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The Cytotoxic Effect of Polyelectrolyte Shells Coated Bacterial Cells on Human Leukemia Cells

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Abstract

Encapsulation of cells in polymeric shells allowing for separation of biological material from produced factors may find application in the systems for biological processes regulation. Inadequate efficiency of existing therapeutic anti-cancer regiments and the rise of multi-drug resistant cancer cells have required investigations into novel anticancer strategies. Enhancement of apoptosis in tumors has been suggested as a new anticancer strategy. Pathogenic microorganisms may have the role as the source of agents for apoptotic therapy.

Modified cells of *Bacillus subtilis* were encapsulated using layer-by-layer technique within polymeric shells for application in local anti-tumor therapy.

The applied shells were modified with incorporated fullerene derivate to ensure the layers stability and integrity. The impact of modified nano-thin shells coated bacterial cells on human leukemia cells was evaluated *in vitro*. It was observed that coating with applied polyelectrolyte layers with incorporated fullerenol allowed for bacterial cells functioning during the culture period and the lethal impact on eukaryotic cells was observed.

Applied membrane conformation allowing for functioning of encapsulated microorganisms may be recommended for coating shells for local anti-tumor treatment purposes.

Keywords: Encapsulation; Polyelectrolyte; Modified bacterial cells of *Bacillus Subtilis*; Human leukemia cells; Coating shell

Introduction

Separation of biologically active material from produced factors is receiving a great deal of interest regarding biotechnological usability. A system of biologically active material coated with polymeric shells insuring delivery of intact active factors and interspacing from host immunological system (in case of cell transplantation) may serve for biological processes regulation in biotechnological applications.

In spite of efforts to control cancer, the mortality associated with this disease has been increasing in developed countries in the recent decade. Inadequate efficiency of existing therapeutic regiments and the rise of multi-drug resistant cancer cells are the main factors which require a broadening of investigations into novel anticancer strategies. Enhancement of apoptosis in tumors has been suggested recently as an optional anticancer strategy [1,2] and generation of apoptotic anticancer agents has been receiving attention. Pathogenic microorganisms may have the role as the source of agents for apoptotic therapy.

Encapsulation in polyelectrolyte membranes may be proposed to separate microorganisms from produced factors and to separate the host from the bacterial cells. Also, the coating shell allows protecting the coated material against the host immunological system.

The layer-by-layer technique (LbL), using self assembly processes of polymers involving electrostatic interactions was applied to immobilize the microorganisms within the membranes. The alternate deposition of differently charged polyions onto charged substrates allows building up polyelectrolyte multilayers. Different polyelectrolyte materials have been applied to form the layers on biological material e.g. poly (styrene sulfonate) and poly(allylamine), poly(ethylene glycol) and poly(vinyl alcohol), hyaluronic acid and poly(L-lysine) [3,4]; chitosan and carboxymethyl cellulose [5], poly(acrylic acid) poly(ethylene oxide)-block-poly(caprolactone) [6]. In our experiments, the modified *Bacillus subtilis* bacterial cells were coated using the polyelectrolyte (PE) shells, modified with the fullerenol introduced into the shells to increase integrity of the system.

Bacillus subtilis is a model Gram-positive bacterium which products are generally regarded as safe (GRAS). This microorganism is widely used in biotechnology because of efficient proteins production as well as the possibility of gaining GRAS status for *B. subtilis*-derived proteins [7,8]. *B. subtilis* was the first platform for heterologous expression of listeriolysin O (LLO), a hemolysin from the pathogenic bacterium *Listeria monocytogenes* [9,10]. LLO is a protein of 58 kDa molecular weight encoded by gene hly. Its physiological function relies on pores performing in endosome membrane allowing for bacteria release to the cytoplasm causing lethal for the cell [9,10].

The bacterial cells producing LLO were immobilized within modified nano-thin polyelectrolyte membrane coatings as a system for anticancer agent production. Their impact on human leukemia cells was evaluated *in vitro* to assess the bacterial lethal impact on target leukemia cells.

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Materials and Methods

Reagents

poly-L-lysine hydrobromide (MW 15-30 kD) (Sigma, USA), poly-L-lysine FITC labeled hydrobromide (MW 15-30 kD) (Sigma, USA), poly(ethylenimine) (MW 60 kD) (Aldrich, USA), Polyhydroxy small gap fullerenes, hydrated (Sigma, USA); (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, USA); Live/Dead® BacLight™ Bacterial Viability and Counting Kit (L34856) (Molecular Probes, USA); Isopropyl β-D-1-thiogalactopyranozyd (IPTG) (ICN, USA).

Media RPMI 1640 (Gibco, USA); Luria-Bertan (LB) Broth, Miller (Difco, USA); Brain Heart Infusion Medium (BHI) (Becton-Dickinson, USA), Newborn calf serum (NCS) (Gibco, USA).

Culture media: RPMI-1640 supplemented 10% NCS (RPMI-1640/10% NCS).

Culture medium for bacteria: BHI supplemented with 100 μg/ml erythromycin and 100 μg/ml chloramphenicol.

Cell line: Jurkat-human leukemia T-lymphocyte cell line.

The Jurkat human leukemia T-lymphocyte cell line (American Type Culture Collection, Rockville, MD, USA).

Peripheral blood mononuclear cells: They are obtained from chronic lymphocytic B leukemia patients. Blood collection was approved by patients.

Bacterial strains: The *B. subtilis* strains used in this study are derived from an asporogenic strain containing a spoIIE: Tn9I7fQHU181::1ac55 insertion designated MB4 [11]. *B. subtilis* strain BR1-S is a recombinant strain expressing hemolysin (LLO). Gene hly encoding LLO from *L. monocytogenes* strain 10403S was cloned in vector pAG58 and transformed into MB4 resulting in strain BR1-S [12]. Plasmid pAG58-hly was engineered to remove lacI repressor coding sequence. Obtained vector was used to transform MB4 strain producing BR1-C strain, capable of IPTG independent LLO synthesis.

Zeta potential measurement

Evaluation was performed in Zeta Potential Analyzer Zetasizer Nano Z (Malvern Instruments, UK) at parameters: electrical field 8-10 V/cm, measure time 20-120 s, temperature measure 20°C, conductivity 120-150 mS/cm.

Fluorescent staining of bacterial cells

Live/Dead BacLight Kit (Molecular Probes) containing the mixture of two nucleic acid stains–green-fluorescent Syto 9 dye (dying alive cells) and red-fluorescent propidium iodide (dying necrotic cells) was used to examine the viability of bacterial cells. Kit was used according to the producer's recommendations. The preparations were covered with a cover glass and observed with a fluorescence microscope IX70 (Olympus).

Confocal laser scanning microscopy (CLSM)

CLSM cells were transferred to 8-well Labtek II chambered coverglasses (Nunc). Imaging was performed on an FV1000 system with spectral detectors (Olympus), using a 60x/1.20 water immersion objective lens. Fluorescence of FITC was excited with the 488 nm line of an argon-ion laser and collected in the 500-600 nm range. Images were processed using the Fluoview and Fiji software.

Flow cytometry

The presence of organisms was assessed using Canto II flow cytometer (Becton Dickinson Immunocytochemistry Systems, USA). The results were processed by the FACS Diva software system (Becton Dickinson, USA). Evaluated objects were separated from other events on light scatter characteristics.

Spectroscopic evaluation

The PE layers presence was confirmed by evaluation of spectrum of absorption for red irradiation (FTIR). The FTS3000MX (BioRadExcalibur, USA) device was applied. The 16 scans were collected with resolution 4 cm^{-1}.

Statistics

Mean values and standard deviations as well as significance of difference were calculated in the Statistica 7.1 software. The values of $p < 0.05$ were assumed as significant.

Procedures

Bacterial strains growth conditions: Bacteria were grown in LB and BHI supplemented with erythromycin (1 μg/ml) and chloramphenicol (3 μg/ml) at 37°C with 120 rpm shaking. *B. subtilis* overnight cultures grown in LB were diluted and were grown in BHI until OD=1.5 was reached. In order to induce LLO synthesis (in BR1-S) IPTG was added to a final concentration of 1 mM. All strains had similar OD values when cells were taken for the experiments.

Jurkat cells culture: The Jurkat human leukemia T-lymphocyte cell line was maintained in RPMI-1640 supplemented with 10% newborn calf serum and 1% penicillin and streptomycin. Cells were passaged every third day by diluting to the concentration of about 0.5×10^6. The cells were grown at 37°C in atmosphere of 5% CO_2 in air.

Human peripheral blood mononuclear cells preparation: The suspension of peripheral blood mononuclear cells was obtained from WBC of chronic lymphocytic B leukemia patients at 40,000 to 100,000 leukocytes/μl. The patient's leukemia B cells represented at least 90% of the whole leukocytes. The blood was diluted twice with physiological saline, mixed, and layered on Accu-Prep reagent for mononuclear cells isolation at the volume ratio of 3:1, centrifuged at 2,000 rpm for 30 minutes. The mononuclear cells suspension was collected from interphase, washed with RPMI-1640/10% CS culture medium, centrifuged at 1,200 rpm for 15 minutes and then suspended for culture. The isolated cells were initially cultured for 7 days to eliminate broken cells as well as trace erythrocytes and monocytes. Blood collection was approved by patients, and the procedure was approved on 27 February, 2008 by the Local Ethics Committee of the Medical Center of Postgraduate Education, Warsaw, Poland.

Preparation of complexes with fullerenol: The polyethyleneimine (PEI) with fullerenol complex preparation was obtained accordingly to the previously described procedure [13]. The complexes containing fullerenol at concentrations 0.250 mg/ml were obtained (FUOL).

Coating of bacterial cells with polyelectrolytes: 5×10^8 bacterial cells *Bacillus subtilis* BR1-S or BR1-C were incubated with polylysine (PLL) solution in 0.1 M NaCl at concentration 1 mg/ml at pH 7.2. After 3 min deposition time cells were washed with RPMI-1640 at 8000 rpm for 3 minutes to remove unabsorbed polyelectrolyte.

Encapsulated PLL bacterial cells were incubated with solution of PEI with incorporated fullerenol in 0.1 M NaCl at concentration 1 mg/ml at pH 7.2.

After 4 min deposition time, cells were washed 2-times with RPMI-1640 at 8000 rpm for 3 minutes to remove unabsorbed polyelectrolyte. The layers PLL/FUOL were produced.

Evaluation of encapsulated bacterial cells impact on human leukemia cells: The suspension of human leukemia cells Jurkat or B-CLL at concentration 1.5×10^6 cells/ml was cultured in culture medium RPMI-1640/10% NCS with addition of erythromycin (3 μg/ml) in presence of the immobilized polymeric shells, bacterial cells BR1-S or BR1-C reached a concentration of about 10^8 nanocapsules/ml. To induce LLO production and release by the immobilized BR1-S bacterial cells the IPTG at concentration 1 μg/ml was added to the culture. As a negative control the leukemia cells were cultured without presence of immobilized bacteria. After 2 or 24 hours incubation the samples of cell suspension were centrifuged at 1200 rpm for 3 minutes and washed with RPMI 1640/10%CS. Then, the pellet was suspended in the culture medium to assess cell viability. The viability of cells was evaluated using cytochemical reaction with propidium iodide (PI) in flow cytometer.

The coating efficiency assessment: The release of bacterial cells from coating shell was assessed: After 24-hour culture, the suspension of leukemia cells cultured in presence of coated bacteria was centrifuged at 1200 rpm for 4 minutes, washed with RPMI-1640/10% CS suspended in the culture medium and cultured for 24 hours to assess the cell viability using cytochemical reaction with PI in flow cytometer.

Microscopic evaluation: Bacterial cell viability assessment was made on the basis of a set of photos of preparations stained LIVE/DEAD® BacLight Kit. Live cells were distinguished by the presence of CYTO-9. It permeates through untouched cell membrane, binds to nucleic acids expressing intensive fluorescence. PI enters cells with damaged cell membrane and binds to nucleic acids, thereby producing a red fluorescence of dead cells (495 nm/635 nm). Photographs were taken after 24 hour culture.

Results

The coating shell layers presence was confirmed using FTIR evaluation in spectrum 4000-666 [cm^{-1}] which was described previously. The analysis of Zeta potential demonstrated that fullerenol incorporation to the layer diminished membrane potential Zeta about 17% as compared to the membrane without incorporated fullerenol. Nevertheless, there was no impact of that difference on bacterial cells viability observed during short-time experiment *in vitro*.

To visualize the polyelectrolyte layer attached to the bacterial cell, the fluorescently labeled polylysine was used. The bacterial cells covered with PLL-FITC shell, visualized using confocal microscopy are presented in the figure 1. The fluorescent surface of shell covering the bacterial cell was demonstrated.

The bacterial cells, BR1-S and BR1-C viability was examined using fluorescence microscopy after coating with PE shell. The bacterial cells BR1-S or BR1-C encapsulated within PLL/FUOL layers exhibited intensive homogeneous green fluorescence of CYTO-9. The lack of red fluorescence proved the maintenance of intact cell membrane. Figure 2 represents the fluorescence microscope visualization of the suspension of bacterial cells BR1-S coated with modified polylysine polyethyleneimine bilayer after 24 hour culture. The intensive fluorescence of coated living bacterial cells was observed.

The applied coating provided protection from bacteria escaping through the coating shell during the experiment. It was observed that after removal of coated bacterial cells the number of viable cells

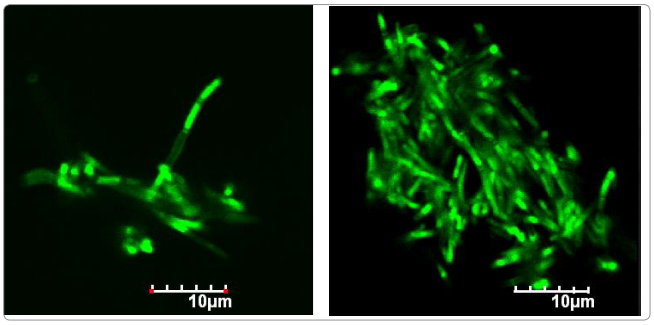

Figure 1: Visualization of coating shell: confocal laser scanning microscopy images showing green fluorescence of the polylysine FITC labeled layer.

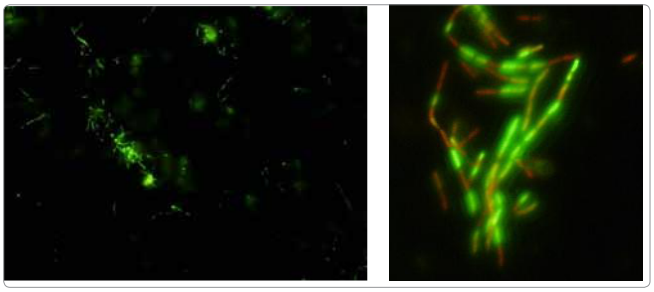

Figure 2: The fluorescence microscope visualization of the suspension of bacterial cells BR1-C coated with modified polylysine-polyethyleneimine bilayer after staining with Live/Dead BacLight Kit, applying the mixture of two nucleic acid stains green fluorescent Syto 9 dye (dying alive cells) and red-fluorescent propidium iodide (dying necrotic cells). In the left: the bacterial cells are shown exhibiting fluorescence bright green, indicating the intact cell membrane presence. In the right (the left picture fragment enlargement): the bacterial cells are shown exhibiting mainly fluorescence bright green, however some organisms revealed red fluorescence.

increased during the culture up to about 80% of initial value after 24 hour culture.

The impact of coated with PE shells bacterial cells on eukaryotic cells was assessed, whereas the applied bacteria production activity was presumed to act up to 48 hours.

It was observed that the nano-thin shell coated bacterial cells producing LLO exhibited the lethal impact on human leukemia Jurkat cells. After 2 hour culture of Jurkat cells in presence of coated BR1-S or BR1-C, there were respectively about 81% or 72% living cells less as compared to a negative control. After 24 -hour culture of Jurkat cells in presence of coated BR1-S or BR1-C, there were respectively about 72% or 64% living cells less as compared to a negative control (figure 3). Analyzing the lethal impact of both coated strains BR1-S or BR1-C, it was observed that the coated BR1-S seemed to exert meanly higher impact on Jurkat cells as compared to the coated BR1-C strain during the incubation. There were meanly 6.95% or 8.35% viable cells less in 2 or 24 hour culture with coated BR1-S presence respectively. Nevertheless, no statistical difference was observed after 24 hour culture.

Assessing the lethal impact of both coated strains BR1-S or BR1-C on B-CLL cells, it was observed that both coated strains exhibited lethal impact on B-CLL cells during incubation (Figure 4). After 24 hours, the percent number of viable cells B-CLL increased as compared to 2 hour incubation. It might be caused by bacterial production activity decline.

There was statistical difference observed between the percent number of viable B-CLL cells after 2 hour or 24 hour incubation in presence of coated BR1-S ($p=0.026<0.05$) or BR1-C ($p=0.002<0.05$) strains.

The coated BR1-S exerted meanly higher impact on B-CLL cells as compared to the coated BR1-C strain during the incubation. There were meanly 11.0% or 8.9% viable cells less in culture with coated BR1-S presence as compared to the culture with coated BR1-C after 2 or 24 hour incubation respectively. There was statistical difference observed between the number of viable cells cultured in presence of those two strains after 2 hours ($p=0.001<0.05$) or 24 hours ($p=0.004<0.05$).

Figure 5 represents the example result of cytometric assessment of PI fluorescence of Jurkat cells after 24-hour culture in presence of coated within polyelectrolyte shells BR1-C bacterial cells. The PI binds to nucleic acids entering cells with damaged cell membrane, thereby producing a red fluorescence of dead cells.

Discussion

The experiments demonstrated that the polylysine-polyethyleneimine membrane with incorporated fullerenol coating allowed for encapsulated bacterial cells functioning in *in vitro* experiment. Applied *B. subtilis* strains exhibited lethal impact on human leukemia cells during 24-hour incubation. The effect was visible after 2 hour incubation and was sustained during the whole time of culture. There were some differences in mean percent number of viable leukemia cells cultured in presence of strains BR1-S or BR1-C. However, it did not exceed about 10%. The applied shells allowed avoiding bacterial cells release during the experiment period.

Masking the bacterial cells immunogenicity with a polymer coating shell is an approach that can be used to inhibit antibody-mediated bacteria neutralization in future biomedical application.

Microorganisms carrying a transfected gene of another organism may become a source of factors affecting eukaryotic cells which may be thus used in local anti-tumor applications.

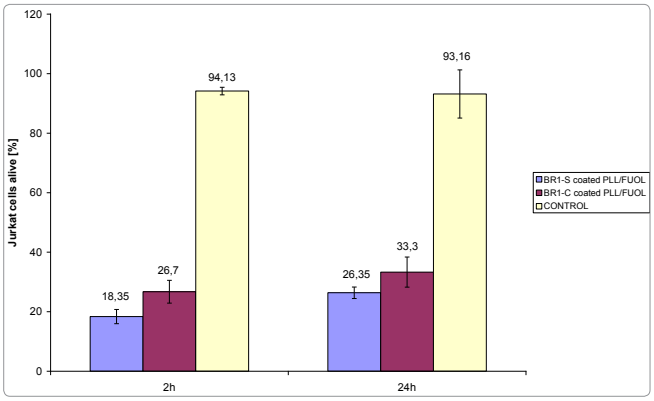

Figure 3: The percent number of viable human leukemia cells Jurkat cultured in presence of *B. subtilis* BR1-S or BR1-C coated with polylysine-polyethyleneimine modified with fullerenol bilayer after 2 or 24hour culture. As a negative control, the Jurkat cells were cultured without coated bacterial cells. The values are presented in form: mean ± SD.

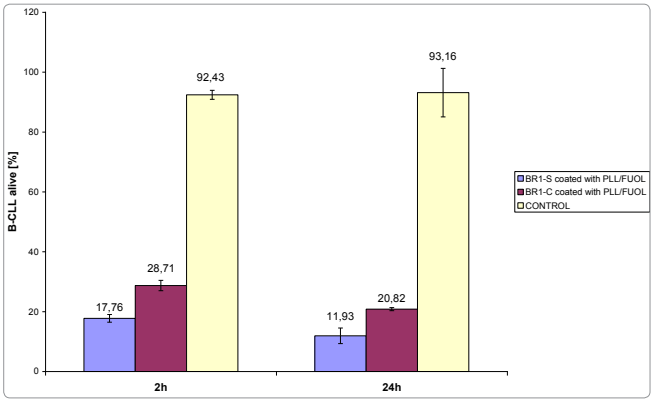

Figure 4: The percent number of viable human leukemia cells B-CLL cultured in presence of *B. subtilis* BR1-S or BR1-C coated with polylysine-polyethyleneimine modified with fullerenol bilayer after 2 or 24 hour culture. As a negative control, the B-CLL cells were cultured in the culture medium. The values are presented in form: mean ± SD.

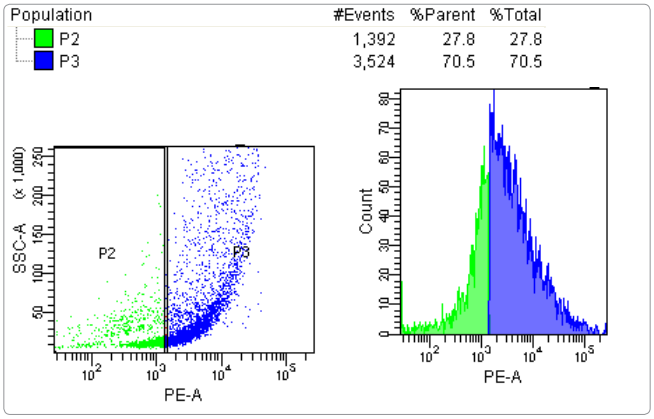

Figure 5: Assessment of PI fluorescence (gate P2 and P3) of Jurkat cells after 24-hour culture in presence of coated within polyelectrolyte shells BR1-C bacterial cells. There were 70.5% PI positive cells (gate P3) and 27.8% PI negative cells (gate P2) in the Jurkat cells population.

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