Expert Opinion



# Bacterial ghosts as a novel advanced targeting system for drug and DNA delivery

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Although there are powerful drugs against infectious diseases and cancer on the market, delivery systems are needed to decrease serious toxic and noncurative side effects. In order to enhance compliance, several delivery systems such as polymeric micro- and nanoparticles, liposomal systems and erythrocyte ghosts have been developed. Bacterial ghosts representing novel advanced delivery and targeting vehicles suitable for the delivery of hydrophobic or water-soluble drugs, are the main focus of this review. They are useful nonliving carriers, as they can carry different active substances in more than one cellular location separately and simultaneously. Bacterial ghosts combine excellent natural or engineered adhesion properties with versatile carrier functions for drugs, proteins and DNA plasmids or DNA minicircles. The simplicity of both bacterial ghost production and packaging of drugs and/or DNA makes them particularly suitable for the use as a delivery system. Further advantages of bacterial ghost delivery vehicles include high bioavailability and a long shelf life without the need of cold-chain storage due to the possibility to freeze-dry the material.

Keywords: advanced drug delivery system, antigen-presenting cells, bacterial ghosts, doxorubicin, DNA carrier, drug targeting, transfection, tumour therapy

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## 1. Introduction

In general, advanced targeting systems deliver a drug or other active substances to cells or tissues if the carrier is able to recognise given receptors. Targeting of active substances is the major goal of advanced drug delivery systems (ADDS). The delivery of drugs, DNA or small inhibitory RNA (siRNA) represents important challenges for cell-based therapy.

The idea of drug targeting dates back to the concept of Paul Ehrlich one century ago. He considered a hypothetical 'magic bullet' consisting of one targeting part and a second component providing the therapeutic action at the target site. In this sense, the application of drug targeting vehicles should result in reduced drug doses, protection of the drug against harmful environmental influences, and higher local drug concentrations. Therefore, potential side effects compared with systemic applications are reduced, better pharmacokinetics obtained, and frequent doses or injections are unnecessary.

Ideal drug targeting vehicles are composed of three parts including the therapeutic drug, the targeting moiety for recognising and binding to the target, and a carrier for multiplication of the number of drug molecules per targeting moiety. Currently used targeting protocols do not necessarily involve the use of specific targeting moieties, but rather physical principles or physiological features of the target tissue or organ. These approaches include:

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- the direct application of the drug into the target site;
- the passive targeting by the natural pattern of the drug distribution (e.g., through leaky vasculature in tumours and sites of inflammation or the particle uptake by the lung endothelium);
- the physical targeting, which is based on the change of physical parameters such as temperature or pH from outside in to the target zone or on an external magnetic field (e.g., hyperthermic treatment, magnet particles).

Active targeting involves vector moieties capable of targeting specific tissues; for example, antibodies and their fragments, lectins and other proteins, lipoproteins, hormones, charged molecules, and mono-, oligo- and polysaccharides with high binding affinity to the target tissue [1]. One principle of drug targeting is the direct coupling of the drug moiety to the binding moiety; for example, immunotoxins, in which the toxic moiety is fused to an antibody. Another alternative is the coupling of a carrier or a drug reservoir to the binding moiety via biodegradable bonds. The advantages of these reservoir-type systems are a maximum volume at a given surface, few targeting moieties necessary to carry multiple drug molecules loaded into the reservoir, and the possibility to control the particle size and permeability.

The first soluble carrier system introduced by Ringsdorf was a polymeric carrier system of polyorthoesters [2]. Other biodegradable polymeric materials include poly(lactide-co-glycolide)s, polyhydroxybutyrate, polyhydroxyalcanoates, block polymers of poly(lactide-co-glycolide) with polyoxyethylene (poly[ethylene glycol]) or polyethylenimine [3].

The latter examples belong to the soluble systems. This review, however, focuses on the insoluble carrier systems with emphasis on the use of bacterial ghosts (BGs). Other insoluble carrier systems include microparticles, nanoparticles, micelles, erythrocyte ghosts and liposomes, which provide different qualities for drug targeting. In comparison with BGs, the other insoluble carrier systems are briefly described in the following sections mainly with regard to their ability to carry the moderate water-soluble drug doxorubicin (DOX), which was used as a model substance for the carrier function of BGs.

The size of microparticles is, according to definition, between 1 and 1000 µm. Microspheres [4] are monolithic or matrix-type microparticles, whereas microcapsules are of reservoir type. With 1 – 2 µm length and 0.5 – 1 µm width, BGs belong to the fraction of small microparticles. Nanoparticles, of size ranging 10 – 1000 nm, is a collective name for nanospheres and nanocapsules having a matrix structure to which drugs or tracers may be adsorbed at their surface or entrapped in the particle [5].

As the moderate water solubility of some drugs (e.g., DOX) made it difficult to load it physically within polymeric micelles [6] in a sufficient amount, the drugs were chemically conjugated to the polymer block; for example, polymeric nanoparticles composed of poly(L-lactic acid) and methoxy-poly(ethylene glycol) or poly(lactide-coglycolide)/poly(ethylene glycol) di-block polymer were coupled to DOX by an amide or ester linkage. DOX released from these particles was shown to be more cytotoxic against cancer cells than free DOX [7]. The superior quality of BG as a carrier of DOX and its targeting to human colon carcinoma cells will be discussed in Section 2.

Erythrocyte ghosts are cell envelopes devoid of cytoplasma [8], a feature in which they resemble BGs derived from Gram-negative bacteria. Erythrocyte ghosts can be filled with proteins [9], DNA [10,11] or the desired drugs, and can subsequently be resealed due to spontaneous membrane fusion of the flexible erythrocyte ghost membrane [12,13]. Erythrocyte ghosts filled with superoxide dismutase, DOX or methotraxate decrease the viability of monkey kidney cells and inhibit the growth of human cervical tumour cells (HeLa) or macrophages, respectively [14,15].

Smaller in size, liposomes are spherical vesicles ranging 50 – 1000 nm in diameter and are composed of one or more phospholipid bilayers [16-18]. Lipophilic drugs (e.g., amphotericin B, which is used in the treatment of visceral leishmaniasis) can be incorporated into the lipid bilayers [19], whereas hydrophilic drugs are carried in the inner aqueous core. Drug release, *in vivo* stability and biodistribution are determined by size, surface charge, surface hydrophobicity and membrane fluidity of the liposomes [18]. Without modification there is only passive targeting to the liver, spleen or tumours. Administration of liposomes containing DOX resulted in remission of the colon carcinoma C26 solid tumour, although this tumour in mice is practically insensitive to DOX. Furthermore, liposomal DOX can arrest the growth of human lung tumours in severe combined immunodeficient mice and can reduce the incidence of metastases from mammary carcinoma. Moreover, in clinical studies, patients with AIDS-related Karposi's sarcoma treated with liposomal DOX showed good response with minimal toxicity [20,21]. These limited examples of insoluble carrier systems showed that an empty envelope derived from biological particles (red cell ghosts) or material (microspheres or liposomes) can have important carrier functions for drugs and, depending on the size and surface composition, can target various tumours.

The application of BG for active particle-based drug delivery is an alternative approach to the system describe above using a more complex natural biological particle. Derived from the envelope complex of Gram-negative bacteria, BGs are highly versatile for easy modification by genetic engineering of the producer bacteria. And using this technology, BG carrier vehicles can be built on a common platform with modifications for specific purposes of targeting or carrier functions.

## 2. Bacterial ghosts

BGs are cytoplasm-free nondenatured Gram-negative bacterial cell envelopes that are formed by the conditional expression of plasmid encoded gene E derived from bacteriophage PhiX174 [22-24]. By integration of the 91 amino-acid

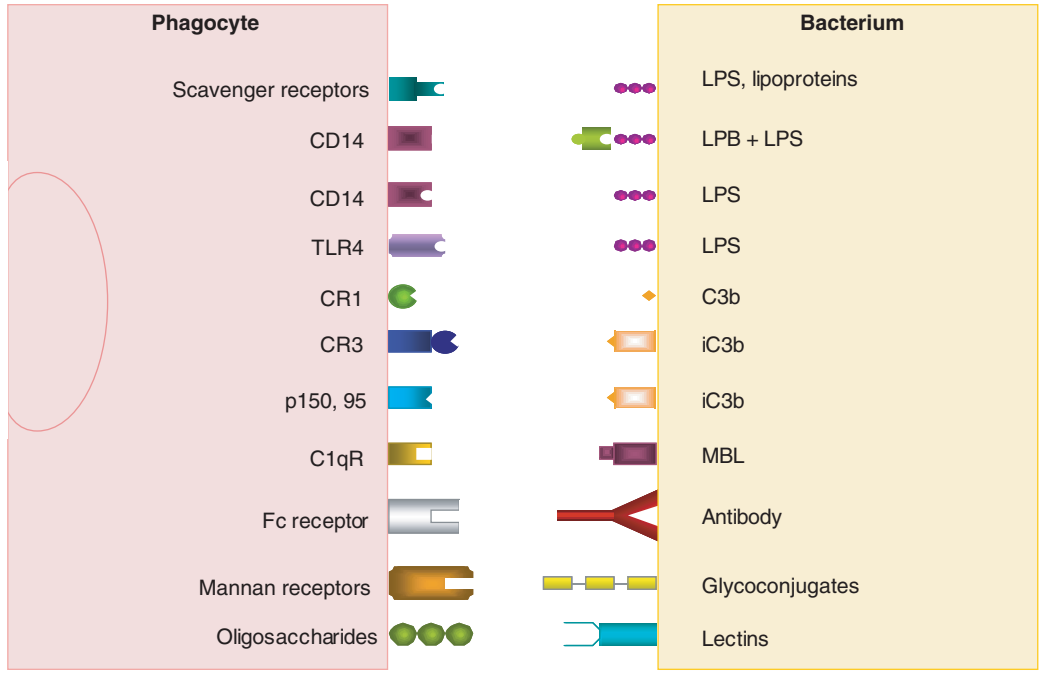


**Figure 1. Interaction of Gram-negative bacteria and phagocytic cells.** A variety of molecules facilitate the adhesion of bacteria to the phagocyte membrane. These include LPS, lectins and glycoconjugates on the bacterial surface and TLR, complement binding receptors (CR1, CR3, p150,95), a receptor ($C1q^R$) for the binding of the MBL, Fc and mannan receptors on the phagocytes surface.
Fc: Crystallisable fragment; LPB: Lipopolysaccharide binding protein; LPS: Lipopolysaccharide; MBL: Mannose-binding lectine; TLR: Toll-like receptors.



polypeptide E in the envelope complex of Gram-negative bacteria a fusion process of the inner and outer membrane is induced, resulting in the formation of a transmembrane tunnel structure of 40 – 200 nm diameter through which the cytoplasmic content is expelled. This cell lysis does not cause any physical or chemical denaturation of BGs providing fully intact surface structures. The application of BGs as vaccines themselves or as carriers of antigens has been extensively investigated [25-38]. The ability of BGs to carry drugs and nucleic acids, and to target various cells and tissues, of animal or human, is discussed in the following section.

## 2.1 Cell targeting with bacterial ghosts

### *2.1.1 Adhesion and uptake rates*

BGs are efficiently internalised by phagocytic cells such as macrophages [36,37] and dendritic cells (DCs) [26,38]. The BG adhesion and uptake rates by targeted cells can be quantified using fluorescein isothiocyanate or Alexa-labelled BGs and flow cytometry. For *Escherichia coli* ghosts the adherence rates (percentage of cells with adherent BGs) for murine macrophages (RAW 264.7) were 83%, and the uptake rates (percentage of cells with internalised BGs) were 89%, respectively [39].

For human monocyte-derived DCs the uptake of *E. coli* ghosts was only one-third compared with *Mannheimia haemolytica* ghosts [38], and a similar observation has been made for Caco-2 human colon cancer cells [40]. The adherence and uptake rates by Caco-2 cells using the same ghost:cell ratios (GCR) of 50 were, 15 and 6% for *E. coli* ghosts, and 64 and 35% for *M. haemolytica*, respectively. For other human colon carcinoma cell lines (e.g., Colo320, Colo205, Colo201 and HT29) using *M. haemolytica* ghosts at a GCR of 1 – 500, adherence rates of ≤ 99% could be determined depending on the cell line and differentiation status of the cells.

The explanation for these varying results lies in the different surface make-up of *E. coli* and *M. haemolytica* ghosts, and of differences in differentiation of the tissue culture cells, respectively (Figure 1, Table 1). For the human melanoma cell lines WM-75, WM-164, WM-239, WM-373, Bowes, SK-Mel-28, A-375, 1F6 and 1F6m, only minor differences in the uptake rates for the three different BGs from *E. coli*, *M. haemolytica* and *Vibrio cholerae* were measured. It was remarkable that the presence of a single surface compound such as the toxin coregulated pilus (TCP) on *V. cholerae* ghosts increased the uptake rates compared with TCP minus *V. cholerae* ghosts by one order of magnitude (Kudela and Lubitz, unpublished data).

### *2.1.2 Targeting structures of the bacterial ghosts*

BGs have been produced from different *E. coli* K12 strains, enterohaemorrhagic (EHEC) and entertoxicgenic strains, *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, *Erwinia cypripedii*, *Helicobacter pylori*, *Klebsiella pneumoniae*, *M. haemolytica*, *Pasteurella multocida*, *Pseudomonas putida*,

**Table 1. Specific adhesins of selected Gram-negative bacteria and receptors on host cells .**

| Bacteria | Adhesin/gene locus | Receptor | Tissue/organ (pathogenicity) | Ref. |
|---|---|---|---|---|
| ***Fimbrial adhesins*** | | | | |
| *Escherichia coli; Klebsiella, Serratia; Shigella; Enterobacter; Salmonella; Citrobacter* | Type I fimbriae/*fimH* | D-mannose | Bladder (cystitis) | [43,46,73] |
| *Escherichia coli* | P pil/*papG* | Digalactoside containing receptors; part of globoseries glycolipid receptors | Kidney (pyelonephritis) | [43,73,74] |
| *Escherichia coli* (uropathogenic) | P pili | GalNacα1-3GalNacβ1-3Galα1-4Galβ1-4GlcCer (globotetrasylceramide) | Kidney (pyelonephritis) | [43] |
| *Escherichia coli* K99 and 987 | K99, K987 fimbriae | GalNacβ1-4Galβ1-4GlcCer | Intestine of pigs (diarrhoea) | [43] |
| *Escherichia coli* K88 | K88 fimbriae | β-D-Gal or GalNac and GlcNac | Intestine of pigs (diarrhoea) | [43] |
| *Escherichia coli* CFA/I and CFA/II | CFA/I, CFA/II fimbriae colonisation factor antigen | βGalNacβ1-4Galα1-4GlcCer ($GM_2$ ganglioside) | Small intestine (diarrhoea) | [43,46] |
| *Escherichia coli/Salmonella sp.* | GVVPQ fimbriae (curli)/*csgA* | | Colon (diarrhoea) | [46] |
| *Salmonella enteritica seovar enteritis* | SEF14, SEF17, SEF21/*agfA* | Glucosylceramide, ganglioside | Human small intestine (diarrhoea), chicken intestinal mucosa | [75-77] |
| *Escherichia coli* | S fimbriae *sfaS* | Sialic acid | ND | [46,78] |
| *Escherichia coli* | F1C fimbriae<br>F18 fimbriae/*fedF* | ND<br>ND | Porcine epithelial cells | [46] |
| *Escherichia coli (ETEC, EPEC)* | CS1, CS2, CS15, fimbrilliae like/*cfaB, csoA, cotA* | ND | Colon (diarrhoea) | [46] |
| *Mannheimia haemolytica A-1* | Large fimbriae | ND | Respiratory epithelial cells; pulmonary alveolus | [79-81] |
| *Vibrio cholerae* | Type IV fimbriae/TCP | Fucose and mannose | Colon (cholera) | [43,46,82] |
| *Neisseria gonorrhoeae, Neisseria meningitidis, Moraxella bovis, Pseudomonas aeroginosa, Dichelobacter nodosus* | Type IV fimbriae; nonhomologous sequences but similar biogenesis | Galβ1-3GalNacβ1-4Gal | Urogenital epithelium, respiratory tract | [43,46] |
| *Bordetella pertussis* | Fimbriae | Sterol | Respiratory tract (whooping cough) | [43] |
| ***Nonfimbrial adhesins*** | | | | |
| Gram-negative bacteria | LPS | CD14, sCD14, LPB | Serum proteins, endothelial cells, reticuloentothelial cells | [51,54] |
| *Escherichia coli* (DAEC) | Dr family adhesins: Dr (haemagglutinin), Afa-I, Afa-III, F1845 | SCR-3 domain of DAF | Human colonic epithelial cells (urinary tract and intestinal infection) | [83-85] |
| *Escherichia coli* (DAEC) | Dr-II family (e.g., *draE2*) | DAF receptor | Tubular basement membranes, Bowman's capsule | [86] |
| *Escherichia coli* (ETEC) | CS22 fimbrilliae like; non fimbrial | ND | Colon (diarrhoea) | [75,87] |

BG: Bacterial ghost; Cer: Ceramide; CFA: Colonisation factor antigen; DAEC: Diffusely adhering *Escherichia coli*; DAF: Decay accelerating factor; EHEC: Enterohaemorrhagic; EPEC: Enteropathogenic *Escherichia coli*; ETEC: Entertoxicgenic; Fha: Filamentous haemagglutinin; Gal: Galactose; GalNac: *N*-acetylgalactosamine; Glc: Glucose; HMW: High molecular weight; HSPG: Heparan sulfate proteoglycan; LPS: Lipopolysaccharide; ND: Not determined; NT: Nontypeable; PAF: Putative adhesion factor; SCR: Short consensus repeat; STEC: Shiga-toxigenic *Escherichia coli*; TCP: Toxin coregulated pilus.

**Table 1. Specific adhesins of selected Gram-negative bacteria and receptors on host cells (continued).**

| Bacteria | Adhesin/gene locus | Receptor | Tissue/organ (pathogenicity) | Ref. |
|---|---|---|---|---|
| *Escherichia coli* (EHEC, STEC) | Intimin (*eaeA*) and others encoded in the locus of enterocyte effacement | Tir (intimin receptor); β1-integrins, nucleoin | Colon (diarrhoea) | [48-50] |
| *Escherichia coli* (EHEC, EPEC) | Putative adhesions *bfpA, lpfA, ihA, lifA* | ND | Colon (diarrhoea) | [88] |
| *NT Haemophilus influenzae* | HMW adhesins Hia, HMW1, HMW2, Hsf | ND | Human nonciliated epithelial cells, mononuclear cells | [89,90] |
| *Mannheimia haemolytica A1* | Adh1 | ND | Respiratory epithelial cells | [89] |
| *NT Haemophilus influenzae* | P2, P5 outer membrane proteins | PAF; β-glucan receptor | Upper respiratory tract (otitis media) | [90,91] |
| *Haemophilus ducreyi* | LspA1, LspA2 DsrA (outer membrane protein) | Vitronectin | Keratinocytes; (sexually transmitted genital ulcer disease chancroid) | [92,93] |
| *Helicobacter pylori* | Hp BabA adhesin | MUC5AC mucin | Stomach epithelial cells | [94,95] |
| *Neisseria gonorrhoeae* | Opa protein | HSPG; $\alpha_5\beta_1$ integrins via vitronectin or fibronectin | Human epithelium of urogenital tract, nasopharynx | [43,46,96] |
| *Salmonella typhimurium* | Invasin/*invH* | $\alpha_5\beta_1$ integrins; CD44; β2-microglobulin | Colonic epithelial cells (salmonellosis) | [97,98] |
| *Shigella flexneri* | Ipa proteins | $\alpha_5\beta_1$ integrins | Colonic epithelial cells (bacillary dysentery) | [99,100] |
| *Bordetella pertussis* | Fha | Galactose containing glucoconjugates; integrin CR3 | Respiratory tract (whooping cough) | [98-101] |

BG: Bacterial ghost; Cer: Ceramide; CFA: Colonisation factor antigen; DAEC: Diffusely adhering *Escherichia coli*; DAF: Decay accelerating factor; EHEC: Enterohaemorrhagic; EPEC: Enteropathogenic *Escherichia coli*; ETEC: Entertoxicgenic; Fha: Filamentous haemagglutinin; Gal: Galactose; GalNac: *N*-acetylgalactosamine; Glc: Glucose; HMW: High molecular weight; HSPG: Heparan sulfate proteoglycan; LPS: Lipopolysaccharide; ND: Not determined; NT: Nontypeable; PAF: Putative adhesion factor; SCR: Short consensus repeat; STEC: Shiga-toxigenic *Escherichia coli*; TCP: Toxin coregulated pilus.



*Ralstonia eutropha*, *Salmonella typhimurium*, *Salmonella enteritidis* and *V. cholerae*. This broad spectrum of bacteria shows that E-mediated lysis most probably works in every Gram-negative bacterium, provided that the E-specific cassette can be introduced into the new recipient by an appropriate carrier plasmid allowing tight repression and induction control of the lethal gene E activity [41,42].

The outer surface of BGs is dependent on the bacterial species used and provides a variety of adhesion structures (adhesins) to mammalian cells (Figure 1). These include the lipopolysaccharide (LPS), lipoproteins, porins, fimbriae and lectins, which are often also associated with pathogenicity. Per definition these adhesins bind to receptors on the host cell (Table 1). The interaction of adhesins with host receptors is highly specific for the species (species specificity), for the host tissues (tissue tropism) and individual gene-encoded receptors (genetic specificity) [43]. Adhesins mediate the adherence of bacteria and, therefore, of BGs to host cells. Once the bonds between adhesins and receptors are formed, the BG attachment to the host cell under physiological conditions becomes virtually irreversible. Even if each bond, by itself, is relatively weak, the likelihood that all the bonds break at the same time is very small [44].

### 2.1.3 *Adhesins and receptors of bacterial ghosts for targeting endo- and epithelial cells*

The most effective adherence factors of pathogenic *E. coli* and other Gram-negative bacteria to endo- and epithelial cells are surface fimbriae; although bacteria without fimbriae are also capable of adhering to, epithelial colon cancer cells for example [45]. Generally, fimbriae are peritrichous, nonflagellar, filamentous, rod-like structures 5 – 7 nm in diameter and are classified as type-I fimbriae (common type fimbriae), P pilli and type IV fimbriae among others (Table 1).

The K88 and K99 fimbriae, for example, are found on various strains of enteropathogenic *E. coli* (EPEC), which bind to the brush border of epithelial cells in the gut of pigs and humans and cause severe diarrhoea [43,46]. These were used by Lubitz *et al.* for drug-targeting purposes [47]. K88, K99 and CFA/I fimbriae are composed of one type of subunit. In CFA/I fimbriae, the subunit located at the tip has an exposed receptor-binding site, whereas K88 and K99 fimbriae have multivalent receptor-binding sites along their length. P fimbriae (also P pili), which bind to the P blood group antigen, are composed of several subunits

encoded by the *pap* operon and present the adhesin PapG to the specific cell receptor. Although fimbriae often play a major role in the adherence of EPEC to mammalian cells, other outer membrane proteins such as intimin of EHEC cause 'intimate' attachment to epithelial cells [48-50].

LPS is another nonfimbrial adhesin that is recognised by endothelial cells through the LPS-binding protein (LBP) and subsequently binding to CD14 receptors; a mechanism that was also reported for the attachment of *E. coli* K12 ghosts [51]. By a different mechanism, the attachment of *E. coli* K12 ghosts to the dermal microvascular endothelial cells (DMECs) is mediated by mannose in the LPS, which is recognised by the mannose receptors on the surface of DMEC [52]. As *V. cholerae* does not possess mannose in its LPS, BGs from this strain do not bind to DMEC [52]. As summarised in Table 1, various other nonfimbrial or non-LPS adhesins are present in the outer membrane of Gram-negative bacteria, which facilitates specific attachment to mammalian cells and tissues.

#### 2.1.4 *Adhesins and receptors for targeting antigen-presenting cells*

Antigen-presenting cells (APCs), macrophages and DCs, are phagocytic cells mainly responsible for 'cleansing' the organism from bacteria and other invading microorganisms. Therefore, the attachment of invading bacteria to phagocytes is an important interaction [53,54]. Lubitz *et al.* have shown that macrophages and DCs efficiently bind and internalise BGs derived from *A. pleuropneumoniae* and *V. cholerae* [26,36].

Host cell lectins, which are proteins capable of binding specific carbohydrate moieties, can bind to β-glucans and to LPS [55,56]. Of particular interest are the complement receptors CR3 and p150,95 and the related molecule leukocyte functional antigen-1 (LFA-1) on the surface of APCs, which have multiple binding sites specific for different carbohydrate moieties. Other receptors on the APC surface are (e.g., C1q) binding mannose-binding lectins, which are present in the serum and recognise bacterial mannose and Fc parts of antibodies bound to bacteria (Figure 1).

Furthermore, a series of Toll-like receptors (TLRs) are present on macrophage and DC surfaces recognising several different bacterial components [57]. Among them is TLR4 recognising the bacterial LPS and so recognising BGs. Bacterial DNA (unmethylated cytosine phosphoryl guanine DNA) is recognised by TLR9 which is selectively expressed on plasmacytoid DCs but not on monocyte-derived DCs [58-60]. Receptor presentation is also dependent on the differentiation status of the APCs; for example, the expression of the human macrophage lectin specific for galactose/*N*-acetylglactosamine [61]. Type A scavenger receptors of macrophages (SR-A) usually implicated in the recognition of apoptotic cells are also capable of binding modified lipoproteins and other polyanionic ligands including LPS [54].

### 2.2 Specific examples for bacterial ghosts as drug-targeting vehicles

#### 2.2.1 *Immobilisation of proteins and drugs in the bacterial ghosts*

The extended BG system [62] provides various possibilities of immobilising proteins and drugs within the BG. Foreign proteins can be tethered to the outer membrane (OM), exported into the periplasmic space (PPS) or inner membrane (IM) (Figure 2). The OM is an asymmetric lipid bilayer with LPS in the outer leaflet and phospholipids in the inner leaflet. The polysaccharide moieties of LPS, filaments and pili extend from the OM to the environment and are important structures to bind to receptors on cell surfaces.

Outer membrane proteins (OMPs) or pili structures can be modified to incorporate sequences that can be used for specific receptor recognition (Figure 2A). Localisation of active substances or proteins in the PPS exported to this compartment (Figure 2C) are not only protected from external degradation processes, but are also immersed in a sugar-rich environment of membrane-derived oligosaccharides, which protects the foreign proteins or substances during lyophilisation. Furthermore, soluble proteins can be expressed in the PPS of BGs as the E-lysis tunnel seals the IM and OM.

The cytoplasmic space of BGs can be filled either with water-soluble proteins, drugs, other active substances or emulsions such that the target protein itself or a matrix can be coupled to appropriate anchors on the inside of the IM of BGs (Figure 2B and D). For example, BGs with streptavidin anchored on the inside of the IM can be filled by resuspending lyophilised BGs in solutions carrying biotinylated proteins or biotinylated matrices [63,64].

For membrane anchoring of target proteins or of acceptor proteins such as streptavidin to the cytoplasmic side of the IM, a membrane targeting system was developed [64]. By cloning foreign DNA sequences into the membrane targeting vector pMTV5, any gene of interest can be expressed as a hybrid protein with N-, C- or N-/C-terminal membrane anchors directing and attaching the fusion proteins to the cytoplasmic side of the IM of the bacteria prior to E-mediated lysis (Figure 2B – D). The current list of membrane-anchored target proteins comprises various viral core or envelope proteins and bacterial target antigens or enzymes. For the latter, it could be shown that the enzymatic activities of β-galactosidase, polyhydroxybutyrate-synthase or alkaline phosphatase were not impaired, thus indicating that the membrane anchors do not interfere with the proper folding of the target proteins and that clustering and self-assembly (e.g., for β-galactosidase) is possible [63].

BGs have been developed more recently for the delivery of DNA. The internal space of BGs can be filled with a substituted matrix (e.g., biotinylated dextran or polylysine), which then binds the nucleic acid of interest (Figure 2B), and if lac repressor proteins (LacI) are membrane anchored they recognise the corresponding operator sequence carried on plasmid DNA.

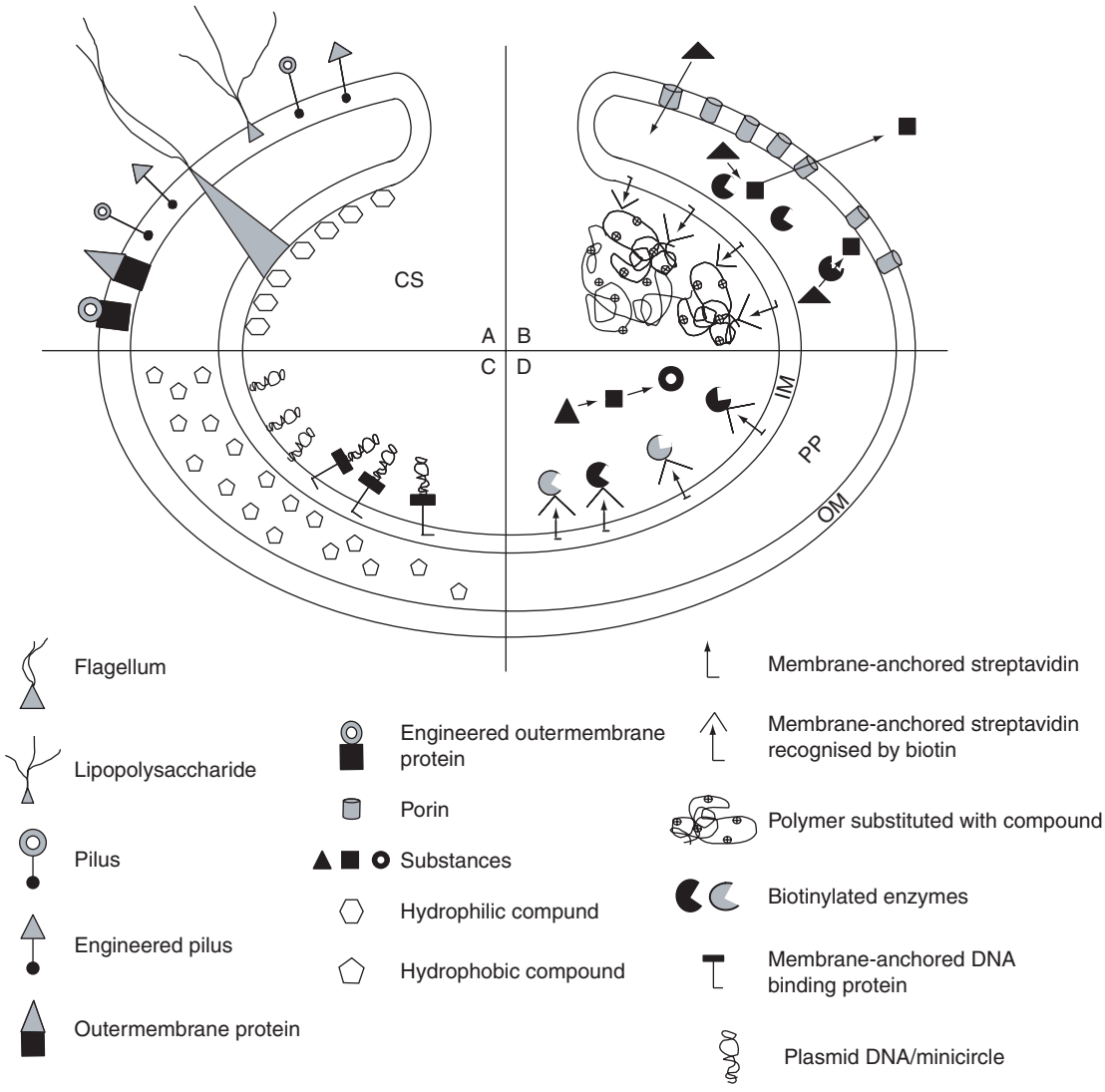


**Figure 2. Bacterial ghosts as advanced drug delivery systems.** Bacterial ghosts can divide into four different compartments, namely the OM, PS, IM and CS. They carry natural (quarter A; e.g., flagella, pili, OM proteins, IM proteins) or engineered components (quarters A – D; e.g., engineered OM or IM proteins, engineered pili, enzymes exported to the PP, membrane-anchored streptavidin and/or DNA binding proteins). Membrane-anchored streptavidin can bind any biotinylated protein/enzymes (quarter D) or polymer (quarter D). By loading the bacterial lumen (CS) with hydrophobic drugs (quarter A) or DNA the compounds can associate with the IM. Hydrophilic drugs or substances can either be loaded in the PS (quarter B) or CS (quarter D), with or without sealing the E-specific transmembrane tunnel structure (quarter A, B).

CS: Cytoplasmic space; IM: Inner membrane; OM: Outer membrane; PP: Periplasma; PS: Periplasmic space.

Plasmids bound to the membrane by this specific interaction are retained in BGs and are not expelled to the culture medium following induction of E-mediated lysis [65].

Recently, the feasibility of plugging the E-lysis tunnel of BGs to entrap water-soluble substances in the cytoplasmic space has been assessed [39]. Using a vesicle-to-ghost membrane fusion system, BGs can be plugged in order to use BGs as carrier systems for soluble, unattached, hydrophilic substances. The sealing process of ghosts requires inside-out vesicles of Gram-negative bacteria and fuses the vesicles to the IM at the edges of the lysis tunnel of the ghost carrier. Orthonitrophenyl-galactoside, calcein and fluorescein-labelled DNA were used as reporter substances to test that BGs can be sealed by restoring membrane integrity [39].

The technique of loosely closing BGs is under optimisation by targeting a vesicle on top of the E-specific transmembrane tunnel. In the most simple model, vesicles can be targeted to the E-specific transmembrane tunnel by specific interaction of biotinylated protein E with membrane-anchored streptavidin on the surface of inside out vesicles, or *vice versa*, by using E-streptavidin fusion proteins for the creation of the E-specific transmembrane tunnel and inside-out vesicles with

membrane-anchored biotinylated receptor sequences. In an alternative model, both receptor sequences on the BGs as well as on the inside-out vesicle display streptavidin on the surface; free biotin is used as a coupling agent. This traps the vesicle on top of the E-specific transmembrane tunnel and can be used to construct BGs carrying back packed carrier and targeting structures, being either biotinylated or modified with streptavidin [66].

#### 2.2.2 Doxorubicin-loaded bacterial ghosts targeting colon carcinoma cells

DOX, a cytotoxic drug commonly used in cancer therapy, was used as a model substance to demonstrate the delivery of moderate water-soluble drugs by BGs [40]. DOX is one of the most frequently used anticancer drugs, which is approved by the FDA but has serious cardiotoxic side effects [67]. Due to the hydrophobic nature of DOX, the lipid environment provided by BGs interacts with DOX, thus providing nonspecific binding of ~ 9 weight DOX/weight BG. Confocal laser scanning microscopy localised DOX in the inner lumen of the BGs (Figure 2A). After uptake of DOX-loaded BGs by Caco2 cells, the drug was released from the lysoendosomal compartment and accumulated in the nucleus of the human colon cancer cells [40]. Compared with free DOX, the application of DOX with BGs reduced the viability of the cells to the same extent at concentrations two orders of magnitude lower than the free drug. In order to simulate an *in vivo* targeting situation, BGs were allowed to adhere to the Caco2 cells for only 10 min, which reduced the viability of the cells considerably at conditions where the free drug had no effect [40].

Another example for loading BGs with a poorly water-soluble drug has been given recently for the delivery of pesticides [68].

#### 2.2.3 Bacterial ghosts as carriers for DNA

DNA as an active agent for somatic gene therapy, and vaccination promises many applications in medical and veterinary science. Important improvements have been made for the DNA vector systems used and the regulation of the DNA-encoded gene expression for protein or siRNA. The procedure of loading BGs with DNA plasmids is simple and can be achieved by resuspending lyophilised BGs in DNA solutions followed by a couple of washing steps. *In situ* DNA–DNA hybridisation and confocal laser scanning microscopy demonstrated the localisation of the external loaded DNA in close association with the inner membrane of BGs (Figure 2C). Depending on the concentration of the DNA solution used, BGs can be loaded with 4000 – 6000 plasmids per ghost [38,69].

*E. coli* and *M. haemolytica* ghosts loaded with plasmid DNA encoding the enhanced green fluorescent protein showed very high transfection efficiencies of ~ 60% for murine macrophages [69], and ~ 85% for human monocyte-derived DCs [38]. Intradermal and intramuscular immunisation studies of Balb/c mice with BG loaded with pCMVβ DNA encoding the model antigen β-galactosidase demonstrated efficient humoral and cellular immune responses against the antigen. Notably for DNA vaccines, and as a direct consequence of the BG carrier used, the T-helper cell ($T_H$) response was modulated from a mixed $T_H1/T_H2$ to a more dominant $T_H2$ pattern [70]. In model studies for potential human applications of the BG–DNA technology, intravenous immunisation of mice with autologous dendritic cells transfected *ex vivo* with pCMVβ-loaded BGs elicited anti-β-galactosidase-specific immune responses [70].

Recently, the multistep procedure of loading BGs with DNA plasmids could be simplified into a one-step procedure with the improvement of *in vivo* production of minicircle DNA and binding to membrane-anchored DNA-binding proteins [65,71] (Figure 2C).

For this application, a site-specific recombination system based on the ParA resolvase encoded by the self-immobilising plasmid system is combined with the protein E-specific lysis technology to produce nonliving bacterial carrier vehicles loaded with minicircle DNA. The *in vivo* recombination process completely divides an origin plasmid into a minicircle and a miniplasmid. The replicative miniplasmid containing the origin of replication and the antibiotic resistance gene is lost during the subsequently induced E-mediated lysis. The minicircle DNA is retained in the BG during the membrane-anchored DNA-binding protein for which the corresponding operator site is encoded on the minicircle DNA. Using this novel platform technology, a DNA delivery vehicle, consisting of a safe bacterial carrier and minicircle DNA with an optimised safety profile, can be produced *in vivo* in a continuous process. This latter system has to be tested for efficacy in comparison with *ex vivo* DNA-loaded BGs. The major advantage of the novel technology lies in the establishment of a simple one-step GMP process for the production of minicircle DNA already carried by its nonliving targeting vector for DNA vaccination and somatic gene transfer.

## 3. Expert opinion and conclusion

The BG system represents a platform technology for antigen, nucleic acid and drug delivery. BGs have significant advantages over other engineered biological delivery particles, owing to their intrinsic cellular and tissue tropic abilities, ease of production and the fact that they can be stored and processed without the need for refrigeration. These particles have found both veterinary and medical applications for the vaccination. The development of a safer and more efficient ADDS based on BGs is a major goal for future applications of the BG platform technology. BGs represent empty nondenaturated nonliving envelopes derived from Gram-negative bacteria for which model studies have shown that they can be used as novel delivery system for drugs, nucleic acids and soluble active compounds for various medical applications.

Current work with BGs concentrates on investigations of the carrier capacity of the cytoplasmic lumen and periplasmic space of the cell envelope. These two separated spaces of BGs

can be filled either with hydrophilic or hydrophobic substances, or emulsions thereof. Proteins and polypeptides of interest can either be produced and anchored in the envelope, or coupled to streptavidin anchored on the inside of the cytoplasmic membrane. The cytoplasmic lumen of BGs can be filled for the delivery of fluid or nonanchored substances, and for this purpose the inner membrane integrity can either be restored by sealing with membrane vesicles or plugged leaving clefts in nanometer ranges. The periplasmic space can be filled with fusion proteins using export sequences from periplasmic proteins, and specific metabolic activities can be stored in this cell compartment. After targeting various cells by the bioadhaesive structures of BGs, uptake of BGs into endosomes and the subsequent fusion to lysosomes are the next events involved in the release of the drug in to the endosomal or lysoendosomal compartments. Depending on their nature, the drugs find their way into the cytoplasma and to the nucleus.

Enzymes or proteins have plenty of room for displaying themselves in the BG compartment, and may be used for modifying the cellular functions of endosomal or endolysosomal organelles. In more speculative future applications the development of new organelles can be envisaged using the BG system carrying genetic information for a minimum of vital functions and active substances.

BGs can be loaded with plasmids either *in vivo* or *in vitro*. The BG-mediated gene transfer to macrophages, which themselves have been proposed as cellular delivery vehicles for adoptive immunotherapy as they localise to sites of inflammation and tumours, is one aim of future applications. Other applications of macrophage transfection include gene-dependent enzyme prodrug therapy or the expression of cytokines for the stimulation of macrophage tumouricidal activity. Therefore, increasing emphasis is given for targeting of macrophages by BG–DNA delivery vehicles.

The vast experience of using BGs as multi-vaccine vehicles based on a combination of proteomic and bioinformatic strategies [72] can be used for the development of BG vesicles with novel target specificities.

Specific emphasis is currently given to the development of mucosal (oral, rectal, intravaginal, aerogen) or noninvasive topical surface applications of BGs. These routes of BG delivery are safe and highly specific for the cells or organs being targeted



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