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Autonomous bacterial nanoswimmers target cancer

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ABSTRACT

Injectable drug delivery systems that autonomously detect, propel towards, and ultimately treat the cancerous tissue, are the future of targeted medicine. Here, we developed a drug delivery system that swims autonomously towards cancer cells, where it releases a therapeutic cargo. This platform is based on viable bacteria, loaded with nanoparticles that contain the chemotherapeutic-antibiotic drug doxorubicin. The bacteria ferry across media and invade the cancer cells, increasing their velocity in the presence of nutrients that are present within the tumor microenvironment. Inside the cancer cells, doxorubicin is released from the nanoparticles, destroying the bacterial swimmer (antibiotic activity) and executing the therapeutic activity against the cancer cells (chemotherapeutic activity). This mode of delivery, where both the carrier and the cancer cell are destroyed, supports implementing nanoswimmers in drug delivery (Fig. 1).

1. Introduction

Nanotechnologies have become important clinical tools, enabling therapeutic precision and functionality that cannot be attained using systems of a larger scale. Newly developed nanotechnologies have the potential to revolutionize diagnosis and care, with >40 nanomedicines already approved for clinical use [1].

The application of nanotechnology has proven to be especially advantageous in cancer therapy. Anti-cancer drugs, such as doxorubicin and paclitaxel, have harsh side effects when used systemically, including vomiting, alopecia and cardiotoxicity [2]. By using nanoparticles that accumulate in the tumor, there is an increase in the effective therapeutic dose at the disease site and reduced adverse side-effects caused by systemic application [3].

Liposomes, vesicles with a lipid bilayer membrane which surrounds an aqueous core, are common nanoscale drug carriers [4]. This structure enables loading hydrophilic drugs into the aqueous core and hydrophobic drugs into the lipid bilayer. The liposome membrane can carry a neutral, positive, or negative charge, by enriching the membrane with cationic or anionic lipids. The liposomal corona can be decorated with targeting moieties to increase accumulation at the target site [5], or, alternatively, creating a stealth polyethylene glycol corona, to disguise the particle from the immune system [6].

While most nanomedicines are dependent on blood flow trafficking to reach their target site, adding a propulsion modality that will actively drive the nanomedicines to the disease site, will improve targeting [7].

Several strategies have been employed to physically drive nanomedicines towards diseased tissues [8–10], including the use of magnets positioned above the disease site or ultrasonic waves that propel drug-loaded microbubbles towards tumors [11,12].

Micro- and nano-swimmers are systems that propel autonomously, or under an external force, towards a target site. In a recent study, biohybrid materials with a sperm-like structure were developed to self-propel in media by using filaments composed of a rigid head and elastic tail, covered with contractile cardiomyocytes [13]. Adapting autonomous swimmers for drug delivery has been suggested, however, shown only recently [14–16].

Since the 1800′s, bacteria have been used as vehicles for therapeutic applications [16–22]. Most bacteria propel using flagella, 10 μm long and 20 nm in diameter filaments that extend from the bacterial body [23,24]. Flagella rotation can be either clockwise or counterclockwise, for forward or backward swimming [25]. Bacteria use their aptitude of motility to survive and move towards favorable, or flee from unfavorable, microenvironments. They source their power from nutrients in their environment by converting chemical energy to mechanical energy. Bacteria use chemoreceptors that sense favorable nutrient-rich environments, and then navigate towards these sites, in a process called chemotaxis [22]. This motility enables them to overcome diffusion resistances, which is beneficial for improving tissue invasion [26].

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In particular, bacteria favor nutrients found in the tumor microenvironment [21,27,28]. Pre-clinical and clinical studies have demonstrated that bacteria accumulate preferentially in cancerous tissue [29–31]. In-vivo experiments have demonstrated that bacteria are attracted to the cancerous tissue by a unique set of nutrients that are secreted metabolically in the tumor microenvironment, including – serine, aspartate, ribose, leucine and arginine [20–22,27,28,32,33,35]. The extracellular matrix of the tumor microenvironment is used by some types of bacteria as anchoring points for propagating within the tissue [36].

Because of their easily manipulated genetics, bacteria can be engineered to overcome toxicity and systemic implications when injected into the human body. *Salmonella* with a modified lipid A (strain vnP20009) is non-toxic but colonizes in tumors upon injection [1,37–40].

To invade cells, bacteria either secrete a set of proteins that puncture the host cell, causing cytoskeletal rearrangement, resulting in bacterial uptake. Otherwise, invasion can occur when the bacteria binds to receptors on the host cell, and is then taken up via cytoskeletal-mediated rearrangements of the cell plasma membrane around the bacteria [26,41].

This study examines the potential of bacteria as carriers of nanomedicine for targeted delivery of cancer drugs. We loaded bacteria with nanomedicines, to autonomously target malignant cells (Fig. 1).

2. Materials and methods

Hydrogenated soy phosphatidylcholine, HSPC, was purchased from Lipoid GmbH (Ludwigshafen, Germany); 1,2-dioleoyl-3-trimethylammonium-propane, DOTAP; 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol), DSPG; 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine-B sulfonyl) ammonium salt, 18:1 Liss Rhod PE, were purchased from Avanti Polar Lipids (USA); cholesterol (Ch), sulforhodamine-B, ampicillin (amp) and doxurubicin-HCl were purchased from Sigma-Aldrich.

2.1. Bacterial strains and growth conditions

Salmonella enterica subspecies *enterica* serovar *Typhimurium* LT2 (ATCC 700720) were transformed with pGFP plasmid (Clontech, CA, USA) by electroporation using MicroPulser electroporator (Bio-Rad Laboratories, Ca, USA). Transformants were selected on Luria-Bertani (LB) agar plates (1.5% w/v) supplemented with ampicillin (100 μg/mL) and stored at −80 °C in LB medium supplemented with 30% glycerol (%v/v).

Escherichia coli (ATCC 53323), JM109 (F′ traD36 proA+ proB+ laclq Δ(lacZ)M15 Δ (lacproAB)supE44 hsdR17 recA1 gyrA96 thi1endA1 relA1 e14λ) was grown on LB medium. Both strains were examined in motility assays. *Salmonella typhimurium* LT2 was further examined as carrier in in vitro experiments.

2.2. Motility assay

Bacteria glycerol stocks were streaked on LB plates supplemented with 100 μg/mL ampicillin (amp100) and grown at 37 °C overnight (O/N). A single colony was inoculated in fresh LB media and grown O/N at 37 °C, at 250 rpm in a TU400 orbital incubator shaker (MRC, Holon, Israel). This was used as a starter to inoculate fresh LB media the following day at a ratio of 1:10 (starter:media). The culture was grown at 37 °C until an OD_{600} ~0.5–0.7 (mid-log phase) was reached; then, centrifuged and washed three times with potassium phosphate buffer (0.1 M at the appropriate pH). The desired glucose concentration was added afterwards. Bacteria were observed in bright field using an inverted Nikon Eclipse TS100 microscope (NU, USA). Velocity was measured as distance/time, where distance was measured using data analysis software NIS-Elements D4.10.00.

2.3. Effect of doxorubicin on bacteria

The antibacterial effect of DOX on bacteria was examined adding increasing concentrations of DOX to *Salmonella* in suspension (OD_{600} = 1). DOX concentration was at range 1.2 ng/mL up to 12 mg/mL. The viability of *Salmonella* cells was evaluated by live cell count. Decimal dilutions of bacterial suspension were seeded in 20 μL droplets on LBamp100 plates. The plates were incubated O/N at 37 °C. The next-day bacterial colonies were counted in each of the DOX dilutions. Colony forming unit (CFU)/mL was determined according to: $\sum$ colonies / ($\sum$ volume of droplets × $\sum$ dilutions), cfu/mL.

2.4. Invasion of Salmonella into 4 T1 cells

1×10^5 cells/well (2 mL) of triple negative breast cancer mouse cell-line (4 T1; ATCC CRL2539) were seeded in 6-well plates in a RPMI 1640 medium (Sigma-Aldrich), supplemented with 10% heat inactivated Fetal Bovine Serum (Biological Industries, Beit-Haemek, Israel), 2 mM of L-Glutamine Solution, 10 U/mL of penicillin G sodium salt and 0.01 mg/mL of streptomycin sulfate for 24 h at 37 °C in a 5% CO_2 humid atmosphere. The following day, the 4 T1 cells were washed, to remove remnants of antibiotics, and *Salmonella* at multiplicity of infection (MOI) 100:1 was added for 3 h. The 4 T1 cells were washed with PBS and gentamycin sulfate at concentration of 20 μg/mL (Biological industries, Israel) to remove external bacteria. Cells were incubated with Hoescht 33342 (Sigma-Aldrich) and Dil (life technologies, Israel) to

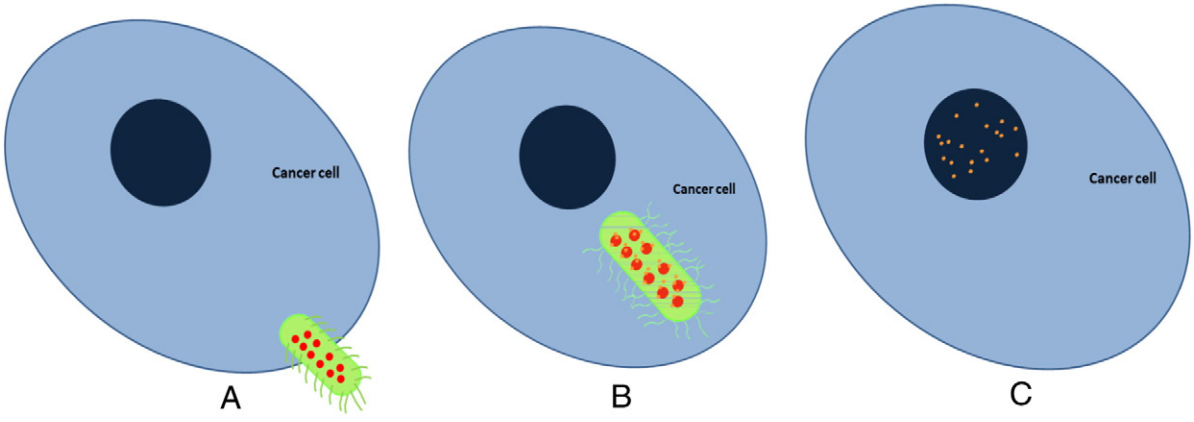

Fig. 1. Therapeutic nano-swimmers. (A) Viable bacteria, with the capacity to autonomously swim in media, are transformed into carriers of anti-cancer agents. The bacteria are loaded with nanoparticles that contain the anti-cancer agent and antibiotic doxorubicin. (B) Cancer cells secret a unique set of nutrients that are strong attractants to the bacterial swimmer. The drug-loaded bacteria swim towards and invade the cancer cell. (C) Inside the cell, the drug releases from the nanoparticle, killing the bacteria and destroying its envelope. Following bacterial death the doxorubicin executes its activity against the invaded cancer cell. Bacteria are shown in green, liposomes in red and doxorubicin in orange.