Nanoencapsulation for Probiotic Delivery

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ABSTRACT: Gut microbiota, the most abundant organisms in the human body, dynamically participates in diverse physiological activities. A range of factors associated with the highly complex intestinal flora ecosystem, pose challenges in regulating the homeostasis of microbiota. In principle, the consumption of live probiotic bacteria can address these challenges and confer a number of health benefits. In this context, one of the major problems is the survival of probiotic cells against physical and chemical assaults during their intake and subsequent gastrointestinal passage to the gut. Advances in the field have focused on improving the conventional encapsulation techniques in the microscale to achieve high cell viability, gastric and temperature resistance, and longer shelf-life. However, these microencapsulation approaches are known to have limitations and translation into clinical benefits. In this perspective, we present a brief overview of the current progress of different probiotic encapsulation methods and focus mostly on the contemporary and emerging single-cell encapsulation strategies using nanocoatings for individual probiotic cells. Finally, we discuss the advantages of various nanoencapsulation approaches and highlight the future trend to develop coated probiotics with advanced features and health benefits.

Intestinal human microbiota represents the largest microbial ecosystem with thousands of microbial cells that regulate multiple biological functions such as maturation of immune system, host cell proliferation, neurologic signal, bone density, and hormone biosynthesis.[1-3] When this delicate balance is disturbed by endogenous and external determinants (food, antibiotic therapy, pathogens and others),[2, 4, 5] a condition called "gut dysbiosis" may result in the host, inducing a set of digestive disorders including diarrhea, cramping, constipation and other metabolic syndromes.[6, 7]

Probiotics, defined as live microorganisms, when administered in adequate amount, confer health benefits to the host.[8] For example, the lactic acid bacteria – one of the most studied genus, owing to their wide distribution in foods, plants, soils, and human hosts are attractive candidates for commercial food products, dietary supplements, and pharmaceutical formulations. These probiotic formulations are expected to provide beneficial functions to the host via modification of the intestinal microbiota, generation of metabolic entities, neutralization of dietary carcinogens, induction of cytokine synthesis and control of pathogens.[9] However, to achieve these health effects, probiotics need to survive a set of environmental assaults such as low pH in the stomach, enzymatic degradation, antimicrobial activity of bile salts, competition with other bacteria, and at the same time, be able to efficiently attach to the gut epithelium.[10, 11] As such, encapsulation strategies are necessary to simultaneously protect and promote their delivery into the target site.

Conventionally, the most common encapsulation approaches for the protection of probiotic cells (Figure 1a) have focused on the use of micro encapsulation techniques where the probiotics are embedded within a protective matrix before being delivered. Among them, the most widely used ones are extrusion, emulsion, and spray-drying methods,[12] where the encapsulation of probiotic cells works via different mechanisms such as sol-gel immobilization, ionic coacervation, and emulsion polymerization, using polysaccharides and proteins as the most common matrix materials.[13] Although, these approaches have been somewhat successful to increase the viability of probiotic cells, the lack of a control in particle size,[14] leakage[15, 16], and low in-vivo efficiency of these formulations remains to be the critical bottlenecks.[17, 18] In addition, the appropriate combination of bulk encapsulated microbiota with food ingredients as supplements, is difficult to achieve. In recent years, progress has been made in developing alternative encapsulation systems to address the existing challenges where the individual cell encapsulation via nanocoatings has emerged as the most attractive and viable alternative. However, a significant amount future works is required as the target remains far from being achieved.
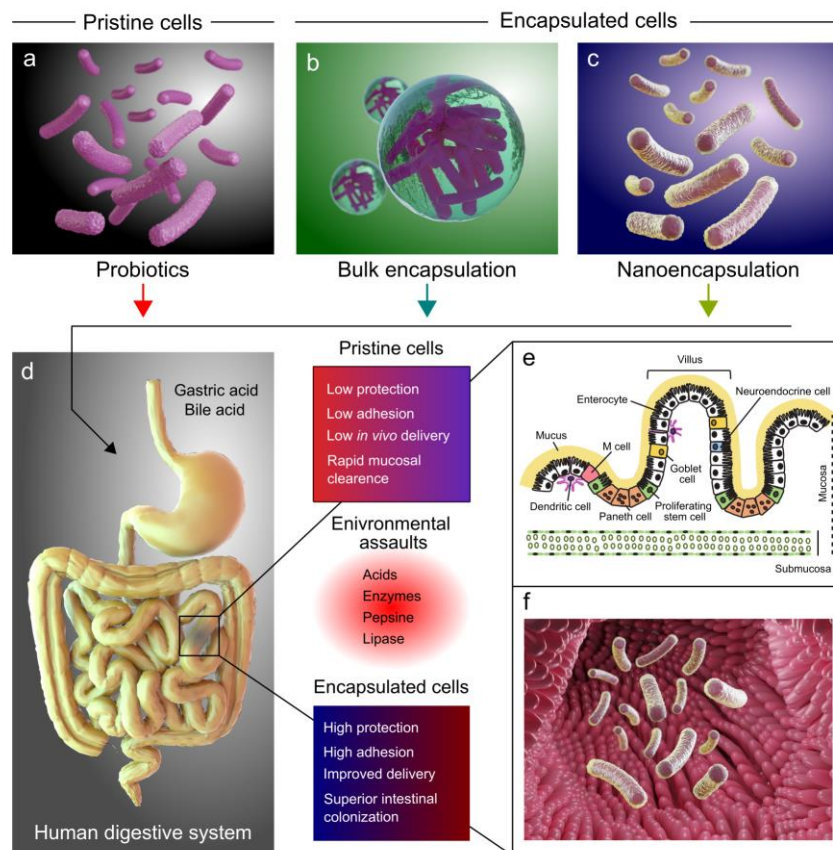
Figure 1. Schematic of different probiotic encapsulation methods. (a) Representation of probiotic cells without protection. (b) Bulk encapsulation of probiotic cells in the micrometer scale. (c). Nanoencapsulation of probiotic cells in the nanometer scale. (d) Gut bacteria pathway via the gastrointestinal tract and the importance of probiotic encapsulation. (e, f) Schematics of the intestinal villi showing the composition and three-dimensional view with coated probiotic cells.

Here, we first briefly highlight the systems where the probiotics are encapsulated in a bulk (Figure 1b) or microscale matrix (e.g., hydrogel system), designated as "bulk encapsulation" and provide an overview about the current progress and limitations of these systems. Consequently, we discuss the technologies where the probiotics are encapsulated in a nanoshell (Figure 1c) matrix (e.g., single-cell encapsulation via nanocoatings) with advanced properties that are able to overcome the delivery challenges (Figure 1 d-f) and provide additional features such as the prevention of pathogenic colonization.[1]

1. Bulk encapsulation systems

The microencapsulation of probiotics using hydrocolloids system (Figure 2a) have been widely used as an effective system to increase the probiotic survival from environmental stresses such as low pH.[19] In this method, probiotics are packed in sealed capsules; that are semi-permeable and spherical with a size in the range of a few microns; that can release the content when they are exposed to specific conditions.[20] The usual encapsulating materials are food-grade polymers, mostly derived from polysaccharides (Figure 2b), proteins (Figure 2c), and lipids, where the choice depends on several factors including compatibility and the desired properties.[21]

1.1. Polysaccharide hydrogels

Polysaccharides are one the most studied materials for probiotic encapsulation. A combination of various useful features such as biocompatibility, biodegradability, low cost and ready availability, make them an attractive candidate for different cell encapsulation techniques.[21] For example, alginate, a natural polymer extracted from seaweed, have been extensively investigated for probiotic encapsulation due to their ability to absorb water and ion-gelation properties. When alginate polymers interact with ionic crosslinking agents, a hydrogel matrix is formed that is able to resist harsh conditions such as the stomach pH (Figure 2 b-f). In addition, the enteric dissolution property enables the alginate microcapsules to release the content in the intestinal environment.[22] However, the current limitations of polysaccharide hydrogel systems include, (1) the presence of high porosity in the matrix, leading to an early release

of the encapsulated probiotics, (2) the integrity of alginate gels can be affected by chelating agents that deteriorate the calcium crosslinked network, and (3) a low encapsulation efficiency that is difficult to scale up.[21, 23]

1.2. Protein hydrogels

Probiotic encapsulation with milk proteins involves the confinement of probiotic cells in an internal microenvironment derived from milk proteins. Milk proteins, as an encapsulating agent, have good solubility, gelling and film-forming properties (Figure 2c). These features make milk proteins (e.g., caseins) a versatile platform to stabilize different emulsion systems. Compared to polysaccharide systems, milk proteins are also nutritive and bioactive.[24] In particular, dense milk proteins with buffering capacity are suitable to produce microbeads that can resist the gastric conditions. Note that milk proteins can easily form gels via heat treatment, however, this treatment can also affect the heat-sensitive materials such as probiotic cells, resulting in a loss of viability. Therefore, the production of milk protein microbeads is commonly performed via extrusion, coacervation, electrospinning, fluidized bed and spray coating methods.[10]
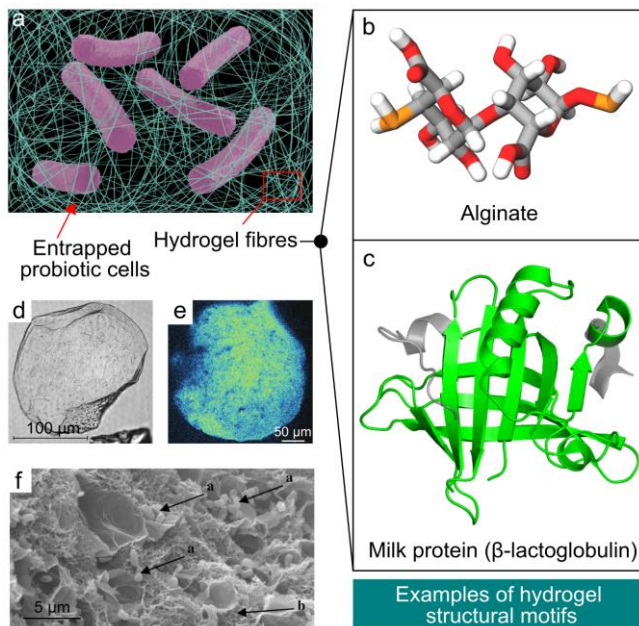

Figure 2. Bulk encapsulation of probiotic cells. (a) Probiotic cells entrapped in a hydrogel network. (b) Structure of conventional encapsulating materials based on (b) alginate and (c) milk proteins. (d) Optical and (e) confocal images of alginate hydrogels filled with probiotic cells. (f) Cross-section of alginate hydrogels filled with probiotic cells – as indicated by the arrows.

Depending on the encapsulating method, it was found the encapsulation using whey proteins produces relatively large microbeads using the extrusion technique, and the size control is determined by the extrusion conditions. Emulsion technique has shown to produce capsules with a relatively lower size than the extrusion method, but factors such as emulsion instability and vigorous stirring are detrimental for the cells. More recent approaches include the use of an enzyme that is capable of inducing the gelation of proteins (e.g., transglutaminase). The ability of these enzymes to crosslink proteins such as casein under mild conditions, making possible to encapsulate living cells inside the gel matrix.[10, 25] In summary, the use of milk proteins for probiotic encapsulation has shown a strong protection in acidic conditions. However, there is a need of multiple optimization processes including heating conditions for denaturation of milk proteins, pH, effect of conformational behavior of milk protein (fibrillar and capsular), and interaction between the surface components of probiotic cells and milk proteins.[10]

1.3. Coated hydrogels

A common approach to deal with the current limitations of single hydrogels systems is the use of a second polymer to coat the hydrogel beads. In this category, chitosan, as a cationic polysaccharide which is non-toxic, biocompatible, and biodegradable, interact with alginate forming a polyelectrolyte complex among the positive and negative charge between these polymers. As a result, the alginate microcapsules are coated with a semipermeable membrane that possess a reduced porosity which leads to a reduced leaking of probiotic, and a wide stability in different pH ranges. It is important to note that this last approach of creating a single layer of chitosan on alginate beads can be repeated forming multiple layers which can enhance the encapsulation system.[22, 26] Similarly, alginate/poly-L-lysine capsules were developed forming a polyanion-polycation complex membrane which reduce the porosity and swelling of alginate beads. However, a special care should be considered due to a cellular attachment of poly-L-lysine capsules to cells and any immune reaction from the hots.[27, 28] Overall, all the bulk encapsulations can effectively protect probiotic cells from acidic conditions. However, these methods are subjected to achieve an effective control of the size of the microcapsule mainly because this factor will influence in the stability and efficacy of the entrapped probiotic.

2. Single-cell encapsulation systems

In nature, microorganisms have evolved trough millions of years with adaptative mechanisms to survive in a wide range of harsh conditions. For instance, *Bacillus subtilis*, an aerobic Gram-positive bacterium, is known to sporulate and produce a protective shell against aggressive environments including heat, desiccation, radiation, and oxidation. Taking inspiration from such example, today a range of biomimetic approaches are available to fabricate an artificial shell around living cells
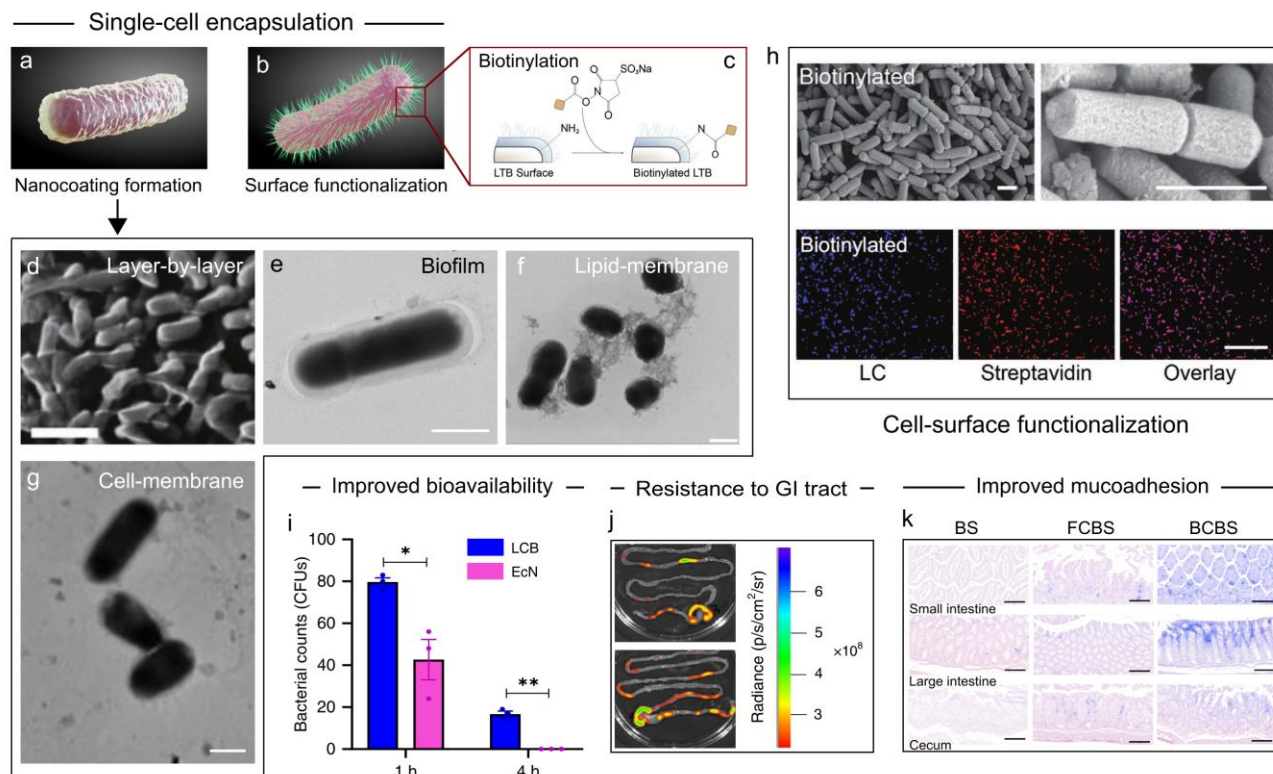
Figure 3. Methods of single-cell encapsulation via nanocoating formation or surface functionalization through conjugation chemistry. (a) Encapsulation of individual probiotic cells via nanocoating formation. (b, c) Surface functionalization of gut bacteria *via* biotinylation and the underlying chemistry for the bioconjugation of biotin. (d) SEM images of coated *Bacillus coagulans* using chitosan and alginate via LbL assembly. (e–g) TEM images of coated *E. coli Nissle via* biofilm, lipid-membrane, and cell-membrane coatings. (h) SEM and epi-fluorescence images of biotinylated gut bacteria. (i) Gastric acid (pH 2) resistance of uncoated (EcN) and lipid-membrane coated EcN. (j) Representative IVIS images of intestinal tracts from mice after oral gavage for 4 h of uncoated and lipid-membrane coated EcN. (k) Microscopic images of Gram staining of the intestinal tissues harvested from mice, orally administered with 1×10^7 CFUs of bacteria after 24 h – uncoated (BS), biofilm fragment (FCB) and biofilm-coated (BCBS) bacteria.

(known as single-cell encapsulation) to improve the cell resistance to physicochemical stresses and provide additional biological functions to the native cells.[29] In the field of single-cell encapsulation, different cytoprotective approaches based on silica,[30] graphene,[31] polydopamine,[32] metal-organic frameworks.[33] metal-polyphenol nanoshell[34] have been developed. Regardless of the materials used, these encapsulation approaches have been developed based on mild chemical conditions without significantly affecting cell viability. The generation of protective and degradable shells,[34] via these approaches can potentially improve the cell resistance to external stresses,[30-35] transport of essential nutrients[35], and also open up the opportunity for post-functionalization.

In contrast to the bulk encapsulation methods based on the immobilization of gut bacteria into a gel matrix in a micrometer scale, single-cell encapsulation is based on the formation of nanofilms around individual probiotic cell that can result in several advantages for probiotic delivery such as providing a cytoprotective

suit which can display new functionalities including improved adhesion, *in vivo* resistance and even prevention of diseases.[3]

In the next section, we will present an overview of the contemporary single-cell encapsulation methods (Figure 3) used for probiotic cells including layer-by-layer approach,[36] chemical conjugation,[37] encapsulation in cell membranes,[38] and lipid self-assembly[39]. We also briefly discuss the distinct features and properties achieved by the single-cell encapsulation approaches. We note that although these strategies have been investigated for a variety of other living cells, their applications for probiotic encapsulation are currently limited and require future explorations.

2.1. Layer-by-Layer encapsulation

Nanoscale thin films of charged polymers (polyelectrolytes) can be prepared by the alternating deposition of polyanions and polycations on substrate surfaces.[40] This concept of sequential deposition oppositely charged polymers typically driven by electrostatic

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interactions, is better known as the layer-by-layer (LbL) assembly method and can be applied to planar and colloidal substrates. Initially demonstrated by R. K. Iler[41] in 1966 by the alternative adsorption of positively and negatively charged colloidal particles, Decher et al.[42] provided the concept with oppositely charged polyelectrolyte pairs on planar substrates. This concept was first introduced to colloidal supports by Donath et al.[43] and later developed by Sukhorukov et al.[44] and Caruso et al.[45]

Although the LbL strategies with charged polymers to form multilayered nanoscale films have been adopted for different types of living cells, their application for the encapsulation of the probiotic cells in particular is rather limited. Anselmo et. al.[30] demonstrated the single-cell encapsulation of probiotic cells using a combination of cationic polysaccharide (e.g., chitosan) and an anionic polymer (e.g., alginate). The morphology of the coated probiotic cells was not significantly altered by the deposition of the polymer layers due to their smooth nanoscale features (Figure 3d). The cell division of the coated probiotics was observed to be delayed as a function of the number of polymer layers. Furthermore, this strategy showed an improved protection and controlled release of the probiotic cells under gastrointestinal conditions.[36, 46] However, despite the effective protection from physical and chemical insults,[36, 47] the adhesion properties exhibited by the coated probiotics require to be much improved. Finally, the LBL assembly method is typically time consuming, and the automation of the polymer deposition steps are difficult to scale-up.

2.2. Protective coatings via self-assembly

Self-assembly, one of nature's wonder design principles, can be defined as the spontaneous arrangement of molecular components into ordered hierarchical structures.[48] The formation of many sophisticated biological structures ranging from proteins to viruses and cell membranes, use a process of dynamic self-assembly that involves a series of assembly and disassembly steps consuming energy from the environment. Such a process delicately controls the aggregation of biomolecules to form various cellular components such as filaments, membranes and organelles that performs a complex set of biochemical reactions – central to life.[49]

Inspired by the concept of natural self-assembly, two different approaches have been adopted recently for the single-cell encapsulation of probiotics. First, a biofilm (defined as organized conglomerate of microorganism living in a self-produced matrix[49]) approach, where the self-produced biofilms can serve as the protective encapsulation matrix for the probiotic cells. For example, Wang et al.[50] reported that *Bacillus subtilis* can secrete a large quantity of exopolysaccharides and proteins that can trigger the formation of a self-

assembled biofilm (Figure 3e) on the cell wall when cultured in appropriate conditions.[50] The use of exopolysaccharides as an encapsulating agent, thus promotes intestinal health, improve cell adherence and resistance to harsh conditions.[48, 51]

In the second approach, the probiotic bacteria can be wrapped into a thin lipid membrane via a biointerfacial supramolecular assembly (Figure 3f) of dioleoylphosphatydic acid on the negatively charged surface of the bacterial wall.[39] The use of this natural phospholipids provide properties including a great chemical biostability against different enzymes like phospholipases, esterases, bile salts, and resistance to serum proteins. These lead to higher thermodynamic stability against alkaline pH, high temperature, and oxidative stress conditions. Furthermore, phospholipids can be degraded by lipolysis which results in low toxicity.[39, 52] A relevant strategy has also been reported to generate stealth bacteria by camouflaging with cell-membrane.[38] This cell-membrane coated bacteria (CMCB, Figure 3g), was prepared by simply extruding the erythrocyte membranes with bacteria. The erythrocyte membranes were chosen because of their low immunogenicity and long circulation properties. As demonstrated, this approach (1) lowered the inflammatory reaction and side effects of CMCB as the bacterial immunogens are camouflaged and (2) decreased their body clearance because of the anti-phagocytic nature of the erythrocyte membrane coatings.

2.3. Coordination-driven assembly and cell-surface functionalization

Phenolic compounds ubiquitous in plant kingdom containing catechol or gallol functional groups are well known for their universal adhesion and metal chelation ability.[53] The versatile coordination chemistry of metal-phenolic complexes has become a key synthetic strategy for surface engineering in recent years. In particular, the incorporation of the catechol moiety into synthetic materials and subsequent crosslinking by transition metal ions have been a subject of intensive research to develop biomimetic functional materials. In 2013, Ejima et al.[54] pioneered the versatile metal-phenolic network (MPN) method for surface film formation exploiting a coordination driven assembly process. The process involves mixing of tannic acid (a natural polyphenol, TA) and iron(III) (Fe^{III}) ions in the presence of different substrates resulting in instantaneous film formation (~10 nm) on the substrate surfaces.

Although the MPN assembly approach has been employed as cytoprotective nanocoatings for various living cells,[34] only recently, Liu et al.[55] used the MPN coatings for probiotic encapsulation. In their double layer coating strategy, EcN was sequentially encapsulated in TA/Fe^{III} MPN and enteric L100 layers (outer layer). The double layered coatings exhibit excellent resistances toward the harsh environment of the GI

tract. In addition, the pH-responsive disassembly of the outer L100 layer, facilitates the selective delivery of the MPN-EcN to the intestine, where the strong mucoadhesive properties of the outer crosslinked TA networks prolong the cell retention time without compromising their viability and proliferation capabilities.

Like the versatile MPN assembly, the avidin-biotin interactions has been widely used in biochemical assays, diagnosis, and drug delivery.[56] Avidin is a tetrameric glycoprotein isolated from egg white consists of terminal N-acetyl glucosamine and mannose moieties, and each monomer can bind to biotin. This type of non-covalent interaction, one of the most specific, employs multiple hydrogen bonding and hydrophobic interactions, providing an affinity of ~10^{-15} M. Similarly, streptavidin, a purified protein isolated from *Streptomyces avidinii*, can also bind to biotin with a high affinity. Both avidin and streptavidin can be conjugated to other proteins *via* the covalent addition of a sulfo-N-hydroxy-succinimide (Sulfo-NHS) to biotin.[57, 58] Using this chemistry (Figure 3b,c), a surface functionalization method has been adopted for live therapeutic bacteria as shown in Figure 3h. In this work,[37] the bacteria wall was chemically modified with biotin to produce artificial adhesins which can adhere into the gastrointestinal tract. During this surface modification process, the conditions used for the conjugation chemistry was not deleterious for the bacteria viability, and the metabolic activity remained unchanged. Furthermore, these synthetic adhesins improve the *in vivo* pharmacokinetics and colonization rate of probiotic cells.

3. Distinct features of single cell encapsulation

Unlike bulk encapsulation, single-cell encapsulation of probiotics can offer several advantages including improved bioavailability against environmental assaults, improved mucoadhesion, *in-vivo* resistance, and can potentially play a role in the prevention and treatment of diseases, at a cellular level. Although bulk encapsulation methods have shown a successful probiotic protection from the gastrointestinal environment, the challenges associated with their synthesis and desired performance demands alternative approaches as discussed before. In this regard, single-cell encapsulation of probiotics has surged as an advanced approach where the individual probiotic cells are coated with nanomaterials that can provide different types of protection like pH resistance and enzymatic activity, antibiotic resistance, and even some degree of protection against chemicals (e.g., ethanol).[36, 39]

As the main target of probiotic cells is to reach the intestinal cells, there is only a handful of reports about the adhesion benefits from bulk encapsulation systems where some materials (e.g., chitosan) has shown to increase the adherence of coated microcapsules into the gut according to the *in-vitro* studies.[59] In contrast, single-cell encapsulation methods showed a significant increase of the coated bacteria in the intestinal tract and translation to *in-vivo* studies in animal models, which has started to show an exciting progress in the field.[36, 39] For example, the lipid membrane-coated EcN showed resistance to strong acidic condition (pH 2, Figure 3i) in vitro and in GI tract environment in vivo (Figure 3j). Furthermore, the biofilm-coated bacteria showed improved mucoadhesion in-vivo as shown in the microscopic images of Gram staining of the intestinal tissues harvested from mice after 24 h of oral administration (Figure 3k).

Furthermore, the application of probiotic bacteria to prevent or fight against other microorganisms such as pathogenic bacteria (*i.e., S. aureus, S typhimurium*) have been proposed via different mechanisms such as interfering with the colonization, nutrient competition, and secreting specific low molecular weight antimicrobial substances. It is worth mentioning that single-cell encapsulation methods also provide new insights into different biological features to tackle and prevent diseases. [37, 39, 50, 60]

4. Conclusions and outlook

The global market for probiotic food supplements has been growing quite rapidly. Even amidst the COVID-19 pandemic, this market has been estimated to be US$ 4.6 Billion in 2020 with a projected size of US$ 7.1 Billion to be reached by 2027. As such, future research efforts must center on the challenges concerning the protection and efficient delivery of probiotics to accentuate the health benefits they offer.

Despite the progress we discussed here, the field of nanoencapsulation of probiotic cells is still in its infancy. The approaches presented here with the emphasis on single-cell encapsulation by nanocoating formation include a number of benefits overcoming the conventional microencapsulation strategies for probiotic delivery, and offer unique features such as enhanced colonization, *in-vivo* gastric resistance, and a significant role in prevention and treatment of diseases. Furthermore, single-cell encapsulation uses a minimal quantity of starting materials that can be engineered to produce a biofriendly nanocoating on probiotic cells, without the need of using acid-resistance microcapsules or complex technologies.

There are still extraordinary opportunities in this field to explore in relation to the vast array of biocompatible materials that can provide protection to living cells. Screening of these future coating materials in terms of their coating ability, toxicity, compatibility of coating conditions with the living cells, adhesion properties, cost-effectiveness, ease of operation, and stimuli responsiveness, will play a pivotal role in going forward. Additionally, the development of synbiotics that combine probiotics and prebiotics, will require mutual biocompatibility of the coating chemistry to be applied.

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addressing the more fundamental questions in regard to the physicochemical interactions between the bacteria cell wall and the coating materials, will facilitate in selecting the choice of materials with an adequate balance between the coating reaction and bacterial viability, and optimum coating conditions. Detailed investigations are also necessary for the activation control parameters, i.e., when the coated probiotics are released in the gut. With the advancement of nanotechnology in the context of synthesis tools and bio-nano characterization, the field of nanocoated probiotics will likely provide solutions to the existing limitations and ensure the promised health benefits of probiotics.

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Notes

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ABBREVIATIONS

CCR2, CC chemokine receptor 2; CCL2, CC chemokine ligand 2; CCR5, CC chemokine receptor 5; TLC, thin layer chromatography.

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