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Bacterial Components as Naturally Inspired Nano-Carriers for Drug/Gene Delivery and Immunization: Set the Bugs to Work?

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Abstract

Drug delivery is a rapidly growing area of research motivated by the nanotechnology revolution, the ideal of personalized medicine, and the desire to reduce the side effects of toxic anti-cancer drugs. Amongst a bewildering array of different nanostructures and nanocarriers, those examples that are fundamentally bio-inspired and derived from natural sources are particularly preferred. Delivery of vaccines is also an active area of research in this field. Bacterial cells and their components that have been used for drug delivery, include the crystalline cell-surface layer known as “S-layer”, bacterial ghosts, bacterial outer membrane vesicles, and bacterial products or derivatives (*e.g.* spores, polymers, and magnetic nanoparticles). Considering the origin of these

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components from potentially pathogenic microorganisms, it is not surprising that they have been applied for vaccines and immunization. The present review critically summarizes their applications focusing on their advantages for delivery of drugs, genes, and vaccines.

Keywords

Nanomedicine; Bacterial Components; Drug Delivery System; Immunization; Slayer; Bacterial ghosts; Endospores; Bacterial polymers

1. Introduction

In recent decades, the design of drug and gene delivery systems, especially those endowed with the ability to respond to environmental stimuli (so-called “smart delivery vehicles”), has attracted much attention from researchers worldwide(Karimi, M. et al., 2016; Karimi, Mahdi et al., 2016b; Karimi et al., 2015a; Karimi et al., 2015b). This multidisciplinary field requires contributions from chemists, physicists, chemical engineers, biologists and physicians due to the complexity of these targeted and smart systems(Choi et al., 2011; M Karimi, 2015; Matricardi et al., 2006). One of the most intriguing approaches in this field is the use of bacterial-based nanocarriers. Bacteria are simple unicellular organisms without a nucleus or membrane enclosed organelles. It is intellectually satisfying to employ the agents that are so frequently suspected or known to cause human disease, by turning the tables (so to speak) and set them to work to cure some diseases. Several bacterial species, specifically some facultative anaerobes, such as *Salmonella spp* and *Escherichia coli* are known to actively target specific tissues and organs. Genetically engineered versions of anaerobic bacteria expressing particular proteins could show enhanced uptake by mammalian cells, and could be used to deliver therapeutic cargos (Gong et al., 2011). Evolution has designed the mammalian immune system to respond to bacteria and their components with exquisite sensitivity, often based on Toll-like receptors (TLRs). TLRs have a crucial role in recognizing bacterial flagellin and other microbial molecules, and these motifs are often used as a component of vaccines or adjuvant systems (Cui et al., 2014). However, the use of living engineered bacteria in therapeutic applications is fraught with dangers and difficulty. Not only could living bacterial-based nanovehicles cause infections themselves, but they could also transfer antibiotic resistance genes to other bacteria resident in the body. Therefore care should be taken to avoid unwanted side effects in applications of therapeutic bacteria(George and Abraham, 2007).

In targeted-delivery, the main aim is the delivery of drugs and different therapeutic cargos to target cells, either to cause cytotoxicity or to alter gene expression profiles(Berditsch et al., 2017; Karimi et al., 2017; Karimi, Mahdi et al., 2016b; Karimi et al., 2015c; Yoo et al., 2011). Recent advances in immunization have focused on the use of DNA vaccines and multivalent peptides(Negahdaripour et al., 2017), but these agents have several drawbacks such as the lack of an efficient delivery technology to get them inside cells. Recombinant protein and DNA vaccines also have drawbacks such as low immunogenicity. The effectiveness of viral vectors to carry out immunization has led to their being considered the first choice for inducing T lymphocyte responses, but it is necessary to design an optimized

delivery system and appropriate vaccine adjuvants (Moon et al., 2011). Nowadays new advances in nanotechnology have led to the use of a variety of nanoparticles (NPs) to deliver different cargos into cells for diagnostic, imaging and therapeutic applications(Jozala et al., 2016; Karimi, Mahdi et al., 2016a; Liang et al., 2016). Bacterial cells and their components (S-layer, bacterial ghosts, endospores, etc.) represent potentially useful carriers for the delivery of nucleic acids, antigens and some drugs (Gong et al., 2011). Bacterial components (Scheme 1) can be considered biomimetic structures either with intrinsic targeting ability or else the ability to be equipped with the desired target-specific ligands. The biomimetic characteristics of bacteria and their ability to be recognized by and to stimulate the host immune system, is a fundamental concept in immunization. This property has been highlighted in the case of entero-invasive species such as *Listeria monocytogenes* and *Yersinia pseudotuberculosis* that can vaccinate through mucosal surfaces (Mattheolabakis et al., 2015).

The bacterial S-layer (Surface Layer) functions as a cell surface envelope in nearly all archaea and many bacterial species (both Gram-positive and Gram-negative). It is made up from proteins or glycoproteins depending on the species. The beneficial features of the S-layer, such as self-assembly, structural regularity, mechanical and osmotic stability, predictable physicochemical properties, have led to its exploitation in many drug delivery systems. Carriers containing the Slayer have found applications for delivery of immunogens and vaccines, and as platforms for targeting biological therapeutic cargos. In addition, S-layers have shown to be useful for some promising approaches such as supramolecular engineering due to their tunable molecular structure and the ability to be recrystallized. The uniform patterning on their surface, their unique morphology, and the presence of functional groups, make them useful biopolymers which can play an important role in biotechnology applications such as designer matrices, porous ultrafiltration membranes, and substrates for the immobilization of active compounds(Sleytr et al., 1997).

The bacterial ghost (BG) is an empty non-denatured envelope originating from Gram-negative bacteria. BGs lack all of the cytoplasmic contents of bacteria, but still maintain and preserve their overall cellular morphology and contain all the structures of the original cell surface. BGs also have a high loading capacity, which makes them a promising approach to design new polyvalent vaccines. In addition the PhiX174 protein E-mediated lysis of Gram-negative bacteria leads to the loss of the cytoplasm as well as most enzymatic activities (Langemann et al., 2010). In recent years, BGs have attracted increasing interest as non-living carriers of nucleic acids, proteins/antigens, drugs, and soluble cargos. The principle advantages of using BGs as carriers is that no denaturing conditions are needed to avoid the possibility of infectivity, and therefore their ability to stimulate immune response remains as potent as that of living bacteria. BGs are excellent at stimulating the immune system and display inherent adjuvant properties. For example, BGs from *Pantoea cypripedii* have been used as novel pesticide delivery systems containing the lipophilic fungizide, tebuconazole. This compound is useful due to its persistence against being washed away with rainfall, and its high activity against agricultural plant pathogens. Generally organic ring structures can bind best to the BG membrane, which was shown by loading BGs with resveratrol, and showing that this construct could prevent nitric oxide release from RAW264.7 macrophages

caused by the BG itself. Loaded resveratrol was ten times more efficient than free reveratrol (Koller et al., 2013; Lubitz et al., 2009).

Outer membrane vesicles (OMV) are nano-sized spherical lipid bilayer membrane blebs produced by the vesicular secretory process from some Gram-negative bacteria, which contain pathogenic markers and virulence factors. OMVs contain bacterial membrane proteins (*e.g.* latent membrane protein, lipoproteins, exogenous protein epitopes) and also some genomic material (*i.e.* DNA and RNA). OMV-based vehicles such as VSSP can be used for therapeutic purposes and vaccine production (Acevedo et al., 2014b; Gujrati et al., 2014).

Bacterial endospores are another delivery system that has recently been applied for delivery of therapeutic proteins, anticancer drugs, cytotoxic genes, and as vectors with great potential for gene therapy.

The biosynthesis of naturally occurring polymers by bacteria has created new materials for medical applications. Various polymers can be isolated from bacteria, such as polysaccharides (*i.e.* alginate, cellulose, dextran and starch), polyamides (*i.e.* poly (γ-glutamic acid), polyoxoesters (*i.e.* polyhydroxyalkanoates (PHA)), polyesters and inorganic polyanhydrides and have all been successfully tested for drug delivery systems (DDSs) and controlled release of cargo. Generally speaking, these polymeric platforms can improve drug distribution and interaction with their target sites (Rehm, 2010; Vilar et al., 2012).

Magnetotactic bacteria (discovered in 1970s by Richard P. Blakemore at Woods Hole Oceanographic Institute (Frankel et al., 1979)) take up orientations along the direction of the Earth's magnetic field lines (Jacob and Suthindhiran, 2016). This ability is derived from unique intracellular organelles called magnetosomes, which contain nanometer-sized crystals of magnetic iron-containing minerals (magnetite) (Uebe and Schuler, 2016). These magnetosomes can be isolated and used as naturally-occurring magnetic nanoparticles (Alphandéry, 2014), and have been used as potent nanocarriers in drug, gene delivery and theranostics (Angelakeris, 2017).

The present article aims to review recent applications of bacterial cells and their components as therapeutic cargo delivery vehicles. The investigation of bacterial-based gene vectors and systems for delivery of antigens and adjuvants is also within the scope of this review.

2. Bacterial S-layer

The outer membrane of bacteria (known as the surface layer or S-layer) forms the cell surface envelope or outermost layer, and is considered to be one of the simplest and least complex organized biological structures(Sleytr et al., 2014). S-layer proteins account for large fractions of the total protein in bacterial cells, and are one of most abundant biopolymers in living organisms composed of single protein or glycoproteins (Sára and Sleytr, 2000). The S-layer protein structure is a highly porous meshwork, with 30–70% porosity, having a unit cell size between 3 to 30 nm. With its thicknesses approximately 10 nm this gives pore sizes of 2–8 nm (Pum et al., 2013). As revealed by electron microscopy, S-layers form regular repeats with oblique, square or hexagonal geometry. S-Layer proteins

have inherently tunable structures that can be genetically engineered to endow specific features. For instance, the S-Layer protein SbsB from *Geobacillus stearothermophilus* fused to streptavidin conjugated to the C terminus. The proteins were refolded to heterotetramers comprising of a chain of fused protein and three other chains of streptavidin providing the suceptibility to bind with D-biotin and biotinylated marker proteins, peroxidase and ferritin, in *E. coli* (Mader et al., 2004; Moll et al., 2002)

2.1. Cell Surface Layer and Self-assembly

The S-layer has properties and functions generally consistent with the requirements of its bacterial host. In addition to mediating virulence and pathogenicity determinants, it governs the overall cell shape, provides an attachment point for exo-enzymes, forms a more stable membrane for improved thermal resistance, acts as a precise molecular sieve to restrict entry of different sized molecules, and regulates interactions with the external environment. In addition, in some system, enzymes can bind to S-layers, which have been deposited on porous membranes, and these systems can be utilized for amperometric biosensors. The sensing layer can be constructed by sputtering a thin layer of a metal (such as gold) onto the porous surface(Salyers and Whitt, 2002). The modern ability to use genetic engineering to tailor the precise chemical structure, geometry, ability for self-assembly, means that the S-layer has found a wide array of applications in nano-biotechnology, including for sensors and diagnostics, vaccines, biomimetic processes, bio-markers, heavy metal adsorption and targeted drug delivery. One of the important features is the ability of S-layer proteins to be recrystallized in a single layer on a solid porous support. These porous constructs can be used as affinity matrices, diagnostic tools, and biosensors (Pum and Sleytr, 1999).

The amino acids of the proteins in the S-layer are mostly comprised of hydrophobic and acidic residues, while lysine is the most common basic residue; histidine and methionine are only found in low abundance, and cysteine is very rare. Based on recent research, most of the S-layer proteins are active in weak acidic solutions, except *Methanothermus fervidus.* Based on circular dichroism studies, nearly 20% of its amino-acids are arranged as α-helices and most of them are found in the *N*-terminal segments of the proteins. About twice that percentage of amino-acids are arranged as β-helices connected together by short loops. The S-layer proteins can be covalently bound to carbohydrate polymers consisting of glycan chains usually comprising (2–4 linked) sugar units via *O*- or *N*- glycolysidic linkages. The presence of these functional groups make Slayers capable of responding to environmental stimuli, and also provide susceptible attachment points for conjugation of different ligands, including lipoproteins, autotransporters, outer membrane proteins (OMP), and subunits of other bacteria(Sára and Sleytr, 2000). Since early studies described how the S-layer could be genetically engineered to produce fusion proteins, further studies based on attachment of foreign proteins such as antibodies or nanobodies to the Slayer have been reported, especially for the production of bioanalytical sensors and affinity particles or membranes(Schuster and Sleytr, 2014).

The isolation of S-layer proteins from bacteria can be performed by treatment with detergents combined with agents that disrupt hydrogen bonds *(e.g.* guanidine HCl) using a high salt concentration, followed by a sharp drop in pH to solubilize the S-layer. These steps

will allow the cell envelopes of most bacteria to release the S-layer constituents, which then spontaneously reassemble. For instance, some *Lactobacillus* species possess an S-layer that can be used as a model for putative adhesions, which are molecules involved in tissue adherence. *L. brevis* Slayer proteins were isolated by biochemical and genetic screening methods, and polymerase chain reaction (PCR) analysis was carried out. BLAST-P analysis confirmed the *C*-terminal fragment was conserved, and included genes homologous to known S-layer protein genes (Cai et al., 2010). In this manner, genes encoding S-layer proteins can be computationally designed to arrive at the optimum amino acid sequence to allow self-assembly into extended ordered periodic arrays(Schuster and Sleytr, 2013). The resulting arranged polypeptide mesh-works showed balanced rotational symmetry axes, since the chirality of these proteins does not allow the mirror-image glide planes or inversion centers so only an *n*-fold rotation axis is allowed(Pum et al., 2013).

There are potential applications of S-layer-based nanostructures for DDSs and vaccination vehicles, and also some industrial applications such as membranes for ultrafiltration, support layers for specific attachment of functional molecules. S-layers can be combined with internal layers into the structures that resemble the cell wall membranes of Gram-negative bacteria. These can be used to stabilize the lipid membrane of liposomes, and as templates for the formation of nanowires, and other topics in nano-geometry or nano-topology (Sleytr et al., 2007).

In one study, the biosynthesis of the antimicrobial peptide, gramicidin S (GS) was investigated in *Aneurinibacillus migulanus*, and they found a new type of high-energy phosphate storage granules was involved in the process. Electron microscopy showed that the cationic GS granules complexed with acidic S-Layer proteins. In addition, according to the SEM micrographs, by estimating the size of the granules and the fusion mechanism of nano-globules into larger granules during the maturation process, it was suggested they contained the newly produced GS. (Berditsch et al., 2017) (Figure 1).

2.2 Applications of S-layers as Nano-carriers

S-layers can be considered as the single most important bacterial component that is used as a building block part for the design of biomimetic NPs for therapeutic applications. In the following sections we discuss examples of these drug NCs that have S-layers on their surfaces.

2.3. S-layer Coated Lipid Membranes (Emulsomes and Liposomes)

Solid lipid NPs are a new category of NCs which have found many applications in drug delivery, especially for poorly water-soluble drugs which are encapsulated by the lipophilic region of the micelles(Buse and El-Aneed, 2010; Mäder, 2006). Isolated S-layer proteins can be recrystallized on the phospholipid interface that may offer a more biomimetic approach (Wetzer et al., 1997). Moreover the S-layer coated lipid membrane is more stable due to nano-patterned fluidity (Mader et al., 2000a). S-layer binding to lipid-based NPs can be achieved by physical or chemical bonding approaches such as electrostatic interactions, lectin-carbohydrate type non-covalent binding with bacterial sugars, biotinylation and avidin binding, or covalent conjugation via amide linkages (Ilk et al., 2008).

One of the new classes of lipid-based NPs is known as “emulsomes”, composed of a solid or crystalline lipid core covered with a shell of additional phospholipid bilayers. These NPs have found wide applications in various drug delivery fields (*i.e.* oral, rectal, ocular, topical and vaginal routes) and can be used with both hydrophilic, or hydrophobic drugs. For instance an anti-Leishmanial agent, amphotericin B, formulated in trilaurin-based lipid particles was able to remove unwanted macrophages in autoimmune diseasess(Suri et al., 2007). The lipid core of emulsomes can be loaded with hydrophobic agents, and the aqueous compartment of the phospholipid bilayer can be loaded with hydrophilic agents(Suri et al., 2007). On the other hand, the incorporation of an S-layer on the outside of the emulsomes could provide targeting features, and additional attachment points for conjugation of other bioactive motifs such as enzymes, antibodies and antigens. This modification could protect cells from membrane damage and oxidative stress that can be undesirable side-effects of conventional emulsomes used in drug delivery (H Ucisik et al., 2015).

Nano-patterned emulsomes were prepared comprised of a solid tripalmitin core, coated with three shells of phospholipid bearing an outer envelope of S-layer protein derived from *G. stearothermophilus* PV72/p2 in order to stabilize the core (Figure 2). The S-layer proteins provided a more compatible interface between the emulsomes and the external aqueous medium. In vitro studies with a human liver carcinoma cell line (HepG2) confirmed these structures could act as biocompatible nano-structures in drug delivery(Ucisik et al., 2013).

The possibility of genetic modification of S-layer proteins, for instance engineering of terminal functional groups to produce modified S-layer proteins, has opened new horizons for designing new structural features in fusion proteins or chimeric constructs. The genetic information coding for S-layer proteins shows high tolerance for the insertion of extra DNA coding for foreign proteins or domains. Homologous expression and secretion, leads to production of these modified S-layers, and the exact geometry of the layers can be predicted and investigated. Highly flexible S-layer arrays can be prepared by tuning the functional groups (Ilk et al., 2011a). This approach, was used to increase the solubility of the highly lipophilic antioxidant, curcumin. The resulting DDS based on S-layer-coated emulsomes was called “CurcuEmulsomes” (Ucisik et al., 2015). The S-layer from *Lysinibacillus sphaericus* CCM 2177was fused with rSbpA-GG having two protein G domains. Protein G is a cell-wall protein derived from group G Streptococci that has a high affinity for immunoglubin G (IgG), which is used as a target in cancer and inflammation. The S-layer rSbpA-GG fusion protein was conjugated to the phospholipid at the *N*-terminus, to the GG domains at the *C*-terminus. This orientation prevented any hydrophobic interactions between protein G and the lipid surface.(Ucisik et al., 2015).

Another approach is the modification of liposomes with S-layers, due to their ability to encapsulate both lipophilic and hydrophilic compounds. S-layer modification could enhance the blood circulation time, and increase the stability of liposomes by decreasing *in vivo* opsonization with serum components. Liposomes are closed continuous vesicular structures made of lipid bilayers containing cholesterol and phospholipids such as dipalmitoylphosphatidylcholine (DPPC), phosphatidylethanolamine hexadecylamine (HDA) and stearylamine. Liposomes can encapsulate hydrophobic drugs in the bilayer membrane, and hydrophilic drugs in the aqueous interior (Zhang et al., 2017). Several methods have

been used to attach proteins to liposomes, such as: electrostatic attraction, binding with streptavidin-biotin, or lectin-polysaccharide interactions. The optimum method depends on the terminal functional groups available and on the size of the liposomes (Velev, 1997).

Liposomes coated with S-layer were functionalized with biotin using p-diazobenzoyl biocytin which reacts with the histidine imidazole ring or the phenolic residue of tyrosine, and then a layer of streptavidin was added that acted as a bridge to allow further conjugation with biotinylated ferritin or with biotinylated human IgG antibodies. In the case of biotinylated ferritin the formation of a monomolecular ferritin layer on was visualized and confirmed by electron microscopy (Mader et al., 2000b). Another method that has been used is electrostatic interaction between the carboxylic acid groups on the S-layers of various bacteria such as *Bacillus coagulans E38–66*(Magnotti and Conticello, 2016), *L. kefir* (Hollmann et al., 2017) and *B. stearothermophilus* PV72/p2(Mader et al., 1999), and cationic liposomes. The covalent conjugation of targeting agents such as ferritin or therapeutic cargos could be accomplished through bioactive matrixes of porous S-layer available on liposome (Küpcü et al., 1995; Wang et al., 2017). The S-layer of SbpA derived from *B. sphaericus CCM 2177* was genetically modified with enhanced green fluorescent proteins (EGFP) using plasmid pET28a, and cloned in *E. coli.* The *B. sphaericus CCM 2177* S-layer has a significant cross-reaction with polyclonal rabbit antiserum, that is critical for immunoblot analysis(Ilk et al., 2004). The S-layer fusion protein was attached to the liposomes by a streptavidin-biotin interaction. As monitored by EGFP fluorescence, the positive charge on the SbpA-S-layer coated liposomes led to high uptake in HeLa human cancer cells (Ilk et al., 2004). Using a similar chimeric fusion protein, the fluorescence of S-layer of $rSbpA_{31–1068}$/EGFP and a mixture of EGFP with rSbpA were investigated as functions of temperature, concentration of denaturing agent (guanidine hydrochloride), and pH. The results showed that the fluorescence was highly sensitive to variations of the aforementioned parameters suggesting this system could be used to monitor Slayer interactions with other biological components (Toca-Herrera et al., 2006).

The attachment of S-layers to liposomes was investigated using S-layers from *L. kefir* and *L. brevis* and positively charged liposomes prepared from dipalmitoylphosphatidylcholine or soybean lecithin. By varying the Zeta potentials at different lipid-protein ratios, proteins were become able to attach to the surface. The positive Zeta potentials were retained after coating the liposome surface with the S-layer proteins, suggesting they could function as a vaccine carrier for oral administration (Hollmann et al., 2007).

Nanostructured polymer-based capsules are another efficient carrier for DDS applications. It was shown that polyelectrolytes could form capsules via electrostatic interaction between sodium polystyrene sulfonate and poly(allylamine hydrochloride), and could then be modified by attaching the S-layer from *B. thuringiensis* which had been further modified by adding an IgG antibody. The functionalization procedure was carried out on these hollow polyelectrolyte capsules, by the alternating deposition of polyelectrolytes with opposite charge onto the surface of the template(Habibi et al., 2011). The high stability and good biocompatibility of this system was shown, and the polymer degradation rate was decreased (Habibi et al., 2016).

2.4. Application of S-layers for Vaccination and Antigen Delivery

S-layer proteins have been used as carriers for delivering antigens and different types of vaccines in three main disease areas: cancer, allergies and antibacterial(Sleytr et al., 1997). Over millions of years of evolution, the mammalian immune system has developed highly sophisticated and pleiotropic ways of detecting and reacting to external threats from different kinds of infectious microorganisms. This is largely accomplished by the recognition process of “pathogen-associated molecular patterns (PAMPs)”. This recognition is orchestrated by multiple type of germline-encoded pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs), retinoic-acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain (Nod)-like receptors (NLRs) (Cui et al., 2014). Immune cells patrol their local environment continuously sampling their surroundings using cell surface PRRs, but internal cytosolic PRRs also have a critical role in providing redundant identification of microbial signals or PAMPs. These PRRs also recognize molecules derived from the host tissue itself, known as “danger-associated molecular patterns, DAMPs” or alarmins (Matta et al., 2017). Although TLRs are the best known examples of these PRRs, other examples include C-type lectins, NLRs and RLRs (Raymond et al., 2017). The cells which are most prominent in expressing these PRRs, and react most vigorously to their engagement with microbial ligands, are the various specialized cells belonging to the adaptive and innate arms of the immune system. PAMPs consist of a wide range of molecules derived from different classes of pathogen such as Gram-positive bacteria, Gram-negative bacteria, fungal cells, a wide range of viruses, and even other microbes such as parasites. The idea is that these components are released or shed from invading microbial cells and can act as early warning systems before invasion is fully established. The most important class of immune cell in this regard is that of antigen-presenting cells (APC) such as dendritic cells and macrophages both of which have high levels of PRR expression. Since APCs are precisely the cell type that is desired to be targeted in the process of vaccination, it makes sense to incorporate PAMPs as components of vaccines and adjuvants.

Bacterial S-layers act as PAMPs, and can activate TLRs on dendritic cells and other APC, meaning they have intrinsic adjuvant properties(Malcolm et al., 1993; Yoneda et al., 2003). In addition, the S-layer is considered as a good candidate for an antigen delivery platform, due to its ability to display amino acid epitopes incorporated in its surface proteins. Several examples of bacterial S-layer proteins that have been considered for vaccine development are; SbpA of *B. sphaericus CCM 2177*(Ilk et al., 2002), SbpA of *L. sphaericus*(Ilk et al., 2011b), SLH-EA1 (surface layer homology-extracellular antigen 1) of *B. anthracis*(Mesnage et al., 1999), and RsaA of *Caulobacter crescentus*(Umelo-Njaka et al., 2001).

Other than proteins or recombinant proteins derived from the S-layer, other examples of closely related S-layer components have been used in vaccine preparation. For example, the oligosaccharides, which are derived from the capsule of *Streptococcus pneumonia* type 8 have been applied as antigens (while still conjugated to the S-layer) and they showed protective responses against pneumococcal disease. In addition, *S. pneumonia* type 8 antigens were selected to overcome some defects of capsular polysaccharides (such as poor response in immune-compromised patients) due to their ability to bind strongly to non-

cross-linked S-layer proteins making them more effective in eliciting a protective antibody response(Jahn-Schmid et al., 1996b).

Allergies can be treated by the process of “desensitization” by switching the immune response away from the unhealthy T helper cell type 2 (Th2) phenotype found in atopic individuals, to the more normal Th1 phenotype found in healthy individuals (Akdis and Akdis, 2009, 2015). Recombinant allergens and adjuvants can be used to rebalance the immune system for treatment of type I allergies. In a study by Jahn-Schmid *et al*, the major recombinant birch pollen allergen, Bet-v1 was immobilized on the S-layer of *B. sphaericus* CCM 2 177 and *Thermoanaerobacter hydrosulfuricus* L 111-69 and L 1I O-69. It should be mentioned that the overall T-cell response correlated to the total amount of Bet-v1 rather than the Bet-v1 bound to the nanocarrier. The balance of cytokines between Th2 (unhealthy) and Th0/Th1 (healthy) was studied by in vitro stimulation of human allergen-specific Th2 cells with the S-layer-conjugated Bet-v1(Jahn-Schmid et al., 1996a).

In a similar study, the same gene (Bet-v1) was inserted into the gene coding for the S-layer of *G. stearothermophilus*. This insertion led to production of recombinant protein, rSbsC-Bet-v1. Although rSbsC-Bet-v1 stimulated a lower release of histamine compared to Bet-v1 alone, the fact that it contained T cell epitopes meant that cells from birch pollen-allergic individuals produced interferon gamma (IFNγ) on stimulation with rSbsC-Bet-v1 (along with interleukin-10), but no Th2-like responses. Intracellular cytokine staining showed that rSbsC-Bet-v1 promoted Th cells production by IFNγ. Furthermore, rSbsC-Bet-v-1 induced IFNγ synthesis in Bet-v1-specific Th2 cells, and the more important is an increase in IL-10 production in these cells (Bohle et al., 2004; Gerstmayr et al., 2007).

3. Bacterial Ghosts

Bacterial ghosts are the relatively intact envelopes of bacterial cells, from which the majority of the contents have been removed. This is accomplished by creation of a tunnel in the cell membrane mediated by protein E, which is encoded by a gene of bacteriophage PhiX174 (in the case of *E. coli)*. The lysis process occurs when protein E induces the formation of a transmembrane tunnel through the outer membrane and the cell envelope. The tunnel is located preferentially at the polar regions of the cell or the septum. Genetic investigations have shown that a single lysis gene of the phage is sufficient to trigger the lysis process(Witte et al., 1992). Additionally, the geometry of the individual tunnel structures differs greatly, disturbing the rigidity of the bacterial cell wall structure. The shape of the individual lysis tunnels are governed by the pores produced in the peptidoglycan meshwork, and the force exerted by the outflow of the cytoplasmic contents through the tunnels. The osmotic pressure arising from the difference in salt concentration between the cytoplasm and the external medium provides the driving force, which is needed for the lysis process. The negative pressure, which is produced during lysis, causes the outward partial expansion of the cytoplasmic membrane, which is only weakly placed on the cell envelope. Evidence has shown that the release of cytoplasmic material from the cells begins after tunnel structure formation of the transmembrane, and could be stopped by addition of about 20% sucrose to the external medium. After cell lysis, the inner cell space of the rigid bacterial ghost can fill up by means of diffusion with the external medium (Witte et al., 1992). BGs are mostly

produced from Gram-negative bacterial species, and possess all the structural characteristic of the original bacterial cells, particularly their immunogenic and bio-adhesive properties. Moreover, genetically engineered variants of these species such as *Edwardsiella tarda* ghosts (ETG) that were generated by protein E mediated lysis, possessed all the structural features of the BGs from wild-type bacteria. Plasmid vectors containing the PhiX174 E gene can be used to allow BGs to be constructed from other bacterial species, and these can be introduced into the cells by electroporation (Lee et al., 2008). Consequently, BGs can serve as novel carriers for antigens in vaccines, and also as biocompatible drug delivery vehicles because the production of BGs is possible from various types of Gram-negative bacteria. In addition, their high loading capacity makes them a good choice in drug delivery systems. (Kudela et al., 2010).

BG production technology can takes place in large-scale fermenters involving several steps, including (a) bacterial growth phase, (b) E-lysis, and (c) downstream processing. The downstream processing includes harvesting via filtration, followed by an inactivation step using β-propiolactone (BPL) to sterilize the culture. Finally, the BPL-treated broth should go through a washing process to replace the residual cytoplasmic contents by water. The final preparation of BGs is divided into aliquots and lyophilized, and the ultimate, stable BG product is produced by freeze-drying. BGs produced via the fore-mentioned procedure could play a role as micro-bioreactors that could lead to production enantiomerically pure fine chemical intermediates, because the internal lumen can act as a reactor (Langemann et al., 2010).

BGs retain the immunogenic properties of the bacteria such as lipopolysaccharide (LPS), OMP, and inner membrane proteins (Lubitz et al., 2009). BGs can trigger the production of pro-inflammatory cytokines, the expression of antimicrobial peptides, and finally leads to the stimulation of both cellular and humoral immunity(Lubitz et al., 2009). BGs have inherently bio-adhesive properties, and are excellent cargo delivery vehicles because they can entrap biomolecular payloads within themselves. These payloads can gain entry through their tunnels but cannot easily escape. Moreover BGs derived from *Salmonella enteritidis* and *Pasteurella haemolytica* have been used as candidates in immunization vehicles(Ganeshpurkar et al., 2014). Other advantages of BGs include; easy production through a fermentation processes, long shelf life of the lyophilized BGs at ambient temperatures, and the ability to serve as bioreactors in enzymatic reactions(Langemann et al., 2010).

3.1. Application of BGs in Drug Delivery

One of the major drawbacks of cytotoxic chemotherapy as a cancer treatment, is the tendency to damage non-cancerous healthy cells in the target tissue. An important advantage of nanomedicine-based DDSs, such as BGs, is their drug-targeting capability to various types of cancer cells. The drug can be encapsulated inside the hollow BGs where it can non-covalently bind to the inner surface through electrostatic charge interactions. Efficient endocytosis of BGs by colon carcinoma, leukemia and melanoma cells has been reported(Kudela et al., 2010). Some recent reports of the application of BGs in DDS are summarized below.

In one study, BGs derived from recombinant *E. coli* were investigated for immobilization of active substances, and increasing the binding capacity for cargo. Encoding plasmid pAV1, by streptavidin fusion protein with anchor sequence of N-terminal membrane anchor, has led to the attachment of streptavidin to the cytoplasmic membrane inner side. This streptavidin-BG could accommodate even large biotinylated compounds such as alkaline phosphatase. In other examples, biotinylated dextran and fluorescent biotinylated DNA were loaded as well to demonstrate the fixation of such compounds in SA-ghosts for particular drug delivery systems. These is another critical perspective to use dextran, as a highly branched and inert polymer, is to enhance the efficiency of SA-ghosts by their functional sites which can make a strong interaction with a therapeutic agents at their active sites(Huter et al., 1999). BG from the colonic commensal bacteria *Mannheimia haemolytica* also could be loaded with the cytostatic drug, doxorubicin (DOX) and utilized in targeted delivery to human colorectal adenocarcinoma cells Caco-2. It was shown that the contents of the BG were released to the cytoplasm of the cancer cells and the DOX mainly accumulated in the nucleus. Anti-proliferation assessment showed that the effects were 2–3 orders of magnitude higher than the results obtained with free DOX using BrdU incorporation assays. Similar results were found with different leukemia cell lines (Paukner et al., 2004). Some hydrophobic drugs have an intrinsic binding affinity to the outer membranes of BGs, while other different drugs are contained within the cytoplasmic space. In a recent investigation on the binding of phenolic compounds such as resveratrol, to BG, it was shown that molecules with an organic ring structure could bind to the inner membrane segments of BG. The system was capable of modulating nitric oxide production and acting as a targeted antioxidant delivery vehicle and it could enhance tumor infiltration by some specific T cells in tumor micro-environment by using BGs(Koller et al., 2013). Furthermore, BGs from *E. coli* Nissle 1917 (EcN) were produced, and were taken up by human conjunctival epithelial (HCjE) cells in vitro, and by guinea pig conjunctival epithelial cells in vivo. The results of this study demonstrated that EcN BG could be an effective non-toxic carrier to target superficial ocular diseases(Stein et al., 2013).

3.2. Application of BGs in Vaccination

Because of the weakly immunogenic properties of many foreign antigens used in vaccines, the it is necessary to combine them with specific carriers and adjuvants that can enhance the immune response to obtain sufficient protection. BGs can serve as a novel delivery system as well as acting as an intrinsic adjuvant for foreign antigens (without the use of any exogenous adjuvants). BGs have no limitations of size for the encapsulated immunogens, and there exist various strategies for loading the antigens into the BGs. Genetic engineering can create fusion proteins between the antigen and OMP-A or with pili structures, or else the antigens can be directly attached to the periplasmic space via maltose binding protein (MBP), or they can be biotinylated and attached to a streptavidin fusion site (Figure 3). SbsA and SbsB (S-layer protein matrices) can form sheet-like structures within the BG(Chen et al., 2014; Park et al., 2016). Another method to incorporate foreign proteins into BGs is through the direct fusion of the target antigen with the inner-layer membrane proteins via E′, L′ or E′/L′-anchoring (Figure 3) (Kudela et al., 2011; Muhammad et al., 2012). The cytoplasmic space of the BGs has also been loaded with emulsions and water soluble antigens by a resuspension technique(Mayr et al., 2005). In one chimeric study, the OMP-A of *E. coli* BG

was fused with the antigen of the hepatitis B core virus (HBc)-149 and induced efficient protective immunity in mice compared to only using HBc-0149 alone. Similar fusion proteins (MalE) have been used as part of an S-layer self-assembled structure with flexible acceptor sites to bind foreign antigens (Jechlinger et al., 2005b; Mayr et al., 2005). In another study, recombinant *Vibrio cholerae* BGs were used as an anti-chlamydia vaccine. The efficacy of this vaccine was evaluated in a murine model, and could stimulate systemic Th1 responses(Eko et al., 2003). *Helicobacter pylori* BGs, were produced and loaded with additional recombinant *H. pylori* OMP18 and cholera toxin, and tested for immunizing *H. pylori*-infected mice showing significant reduction of gastric *H. pylori* colonization(Talebkhan et al., 2010).

Vibrio alginolyticus has been considered to be a pathogen both for humans and marine life forms. An effective vaccine for control of the disease caused by *V. alginolyticus* is urgently needed. The bacterial strain 16-3 isolated from a lesion on the fish *Larimichtys crocea* was proved to be *V. alginolyticus* based on biochemical and morphological analysis. Generation of *V. alginolyticus* bacterial ghosts (VaBGs) by induction of lysis gene E expression was investigated as a vaccine strategy in mice. The results showed that VaBGs isolated from formalin killed *V. alginolyticus* cells were safe and effective. The structure of BGs and the apparent loss of cytoplasmic materials and the size of the transmembrane lysis pores (40 to 131 nm) were shown by TEM (Figure 4) (Cao et al., 2018)

The BG delivery system is a highly safe and efficient gene transfer technology compared to delivery using inactivated whole bacterial systems. BG-based DNA vaccines based on bacterial derived components led to the induction of effective immune responses (both cellular and humoral) due to the additional adjuvant properties of BGs. DNA (ranging from 10–10000 individual copies of plasmid DNA) can be loaded into BGs(Jalava et al., 2003). The immobilization of mini-circular DNA species in the BG was shown to act as a novel platform for DNA delivery with adjuvant properties(Jechlinger et al., 2005a). In another study, self-immobilizing DNA plasmids were successfully anchored to the inner membrane of BGs to create non-living DNA carriers for vaccine production(Mayrhofer et al., 2005). In vitro studies used a plasmid carrying the gene encoding GFP and *M. haemolytica* ghosts indicated that these BG particles were internalized by antigen presenting cells with high efficiency. In vivo assessment in BALB/c mice proved that DNA could be delivered using *M. haemolytica* ghosts by two different routes of inoculation (intradermal or intramuscular). They used a eukaryotic expression plasmid encoding beta-galactosidase, that stimulated both arms of the immune system in comparison with naked DNA. BG targeted the DNA vaccine to antigen presenting cells while at the same time providing a stronger danger signal, thereby improving their effectiveness (Ebensen et al., 2004).

The mucosal surfaces within the eyes, have features such as easy accessibility (even by untrained personnel using eye-drops), and could therefore be considered as an administration route for vaccines. The efficiency of BGs derived from different bacterial sources was evaluated to target HCjE cells and showed a high rate of internalization with low cytotoxicity for all types of BGs. Therefore, it seems that this delivery system could find various applications in vaccines for eye diseases and for drug delivery to the ocular surface with high accuracy (Kudela et al., 2011).

4. Outer Membrane Vesicles (OMVs)

Bacterial communicate between themselves, or with other living organisms in their extracellular environment by employing outer membrane vesicles (OMV). These OMVs originate from Gram-negative bacteria, and are released by “pinching-off” segments of the outer membrane. Despite the fact that Gram-positive bacteria have no outer membrane, they can also produce OMVs from the peptidoglycan cell wall by mechanisms that are still under investigation (Brown et al., 2015). OMVs used for DDS purposes are usually nano-sized (10–300 nm diameter) spherical bilayer membrane vesicles, and contain bacterial virulence factors (Baker et al., 2014). The phenomenon of shedding of these OMVs was discovered approximately 40 years ago during the study of bacterial structures by electron microscopy (Takeo et al., 1973). OMVs contains different components including latent membrane protein (LMP), lipoproteins, phospholipids, exogenous protein epitopes, DNA, RNA, flagellin, and peptidoglycan (Kaparakis-Liaskos and Ferrero, 2015). OMVs and other bacterial vesicles such as very small size proteoliposomes (VSSP) can be used as promising carriers for cancer therapy and other medical applications (Choi, K.S. et al., 2014; Fantappiè et al., 2014). OMVs are produced during normal bacterial growth, while even more are formed under stressful conditions such as host-bacterial interactions and nutrient depletion. This process is not accompanied by cell lysis because OMV are composed of only a small fraction of the total inner membrane proteins (Klimentová and Stulík, 2015). Therapeutic applications of Gram-negative OMVs should be accompanied by a detoxification process in order to remove lipopolysaccharide (LPS) (Acevedo et al., 2014a). LPS is a very powerful activator of Toll-like receptor 4 (TLR4) that mediates the host innate immune response (Acevedo et al., 2014a). The isolation of OMVs is performed a multi-step process including: (a) bacterial cultivation; (b) isolation of OMV from the culture filtrate; (c) pre-concentration; and (d) purification (Klimentová and Stulík, 2015).

4.1. Therapeutic Applications of OMVs

One of the important applications of OMV is delivery of therapeutic cargos such as drugs, proteins and nucleic acids. Gujrati *et al.* investigated the application of bioengineered OMVs as a nano-carrier in targeted drug delivery. They constructed a recombinant protein OMV expressed by *E. coli* containing cytolysin A (ClyA) that could be loaded with siRNA (Figure 5). ClyA is naturally located on the outside of OMVs, and was fused to a human epidermal growth factor receptor 2 (HER2) specific affibody designed to target cancer cells. The resulting OMVs were loaded by a siRNA construct targeting the kinesin spindle protein (KSP) using an electroporation method. The system showed good in vitro cytotoxicity, and caused retardation of tumor growth in vivo (Gujrati et al., 2014).

Other advantages of OMVs as carrier vehicles include: the cargos are protected from extracellular degradation by DNase, RNase, protease and extreme pH; OMV can adhere to host cells and fuse with cell membranes (Gholami et al., 2016).

4.2. Application of OMV in Immunization

OMVs started to be developed for use in vaccines about twenty years ago, due to the presence of several immunogenic components within their structure (Doi, Yoshiharu, 2002).

The two major areas of OMV-based vaccine research are: 1) engineered OMV derived from some pathogenic bacteria designed to elicit protective immunity against themselves; 2) structurally engineered OMV coated by heterologous antigens (Acevedo et al., 2014b). OMV vaccines have been investigated against different pathogenic bacteria such as *Bordetella pertussis*, *Neisseria meningitides*, *Mycobacterium tuberculosis*, *Chlamydia spp*, enterohemorrhagic *E. coli* (*EHEC* O157:H7) and *Burkholderia pseudomallei,* all of which resulted in induction of immune responses. Some derived OMV derived from non-pathogenic bacteria have also been obtained, for instance *M. smegmatis* has noteworthy antigenic homology with *M. tuberculosis* (MTB), and it was shown that proteoliposomes derived from *M. smegmatis* could induce immune response against MTB (Doi, Yoshiharu, 2002). OMV based vaccines have great potential for stimulation of immune responses and can activate both arms of the host immune system. OMVs from *Brucella melitensis* induced expression of IFNγ and IL-12 by dendritic cells, and could stimulate protective immunity against this bacterial species (Avila-Calderón et al., 2011).

In an important clinical trial in healthy adults, a novel OMV-based vaccine against *Neisseria meningitides* group B (MenB) was tested. An OMV based vaccine was designed that expressed a defined amount of ferric enterobactin receptor (FetA). The results demonstrated that this vaccine was immunogenic, safe, and effectively induced specific immune response against FetA and OMPs. This vaccine could have extensive applications in the prevention and treatment of bacterial meningitis which is a major health problem (Marsay et al., 2015). In a similar study, it was shown that the heterologous antigens of engineered *E. coli* OMVs induced an antibody response against this bacteria (Fantappiè et al., 2014). In addition, it was observed that the OMVs of *Pseudomonas aeruginosa* could trigger the production of cytokines from macrophages and epithelial cells(Ellis et al., 2010). Other researchers used *Salmonella*-derived OMVs, which were decorated with pneumococcal surface protein A and pneumolysin as vaccines to prevent colonization with *Streptococcus pneumoniae*. Their results demonstrated that intranasal administration of this vaccine resulted in local production of IL-17A and protection against *S. pneumoniae* (Kuipers et al., 2015). In another investigation, glycosylated OMVs were constructed with the ability to be decorated with pathogen-mimetic glycotopes, and BALB/c mice were immunized and showed protection against infection(Chen et al., 2016).

4.3. Very Small Size Proteoliposomes (VSSPs)

VSSPs were first derived from bacterial OMV (most often *N. meningitides)* by treatment with an anionic detergent, leading to incorporation of added monosialodihexosylganglioside (GM3) into the vesicles (Estevez et al., 1999; Mesa et al., 2006). Gangliosides are sialylated glycosphingolipids present on the plasma membrane of mammalian cells, and are considered as a target for cancer immunotherapy. Gangliosides generally function as autoantigens that are immunologically tolerated to a greater or lesser degree, and are not recognized by most cytotoxic T-cells. In order to induce immune recognition against gangliosides, the adjuvant ability of VSSP may show a benefit. The N-glycosylated form of GM3 ganglioside (NGcGM3) is a target for immunotherapy of specific human tumors such as breast cancer and metastatic melanoma that is only found on tumor cells. The NGcGM3 based vaccine had been tested in several clinical trials with VSSPs as an essential component. Results of

vaccination with N-glycolyl GM3/VSSP in phase I/II clinical trials clearly showed that it was safe, there was increased anti-NGcGM3 antibody titers and some evidence of antitumor response (Mulens et al., 2010; Osorio et al., 2012; Pérez et al., 2013).

VSSP derived from OMV contain Toll-like receptor 2 ligands and function as an adjuvant capable of inducing dendritic cell maturation and cross-presentation of antigen to $CD8^+$ T cells in mice. It was shown that this vaccine could stimulate cytotoxic T lymphocyte responses (Fernández et al., 2011). By using these VSSP-based approaches, four therapeutic cancer vaccines have been developed and tested in clinical trials. Two of these formulations, based on recombinant proteins from epidermal growth factor receptor(Ramírez et al., 2006) and vascular endothelial growth factor (Bequet-Romero et al., 2012) are in Phase I clinical trials. The two others are vaccines against human papilloma virus peptide (Solares et al., 2011) and gonadotropin releasing hormone (VSSP/Montanide) (Aguilar et al., 2012), that are in Phase II trials. On the other hand, it has been shown that VSSP can be used in immunotherapy to modulate myeloid-derived suppressor cells which mediate an important suppression of antitumor immune responses(Fernández et al., 2014).

5. Endospores

Endospores are only produced in some bacteria having sporulation-specific genes, and function as a preservation mechanism against harsh environmental conditions, enabling the bacteria to survive without nutrients for long periods. This survival mechanism is only available in certain bacterial species such as: *Bacillus, Clostridium, Sporosarcina spp,* and *Sporolactobacillus* (Leggett et al., 2012). Bacterial spores are able to endure severe drought, withstand ultraviolet and ionizing radiation, and survive at temperatures ≥ 100 °C; they are not harmed by many toxic chemicals used as disinfectants. They are able to germinate when conditions become suitable again (Jones, 2009). The spore coat is a thick cross-linked protein layer which contains about 70 different proteins and plays a role to protect the bacterial genetic material from degradation by harsh environmental conditions(Wu et al., 2015). Some studies have explored spores, and vehicles constructed from the spore protein coat, for many applications such as presentation of heterologous antigens, protective immunization (vaccination), drug, enzyme and for delivery of protein based therapeutics, as well as a source of novel self-assembling proteins (Ricca and Cutting, 2003).

The direct use of intact bacterial spores may appear dangerous, as conditions in the human body are favorable for spore germination (awakening the bacteria from their dormant state) and the proliferating bacteria may be dangerous before reaching the desired tissue. However, studies have been conducted using endospores from *Clostridium* species, because this species shows potential for direct therapy of some tumors by forming colonies around cancerous cells. About 60 years ago spores of *C. histolyticum* were directly injected into mice with transplanted sarcomas by *Parker et al.* These workers showed that spores of anaerobic bacteria such as *Clostridium* species could preferentially germinate in the hypoxic regions of tumors where pO2 levels are remarkably low. When the spores become vegetative bacterial cells, tumor lysis can occur(Minton, 2003). The intratumoral injection of *C. novyi* NT spores for treating solid tumors was evaluated in studies looking at spontaneous soft tissue sarcomas in dogs, and in one human patient with advanced leiomyosarcoma.

Objective responses (tumor shrinkage) were seen in 37.5% of the dogs, and the human patient showed tumor regression in the tumor within and surrounding the bone(Roberts et al., 2014).

Gene therapy using *Clostridium* spores can also take advantage of the preferential germination in hypoxic tumors(Lambin et al., 1998). For example, in a *Clostridium* directed enzyme prodrug therapy (CDEPT) protocol, *Clostridium spp* was genetically engineered to produce a specific enzyme (cytosine deaminase). This enzyme can produce a potent anti-cancer drug from a prodrug precursor (i.e. 5-fluorouracil from 5-fluorocytosine) which is effective in treating tumors. Figure 6 shows a schematic illustration of how the recombinant vector of the prodrug converting enzyme was generated by insertion of the relevant gene, and the spores from the recombinant cells were isolated for tumor therapy in mice. The resulting spores are dispersed throughout the body after injection, but those encountering the hypoxic tissue experience optimum conditions for germination and can colonize the tumor. Two weeks later a prodrug (i.e. 5-fluorocytosine) was administered and converted to 5-fluorouracil within the tumor (Fox et al., 1996; Malmgren and Flanigan, 1955). A similar approach was taken by Heap *et al* (Heap et al., 2014) who engineered *C. sporogenes* to express a novel nitro reductase enzyme isolated from *N. meningitidis*, which was able to activate the anti-cancer prodrug CB1954 at clinically-achievable serum concentrations. The nitro group of CB1954 can be reduced to give DNA-alkylating reactive N-hydroxylamine compounds. CDEPT using this system produced tumor suppression in a mouse xenograft model of human colon carcinoma.

5.1. Spore Vaccines

Recombinant bacterial spores can also be utilized as a thermostable vaccine platform. Due to difficulties experienced in the storage of conventional vaccines, this nanocarrier could be very useful for immunization procedures in the developing world (Jiang et al., 2009).

Bacterial spores were reported as a vaccine platform for the first time by Duc *et al*. (Duc et al., 2003). In another investigation tetanus toxin fragment C (TTFC) was used to test several strategies for antigen presentation. There are three ways that the TTFC antigens could be delivered by the spore: (a) coated on the outside of the spore surface; (b) expressed in the germinating spore, and (c) a combination of both of these. It was shown that the expression of the antigen in the germinating spore provided a more rapid development of immune response than coating the antigen on the spore surface. This study demonstrated that spores could be useful for the preparation of oral vaccines in human. The main problem with using this platform was the number of doses that were required for full protection (Uyen et al., 2007). In another investigation, the spore coat protein C (CotC), a major protein of *Bacillus subtilis* spores, was used as a dock for allowing the attachment of two different antigens, TTFC or the B subunit of the heat-labile toxin of *E. coli* onto the spore surface. This recombinant vaccine was used for oral administration in mice, and led to systemic and mucosal immune responses. The results showed that various antigens could be presented by this platform for mucosal immunization (Mauriello et al., 2004).

In one study the tuberculosis (TB) antigen, MPT64, was expressed in spores of *B. subtilis* as a vaccination against tuberculosis. A chimeric fusion between the spore coat gene, cotB, and

MPT64 antigen led to expression of a stable CotB-MPT64 hybrid protein in *B.* subtilis *spores*. C57BL/6 mice were immunized with 2X10(9) HU58 spores administered intranasally on days 15, 36 and 57. The Bacillus Calmette-Guerin (BCG) control group was immunized subcutaneously with 5X10(5) BCG Pasteur on day 1. The results showed that the recombinant spore vaccine caused a more pronounced reduction in the bacterial load when the mice were challenged intra nasally with virulent *M. tuberculosis* H37Rv compared to the conventional BCG vaccine. To examine cellular responses, the poly-functional T-cell analysis and the enzyme-linked immuno-spot (ELISPOT) assay were used to show the spore vaccine elicited a Th1 response (Sibley et al., 2014). In another investigation to enhance the efficacy of BCG vaccines, a recombinant spore-based vaccine was constructed with CotC-Ag85B-CFP10 expressed on the spore coat of *B. subtilis*. Splenocyte analysis of immunized mice showed that the Th1 cytokines and antigen specific proliferation of IFNγ producing cells were significantly higher than in the splenocytes of controlled mice (Das et al., 2016).

6. Bacterial Polymers

Bacterial polymers were first discovered by Louis Pasteur in the mid nineteenth century when a insoluble product was isolated from wine which turned out to be a bio-polymer that became known as dextran (Pasteur, 1861). Subsequently Van Tieghem discovered the bacterium "*Leuconostoc mesenteriodes*" could produce dextran in high yield (Van Tieghem, 1878). These discoveries resulted in the production of cellulose from *Acetobacter xylinumin* (a soil bacterium commonly found on rotting fallen fruit) by Brown in 1886 (Brown, 1886). Bacteria are now known to be able to synthesize a vast variety of biopolymers, which can be used in many medical applications. Recent research in the field of bacterial biopolymers has elucidated the molecular mechanisms of biosynthesis of these biopolymers (Rehm, 2010).

There are many bacteria, which can transform various carbon sources into a diverse range of biopolymers. For example some species accumulate polyoxoesters contained in water-insoluble granules in the cytoplasm which function as an intracellular reservoir of carbon and energy(Doi, Yoshiharu, 2002; Gholami et al., 2016). An example of this, is polyhydroxybutyrate that is produced by *Ralstonia eutropha*, (York et al., 2001) which can also accumulate polymers containing a mixture of thioester and oxoester bonds when the medium contains mercaptoalkanoic acids (Doi, Y., 2002). Other classes of polymers which can be synthesized by bacteria include: polysaccharides (*i.e.* glycogen, xanthan, K30 antigen, alginate, cellulose, dextran and starch), polyamides (*i.e.* proteins and polypeptides, poly(γ-glutamic acid), polyphenols (*i.e.* lignin), polyesters (*i.e.* polyhydroxyalkanoates and polythioesters (Lutke-Eversloh et al., 2002)) and inorganic polyesters (polyphosphate) (Chatterjee and Chaudhuri, 2012; Steinbüchel, 2001).

These biopolymers perform different fundamental functions which are essential for bacterial growth and survival, such as catalysis of reactions, protection against the environment, defense against attacks by other cells, expression of genetic information, sensing of both abiotic and biotic factors, communication with the surrounding environment and other organisms, interruption of the adhesion to surfaces by other competing cells, and many other functions (Steinbüchel, 2001). Biopolymers are generally non-toxic natural materials so they are inherently biocompatible and biodegradable. Moreover, applications related to

replacement of these biopolymers in place of conventional petroleum-based polymers have become more prominent due to the recent rise of the green movement. Bacterial biopolymers have found applications in different fields such as agriculture, food product, environment and medicals including drug delivery, tissue engineering and wound dressing(Rehm, 2010).

Bacterial polymers can be used to produce nanoparticles for encapsulation of drugs in DDS. Among these types of polymers, bacterial polysaccharides such as xanthan gum, hyaluronic acid (HA), gellan gum, and fructans such as levan, can be used in controlled drug release. These polysaccharides have been used either alone or in combination with other polymers to construct nanoparticles, microspheres, hydrogels, beads and liposomes (Alves et al., 2016). For example, xanthan gum has been widely used as a supporting hydrogel or as an excipient in tablets, and can be combined with other polymers for improving the desired properties(Benny et al., 2014). A hydrogel prepared with xanthan and locust bean gum was used to develop gel-embedded vesicular formulations, which could remain stable for one year without any preservatives(Coviello et al., 2015). Chitosan-xanthan gum has been coated onto liposomes to overcome the poor stability of liposomes when used for rifampicin delivery to the lungs by inhalation(Manca et al., 2012). In the following sections, a brief introduction to bacterial-based biopolymers with an illustration of chemical structure of alginate, cellulose, hyaluronic acid, polyglutaric acid and polylysine (Figure 7) and applications in drug and gene delivery is presented.

6.1. Alginate

Bacterial alginate is produced by *P. aeruginosa* and some other *Pseudomonas spp,* which are human pathogens and phytopathogens respectively. Alginate is comprised of alternating blocks of β-1,4-linked mannuronic acids and its epimer, α-L-guluronic acid (Figure 7) (Hay et al., 2014). The so-called mucoid variants of *P. aeruginosa* over-produce alginate, which is a component of their biofilms, and is regarded as a virulence factor in this species (Mann and Wozniak, 2012).

Several modifications have been applied to enhance the efficiency of alginate for applications in DDS. Some modified alginate derivatives have included, thiolated alginate-albumin (Martínez et al., 2011) and micro-particles comprising alginate-poloxamer(Moebus et al., 2009). Other alginate derivatives have involved combinations of alginate with chitosan(Li et al., 2008), micro-environmental interaction of alginate with calcium(Chan et al., 2002), nanogel and micro-hydrogel matrices of alginate-poly(lactic-co-glycolic acid) (Qi et al., 2009), heparin-alginate hydrogels (Jeon et al., 2011), polyethylene glycol/anthracene/alginate composites (Wells and Sheardown, 2011), scleroglucan/alginate/borax gels, dual cross-linked alginate/N-α-glutaric acid/chitosan (Matricardi et al., 2006), gel beads of micelles formed from sodium-alginate composites (Huang et al., 2012), and alginate- guar-gum hydrogels(Yang et al., 2013).

Alginate/chitosan-based nano-emulsions have been used for *in vivo* insulin delivery. This approach resulted in sustained hypoglycemic effects when the nano-emulsion was administered orally, instead of being injected subcutaneously(Li et al., 2013). Alginate microspheres were used as a controlled-release dexamethasone carrier and it was shown that approximately 100% of entrapped drug was released at a stable rate over a one-month

period(Jayant et al., 2009). Alginate/chitosan nano-particles were synthesized and loaded with tamoxifen, and it was found that the tamoxifen release was better when a higher proportion of alginate was employed (Martinez et al., 2013). An alginate/poly (gamma-glutamic acid) gel which could be used as a replacement for bone tissue was prepared and a suitable composition of Pluronic F-127 and alginate-calcium- poly (gamma-glutamic acid) was developed that could be investigated as an injectable biomaterial for repair of bone defects (Chan, 2015). Alginate microspheres were synthesized by an emulsification/internal gelation procedure as a carrier of nystatin for treatment of cutaneous and mucocutaneus fungal infections infection (Martín et al., 2015).

6.2. Cellulose

Cellulose is a highly insoluble and inelastic carbohydrate homopolymer composed of β-D-glucopyranose units which are connected by β-1,4-glycosidic linkage (Figure 7) (Moon et al., 2011). Bacteria of different genera such as *Azotobacter*, *Gluconacetobacter* (previously, *Acetobacter), Aerobacter, Achromobacter, Rhizobium, Sarcina, Agrobacterium* and *Salmonella* can biosynthesize the exopolysaccharide cellulose (Cherian et al., 2013). In spite of considerable interest for potential application of bacterial nanocellulose for drug delivery, only a limited number of reports have so far been published (Jozala et al., 2016). Müller et al. reported the application of bacterial nanocellulose for loading serum albumin. They concluded that this could be a new and an interesting biopolymer for designing DDSs offering advantages such as biocompatibility and hydrophilicity (Müller et al., 2013). Bacterial nanocellulose was employed to prepare an antimicrobial wound dressing by loading povidone-iodine and polyhexanide antiseptics and functioned as a controlled drug release system (Wiegand et al., 2015). Another drug delivery system was developed by formulating bacterial cellulose as a gel that could incorporate a hydrophobic nanoparticle-encapsulated active ingredient. Poly(ethyleneoxide)-*b*-poly(caprolactone) nanoparticles were used as an amphiphilic block copolymer for encapsulating retinol in the bacterial cellulose gel(Numata et al., 2015).

6.3. Hyaluronic Acid

Hyaluronic acid (HA) is a linear polysaccharide composed of an alternating sequence of d-glucuronic acid and N-acetyl-d-glucosamine units linked by (1→4) inter-glycosidic bonds (Figure 7). It can be obtained from various biological sources such as rooster combs, umbilical cords and shark skin, Furthermore it can be extracted from *Streptococcus* bacteria which produce a capsular material containing hyaluronic acid(Van Brunt, 1986). The molecular weight can vary between 5000 to 10,000,000 Da. Hyaluronic acid is water-soluble, biodegradable and viscoelastic and its water-absorbent property makes it homeostatic and hygroscopic. The chain length, cross-linking, chemical modifications and external pH are all factors that affect its structure and viscosity. The characteristics of hyaluronic acid suggest its use in non-parenteral drug delivery routes especially for ocular and nasal delivery(Jin et al., 2010). CD44 is a cell surface protein known as the HA receptor, and is over-expressed on several tumor cells, making HA an appropriate ligand for targeted anti-cancer drug delivery (Mattheolabakis et al., 2015). Different nanoparticles loaded with DOX (Cai et al., 2010), mitomycin C (Gujrati et al., 2014), siRNA (Jiang et al., 2009) have been physically or chemically attached to HA for tumor targeting. These carriers have been

found to be efficient in targeting tumor cells and sparing normal cells. A nanoparticle structure that was stable under physiological conditions was synthesized by conjugating amphiphilic HA with hydrophobic bile acids. In a cell culture system, SCC7 cells could internalize HA nanoparticles by means of CD44 receptor-mediated endocytosis, (Choi et al., 2009). A HA-paclitaxel nanoconjugate was proposed as a novel and useful nanocarrier (Leonelli et al., 2008). Furthermore, thiolated HA was chemically linked to gold nanoparticles, and bound to IFNα by a combination of electrostatic and hydrophobic interactions, for delivery to the liver (Mero and Campisi, 2014). Graphene oxide-HA has been applied successfully for cancer therapy based on uptake by endocytosis mediated by the HA receptor (CD44). This construct also showed pH-dependent epirubicin release in acidic lysosomes(Jung et al., 2014). It was shown that PEGylation of HA reduced cellular uptake, which may prevent excessive accumulation in the liver after systemic administration(Choi et al., 2011). A delivery system for the corticosteroid, dexamethasone, into the inner ear was studied using a HA-based gel combined with PEGylated liposomes which showed good injectability(El Kechai et al., 2016). A ternary system was constructed from HA-epigallocatechin gallate-linear polyethylenimine, and developed for the targeted intracellular delivery of proteins such as granzyme B and lysozyme into cancer cells (Liang et al., 2016). Epigallocatechin gallate is an anti-cancer and anti-oxidative component of green tea.

A HA-based nanoparticle system was applied for tumor-specific delivery of siRNA. This system was based upon cholesterol-hyaluronic acid conjugates attached to the 2b RNA-binding protein. The 2b proteins, derived from tomato aspermy virus, are known to bind dsRNA to counter host defense during viral infection (Park et al., 2014). The HA-cholesterol provided a hydrophobic core which contained the 2b protein/siRNA complexes, and could leads to neutralization of the highly negatively-charged siRNA(Choi, K.-m. et al., 2014). The construct was taken up by tumor cells expressing up-regulated CD44 receptors and suppressed target gene expression. In addition, siRNA controlled release was observed in the endocytic compartments, whereas the encapsulated 2b proteins remained within the HA-cholesterol nanoparticles.

6.4. Poly (γ-glutamic acid)

Poly (γ-glutamic acid) (PGGA) is a polymer of glutamic acid with amide bonds between α-carboxyl and γ-amino groups (Figure 7) having 10 to 10000 kDa molecular mass depending on the microorganism producing it. It is a biological resource, biodegradable, and even an edible substance that is produced primarily by *B. subtilis* bacteria (Bae et al., 2010; Luo et al., 2016). It is a major constituent of the Japanese breakfast food called “natto”. Chemical modifications can be carried out to change the water-solubility of PGGA. It was reported that increasing the hydrophobicity by using alkylated PGGA, methylated PGGA or ethylated PGGA, allowed spheroidal nanoparticles to be formed. These nanoparticles were degraded within 15 to 40 days in physiological conditions with a linear kinetic profile. Furthermore they could encapsulate the antibiotic erythromycin efficiently, and in the case of methylation, maximum drug loading was achieved (Portilla-Arias et al., 2009). The oral delivery of insulin was investigated mediated by chitosan and N-trimethyl chitosan/PGGA nanoparticles that provided a protective delivery vehicle with short-term stability across a

broad pH range and a steady release profile at pH 7.4. Further studies showed that this combination could help the transportation of insulin by crossing the tight junctions of Caco. 2 cells; the insulin was transported from the small intestine through the paracellular pathway(Mi et al., 2008). Delivery of the model antigen ovalbumin (OVA) into dendritic cells was enhanced by PGGA nanoparticles. OVA antigen-specific T cells produced IFNγ showing they were specifically activated when exposed to dendritic cells that had been administered antigen by this method. This system was proposed as a protein type vaccine against infectious diseases like HIV-1/human immunodeficiency virus(Wang et al., 2008). The delivery of an anti-inflammatory drug, diclofenac was also achieved via chitosan/PGGA nanoparticles(Gonçalves et al., 2015). These types of nanoparticles were also designed as vehicles for a delivery system for a nitric oxide releasing agent combined with antimicrobial peptides (LL-37) (Sun et al., 2015). PGGA nanoparticles and an albumin adjuvant were used to stimulate immune response against Japanese encephalitis virus. They observed that delivery of the virus envelope protein via PGGA nanoparticles provided greater immunity (Okamoto et al., 2012).

6.5. ε-poly-L-lysine

ε-poly-L-lysine is another linear homopolymer between the α-carboxyl and ε-amino functional groups of lysine monomers (Figure 7) which is synthesized by different species of *Streptomycetaceae* actinobacteria, and it is more common compared to its isomer α-poly-L-lysine(Shima and Sakai, 1981). Several applications of ε-poly-L-lysine have been reported. ε-Poly-L-lysine compounds with poly-riboinosinic-poly-ribocytidylic acid were reported to induce endogenous interferon. Conjugation of ε-poly-L-lysine with methotrexate or other anticancer drugs can be used for delivery to sarcoma, leukemia and other neoplastic cells. As a poly cationic polymer, ε-poly-L-lysine has been explored for applications where it needs to bind to polyanionic molecules such as the delivery of non-viral gene vectors, and elimination of lipopolysaccharide endotoxin (Shih et al., 2006).

The use of dendritic or branched alpha ε-poly-L-lysine (DPL) as a nanocarrier of antisense oligonucleotides was investigated. The DPL had moderate cytotoxicity when complexed with antisense oligonucleotides. Dendrimers with a high generation number appeared effective for oligonucleotide delivery with promise for future therapeutic application(Eom et al., 2007). A delivery system based on heparin and ε-poly-L-lysine was developed for controlled release of nerve growth factor combined with basic fibroblast growth factor. The continuous and slow release of these growth factors enhanced the regeneration of peripheral nerve injury and also simulated the microenvironment of natural nerve cells(Zhang et al., 2014). Hydrogel systems containing ε-poly-lysine-PGGA were studied as a wound dressing with inherent antimicrobial and anti-infective properties and a good tissue adhesive behavior (Wang et al., 2016). A bio-adhesive self-biodegradable hydrogel was also developed as a nonviral gene carrier by blending poly(L-lysine) and aldehyde-functionalized dextran. Poly(L-lysine) is non-antigenic and forms complexes with DNA that can provide controlled release by controlling the speed of gel degradation (Togo et al., 2013).

7. Magnetosomes

As early as 1963, Salvatore Bellini was the first person to observe bacteria responding to the Earth's geomagnetic field. While carrying out microscopy studies of bog sediments in Italy, he discovered a group of bacteria that aligned themselves in a unique direction. Eventually he realized these bacteria took up positions relative to the North Pole, and effectively behaved like a magnetic compass. Bellini named them "magnetosensitive bacteria" (Jogler and Schüler, 2009). What are now known as magnetotactic bacteria (BTM) comprise a variety of bacterial species including *Magnetospirillumm agneticum* and *M. bavaricum.* Magnetic iron-containing minerals such as magnetite (Fe_3O_4) and greigite (Fe_3S_4) are contained within sub-cellular organelles called magnetosomes inside the bacteria. These magnetic particles are organized into chains, therefore the sum of the magnetic dipoles of each individual magnetosome represents the total magnetic dipole of the cell, which is sufficient to overcome the Brownian motion found in an aqueous environment, and thus passively orient the cells in the medium (Alphandéry, 2014; Bazylinski and Williams, 2007; Lefevre et al., 2011).

7.1. Applications of Magnetic Nanoparticles

It was in the 1960s that Freeman *et al.* introduced the then novel concept of using magnetism in medicine (Freeman et al., 1960). Since 1975, magnetic nanoparticles have attracted lots of attention, and many researchers have designed different magnetic particles to serve as drug and gene delivery vehicles that can be manipulated inside the body by using externally applied magnetic fields(McBain et al., 2008; Wells and Sheardown, 2011). Magnetic nanoparticles such as superparamagnetic iron oxide (known as SPIONs) have found prominent industrial and biomedical applications, mainly due to their low toxicity, facile surface modification, biocompatibility, and their magnetic sensitivity. SPIONs with a magnetic core coated with special biocompatible polymers provide an appropriate surface for chemical modification to allow them to be loaded with therapeutic agents' (Moebus et al., 2009). SPIONs have applications in drug delivery (George and Abraham, 2006; Pongjanyakul and Puttipipatkhachorn, 2007), gene delivery (El-Sherbiny et al., 2011) and theranostics(Jeon et al., 2010).

7.2 Drug and Gene Delivery

Magnetosomes have some unique features that make them particularly suitable for applications in nanomedicine(Mathuriya, 2016). They have nanoscale dimensions with sizes between 40–120 nm, but with a with narrow size distribution depending on the specific microorganism that produces them. The size range of magnetosomes is ideal for accumulation in tumors, and other targeted organs, after intravenous injection, because of the enhanced permeability and retention (EPR) effect (Maeda, 2015). They also have a consistent shape, and already come equipped with their own membrane that is composed of proteins and lipids. The magnetosome envelope provides easy coupling of bioactive substances to the surface, an important characteristic for drug delivery applications(Faivre and Schuler, 2008). However, for magnetosomes to reach their true potential, large-scale commercial production would be necessary. This would require industrial scaled-up culture of magnetotactic bacteria, or alternatively the introduction and expression of the relevent

genes into a biotechnology production species leading to magnetosome biosynthesis. e.g., *E. coli*, can be grown in very large yields. Neither of these goals has yet been satisfactorily achieved.

Magnetosomes isolated from *M. gryphiswaldense* were studied as carriers for the anticancer drug DOX against hepatic cancer. DOX-conjugated to the magnetosome surface showed only a slight increase in the cytotoxic activity toward cancer cells (from 79% for free DOX up to 87% for DOX-magnetosomes) (Sun et al., 2007; Sun et al., 2008). However it was found that the mortality rate of tumor-bearing mice treated with free DOX was 80%, while the mortality rate for DOX-magnetosomes was only 20% which showed that the drug coupled to magnetosomes was less toxic to healthy organs, but with equivalent efficacy against the tumor(Sun et al., 2007). In another investigation, a novel anti-tumor targeted DDS based on bacterial magnetosomes from *M. magneticum* AMB-1 was investigated for treatment of acute leukemia using cytosine arabinoside (Ara-C) covalently linked to the membranes of bacterial magnetosomes(Deng et al., 2013). In another study, Ara-C was loaded into magnetosomes using genipin (an aglycone derived from a Gardenia glycoside) and poly-l-glutamic acid as dual cross-linkers (Liu et al., 2015).

Oh and colleagues prepared “magnetic micro-droplets” using magnetosomes and sodium alginate that could be loaded with calcium and guided into different channels in a microfluidic device using externally applied magnetic fields (Oh et al., 2014). Cheng et al (Cheng et al., 2016) constructed a eukaryotic plasmid encoding heat shock protein 70-polo-like kinase 1-short hairpin RNA (pHSP70-Plk1-shRNA) under the specific ambient-temperature transcriptional control of a thermosensitive promoter (human HSP70 promoter). The magnetosomes were heated up by external application of an alternating magnetic field (AMF), and enhancing the temperature (43°C within 3 minutes) led to controlled release of the DOX and pHSP70-Plk1-shRNA. The expression of Plk1 (mRNA and protein) were remarkably reduced in cells which were treated with BM-DOX-shPlk1 (Cheng et al., 2016). After AMF hyperthermia treatment, tumor cell cytotoxicity was also potentiated by AMF due to hyperthermia mediated DOX release.

8. Conclusions

Drug delivery, controlled release, and targeted nano-carriers are becoming ever more important in the present age of personalized medicine, theranostics, and nanomedicine. This is particularly true in cancer therapy, where the indiscriminate toxicity of many chemotherapeutic agents is well known. Meanwhile, the action of some other drugs is limited due to degradation by enzymes they encounter en-route to their intended target, by interaction with other neighboring cells, or by their inability to penetrate tissues. To overcome these problems, researchers are conducting studies to develop an arsenal of strategies to allow a variety of drugs and other therapeutic payloads to be safely delivered to the target sites. Many of these strategies include rationally-designed nano-carriers constructed from polymeric and inorganic nanomaterials and even pure elements such as carbon and silicon. However, another strategy prefers to concentrate on nano-carriers derived from materials isolated from purely natural origins. One of the most fertile sources in nature for constructing these nano-carriers are derived from the wide range of microorganisms,

which are an attractive source in terms of toxicity and abundance. Since pathogenic microorganisms are often an important source of human disease, it appears to be only poetic justice that derivatives of these life forms should serve as the means to engineer their own destruction. Immunization and vaccines are increasingly being used as countermeasures against a wide range of different diseases that lie outside the traditional vaccine area of infectious disease. Cancer is an obvious example of this, but vaccines are also being studied for treatment of allergies, cardiovascular disease, hypertension, diabetes, addictions, and even to limit fertility (contraception). Due to the inherent ability of bacterial-derived nanocarriers to be recognized by the innate immune system, they have been widely studied as delivery vehicles for different kinds of antigens, and as naturally occurring vaccine adjuvants. Bacterial components like S-layer, BGs, outer membrane vesicles, endospores, all fall into this class, while bacterial-derived polymers and bacterial magnetosomes could replace and improve upon, traditional nanotechnology-based materials for construction of DDS. Despite concerns about the use of potentially infectious agents, bacterial-derived nano vehicles appear to be relatively safe so far, and future studies that are ongoing that are expected to lead to even more clinical applications.

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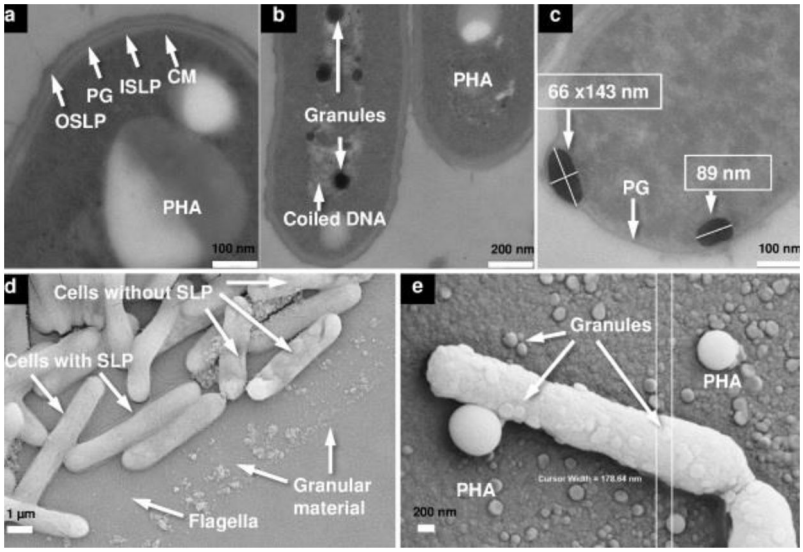


Figure 1.

Subcellular morphology of *A. migulanus.* (a) cell wall consisting of outer S-layer protein (OSLP), peptidoglycan (PG), inner S-layer protein (ISLP) and cell membrane (CM), (b) localization of Gramicidin S containing granules like polyhydroxyalkanoates (PHA), (c) size estimation of the granules, (d) cells that are covered with S-layer proteins are found to be intact in the SEM, whereas those cells that have shed their S-layer proteins are seen to be disrupted and have release their granules into the environment. (e) Two types of granules are distributed over the silicon support when the S-layer protein was washed off and the neighboring cells got ruptured (Berditsch et al., 2017). no permission required

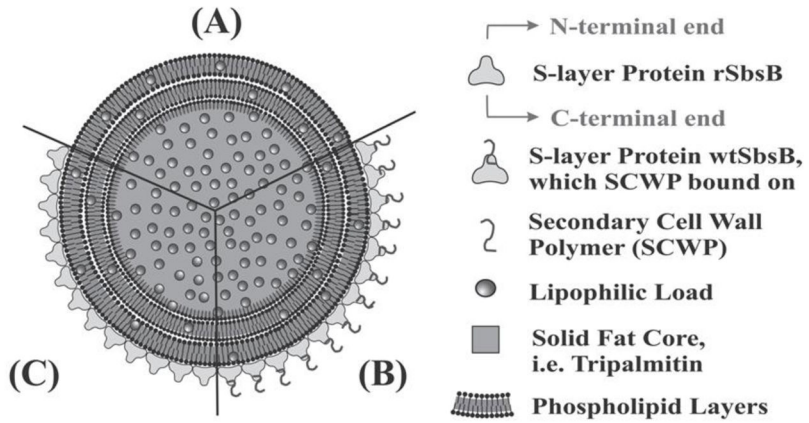


Figure 2.

S-layer functionalized emulsomes showing: (A) naked emulsomes structure (B) emulsomes coated with S-layer protein/ wtSbsB (C) S-layer protein/ rSbs B coated emulsomes. Reprinted with permission from Wiley (Ucisik et al., 2013).

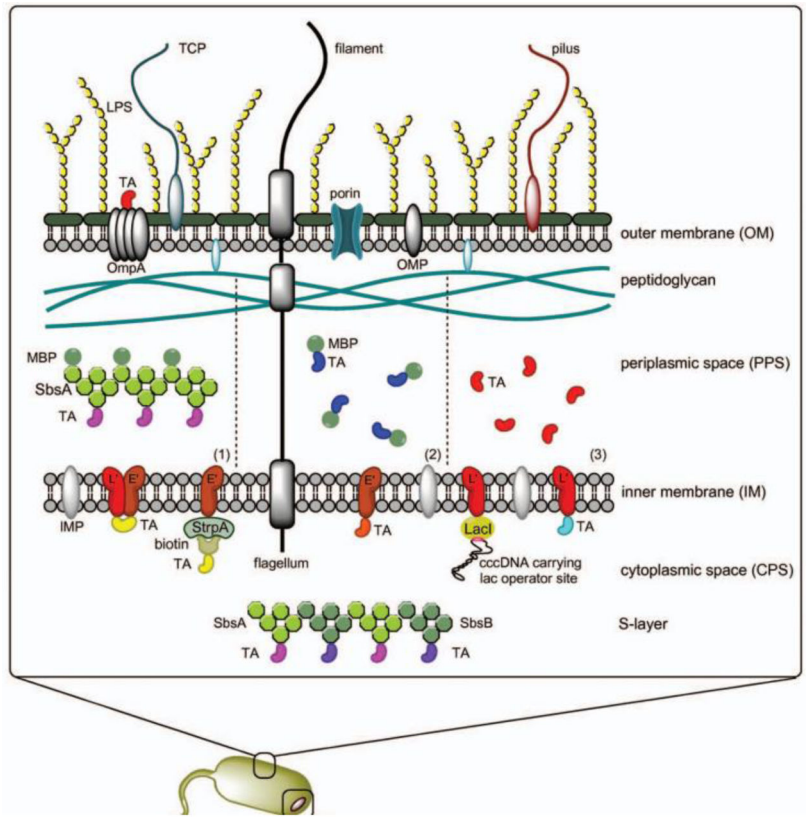


Figure 3.

There are several methods to incorporate antigens in BGs. A) through fusion between TA with MBP, B) TA using the gene III signal sequence, C) binding to TA which attached to E′, L′ or E′/L′-anchoring or binding to E′-FXa-StrpA membrane anchors if biotinylated. (TA = Teichoic acid) (Langemann et al., 2010). no permission required

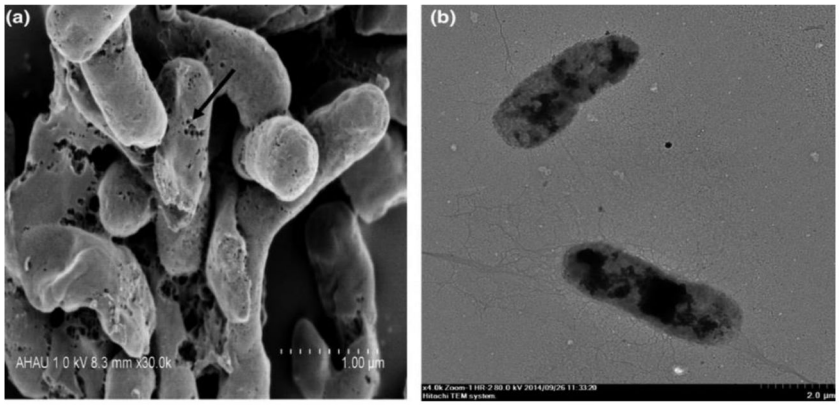


Figure 4.

V. alginolyticus BGs under TEM, (a) 30,000× scanning electron micrograph including transmembrane tunnels. (b) 4,000× transmission electron micrograph (Cao et al., 2018) no permission required

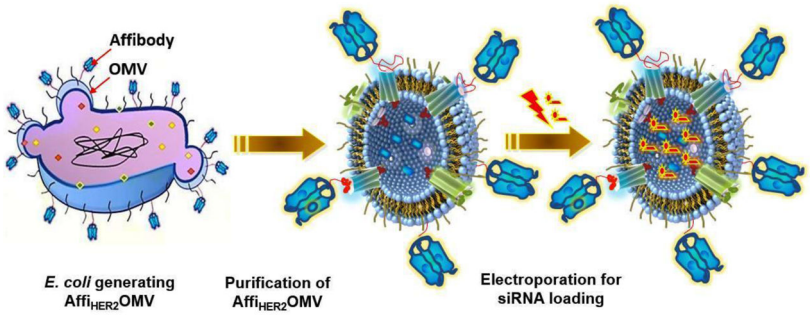


Figure 5.

Schematic illustration of use of an anti-HER2 specific affibody on OMVs loaded with siRNA. Reprinted with permission from American Chemical Society (Gujrati et al., 2014).

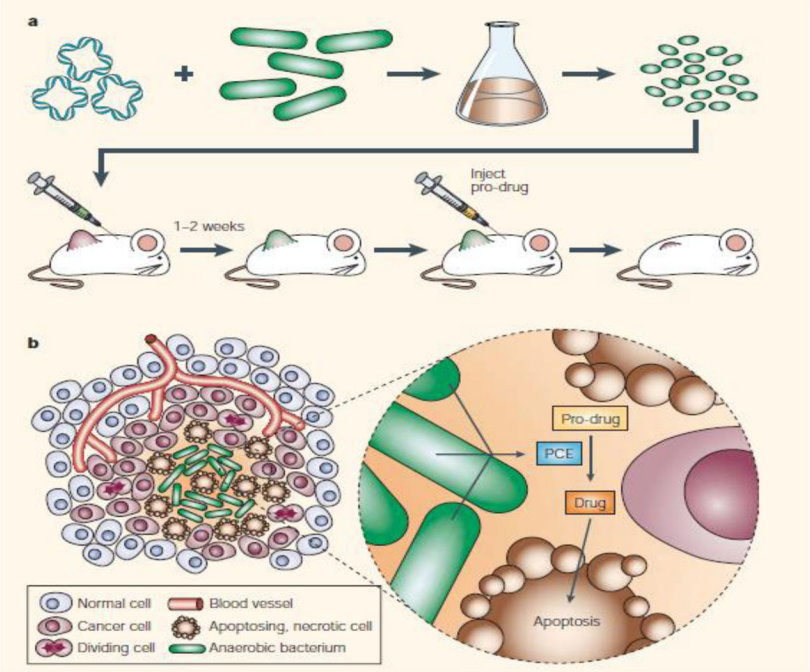


Figure 6.

Prodrug-converting enzyme gene (cytosine deaminase) was inserted into a plasmid which was transformed into *Clostridium*. Spores produced by the bacteria were collected, prepared and directly injected into a tumor-bearing animal. The spores spread in the body, but germination only occurred in hypoxic areas of the tumor. After 1–2 weeks (the time required for colonization), the prodrug (5-fluorocytosine) was administered. The conversion of the prodrug into the active drug (5-fluorouracil) caused tumor regression (Minton, 2003). Reprinted with permission from Nature Reviews Microbiology.

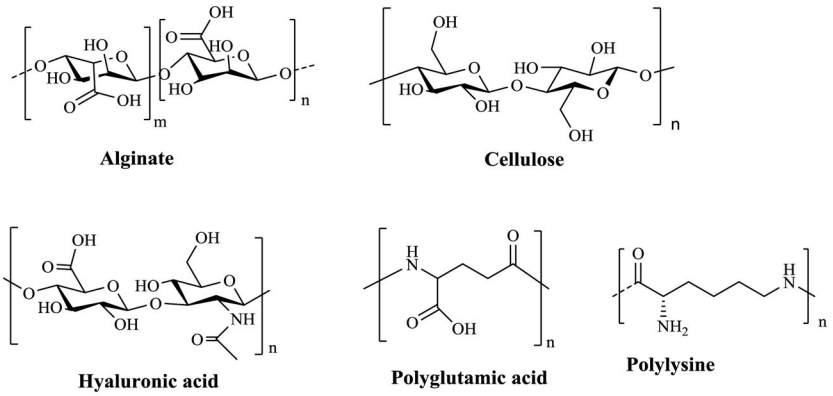
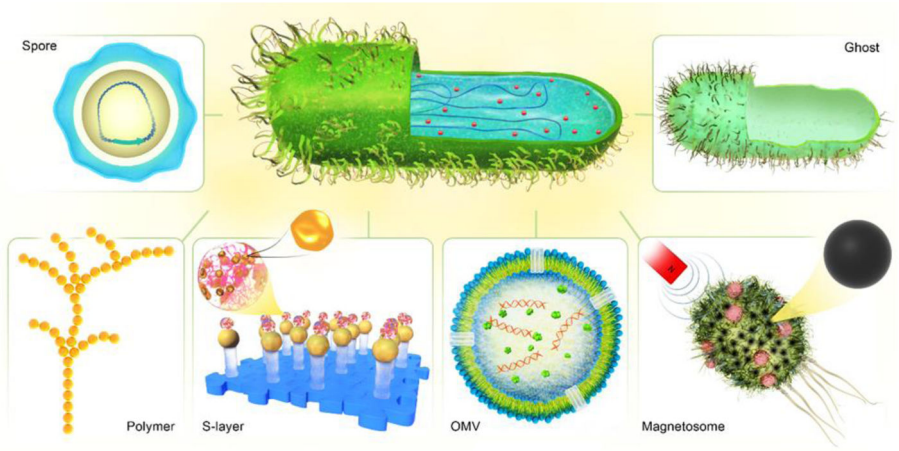


Figure 7.
Chemical structures of some bacterial-based biopolymers



Scheme 1.
Schematic illustration of bacteria and bacterial components