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Bacterial ghost: A novel strategy for medicinal applications

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Abstract

Bacterial ghost (BG) is an empty cell envelope of bacteria devoid of chromosomal and plasmid DNA and cytoplasmic content. However, it maintains the cellular morphology with native surface characteristics and bio adhesive properties. The widely used method to prepare BG is the protein E-mediated lysis method, which forms transmembrane tunnels through the inner and outer membranes of bacteria. There are also chemical and mechanical methods that lead to the formation of bacterial ghosts without damaging the necessary components and structure of bacteria. Alongside using NaOH, SDS, H_2O_2, tween 80, β-propiolactone, and 60% ethanol, hydrostatic pressure is also used to expel the cytoplasmic content resulting in pure envelop ghost. Recently developed holin-mediated inactivation, lysozyme, and protein kinase K are also used to kill bacteria, leaving them as BG. It is used in vaccines, immunotherapy of cancer, and a widely accepted delivery system of drugs, antigens, and other active components. The other efficient use of bacterial ghosts is to stimulate humoral and cell-mediated immunity through antigen-presenting cells. Taken together, BG represents a novel and emerging method for developing potential disease prevention and public health.

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1. Introduction

The Bacterial Ghost (BG) platform technology is a cutting-edge method for administering vaccines, medications, or other active ingredients, as well as for technical uses in the field of present-day medicine and biotechnology. BG is a non-living bacterial cell containing envelopes in its structure and maintains the cellular morphology resembling native

bacteria, where cell surface structures like flagella, pili, membrane proteins, lipopolysaccharide (LPS), peptidoglycans, and adhesins are preserved (Lubitz, 2009). As BG is empty, it can be filled with proteins, DNA, and drugs and can act as a carrier to deliver into the target site without hampering the host (Huter, 1999; Paukner, 2003). It shows intrinsic adjuvant properties and can trigger cellular and humoral immune responses to target antigens (Paukner, 2003). On the basis of bacterial species from which BG is formed, their size of distribution is almost uniform, ranging from 0.5μm to 2.0 μm wide and 1 to 2 μm long (Chen, 2021).

To prepare the BG, several innovative and useful techniques are employed. The cloned gene produced from bacteriophage *PhiX174* is used to lyse the bacterial cell, resulting in the bacterial ghost with the transmembrane tunnels. The morphology of bacteria, including cell surface structure, is not hampered by the lysis method. Using bacteriophage *PhiX174*, which was the first method to produce BG, but nowadays, much more efficient, economical, and less time-consuming methods are used. Subsequently, many other methods have been discovered by using various chemicals and applying mechanical pressure. Interestingly, not only Gram-positive but also Gram-negative bacteria are used to prepare ghosts. In Gram-positive bacteria, without cell killing by lysis, the E-mediated method is used depending on the fusion of inner and outer membranes (Halfmann, 1993). There are many different specious Gram-negative bacteria like *Escherichia, Haemophilus, Klebsiella, Salmonella, Pseudomonas, Enterobacter, Vibrio, Pasteurella, Bordetella, Helicobacter, Brambamella, Actinobacillus, Pseudomonas, Ralstopia, Ervinia; and* Gram-positive bacteria such as *Bacillus, Streptococcus, Staphylococcus, and Lactobacillus* are used to prepare bacterial ghost (Jalava, 2002; Wu, 2017).

There are a number of applications of BG in the field of modern medical technology. For use in medical applications, potential vaccine candidates that function as foreign antigen carriers have been developed, with a focus on immunizations for children against enteric illnesses (Mayr, 2005a). Functional vaccinations are, without a doubt, the most efficient medical interventions to save lives and lower healthcare costs. Numerous studies have demonstrated that various inactivated bacteria can modulate immune responses by altering the immune system of the host (Taverniti & Guglielmetti, 2011; Lim, 2019). It has been reported that dendritic cells are affected by *Vibrio cholerae* ghost in a way that modulates their immune system to improve antigen presentation and trigger the protective response (Eko, 2014). It is possible to actively target cancer cells with attenuated strains of microbes. BG has strong synergistic anticancer

effects because it possesses the capability to stimulate T-lymphocytes that trigger the efficient antitumor effects (Groza, 2019). BG is also widely used in targeted drug delivery systems. As BG provides the empty inside space, it can be filled with various drugs, nucleic acids, and antigens to deliver to the targeted sites (Chen, 2021). The overall potential benefits of BG have been summarized in Table I.

Table I
The potential benefits of bacterial ghost (BG) in contrast with live bacteria.

Characteristics	Benefits
Safety profile	No risk of reversal to pathogenic form. Capability to express recombinant proteins in the various positions on the bacterial ghost. Provides cross protection against heterogeneous strains. Other than parenteral routes BG can be administered by oral, nasal, conjunctival or rectal routes No clinical side effects.
Physiological properties	Excellent uptake and recognition by antigen-presenting cells (APC). Robust adjuvant properties. Loading capability is high for DNA Good targeting properties for different cells and tissues.
Economical features	Lower production cost. Processing time is less.
Pharmaceutical features	Longer shelf-life in room temperature if lyophilized. Easy to handle and transport.

This review discusses the production and application of bacterial ghosts based on the available published literature and encompasses the different modes of preparation and the potential role of BG in vaccines, immunomodulation, cancer treatment, and drug delivery systems (Figure 1).

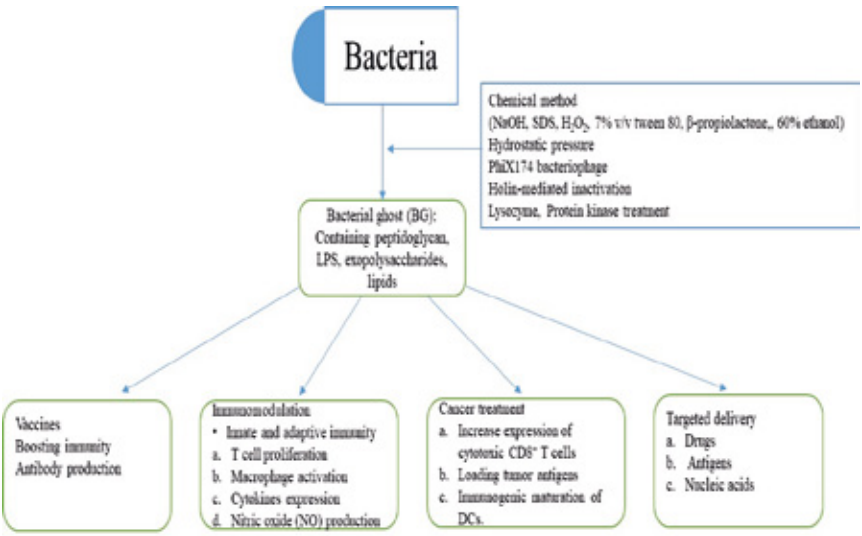

Figure 1
Schematic presentation of bacterial ghost preparation and potential applications.

2. Methods of BG preparation

The BG preparation principle is to disrupt the cell walls and selectively remove the internal content of bacteria while preserving the cell envelope. The method begins by culturing the bacteria in suitable conditions, followed by a specific multistage preparation method, such as treating them with a particular chemical, applying optimal pressure, or genetically modifying them. Thus, producing BGs preserves their functional properties, including immunogenic characteristics (Vinod, 2015). Recently, several methods have been developed to prepare BG. However, the main aim of every method is to maintain the internal content intact from preparation to ultimate use. Among many others, the following methods, such as chemical, mechanical, and genetic engineering, are widely used for the preparation of BG. The summarized concept of preparation of bacterial ghost (BG) has been depicted in Figure 2.

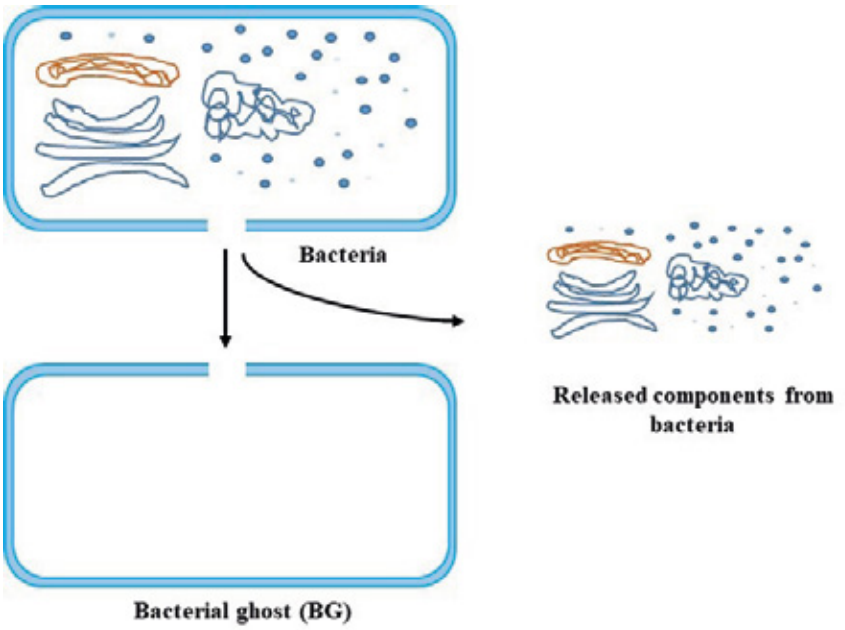

Figure 2
The preparation of bacterial ghost (BG). The upper figure represents the intact bacteria, and the lower figure represents the empty bacterial cell, i.e., BG.

2.1 Chemical method

There are a number of chemicals, such as NaOH, SDS, and H2O2 are used to prepare ghosts. Moreover, it has been studied that the produced ghosts have been tested as vaccines because they have immune protection capabilities against the virulent (Vinod, 2015; Park, 2016; Wu, 2017). The preparation of empty bacterial cells using NaOH, SDS, and H_2O_2 has been used to prepare *Escherichia coli* JM109 strain ghost (Amro, 2014; Hajam, 2015). A modified method based on critical chemical concentrations called 'sponge-like reduced protocol' has been used to prepare bacterial ghost and evaluate the surface antigen of *the Salmonella* Typhimurium ATCC 14028 strain (Amro, 2014). A recent method has been used to prepare bacterial ghost of *Salmonella enterica* serovar Typhimurium ATCC 13311 strain by applying 7% v/v tween 80 solution in Muller-Hinton broth, which is very simple, economical, and efficient (Rabea, 2018). For the preparation of an inactivated whole-cell vaccine, β-propiolcatone (BPL) can be used where the function of BPL is to alkylate DNA and ultimately inhibit DNA from replication and transcription (Langemann, 2011). Using 60% ethanol, it is

possible to eliminate bacterial cell content, including salts, DNA, and RNA, making the virtually inactive cell (Amara, 2013).

2.2 Mechanical method

A large variety of bacteria can be quickly and effectively lysed by mechanical lytic methods. Since cells are broken down during mechanical lysis, all intracellular components, including nucleic acids, are easily liberated. The majority of mechanical lysis techniques, such as the French press and bead pounding, are efficient and let the bacteria to be broken easily (Bahamonde, 2021). A new type of *Actinobacillus pleuropneumoniae* ghost has been prepared by the combination of high hydrostatic pressure and limulus antimicrobial peptides (Li, 2012). Mild hydrostatic pressure is employed to generate an inactivated, empty-shelled *E. coli* bacterial ghost, which is used as a vaccine (Vanlint, 2008).

2.3 Genetic engineering

The preparation of killed bacteria with risk-free envelopes and unique characteristics can be obtained using bacteriophage *PhiX174* lysis gene E (Halfmann, 1993; Szostak, 1996; Lubitz, 1999; Panthel, 2003; Kudela, 2005; Mayr, 2005b; Zhu, 2012a, 2017b; Hajam, 2015; Won, 2017). Thus, producing nucleic acid-free *E. coli* ghost, inactivation can be achieved by adding *Staphylococcus* nuclease A (SNUC) along with lysis by E-lysis, where SNUC acts as cleaner of the residual DNA below 100 base pairs (Haidinger, 2003). It has been shown that *E. tarda* ghost's cell walls maintain excellent antigenicity (Kwon, 2005). By controlling the expression of lysis protein E, which is used in the preparation of *Mannheimia haemolytica* ghost, it can be efficiently loaded with plasmid DNA (Ebensen, 2004). A slightly modified method of using the E-lysis technique has been practiced by using a high concentration of MgSO4 to reduce the fluidity of the outer membrane ion bridge 3-deoxy-D-manno-2-oclutosonic acid units of LPS for further inhibiting the E-specific transmembrane tunnel formation of already primed E-mediated lysed bacterial cells (Witte, 1992). The cell lysis method by using *PhiX174* leads to the creation of a transmembrane tunnel in the bacteria, releasing the content of cytoplasm, which is a little bit time-consuming since the bacteria have to be loaded with the lysis plasmid and after the completion of lysis, bacteria need to be reloaded with a new plasmid or DNA of interest. To overcome this, another method has been described where cell-penetrating peptides traverse the cell membrane by the process called endocytosis (Palm-Apergi & Hällbrink, 2008). The holin-mediated inactivation strategy is used to construct *Lactobacillus casei* ghost, which can be applicable in the delivery of DNA vaccine (Hou, 2018). Lysozyme,

whose function is to maintain the defense against pathogens, can be used to lysis the cell wall by targeting bacterial exopolysaccharide and Protein kinase K, which are able to denature the protein, and both of these two compounds are used to prepare BG (Morihara & Tsuzuki, 1975).

The production of BGs is still being developed using different methods. Among many other methods, the chemically induced BGs provide several benefits in biomedical applications, especially in vaccine development. These advantages include effective delivery of antigens, increased ability to stimulate an immune response, inherent adjuvant qualities, safety, and scaling up the preparation (Park, 2023). The BGs are produced by selectively removing the cytoplasmic content of bacterial cells using chemical treatment, maintaining the structural integrity of the cell membrane (Vinod et al., 2015). This approach yields bacterial envelopes that are devoid of internal content, creating a larger inner surface that is very effective for drug delivery (Amara et al., 2013). The chemical method does not necessitate personnel with advanced training, unlike mechanical and genetic engineering methods. Moreover, the chemicals used in this process are cheap and available (Rabea et al., 2018). The chemical treatment method is quick, while the mechanical and genetic engineering techniques are relatively time-consuming. In addition, the potential toxicity of the chemicals used in the BGs can be eliminated by subsequent washing by centrifugation (Vinod et al., 2015). In most cases, the used chemical is washed out through the pores of bacteria created by applying specific chemicals, leaving safe BGs.

3. Applications of BG

3.1 In vaccine preparation

There is no doubt that effective vaccines are the most efficient, reliable medical interference to save lives and reduce the unbearable treatment cost than the treatment started after the introduction of disease. The traditional vaccines are expensive, difficult to produce, may have multiple serotypes, and may not be suitable for all ages (Josefsberg & Buckland, 2012). However, the BG vaccine could be an excellent alternative, providing these criteria. The fact that bacterial ghost contains many well-known immune-stimulatory components such as peptidoglycans, lipopolysaccharides, exopolysaccharides, and lipids which can enhance the immunity of the host (Szostak, 1996). BG contains a lot of periplasmic, inner, and outer membranes in the internal lumen, which can carry DNA, antigens, or mediators of immune response (Walcher, 2004). The presence of especial non-denatured proteins on the surface of the cells, which stimulate the immune cells of the host to engulf them instantly, is the vital point of using BG (Nik, 2015). An *in vitro* study showed that the empty

bacterial cell has the capability to be loaded with DNA vaccine, producing high transfection to deliver the content into the target cells (Ebensen, 2004). The presence of endotoxins in the bacterial cell cannot produce potential health hazards when using whole cells or enveloping bacterial ghosts. As DNA plays a crucial role as an active agent for vaccination, the site-specific recombination system based on the preparation of plasmid containing minicircle DNA in bacterial ghosts provides safe application in non-viral DNA delivery systems (Jechlinger, 2004). In the DNA vaccine, immobilized attachment of DNA in the cytoplasmic membrane of inactivated bacteria provides an efficient tool for vaccine therapy (Hoffelner & Haas, 2004; Mayrhofer, 2005). Bacterial ghost vaccine technology is used in immunocontraceptive vaccine for birth control by immunization of female against possum zona pellucida protein-2, which reduce embryo production (Walcher, 2008). The inactivated form of *E. coli* O157:H7 is used as a vaccine for controlling hemolytic uremia syndrome and hemorrhagic colitis caused by *E. coli* (Sanchez-Villamil, 2019). With the ample space provided by membranes, periplasm, and internal lumen of the bacterial ghost, it is possible to make a recombinant ghost, which could be a new tool for adjuvant-free unification of vaccines (Lubitz, 1999). *Pasteurella* is an animal pathogenic bacteria that has been used to produce bacterial ghosts and tested for immunization in rabbits by administering subcutaneously, where *P. multocida* ghost developed antibodies and demonstrated resistance against *Pasteurella* strains (Marchart, 2003). The *Helicobacter pyroli* (Hp) ghost has been used as a vaccine loaded with recombinant OMP18, a peptidoglycan-associated lipoprotein precursor that decreases the Hp colonization in experimental mice (Talebkhan, 2010). Research on the field of HIV vaccine using BG could be a promising strategy. The *S. typhi* Ty21a strain BG has been explored for its capacity to deliver plasmid DNA HIV-1 vaccine *in vitro* and *in vivo*, where BG-DNA shows superior immune response compared to naked DNA (Wen, 2012). Gonorrhoea is a sexually transmitted disease creating public health problems worldwide. It is evident in the mouse model that the *Neisseria gonorrhoeae* DNA vaccine delivered by *Salmonella enteritidis* ghost triggered the higher production of IgG, which could be a potential target for preventing this disease in humans (Jiao, 2018). Trachoma is a preventable disease affecting many people worldwide, making them blind. *Chlamydia trachomatis* is responsible for this devastating ocular disease. *The Chlamydia trachomatis* ghost vaccine could be a potential tool to target *C. trachomatis* and ultimately help eradicate human blindness caused by this bacterium (Eko & Barisani-Asenbauer, 2008).

3.2 As an immunomodulating agent

Bacterial ghost plays an important role in maintaining the health of the host by modulating the immune system. It could stimulate both innate and adaptive immunity by controlling the expression of different inflammatory mediators (Karasawa, 2013). BG has the capability to induce dendritic cells (DCs), leading to augmenting T cell proliferation and differentiation, and the ability to increase macrophage activation (Haslberger, 2000), triggering nitric oxide, a well-known weapon to fight against bacteria (Lim, 2019). The 'killing' by removing all the content of one serotype, *E. coli* O78:K80, and subsequent administration into Ross 308 broiler chicken, which stimulates the immune system by producing IFN-γ, IgA, and IgY, shows inhibition against avian colibacillosis (Ebrahimi-Nik, 2018). Making another *E. coli* O157:H7 strain as a ghost has shown the immunogenic prevention against its infection in the mouse model (Cai, 2015). The *Salmonella typhi* bacterial ghost can act on bone marrow-derived dendritic cells to activate adaptive immunity by enhancing the expression of various cytokines (Won, 2018). BG generated from *E. coli* could elicit an immune response in human keratinocytes (Abtin, 2010). The ghost antigen produced from *Actinobacillus pleuropneumoniae* revealed the robust-cell response by activating the antigen-presenting cells (APC) macrophage and dendritic cells (Felnerova, 2004). The experiment showed that bacterial ghost from *E. coli* NM522 stimulates murine macrophage cells to produce nitric oxide (NO) in a dose-dependent manner (Koller, 2013).

3.3 In cancer treatment

The deadly disease–cancer can be treated by bacterial ghost therapy. Using bacterial ghost, a novel delivery system might improve cancer treatment (Afkhami-Poustchi & Matin, 2015). Because of the immunogenic properties of BG, the expression of $CD8^+$ T cells significantly increases, and ultimately, uncontrolled proliferation and metastasis of cancer cells are reduced (Kraśko, 2017). BG also improves the activity of anticancer drugs (Groza, 2019). Due to having higher transfection efficiency of BG loaded with DNA, the gene transfer to the melanoma cells using *E. coli* NM522 and *Mannheimia haemolytica* ghosts provide effective tools for the treatment of this cancer (Kudela, 2008). BG is emerging as a tremendous platform for both loading with tumor antigens and the induction of immunogenic maturation of dendritic cells (Michalek, 2017; Dobrovolskienė et, 2018).

3.4 In drug delivery systems

Bacterial ghosts can encapsulate the drugs and can specifically attach to mammalian cells or tissues. These kinds of properties act as true drug

delivery system allowing the drug to reach into the tissue of specific targeting. In order to inhibit the rapid leakage of loaded water-soluble drugs, the bacterial ghost can be sealed by the fusion of the cell membrane (Paukner, 2003). The targeted delivery of water-soluble drugs, antigens, and nucleic acids by using the empty cell is possible (Jalava, 2003; Tabrizi, 2004). The *E. coli* Nissle 1917 BG can be used as a carrier to reach conjunctiva for the treatment or prevention of ocular surface diseases (Stein, 2013). The bacterial ghost provided an excellent carrier system for antigen delivery. *E. coli* ghost encoring Hepatitis B virus antigen in its envelop produces excellent immunogenicity in the host against Hepatitis B (Jechlinger, 2005). A recent study confirmed that BG can recognize different types of tumor cells and efficiently target and treat leukemia, melanoma, and colorectal carcinoma (Kudela, 2010). The delivery system using bacterial ghosts can transport the chemotherapeutic drugs in the cytosol and nucleus of the target cells. This pinpoint target of delivery of drugs into target sites can minimize the negative impact on the other tissues and decrease the drug dosages (Paukner, 2004). Moreover, due to the presence of a pathogen-associated molecular pattern (PAMP) on the surface of BG, the targeting of tumor cells with BG loaded with chemotherapeutic drugs is increased (Hajam, 2017). In the case of DNA loading, BG provides an excellent capacity to be loaded, ranging from 4000 to 6000 plasmid copies per BG, and APCs and cancer cells efficiently internalize DNA-loaded BG (Kudela, 2008).

4. Safety profiles of BG

In case of safety issues, BG provides an excellent safety profile because they are no-loving with no pathogenic threat or danger of horizontal gene transfer and do not require additional adjuvants (Eko & Barisani-Asenbauer, 2008; Kudela, 2010). There are chances of risk of infection while using live bacteria. So, the killed or attenuated bacteria are being used for therapeutic purposes. Unfortunately, sometimes, during the inactivation process, the essential structure and immunogenic characteristics of bacteria can be destroyed, resulting in lessened function and no or less effective immune response (Bergmann-Leitner & Leitner, 2014). To overcome these problems, during the preparation of BG, it is essential to ensure that the essential components are not destroyed. The proper quality control system should be maintained to get the full activity of BG with the essential components.

5. Concluding remarks

The interest in bacterial ghosts is gaining the attention of the scientific community, clinicians, patients, and pharmaceutical companies. In this respect, different bacterial ghost, their key compounds, and components are being developed and marketed for certain indications, particularly in the area of vaccines.

However, there are still some issues to be tackled while preparing bacterial ghosts from new genera as well as existing bacteria, and any of their potential risks should be taken into consideration. The bacterial ghost used as a vaccine may have a reduction of providing complete immunity by being destroyed during preparation by applying chemicals or mechanical methods (Cao, 2018). More attention is to be paid to the different areas of using BG and various components derived after the lysis of bacteria. The physiological effect that inactivated strains can exert *in vivo* should be considered since the substantially disrupted cells may produce unwanted or may be less effective for the target use. Moreover, the *in vitro* and *in vivo* results should be interpreted correctly considering the specific conditions of the humans or animals where the BG will be used.

Nevertheless, the information reviewed in this article shows that BG has favorable clinical effects on its use in different aspects, representing a new, safe, and effective service area. As reviewed, the evidence may provide the idea of the safe use of BG in the vaccine, immunomodulation, and targeted drug delivery systems. Proper purification, quantification, and standardization may lead to highly specific and safe products intended for patient specific therapies. Currently existing evidence is that BG is the most widely explored and promising therapy as a vaccine (Eko, 1999; Hoffelner & Haas, 2004; Mayrhofer, 2005; Eko & Barisani-Asenbauer, 2008; Walcher, 2008; Talebkhan, 2010). Not only in the field of vaccines but also in other aspects, the demand has been increasing day by day. The interested field of using BG in the delivery of antigens (Huter, 2000; Samuelson, 2002; Ebensen, 2004; Mauriello, 2004; Paukner, 2004; Tabrizi, 2004), drugs (Huter, 2000; Paukner, 2004) and nucleic acid (Ebensen et, 2004) may provide promising future in medical biotechnology.

Overall, the reviewed information indicates that BG could be an interesting choice for vaccination, immunomodulation, drug delivery, cancer treatment, and beyond.

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