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Toward a New Generation of Therapeutics

Artificial Cell Targeted Delivery of Live Cells for Therapy

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Abstract

Scientific evidence in the prevention and treatment of various disorders is accumulating regarding probiotics. The health benefits supported by adequate clinical data include increased resistance to infectious disease, decreased duration of diarrhea, management of inflammatory bowel disease, reduction of serum cholesterol, prevention of allergy, modulation of cytokine gene expression, and suppression of carcinogen production. Recent ventures in metabolic engineering and heterologous protein expression have enhanced the enzymatic and immunomodulatory effects of probiotics and, with time, may allow more active intervention among critical care patients. In addition, a number of approaches are currently being explored, including the physical and chemical protection of cells, to increase probiotic viability and its health benefits. Traditional immobilization of probiotics in gel matrices, most notably calcium alginate and κ-carrageenan, has frequently been employed, with noted improvements in viability during freezing and storage. Conflicting reports exist, however, on the protection offered by immobilization from harsh physiologic environments. An alternative approach, microencapsulation in "artificial cells," builds on immobilization technologies by combining enhanced mechanical stability of the capsule membrane with improved mass transport, increased cell loading, and greater control of parameters. This review summarizes the current clinical status of probiotics, examines the promises and challenges of current immobilization technologies, and presents the concept of artificial cells for effective delivery of therapeutic bacterial cells.

Index Entries: Artificial cells; biotherapy; probiotic; gastrointestinal tract; immobilization; microencapsulation; biopharmaceutics.

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Introduction

Probiotics refer to ingestion of live microorganisms for health benefits *(1)*. Nearly a century ago, Russian physiologist Eli Metchnikoff proposed ingesting fermented milk on a daily basis to maintain a proper equilibrium of intestinal microflora *(2)*. More recently, probiotic bacteria have been the focus of much scientific and commercial interest owing to a myriad of preventative and curative effects. Most probiotics belong to a large group of bacteria designated as lactic acid bacteria, especially but not exclusively lactobacilli and bifidobacteria, which are important components of the human gastrointestinal (GI) microflora. In addition, new probiotics include a variety of different microbes including engineered microorganisms with acquired or enhanced health-promoting properties. The probiotic concept yet remains ineffective partly as a result of viability losses in traditional probiotic products.

Beyond selection of strain, a number of approaches are currently being explored to increase viability, including stress adaptation to simulated environments, incorporation of micronutrients, and two-step fermentation processes *(3)*. In addition, the physical and chemical protection of cells has been proposed. Immobilization of probiotics in gel matrices, most notably calcium alginate and κ-carrageenan, is a mild process that has been used to protect cells from storage and GI transit *(4,5)*. However, many current gel-based entrapment carriers are acid sensitive *(6)* and provide a suboptimal environment for cell proliferation and metabolic activity *(7)*.

An alternative approach for probiotic protection utilizes semipermeable microcapsules with strong and thin multilayer membrane components with specific mass transport properties. The concept of microencapsulation in "artificial cells" was first discovered by Chang in the early 1960s for the encasement of biologic materials such as transplanted cells, enzymes, and adsorbents *(8)*. Since then, numerous other applications have emerged, including the encapsulation of probiotics for oral administration. The permselective microcapsule environment has been shown to support cellular metabolism and proliferation and to permit higher cell loading. Furthermore, the final microcapsule properties, namely the permeability and composition of the membrane, can be varied to suit the needs of a particular application.

The review that follows focuses on the current clinical status of probiotic therapy, the development of immobilization technologies for the entrapment and protection of viable cells, and the introduction of microencapsulation in artificial cells as a probiotic delivery vehicle toward designing a new generation of therapeutics.

Probiotic Therapy

The human GI tract represents a complex ecosystem in which a fine balance exists between the intestinal microflora and the host. The vast communities of microorganisms, representing up to 500 species, are in constant

interaction with their environment, including other bacteria, the gut epithelium, and the mucosal immune system *(9)*. Functionally, the indigenous flora has significant metabolic activity and is crucial for maturation of the immune system and the development of normal intestinal morphology *(10)*. For several decades, probiotic therapy has been stressed as a means to optimize indigenous flora, enhance the metabolic activity of the GI tract and reinforce the natural defense mechanisms of the host. More recently, probiotic bacteria have been the focus of much scientific and commercial interest owing to a myriad of preventative and curative effects.

Present Status in Clinical Practice

The consumption of specific probiotic strains is associated with a range of clinically relevant health benefits. Placebo-controlled clinical trials have shown *Lactobacillus reuteri*, *Lactobacillus rhamnosus GG*, *Lactobacillus casei*, and *Saccharomyces boulardii* to be effective in reducing the duration of acute diarrhea *(11,12)*. *L. rhamnosus GG* administered to infants reduced the risk of nosocomial diarrhea and rotavirus gastroenteritis *(13)*. Studies by Aso et al. *(14)* revealed that *L. casei* Shirota increases the percentage of T-helper cells and natural killer cells in adult patients with colorectal cancer and has a protective effect on the recurrence of superficial bladder cancer. In addition, select strains of lactobacilli have been shown to significantly suppress intestinal tumors by chemical mutagens *(15,16)*. Lactic acid bacteria have been administered to prevent sepsis in patients with severe acute pancreatitis *(9)*. A randomized study by Rayes et al. *(17)* involving liver transplant patients revealed that postoperative infections were significantly reduced by feeding live *Lactobacillus plantarum* cells in comparison to standard antibiotic treatment *(9)*. As a means of preventing allergy, a randomized controlled study by Lodinova-Zadnikova and Sonnenborn *(18)* investigated the effect of at-birth colonization with nonpathogenic *Escherichia coli* Nissle 1917. Subjects inoculated with the *E. coli* strain showed significantly reduced colonization of bacterial pathogens as well as significantly lower incidence of allergies after 10 and 20 yr in comparison with control subjects *(18)*. Several species of lactobacilli have been implicated in lowering cholesterol. For example, administration of *L. plantarum* 299v lowered total and low-density lipoprotein cholesterol levels in a placebo-controlled study *(19)*.

Probiotics have been used as treatment options for managing inflammatory bowel diseases (IBDs) such as Crohn disease, ulcerative colitis, and pouchitis. Recently, genetically modified food-grade microorganisms have been shown to appropriately target therapeutic proteins to the gut mucosa. In an animal model, *Lactococcus lactis*, genetically engineered to secrete the anti-inflammatory cytokine interleukin-10, was shown to cure or prevent experimental enterocolitis, a model designed to mimic human IBD *(20,21)*. Future research should continue to elucidate live normal or genetically engineered probiotics with specific health benefits and, in doing so, isolate mechanistic pathways by which they function (*see* Table 1). However, the

Table 1
Mechanisms of Action of Select Probiotic Therapies[a]

- ***Enhancement of immune system:*** Probiotic studies have reported increased production of a vast array of cytokines such as IL-1, IL-2, IL-6, IL-10, IL-12, IL-18, TNF-α, and IFN-γ *(22,23)*. These cytokines are known to exert a range of immunomodulatory functions. For example, IL-12 and IL-18 induce IFN-γ production, which, in turn, enhances phagocyte-mediated clearance of microbes and augments the cytotoxic capacity of T-cells and NK cells. By comparison, TNF-α increases macrophage activity, IL-1 stimulates proliferation of T- and B-cells, and IL-10 possesses potent anti-inflammatory properties *(9,24)*.
- ***Management of IBD:*** In most chronic cases, abnormal activation of the mucosal immune system against the enteric flora is the key event triggering inflammatory mechanisms. Probiotic therapy increases the gut IgA immune response, promoting the gut's immunogenic barrier *(25)*.
- ***Cancer therapy:*** Studies in human subjects have revealed that probiotic therapy may reduce the risk of colon cancer by inhibiting transformation of procarcinogen to active carcinogens, binding/inactivating mutagenic compounds, suppressing the growth of procarcinogenic bacteria, reducing the absorption of mutagens from the intestine, and enhancing host immune response *(9,26)*.
- ***Allergy prevention:*** Administration of probiotic bacteria has contributed to antiallergy effects by strengthening mucosal defenses (through enhanced gut-specific IgA responses); promoting gut mucosal barrier function; down-regulating inflammatory responses; and increasing the production of TGF-β, IL-10, and cytokines known to produce IgE antibodies *(27,28)*.
- ***Lowering of cholesterol:*** Potential mechanisms by which probiotics lower cholesterol levels include assimilation of cholesterol by bacterial cells, binding of cholesterol to bacterial cell walls, and deconjugation of bile salts by the bacterial enzyme bile salt hydrolase. In addition, short-chain fatty acids, the end product of carbohydrate fermentation in the gut, could prompt inhibition of hepatic cholesterol synthesis and/or redistribution of cholesterol from plasma to the liver *(29,30)*.

[a]Initial and ongoing efforts in probiotic research have been focused on studying their effectiveness in placebo-controlled clinical trials. However, of increasing interest is the identification of mechanisms of action. IL, interleukin; TNF-α, tumor necrosing factor-α; IFN-γ, interferon-γ; NK, natural killer; TGF-β, transforming growth factor-β.

aforementioned clinical data highlight the important potential of probiotic therapy.

Limiting Factors

Although probiotic therapy shows significant therapeutic potential, there are limitations precluding its use in clinical or supplemental therapy. Probiotic-containing products, such as yogurts and other dairy-based beverages, tablets, or health foods, present unique challenges to manufacturers. To provide functional properties, the minimum level of viable bacteria is approx 10^6 CFU/g of product, and the suggested therapeutic dose is 10^8–10^9 viable cells/d *(3)*. However, the stability of probiotic cultures is often significantly diminished prior to administration. Many probiotic

cultures (e.g., bifidobacteria) are fastidious, noncompetitive, and very sensitive to environmental parameters such as oxygen and low pH. In addition, probiotics in storage cultures encounter inhibitory metabolic byproducts such as lactic and acetic acid, hydrogen peroxide, and bacteriocins *(3,31)*. Tablets incorporating freeze-dried microorganisms prolong shelf life especially with added protectants; however, viability losses are nevertheless observed *(32)*.

Orally administered probiotics must also circumvent passage from the mouth to the intestine. Physiologic conditions such as acidic pH, mechanical stresses, digestive enzymes (e.g., pepsin and pancreatin), bile acids, and oxygen provide an effective barrier against entry *(33,34)*. Selection of strain for therapy is therefore dependent on the tolerance of the probiotic to the aforementioned stresses as well as the ability to persist and potentially colonize the human GI tract. Recently, with the advent of genetic engineering, strategies have been developed to accelerate improvement of strains. However, novel microorganisms may present a risk of systemic infections, deleterious metabolic activities, adjuvant side effects, immunomodulation, and gene transfer *(35)*. Furthermore, repeated large doses could result in their replacing the normal intestinal flora *(36–39)*. Therefore, although there have been many publications describing the use of probiotics to prevent and treat GI disorders, safety and efficacy issues have resulted in only a few studies contributing significantly to the knowledge of health effects in humans.

Technologies for Improving Probiotic Survival

Beyond selection of strain, a number of approaches are currently being explored to increase the viability of probiotics in commercial or experimental products. Stress adaptation to simulated environments; incorporation of micronutrients such as peptides and amino acids; and two-step fermentation, whereby probiotic bacteria are permitted to proliferate prior to the addition of yogurt starter cultures, have proven useful in improving probiotic survival *(3)*. In addition, the use of immobilization and microencapsulation technologies to retain high cell concentrations during storage and gastric transit, whereby the probiotic is separated from its environment by a protective coating, has been proposed.

Immobilization Technologies

Immobilization of whole cells has been defined as the physical confinement or localization of intact cells to a defined region of space without appreciable loss of catalytic activity *(40)*. Currently, a myriad of methods are available for immobilization of probiotics, with selection of a particular application dependent on the nature of the microorganism being immobilized as well as the availability and cost of the carrier material. Adsorption to a preformed carrier and ionic bonding methods are mild and inexpen-

sive, utilizing weak interaction forces to immobilize cells. However, the beads are highly sensitive to pH and prone to cell leakage *(41)*. By comparison, covalent binding and crosslinking offer significant improvements in strength over methods employing weak bonds; however, potential toxicity of reagents employed during processing has limited this method's applicability *(42)*. As a result, the physical retention of probiotics has far outnumbered attempts at binding cells to carriers in food- and medical-based applications. Of these, the most widely used immobilization technique is the entrapment of cells within a gel matrix.

Application of Probiotic Immobilization in Commercial and Experimental Products

Entrapment techniques based on the formation of thermal and ionotropic gels have proven useful for probiotic applications. An array of polymers, such as agarose, alginate, chitosan, cellulose, gellan, and κ-carrageenan, have been chosen as the support materials for the gel matrix. These are generally nontoxic, readily available, and acceptable for use as additives in the food and dairy industries.

Probiotic entrapment by alginate and κ-carrageenan is widely encountered in the literature. Alginate forms a gel matrix in the presence of polyvalent cations such as Ca^{2+} or Ba^{2+} and κ-carrageenan solidifies on exposure to potassium ions. The mild yet sturdy properties of the gel-like matrix allow the probiotic to remain viable with retention of enzymatic activity for extended periods of time. A study by Adhikari et al. *(43)* revealed that bifidobacteria entrapped in κ-carrageenan appeared to increase their viability in yogurt over a 30-d storage period. Sheu and Marshall *(44)* observed that lactobacilli immobilized in calcium alginate survived 40% better than free cells during the freezing of ice milk. Furthermore, Kebary et al. *(45)* reported that entrapping *Bifidobacterium bifidum* and *Bifidobacterium infantis* in alginate or κ-carrageenan beads improved their viability in frozen ice milk over 10 wk. Sultana et al. *(46)* modified the method of calcium alginate immobilization and found that incorporation of starch as a prebiotic improved probiotic survival over an 8-wk storage period in yogurt. In addition, gel entrapment of lactobacilli and bifidobacteria has been reported to protect probiotic cells from lyophilization and rehydration *(47)*.

Conflicting reports exist, however, on the protection offered by immobilization from gastric conditions. Shah and Ravula *(48)* reported that probiotic bacteria immobilized in calcium alginate were able to survive at pH 2.5. Similarly, when *Bifidobacterium longum* entrapped in calcium alginate beads were exposed to simulated gastric juice and bile salt solution, the death rate of the cells in the beads decreased proportionally with an increase in both the alginate gel concentration and the bead size *(49)*. Other studies, however, indicate that calcium alginate beads are acid sensitive

and are not able to prevent cell death at low pH *(6)*. By contrast, κ-carrageenan/locust bean gum gel beads have been shown to be more resistant to acidic conditions; however, potassium ions required during processing are damaging to certain probiotic strains *(50,51)*. Sun and Griffiths *(52)* reported that gellan gum/xanthan gum form spherical beads in the presence of calcium ions at room temperature and effectively protect bifidobacteria in yogurt storage and gastric juice. However, survival at acidic pH was strongly dependent on the strain employed. Among other formulations, gelatin and polymer-coated gelatin capsules have been studied for oral delivery of probiotic cells *(53,54)*. The latter, with 20% (w/v) of the polymer, has shown promising results in vitro *(54)*. Attempts have been made at adhering probiotic bacteria to prebiotics *(55)*. The presence of high-amylase maize-resistant starch was shown to increase survival of bifidobacteria at low pH, in bile, and during intestinal transit in mice *(56)*. However, Crittenden et al. *(55)* reported that adhesion of bifidobacteria to starch is sensitive to acid and protease activity and likely would not survive through the stomach. A host of other formulations has been proposed for bead entrapment (Table 2); however, general limitations persist.

General Limitations of Immobilization Technologies

Reports on the added protective benefit of gel entrapment in the oral delivery of probiotics are, as we have described, frequently inconclusive. Although probiotic survival in the gel matrix has been shown to be enhanced in response to environmental stresses (freezing, storage, and simulated gastric transit), reports are often conflicting and dependent on the strain employed. It has therefore become difficult to isolate a particular entrapment procedure as a candidate for rigorous optimization studies and eventual scale-up. Support materials for gel entrapment are also insufficiently immunoprotective for novel microorganisms as well as traditional probiotics that have shown in certain cases to instigate an inflammatory response *(35)*. In addition, diffusional properties and inadequate mechanical strength limit the proliferation of the entrapped probiotic. Encased cells have been reported to leak; escape from the gel matrix; and, as a result, grow in the surrounding solution *(7)*. Growth in gel beads is restricted, especially in larger beads, in which proliferation occurs only in the periphery, owing to substrate limitation. In many cases, the maximum cell loading of entrapped gel beads is limited to 25% by volume, owing to weak mechanical strength *(7)*. Furthermore, diffusional limitations of both substrates and metabolic byproducts can lead to the development of steep gradients regarding pH and inhibitory products that can hinder the viability and metabolic activity of the entrapped probiotic. Therefore, despite reported advantages, the associated drawbacks of immobilizing probiotics in gel matrices have limited the influence of this technology in commercial and experimental products.

Table 2
Gel Entrapment for Probiotic Delivery[a]

Material	Probiotic	Strengths	Limitations	References
Calcium alginate	*L. acidophilus* *L. casei* *L. lactis* *B. bifidum* *B. longum* *B. infantis*	Acceptable food additive Cell and tissue compatible Mild reaction conditions Low cost Ease of control over parameters Low cost Starch useful as prebiotic	Susceptible to acid Reduced mechanical stability during lactic fermentation Insufficient immunoprotection	*5, 6, 44, 57*
κ-Carrageenan/ locust bean gum gel	*L. casei* *L. lactis* *B. bifidum* *B. infantis* *B. longum*	Strong, rigid gel Acid resistant Thermoreversible Good results in cryopreservation studies	Less biocompatible than alginate Potassium ions damaging to cells and potentially host electrolyte composition Insufficient immunoprotection	*4, 50, 51*
Cellulose acetate phthalate	*L. acidophilus* *B. lactis* *B. pseudolongum*	Resistant to gastric acid conditions Established enteric coating material for controlled release Readily dissolves in mildly acidic to neutral environment of small intestine	Probiotic viability during membrane formation limited by harsh reaction conditions (HCl) Nonporous membrane Limited access to substrate during storage	*58, 59*
Gellan gum/ xanthan gum	*B. adolescentis* *B. bifidum* *B. breve* *B. infantis* *B. longum*	Acid resistant Stabilized by calcium ions Easy to mix bacterial suspension with gum prior to gelation Economical processing Retention of cellular viability in pasteurized yogurt No shrinkage in lactic and acetic acid solutions	High-setting temperature required by gellan gum Acid survival dependent on strain Poor viability in storage indicated in some reports Insufficient immunoprotection	*52, 60*

Agarose	Bacillus Calmette Guerin	Prolonged stability in storage Cell and tissue compatible Mild reaction conditions Narrow size distribution of beads Low cost/readily available	Limited mechanical stability Insufficient immunoprotection owing to cellular protrusion and absence of permselective layer	*61, 62*
Artificial sesame oil	*L. delbrueckii* ssp. *bulgaricus*	Acceptable food additive Lactic acid viability retained in storage Acid and bile resistant	Sensitive to mechanical stress Wide size distribution High temperatures required for processing	*63*
Starch	*B. adolescentis* *B. bifidum* *B. breve* *B. lactis* *B. longum* *B. pseudolongum*	Natural adhesion (prebiotic) Exhibits good spray drying properties, allowing for easy scale-up and economical processing Small microparticles with excellent cell coverage	Limited protection against acid stress, protease activity, and pancreatin Poor survival in foods Nature of adhesion dependent on strain (presence of cell-surface protein)	*55, 56, 64*

[a]Several different probiotic strains have been immobilized in food-grade porous polymeric matrices. Ideal carriers are nontoxic, readily available, and inexpensive. Other qualities that are assessed include cell loading, cell viability in the support material, and protection from environmental stresses.

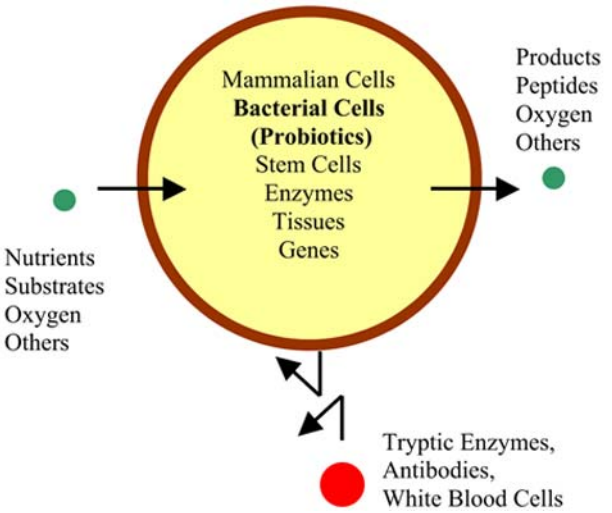

Fig. 1. Semipermeable artificial cells. Biologically active material such as tissues, cells, or cellular constituents are enclosed within a microscopic, semipermeable polymer membrane. The latter allows bidirectional diffusion of small molecules necessary for cell survival and proliferation (e.g., nutrients, environmental substrates, therapeutic protein products, metabolic waste materials). Larger molecules such as tryptic enzymes and antibodies are blocked from entry. By applying different membrane materials and encapsulation parameters, the permeability and surface chemistry can be varied over a large range. This flexibility allows extensive possibilities to fit a desired application.

Microencapsulation in Semipermeable Artificial Cells

Artificial cell microencapsulation of probiotic bacteria is currently being studied to confront limitations with traditional immobilization technologies. The concept behind microencapsulation involves utilizing a spherical, semipermeable, thin, and strong membrane to surround a liquid core containing biologic material (Fig. 1). The first semipermeable microcapsules or "artificial cells," discovered by Chang in the 1960s, contained hemoglobin (for use as blood substitutes), enzymes (to treat inborn errors of metabolism), or adsorbents (to treat drug overdoses) *(8,65)*. They were of cellular dimensions with membrane materials composed primarily of semipermeable polymers such as cellulose nitrate or polyamide *(8,65)*. Beginning in the late 1970s, pancreatic islet cells and hepatocytes were effectively encapsulated into millimeter-size artificial cells and implanted for treatment of diabetes mellitus and liver failure, respectively *(66)*. In 1980, Lim developed the alginate-poly-L-lysine-alginate (APA) membrane system for islet encapsulation *(67)*, a protocol that is widely regarded for providing the impetus for the cell encapsulation field and has been applied to encapsulate probiotics effectively *(68–70)*.

Artificial Cell Membrane Design

A number of microencapsulation techniques have been developed by independent laboratories in recent years. The most widely used among them is polyelectrolyte complexation, a technique utilizing the interaction of oppositely charged polymers to form a physical membrane around the probiotic *(71)*. Choice material can be natural or synthetic, with natural polymers exhibiting greater cell compatibility and milder conditions (Table 3). Other techniques employ interfacial polymerization, coacervation, or conformal coating for production of single- or multilayered membranes that can range in surface chemistry from hydrophilic to lipophilic. The use of different membranes allows variations in permeability, mass transfer, mechanical stability, biocompatibility, and buffering capability that can be exploited to fit a desired application. The mass transport properties of a membrane are critical because the influx rate of molecules, essential for cell survival and proliferation, and the outflow rate of metabolic waste ultimately determine the viability of encapsulated cells. Frequently, membrane permeability is defined by the molecular weight cutoff, the maximum molecular weight of a molecule that may freely pass through the pores of the capsule membrane (Fig. 2). The molecular weight cutoff of orally delivered microcapsules must allow passage of substrates from the GI tract as well as unwanted metabolites from the plasma and then either facilitate the subsequent removal of the altered molecule or provide for its storage. Figure 2 outlines a variety of microcapsule membrane materials that have historically been used with their mass transport properties. The versatility of various microcapsule membranes has been well documented, with many novel formulations coming to fruition today, leading to a range of preparation methods for probiotic therapy.

APA Membrane System

The well-established interaction between alginate and poly-L-lysine for the production of APA microcapsules utilizes polyelectrolyte complexation. Calcium alginate beads are first prepared according to traditional gel entrapment procedures. Binding of poly-L-lysine to alginate occurs electrostatically by long-chain alkyl amino groups that extend from the polyamide backbone of poly-L-lysine and interact with carboxyl groups of the calcium alginate bead. Reexposure of the crosslinked bead to dilute alginate neutralizes positively charged poly-L-lysine residues still present at the capsule surface. The calcium-alginate core is then liquefied by exposing the freshly made microcapsules to a chelating agent such as sodium citrate.

APA microcapsules have been used to encase probiotic bacteria, with several advantages noted over traditional immobilization technologies (Fig. 3). The aqueous core provides minimal mass transfer resistance, which, coupled with the large surface area-to-volume ratio of the semipermeable membrane, allows permeant substrates and products to diffuse rapidly *(7)*. In addition, microorganisms have a larger accessible volume to grow and

Table 3
Artificial Cell Membrane Materials for Microencapsulation and Oral Delivery of Probiotics[a]

Membrane material	Features	References
APA	Established synthesis protocols, mild encapsulation conditions, cell-compatible core, flexible membrane permeability, in vivo effectiveness for oral bacterial delivery, low inflammatory response if coated with PEG, mechanical instability in simulated gastric conditions, inflammatory response in certain instances if polycation inadequately neutralized	*73, 82, 83*
AC	Better biocompatibility in polycation component than APA; improved biocompatibility and immunocompatibility when coated with PEG; superior capsule strength, flexibility, and biocompatibility when coated with glutaraldehyde; crosslinking with genipin shown to improve membrane strength; mechanical instability in noncoated capsule	*77, 84, 85*
APPPA	Performed well in GI stability tests, showing increased resistance to acidic and basic conditions, as well as in the presence of ion chelators; greater control over membrane permeability than traditional APA capsules	*80*
ACPPA	Superior to APA and AC in cell viability studies, allowed outflow of small proteins, similar to APA in terms of membrane strength, good retention of cellular viability and metabolic activity in cryopreservation studies	*86*
A-PMCG-A	Flexible membrane permeability; better mechanical stability than APA; independent adjustment of capsule size, wall thickness, and mechanical strength; in vivo mechanical stability yet to be studied	*79, 87, 88*
HEMA-MMA	Synthetic, thermoreversible, water-insoluble system; improved mechanical stability owing to insolubility in aqueous conditions; easy scale-up; control over desired properties; reaction conditions potentially damaging to probiotics; diffusion properties of aqueous solutes limited	*81, 89*

[a]ACPPA, alginate-chitosan-polyethylene glycol-poly-L-lysine-alginate; A-PMCG-A, alginate-cellulose sulfate-polymethylene-co-guanidine-alginate; PEG, polyethylene glycol.

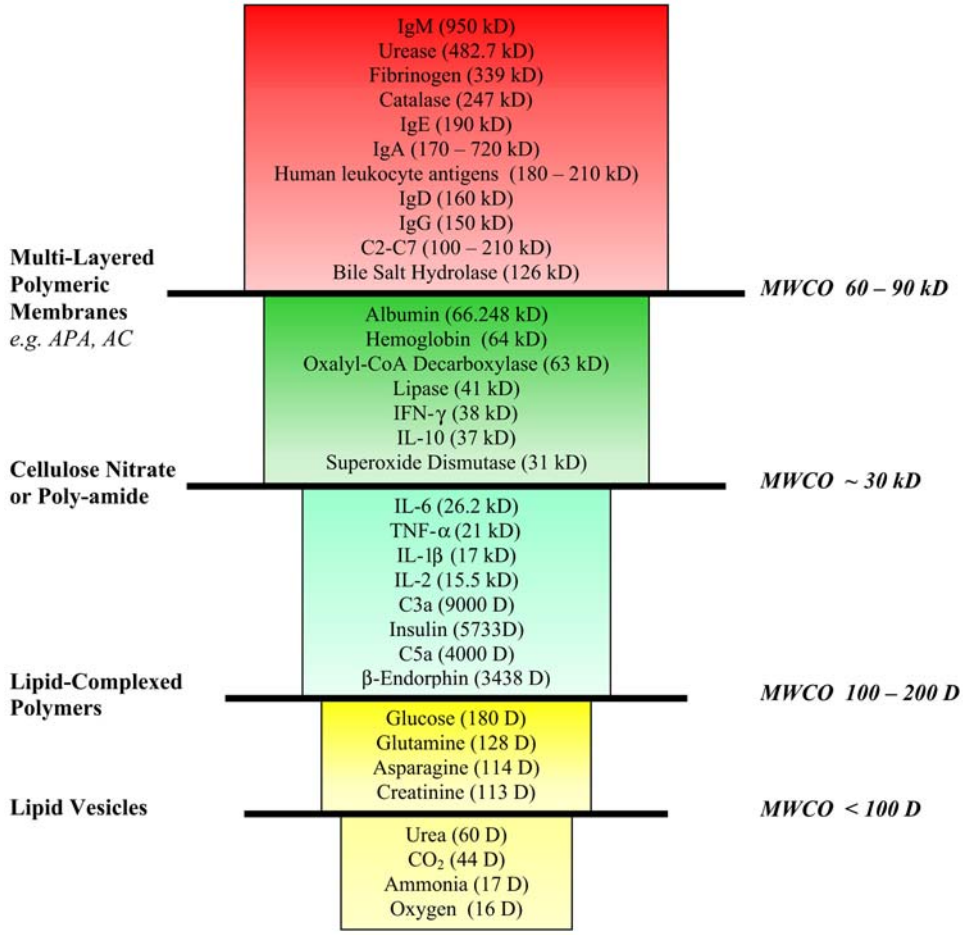

Fig. 2. Molecular weight cutoff (MWCO) for various membrane materials that have traditionally been employed for microencapsulation of cells. The mass transport of a particular molecule through a given membrane is dependent on a number of factors including membrane pore size, wall thickness, and surface charge. Currently, multi-layered polymeric membranes are considered advantageous owing to their inherent ability to have mass transport characteristics tailored to fit a desired application by varying encapsulation parameters.

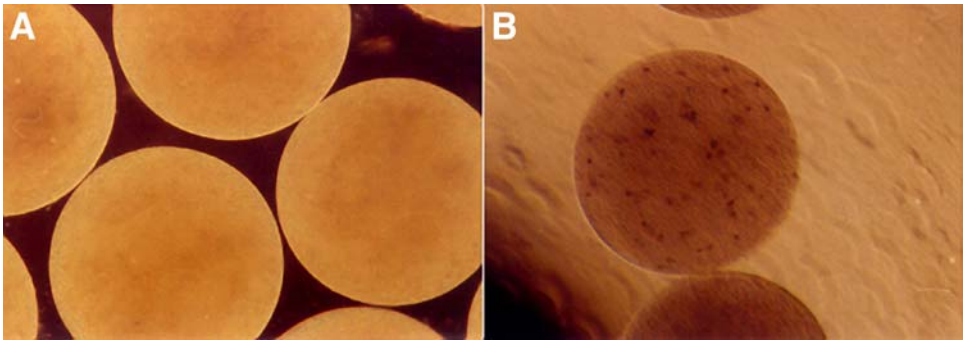

Fig. 3. Semipermeable microcapsules utilizing APA membrane system for encapsulation and oral delivery of probiotics: **(A)** empty APA microcapsules; **(B)** APA microcapsules containing *E. coli* DH5 cells. (Reproduced from refs. *66* and *68* with permission.)

proliferate with no compromise of membrane stability. Poly-L-lysine provides a permselective layer that can be quantified for mass transport, which, in turn, can be controlled by adjusting the reaction time and poly-L-lysine concentration *(71)*. For oral delivery, alginate is perhaps the best suited polymer for capsular design, in part because of its excellent biocompatibility and status as a Food and Drug Administration-approved food additive *(72)*. Processing of the APA microcapsule uses mild and aqueous-based conditions that do not compromise viability, and it has been shown to be immunoprotective for orally administered genetically engineered probiotic cells for therapy *(73)*. Reports on the mechanical stability of APA, however, are conflicting. Prakash and Chang observed APA microcapsules with encased *E. coli* DH5 to be stable when agitated up to 210 rpm for 7 h. Furthermore, no leakage of encapsulated bacteria was observed in overnight incubations for up to three cycles. However, other reports found APA microcapsules unstable in simulated GI conditions *(74)* and susceptible to enzymatic hydrolysis *(75)*. To overcome this, researchers used a higher concentration of alginate crosslinked with barium instead of calcium *(76)*. This modification prolonged the stability of the microcapsule for systemic delivery applications in canine models, but not for oral delivery.

Alternative Membrane Systems

Chitosan and poly-L-ornithine have been employed as polycationic replacements for poly-L-lysine, with noted improvements in biocompatibility *(77,78)*. Alginate-chitosan (AC) microcapsules were found to have superior strength, flexibility, and biocompatibility when coated with glutaraldehyde. In addition, crosslinking of AC microcapsules with naturally derived genipin improved performance in membrane-strength tests. A novel design utilizing alginate, cellulose sulfate, and polymethylene-co-guanidine introduced both weak (alginate) and strong (cellulose sulfate) interactions with the polycation (polymethylene-co-guanidine) and was found to have greatly enhanced mechanical strength and capsule durability over traditional APA capsules *(79)*.

Other intricate membrane systems utilizing polyelectrolyte complexation have been proposed for encapsulating microorganisms. For instance, alginate-polylysine-pectin-polylysine-alginate (APPPA) membranes have been prepared and tested for stability in simulated GI fluid *(80)*. Results indicate increased resistance to acidic and basic conditions, as well as in the presence of ion chelators, while allowing for more precise control over membrane permeability than traditional APA capsules. Synthetic polymer systems such as hydroxymethyl methacrylate-methyl methacrylate (HEMA-MMA) processed by interfacial polymerization have been shown to have easily adjustable parameters and improved mass transfer, stability, and durability *(81)*. However, the reaction conditions required for its formation are damaging to cells. Current research should continue to see adaptation of the initial APA membrane system as well as the development of superior microencapsulation methods. In doing so, systems will be optimized for bacterial cell-based encapsulation with enhanced delivery properties.

Potential of Microencapsulation Technology in the Probiotic Industry

Two-Step Encapsulation Method for Improved Retention of Probiotic

The artificial cell membranes can provide a larger, milder, and more comfortable working environment for the encased probiotic. Cell density can be maximized, which, in turn, increases volumetric productivity, while mass transport resistance is reduced. Other advantages of using artificial cells include high process stability over long fermentation periods and the retention of plasmid-bearing cells. Furthermore, other technologies such as a two-step encapsulation method developed by Wong and Chang *(90)* has been applied to improve cell retention and minimize leakage into the surrounding environment. This method is based on the formation of very small alginate gel microspheres containing cells, which are subsequently enclosed within larger gel spheres. Following membrane formation of the outer sphere, the small alginate gel microspheres are dissolved to release the cells. The probiotic is thus freely dispersed inside the artificial cell, avoiding the extra diffusion barrier while preventing cell protrusion and leakage.

Large-Scale Production

Large-scale production of sterile microcapsules containing probiotics can be done using automated, medical-grade encapsulators such as the Inotech Encapsulator produced by Biosystems International (Rockville, MD). The technology utilizes vibrational frequency for shearing of an alginate-probiotic suspension into equally sized droplets. An electrostatic charge is initially imparted onto the newly sheared droplets to prevent contact during flight and entry into the hardening solution. In this way, microcapsule homogeneity >95% is achieved and is reproducible from run to run. Furthermore, automated, medical-grade encapsulators reduce production time over traditional syringe-based methods, increase robustness, heighten control over parameters, and provide an end product with narrow size distribution. Thus, there is available technology for manufacturing large numbers of superior quality microcapsules without compromising probiotic viability.

Potential for Storage and Administration in Probiotic Products

Considerations for the production and storage of artificial cell-containing probiotics have been outlined and are continually addressed *(91)*. It is implied that the design of artificial cell systems be protective against environmental stresses in storage conditions such as inhibitory metabolic byproducts and low pH. Furthermore, designers must minimize stresses imparted owing to long exposure times to stomach acid, antimicrobial compounds, digestive enzymes, and bile acids in the GI system. A variety of formulation modalities are now available that provide for effective storage and delivery of artificial cell-containing probiotics without compromising consumer taste. Specifically, microencapsulated probiotics can be incorpo-

rated into foodstuffs such as fermented milk products (yogurt, ice cream, cheeses) as active ingredients or, alternatively, be dehydrated and incorporated into capsules or pill form with a shelf life up to 2 yr *(92)*. Furthermore, artificial cells can be stored for extended periods at low temperature in minimal solution and subsequently delivered in drink format. Future work will continue to investigate industrial platforms for storage and administration, which should increase delivery rates as well as palatability.

Clinical Applications of Artificial Cells Containing Live Bacterial Cells

Early developments of microencapsulated bacterial therapy utilizing genetically engineered microorganisms with novel or enhanced probiotic properties are emerging. For instance, researchers have developed inducible expression promoters for high-level production of heterologous proteins. In this way, it is possible to control gene expression in engineered lactic acid bacteria by an inductor; a repressor; or environmental factors such as temperature, pH, or ion concentrations *(93)*. Additionally, one may expand the range of possible active components beyond protein therapeutics by integrating foreign enzymes.

To accomplish this end, Prakash and Chang *(68)* developed nonpathogenic *E. coli* strain DH5 expressing *Klebsiella aerogenes* urease for use as an oral artificial kidney substitute. For patients with chronic renal failure, dialysis is conventionally used for the removal of waste metabolites from plasma; however, it is time-consuming and uncomfortable as well as inaccessible to low-income households. This study shows that the oral administration of genetically engineered *E. coli* encased in APA artificial cells decomposes urea that diffuses into the capsule from the GI tract into ammonia, which the bacteria can utilize for its biosynthesis *(68)*. When orally administered daily to rats with surgically induced renal failure, APA artificial cells containing *E. coli* reduced plasma urea from 52 to 9 mg and subsequently maintained the normal level throughout a 21-d experimental period *(68)*. Extrapolation of these results to a 70-kg uremic patient reveals that approx 4 g of microcapsules containing *E. coli* DH5 would be required for effective daily therapy, significantly less than alternative urea absorption protocols such as oxystarch or microencapsulated zirconium phosphate–urease *(68)*.

Recent interest in characterizing oxalate-degrading enzymes in Bifidobacterium has given rise to microencapsulating such bacteria for oral probiotic therapy. Oxalate, a component of fruits and vegetables consumed in normal human diets, represents a highly oxidized organic compound with strong chelating capacity and limited potential for catabolic removal *(94)*. As such, accumulation in humans can prove toxic and result in a number of pathologic conditions including hyperoxaluria, calcium oxalate nephrolithiasis, and cardiomyopathy *(95,96)*. The degradation of intestinal oxalate is dependent on the persistence of oxalate-degrading microorganisms in the gut, the most important being *Oxalobacter formigenes*, whose intestinal absence has been associated with the aforementioned pathologic

conditions *(97)*. The key enzyme, oxalyl-CoA decarboxylase has been similarly characterized and heterologously expressed in *Bifidobacterium lactis* by Federici et al. *(94)*. Unlike *O. formigenes*, however, Bifidobacteria do not depend on oxalate for growth and thus pose fewer limitations in storage and administration. The potential exists to microencapsulate *B. lactis* with heterologous expression of oxalyl-CoA decarboxylase for colon-targeting oral delivery. The polymeric membrane may be designed to allow secretion of oxalyl-CoA decarboxylase, with a molecular mass of about 63 kDa *(94)*, into the surrounding environment while simultaneously protecting the encased microorganism. As such, artificial cells can help regulate oxalic acid levels in the colon as well as in plasma and urine for individuals deficient in intestinal oxalate-degrading microorganisms.

Several additional probiotic therapies have been reported to use artificial cells both in vitro and in vivo (Table 4). Others remain prime candidates. Because of newly designed probiotic strains as well as advances in heterologous protein expression, metabolic induction, and genetic engineering, it is expected that a number of other novel therapeutic applications will soon emerge.

Future Directions

Recent advances in biotechnology and molecular biology will continue to generate valuable advances in the probiotic industry. The isolation of novel strains as well as progress in the applications of metabolic induction, heterologous protein expression, and genetic engineering will lead to probiotics with improved properties and enhanced health benefits. There is therefore a need to look for new technologies to meet the present demands and future challenges of probiotics. Entrapment techniques in food-based polymeric matrices have proven useful for probiotic applications. They are generally nontoxic and readily available and have been reported to protect cells from storage, lyophilization, and rehydration. Reports on gastric protection are conflicting, however, and conditions for cell loading and retention are suboptimal. Initial research with artificial cells indicates that they are a more suitable vehicle for probiotic delivery than previously applied methods. They provide a larger, milder environment for encased cells with enhanced cell loading and minimized mass transfer resistance. Furthermore, greater control over parameters such as membrane permeability and surface chemistry allows extensive possibilities to fit a desired application. However, to apply artificial cells to specific probiotic therapies effectively, research must be directed at optimizing methodology as well as membrane materials and purification techniques. In addition, low-cost methods that provide stable storage must be examined to best preserve the potency of probiotics to be delivered. However, microencapsulation in artificial cells holds significant promise and appears to better satisfy delivery requirements over other entrapment methods on the market today.

Table 4
Clinical Applications of Probiotics Utilizing Artificial Cell Microencapsulation

Disease	Probiotic	Therapeutic mode of action	References
Renal failure	*E. coli* DH5 *L. delbrueckii*	Overexpression of *K. aerogenes* urease gene in microencapsulated *E. coli* DH5 cells has been applied for use as an oral artificial kidney. An alternative approach utilizes metabolic induction of microencapsulated *L. delbrueckii* for increased enzymatic activity in lowering plasma urea.	*68, 98, 99*
Accumulation of oxalate	*O. formigenes* *B. lactis*	The key oxalate-degrading enzyme oxalyl-CoA decarboxylase is inherent to *O. formigenes*, which can subsequently be administered to remove accumulated oxalate, a major risk factor for renal stone formation in pathologic cases. An alternative approach utilizes heterologous expression of oxalyl-CoA decarboxylase enzyme in *B. lactis.*	*94, 100*
Elevated serum cholesterol	*L. plantarum* 80 (pCBH1) *L. reuteri*	Overexpression of bile salt hydrolase gene in microencapsulated *L. plantarum* 80 cells has been applied to increase bile salt deconjugation and lower serum cholesterol levels (see Table 1). Microencapsulated nonengineered *L. reuteri* has shown similar heightened bile salt hydrolase activity.	*101, 102*
Colon cancer	*L. reuteri*	Certain strains of *L. reuteri* have shown protective effects against bile salt cytotoxicity by precipitating deconjugated bile salts and physically binding them, thus decreasing their bioavailability in the colon.	*102–104*

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