products may occur during their life cycle, from synthesis, production approach may be required for the risk assessment of nanomaterials. Governments and regulatory bodies are responsible nanotechnologies in the food and drink sector. Due to their modified physico-chemical characteristics, a specific European Commission has already made certified reference material for size determination (SiO2) available and hosts a published a Scientific Opinion on the potential risks arising from nanoscience and nanotechnology on food safety [47]. Some of them are authorised for use in plastic food contact materials. The European Food Safety Authority (EFSA) products coming to the market [127, 128]. The EC demands that nanomaterials must be assessed on an individual basis. clear identification. This is needed to facilitate implementation of labelling requirements, but also to ensure the safety of properly. Regulation specific to nanomaterials requires standardised and harmonised analytical test methods allowing assessment of nanomaterials as evidence that the safety of nanoscale materials may not be directly inferred from data on (as seen for e.g. carbonic anhydrase II) [118, 119].

Nanomaterials may enter multicellular biological organisms via the respective alimentary (ingestional, gastrointestinal), respiratory and integumentary (cuticle, skin) tracts systems and are subsequently translocated, and in cases even reach biochemically protected organs, e.g. by crossing to blood-brain barrier [33, 34]. Nonetheless, exposure takes place as cellular events also in these cases. Alimentary exposure is considered most typical food safety concerns, yet other routes of exposure have also to be considered. Toxicity of inhaled carbon black nanoparticles have been shown to correlate with particle size [120], while regional deposition capacity (and corresponding respiratory toxicity) of particulate matter from Diesel exhaust in the body shows dual maxima at size regions in the nanoparticle (1-50 nm) and microparticle (10 μm) region [121]. Complex toxic effects exerted on the organisms may be of genetic, carcinogenic, immunotoxic or reprotoxic character. Certain nanoparticles (e.g. high aspect ratio nanoparticles nanotubes) exert pulmonary effects similar to asbestos [122-124] due to their size and elongated shape, and therefore, health consequences as severe and long-lasting as for asbestos are being anticipated [125].

As seen, the toxicological profiles of nanomaterials are complex, very much related to given physicochemical and biochemical characteristics, and difficult to predict. Therefore, the approach of the precautionary principle in their safety assessment is to be followed, the regulatory system of the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) has to be extended to cover these substances, and the establishment of a European nanoproduct database is required [126]. To avoid the situation in which the technological advantages may be counterbalanced by long term drawbacks, it is important to possess the means of evaluating any possible associated risks. The areas of concern resemble the debates surrounding previous emerging technologies. Communication is the key to the future of nanotechnologies in the food and drink sector. Due to their modified physico-chemical characteristics, a specific approach may be required for the risk assessment of nanomaterials. Governments and regulatory bodies are responsible to set guidelines or controls to protect those involved in the manufacture or use of nanomaterials. Human exposure to manufactured nanomaterials used in consumer products may occur during their life cycle, from synthesis, production and inclusion in products and the release of these nanomaterials into the environment. There is a clear need to support research into evaluation of exposures to engineered nanomaterials and increased understanding of engineered nanoscale additives and ingredients.

In order to apply nanotechnology safely, legislation and regulation should be effective worldwide. The European Commission (EC) has recently adopted a recommendation for the definition of nanomaterials in order to identify them properly. Regulation specific to nanomaterials requires standardised and harmonised analytical test methods allowing clear identification. This is needed to facilitate implementation of labelling requirements, but also to ensure the safety of products coming to the market [127, 128]. The EC demands that nanomaterials must be assessed on an individual basis. Some of them are authorised for use in plastic food contact materials. The European Food Safety Authority (EFSA) published a Scientific Opinion on the potential risks arising from nanoscience and nanotechnology on food safety [47]. A subsequent EFSA document published guidance on risk assessment [129]. The Joint Research Center (JRC) of the European Commission has already made certified reference material for size determination (SiO2) available and hosts a Repository of Representative Nanomaterials as tools to improve the quality of testing. The FDA issued guidelines in April 2012 outlining nanotechnology [130]. The FDA’s guidance points to the uncertainty regarding toxicity testing and assessment of nanomaterials as evidence that the safety of nanoscale materials may not be directly inferred from data on their traditionally manufactured counterparts [131].

The impact of nanoparticle exposure on laboratory animals (mainly mice and rats) have been tested in several EU funded research programs recently, results even from an in vitro testing system have been validated by in vivo studies (e.g. the InLiveTox project), and the potential genotoxicity of nanomaterials have been assessed (e.g. the Nanogenotox program). In the U.S.A., the Environmental Protection Agency (EPA) and the Consumer Product Safety Commission (CDSC) are working together in research to assess the potential impact of nanomaterials on human and environmental health. Most recently, researchers of the NIOSH, Center for Disease Control and Prevention (CDC) reported preliminary findings in which mice were exposed by inhalation to multiwalled carbon nanotubes, indicating potential to promote cancer as eventual occupational risk [132]. The Federal Institute for Risk Assessment (BfR) and the Federal Environmental Agency (UBA) in Germany also expressed that – despite existing uncertainties – findings on the carcinogenic potential of certain nanomaterials are of serious concern. The U.S. EPA published an „emerging contaminant” fact sheet summarizing, among others, how nanomaterials may impact the environment, what are the
routes of exposure to nanomaterials, and what health effects of they might exert [133]. EPA, in collaboration with the Organisation for Economic Co-operation and Development (OECD), has identified types of nanomaterials of current or potential wide use, including carbon nanotubes, cerium oxide, titanium dioxide, nanosilver, nanotin and micronised copper, for investigation, and renders these nanomaterials to be assessed in detail for their possible effects on human and ecosystem health.

Realistic exposure scenarios and subsequent exposure assessment are crucial prerequisites for risk management for products containing nanomaterials with possible direct contact to consumers such as food and cosmetic products. In case of food packaging, where the nanomaterials are embedded in the packaging material, it is necessary to determine whether their release and migration in the food products might occur. There is concern that anti-microbial agents used in food contact materials or their use in the medical area may contribute to increased bacterial resistance by a widespread low-level exposure. The long-run effect of nanoparticles originating i.e. from packaging wastes on the natural microbiota of the soil and natural waters, and that of nano-medicals on the natural microbial community of human body should also be considered.

Acknowledgements For further studies, the financial support will be received from project SPICED “Securing the spices and herbs commodity chains in Europe against deliberate, accidental or natural biological and chemical contamination” within EU Cooperation Programme FP7-SEC-2012-1 (Grant Agreement No: 312631).

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