Sensors and Actuators B 224 (2016) 217–224

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

Active tumor-therapeutic liposomal bacteriobot combining a drug (paclitaxel)-encapsulated liposome with targeting bacteria (*Salmonella Typhimurium*)

Van Du Nguyen, Ji-Won Han, Young Jin Choi, Sunghoon Cho, Shaohui Zheng, Seong Young Ko, Jong-Oh Park*, Sukho Park*

School of Mechanical Engineering, Chonnam National University, Gwangju 500-757, Republic of Korea

ARTICLE INFO

Article history:
Received 7 April 2015
Received in revised form 27 July 2015
Accepted 7 September 2015
Available online 9 September 2015

Keywords:
Liposome
Microrobot
Salmonella Typhimurium
Paclitaxel
Tumor therapy
Bacterial actuation

ABSTRACT

We propose a new tumor-therapeutic bacteria-based microrobot (bacteriobot) combining a paclitaxel-loaded liposomal microcargo with tumor-targeting *Salmonella Typhimurium* bacteria. The tumor-therapeutic liposomal bacteriobot was constructed by binding biotin molecules displayed on the outer membrane proteins of the bacteria and streptavidin coated on the drug-loaded liposomes. First, we performed a motility analysis of the bacteriobot, where the bacteria-actuated liposomes showed much higher average velocity (3.09 ± 0.44 μm/s) than the liposomes without bacterial actuation (0.40 ± 0.14 μm/s). Second, we performed a cytotoxicity test using a breast cancer cell line (4T1) to check the tumor-therapeutic efficacy of the bacteriobots. The drug-loaded bacteriobots ($IC_{50} = 16.48 \pm 0.43$ μg/ml) showed better tumor-killing ability than the drug-containing liposomes ($IC_{50} = 21.91 \pm 0.74$ μg/ml). Moreover, the bacteriobots showed strong tumor-targeting and killing properties in a simple co-culture chamber containing normal cells (NIH/3T3) and cancer cells (4T1). These results revealed that the constructed bacteriobots can be used for active tumor therapy.

1. Introduction

Recently, many research groups have focused on developing prototypes of microrobots for various medical applications [1–8], especially in anticancer therapy, because of their potential advantages over conventional methods such as chemotherapy and radiotherapy [6]. In addition, some proposed microrobots have special abilities to target the inaccessible parts of the human body to perform diagnoses or minimally invasive operations [9]. Among the various types of microrobots, bacteria-based microrobots (so-called "bacteriobots") that consist of a microcargo and attached flagellated bacteria have been proposed. They can offer the intrinsic valuable characteristics of bacterial microorganisms, such as self-sensing, self-propulsion, fluorescence, and chemotaxis toward tumor sites. They can also have tumor-therapeutic effects through the loading of anti-cancer drugs into their microcargos. Therefore, in the design of a bacteriobot, the most important thing is the selection of a bacterial strain with characteristics of a directional actuator to target the tumor region. Various bacterial strains, such as *Escherichia coli*, *Serratia marcescens*, magnetotatic bacteria (MTB) and *Salmonella Typhimurium* (*S. Typhimurium*), have been used to develop bacteriobots [7,10,11]. However, there are some limitations regarding their use in biomedical applications, such as pathogenicity, antibiotic resistance (*E. coli* and *S. marcescens*), a difficult culturing process, and complicated coiling system requirements (MTB) [12,13]. Therefore, an attenuated *S. Typhimurium* bacterial strain has recently been used in the diagnosis and treatment of colorectal and breast cancers because of its ability to target the tumor microenvironment using its flagellated actuation and chemotactic receptors. In addition, it has been reported that the attenuated *S. Typhimurium* bacterial strain has anti-cancer effects [6,14–16].

Until now, in various prototypes of bacteriobots the bacteria were attached to microstructures using several different bacterial attachment methods. For instance, Behkam and Sitti used a plasma reactive ion etching technique to attach bacteria onto a microbead [17]. Our group proposed a method of bacteria patterning on biocompatible Polyethylene glycol (PEG) microbeads by selectively using poly-L-lysine (PLL) coating [18,19]. We also developed a different method for adhering bacteria to alginate microbeads by

* Corresponding authors.
E-mail addresses: jop@jnu.ac.kr (J.-O. Park), spark@jnu.ac.kr (S. Park).

http://dx.doi.org/10.1016/j.snb.2015.09.034
0925-4005/

coating the beads with 1% chitosan and proved that the flagellated bacteria showed enhanced adhesion onto the chitosan-coated alginate microbeads [20]. In addition, the high affinity between biotin and streptavidin was also used to adhere bacteria to microstructures [6,21].

For the tumor-therapeutic function of the bacteriobots, liposomes for anti-tumor drug loading have been considered as microcargos of the bacteriobots. Recently, Mhanna et al. proposed artificial bacterial flagella that can adsorb calcein-loaded liposomes for single-cell drug delivery [22]. Qiu et al. reported the use of artificial bacterial flagella attached with temperature-sensitive liposomes for the controlled release of a drug model (calcein) [23]. Kojima et al. [21] reported a liposome driven by bacteria as a new drug delivery system (DDS). They used a raft domain binding technique to attach a *Vibrio alginolyticus* mutant strain (VIO5) to a liposome employing the high-binding affinity between biotin and streptavidin. Kojima et al. [24] and Zhang et al. [25] also proposed another attachment method involving the same bacterial strain (VIO5) with liposomes using an antibody and showed increased liposomal mobility through the bacterial adhesion. However, because these proposed bacteriobots did not contain any anti-tumor drug, they could not induce any tumor-therapeutic effect.

Therefore, we aim to develop the first active tumor-therapeutic liposomal bacteriobot that utilizes a tumor-targeting bacterial strain (*S. Typhimurium*) to directionally drive an anti-tumor drug (paclitaxel)-loaded liposomal microcargo. We analyze the motility of the liposomal bacteriobots and evaluate their therapeutic effect using cytotoxicity tests. By conducting tumor-targeting and killing tests in a simple co-culture chamber with normal cells (NIH/3T3) and tumor cells (4T1), we show that our proposed liposomal bacteriobots have a better therapeutic effect on the murine mammary carcinoma cell line (4T1) compared to conventional drug-loaded liposomes.

2. Materials and methods

2.1. Design of drug-loaded liposomal bacteriobot (DL bacteriobot)

Fig. 1 shows a schematic diagram of a drug-loaded liposomal bacteriobot (DL bacteriobot). The DL bacteriobot consists of an L-α-phosphatidylcholine (Egg PC, Sigma–Aldrich Chemical, St. Louis, MO)/1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000]-ammonium salt (DSPE-PEG(2000)-biotin, Avanti, Alabaster, AL) liposome loaded with an anti-tumor drug (Paclitaxel) and tumor-targeting bacteria (*S. Typhimurium*). The attachment between the bacteria and the drug-loaded liposome of the DL bacteriobot was performed using a streptavidin–biotin binding mechanism [6,21]. The bacteria were engineered to display biotin molecules on their outer membrane proteins and were incubated with an Egg PC/DSPE-PEG(2000)-biotin liposome coated with streptavidin.

2.2. Anti-cancer drug-loaded liposomes (DLs)

2.2.1. Preparation of liposome cargo

Liposomes were prepared using the thin film hydration [26] and sonication method. A lipid solution with a concentration of 10 mg/ml was prepared using Egg PC and DSPE-PEG(2000)-biotin with a molar ratio of 99.5:0.5 and a chloroform/methanol (9:1, v/v) solvent. The organic solvent for the lipid solution was evaporated overnight in a fume hood and completely removed in a vacuum for an additional 2 h to obtain a dry film. The dry film was hydrated using phosphate-buffered saline (PBS, pH 7.4) for 4 h to form multi-lamellar vesicles that were then homogenized by sonication for 60 s (VC750, Sonics & Materials, Newtown, CT). The vesicle solution was washed two times with PBS by centrifugation for 10 min at 12,000 rpm and incubated with 500 μg/ml streptavidin (Sigma–Aldrich Chemical) for 30 min at room temperature. The final liposomes were then washed two times to remove unbound streptavidin and stored in a refrigerator at 4 °C. The average size of the liposomes was evaluated using an inverted microscope (Ti-U, Nikon USA, Melville, NY) and a MATLAB program after a 40-fold dilution with PBS. The surface morphology of the liposome was investigated using a scanning electron microscope (SEM, SS-550, Shimadzu, Kyoto, Japan). Briefly, 5 μl of the liposome solution was placed on a coverslip and air dried in a fume hood at room temperature for 1.5 h. The sample was then examined in the SEM system. In addition, to load the anti-tumor drug into the liposome before hydration of the dry film, paclitaxel (6 mg/ml) (Shinpoong, Seoul, Korea) was diluted from the stock solution to 200 μg/ml and used as the hydration solution.

2.2.2. In vitro drug release study

The in vitro drug release of paclitaxel from the DLs and DL bacteriobots was examined using a dialysis technique [27]. To accomplish this, 50 μg of the paclitaxel formulated in DLs and the same amount of paclitaxel formulated in DL bacteriobots were added to dialysis bags (molecular weight cutoff of 3500 Da) that were immersed in 20 ml PBS solution in 50 ml falcon tubes. The tubes were shaken in a shaking incubator at 100 rpm and 37 °C. At each predetermined time interval, a 1 ml sample was harvested and the same volume of PBS was refilled. Each sample was added to 1 ml of dichloromethane (DCM). Next, the PBS was removed and the remaining DCM was evaporated overnight in a fume hood to obtain the eluted drug volume in the bottom of the sample vial. The amount of paclitaxel in each sample was measured using high performance liquid chromatography (HPLC, Shimadzu). Based on the obtained HPLC results, the drug release profiles of DLs and DL bacteriobots were calculated.

2.3. Drug-loaded liposomal bacteriobots (DL bacteriobots)

2.3.1. Culture of tumor-targeting bacteria

The *S. Typhimurium* strain (SHJ2037) was genetically modified by the defection of guanosine 5′-diphosphate-3′-diphosphate (ppGpp) for attenuation. The bacteria were cultured for 12 h in a Luria–Bertani (LB) broth medium (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl) with the addition of 50 μg/ml ampicillin and 50 μg/ml kanamycin. Before use, the bacteria were further cultured in a solid medium (4 g tryptone, 2 g yeast extract, 4 g NaCl, 6 g agar, 400 ml of DI water; pH 7.0) for 12 h. Next, bacteria colonies were harvested and incubated again in the liquid medium for 4 h. In addition, bacteria density was measured using a spectrophotometer at a wavelength of 600 nm (UV mini-1240, Shimadzu).

2.3.2. Attachment of bacteria to drug-loaded liposomes

For the surface modification of the bacteria, we used EZ-link NHS-LC-Biotin (Thermo Scientific, Rockford, IL) to display biotin molecules on the outer membrane proteins of *S. Typhimurium*. Briefly, 1 ml of the bacterial solution (3×10^8 CFU/ml) was exposed to 500 μg of EZ-link NHS-LC-biotin at room temperature for 30 min. Next, the biotin-displaying bacteria were washed two times with PBS and further incubated with streptavidin coated liposomes containing paclitaxel, which were fabricated as mentioned in Section 2.2, for 30 min at room temperature to make the DL bacteriobots. The final optical density of bacteria was 0.1 and the ratio of bacteria and liposomes was 5:1 (v/v). The bacteria and the liposomes were then adhered as a result of the high interaction between biotin and