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Review Article

Prospects of using nanotechnology for food preservation, safety, and security

Vivek K. Bajpai [a], *Madhu Kamle* [b], *Shruti Shukla* [a], *Dipendra Kumar Mahato* [c], *Pranjal Chandra* [d], *Seung Kyu Hwang* [e], *Pradeep Kumar* [b,*], *Yun Suk Huh* [e,**], *Young-Kyu Han* [a,***]

[a] *Department of Energy and Materials Engineering, Dongguk University-Seoul, 30 Pildong-ro 1-gil, Seoul, 04620, South Korea*

[b] *Department of Forestry, North Eastern Regional Institute of Science and Technology, Nirjuli, 791109, Arunachal Pradesh, India*

[c] *Department of Agriculture and Food Engineering, Indian Institute of Technology Kharagpur, West Bengal, 721302, India*

[d] *Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, 781039, Assam, India*

[e] *Department of Biological Engineering, Biohybrid Systems Research Center (BSRC), Inha University, 100 Inha-ro, Nam-gu, Incheon, 22212, South Korea*

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ABSTRACT

The rapid development of nanotechnology has transformed many domains of food science, especially those that involve the processing, packaging, storage, transportation, functionality, and other safety aspects of food. A wide range of nanostructured materials (NSMs), from inorganic metal, metal oxides, and their nanocomposites to nano-organic materials with bioactive agents, has been applied to the food industry. Despite the huge benefits nanotechnology has to offer, there are emerging concerns regarding the use of nanotechnology, as the accumulation of NSMs in human bodies and in the environment can cause several health and safety hazards. Therefore, safety and health concerns as well as regulatory policies must be considered while manufacturing, processing, intelligently and actively packaging, and consuming nano-processed food products. This review aims to provide a basic understanding regarding the applications of nanotechnology in the food packaging and processing industries and to identify the future prospects and potential risks associated with the use of NSMs.

* *Corresponding author.*
** *Corresponding author.*
*** *Corresponding author.*
E-mail addresses: pkbiotech@gmail.com (P. Kumar), yunsuk.huh@inha.ac.kr (Y.S. Huh), ykenergy@dongguk.edu (Y.-K. Han).

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1. Introduction

Nanotechnology integrates several disciplines, including physics, chemistry, biotechnology, and engineering, and refers to the use of nanomaterials whose nanoscale structures range from 1 to 100 nm [1]. In these nano-sizes, materials adopt unique properties that were not present when the materials were in their original form. Understanding these unique properties in order to develop new and improved products through green processes is the goal of nanoscientists across the globe [2].

Nanomaterials or nanostructure materials exhibit different dimensions for their structural elements, clusters, molecules, or crystallites, such as zero dimensions (nanoparticles, nanoclusters, and quantum dots), one dimension (nanorods and nanotubes), two dimensions (nano-thin films), and three dimensions (nanomaterials) in the 1–100 nm range [3,4]. Combining nanostructure materials with other polymer, biomolecule, and other nanostructure material or existing in the aggregate form can result relatively in a larger particle size material (>100 nm) leading to the formation of nanocomposite [5]. These nanomaterials having high surface volume ratio exhibit inimitable physio-chemical characteristics, such as solubility, toxicity, strength, magnetism, diffusivity, optics, color, and thermodynamics [6,7].

The applications of these nanomaterials are growing in various sectors, including agriculture, medicine, clothing, cosmetics, food, and public health due to their unique ability to increase solubility and bioavailability and to protect bioactive components while they are being processed and stored [4,8,9]. Due to the excellent physiochemical nature and the antimicrobial potential of nanomaterials, they are widely used against various pathogenic microbes and in healthcare, crop protection, water treatment, food safety, and food preservation [8,10]. In addition, nanostructured materials (NSMs) are being applied in the food industry as a nanosensor, new packaging material, and encapsulated food component. In general, this review concerns the use of nanotechnology, specifically nanoparticles and constructed nanostructures, in the food sector.

Nanotechnology has a wide range of food-related applications. In these applications, a specific type of nanomaterial is incorporated into a specific food product in order for that food product to develop certain desired properties [2]. The field of nanotechnology has also been an integral part of research and development for the large-scale manufacturing of agricultural products and processed foods and drinks, as well as for food packaging across the world [3]. Several reports confirm that these nanomaterials can successfully improve food safety by enhancing the efficacy of food packaging, shelf-life, and nutritional value as additives without changing the taste and physical characteristics of food products [13,14]. Although they have much potential to frame innovative products and production processes in the food industry, nanotechniques are facing a major challenge in using cost-effective processing operations to create edible and non-toxic nano-delivery systems and to develop effective formulations that are safe for human consumption. Therefore, due to the increased use of these NSMs, there have been growing concerns regarding the development of biocompatible, safe, and non-toxic nanostructures from food-grade ingredients using simple, green, and cost-effective strategies, including the layer-by-layer technique [15].

United states-based safety evaluation agencies such as FDA and EPA, including the Directorate of European Health and Consumer Protection, and many other regulatory authorities, have released several guidelines on potential human health risks associated with the use of nanostructure materials in food [16]. These potential risks are determined by nanoscientists using nanotoxicology, which is a branch of toxicology and an interdisciplinary field that concerns varied toxicity aspects of nanomaterials [15]. Nanotoxicity mediated via generation of reactive oxygen species (ROS) is considered a crucial mechanism leading to successive human oxidative stress [8]. Also, there is a huge interest in using nanomaterials in various fields, including their safety concerns. Consequently, there is a compelling demand to address these issues so as to expand our knowledge on the use of nanomaterials/nanostructures in terms of their biocompatibility, safety, and toxicity in the food sector.

Although nanotechnology has applied green-synthesized NSMs in several ways in the food sector, on a few occasions, the use of NSMs has been controversial, as NSMs are scientifically uncertain and can impart long-term detrimental effects on human health and the environment [17]. In this regard, the complexity and limitations in the field of nanotechnology in terms of toxicity and accumulation (in appropriate doses) can be overcome only by elucidating the physiochemical and biological properties of NSMs through extensive research [18]. In light of the effects of nanomaterials on the food industry, this review has been formulated as a "snapshot" to address the current status of the techniques and implementations of nanotechnology in food preservation, safety, and security criteria and to identify the underlining mechanistic actions regarding safety or packaging issues. In addition, the review focuses on the current and future prospects of nanotechnology and discusses the strengths and weaknesses (i.e. the risks related to toxicological effects) of existing regulatory authorities. Moreover, the review discusses the current efforts to address these weaknesses and other issues related to the development, understanding, and promotion of nanotechnology. Fig. 1 illustrates an overview of the important usages of nanoparticles/nanomaterials and nanostructures in the food sector.

2. Natural and synthetic nanostructures in the food system

The food system naturally contains a variety of nano-sized elements that include self-assembled higher order structures, such as carbohydrates and fats [19]. These ingredients are different from synthetically manufactured nanomaterials/nanostructures thus can be used to make nanoemulsion, nano-encapsulate, and food-grade polymer [19]. During food processing, manufacturing, or thermal treatments, nanostructures are used to produce, which are not associated with modern nanotechnology (e.g. coagulation, emulsifying, or homogenizing). In brief, food proteins, including polysaccharides and lipids, are globular particles whose size can vary between 10 and several hundred nanometers. Coagulation and emulsification are based on the formation of reticular, zero, one,

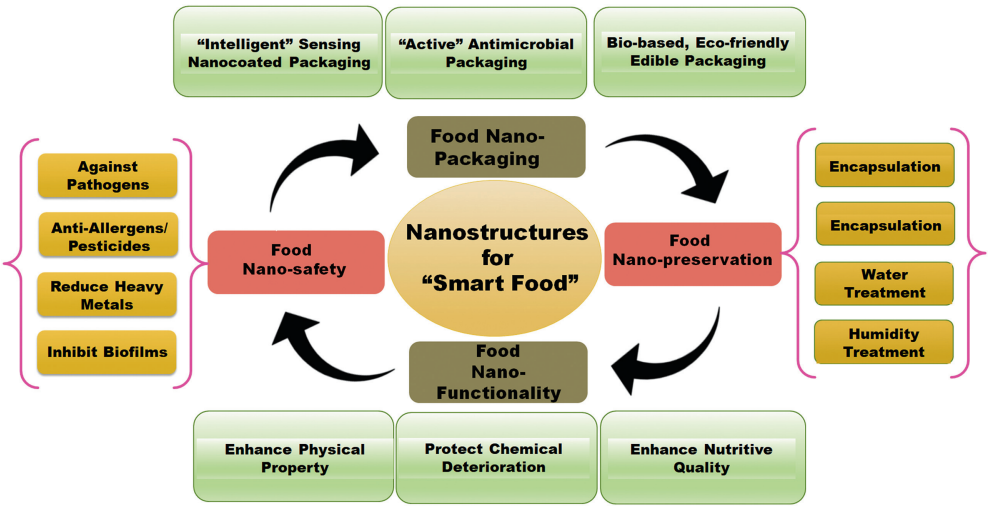

Fig. 1 – Systematic representation of application of nanoparticles in various areas of food industry.

two and three-dimensional nanostructures. When corn starch is boiled to make custard-like food, small, three-dimensional crystalline structures that are only tens of nanometers in thickness are melted [20]. Fresh milk and milk-based products naturally contain nanostructures, such as milk proteins and casein. When milk is homogenized, fat globules that are about 100 nm in size are produced [20].

Nanotechniques play an essential role in the functional and nutraceutical food sectors. Encapsulation of effective molecules or components in NSMs can enhance the bio-availability and solubility of coloring agents and nutritional ingredients such as minerals and vitamins, thus facilitating controlled release and protecting biologically active substances and micro-nutrients during their processing. Currently, the most usable NSMs are nanocapsules (e.g. micelles and liposomes) and nanoemulsions, including a few carbon-based, green-synthesized, ecofriendly nanomaterials.

3. Nanotechnology in food packaging and security

The packaging of food is one of the most critical steps in terms of food safety. Natural substances, atmospheric gases and water vapors are absolutely impermeable to no packaging materials [21,22]. However, completing blocking the migration and permeability of gases is not desirable in the case of packaging fresh fruits and vegetables that undergo cellular respiration [21]. In contrast, the packaging of carbonated beverages should eliminate the flow of oxygen and carbon dioxide (CO_2) to prevent oxidation and de-carbonation [21]. The flow of CO_2, oxygen, and water vapors varies according to the food matrices and the packaging materials that are used. Hence, these complexities in food packaging can be addressed and overcome by employing various nanocomposite materials, including polymers [24]. Nanoparticle having a diameter of less than 100 nm is thousand-times thinner than about 100,000 nm thick book page or hundred-times thinner than about 10,000 nm thick human hair. With an appropriate regulatory processing approval, different structures of nanoparticles can be of significant use in various fields of food and pharmaceutical industries, especially for the research programs associated with food science-based research (Fig. 2).

Recently, the use of nano-biocomposites in food packaging has enhanced the ability of food packaging to act as a barrier against gases [25]. The applications of various nanoparticles in food packaging are briefly shown in Table 1. Recent trends in food packaging are encouraging the use of biodegradable polymers reinforced with nanofillers, which are eco-friendly [26]. However, there is a major concern regarding the

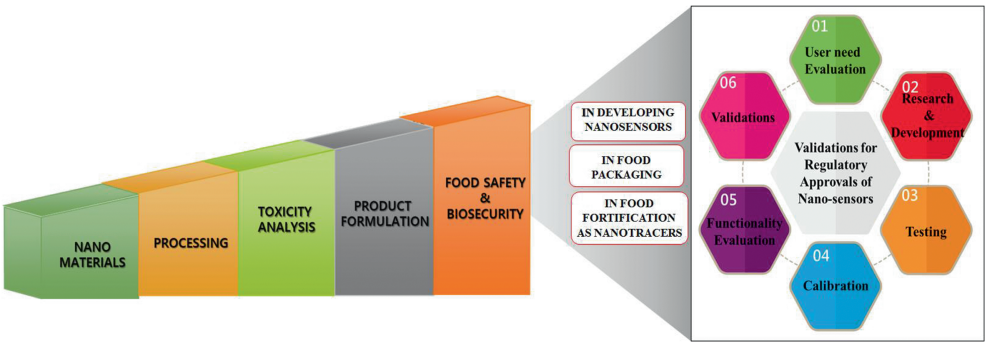

Fig. 2 – Steps for processing and utilizing of nanomaterials in food sectors.

Table 1 – Nanoparticles for application in food packaging.

Types of nanoparticles	Matrix	Application	Reference
Silver	Asparagus, Orange juice, Poultry meat, Fresh-cut melon, Beef meat exudates	Retards the growth of aerobic psychrotrophics, yeasts and molds; antimicrobial effect against *Escherichia coli* and *Staphylococcus aureus*	[165–169]
Zinc oxide	Orange juice, Liquid egg albumen	Effectively reduces *Lactobacillus plantarum*, *Salmonella*, yeast and mold counts without changes in quality parameters	[169–171]
Titanium oxide	Chinese jujube, Strawberry	Reduces browning, slow-down ripening, senescence and decay	[172]
Silver oxide	Apple slice	Retards microbial spoilage	[173]

ingestion of these nano-compounds during consumption of the food. Hence, it is essential to investigate how these nanoparticles migrate within the human body as well as these nanoparticles' toxic and immunogenic effects [27]. Another concern is related to the biodegradability of these nanofilled, biodegradable polymers [28]. These concerns are seriously considered by researchers across the world who are searching for human and environmentally friendly nanomaterials [29].

3.1. *Nanocoatings as intelligent packaging for surfaces*

In food packaging, NSMs, with biopolymers, can either enhance the property characteristics of neat polymer or improve the functional properties of active and intelligent packaging. Packaging can be categorized as "Improved", "Active," and "Intelligent" packaging, which represent which kind of packaging material could be used for what applications [30]. However, official restrictions have been imposed by the European Union (EU) using "active" and "intelligent" packaging materials in foods except using titanium nitride in plastic bottles [31].

For in-packaging detection of oxygen, Mill [32] developed nano-sized titanium dioxide (TiO_2) or tin dioxide particle-based favorable photo-indicator intelligent ink using a redox-activate methylene blue dye, and this detector was gradually able to change the color in response to minor changes in oxygen quantity. In recent years, the packaging industry has focused on the various types of nanostructures and specified nanomaterials, including nano-clay particulates due to their easy accessibility, low-cost, easy process ability, and great performance. In addition, graphene nano-sheets and carbon nano-tubes are also encouragingly developed as a carbon-based nanomaterial [33]. Polymeric substrate-based flexible or static films and bottles (various materials, such as glass and plastic etc.) in combination with vacuum-coated thin inorganic layers have great demand in material packaging due to their ability to block out oxygen and aromas. While aluminum foil and aluminum-metallization used to be the material and method of choice, respectively, new materials are being used more frequently.

Thick and uniform layers (10–100 nm coating thickness) are broadly referred to as "nanocoatings" [34]. In recent years, few researchers have developed an intelligent type of nanocoating film that can indicate whether any contamination has occurred during storage. Gas content and non-invasive detection methods have also shown great ability to continually and easily monitor the gas content, excess moisture, and oxygen content of a package-headspace thus providing effective means to evaluate the quality and safety of food even after the production process [35,36]. Presence of oxygen inside the packaging can cause shelf-life threatening of food due to the ability of oxygen to create healthy environment for microbial growth [37]. Yu et al. [38] developed polyvinyl alcohol/chitosan polymer-based biodegradable and cost-effective films using a nanocomposite of silica *in-situ* which caused significant reduction in the permeability of oxygen and moisture by 25.6% and 10.2%, respectively, suggesting that the films extended the preservation time of cherries by three-fold compared to normal packaging. Swaroop and Shukla [39] developed a food packaging material using a combination of nanostructures of magnesium oxide (MgO) and polylactic acid biopolymer, and found that the material effectively protected against bacterial biofilms. Furthermore, Foltynowicz et al. [40] synthesized zero-valent iron particles to act as oxygen scavengers in food packaging.

3.2. *The mechanisms of nanoparticles/nanocapsules as antimicrobial agents for active packaging*

The outstanding use of nanoparticles with antimicrobial properties has, to some extent, protected our food products from foodborne illness outbreaks due to the consumption of spoiled packaged food. When compared with conventional food-packagings, an active food-packaging not only has the ability of being a passive barrier, but also helps to release antioxidant and antimicrobial compounds through the direct interaction with the food as well as helps to remove some negative factors, such as oxygen or water vapor. These interactions usually improve food stability [41].

Active packaging contains specific molecules which have the ability to absorb or release the components into or from the surrounding environment of the packaged food. Today, active polymer nanomaterials in food packaging have mainly been developed for antimicrobial packaging applications [42]. Overall, in order to improve the functionality and effectiveness of food packages, various bioactive substances can be incorporated into the packaging material by capsulation, capping the material with nanomaterials, or employing other nanotechniques [43]. To effectively apply antimicrobial active packaging to food products, food providers should select effective antimicrobial substances-based unique methodology to ensure the quality of the packaged products, such as their visual appearance and sensory levels [44].

The encapsulation of nutraceuticals and functional antimicrobial ingredients is essential for the preservation and

bioavailability of bioactive ingredients, as it is extremely important in food processing, food storage, and passage through the gastrointestinal tract [5]. The macromolecule-based nanoparticles in food not only improve the bioavailability of bioactive polyphenolics, such as resveratrol, epigallocatechin-3-gallate, and curcumin, but also enhance the solubility of these polyphenols and thus prevent their degradation in the gastrointestinal environment [9]. There are various encapsulation techniques that have been used to produce nano or micro-particulate systems, such as nano-emulsion [45], coacervation [46,47], the extrusion method [48], fluidized bed coating [49], spray cooling [50], and spray drying [51].

Nano-encapsulation allows for the direct contact of nano-materials during the consumption of food. In a commonly known example, silicon dioxide (SiO_2) is widely applied as a fragrance carrier in various food products [52]. Several lipid-based, nano-encapsulation systems have been developed to increase the activity of antioxidant components by improving their bio-availability and solubility [53] as well as their targeted, site-specific delivery with efficient absorption [54]. Studies have found that nano-sized edible coating is an effective alternative for preservation and shelf-life extension of food, as well as prevention of food from microbial spoilage [55]. Efficacy of coatings made with gelatin incorporating nano-crystals of cellulose [56], chitosan or nano-silica [57], chitosan with nano-silica [58], and alginate or lysozyme nano-laminate has been found very effective for the preservation of the quality of fresh foods during prolonged storage [59]. Additionally, the nano-packaging-based method that incorporates blended polyethylene with nano-powders such as Ag, kaolin, anatase TiO_2, and rutile TiO_2 has been considered to be a novel and facile technique to preserve fruits, such as strawberries (*Fragaria ananassa* Duch.) [60]. Despite the tremendous research in nano-encapsulation using various materials [61], the precise delivery of foods and their safety has not been studied in detail. Therefore, future research should examine the long-term toxicity of nano-encapsulated foods [62].

In addition to this, Johnston [63] employed nanostructured calcium silicate (NCS) for the absorption of Ag^+ ions from a solution, which formed an NCS–Ag complex that was successfully used as an antimicrobial agent for food packaging purposes. Similarly, TiO_2 is commonly utilized in surface-coating as a photocatalytic disinfecting material [64] and to inactivate several pathogenic bacteria in food [65]. The antibacterial potential of chitosan-capsulated nanoparticles could be mediated through the interaction between the positively-charged chitosan and the negatively-charged cell membranes. These interactions lead to enhanced membrane permeability and cause the rupture and eventual leakage of the intracellular material [66].

Sarwar et al. [67] developed polyvinyl alcohol (PVA), nanocellulose and Ag nanocomposite films and applied them to antimicrobial food packaging. These films profound antimicrobial effects against both Methicillin Resistant *Staphylococcus aureus* (MRSA) and DH5-alpha strain of *Escherichia coli*. Valerini et al. [68] also developed nanostructured, and aluminum-doped zinc oxide-based antimicrobial coatings functionalized with polylactic acid using sputtering power. The coatings exhibited strong antibacterial potential against *E. coli* and were proposed as promising materials for environment-friendly active packaging. Moreover, Lu et al. [69] prepared an antimicrobial nano-emulsion that was 100 nm by encapsulating citral essential oil using ultrasonic power, and as a result, the designed nano-emulsion was significantly effective in delivering an antimicrobial agent (citral essential oil) in the food system.

Metal and metal oxide nanoparticles (NPs)-based active packagings could be effective tools of nano-composites in terms of their antimicrobial potential. Among all metal nanoparticles, silver (Ag^+) nanoparticles are widely used in commercial sectors. Silver nanoparticles are toxic for various food pathogens [70] because silver nanoparticles increase cell membrane permeability via cell surface adherence and by degrading lipopolysaccharide [71]; in this way, the silver nanoparticles can penetrate the bacterial cell, damage its DNA [72], and release antimicrobial Ag^+ ions [73], which bind to sulfur, oxygen, or nitrogen containing electron donor groups in molecules and thus inhibiting synthesis of adenosine triphosphate (ATP) and DNA replication, eventually leading the cell to die [74]. Millimolar concentrations of Ag^+ ions can easily cause cytoplasm shrinkage; separation of the cell wall membrane; the devastation of the peptidoglycan in the cell wall; denaturation of ribosomes; and DNA compression, which inhibits DNA synthesis, ruptures the cell membrane, and causes cell death [74]. Basically, the three major mechanisms of the bacterial toxicity of metal-containing NPs that are widely accepted include the uptake of metal ions, which causes the intracellular ATP depletion; ROS production, which causes oxidative cellular damage, and disruption of bacterial membrane [4].

Similarly, reports have confirmed incorporation, coating, immobilization and surface modification of antimicrobial components onto packaging material [75]. Also, the higher antimicrobial effect can be achieved via direct incorporation of antimicrobial components into packaging films. Similarly, films coated with various effective antimicrobial components can result in higher antimicrobial potential. While incorporating these antimicrobial agents (volatile and non-volatile) into film packaging, these molecules can be dispersed onto the food surfaces via adopting migration, evaporation or diffusion abilities [76]. Recently, Arfat et al. [77] designed a bio-nanocomposite film based on fish skin gelatin and silver-copper bimetallic NPs. The film showed significant antibacterial potential against *Listeria monocytogenes* and *Salmonella enterica*. Several silver-based, antimicrobial master batches, such as Bactiblock®, Aglon®, Surfacine®, d2p®, IonPure®, Irgaguard®, and Biomaster® have been used to retard the growth of *Campylobacter*, *Salmonella*, and *E. coli*.

4. Nanotechnology in food functionality

4.1. *Nanoparticles for protection from chemical deterioration*

The main reason behind deterioration in the quality of food lies in an array of chemical reactions that occur between various components present in food and the external environment. Researchers have identified several nanomaterials

that can control these unwanted reactions in various food matrices. However, some of these metal and metal oxide nanomaterials cause nano-toxicity, primarily because they can form ROS and cause oxidative stress, which leads to an imbalanced redox state in the cell [78]. Therefore, relatively less reactive nanomaterials are employed as an antioxidant carrier [18,79,80]. Polymeric nanoparticles are suitable for the encapsulation of bioactive compounds, such as vitamins and flavonoids, and for the release of these compounds in an acidic environment, such as in the stomach [81]. The application of antioxidants and edible coatings control the browning, the conversion of phenolics into dark-colored pigments in the presence of oxygen of fresh-cut fruits [82]. Although few nanomaterials have been used directly as anti-browning agents, nano-ZnO has been used as coated active packaging to improve the shelf-life of fresh-cut Fuji apples [83].

Nanotechnology can enhance food functionality either by eliminating chemical toxicants or by enhancing nano-sized nutritional supplements. For instance, some of the nutraceuticals incorporated in the carriers consist of lycopene, β-carotenes, and phytosterols in order to avoid cholesterol accumulation in body [84]. It is well-known that food itself has few nanostructures that are responsible for self-mediated nano-effects. For example, green tea has many health benefits because of its nano-selenium content, which facilitates the effective uptake of selenium.

Nano-encapsulation involves a nanoscale process of packing material by using nanocapsules, and it ensures the functionality of the final product, which includes the controlled release of the core. Therefore, encapsulated forms of ingredients exhibit numerous advantages, including consecutive delivery of multiple active ingredients, shelf-life extension, increased stability, and pH-triggered controlled release [85]. Further, Liang et al. [86] encapsulated epigallocatechin gallate (EGCG in zein/chitosan) nanoparticles for controlled, effective, functional applications in the food system. It was observed that the release of EGCG from zein/chitosan (CS) NPs and the DPPH scavenging ability of the zein/CS NPs was relatively higher than that of the nanoparticles that did not have the zein/CS coating in fatty simulant of 95% ethanol. These findings confirmed that the antioxidant activity and controlled-release of EGCG from zein/CS NPs in fatty simulant of 95% ethanol protect fatty foods from chemical deteriorates via enhancing antioxidant efficiencies and thus may help fatty foods to protect from oxidation for longer period.

4.2. *Nanoparticles for enhancing the physical properties of food and packaging materials*

Nanomaterials developed have shown enormous ability to increase the physical properties of both food and packaging materials [87]. In the 1990s, polymer nanocomposites with layered silicates that had several important characteristics, such as high flame resistance [88] and protection from UV rays, were developed [89]. Moreover, a number of NPs have been developed to increase the physical attributes of food, such as color and appearance. TiO_2 was approved as a coloring food additive by the USFDA with the agreement that the limit of TiO_2 should not exceed 1% (w/w) as an additive ingredient [90]. Besides TiO_2, mixture of color additives may contain SiO_2 and/or Al_2O_3, but the use of carbon black is strictly prohibited by the USFDA as a food coloring additive [91]. On the other hand, SiO_2 is used as an anti-caking agent to maintain the flow properties in powdered products and as a carrier of aroma in food and non-food products. Nano-sized SiO_2 is widely employed in food products and has been registered within the EU as a food additive, as per Directive E551 [52].

β-Carotene is a nutraceutical component that acts as a coloring agent and as provitamin A, but its incorporation into foods is limited because of its low chemical stability and hydrophobicity. To improve the physicochemical stability of β-carotene, Mehrad et al. [92] synthesized nanomaterial by encapsulating it into solid lipid nanoparticles (SLNPs) that contained palmitic acid and corn oil and stabilized the material using a whey protein isolate (WPI). In this nanostructure, the encapsulated β-carotene was protected by a solid shell made by palmitic acid crystals covering the surface of the oil droplets. Corn oil was able to decrease the exclusion of β-carotene from the solid lipid matrix to the surface of the SLNPs, while WPI increased the stability of the colloidal system, thus improving the oxidative stability of the β-carotene.

5. Nanotechnology in food safety

Globally, food safety is a growing public health concern. The primary goal of food safety is to ensure that food will not cause any harm to the consumer during preparation and consumption [93]. Food must be protected from any type of physical, chemical, and biological contamination during processing, handling, and distribution [94]. Recent developments in nanotechnology have revolutionized the food industry with its various applications in food processing, safety, and security, as well as its strides in enhancing nutraceutical value, extending shelf-life, and reducing packaging waste [94]. Today, food safety is a major concern due to rapid changes in food recipes and food habits. Foodborne pathogens, toxins, and other contaminants can cause serious threats to human health. The conventional methods for the detection of pathogens and their toxins are labor intensive and time consuming. Advances in nanotechnology have expedited the addressing of food safety issues in microbial contaminants and have improved toxin detection, shelf-life, and packaging strategies [84]. In addition, nanomaterials, including metal nanoparticles, carbon nanotubes, quantum dots, and other active nanomaterials can be used to develop biosensors for the quantification of microbes and other tests for food safety applications [84,94].

5.1. *Nanoparticles for the detection of foodborne pathogens*

Nano-biosensors are bioanalytical devices that are developed using various NSMs and biological receptors in an integrated system design [96]. Various types of biosensors have been developed to detect foodborne pathogens and food spoiling materials [97,98]. Surface enhanced Raman scattering (SERS) is employed as a nano-biosensing tool to rapidly and accurately detect microbial pathogens [99,100]. Silver nano-

colloids are commonly used in SERS [101] to detect bacteria, as silver nano-colloids enhance Raman signals. Besides silver nano-colloids, graphene oxide [102], magnetic beads [103], carbon nanotubes [104], plasmonic gold [44], and silver nanoparticles [24] are commonly used to detect food-associated bacterial pathogens. In addition, synthetic DNA molecular beacons tagged with color-coded probes are used as nano-barcodes to identify food pathogens [72].

The direct detection of *E. coli* in food samples has also become possible by the measurement and detection of light that is scattered by the cells. This type of sensor operates on the basis of binding with a known protein and is characterized as a bacterium onto a silicon chip, which can further bind with any other *E. coli* bacteria present in the food sample [105]. Chen and Durst [106] devised an array-based immunosorbent assay to detect *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes* using protein G-liposomal nano-vesicles in pure and mixed cultures. The researchers showed that protein G-liposomal nanovesicles can be successfully used in immunoassays for simultaneous detection of foodborne pathogens and demonstrated the effectiveness of protein G-liposomal nanovesicles as universal immunoassay reagents. Further, DeCory et al. [107] developed an immunomagnetic bead-immunoliposome fluorescence assay to rapidly detect *E. coli* O157:H7 in aqueous samples. The findings demonstrated the feasibility of using immunomagnetic beads in combination with sulforhodamine B encapsulated in immunoliposomes to rapidly detect *E. coli* O157:H7 in aqueous samples. Additionally, liposome-based methods have also been investigated by other researchers for pathogen detection [108,109]. Nanosensors, such as nano-cantilevers, employ silicon-based materials to recognize proteins and detect pathogens that vibrate at different frequencies depending on their biomass [110].

In recent years, various new nanoparticle-based detection platforms have been developed. Tominaga [111] developed lateral-flow immune test strips with palladium nanoparticles against *Klebsiella*, which allowed for the specific binding and visualized detection of specific bacteria. Further, Thakur et al. [112] detected single *E. coli* bacterial cell using a reduced graphene, nanoparticle-based, field-effect transistor device. Recently, our research group developed an electrochemical sensing platform based on graphene oxide–gold nanoparticles to detect *Cronobacter sakazakii*, which is a bacterium that is hazardous to infants, in infant formula powder with a detection limit of 2.0×10^1 cfu/mL [113]. Also, Song et al. [114] developed a fluorescence sensing platform using immunomagnetic nanoparticles coupled with liposome nanoparticles to detect *Cronobacter* sp. at the genus level with a detection limit of 5.9×10^3 cfu/mL. Additionally, our research team developed an aptamer and gold nanoparticle-based optical detection platform to detect *Salmonella* in contaminated pork samples [115]. The various types of nanoparticles for the detection of various foodborne pathogens and their detection limits are shown in Table 2.

5.2. *Nanoparticles for protection from allergens*

Nanotechnology has been employed as a basic tool to control and manage food allergens [116,117]. Despite these efforts, certain nanomaterials cause allergic pulmonary inflammation [118,119] in humans. For instance, SiO_2 nanoparticles have been reported to cause allergen-specific, Th2-type immune responses *in vivo* in female BALB/c mice [118]. The immunotherapy of allergies using aluminum hydroxide (alum) as an adjuvant displayed several side effects, including swelling, indurations, erythema, cutaneous nodules, and granulomas at the injection site [119]. To overcome this, researchers have investigated the use of polymeric nanoparticles [120] and substances that target toll-like receptors (TLR) as alternative adjuvants [121]. For instance, the protamine-based nanoparticles with TLR-9 ligand cytosine phosphate guanine (CpG)-oligodeoxynucleotides (ODNs) can be used as an adjuvant. Protamines are arginine-rich peptides of approximately 4 kDa found in the sperm of salmon and are isolated from the mature testicles of salmons. Protamines are biodegradable and efficiently utilized for the reversal of heparin activity during surgical operations [122] or as an insulin-additive [123]. Additionally, protamine-based nanoparticles with CpG-ODN counteract the Th2-dominated immune response induced by an allergen, thus have shown remarkable potential in allergen immunotherapy as a novel carrier system [124].

Table 2 – Nanoparticles employed for the detection of foodborne pathogens.

Nanoparticles	Pathogens	Detection limit	Reference
Gold nanoparticle	*Salmonella enterica serotype Typhi*	98.9 CFU/mL	[174]
Gold/silicon nanorod	*Salmonella enterica serotype Typhi*; Respiratory syncytial virus	Not reported	[175]
Gold nanorod	*Escherichia coli* O157:H7	1–10 CFU/mL	[159]
Quantum dot	*Salmonella enterica serotype Typhi*, *E. coli* O157:H7, *Listeria monocytogenes*	10^3–10^6 cells/mL	[160–163]
Magnetic bead/quantum dot	*E. coli* 0157:H7	10^3 CFU/mL	[176]
RuBpy doped silica	*E. coli* O157:H7	1 cell/mL	[164,176]
Single walled carbon nanotube	*E. coli*	Not reported	[177]
Magnetic nanoparticle	*E. coli* O157:H7, *S. aureus*, *S. epidermidis*	10^4 CFU/mL, 8 CFU/mL, 10 CFU/mL	[177]
Immunomagnetic liposome nanoparticle	*Cronobacter sakazakii*	10^3 CFU/mL	[108]
Aptamer conjugated gold nanoparticles	*Salmonella typhimurium*	10^4 CFU/mL	[115,120]
Liposome nanoparticles	*Salmonella typhimurium*	10^2 CFU/mL	[173]

Similarly, Gamazo et al. [125] reported immunotherapy that employed nanoparticles as an allergen delivery system, which facilitated the administration, reduced the dose, and diminished the allergen exposure to the immunoglobulin E that was bound to mast cells and/or basophils. Moreover, in recent years, bioinspired NSMs are being explored that are known to have little to no toxic effects and can thereby be potentially exploited in the food industry [126]. Localized surface plasmon resonance (LSPR)-based label-free biosensing methods have gained huge attention due to their high sensitivity, simplicity, and relatively low cost efficacy. However, the challenges have been observed using these methods for *in situ* analysis of real samples due to the instability of colloidal nanoparticles at certain levels of pH and salt concentration. Recently, Lee et al. [127] developed a simple and cost-effective optical fiber coated with aptamer-modified gold nanorods for easy detection of ochratoxin A in grape juice samples (food mycotoxin causing allergy) and subjected the fiber to LSPR analysis, which demonstrated a significant detection limit of 12.0 pM. Zhang et al. [128] reported a magnetic, nanoparticle-assisted, aptamer-based fluorescence assay to detect allergens in food matrices. As a result, under optimal conditions, the linear range was recorded as 0.4–5 g/mL ($R^2 = 0.996$), and a low limit of detection was calculated as 77 ng/mL with efficient selectivity. Moreover, Brotons-Canto et al. [129] evaluated the positive effects of poly-(anhydride) nanoparticles (150 nm) as oral vehicles for immunotherapy against experimental peanut allergies. Several other studies demonstrated the promising applications of nanotechnologies in vaccinology and in allergen immunotherapy [130]. In the near future, nanoparticle-based formulations can be applied to allergen immunotherapy to solve its unmet needs [131].

5.3. *Nanoparticles for preventing heavy metal reduction*

The release of heavy metals from nanomaterials poses a high risk of toxic outbreaks [80]. The release of these metals in food products has adverse effects in the case of long-term accumulation. Metal and metal oxide-based nanomaterials, such as ZnO [132], Ag [133], and CuO [134], increase the intracellular ROS level [132] and ultimately cause lipid peroxidation and DNA damage. Cationic surfactant coated silica-modified magnetite nanoparticles act as adsorbents for the micro-extraction and have ability to determine trace amount of Cu, Ni, Co, Cd, Pb, and Mn from environmental samples. The silica-coated NPs synthesized using cetylpyridinium bromide, can solubilize metal ions after being complexed with 8-hydroxyquinoline [135]. While many nanomaterials have tremendous potential for the remediation of contaminants, magnetite nanoparticles have proven to be the most attractive and inexpensive substrates for the recovery of heavy metals [136] from various sources. Zhang et al. [137] fabricated $Fe@Fe_2O_3$ core/shells, nanowires, and nano-necklaces to remove chromium from aqueous solutions.

Recently, Lin et al. [138] demonstrated the ability of aminated magnetic iron oxide nanoparticles, especially Cu^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+}, to act as adsorbents to remove aqueous heavy metal ions. The results of the study displayed that the increase in the degree of amination had a positive effect with a simultaneous increase in the adsorption capacity and the initial rate of adsorption of heavy metal ions. MgO nanoparticles synthesized via sol–gel and calcination processes showed great potential against bacterial infections and were able to remove heavy metal ions from contaminated water samples [139]. These findings anticipated that nano-sized MgO particles may have potential for the treatment of bacterial and heavy metal contaminated wastewater due to their high removal efficiency, low cost, facile preparation, and environmentally friendly characteristics.

In recent years, Lingamdinne et al. [140] demonstrated how synthesized, reusable, recoverable iron oxide nanoparticles could reduce heavy metals without losing their stability. In addition, over the years, various carbon nanoparticles with high fluorescence properties such as carbon dots (C-dots) with a particle size of smaller than 10 nm and carbon nanoparticles (CNPs) of about 10 nm size or larger have shown a broad range of biological properties with low toxicity and good biocompatibility. Recently, through a thermal process in the presence of H_3PO_4, Simpson et al. [141] fabricated, characterized, and approved carbon nanoparticles (66 nm) with glycerol for the detection of heavy metal ions, with a detection limit as low as 0.30 ppm.

5.4. *Nanoparticles for the inhibition of biofilm formation*

Biofilms are tightly-packed bunch of bacterial cells that adhere to many substrates and produce a polymeric extracellular matrix that is extremely difficult to penetrate [142]. The biofilm formation begins with the attachment of free floating microorganisms to the surface by employing van der Waals forces that create problems such as biocorrosion, biofouling, and accumulation in the food processing industries [143]. Glycerol monolaurate (GML), which is recognized as safe by the USFDA, acts as an antimicrobial agent against many Gram-positive bacteria, including *Bacillus anthracis* [144]. GML has been shown to inhibit the biofilm formation of three different strains of *S. aureus* and of MRSA [145]. Besides this, the use of antimicrobials throughout the nano-fibers of filter membranes is employed to prevent biofilm formation [137]. Moreover, Shahrokh and Emtiazi [146] demonstrated that nanosilver particles at low concentrations (0.2 ppm) enhanced bacterial metabolism and thus suggested the use of an optimum concentration of nanosilver particles for various nanomaterials to prevent biofilm formation.

Further, nickel oxide nanoparticles (NiO NPs) have been proposed as prospective antibacterial and anti-tumor agents. Through a green approach and using *Eucalyptus globulus* leaf extract, Saleem et al. [147] synthesized NiO-NPs that were 10–20 nm in size and assessed their anti-biofilm activity. In addition, Gambino et al. [148] demonstrated the bio-applicability of zinc oxide nanoparticles for hindering fungal biofilm (*Alternaria alternata*, *Penicillium chrysogenum*, *P. pinophilum,* and *Aspergillus niger*) that had developed in an ancient Egyptian tomb. Chlorhexidine-conjugated gold nanoparticles exhibited biofilm inhibitory effects against *Klebsiella pneumonia* [149].

Bacillus subtilis has shown enormous potential in various industrial processes for the production of value-added products. Fermentation process of *B. subtilis* is directly related to its ability to form biofilm resulting in operational issues and major process. Ranmadugala et al. [150] evaluated the effects of two types of superparamagnetic iron oxide (IONs), such as naked

IONs and IONs coated with 3-aminopropyltriethoxy silane (IONs@APTES) against *B. subtilis* in terms of their ability to inhibit its biofilm formation, bacterial growth, and cell viabilities. The results showed that a significant reduction in the total biomass of the bacterial biofilm was observed without affecting the cell viabilities. These results suggested that these nanoparticles could be applied in several industries to combat the growth of bacterial biofilm. Moreover, Thuptimdang et al. [151] suggested that controlling the growth conditions to alter the biofilm physical structure was a possible approach to reduce the impact of silver nanoparticles (AgNPs) on biofilms in engineered and natural systems. These recent findings are increasing the demand for researchers to explore the use of nanoparticles in several other industries, including medicine.

6. Are nanotechnology and big data effective enough for next industrial revolution for securing "Smart Food"?

The engineering applications of nanotechnology will herald a new digital future characterized by improvements in food safety, shelf-life performance, and food reliability. These advancements are frequently referred to as big data information and open new fields of research and applications that will increasingly affect all parts of society. There is a huge complicated response to outbreaks associated with foodborne diseases due to the globalization of food supply chains. Generation of big data in the food industry may help to facilitate encouraging advancement in food safety and security at the global scale [156]. Big data is represented as high volume, high velocity, high veracity, and/or high variety information assets requiring new processing formats so as to enhance the process of decision making, process optimization, and insight discovery [157]. Although the big data analysis and large datasets in food safety and quality have focused on improving root cause and retrospective analyses, there is a huge growth in the advancement and utilization of predictive analytics in food security sector [157]. For example, whole genome sequencing data for *L. monocytogenes* isolates obtained from ice cream in Kansas became publicly available soon after a listeriosis outbreak linked to ice cream was reported in early 2015. Other omics datasets, such as metagenomics data, have also been used to identify and characterize food spoilage issues. It is likely that these types of data sources will become increasingly available to personnel in the food industry.

Recently, the World Health Organization (WHO) has enfold the big data information approach to deal with the food-safety process of decision making, which has brought about a new food-safety platform known as "FOSCOLLAB," which integrates different sources from various disciplines [158]. On this platform, organized and nano-structured information from numerous areas, for example, food, horticulture, animals, public health, and economic index, are incorporated and accessible to the users by means of a few committed dashboards [158]. The information sources associated in FOSCOLLAB are assessments of the Joint FAO/WHO Expert Committee on Food Additives and the Joint FAO/WHO Meeting on Pesticide Residues databases on chemical risk assessment, the WHO database on Collaborating Centers, and the Global Environment Monitoring System (GEMS) food databases on chemical occurrence and food consumption. There is no restriction of utilizing big data information in the fields of food safety and food quality to facilitate improved methodologies.

Finally, input food industries often work worldwide, are R&D intensive, and deal with new technologies, including genetics, robotics, informatics, and nanotechnology. Besides the challenge of feeding the world, there is the issue of sustainability, which is mainly an issue of farming and transport. Together with the relationship between food and health (e.g. obesity concerns), food safety directly affects food processors, retailers, and consumers. The ultimate objective is to estimate risks to farms and to build up a preventive way to deal with food safety issues. The use of geographical information system technology (GIST) to create food security has demonstrated incredible practices, for example, helping producers settle on more informed choices about field practices and establish the programs of targeted pathogen surveillances. Overall, the utilization of GIST to yield big data information in food safety investigations has created remarkable measures of new data with respect to the ecology of various life forms in the food environment and data on different contamination episodes. In the era of big data information, GIST is one of the data tools that enables scientists to store, analyze, process, capture, and visualize extensive datasets. Additionally, the use of GIST to complex food safety/security issues is being demonstrated for further integration of various large data sets. Essential components associated with big data requirements in nano-food safety are presented in Fig. 3.

7. Future perspectives and potential risks of nanotechnology

There have been tremendous developments in the application of nanotechnology in food science and research. Nanotechnology helps in the detection of pesticides [159], pathogens [160], and toxins [161], and tracking-tracing-monitoring helps

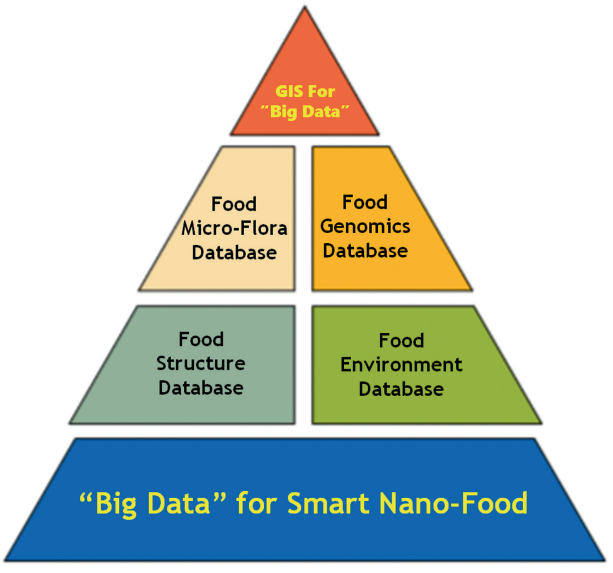

Fig. 3 – Essential components for producing "big data" for safety and security of smart nano-food.

in the maintenance of food quality. A massive amount of interest is being on using carbon nano-tubes for incorporating them into packaging materials for the detection of toxic proteins, microorganisms, and food spoilage [162]. Furthermore, carbon nanotube can transform our future food packaging materials to active and intelligent packaging systems [163]. Along with the incessant research on the application of nanomaterials, several potential risks and toxicity issues associated with the application of nanomaterials have been revealed, and these concerns must be addressed [104,161]. The effect of these nanoscale particles on human beings, animals, and the environment are unpredictable due to changes over time in their properties. Some nanoparticles can even cross biological barriers, such as the blood–brain barrier, and enter various cells and organs [164].

The evidences for the health risks associated with the inhalation of ultrafine and nanoparticles have been increasing every day [60]. In addition, bioaccumulation of nanomaterials, such as nano-silver, derived from either nanopackaging or plants and animals has been confirmed in food and human being [62]. The use of nanomaterials as activation catalysts, pesticides, and microbicides always pose an unknown associated risk, and hence risk assessment procedures must be strictly followed while processing food [57,58]. Even with the advent of nanotechnology, the challenges to the development of a healthy and sustainable food industry remain. With the introduction and development of nanotechnology in the food system, the public should be educated regarding the associated health, safety, and environmental effects of nanotechnology.

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Review Article

Nanoparticle-based laser desorption/ionization mass spectrometric analysis of drugs and metabolites

Han-Wei Chu [a], *Binesh Unnikrishnan* [a], *Anisha Anand* [a], *Ju-Yi Mao* [a,b,c], *Chih-Ching Huang* [a,d,e,*]

[a] *Department of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung, 20224, Taiwan*
[b] *Doctoral Degree Program in Marine Biotechnology, National Taiwan Ocean University, Keelung, 20224, Taiwan*
[c] *Doctoral Degree Program in Marine Biotechnology, Academia Sinica, Taipei, 11529, Taiwan*
[d] *Center of Excellence for the Oceans, National Taiwan Ocean University, Keelung, 20224, Taiwan*
[e] *School of Pharmacy, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, 80708, Taiwan*

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ABSTRACT

Nanoparticle-assisted laser desorption/ionization mass spectrometry (LDI-MS) is a powerful tool for the analysis of a wide range of molecules. Many of the drawbacks in the matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) can be avoided with the application of nanomaterials as matrices as well as substrates for the LDI-MS to achieve a low background noise in low *m/z* region and high reproducibility. Surface-assisted LDI (SALDI)-MS, especially the nanoparticle-based LDI-MS, has emerged as a promising technique for the analysis of trace amounts of substances in various biological samples due to their high surface area for analyte enrichment, efficient desorption/ionization, and homogeneous crystallization of sample. Therefore, it is highly useful in clinical, forensic, medical, food and drug analyses, disease diagnosis, and various other fields. In this review, we briefly discuss the application of various nanomaterials, which include metal-based, carbon-based, silicon-based nanomaterials and nanocomposites, as matrices and substrates for LDI-MS based drug and metabolite analyses and possible detection strategies. Also, we discuss the idea of using "mass tag" for signal amplification for drug and metabolite detection using nanoparticle assisted LDI-MS.

* *Corresponding author*. Department of Bioscience and Biotechnology, National Taiwan Ocean University, 2, Beining Road, Keelung, 20224, Taiwan.
E-mail address: huanging@ntou.edu.tw (C.-C. Huang).
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1. Introduction

Laser desorption/ionization (LDI) is an ionization technique that desorbs and ionizes substances through heating them by pulse laser irradiation. LDI is one of the most prevalent ionization techniques; only second to electrospray-based ionization (ESI), due to its high speed of analysis, simple spectra and its capacity to analyze wide category of substances. Early LDI-mass spectrometry (LDI-MS) techniques had limitations in the type of substances due to low laser absorption and ionization efficiencies, easy fragmentation and thermal degradation of analytes. Organic molecule (nicotinic acid) as matrix to assist the LDI process of the analyte was introduced in the 1980's by Hillenkamp and Karas [1]. The matrix enhances the desorption and ionization of the analytes by absorbing laser energy and transferring it to analytes while preventing their direct fragmentation. This technique, named matrix-assisted laser desorption/ionization (MALDI), has greatly expanded the category of analytes, especially large molecules, that can be analyzed by LDI-MS [2–4]. In a typical process, both the matrix and the analyte molecule are desorbed and ionized by protonation or deprotonation. Although MALDI improves the signal of large biomolecules by preventing fragmentation to an extent and increases ionization efficiency, the matrices create intense fragmentation signals in the <700 m/z region of the mass spectrum [5]. Thus, matrix fragmentation results in poor analysis of smaller molecules even though matrices can be modified to reduce matrix effects [6]. In addition, the uneven crystallization of the matrix and the analyte molecules may cause heterogeneous sample distribution on the MALDI plate and affect the shot-to-shot and sample-to-sample reproducibilities. Another variation of the LDI-MS, the surface assisted laser desorption/ionization (SALDI), has emerged as the technique of choice for the analysis of small molecules, such as drugs and metabolites [7–11]. SALDI is an ionization technique that utilizes inorganic substances, nanomaterials or composites to assist in the ionization of target analyte [12]. SALDI has significantly lower matrix interferences in the low *m/z* region with more homogeneous analyte distribution and higher salt tolerance in comparison with MALDI [13–15].

Many nanostructured inorganic materials such as silica, metal and metal oxide nanoparticles, semiconductor nanoparticles and some carbon-based nanomaterials like graphene oxide are efficient substrates in SALDI-MS analysis for drug and metabolite analysis due to their excellent photoabsorption coefficient and heat transfer efficiency [7,16–22]. In a SALDI process, the applied laser beam heats up the substrate instead of the analyte. The substrate then transfers heat to the analyte, leading to the desorption and ionization of the latter. Thus, the desorption and ionization of the analyte highly depend on the photothermal conversion and heat transfer efficiencies of the substrate [23]. The mechanism of SALDI process is illustrated in Fig. 1. Recently, Picca et al. have reported a concise review on the mechanism of nanophase induced LDI in nanoparticle assisted LDI-MS [24]. Since there is very low fragmentation of nanostructure-based substrates in SALDI process, the interference due to the fragments is highly reduced. As a result, the low background noises of SALDI-MS at low *m/z* region allow for the analysis of a large

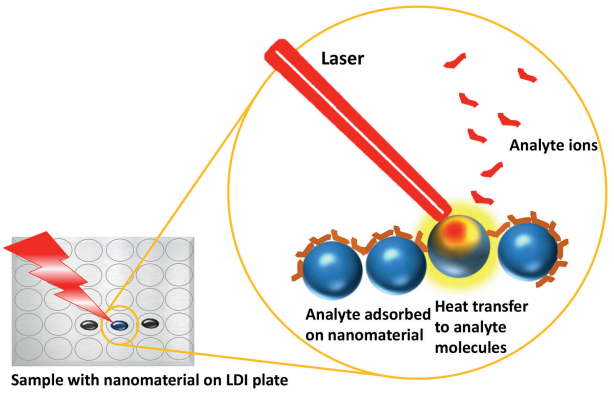

Fig. 1 – Schematic representation of a SALDI process mechanism.

category of small molecules. Common pollutants, illegal or medicinal drugs and their metabolites tend to have small molecular sizes, which can be analyzed by SALDI-MS. Other important aspects in mass spectrometry based detection of metabolite are the metabolomics profiling and targeted approach [25–28]. For selective ionization of the analytes, SALDI matrices are engineered through structural and surface modification, which has the advantages of maximizing the specificity and sensitivity of MS methods. The nanoparticle surfaces modified with specific molecules, such as antibodies, proteins, and aptamers, highly reduce the background noise and interference from the undesired molecules from the biological samples. Therefore, combination of separation or enrichment of analytes and efficient SALDI is critical in nanoparticle-based LDI-MS for drugs and metabolites analysis.

In this review, we mainly discuss the detection of drugs and metabolites using nanomaterial-assisted LDI-MS. A summary of the various types of naomaterial substrates and analytes discussed in this review is presented in Table 1. Based on the category of nanomaterials employed as substrate, different names have been coined to the LDI processes for convenience, by various authors. For example, nano-assisted LDI (NALDI) [16], desorption/ionization on silicon (DIOS) [17], silicon nanopost arrays-assisted LDI (NAPA-LDI) [29], colloidal graphite-assisted LDI (GALDI) [30], carbon dot assisted LDI (CALDI) [31], etc. However, the role of these substrates in the LDI application is almost the same. We briefly discuss the type of nanomaterial substrates and their important properties which improve the LDI process and application of "mass tag" for LDI-MS signal amplification, in drug and metabolite analysis.

2. Nanomaterials as a matrix for SALDI-MS

The major role of nanomaterial in nanomaterial-based LDI-MS is the enrichment of the analyte molecules and their efficient desorption and ionization. Therefore, nanomaterials with large surface area, porous structure, easy functionalization, high photoabsorption properties and heat transfer efficiency are highly favored. Metal nanoparticles, especially gold

Table 1 – Various types of nanomaterial substrates used for the detection of drugs or metabolites by LDI-MS.

Category	Probe/substrate	Nanomaterial properties or LDI process	Analytes	LOD[a]	References
Metal-based	Au nanoshell	plasmonic, generation of hot carriers	amino acids and carbohydrate	3–30 pmol	[8]
	ITO	conductive, nanostructured, and transparent	lipids, glycerol, and creatinine	6 mg L^{-1}	[9]
	TiO_2 nanowire	photoabsorption	benzylpenicillin	0.4 ng mL^{-1}	[18]
	TiO_2 NPs	photoabsorption	18 candidate metabolites from bacteria	n/a	[46]
	HSA-Fe_3O_4 NPs	affinity towards analytes molecules	phenytoin, ibuprofen, camptothecin, warfarin	5 μM	[19]
	ZnO, TiO_2, Fe_2O_3, CeO_2	small size and large surface area, strong UV absorption	amitriptyline hydrochloride, imipramine hydrochloride, nortriptyline hydrochloride, promazine hydrochloride	n/a	[44]
	Au NPs, Ag NPs, Pd NPs, Pt NPs	photoabsorption, binding affinity, internal energy transfer	benzylpyridinium chloride	n/a	[39]
	Au NPs	photoabsorption, high salt tolerance	progesterone, testosterone, cortisol, ribose, glucose, maltose, 5-HIAA, tryptophan, GM1, bradykinin, angiotensin I/II, substance P	46.5–5115.7 nM	[40]
	Au NPs	photoabsorption	palmitic acid, oleic acid, stearic acid, verapamil	n/a	[41]
	Au NPs/KBr	photoabsorption	acetaminophen, noscapine, loratadine, coptisine, berberine, palmatine	n/a	[42]
	ARRO SupraNano™	photoabsorption	cocaine, methadone, aspirin, paracetamol, caffeine	n/a	[43]
	TiO_2-dopamine monolith	dopamine enhanced UV absorbance, selective binding of Lewis bases	fatty acids, cholesterols, ceramides, diacylglycerols, phosphatidylethanolamine, amino acids, alkaloids, peptides, lipids	n/a	[45]
Carbon-based	graphene, GO	photoabsorption, electron transport	scutellarin, wogonin, ferulic acid	n/a	[67]
	aptamer modified GO	enrichment, photoabsorption	cocaine, adenosine	n/a	[21]
	graphite rods	photoabsorption	9-phenylacridine	0.1 pmol	[30]
	C-Dots	*in situ* energy transfer *via* functional groups	mefenamic acid	0.5 ng	[31]
	GO/MWCNT hybrid films	photoabsorption; functional groups of GO	cellobiose, leuenkephaline, glutamine, glucose, leucine, lysine, D-mannitol, phenylalanine	n/a	[33]
	CNT, C_{60}, PGC, G, HOPG, ND	phase transition	nenzylpyridinium halide derivatives	n/a	[34]
	Graphene, GO, rGO	size dependent, photoabsorption	flavonoids, coumarin derivatives	10 ppm	[35]
	Graphene, GO	photoabsorption, electron transport	tetracycline	2 nM	[51]
	Graphite dots	strong UV absorption, hydroxyl groups provide high stability and favorable ΔG for analyte deprotonation	glucose, Ala-Gln, oligosaccharides; puerarin, daidzein, dihydrodaidzein	n/a	[52]
	N-doped graphene	proton transfer, photoabsorbance	fatty acid, peptide, epiandrosterone, testosterone, methyltestosterone, nilotinib	n/a	[53]

(*continued on next page*)

Table 1 – (*continued*)

Category	Probe/substrate	Nanomaterial properties or LDI process	Analytes	LOD[a]	References
Silicon/Silica/Semiconductor	Nanostructured silicon	desorption ionization on porous silicon mass spectrometry imaging (DIOS-MSI)	methadone, heroine, EDDP	n/a	[17]
	Mesoporous germanium	photoabsorption	cocaine	1.7–3.5 ng mL^{-1}	[20]
	Silicon nanopillar arrays	photoabsorption	peptides, methadone	32 ng mL^{-1}	[22]
	Nanoporous GaN–Ag composite	high UV absorption	R6G, adenine	<50 pmoles	[23]
	Silicon nanopost arrays	nanophotonic interaction	cAMP, acetylcholine, glucose, cholesterol, amino acids, small organic acids, phospholipids, fatty acids	n/a	[29]
	Porous silicon	high UV absorption	methadone	14.74–19.50 ng mL^{-1}	[36]
	Nanostructured Silicon	nanostructure-initiator mass spectrometry (NIMS)	rapamycin, tigylglycine, N-acetyl-glutamic acid, uridine monophosphate, isobutyrylcarnitine, stearic acid, D-glucose	n/a	[37]
	Ordered silicon nanocavity arrays	desorption ionization on porous silicon-mass spectrometry (DIOS-MS)	benzylpyridinium salts, DPPC, angiotensin III	n/a	[56]
	Ordered silicon nanocavity arrays	desorption ionization on porous silicon-mass spectrometry (DIOS-MS)	methamphetamine, cocaine, MDMA	n/a	[57]
	Nanoporous silicon microparticles	photoabsorption	methamphetamine, cocaine, MDMA, methadone, EDDP	~20 ng mL^{-1}	[58]
	hydrophobic silica powder	photoabsorption	nicotine	n/a	[60]
	DHB + porous silicon	matrix-enhanced nanostructure initiator mass spectrometry (ME-NIMS)	pentamidine	0.005 μM	[61]
	Nanostructured Silicon	morphology-driven controlled nanostructure-initiator mass spectrometry (NIMS)	arginine, palmitylcarnitine, streptomycin, bradykinin, angiotensin, neurotensin	n/a	[62]
	Nanoporous silicon films	perfluoro coating assisted desalting	taurine, aspartic acid, malic acid, glutamic acid, histidine	~1 μM	[63]
Composite-based	PAN/Nafion®/CNT	photoabsorption	verapamil, methotrimeprazine, propranolol, chloroprothixene	220 fM	[13]
	Au/PAA-GO film	photoabsorption, thermal conductivity	cellobiose, mannitol, glucose, leucine, phenylalanine, glutamine, leuenkephalin	n/a	[50]
	SiO_2@Ag particles	plasmonic resonance	mannitol, glucose	100 ng μL^{-1}	[65]
	Ag-DIOS	silver adduct assisted	bromoisatin, lipids, cholesterol	n/a	[66]
	plasmonic gold chip	plasmon resonance, hot carrier production	amino acids, carbohydrates, metabolites	n/a	[64]
Mass tag	thiolalkane-Au NPs	thermal desorption of ligand	enrofloxacin, ciprofloxacin	50 mg kg^{-1}	[7]
	Fib–Au NPs–MCEM	NP fragmentation	thrombin, anticoagulants	n/a	[47]

[a] n/a: not available.

nanoparticles (Au NPs) were found to transfer heat efficiently to the analyte molecules under pulse laser irradiation, enabling more effective ionization [32]. In addition, Au NPs' surface favors easy functionalization especially with sulfur containing molecules. Nanostructured metal oxides tend to have porous structure which enhances the analyte enrichment. Carbon materials such as carbon nanotube (CNT), graphene oxide (GO), graphite, etc. have hydrophobic as well as hydrophilic properties, and their surface can be easily modified. They are excellent choices as substrates for LDI-MS [33–35]. Similarly, silicon based nanomaterials, especially porous structured materials, are highly useful in enriching analyte molecules from the sample, which are found to be applied for detection of many drugs and metabolites [36,37]. For convenience, the discussion in this review has been divided into sections based on the category of nanomaterials employed for the analysis of drugs and metabolites. Most of the nanomaterial based LDI-MS follow SALDI-principle, in which either analyte ions or mass tag ions are produced; however, in some cases the nanomaterial substrate itself undergoes explosion and produces cluster ions, which are considered as the signal response for analyte determination. A detailed review of such LDI process is available in our earlier review article [32].

2.1. *Metal-based nanomaterial*

Metal-based nanomaterials are one of the earliest nanomaterials to be developed and utilized as matrices in LDI-MS. Tanaka et al. demonstrated the use of ultrafine cobalt powder with a diameter of about 300 Å for the analysis of proteins and polymers with *m/z* values up to 100,000 by LDI-TOF-MS [38]. The discrete electronic levels in small-size metal nanoparticles create quantum effects, and thus they show increased photo-absorption efficiency than that of bulk metals. Many metal nanoparticles have excellent UV absorption profile, and high melting point, which can promote thermal desorption process. Additionally, the ability of metal to form alloys, whether by chemical or physical processes, allows easy control of nanoparticle properties for optimization of analyte desorption. The mechanism of ionization of analytes by metallic nanoparticles are well addressed by Ng et al. who conducted the experiments on different small-size noble metal nanoparticles (Ag, Au, Pd and Pt NPs) [39]. The study concluded that many factors might affect the desorption/ionization efficiency of analytes, including thermal conductivity, internal energy transfer, thermal absorption, melting point and analyte-nanoparticle binding affinity. They suggest that substrates with a low melting point could enhance the ionization through phase transition. Phase transition increases entropy and favors ionization. The high affinity between analyte and the substrate could reduce the ionization efficiency. Therefore, to achieve efficient ionization of the analytes, high photo-absorption and low melting point of the substrate material, and moderate affinity between the analytes and substrate are highly favorable. Metal nanoparticles, Au NPs in particular have a long history in SALDI-MS detection of drugs and metabolites. Wu et al. reported the use of Au NPs in the detection of biomolecules including urea, creatinine, uric acid, and glucose in complicated urine samples [40]. In this report, Au NPs have been proved to be an excellent substrate, providing great shot-to-shot reproducibility [relative standard deviation (RSD) values from 0.03 to 3.48%], and is capable of detecting a multitude of compounds including neutral steroids (testosterone, progesterone and cortisol), carbohydrates (ribose, glucose and maltose), indoleamines (5-hydroxyindole acetic acid and tryptophan), etc. Tang et al. demonstrated that Au NPs when coupled with LDI-MS could also be applied for mass spectrometric detection of endogenous (palmitic acid, oleic acid, and stearic acid) and exogenous (verampil) chemicals in latent fingerprint (LFP) and mass spectrometric imaging of LFP based on the distribution of exogenous compounds [41]. The Au NPs are deposited on the ridges and grooves of the fingerprint with different degree of aggregation, resulting in a clear pattern of distribution. Mass spectrometric molecular imaging could reveal the chemical information of endogenous and exogenous compounds in the fingerprint, and if coupled with optical imaging it allows for personal identification. Chau et al. demonstrated that Au NPs could be directly applied as a coating on over-the-counter drug and traditional concentrated Chinese medicine granules in KBr pellets for LDI-MS detection of their active ingredients, without interfering the usage of the same pellets for FT-IR analysis (Fig. 2) [42]. They showed that the thin layer of Au NPs do not interfere with FT-IR analysis (difference in transmittance less than 2%), and also allow for LDI-MS detection of several minor active ingredients (e.g. noscapine and loratadine) and phytochemicals of *Coptidis rhizoma* (i.e. berberine, palmatine and coptisine) that are undetected by FT-IR.

Metal oxide nanoparticles also assist LDI process and some of them have porous structure and high surface area, which are favorable for the enrichment of analyte molecules. Human serum albumin-modified Fe_3O_4 magnetic nanoparticles (HSA–Fe_3O_4 NPs) are found to be an efficient affinity SALDI probe to capture traces of drugs with low molecular weight, such as phenytoin, ibuprofen, camptothecin, and warfarin present in urine and serum by LDI-MS, whereas traditional MALDI can only detect warfarin [19]. Fig. 3 shows the

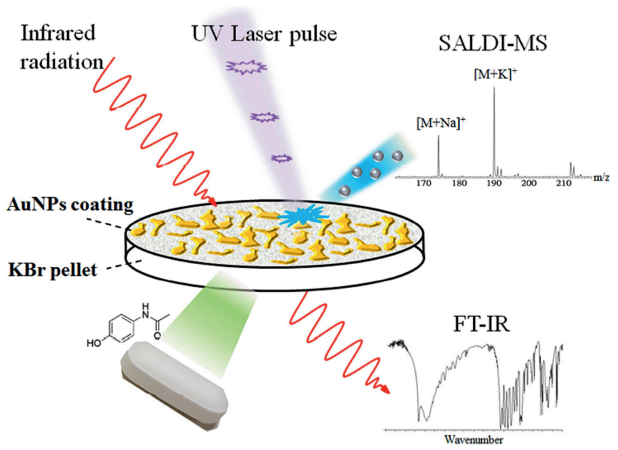

Fig. 2 – Schematic representation of Au NPs coated KBr pellet for SALDI-MS and FT-IR analysis of traditional Chinese medicine. (Reproduced with permission from Ref. [42]).

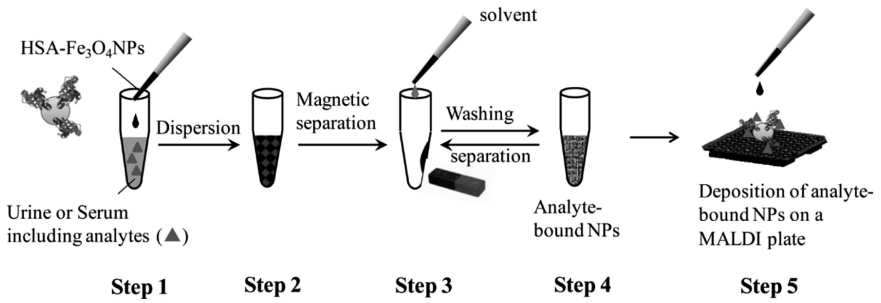

Fig. 3 – Schematic representation of enrichment and detection strategy for the analysis of phenytoin, ibuprofen, camptothecin, and warfarin in urine and serum samples using human serum albumin-modified Fe_2O_3 nanoparticles. (Reproduced with permission from Ref. [19]).

schematic representation of affinity-SALDI-MS procedure for the enrichment and detection of drugs using HSA–Fe_3O_4 NPs as SALDI substrate, in serum or urine samples. The magnetic property of the nanoparticles enables the easy separation of the analytes from the background molecules. In this approach, first the analyte molecules are enriched using the affinity probe-functionalized Fe_3O_4 magnetic nanoparticles, then they are subjected to SALDI process. In such a case, the analysis follows a SALDI principle, in which analyte ions are produced. The authors further demonstrated that denatured-HSA (using $NaBH_4$ for denaturation) exhibits higher sensitivity for the detection of drugs than HSA, probably due to the reduced S–S bonding in denatured-HSA leading to more open structure and enhanced interaction with drugs. Interestingly, LDI-MS can be successfully applied for the detection of drugs in latent fingerprint using the fingerprint powder itself as LDI substrate. Sundar et al. applied LDI-MS for the detection of two drugs of abuse (cocaine and methadone) and three therapeutic drugs (aspirin, paracetamol and caffeine) in latent fingerprint and imaging using commercial ARRO SupraNano™ MS black magnetic powder [43]. Very recently, Amin et al. used various metal oxide nanoparticles, such as ZnO, TiO_2, Fe_2O_3, and CeO_2 nanoparticles for the identification of small drug molecules, such as amitriptyline hydrochloride, imipramine hydrochloride, nortriptyline hydrochloride, promazine hydrochloride in latent fingerprint by SALDI-MS [44]. They found that all the nanoparticles reduce the background noise and increase the signal intensity, among which Fe_2O_3 exhibits the maximum sensitivity.

TiO_2 is widely used in various analytical applications. Kim et al. utilized TiO_2 nanowires for the detection of benzylpenicillin in milk [18]. This report revealed that the optimal TiO_2 crystalline structure to be served as LDI matrix is anatase. The nanowire has proven to be excellent for the detection of two model amino acids (asparagine and alanine). Penicillin spike in milk is detectable after simple centrifugation treatment with a detection limit of 0.4 ng mL^{-1}, which is one order lower than the cut-off value of 4 ng mL^{-1}, according to the EU directive. Dopamine modified TiO_2 (TiO_2-DA) monoliths have been reported to exhibit better UV absorption in comparison with unmodified TiO_2 particles [45]. The TiO_2-DA monolith-assisted LDI-MS imaging of mouse brain tissue exhibits lower background signals, higher selectivity and sensitivity for some Lewis base compounds, such as fatty acids, cholesterols, ceramides, diacylglycerols, and phosphatidylethanolamine, compared to traditional MALDI-MS imaging with 2,5-dihydroxybenzoic acid (DHB) as matrix. The Lewis acid site in TiO_2 (Ti^{4+}) possess very strong affinity to Lewis bases. More than 100 molecules, including amino acids, alkaloids, free fatty acids, peptides, and lipids, localized in mouse brain sections could be detected by TiO_2-DA monolith-assisted LDI-MS imaging. Moreover, this approach demonstrates the analysis of aging-related neurochemical changes in the brain by comparing the presence and localization of those molecules in young and old mouse brains. Recently, Zhang et al. showed the potential of TiO_2-assisted LDI-MS to distinguish drug-resistant bacteria based on the profiling of candidate metabolite biomarkers from intact bacterial cells [46].

"Mass tags" or "amplification tags" are used in the SALDI-MS analysis for signal amplification and to reduce interference. Amplification tags, generally, are easily ionizable molecules immobilized on the probe nanoparticles. Depending on the number of tags on each nanoparticle and the type of assay, the signal can be highly amplified for improving the sensitivity. In this case, instead of monitoring the analyte peaks, the mass tag peaks are monitored. However, the assay preparation is a little more complicated, involving the functionalization of the nanoparticles with mass tags and selective enrichment of the analyte, than that of normal SALDI-MS analysis. Thioalkanes have unique affinity with gold through the strong Au–S bond (180 kJ mol^{-1}) and their ionization efficiency enable them as mass reporter tags in LDI-MS [7]. Ha et al. utilized Au NPs with mass tags of thiolalkane ligands for the detection of enrofloxacin, a commonly used antibiotic, and its metabolite ciprofloxacin (Fig. 4A) [7]. The enrofloxacin or ciprofloxacin molecules are chemically anchored on thiolated-Au NPs with the carboxylate terminal by EDC/NHS reaction, whereas the tri(ethylene glycol)-terminated thioalkanes are used as the mass reporter tags. The analytes (enrofloxacin and ciprofloxacin) and functional Au NPs are competitively captured by antibody coated coverslips and subjected to LDI-TOF MS. Therefore, the tag signal deceases upon increasing the concentration. This competitive assay could selectively detect enrofloxacin and ciprofloxacin with a limit of detection of 0.5 mg kg^{-1}.

Other than organic molecules, metal-based nanoparticles also can be applied as mass tags or amplification tags. In this

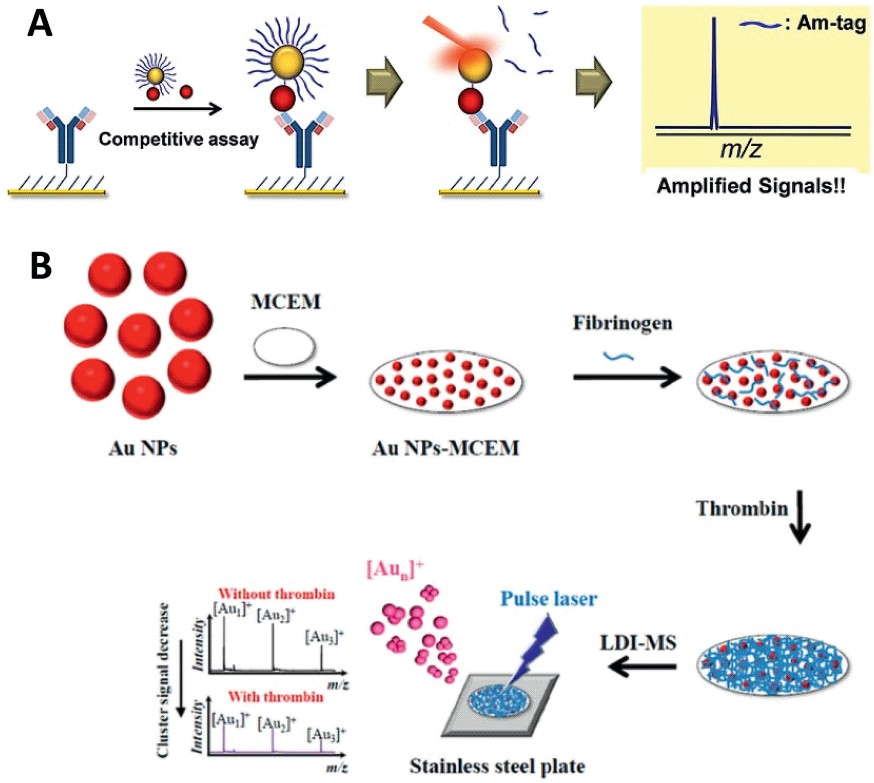

Fig. 4 – Schematic representation of SALDI-MS coupled with (A) thioalkane as amplification tag for detection of enrofloxacin and ciprofloxacin, and (B) gold cluster ions as amplification tag for monitoring of thrombin activity and anticoagulant screening. (Reproduced with permission from Refs. [7] and [47], respectively).

case, the metal nanoparticles serve as substrate as well as amplification tag for LDI-MS. Metallic nanoparticles are known to produce metal cluster ions $[M_n]^+$ under pulse laser irradiation. Several studies have shown that Au NPs can produce intense gold cluster ions ($[Au_n]^+$) signals in LDI-MS [47]. Nano- and femto-second pulsed laser can produce $[Au_n]^+$ by thermal evaporation and columbic explosion, respectively [32]. Fig. 4B shows the principle of the LDI-MS using metallic cluster ions as mass tags. In 2014, our research group demonstrated the use of Au NPs as metallic barcode for anticoagulant drugs screening [47]. We utilized Au NPs-modified mixed cellulose ester membrane (Au NPs–MCEM) conjugated with fibrinogen (Fib) as a probe (Fib/Au NPs–MCEM) for the detection of the activity of thrombin. The Fib/Au NPs–MCEM coupled with LDI–MS sensing system provides very clean mass spectra, allowing the monitoring of Au cluster ions ($[Au_n]^+$; $n = 1–3$) under pulse LDI process. The deposition of fibrin on Au NPs–MCEM triggered by thrombin is found to suppress the signal of $[Au_n]^+$. As a result, the intensity of the $[Au_n]^+$ is correlated with the thrombin concentration. The probe could specifically detect thrombin with a limit of detection of 2.5 pM in human plasma samples. Moreover, the sensing system is employed for the detection of direct thrombin inhibitors (DTI), such as thrombin-binding-aptamers, hirudin and argatroban. Due to the fact that monometallic nanoparticles can only produce certain cluster ions of certain *m/z* after fragmentation, like ligand amplification mass tags, metallic barcodes also have the potential for multiplex detection. Our research group demonstrated the use of different metal nanoparticles (Au, Ag and Pt NPs) for the detection of different proteins (thrombin, VEGF, PDGF) [48]. By using appropriate recognition ligands, multiple metal cluster ion bar codes have huge potential for the analysis of multiple metabolites and drugs simultaneously.

In summary, metal-based nanomaterials including noble metal nanoparticles and oxides of transitional metals are widely used in both global and targeted detection of a variety of metabolites and drugs. Further, they are successfully applied for the detection of drugs in latent fingerprints. Interestingly, metal-based nanomaterials can provide high resolution and visual contrast (due to plasmon resonance) that can be applied for dual modal fingerprint imaging. Many transitional metal oxides are favored for their high melting point and magnetic properties, providing better thermal desorption and easier sample enrichment. Thermal desorption processes are highly important for metal and metal oxide particles; however, recent reports suggest that surface chemical properties and phase transition processes are also important. In addition, metallic nanoparticles and their surface ligands can also act as mass tags for signal amplification. However, for selective enrichment and ionization of analytes in targeted approach, the functionalization of the nanoparticle followed by analyte enrichment usually is complicated, time consuming or expensive.

2.2. *Carbon-based nanomaterials*

Carbon materials as matrices for LDI-MS were developed quite early in the history of SALDI-MS. Sunner et al. demonstrated the use of graphite as SALDI substrate for the analysis of peptides and proteins by SALDI-TOF-MS [49]. They obtained slightly different spectra in wet and dry conditions for peptides and proteins and were comparable to that of conventional MALDI spectra, even though a few background peaks were observed in the low molecular weight region. Carbon nanomaterials, due to their low cost, excellent UV absorption, tunable surface functional groups and electronic properties, have been sought after as potential LDI matrices. One of the greatest advantage of carbon nanomaterial in LDI application is their ease of conjugation and range of interactions with many analytes. Simple oxidation treatment can introduce various functional groups onto the surface of carbon nanomaterials. Functional groups such as carboxylate, epoxy and hydroxyls offer sites for chemical modification/physical adsorption of ligands/analytes. In addition, carbon nanomaterials with sp^2 hybridization allow for physical interaction with analytes through π-π interaction, enabling enrichment. That is, nonoxidized sites can also contribute by interacting with hydrophobic analytes. A hybrid film of poly(allylamine hydrochloride)-functionalized graphene oxide (GO) and Au NP is found to be an efficient substrate for the LDI-MS detection of small molecules [50]. GO can efficiently dissipate the heat and avoid the fragmentation of the analytes. Multi-layer GO films have good photoabsorption and heat transfer efficiency [33]. Tang et al. conducted a study on the SALDI mechanism of carbon-based nanomaterials [carbon nanotubes (CNT), buckminsterfullerene (C_{60}), nanoporous graphitic carbon (PGC), non-porous graphite particles (G), highly oriented pyrolytic graphite (HOPG), or nanodiamonds (ND)] [34]. Their work demonstrated the desorption efficiency is in the order of CNT $\approx$ C_{60} > PGC > G > HOPG > ND, which in general exhibits an opposite trend to the extent of internal energy transfer (CNT < C_{60} $\approx$ PGC < G $\approx$ HOPG < ND). This result indicates that increasing the extent of internal energy transfer in the SALDI process may not enhance the ion desorption efficiency. Furthermore, the authors demonstrated that thermal desorption (determined mainly by photoabsorption efficiencies) accounts for only a small part in the desorption efficiency of carbon substrates, the main mechanism for the improved ionization efficiency may be due to the phase transition/destruction of the substrates. Phase transition in essence is the melting and vaporization of the substrates which eject the adsorbed analyte into the vacuum. Another study further proposed that the thermal-driven desorption plays a significant role in the ion-desorption process [39]. The phase transition of nanoparticles during laser ablation could reduce the activation energy (ΔG) of the ion-desorption of the analyte by increasing the entropy (ΔS) through vaporization and phase explosion of the substrates [39].

Gulbakan et al. have reported the detection of cocaine by cocaine aptamer covalently conjugated GO as the matrix [21]. By using the matrix effect of GO combined with the enrichment ability of cocaine aptamer, they have successfully detected cocaine in plasma samples. Graphene, GO and reduced graphene oxide (rGO) can be used as matrices for the detection of various flavonoids, such as kaempferol, morin hydrate, myricetin, quercitrin, quercetin-3-b-D-glucoside and rutin in the negative ion mode [35]. At low concentrations of flavanoids, GO exhibits higher sensitivity than graphene and reduced GO, due to the abundant carboxyl functional groups, indicating its efficiency for ionizing the analytes. Liu et al. reported the use of graphene and GO for the detection of tetracycline antibiotics (tetracycline, oxytetracycline, demeclocycline, and chlortetracycline) in milk [51]. In addition, due to the large surface area and interaction with the analytes, the enrichment of tetracycline on the matrices are high, enabling a limit of detection as low as 2 nM.

Carbon dots (C-dots) are a new class of interesting carbon materials for LDI-MS analyses. Gedda et al. employed C-dots synthesized from citric acid as matrix for the detection of low molecular weight nonsteroidal anti-inflammatory drug, mefenamic acid (MFA), which is difficult to detect with other spectrometric methods without complex sample preparation [31]. C-dots are found to be effective in detecting MFA in serum in both positive and negative ion mode with a limit of detection of ca. 0.5 ng. Shi et al. conducted a detailed research on using hydroxyl-group dominated graphite dots (GDs) as substrates for the detection, *in situ* imaging and real time monitoring of many small molecules [52]. GDs have been found to be excellent matrices for analysis of extracts of *Morinda officinalis*, which is commonly used in traditional Chinese medicine, and identification of several oligosaccharides [52]. Furthermore, GDs are also capable of detecting puerarin in mouse serum and that by applying GDs on kidneys, metabolites of puerarin (daidzein and dihydrodaidzein) can be tracked *in situ* through mass spectrometry imaging (MSI) (Fig. 5). The hydroxyl rich GDs have favorable energy dissociation pathways for non-carbon groups, which indicates lower background noise and higher structural stability.

Similarly, nitrogen-doped graphene (NG) has been found to be an excellent matrix for the detection of anabolic androgenic steroids (AAS) and anticancer drugs in negative ion mode, which is currently not often used in MS analysis, producing $[M]^-$ and $[M-H]^-$ ions [53]. This study reveals NG as a superior matrix for detection of a wide range of low-molecular weight analytes including amino acids, fatty acids, peptides, anabolic androgenic steroids as well as anticancer drugs, with an extraordinary LDI efficiency over traditional α-cyano-4-hydroxycinnamic acid (CHCA) and other carbon-based materials in the negative ion detection mode. The $[M-H]^-$ ions are proposed to arise mainly from proton transfer from the analyte to the pyridinic nitrogen while the $[M]^-$ are the result of electron transfer from the graphitic nitrogen species to the analyte on the NG. The results show that NG is an excellent matrix in negative ion mode, providing both less matrix interferences and lower matrix fragmentation. The good salt tolerance and high sensitivity of NG based SALDI-MS allow for the detection of anticancer drug nilotinib in the spiked human serum, down to 1 μM, which meets the demand of assessing drug level in the patient serum. The above reports clearly reveal that carbon materials are excellent matrices/substrates for drug and metabolites analysis due to their functional and

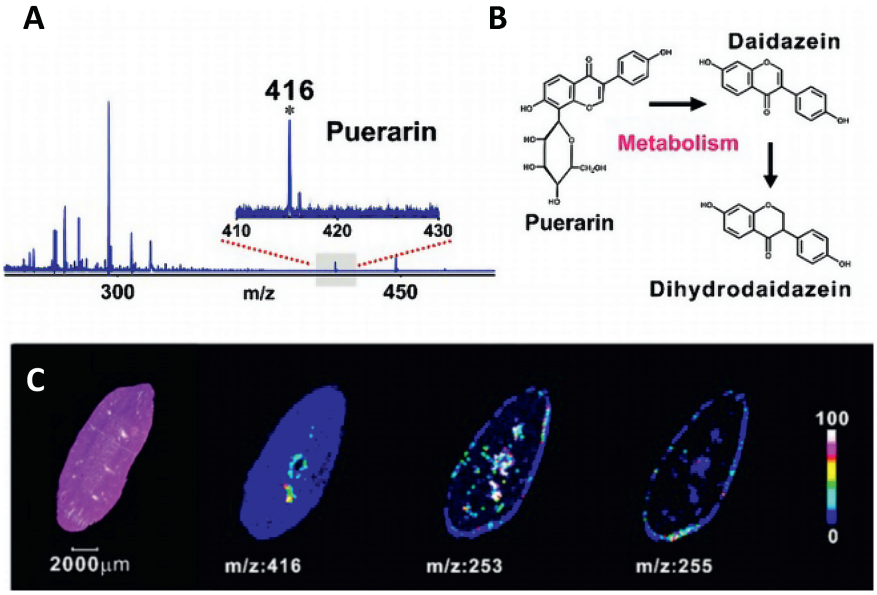

Fig. 5 – MSI of puerarin and its metabolites in rat kidney. (A) MALDI mass spectrum (negative-ion mode) of mouse serum after intraperitoneal administration of puerarin. Inset: Zoomed-in view of the spectrum in *m/z* 410–430, characteristic peak of puerarin: [M–H] at *m/z* 416. (B) Metabolic pathways showing puerarin and its two metabolites, (C) MALDI-MS images of the drug and its metabolites (puerarin, *m/z*: 416; daidzein, *m/z*: 253; and dihydrodaidzein, *m/z*: 255) in a kidney tissue slice with an optical micrograph of an H&E-stained consecutive slice as a reference. The color bar encodes the signal intensities of the three small molecules in MSI. Reproduced with permission from Ref. [52].

hydrophobic properties and high desorption/ionization efficiency emanating from phase transition during laser ablation.

In summary, carbon-based nanomaterials are also excellent SALDI matrices in both global and targeted profiling. Compared to metal nanoparticles, the core structure, phase transition processes and surface functional groups of carbon nanomaterials are shown to play a more significant role in the ionization mechanism. Moreover, carbon nanomaterials are shown to have more potential in both positive and negative ion mode detection. The functional properties of carbon nanomaterials and π-π interaction with analytes are advantages in enrichment of some specific analytes. However, a small disadvantage of using carbon nanomaterials as substrate for LDI-MS is that, they produce carbon cluster peaks in the low molecular weight region at high laser fluence. Although there had been some success in reducing carbon cluster signal by modifying functional groups, precise control of the surface functional groups of carbon materials remains challenging.

2.3. *Silicon-based nanostructures*

Silicon oxides are known to produce porous structures, which provide greater area of contact. Since silica can be easily functionalized with a variety of molecules, it has been used for the extraction of analytes prior to mass spectrometry analysis for better sensitivity and selectivity. For example, Rejeeth et al. reported the application of hyaluronic acid (HA) functionalized Fe_3O_4–SiO_2 core shell nanocomposite for the extraction and profiling of various serum biomarkers by mass spectrometry [54]. The silica shell enables easy functionalization with HA, which is otherwise difficult to functionalize HA directly on Fe_3O_4. Even though silica materials are developed later for LDI-MS [55], they revolutionized SALDI-MS detection and are currently the most used matrices for small molecule analysis. They have a variety of LDI derivatives such as DIOS which operates mainly on direct analyte desorption process (the "dry process") and indirect desorption process from the fast vaporization of residue solvents in the pores (the "wet process") [56]. Dry process dominates when the porosity is low, while wet process dominates when the porosity is high. DIOS chip of thin porous silicon (pSi) film functionalized with pentafluorophenylpropyl chlorodimethyl silane (F5PhPr) has been employed for the detection of nicotine, methamphetamine, codeine, methadone, and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (metabolite of methadone) in fingerprint [17,57]. This DIOS-MSI has been further utilized for the analysis of exogenous and endogenous drug compounds from fingerprints.

Hydrophobically functionalized porous silicon microparticles (pSi-MPs)-based DIOS has been reported as an efficient matrix for the detection of illicit drugs, such as methamphetamine, cocaine and 3,4-methylenedioxy-methamphetamine (Fig. 6) [58]. The study reveals that particle size, pore diameter, pore depth and functionalization of pSi-MP all have effect on the detection of the illicit drugs. The optimized pSi-MPs allow for the extraction and detection of methadone from spiked saliva and urine samples. More recently, the same group further demonstrated pSi-MPs as efficient substrates for the detection of methadone and its metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), in clinical urine and plasma samples [36]. Similarly, hydrophobic silica with

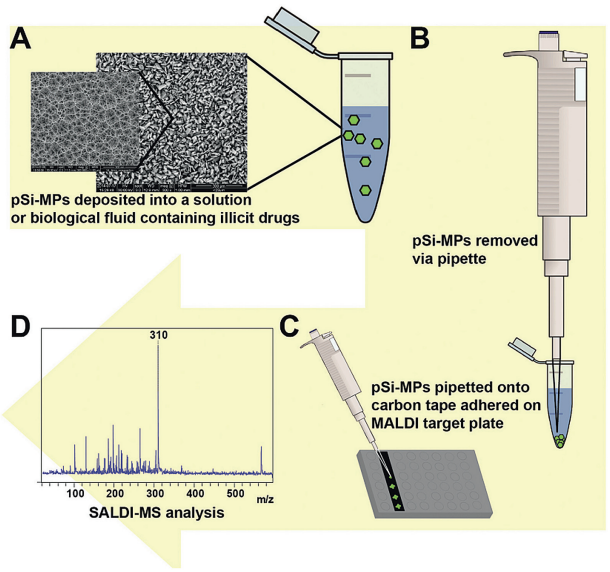

Fig. 6 – Schematic for (A) pSi-MP extraction of illicit drugs compounds from biological fluids, (B) pSi-MPs removed from the sample solution, (C) deposition onto standard MALDI target plate using double sided carbon tape and (D) analysis using SALDI-MS. (Reproduced with permission from Ref. [58]).

sub-micron size doped with carbon black has been found to be an effective fingerprint-developing agent as well as a SALDI substrate for the detection of nicotine and cotinine in latent fingermark of smokers [59,60].

Another strategy for silicon based substrate in LDI-MS is the nanostructure initiator mass spectrometry (NIMS), which uses perfluorinated compounds as liquid initiators [16,61,62]. In addition to better adsorption of analytes due to hydrophobic interaction, these initiators do not absorb UV light, usually do not ionize or crystallize with the analyte allowing for reducing matrix interferences. This technique combines the benefits of traditional MALDI and NIMS, using a porous substrate to improve desorption efficiency at lower flux and providing a proton rich environment with acid matrix. The study by Guinan et al. on the DIOS, NIMS and NALDI process for the detection of amphetamines, benzodiazepines, opiates and tropane alkaloids suggests these matrices have comparable performance, however, cheaper manufacturing cost for DIOS and NIMS may be a better alternative for commercialization [16]. Perfluoro-coated (*1H,1H,2H,2H*-perfluorooctyl) trichlorosilane or (*1H,1H,2H,2H*-perfluorooctyl) dimethylchlorosilane nanostructured surfaces can also be employed to selectively segregate hydrophilic analytes from biofluidic electrolytes [63]. The work demonstrated that by controlling the contact angle of fluorinated silane substrate between 120 and 150°, background electrolytes can be made to segregate from hydrophilic analytes during a drying step on the surface of a highly nanoporous thin film (Fig. 7). By this way, the high concentration salts are separated, while the analytes can enter the pores. Coupling with LDI-MS, this strategy enables more sensitive detection of metabolites such as amino acids even in complicated samples such as cerebrospinal fluid and serum. With this on-chip desalting, histidine spiked in cerebrospinal fluid could be detected within a range from 1 to 50 μM. Moreover, this SALDI-MS shows both good reproducibility for the quantification of five highly polar metabolites (taurine, aspartic acid, malic acid, glutamic acid, and histidine) in serum and the results obtained on the desalted serum sample spots are comparable with those of NMR and liquid chromatography–mass spectrometry. In addition to silicon, some semiconductors such as mesoporous germanium (meso-pGe), synthesized by bipolar electrochemical etching method, has been demonstrated as an efficient matrix for the detection of cocaine by LDI-MS [20]. The report suggests that the substrate has high stability and reproducibility and it is capable of detecting cocaine down to 1.7 ng mL^{-1} in DI water and 5.3 ng mL^{-1} in spiked saliva.

In summary, silicon-based materials are widely used as matrices for analysis of small compounds including drugs and metabolites. The porosity of silicon nanomaterials is easy to control and a variety of materials (such as fluorocarbons) can be functionalized or coated onto the silica materials for improved detection of certain analytes making them extremely useful for small molecule detection. DIOS and its many variants are now a staple for SALDI detection of small molecule. However, preparation of various types of silicon substrates requires a more complicated procedure than that of metal- or carbon-based LDI substrates.

2.4. *Nanocomposites*

Recently, composite nanoparticles have been found to be very useful as substrates for LDI-MS analysis of drugs and metabolites. Nanocomposites having tunable band gap and surface properties for the absorption of laser light and interaction with analytes endow them as potential LDI substrates. Nie et al. demonstrated the use of nanoporous GaN–Ag composite material with moderate band-gap and high density of pores (~10^9 cm^{-2}) as LDI-substrate for the detection of cholesterol and nucleotides [23]. The moderate band gap enables strong photo absorption and GaN–Ag composite has good heat transfer efficiency. In addition, the Ag nanostructures in the porous GaN-Ag composite material act as cationization agents and also facilitate the thermal desorption process, resulting in high efficiency of LDI process. Wei et al. synthesized SiO_2 core with plasmonic gold nanoshells (Au nanoshells) for the detection of serum metabolites [8]. The Au nanoshell exhibits higher ionization efficiency and less fragmentation in the low *m/z* region, than those of gold nanorod, nanosphere and traditional organic matrices. Au nanoshells are shown to be highly effective in the detection of six representative amino acids (valine, lysine, methionine, arginine, tryptophan and phenylalanine) with a detection limit of 3–30 pmol, and also various amino acids and carbohydrates (glucose, mannitol) in serum. A more recent article by Sun et al. reveals the potential of plasmonic gold nanoshells coated microarray chips for the metabolite fingerprinting of biofluids and exosomes [64]. The authors suggest that plasmonic properties of the gold nanoshell with silica core has significant influence in the LDI processes such as hot carrier production, local heating, and photodesorption. Such efficient LDI substrate can handle samples with sample volume as low as 400 pL. The plasmonic gold chip has the potential to diagnose early stage non-small

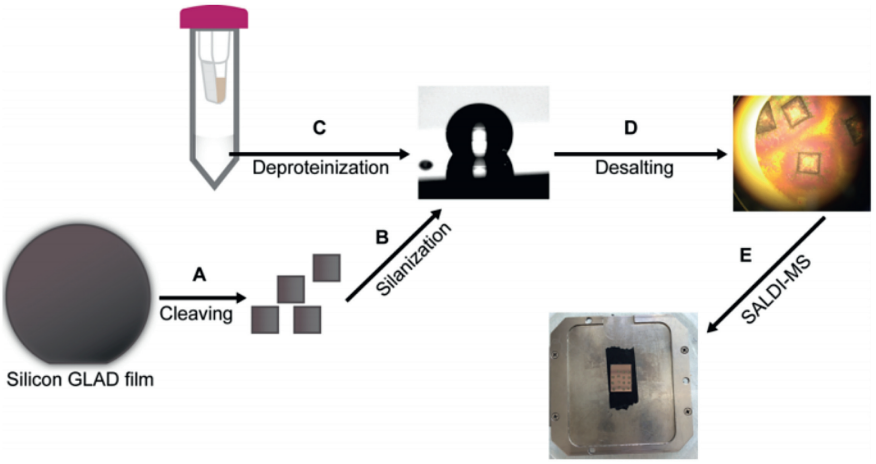

Fig. 7 – Workflow for biofluid analysis by silicon GLAD film. (A) Cleaving silicon GLAD film into workable wafers. (B) Modifying Si GLAD film with $pFMe_2SiCl$ solution to obtain a perfluoro coated surface with optimal contact angle. (C) Preparation of deproteinated human serum sample. (D) On-chip desalting and (E) mounting the SALDI chips on a custom MALDI plate for MS analysis. (Reproduced with permission from Ref. [63]).

cell lung cancer, by differentiating them from healthy controls. Huang et al. reported the use of SiO_2 with plasmonic silver nanoshells (SiO_2@Ag) nanocomposites for the detection of selected metabolites such as glucose and mannitol (dynamic range 100–600 ng μL^{-1}) in biofluid (cerebrospinal fluid and serum) volume of just 0.5 μL (Fig. 8) [65]. Furthermore, their research demonstrated that SiO_2@Ag nanocomposites can be used to determine glucose concentration in cerebrospinal fluid for identifying patients with postoperative brain infection and mannitol distribution in blood and cerebrospinal fluid systems to validate the function and permeability of blood–brain/cerebrospinal fluid-barriers.

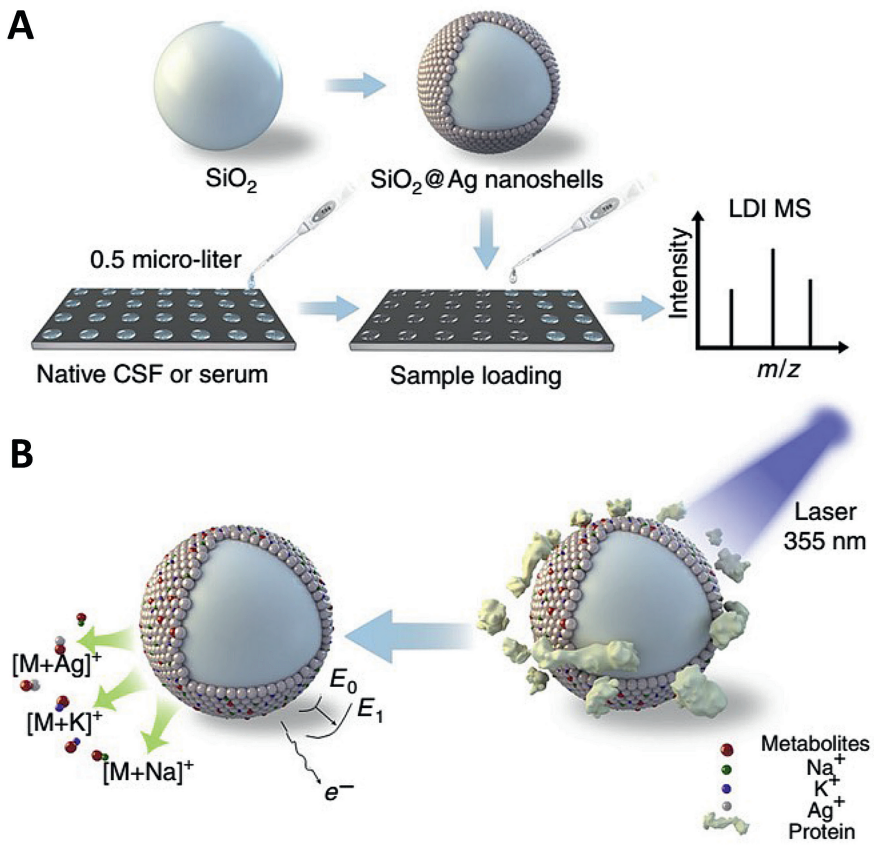

Fig. 8 – Schematic representation of (A) work flow and (B) LDI-MS process using SiO_2@Ag nanoshells as substrate for the detection of drugs and metabolites (Reproduced with permission from Ref. [65]).

Gustafsson et al. reported the use of silver coated desorption/ionization on silicon (Ag-DIOS) for detection and imaging of metabolites with low ionization efficiency such as fatty acids and esters in tissue and fingerprints [66]. Other than the enhanced desorption of the analyte, Ag layer on the DIOS substrate can produce $[Ag_n]^+$ clusters, which can act as calibration standards for improved mass accuracy and can form adducts with compounds such as fatty acids and cholesterol which are hard to detect with DIOS otherwise. Carbon nanotube and polymer composites also have been found to be excellent substrate for LDI-MS analysis of drugs providing clean mass spectra, enabling simultaneous detection. Bian et al. demonstrated electrospun polyacrylonitrile/Nafion®/carbon nanotube (PAN/Nafion®/CNT) fiber as SALDI substrate for the detection of four drugs, namely verapamil, chlorprothixene, methotrimeprazine and propranolol [13]. The carbon nanotube composite material extends the duration of signals of analyte ions. A comparison of the complicated MALDI spectrum using α-cyano-4-hydroxycinnamic acid as matrix and clean SALDI spectrum using PAN/Nafion®/CNT, of four drugs is given in Fig. 9, which clearly indicates the advantages of SALDI-MS using nanocomposite for the detection of low molecular weight molecules over MALDI-MS [13].

Nanocomposites combine several advantages of different materials and are well suited for detection of many drugs and metabolites. However, designing composite materials require in depth knowledge of ionization mechanism, which is rather lacking in current SALDI research. As of present Ag/Au and silicon/silica composite materials appear to be an attractive option, providing easy access to cationization (via Ag^+ addition), controlled plasmonic resonance and/or porous structure. Recent design of SALDI nanocomposite have advanced from nanoparticle dispersions into nanostructured target plate or chip fabrication, which effectively resolves matrix deposition issue such as uneven distribution of substrate and analytes on the plate. The better shot-to-shot reproducibility provided by this design may be a reason why silicon–metal composites are so commonly used, as their nanostructures can be easily controlled by electrochemical etching and sputtering.

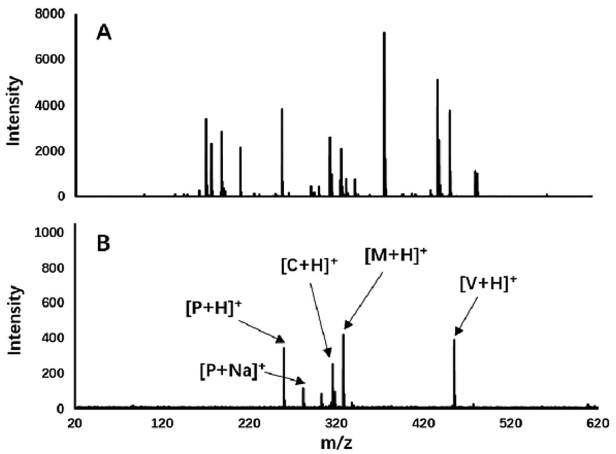

Fig. 9 – (A) MALDI-MS spectrum using α-cyano-4-hydroxycinnamic acid as matrix and (B) SALDI-MS spectrum using polyacrylonitrile/Nafion®/carbon nanotube composite as substrate for the detection of verapamil (V), chlorprothixene (C), methotrimeprazine (M) and propranolol (P). (Reproduced with permission from Ref. [13]).

3. Conclusions

A thorough study of literature suggests that nanomaterial-assisted LDI-MS is a promising technique for the analysis of drugs and metabolites. Nanomaterials offer several advantages including high surface area, homogeneous co-crystallization, low matrix interference, analyte enrichment, and tunable physical properties. Many of these characteristics are absent in traditional organic matrices and often encounter low shot-to-shot and sample-to-sample reproducibility. The nanomaterials serve as substrates and not only reduce the fragmentation of analytes and background noises, but also increase selectivity through enrichment of the analyte on its surface. However, moderate affinity between the nano-substrates and analytes should be maintained for efficient LDI process. Metal based nanoparticles such as Au NPs, Fe_3O_4 NPs, and TiO_2 NPs coupled with LDI-MS is especially useful in analyzing the low molecular weight region. Carbon and silica nanomaterials are also successful SALDI matrices with advantages over metal-based substrates due to their functional properties which enable efficient enrichment of analytes and easy ionization. In particular, silicon nanostructure (such as DIOS, NIMS) have been explored more than other nanomaterials for LDI-MS for drugs and metabolites. Development of new nanomaterials and nanocomposites for LDI-MS and elucidation of their mechanisms are urgent in the future for simultaneous detection of biomacromolecules (protein and DNA) and small molecules in clinical diagnosis. Understanding the nanomaterial-assisted LDI process mechanism, especially that of nanocomposites, will facilitate more systematic and logical design of new effective nanomaterial matrices compared to traditional method of continuous trial and error. On the other hand, employing mass tags as amplification system is a promising leap in applying SALDI-MS for trace analysis of biomolecules in various fields including food, drug, clinical and forensic analyses. However, this technique is relatively new and only a few reports are available, and needs to be explored for its potential use in drugs and metabolite analyses. A selective detection assay coupled with SALDI-MS and mass tag will provide a better strategy to increase the selectivity, sensitivity and reduce the interference.

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Original Article

Kinetics of lactose fermentation in milk with kombucha starter

Katarina Gojko Kanurić, Spasenija Danilo Milanović, Bojana Branko Ikonić, Eva Stjepan Lončar, Mirela Dragoljub Iličić, Vladimir Radovan Vukić, Dajana Vukota Vukić*

University of Novi Sad, Faculty of Technology Novi Sad, Bulevar Cara Lazara 1, 21000, Novi Sad, Serbia

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ABSTRACT

The aim of this research was to investigate the effect of new, non-conventional starter culture on the kinetics of the lactose transformation during milk fermentation by kombucha, at pH 5.8; 5.4; 5.1; 4.8; and 4.6, at two different temperatures 37 °C and 42 °C. Milk fermentation at 42 °C lasted significantly shorter (about 5 h, 30 min) compared to the fermentation at 37 °C. Changes of lactose concentration at the both temperatures are consisting of two retaining stages and very steep decline in–between. The analysis of the rate curves showed that the reaction rate passes through the maximum after 9 h, 30 min at 37 °C and after 4 h at 42 °C. The sigmoidal saturation curve indicates a complex kinetics of lactose fermentation by kombucha starter.

1. Introduction

Kombucha is known as a symbiotic association of yeasts and acetic acid bacteria whose metabolic activities on sweetened tea produce a pleasant slightly sweet, slightly acidic refreshing beverage consumed world wide. The microbial composition of kombucha cannot be given because it depends on the culture origin. Novel research [1] has showed significant presence of lactic acid bacteria in kombucha. The major bacterial genus in 5 kombucha samples was *Gluconacetobacter* (over 85% in most samples) and *Lactobacillus* (up to 30%). Only trace populations of *Acetobacter* were detected (lower than 2%). Radulović et al. [2] also isolated and identificated lactic acid bacteria from kombucha tea.

Traditional substrate for the kombucha cultivation is black or green tea extract sweetened with 5%–8% sucrose. Besides traditional substrates, the possibilities of use of alternative substrates have been established in various studies [3–11]. Reiss [12] found that kombucha fermentation on other sugars (lactose, glucose or fructose) produced beverages slightly different in flavour but significantly different in ethanol and lactic acid quantity, compared to sucrose's sweetened tea. For example, fermentation on lactose gave extremely low quantities of ethanol in comparison to the fermentation on sucrose. Belloso Morales and Hernández-

* *Corresponding author*.
E-mail address: stay@uns.ac.rs (K.G. Kanurić).
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Sánches [13] successfully cultivated kombucha on cheese whey. Malbaša et al. [14] investigated the manufacture of milk-based beverages by application of kombucha starters cultivated on sweetened black and green tea, and top-inambur. Three different inoculums of kombucha starter: cultivated on black tea with addition of sucrose, concentrated by microfiltration and concentrated by evaporation were applied in functional fermented dairy beverages manufacture [15]. Iličić et al. [16] investigated fermentation of lactose from two types of milk, low fat and reduced fat, inoculated with kombucha starter. Milanović et al. [17] revealed that kombucha inoculums cultivated on different tea types could be used in combination with probiotic starter culture in fermented dairy products technology. Vukić et al. [18] also investigated the effect of kombucha starter culture on the rheological properties, texture and microstructure as well as on the protein fractions in different phases of milk fermentation.

Lactose is the main carbohydrate in dairy products. It is a unique disaccharide, composed of glucose and galactose, in the sense that it occurs exclusively in the milk of mammals [19]. Lactose plays an important role in the formation of the neural system and the growth of skin (texture), bone skeleton and cartilage in infants. It also prevents rickets and saprodontia [20]. Lactose fermentation by lactic acid bacteria is widespread in the production of fermented dairy products. In particular, lactose is the principal energy source for the bacteria in the starter culture, while casein, together with calcium and phosphorus gives rise to the basic structure of a gel structure [21]. In general, dairy starter cultures metabolize lactose either through the homo- or hetero-fermentative metabolic pathways. Lactose content is reduced during fermentation (by 20–30 g/100 g of the level in the original milk), while the concentration of lactic acid and some free amino acids increases, for example, proline, serine, alanine, valine, leucine, and histidine [22].

The biochemistry of kombucha fermentation was analysed using green and black teas by Kallel et al. [23]. They followed several biochemical markers of kombucha fermentation for a period of two weeks. The metabolism of carbon was targeted using sucrose, glucose, fructose, cellulose, ethanol and total acetic acid equivalents. Green and black teas exhibited similar kombucha fermentation profiles, but specific biochemical behaviours were observed as well. Lončar et al. [24] defined, by processing the experimental results, two mathematical models for the kinetics of sucrose fermentation by kombucha. It has been shown that both empirical models – one for the change of saccharose concentration during its fermentation, and the other for the rate of the mentioned fermentation, enable better insight into sucrose transformation.

There is no data in available literature about kinetics of lactose conversion during milk fermentation by kombucha starter. Therefore, the aim of this research was to determine the effect of non-conventional starter culture on the kinetics of the lactose transformation during milk fermentation by kombucha, at pH 5.8; 5.4; 5.1; 4.8; and 4.6, at two different temperatures 37 °C and 42 °C.

2. Experimental

2.1. Milk

Homogenized and pasteurized cow's milk from AD Imlek, Division Novi Sad Dairy, was used for the production of kombucha fermented milk products. The composition of milk was as follows: fat content – 2.0 g/100 g, total solids – 10.59 g/100 g, total proteins – 3.30 g/100 g and lactose – 4.60 g/100 g, ash 0.69 g/100 g.

2.2. Kombucha inoculum

Kombucha inoculum was prepared according to procedure published by Milanović et al. [25] (2002). Kombucha is cultivated on a black tea (*Camellia sinensis* – oxidized, 1.5 g/L) with sucrose concentration of 70 g/L. The tea was cooled to room temperature, after which inoculum from a previous fermentation was added in concentration of 10% (v/v). Incubation was performed at 25 °C for 7 days. Inoculum in concentration of 10% (v/v) was used for milk fermentation.

Local domestic Kombucha, used for the fermentation, contains at least five yeast strains (*Saccharomycodes ludwigii, Saccharomyces cerevisiae, Saccharomyces bisporus, Torulopsis* sp. and *Zygosaccharomyces* sp.) as determined by Markov et al. [26]. Total number of viable cells was as follows: approximately 5×10^4 of yeast cells per mL of the reaction mixture and approximately 2×10^5 of bacteria cells per mL of the mentioned mixture [24].

2.3. Samples production

Samples were obtained by addition of 30 mL of kombucha inoculum in 300 mL of milk at 42 °C and 37 °C simultaneously. Samples were taken at pH values: 5.8; 5.4; 5.1; 4.8 and 4.6.

2.4. Methods

2.4.1. pH values

The pH values were determined using a pH-metre (pH Spear, Eutech Instruments Oakton, UK).

2.4.2. Sugar content

The content of lactose and galactose at two temperatures were detected in all samples using specific enzymatic tests K-LACGAR 12/05 (lactose and D-galactose) purchased by Megazyme, Ireland. Products of the reactions were monitored using spectrophotometer (T80 + UV/VIS Spectrometer PG Instruments Ltd, UK).

2.4.3. HPLC analysis of lactic acid

Lactic acid content was determined according to the modified method of Jayablan et al. [27]. Four grams of samples were transferred into a 25 mL volumetric flask, 5 mL of bidestiled water was added and filled up with acetonitrile (Mallinckrodt Baker, Inc., Netherlands). The obtained solution was mixed for 5 min and then was filtered through membrane syringe filter (with diameter pore of 0.45 μm). Agilent 1100 system (USA)

with C-8 column and UV–DAD detector was used for determination. The mobile phase was a mixture of 20 mM potassium dihydrogen phosphate, pH 2.4 and methanol (97:3). The flow rate and column temperature was maintained as 1.0 mL/min and 28 °C, respectively. Detection was carried out at 220 nm. Lactic acid was used as a standard. Each sample was prepared and injected in triplicate.

2.4.4. *Statistical analysis*

Statistical analysis of results was carried out with the computer software program "Statistica 9" and results were expressed as average values with standard deviation.

3. Results and discussion

Milk fermentation process by kombucha, at two temperatures (37 and 42 °C) was followed through measuring of pH value (Fig. 1). Kinetics of lactose fermentation was examined through lactose transformation to products – galactose and lactic acid (Fig. 2). During the fermentation, the lactose is first converted into glucose and galactose which are further converted into other products such as pyruvic acid, lactic acid, carbon dioxide, water, ethanol and others, through a series of consecutive and parallel reactions [22,28]. Reaction mixture changes its qualitative and quantitative composition during the milk fermentation.

Table 1 – Parameters of sigmoidal (Boltzmann) concentration model.

Parameters in equation (1)	Investigated systems	
	37 °C	42 °C
A_1	4.3902	4.3918
A_2	3.5520	3.7459
t_o	9.4869	3.9375
Δt	1.2305	0.6747
R^2	0.99759	0.99896

From Fig. 1 it is evident that the fermentation of milk at 37 °C lasted 13 h, 40 min and at 42 °C 8 h, 15 min. Therefore, milk fermentation at 42 °C lasted significantly shorter (about 5,5 h) compared to the fermentation at 37 °C. After 9 h from the start of fermentation at 37 °C the pH began to decline rapidly, almost linearly, and after 3 h achieved pH 4.95. Fermentation curve at 42 °C is characterized by a slight increase during the first 2 h (from the starting pH 6.07 to pH 6.2). The next 2 h, the pH value gradually decreased. Further decrease in pH value at both temperatures took place until the end of the fermentation. These results are in accordance with

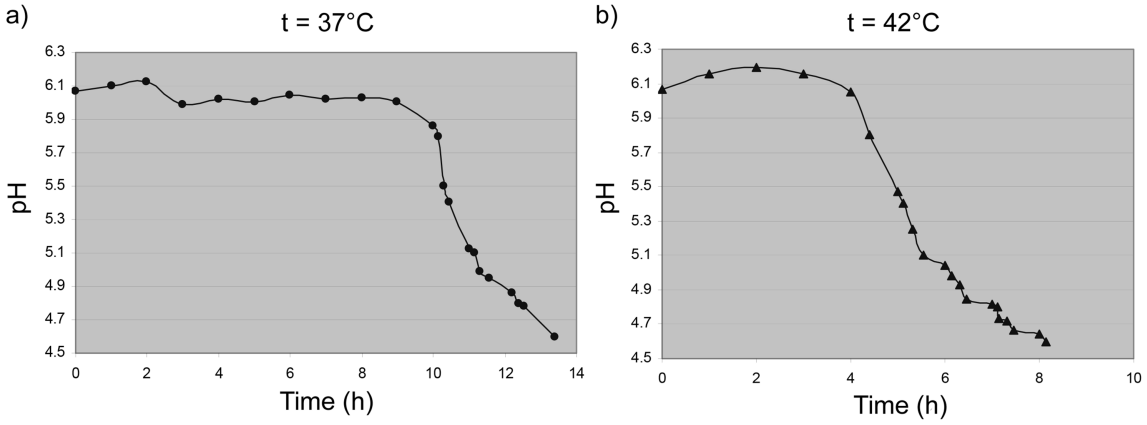

Fig. 1 – Change of pH during milk fermentation at a) 37 and b) 42 °C.

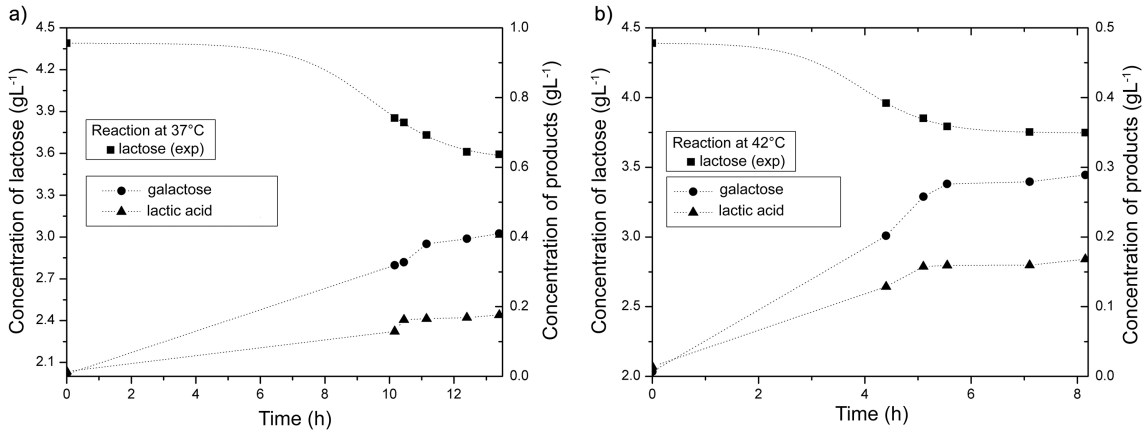

Fig. 2 – Kinetics of lactose fermentation as a function of temperature at a) 37 and b) 42 °C.

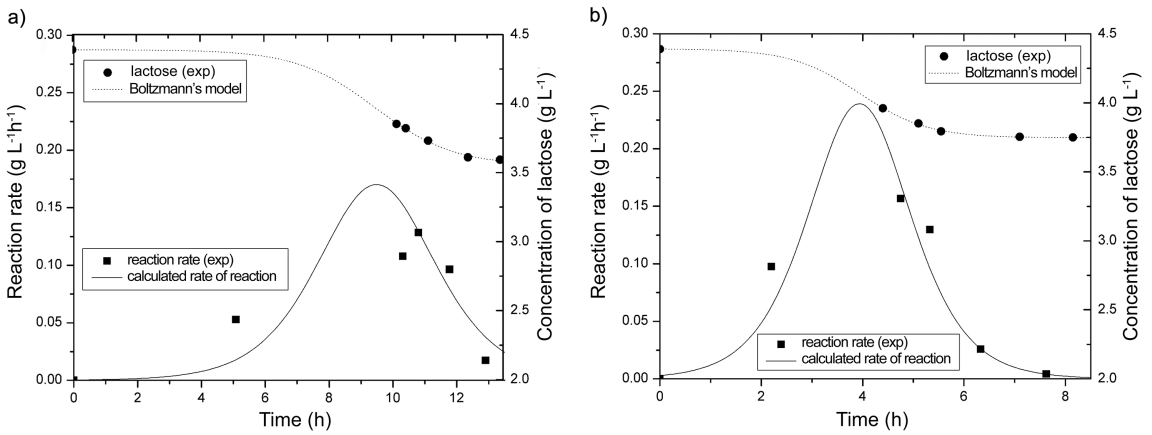

Fig. 3 – Reaction rate as a function of temperature: a) 37 °C and b) 42 °C (Lines show mathematical models, symbols present experimental values).

the results previously reported by Milanović et al. [25] and Milanović et al. [29].

Decrease of pH during the milk fermentation is a result of lactose transformation. Lactose molecules bind to the enzyme's active sites where they are transformed into glucose and galactose, which continued to convert to other products through a number of consecutive and parallel reactions. Seto et al. [30] have reported that *Acetobacter* strains, which are abundant in kombucha medium, consumed preferentially glucose to fructose as carbon source. In line with this, glucose is preferred to galactose as a carbon source by kombucha microflora. At the beginning of the process, lactose content was 4.39 g/100 g and further during the fermentation it decreased continuously. The best model to present the curve of fermentation change of lactose (as well as sucrose, researched by Lončar et al. [24]), is sigmoidal Boltzmann's function. Changes of lactose concentration at the both temperatures (37 and 42 °C) are consisting of two retaining stages and very steep decline in–between (Fig. 2). At 37 °C change in the lactose content is different from the reaction at 42 °C because it had a less pronounced lag phase at the end of the fermentation process. The phase of concentration decrease is between 7th and 12th hours at 37 °C and between 4th and 6th hours at 42 °C.

The best model to present the lactose concentration changes is Boltzmann's (sigmoidal) function:

$$S(t) = A_2 + \frac{A_1 - A_2}{1 + e^{\frac{t - t_0}{\Delta t}}} \quad (1)$$

where S denotes lactose concentration (g L^{-1}), which changes in time t. Parameters A_1 and A_2 correspond to the positions of two asymptotes to the S(t) curve (upper and lower), t_o is the t-coordinate of the point at which the slope has the highest value, while Δt is the width of the step of an exponential decrease. Four parameters (A_1, A_2, t_o and Δt) were determined by applying the Levenberg–Marquardt method (ORIGIN 8.5) over the experimental data (Table 1). The achieved coefficients of determination are very high ($R^2 > 0.99$), indicating a good fit of the data to the selected model within the whole interval of variable values.

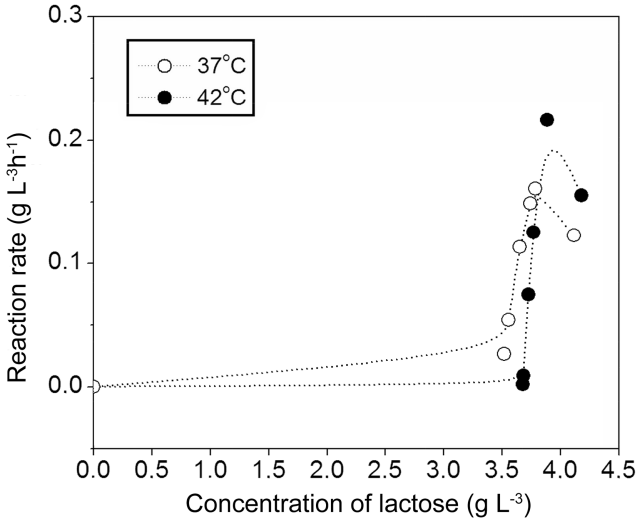

Fig. 4 – Saturation curves – reaction rates versus lactose concentration.

First derivative of the concentration equation (1) defines the rate of reaction:

$$-\frac{ds(t)}{dt} = \frac{e^{\frac{t - t_0}{\Delta t}}(A_1 - A_2)}{\Delta t\left(1 + e^{\frac{t - t_0}{\Delta t}}\right)^2} \quad (2)$$

Since the derivative (2) is obtained analytically, its application does not cause any error in calculations. Derivatives, calculated from equation (2), are graphically presented in Fig. 3 as the rate curves, along with the S(t)-curves (experimental and estimated).

The analysis of the rate curves (Fig. 3) showed that the reaction rate passes through the maximum after 9 h, 30 min h at 37 °C and 4 h at 42 °C. After reaching the maximum, the reaction rate decreases rapidly, probably because of the suppression of metabolic activity of the present microorganisms due to the production of organic acids. Similar results were obtained by Chen and Liu [31] who investigated prolonged fermentation of sucrose by kombucha. The highest reaction

rate (0.24 g/L h) of lactose transformation by kombucha starter was achieved in the system at 42 °C.

The saturation curves (reaction rates *versus* lactose concentration) are presented in Fig. 4. The saturation curves show sigmoidal kinetics at chosen substrate concentration, indicating a complex kinetics of lactose fermentation by kombucha starter.

4. Conclusion

Milk fermentation by kombucha at two temperatures, 37 and 42 °C, exhibited similar profiles but specific biochemical behaviours. The best model to present the curve of fermentation change of lactose is sigmoidal Boltzmann's function. Changes of lactose concentration at the both temperatures (37 and 42 °C) are consisting of two retaining stages and very steep decline in–between. At 37 °C change in the lactose content is different from the reaction at 42 °C because it has a less pronounced lag phase at the end of the fermentation. The saturation curves show sigmoidal kinetics at chosen substrate concentration, indicating a complex kinetics of lactose fermentation by kombucha starter.

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