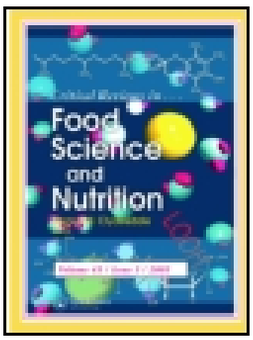

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REVIEW

Current research progress of mammalian cell-based biosensors on the detection of foodborne pathogens and toxins

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ABSTRACT

Foodborne diseases caused by pathogens and toxins are a serious threat to food safety and human health; thus, they are major concern to society. Existing conventional foodborne pathogen or toxin detection methods, including microbiological assay, nucleic acid-based assays, immunological assays, and instrumental analytical method, are time-consuming, labor-intensive and expensive. Because of the fast response and high sensitivity, cell-based biosensors are promising novel tools for food safety risk assessment and monitoring. This review focuses on the properties of mammalian cell-based biosensors and applications in the detection of foodborne pathogens (bacteria and viruses) and toxins (bacterial toxins, mycotoxins and marine toxins). We discuss mammalian cell adhesion and how it is involved in the establishment of 3D cell culture models for mammalian cell-based biosensors, as well as evaluate their limitations for commercialization and further development prospects.

KEYWORDS

Mammalian cell-based biosensor; cell immobilization; 3D cell culture; foodborne pathogen; toxin

Introduction

Food safety is one of the greatest challenges faced by society worldwide. Food contamination caused by pathogens and toxins remains a major concern (Martinovic et al. 2016; Yeni et al. 2016). According to the World Health Organization (WHO 2015), foodborne pathogens caused approximately 600 million illnesses and 420,000 deaths in 2010. In recent years, the incidence of foodborne diseases at home and abroad has increased significantly, and the incidence of foodborne diseases reported in many developed countries has also shown an upward trend. Centers for Disease Control and Prevention (CDC) estimates that approximately 48 million people get sick from a foodborne illness each year in the United States. Of these, 128,000 require hospitalization, and 3000 people die from foodborne diseases (Ma et al. 2019). There are many ways to cause foodborne diseases. The viruses, bacteria, parasites, toxins and chemicals in food are the main biological hazards causing foodborne diseases (Todd 2014). According to the list of food borne outbreaks from CDC's National Outbreak Reporting System (2019), *Escherichia coli* (*E. coli*), *Salmonella* and *Listeria* are the most common bacterial pathogens causing foodborne outbreaks. Aflatoxins (caused by fungi in cereals) are the main cause of death from foodborne diseases, with 70% of people who become ill from aflatoxin worldwide are in the Western Pacific Region (Shimizu 2015). However, the reported incidence of foodborne diseases is still significantly lower than the actual incidence. The reason is that most people with foodborne diseases lack the knowledge of foodborne diseases, fail to go to the hospital and report to the relevant health departments in time; secondly, the diagnosis of some foodborne diseases still lacks laboratory testing technology or inspection equipment.

Therefore, accurate and rapid monitoring of agents that can harm humans, such as pathogens and toxins, is essential throughout the various stages of food production, including consumption (D'Souza, Kumari, and Banerjee 2017; Rivera et al. 2018). However, as a reliable standard for food microbiological detection, conventional microbiological methods that detect and identify foodborne pathogens involve a series of steps, which are time-consuming and labor-intensive; thus, it fails in the timely response needed to identify possible risks (Rohde et al. 2017; Zhao et al. 2006). Various methods have been proposed for the qualitative and quantitative analysis of pathogens and toxins in foods, where qualitative detection is to identify the presence of analyte; quantitative detection is to determine the specific amount of analyte. (Hill and Wachsmuth 1996; Kant et al. 2018). However, these methods still have certain limitations (Lv et al. 2018; Yang and Bashir 2008). For example, the immunochromatographic test strip (ICTS) has the advantages of low cost, simple operation, convenient use and rapid response (Wang and Ouyang 2019), but it cannot achieve quantitative detection and has low sensitivity. Although enzyme-linked immunosorbent assay (ELISA) has

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Table 1. Comparative properties of different types of biosensors (Kintzios and Banerjee 2015).

	Biorecognition		
Trait	Mammalian cells	Enzyme	Antibody
Sensitivity	Very high	Medium to high	Low to medium
Specificity	Low	Low, prone to interferences	Medium to high
Stability	High	High under certain conditions	
Reproducibility	Low	High	Low to medium
Storability	Low	Low to medium	Low to medium
Speed[a]	Low to very high	High	Low to medium

[a]The speed of the assay usually depends on the transducer and not the biorecognition element *per se*.

beenindustrialized as commercial kits and widely used, the preparation of antibodies has to go through a series of complex procedures such as immunization of animals, cell fusion, etc., which is time-consuming and expensive. Moreover, the whole antigens used in the competitive reaction for detecting small molecules are also prepared by a series of chemical synthesis. Therefore, there is an increasing demand for rapid response, high sensitivity and environmentally friendly food risk assessment methods.

A biosensor is an instrument that consists of a bio-sensitive material as the recognition components, an appropriate physicochemical detecting transducer, and a signal amplification device (Liu, Wu, et al. 2014; Narwal et al. 2019). In recent years, due to its high selectivity and sensitivity, fast analytic speed, low cost, and continuous online monitoring in complex systems, in particular its high degree of automation, miniaturization and integration, the use of biosensors in various fields has rapidly expanded (Abolhasan et al. 2019; Ahmad and Moore 2009; Pundir, Jakhar, and Narwal 2019). According to their identification elements, biosensors can be classified into three categories, namely, molecular biosensors, cell biosensors, and tissue biosensors (Gui et al. 2017; Pancrazio et al. 1999; Xu 2002).

Cell-based biosensors (CBBs) combine fixed or unfixed live cells with electrodes or other transducers, in which the live cells function as the sensory elements, to gather information on biologically active analytes (Jiang, Jiang, Shao, et al. 2016; Wang et al. 2005; Ziegler 2000). At present, CBBs are of great interest in the field of biosensor research. Compared with molecular biosensors, CBBs are highly sensitive instruments capable of rapid response detection, wide-spectrum signal capture, and noninvasive long-term recording. CBBs not only testify the presence of particular pathogen or toxin but also provide information about the functionality of pathogen or toxin. In contrast, immunological and nucleic acid-based biosensors for detecting pathogens and toxins primarily rely on chemical properties or molecular recognition to identify a particular agent. As a result, no functional (biological and physiological) information can be obtained from such assays (Gui et al. 2017). In terms of their overall performance, CBBs are distinguished from other types in a number of traits, as summarized in Table 1 (Kintzios and Banerjee 2015). CBBs respond optimally to biologically active analytes; thus, they have been used in various fields, including food safety risk assessment and quality control, environmental monitoring, drug evaluation, and medical diagnosis (Aravanis et al. 2001; Debusschere and Kovacs 2001; Notingher 2007; Wei et al. 2019).

In this review, we focus mainly on mammalian CBBs, in particular their characteristics, performances, and applications in the detection of foodborne pathogens (bacteria and viruses) and toxins (bacterial toxins, mycotoxins and marine toxins), where cell adhesion and the establishment of a 3D model are emphasized. We also discuss their limitations for commercialization and provide perspectives on the further development of mammalian CBBs. Unless explicitly stated, the cells mentioned in this review are all mammalian cells.

Mammalian cell-based biosensors

Mammalian cell-based biosensors incorporate mammalian cell-mediated biological recognition systems and signal transducing elements. When the live cells are stimulated by the analytes, changes in the physiological parameters (e.g., cellular metabolism, impedance, and action potential) or biological responses are directly converted into a quantifiable and processable signal by the transducer, thereby achieving the purpose of detection and analysis (Xu 2002; Xu et al. 2005). Mammalian CBBs can broadly be classified into different types as listed in Table 2, according to the cell type, secondary sensor function, and regulatory mechanism (Banerjee and Bhunia 2009). Among them, the most compelling mammalian CBBs for the detection of pathogenic microorganisms and toxins builds on the electric cell-substrate impedance sensing (ECIS) technology (Szulcek, Bogaard, and van Nieuw Amerongen 2014; Tan and Schirmer 2017). Due to the adhesive properties of mammalian cells cultured in vitro, the number or the morphology of the cells adhering to the metal microelectrodes can change slightly, thereby causing changes in the impedance of the electrode interface (Zhang et al. 2017). Based on this phenomenon, Giaever and Keese (1993) designed an electrical cell-substrate impedance sensor using a microelectrode array, which could track the changes in cell morphology, proliferation, and differentiation in real time, noninvasively, continuously, and quantitatively. This was one of the initial breakthroughs that contributed to the concept of a mammalian CBBs for commercial and research use (Banerjee and Bhunia 2009). In 1991, as the technology advanced, Giaever and Keese founded Applied Biophysics, which was dedicated to the development of an ECIS-based cell dynamic analyzer (Kintzios and Banerjee 2015). Applied Biophysics provided chips and systems for cell impedance measurements, greatly simplifying the study of cell behavior. Their commercialization has made this technique more widely available to researchers without an engineering background (Jang 2018; Pradhan et al. 2014). The simple precept of the ECIS system is proposed in Figure 1A and B (Giaever and Keese 1993; Szulcek, Bogaard, and van Nieuw Amerongen 2014), and readers should refer to the paper by Janshoff et al. (2010) for detailed information on the principle and operation of ECIS.

Table 2. Mammalian cell-based biosensors.

Cell types	Device/methods	Biosensing mechanism	Targets	References
Neurons Cardiac cells Cardiomyocytes	LAPS	Extracellular potentials of excitable cells	Odorant stimulations Heavy metal ions Drug-induced cardiotoxity	(Du et al. 2013; Liu et al. 2007; Liu, Hu, et al. 2011)
HepG2 cells Olfactory cells Pheochromocytoma cells (PC12) Neural network	MEAs	Electrical responses, Cell membrane potential	Ascorbic acid Odorant stimulations Dopamine Insecticide (chlorpyrifos oxon and deltamethrin) Domoic acid	(Liu et al. 2015; Liu, Yu, et al. 2011; Vassallo et al. 2017; Wang et al. 2014)
Hela cells Mouse embryonic fibroblast cells Chinese hamster ovary cells Bovine aortic endothelial cells Rat fat pad endothelial cells Rat basophilic leukemia cells ANA-1 macrophage	ECIS	Impedance measurement	Drug toxicity screening Cytotoxicity of cigarette smoke Toxicants (nicotine, phenol, ammonia and aldicarb) Food allergen	(An et al. 2019; Jiang, Jiang, Zhu, et al. 2016; Tran, Cho, and Min 2013; Zhang et al. 2015)
Fibroblast cells Monkey African green kidney cells HaK hamster cells Neuroblastoma N2a cells	BERA	Antigen-antibody immunoassay Fluorescence microscopy assay Cell membrane potential	Plant viruses Aflatoxin M_1 2,4,6-trichloroanisole Pesticide residues (abamectin, pyrethroid, α-cypermethrin, organophosphates and dithiocarbamates) Superoxide radical	(Ferentinos et al. 2012; Flampouri et al. 2010; Larou et al. 2013; Moschopoulou and Kintzios 2006; Perdikaris et al., 2011; Varelas et al., 2011)
Mouse embryonic fibroblast cells Human colon cancer cells Hela cells	Reporter gene	Reporter gene assay Optical methods	Cytotoxicity detection Measure the nanotoxicity (TiO_2 nanoparticles, CdTe, CdSe/ZnS) Screen anti-colon cancer drugs	(Chen et al. 2011; Choi et al. 2013; Fukushima, Sugiyama, and Sato 2018; Taniguchi 2010)

Abbreviations: BERA, bioelectric recognition assay; ECIS, electric cell-substrate impedance sensing; LAPS, light-addressable potentiometric sensor; MEAs, microelectrode array sensor.

Compared with conventional methods, such as ordinary optical microscopy and fluorescent label observation, the impedance test of CBBs simplifies studies on the morphology of cells, and it is relatively easier to obtain real-time monitoring results. However, there are several intrinsic disadvantages of using live cells as the primary element, including their immobilization, culture and maintenance. These challenges involved in the optimization of CBBs include maintaining the stability of cells, improving the selectivity of the special sensor design, successfully coupling live cells with the secondary sensor, and prolonging the viability of cells (Liu, Wu, et al. 2014). The following sections highlight the key points in the preparation of mammalian CBBs.

Cell immobilization and surface modification of mammalian CBBs

The effective immobilization of cells onto the sensing interface is one of the main challenges of the cell sensing technology, and it affects the performance of the entire cell sensing system. The formation of a uniform adherent layer is one of the most commonly used methods for facilitating the immobilization of cells onto the biosensor surface. Therefore, the modification of the sensing interface by adhesion factors that can specifically adhere to cells without affecting cell function is critical for cell sensing analysis (Liu, Wu, et al. 2014). Extracellular matrix components, peptides, self-assembly monolayers, ion implants and nanomaterials have been employed to improve the cell immobilization efficiency and performance (Figure 2A). It has been reported that the surface modification by (extracellular matrix) ECM components can maintain cell viability while immobilizing cells evenly, which is a crucial factor in many biosensor applications (Gupta et al. 2019). ECM (collagen, fibronectin and laminin), ECM-derived molecules (cell adhesion peptides), and (self-assembled monolayers) SAMs have been predominantly used for the immobilization of various cell types (Table 3).

Extracellular matrix

The ECM is a complex network composed of various macromolecules that are secreted by cells. Most ECM proteins, such as fibronectin, collagen, and laminin, contain cell adhesion sequences that are recognized by cell membrane adhesion receptors (Huxley-Jones, Robertson, and Boot-Handford 2007). Fibronectin, a critical component of the ECM, is a multifunctional glycoprotein mediating ECM interactions; thus, it plays an important role in cell adhesion. Therefore, fibronectin is often used as a physisorbed surface coating in cell culture experiments (Kandel et al. 2014). Cabezas, Mirkin, and Mrksich (2017) reported monolayers that are nanopatterned with fibronectin can support cell adhesion and enable cell-based assays for evaluating the biological effects of small molecules in cell cultures. In this study, HeLa cells were seeded and cultured on a fibronectin nanopatterned substrate, and they fully diffused in a 10×10 nanoarrays of fibronectin and remained adherent during the culture. Other ECM proteins, such as collagen, have also been exploited for their cell adhesive properties. At present, cell patterning culture using ECM proteins has been extensively studied, but there are still several unresolvable issues such as the uncontrollable thickness of ECM proteins which

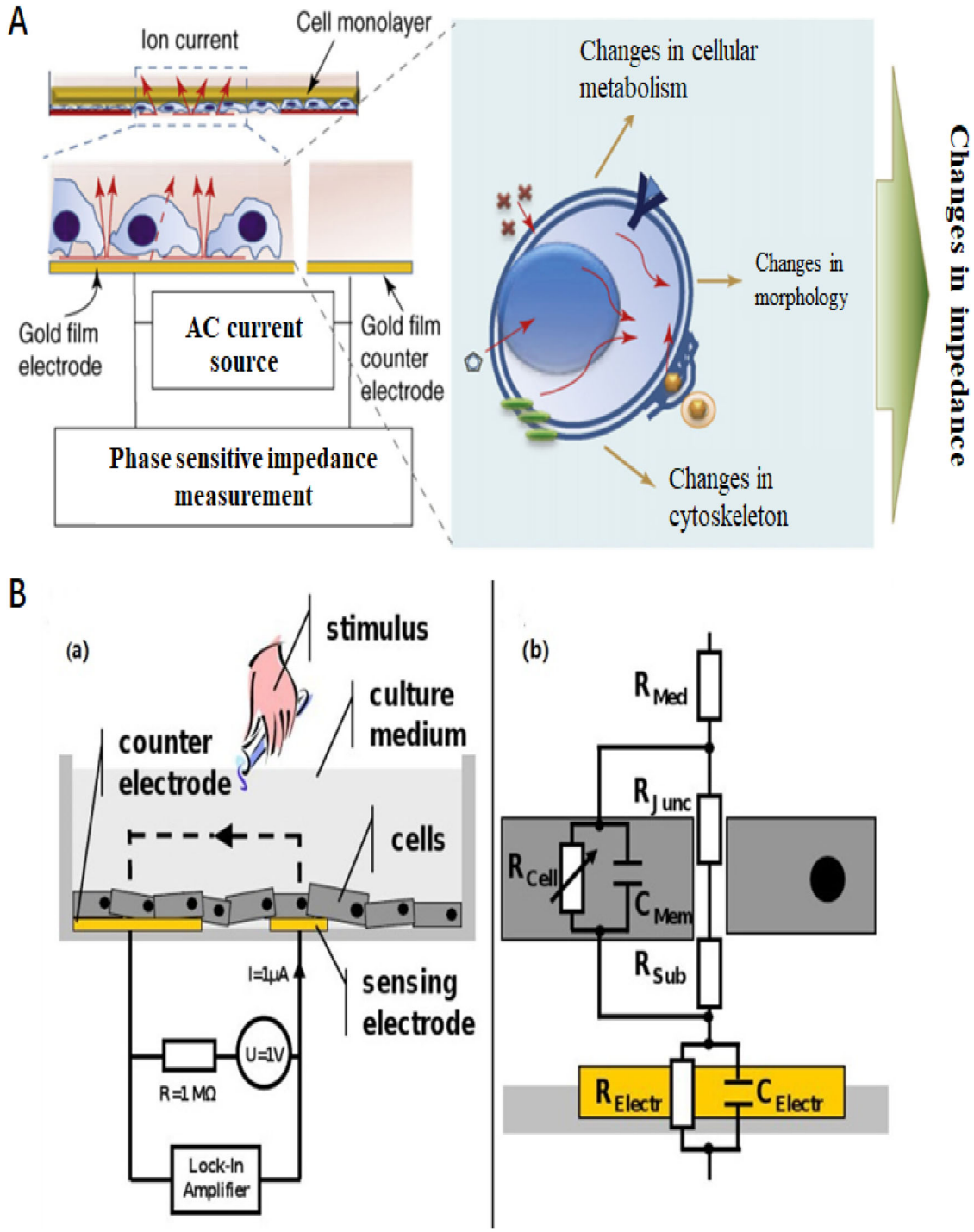

Figure 1. A. Schematic of the ECIS system (Giaever and Keese 1993); B. ECIS measures the changes of impedance under analyte stimulation and representative equivalent circuit for an adherent growing cells layer (Szulcek, Bogaard, and van Nieuw Amerongen 2014).

can reduce the electrochemical sensitivity of electrodes (Soylemez et al. 2016).

Peptides

As a substitute for natural ECM proteins, cell adhesion peptides (CAPs) play an important role in the design of cell culture systems. CAPs are the minimal amino acid sequences that specifically bind to cell-adhesive receptors (Huettner, Dargaville, and Forget 2018). At present, the minimal-binding-domain short peptide sequences from ECM proteins, such as fibronectin, laminin, and elastin, have been isolated and used to improve the biocompatibility of biofunctional surfaces. Several studies on cell growth factors and ECM proteins have revealed that these peptides, including RGD (Arg-Gly-Asp), IKVAV (Ile-Lys-Val-Ala-Val), and YIGSR (Tyr-Ile-Gly-Ser-Arg), can control the properties of other factors or the entire matrix. These CAPs have been investigated extensively in terms of surface modifications to allow or enhance cell adhesion (Mann, Tsai, and West 1999).

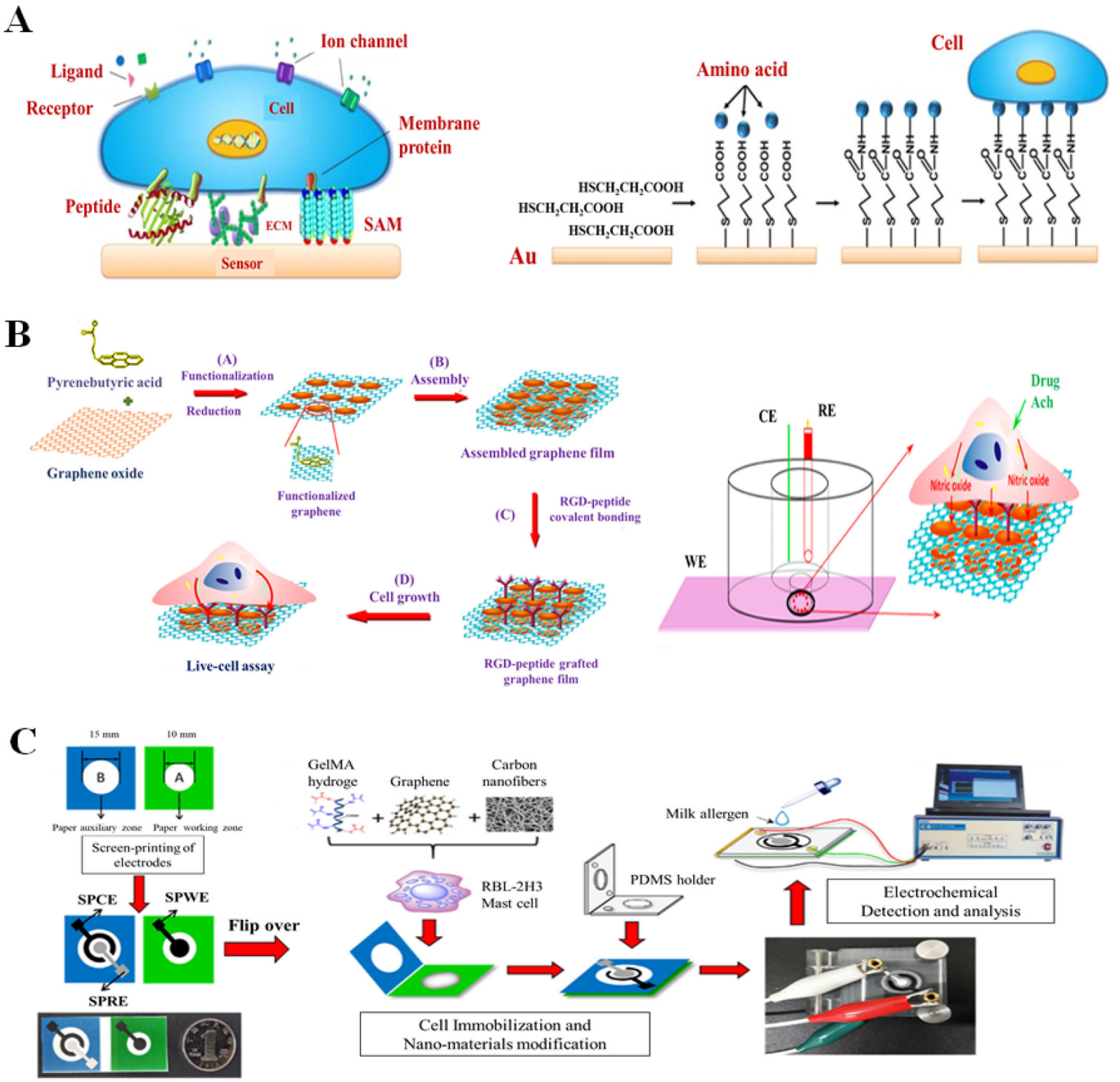

Figure 2. A. Different chemical modifications (ECM, peptide, SAM) of a sensor surface for improving the cell immobilization efficiency (Liu, Wu, et al. 2014); B. Schematic of RGD peptide functionalized graphene biomimetic live-cell sensor for detecting different drugs (Guo et al. 2012); C. Schematic representation the fabrication and assay procedure of the electrochemical mast cell-based paper sensor (Jiang et al. 2019).

It has been reported that RGD peptide with the arginine-glycine-aspartic acid sequences, which is found in many ECM proteins, such as fibronectin and vitronectin, is the binding motif for cell-adhesive receptors and essential for the immobilization of many cells (Kugo et al. 1994). Huettner, Dargaville, and Forget (2018) performed a search on three databases (PubMed, Scopus, and Web of Science) for articles published between 1970 and early 2018 on CAPs. The results show that most studies (89%) have reported the use of the RGD sequence, which targets the integrin receptor. The RGD sequence, which is an indicator of interaction between the receptor and its ligand, can also promote cell proliferation. About 10% of studies have reported IKVAV and YIGSR peptides derived from the laminin sequence. It was found that the covalent binding of YIGSR peptides with glass substrates could promote the proliferation and migration of bovine pulmonary artery cells (Mann, Tsai, and West 1999). Other CAPs, such as DGEA (Asp-Gly-Glu-Ala), PRARI (Pro-Arg-Ala-Arg-Ile), and PHRSN (Pro-His-Arg-Ser-Asn), account for less than 1% of publications. Huber et al. (1998) reported that these peptides have a higher stability compared with that of native laminin. With the immobilization of RGD and YIGSR peptides on ethylene-acrylic co-polymer film, the adhesion of several cell lines was enhanced. In a study by Thein et al. (2010), individual fibroblasts (NIH3T3) were immobilized onto either KRGD (Lys-

Table 3. Cell adhesion strategies in mammalian CBBs.

Cell adhesion molecules		Cell type	Material surface	Reference
ECMs	Collagen type I, laminin and tongue extracellular matrix	Taste cells and neurons	Poly(-dimethylsiloxane) (PDMS)	Lee et al. (2018)
	Laminin	Mouse tongue isolated taste bud (MTITB) cells and human embryonic kidney 293 cell lines (HEK293)	Carbon screen printed electrode	Deng et al. (2019)
CAPs	RGD-peptide	Human endothelial cells	Pyrenebutyric acid functionalized graphene film	Guo et al. (2012)
	RGD-peptide	Human lung cancer cell A549	Indium tin oxide (ITO) surface	Li and Yu (2017)
SAMs	Alkanethiols and polymeric poly-L-lysine-grafted-poly(ethylene glycol)	MCF-7 cells	Gold-silicate interfaces	Wu et al. (2020)
	11-mercapto-1-undecanol, propyl-trimethoxysilane, 11-mercaptoundecanolc acid	Human bladder cancer (5637) cells	The gold-plated glass plates	Cao et al. (2020)

Arg-Gly-Asp) peptide-modified or fibronectin-modified microelectrodes, followed by the recording of the impedance variations over a frequency ranging from 1 to 10 kHz. Theresults demonstrated that the biofunctionalization of the microelectrode surface by the covalent attachment of CAPs or fibronectin proteins could achieve robust and stable cell adhesion.

In addition to RGD, IKVAV, and YIGSR peptides with good characteristics, other adhesive CAPs derived from ECM proteins have also been identified (Huettner, Dargaville, and Forget 2018). RGDS (Arg-Gly-Asp-Ser) peptides have been applied to different surfaces. Besides the RGDS motif, another sequence found in fibronectin, namely, the REDV (Arg-Glu-Asp-Val) peptide, allows specific adhesion of endothelial cells. One study reported that the functionalized coating of RGDS, REDV or YIGSR peptides, or their combinations can promote endothelial cell adhesion and proliferation (Castellanos et al. 2017).

The adhesion peptide can be coated on a specific electrode material (metal, carbon material, semiconductor) by a specific chemical coupling method (e.g., self-assembled monolayer of cysteine and gold, amide reaction of amino group and carboxyl group) (Huber et al. 1998). Typically, thiol groups on peptides can form gold-sulfur bonds with the gold material, thus realizing the modification of CAPs on the surface of electrodes. This property suggests that cysteine residues play an important role in biomolecular anchoring to the surfaces (Soylemez et al. 2016). Therefore, RGDC (Arg-Gly-Asp-Cys) was designed with the goal of being used in the immobilization of the RGD peptide onto a gold surface with thiol affinity or maleimide functionalized surface. For example, the RGDC peptide was successfully immobilized onto a maleimide-grafted titanium substrate by Xiao et al. (1997). In a study by Popat, Leary Swan, and Desai (2005), the RGDC peptide was also covalently immobilized on a maleimide-grafted alumina substrate to study bone cell growth. Similarly, gold surfaces can also be modified with RGDC to facilitate cell adhesion.

Self-assembled monolayers

Several methods for immobilizing cells on a variety of substrates have been reported. Among them, SAMs or SAMs combined with ECM-derived biomolecules are commonly preferred (Soylemez et al. 2016). SAMs are particularly advantageous for well-defined, hypothesis driven cell culture experiments, as they provide chemically well-defined substrates that can be precisely tailored for specific cell culture applications (Hudalla and Murphy 2011). This section will introduce the chemical molecules (*e.g.* alkanethiolates, bioinert polymer chains,) of the SAMs patterned on the interface for precisely immobilization of cell adhesion.

The formation of strong and specific coordination bonds between thiol groups and gold materials was reported by Nuzzo and Allara (1983). Subsequently, Bain et al. (1989) found that the thiol-terminated alkyl chains can self-assemble into an ordered monolayer on the surface of a gold material through the synergistic effect of sulfur-gold coordination bonds and the van der Waals interactions between alkyl chains. A unique distinction of SAMs is that alkanethiolates terminated with a wide variety of chemical functionalities can be readily synthesized, and these functionalized alkanethiolates efficiently pack into SAMs on gold. Cao et al. (2020) compared the differences in culturing human bladder cancer cells on mercaptan SAMs terminated with three different chemical groups (-CH_3, -OH, -COOH) and found that -CH_3 group is more conducive to cells adhesion onto the SAMs. Li et al. (2020) described the difference in cell adhesion capability of vitronectin (Vn) adsorbed onto SAMs terminating with -COOH, -NH_2, -CH_3, and -OH. The Vn-adsorbed SAMs capped with -COOH and -CH_3 groups has a better adhesion effect to human mesenchymal stem cells.

Furthermore, the particular advantage of SAMs of alkanethiolates on gold is their ability to present ECM-derived biomolecules in a chemically well-defined environment (Hudalla and Murphy 2011). By combining with aminopropyl triethoxysilane (APTES) and p-tolyl isocyanate molecules successively, the surface of titanium alloy (Ti6Al4V) with hybrid SAMs was prepared, which contain both hydrophobic (CH_3) and hydrophilic (NH) groups were formed in the same chain (molecule). Subsequently, osteoblasts adhered to the SAMs surface hybrid surface pre-adsorbed with collagen-I (Col-I) exhibited better cell adhesion (Hasan and Pandey 2020).

In addition, SAMs that formed alkanethiolates terminated with an oligo (ethylene glycol) on a gold substrate were

Table 4. Advantages and disadvantages of 2D and 3D cell cultures in mammalian CBBs (Foglietta et al. 2020).

	Advantages	Disadvantages
2D cell cultures	Less expensive	Cells only adhere to one surface
	Proliferation rate is faster than under in vivo conditions	Few cell-cell and cell-matrix interactions
	Easy to use and convenient	
	Easy downstream processing	
3D cell cultures	Increased cell-cell/matrix interactions	More expensive
	Easy to control and monitor the microenvironmental parameters of growing cells	More complex culture models
	Easy to maintain cell viability and three-dimensional shape	Further complex downstream processing
	Better simulate internal physiological conditions and the response of cells in the body to external stimuli	
	Proliferation kinetics are higher or lower than in 2D cells according to cell type and/or type of model used	

demonstrated to resist nonspecific protein adsorption (Pale-Grosdemange et al. 1991). Therefore, according to the different surface chemical properties, the cells were patterned and adhered on the substrate. Functionalized surfaces generated by the SAMs technique can effectively guide cells to attach to the target region (Ostuni, Yan, and Whitesides 1999). For example, the patterned self-assembly of alkanethiols and poly-L-lysine-grafted-poly(ethylene glycol) (PLL-PEG) molecules was performed that can specifically attach to gold and silicate, respectively. The gold array elements were functionalized with a range of alkanethiols and PLL-PEG was used to provide resistance against nonspecific protein and cell adsorption and attached exclusively to the silicate. MCF-7 cells selectively adhere to the gold areas of the gold-silicate surface, resulting in cell microarray (Wu et al. 2020).

Moreover, the experimental steps of covalent modification with SAMs are simple and convenient, which further improve the robustness of the surface to thermal, mechanical, and solvolytic instabilities (Liu, Wu, et al. 2014). Bugga and Mrksich (2019) reported a photoactive surface for cell culture, adhesion, and migration that could be repeatedly activated. The method used SAMs to present maleimide groups, thereby covalently binding RGD peptides capable of adhering to cells.

Hydroxyl ion implantation

Ion implantation has numerous advantages in the treatment of biomaterial surfaces because it can enhance interactions between biomaterials. For example, the degree of cell adhesion and proliferation on the F^+-implanted HA-coated Ti substrate is 1.7 times higher than that in the absence of F^+ injection (Rautray et al. 2010). The adsorption of proteins depends directly on the presence of hydroxyl groups on the surface. Therefore, hydrophilicity is indicative of an increased surface adsorption of hydroxyl groups, followed by a higher probability of available proteins on the surface, which also increases the probability of reactions between proteins and cells, and thus, increases the biocompatibility. The insertion of hydroxyl ions greatly affects the structural properties of the substrate surface and its hydrophilicity, which can improve cell attachment and proliferation. On a silicon surface treated with the hydroxyl ion, for example, cell attachment was increased (Fan et al. 2000).

Modification with nanomaterials

Covalently immobilized and SAMs facilitate cell adhesion, but increase electron transport resistance across the interface, thereby leading to a decrease in the signal-to-noise ratio (Fan et al. 2000). Functional nanomaterials, such as graphenes, carbon nanotubes, and gold nanoparticles, have been explored for use in CBBs to improve their performance (Gu et al. 2015; Guo et al. 2012; Justino et al. 2017).

Graphene, a single-atom-thickness two-dimensional nanomaterial, has shown great promise in the development of various sensitive biosensors due to its remarkable electronic, optical, and thermal properties (Taniselass, Arshad, and Gopinath 2019; Vlăsceanu et al. 2018; Wang et al. 2017). Guo et al. (2012) reported that a smart functional graphene film covalently bound to the RGD peptide could enhance cell adhesion and growth for the real-time detection of nitric oxide molecules released from adherent human cells under various drug stimulations (Figure 2B). The study reported the construction of a biomimetic graphene-based film by covalently binding the RGD peptide onto the surface of pyrenebutyric acid functionalized graphene film, which can promote cell attachment and growth. Jiang et al. (2019) developed an electrochemical mast cell-based paper sensor to detect the major milk allergen casein, in which the graphene/carbon nanofiber/gelatin methacryloyl composite material was modified to improve the electrical conductivity for the immobilization of rat basophilic leukemia mast cells (Figure 2C).

Carbon nanotubes (CNTs) have been extensively studied and used for the construction of biosensors owing to their large surface-to-volume ratio, high conductivity and good biocompatibility (Zhou, Fang, and Ramasamy 2019). Depending on their number of walls, CNTs are designated as single-walled carbon nanotubes (SWCNTs) or multi-walled carbon nanotubes (MWCNTs). It is worth noting that the properties of CNTs may differ significantly between MWCNTs and SWCNTs (Tilmaciu and Morris 2015). CNTs can maintain and promote neuronal electrical activity in cultured cell networks. Cellot et al. (2008) reported the mechanism to explain how CNTs affect the collective electrical activity of neuronal networks. Correa-Duarte et al. (2004) reported the growth of the mouse fibroblast cell line L929 on a 3D network based on interconnected MWCNTs arrays.

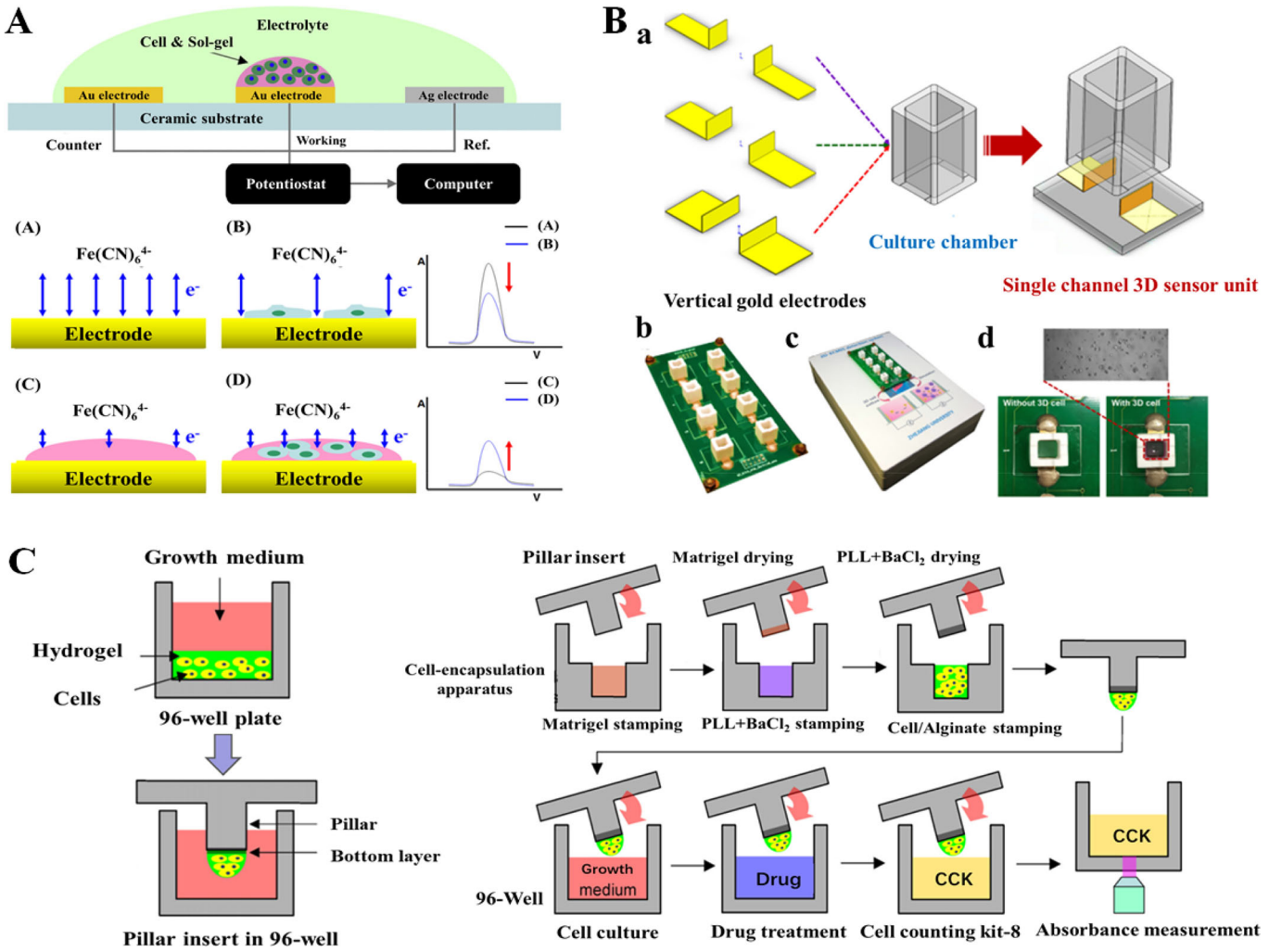

Figure 3. A. Schematics of an electrochemical biosensor applying 3D cell culture and comparison of electrical signal differences between 2D and 3D cultures (Jeong et al. 2012). B. Fabrication of a 3D impedance biosensor for monitoring the activities of 3D-cultured cells (Pan et al. 2019). C. Schematics of experimental procedures to encapsulate 3D cell cultures in hydrogel on the tip of pillar inserts and to test the cytotoxicity of compounds in 96-well plates (Lee et al. 2013).

Sensing element-3D cell cultures in mammalian CBBs

The cultured cell sensitive element is the most important part of a cell-based biosensor, as it senses and detects fluctuations in analytes within the extracellular microenvironment, which directly affects the stability, sensitivity, and accuracy of the cell sensor. The growth of cells in 2D and 3D models, each model brings its own advantages and disadvantages, as shown in Table 4 (Foglietta et al. 2020). The 3D cell model is a superior sensing element in CBBs compared to the 2D cell model because it can simulate complex in vivo microenvironments and replicate cellular responses to external stimuli (Caluori et al. 2019; Dubiak-Szepietowska et al. 2016; Pan et al. 2019; Wei et al. 2019). A cytotoxicity assay that employed a 3D cell model in an electrochemical biosensor was reported by Jeong et al. (2012). In the 2D cell model, the cell layer interfered with the flow of the electrons between the redox molecules and the electrode, and the peak value of the current decreased as the cells proliferated on the electrode. In the 3D culture, the gel acted as the nonconductor, whereas live cells in the gel were considered as the conductor due to the fact that they were stimulated by an external electrical field. Therefore, as the number of live cells was increased in the 3D culture, the electrical signal also increased, as shown in Figure 3A. The results show the feasibility of the 3D cell electrochemical biosensor for use in cytotoxicity assays (Edmondson et al. 2014).

The scaffold is the key supporting element in 3D cell cultures, and different types of scaffolds can be utilized depending on the conditions and intended goals (Wang et al. 2018). In the past few years, researchers have focused on the development of 3D cell culture scaffolds that mimic the natural ECM (Xiao et al. 2019). In addition to some natural polymers, such as polysaccharides, gels are materials that show good mechanical properties, and they are commonly used as scaffolds because they possess a tissue-like stiffness and perfectly mimic the ECM to a certain extent. Various hydrogel-based biomaterials, such as alginate, hyaluronic acid, and chitosan, with controllable properties or cellular compatibility, have been used to fabricate 3D cell culture biosensors (Intini et al. 2018; Murphy et al. 2017). In addition, synthetic biopolymers, such as polyester, polyethylene glycol, and polyamide, have been tested in the fabrication of scaffolds and 3D culture of cells due to their good

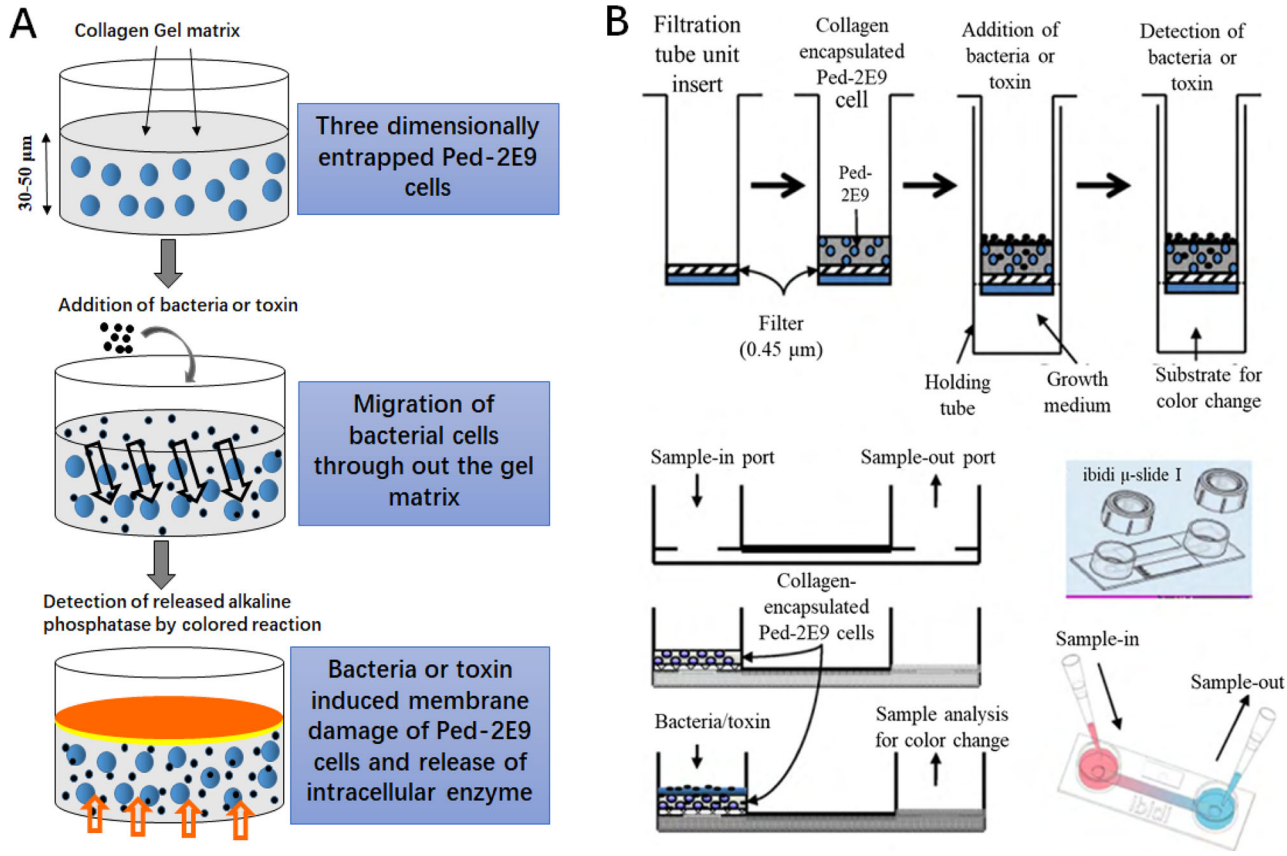

Figure 4. A. Schematic design of gel-entrapped Ped-2E9 cells and the assay procedure (Banerjee et al. 2008); B. Design of different CBBs devices based on the filtration tube and μ-slide (Banerjee and Bhunia 2010).

biocompatibility and excellent mechanical properties (Edmondson et al. 2014; Xiao et al. 2019).

However, 3D cell cultures are still a relatively new technology, and researchers have not yet fully grasped their underlying significance. Although there are several obvious disadvantages, such as the fact that the scaffold matrices incorporate compounds from unwanted sources (viruses, soluble factors) that may interfere with cell growth, this may be overcome by new technological advances (Banerjee and Bhunia 2009; Lee et al. 2013). At the same time, new 3D cell culture systems are being introduced. For example, Pan et al. (2019) developed a dynamic and real-time 3D electric cell/Matrigel-substrate impedance sensing (ECMIS) system for the monitoring of cell growth and viability and the screening of drugs. In this study, human hepatoma cells were cultured on a 3D ECMIS chip, in which a pair of vertical gold electrodes were positioned on opposing sidewalls of the culture chamber for in-situ impedance measurements (Figure 3B). Lee et al. (2013) developed a novel method using a plastic pillar insert combined with a 96-well plate to miniaturize 3D cell cultures by forming 3D hydrogel droplets that contained cells (about 1 μL) on the tip of the pillar insert. The formation of hemispherical 3D droplets containing cells is briefly described in Figure 3C. The results showed that, unlike conventional 2D cell monolayer cultures, cells encapsulated in alginate on the pillar insert by simple immersion can better mimic the in vivo cellular microenvironment for toxicology assays.

Electrical and optical joint monitoring in the evaluation of pathogens or toxins

The realization of rapid and sensitive methods to detect foodborne pathogens and toxins is central to the implementation of effective practices that ensure food safety (Law et al. 2014). Mammalian CBBs have emerged as a dependable and promising tool for biosecurity applications in food microbiology due to their unique ability to detect hazardous agents (Banerjee and Bhunia 2010; Velusamy et al. 2010). The section will focus on the use of mammalian CBBs for the detection of foodborne pathogens (bacteria and virus) and toxins (bacterial toxins, mycotoxins and marine toxins).

Banerjee et al. (2008) established the first cell-based colorimetric sensing system using collagen-encapsulated mammalian cells for the rapid detection of live pathogenic *Listeria*, toxin listeriolysin O, and enterotoxin from *Bacillus* in less than 10 h via quantifiable alkaline phosphatase release (Figure 4A). Thereafter, a handheld prototype of CBBs was designed and developed by Banerjee and Bhunia (2010), and the sensing element, B lymphocyte Ped-2E9, was encapsulated within the 3D collagen matrix for detecting a broad range of bacterial pathogens in food and beverages, such as *Listeria monocytogene, enterotoxigenic Bacillus, Serratia* and toxins etc., as shown in Figure 4B. *Listeria monocytogenes* with initial concentrations of 10^1-10^6 CFU/g was inoculated into 25 g of each hotdog and salami, and then enriched in 225 mL of Buffered Listeria Enrichment Broth (BLEB, Oxoid) for 18 h. *Bacillus cereus and Bacillus subtilis* were

inoculated into fried rice or pasteurized milk (10^4 CFU/g or mL) and enriched in Brain Heart Infusion Broth for 18 h. Similarly, α-hemolysin from *Staphylococcus aureus* and phospholipase C from *Clostridium perfringens*, as well as toxins from *Bacillus cereus* and *Bacillus subtilis* were inoculated into milk samples at a concentration of 10 g/mL, and processed as above. The above samples were tested using the developed handheld CBB. The results showed the detection limit was 10-40 ng in 2 h for toxins while 10^3-10^4 CFU/mL in 4-6 h for a model bacterial pathogen, *Listeria monocytogenes,* even in the presence of a mixture of higher concentrations of nonpathogenic species of the same genera or common background microflora. With inoculated food and beverage, the sensor detected Listeria monocytogenes and Bacillus cereus at a low initial concentration of 10^2 -10^4 CFU/g from ready-to-eat meat and rice, and only active toxins at nanogram quantities from rice, milk and water samples. Banerjee and Bhunia (2010) contended that the CBB was unique because it could detect analytes interacting with mammalian cells and distinguish pathogens from non-pathogens as well as active from inactive toxins.

Jiang et al. (2017, 2018) developed a mast cell-based electrochemical sensor to assess spoilage bacterial quorum signaling molecules, N-acylhomoserine lactones (AHLs), in freshwater fish. Rat basophilic leukemia mast cells encapsulated in alginate/graphene oxide hydrogel were used as recognition element to record the cell impedance signal as-influenced by *Pseudomonas aeruginosa* quorum-sensing molecule, *N*-3-oxododecanoyl homoserine lactone (3OC12-HSL). The procedure for the preparation of fish juice and the extraction of AHLs was as follows. Fresh fish fillets (1 kg) were cut in pieces and boiled with 500 mL tap water for 5 min. The fish juice was separated form solid residues and heated to 100 °C for 30 min. After that the *Pseudomonas aeruginosa* (ATCC 27853) was inoculated into the fish juice and cultivated at 37 °C. Then AHLs were extracted from the different incubated fish juice. Then the sample was dissolved in DMEM medium for analyses. The results show that 3OC12-HSL caused a significant decrease in cell viability in a dose-dependent manner within the concentration range of 0.1-1μM. Therefore, the proposed cell sensor can be used to real-time monitoring the spoilage bacteria in freshwater fish production.

At the same time, CBBs represent a novel strategy for detecting or evaluating individual or combined toxins (Ye, Guo, and Sun 2019). Cell viability and cytotoxicity, the main indicators of the presence of toxins in vitro, are assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)-based bioassay. For various CBBs, cell-based impedance sensors have been reported to have a similar detection capability as that of the MTT-based bioassay. As we all know, marine microalgae produce a wide range of phycotoxins, which may bioaccumulate in filter-feeding shellfish and produce human poisoning syndrome due to food-borne poisoning. Many mammalian cells also exhibit significant cytotoxicity when exposed to marine toxins. Okadaic acid (OA) is a marine toxin produced by some unicellular algae from plankton and benthic microalgae. When humans consume a large number of contaminated seafood, gastrointestinal diseases caused by diarrhea shellfish poisoning (DSP) occurs (Zhang et al. 2016).

Wang's group has developed a series of CBBs that detect various marine toxins (Su et al. 2018; Zou et al. 2015; Zou et al. 2016). Seafood products obtained from shellfish beds and local markets were processed according to protocol offered by Ledreux et al. (2012). A 20 g of the homogenate of digestive glands (DG) was extracted three times with 50 mL of acetone. The residual aqueous phase was partitioned with 50 mL of dichloromethane (DCM). The DCM extract was evaporated to dryness and the residue was resuspended in 4 mL methanol to produce a shellfish extract with a matrix concentration of 5 g DG/mL. Subsequently, continuous cell-based impedance recording was used to investigate cytotoxicity. The human hepatoma cell line was employed to develop CBBs for the real-time and sensitive detection of the DSP toxin, OA. All impedance signals associating with cell viability and compound cytotoxicity were recorded by an impedance reader system, as shown in Figure 5A,B. The results indicated the OA induced cytotoxicity in a dose-dependent manner from 10 to 100 μg/L (Hu et al. 2016; Zhang et al. 2016).

Significant advances have also been made in the detection of mycotoxins by mammalian CBBs. Mycotoxins are toxic secondary metabolites of fungi. Known carcinogens can cause food pollution and pose a major threat to economy, trade, and health. The most common mycotoxins are the aflatoxins (AFs), fumonisins (FBs), ochratoxins (OTs), patulin (PAT), the trichothecenes (THs) [deoxynivalenol (DON)] and zearalenone (ZEA) (Adebiyi et al. 2019). Gu et al. (2015) developed the BEL-7402 cell-based electrochemical biosensor to evaluate the combined toxicity of deoxynivalenol (DON) and zearalenone (ZEN) (Figure 5C). The results of electrochemical impedance spectroscopy (EIS) indicated DON and ZEN caused a marked decrease in cell viability in a dose-dependent manner. Subsequently, the combined toxicity of three mycotoxins (DON, ZEN, and AFB_1) was also assessed by the CBB, as shown in Figure 5D. The results showed that different combinations of toxins reduced cell viability either synergistically or antagonistically in a dose-dependent manner (Xia et al. 2017).

Rider et al. (2003) first reported a pathogen identification sensor using genetically engineered B lymphocytes that express calcium-sensitive bioluminescent proteins and membrane-bound antibodies specific to the target pathogen to achieves an optimal combination of speed and sensitivity. Within a few seconds of exposure to specific bacteria and viruses, the sensor could quickly respond to increase intracellular calcium concentration and emit light. Subsequently, the cell sensor was named CANARY (cellular analysis and notification of antigen risks and yields), and it has been used in the detection of foodborne pathogens and toxins (Figure 6A). CANARY can also identify pathogens in complex relevant samples. For the detection of the *Escherichia coli* strain O157:H7 commonly associated with vegetables,

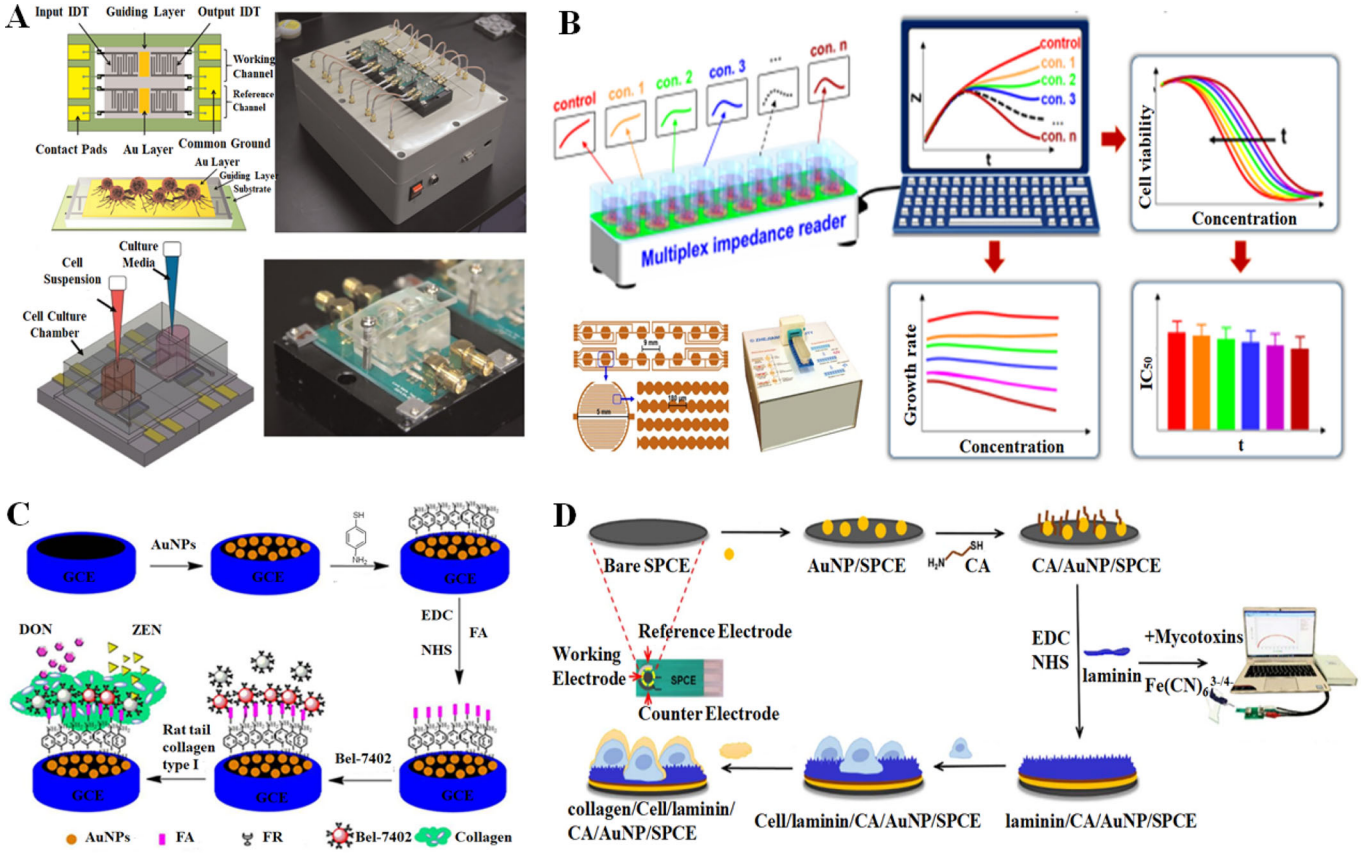

Figure 5. A. The cell-based sensor chip and the 8-channel detection system (Zhang et al. 2016); B. Cell-based interdigitated electrode based impedance sensors and multiplex impedance reader (Hu et al. 2016); C. Schematic representation of the approach for the preparation of the cell-based electrochemical sensor evaluating the combined toxicity of DON and ZEN (Gu et al. 2015); D. Schematic representation of the approach for the preparation of the cell-based electrochemical sensor evaluating the individual and combined toxicity of DON, ZEN, and AFB_1 (Xia et al. 2017).

fruits, and meat products, the specific B cell sensor was developed, which could detect 500 CFU/g in lettuce in less than five minutes (Figure 6B). The results are consistent with reports using PCR protocols to detect bacteria in foods, which takes 30 to 60 min, and the detection limit is 10 to 10000 CFU/g (or CFU/mL). On this basis, fluorescent cell-based sensors were developed to assess the response of cells to pathogens and toxins by monitoring the expression levels of specific reporter genes in genetically engineered cells. Taniguchi (2010) produced a CBB for analyzing cellular cytotoxicity responses, which combined sensor cells transfected with green fluorescence protein (GFP) plasmids derived from HSP70B0 and BTG2 promoters and a microfluidic cell culture system. Qu et al. (2013) developed a RBL CBB, as shown in Figure 6C, which could identify pathogenic viruses within three minutes. H5N1 virus and rabies virus were chosen as model viruses to investigate the feasibility of the RBL CBB. The cell responses were monitored in real-time under a fluorescence microscope. When the analytes bound to specific membrane-bound IgE antibodies, the intracellular calcium concentration increased, causing B cell cytoplasmic aequorin to emit a light almost instantaneously. H5N1 and rabies virus could be specifically recognized in heterogeneous mixture samples with the detection limit of 10^3 $TCID_{50}$/mL for H5N1 virus and 10 LD_{50}/mL for rabies virus.

Limitations and future perspectives of mammalian CBBs

As a promising approach to identify and assess pathogens and toxins, mammalian CBBs have many advantages, and these have been discussed in this review. However, specificity, robustness, and shelf-life are the most serious challenges facing the application of mammalian CBBs.

Mammalian CBBs lack specificity in the identification of the type of analyte; they also fail to discriminate the distinct properties of the analytes. For example, membrane active toxins, such as hemolysins and cytolysins from different microbes, may similarly damage the membranes of mammalian cells. To address this issue, cells transfected with a foreign plasmid expressing specific membrane-bound receptors have been used to target specific analytes, as mentioned above. In addition, another approach has been developed; toxin-specific antibodies have been inserted into the membranes of cultured mammalian cells (e.g., fibroblasts) by using membrane engineering technology, thus improving the specificity of the sensor (Banerjee and Bhunia 2009). Larou et al. (2013) developed a novel biosensor system based on the bioelectric recognition assay (BERA) for the high sensitivity detection of aflatoxin M_1 (AFM_1). Membrane-engineered cells with inserted anti-AFM_1 antibodies, which served as the biorecognition element, were immobilized in a

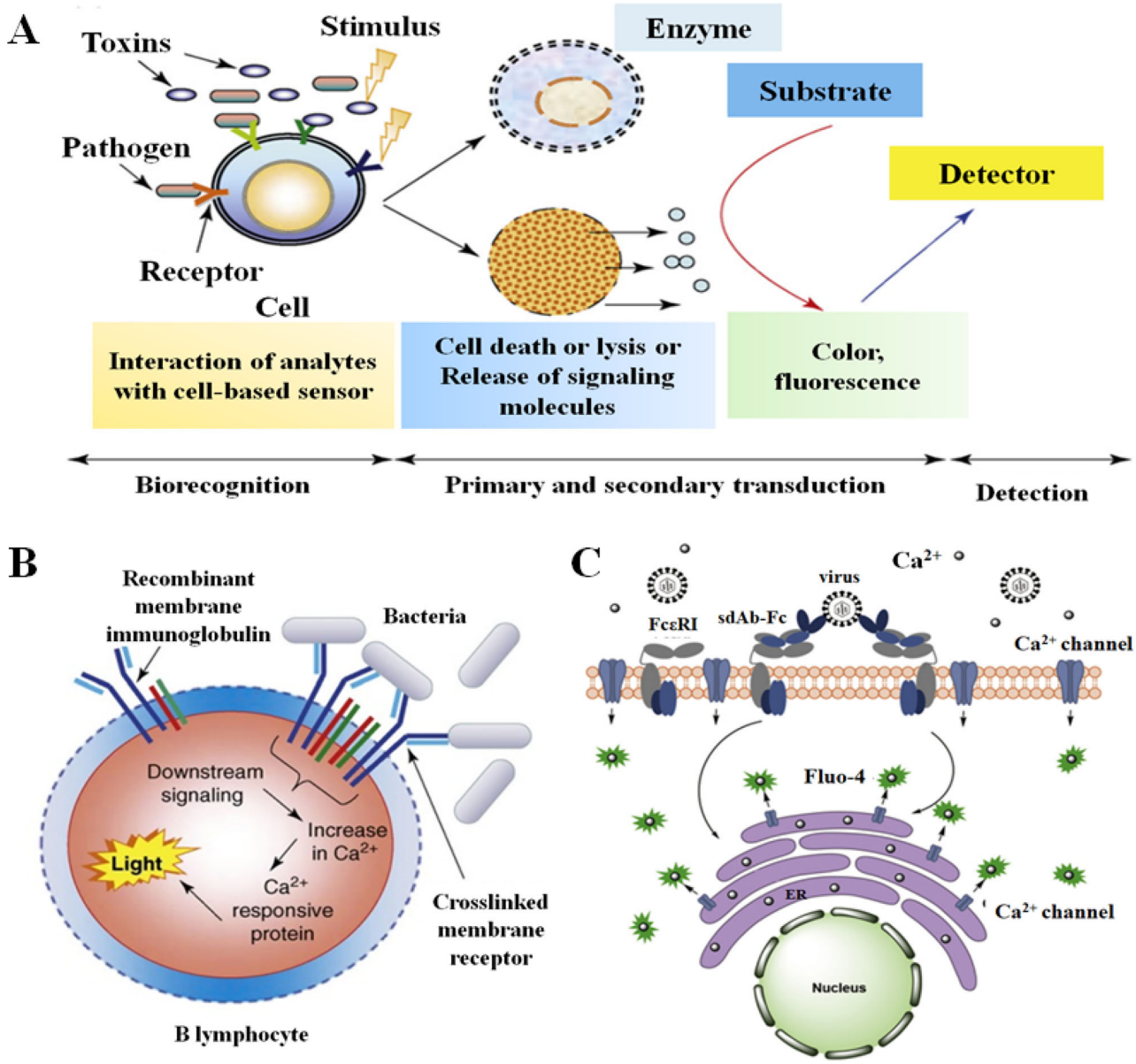

Figure 6. A. Working principle of a cytotoxicity-based biosensor (Banerjee and Bhunia 2009); B. Schematic representation of engineered B lymphocytes based biosensor (Banerjee and Bhunia 2009); C. Schematic illustration of the working principle of the RBL cell sensor (Qu et al. 2013).

calcium alginate gel and used for membrane potential measurements against AFM_1. This BERA-based sensor was able to detect AFM_1 within three minutes at a very low concentration (5 ppt), and it showed excellent selectivity against aflatoxin B_1 and ochratoxin A. (Moschopoulou et al. 2008) created an ultra-sensitive miniature CBB, in which the electroinsertion of cucumber mosaic virus-specific antibodies into the membranes of fibroblast cells improved the specificity of CBBs.

Mammalian CBBs lack robustness and a prolonged shelf-life because mammalian cells are generally fragile and prone to damage, even with minor changes in their growth environment. For CBBs, it is crucial to maintain the cells in a physiologically active state at during exposure to the sample (Roggo and van der Meer 2017). In order to eliminate the limitations of mammalian cell preservation, several strategies for long-term cell storage have been developed such as encapsulating mammalian cells in 3D protective materials and supplementing the cell culture medium with cellular enzyme inhibitors and antioxidants. For example, Petrovick et al., (2009) developed a cell culture medium, in which caspase, a protease inhibitor, a proteasome inhibitor, or the combination of these inhibitors was added to extend the shelf-life of the mammalian cells up to 6 weeks. In the patent application, they developed a strategy for increasing the expression of a quiescence regulatory gene that causes cells to be in a quiescent state, thereby extending their lifespan. In addition, an artificial cell biosensor based on a liposome-doped silica nanocomposite has been developed for the detection of Listeriolysin O (LLO), a pore-forming hemolysin secreted by the foodborne pathogen *L. monocytogenes*. When the liposomes carrying fluorophores were stimulated by membrane bioactive toxic analytes, the fluorophore reporters were released and detected. These

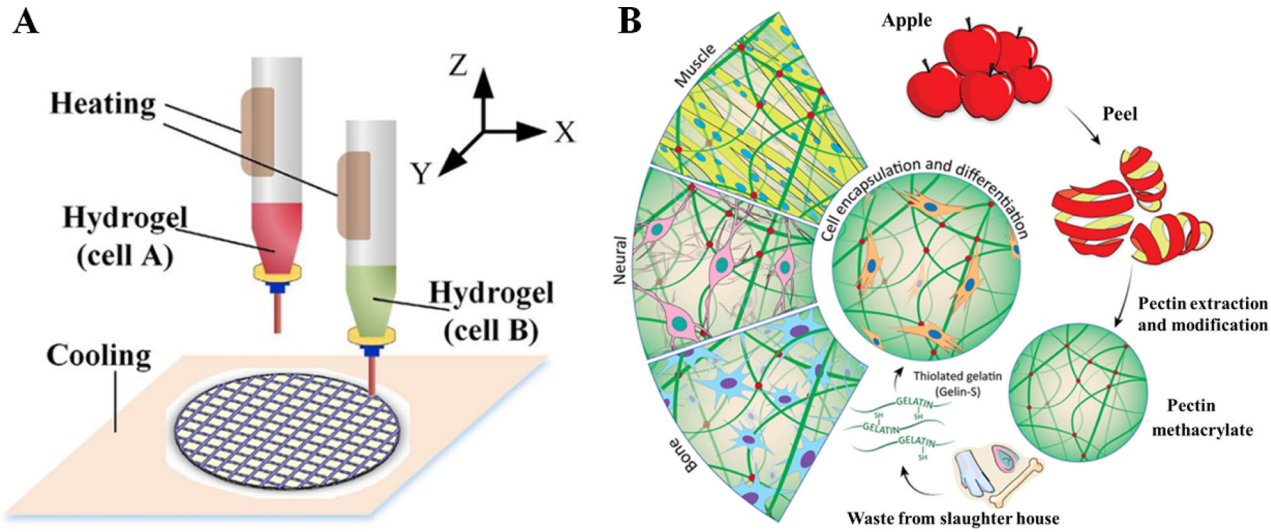

Figure 7. A. Schematic diagram of the 3D bioprinter used to print alginate/gelatin/cells (Gao et al. 2019); B. Pectin from apple waste and gelatin from slaughterhouse waste were combined into a potent hydrogel for 3D cell encapsulation and differentiation (Mehrali et al. 2019).

liposomesilica composites were stable for at least five months in ambient conditions, and thus, extended the shelf-life of synthetic CBBs (Zhao et al. 2006).

Moreover, conventional CBBs generally use cell population recordings to study the behavior of cells, and the recorded data are the collective response of many cells detected by the sensor electrodes. Single cells are systems with non-negligible differences between individual cells; therefore, studying molecules at the single cell level is the most promising way to precisely identify unknown pathogens and toxins, comprehensively investigate the biochemical reactions through molecular biology techniques, and enhance our understanding of the interactions between analytes and cells (Xu et al. 2018).

Despite several limitations, mammalian CBBs are promising analytical tools for detection of foodborne pathogens and toxins. As is the case with any new approach, CBBs are presently in the development stage. Enormous collaborative efforts are being made to appropriately address the shortcomings, as discussed in this review, and to allow their widespread application. Recently, 3D bioprinting has attracted increasing attention because it can automatically fabricate cell-laden structures with multiple cell types and different cell densities. A schematic showing of the 3D bioprinter for printing alginate/gelatin/cells is shown in Figure 7A (Gao et al. 2019). It can automatically print cells encapsulated in 3D hydrogels. Moreover, many excellent hydrogels mixed with cells for 3D bioprinting have been developed (Xie et al. 2019). For example, (Mehrali et al. 2019) employed a new pathway to transform a polysaccharide from apple peel waste, pectin, into an ultraviolet (UV)-cross-linkable pectin methacrylate polymer hydrogel with tunable mechanical properties and stability in physiologically relevant environments (Figure 7B). The results demonstrated the hydrogels can support growth and viability for a wide range of 3D encapsulated cells. Therefore, the construction of mammalian CBBs in combination with 3D bioprinting technology for high-throughput, rapid, and on-site monitoring of food contaminants that are potentially detrimental to human health is a promising approach. Ultimately, one of the most desirable goals is to produce and commercialize mammalian CBBs on a large scale, while making them affordable to users. With their unique properties, mammalian CBBs will undoubtedly revolutionize the functional biosensor field by monitoring the safety of food and assessing its quality.

Disclosure statement

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