J. Microbiol. Biotechnol. (2010), **20**(1), 224–228
doi: 10.4014/jmb.0906.06056
First published online 12 November 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY
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Induced Death of *Escherichia coli* Encapsulated in a Hollow Fiber Membrane as Observed *In Vitro* or After Subcutaneous Implantation

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Received: June 23, 2009 / Revised: August 12, 2009 / Accepted: August 17, 2009

The encapsulation of bacteria may be used to harness them for longer periods of time in order to make them viable, whereas antibiotic treatment would result in controlled release of therapeutic molecules. Encapsulated *Escherichia coli* GFP (green fluorescent protein) (*E. coli* GFP) was used here as a model for therapeutic substance – GFP fragments release (model of bioactive substances). Our aim was to evaluate the performance of bacteria encapsulated in hollow fibers (HFs) treated with antibiotic for induction of cell death. The polypropylene-surface-modified HFs were applied for *E. coli* encapsulation. The encapsulated bacteria were treated with tetracycline *in vitro* or *in vivo* during subcutaneous implantation into mice. The HF content was evaluated in a flow cytometer, to assess the bacteria cell membrane permeability changes induced by tetracycline treatment. It was observed that the applied membranes prevented release of bacteria through the HF wall. The *E. coli* GFP culture encapsulated in HF *in vitro* proved the tetracycline impact on bacteria viability and allows the recognition of the sequence of events within the process of bacteria death. Treatment of the SCID mice with tetracycline for 8 h proved the tetracycline impact on bacteria viability *in vivo*, raising the necrotic bacteria-releasing GFP fragments. It was concluded that the bacteria may be safely enclosed within the HF at the site of implantation, and when the animal is treated with antibiotic, bacteria may act as a local source of fragments of proteins expressed in the bacteria, a hypothetical bioactive factor for the host eukaryotic organism.

Keywords: Encapsulation, hollow fiber, *Escherichia coli*, tetracycline, implantation into mice

Encapsulation of the bacteria within a semipermeable polymer membrane opens several technological possibilities; for example, it improves the bacteria cell's stability during passage under adverse conditions of the gastrointestinal tract in food industry applications [1, 2, 9, 10]. In environmental applications, degradation of petrol hydroxycarbons runs 3 times faster with application of encapsulated bacteria as compared with that non-encapsulated [7]. A similar system allows for avoiding an attack of the implanted microorganisms to the host after implantation into the animal [3]. Some encapsulated microorganisms may carry a transfected gene encoding proteins that may perform a biological function [8].

Encapsulation may be used to harness bacteria for longer-period release of a therapeutic molecule. A polypropylene-surface-modified hollow fiber was applied for bacteria *E. coli* encapsulation. Our aim was to evaluate the performance of bacteria encapsulated in hollow fibers when treated with an antibiotic. The antibiotic application may release the antigens for which its production the bacteria was genetically modified. GFP protein fragments were used here as a model antigen.

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Materials and Methods

Materials

Hollow fibers (HF). Polypropylene K600 PP, Accurel HFs (Membrana, Germany), inner diameter 0.6 mm, wall thickness 0.2 mm, original or surface modified, were used. The modified membrane was composed of a liquid layer of siloxanes on polypropylene support, and their cut-off was tested and estimated for 150 kDa. The modification of the HF membrane improves its biocompatibility and allows to avoid tissue overgrowth after implantation to the animal [3]. Before use, the membrane was sterilized in 70% ethanol for 30 min, and then washed with sterile physiological saline.

Reagents. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) (ICN, U.S.A.), tetracycline (ICN, U.S.A.), propidium iodide (PI) (Sigma, U.S.A.); and microbeads - FluoroSpheres yellow-green-fluorescent excited by 488 nm argon-ion laser, 0.2 µm mean diameter (Invitrogen Molecular Probes, U.S.A.) were used.

Media. RPMI 1640 (Gibco, U.S.A.); Luria–Bertani, Broth, Miller (Difco, U.S.A.); media mixture (LB): Luria-Bertani and RPMI1640

(1:10) supplemented with 100 µg/ml kanamycin and 100 µg/ml streptomycin.

Bacteria. *Escherichia coli (E. coli)* strain SG3103 (Qiagen) was transfected with pQE GFP (green fluorescent protein) plasmid by S. Świeżewski (Institut of Biophysics and Biochemistry, PAS, Poland) as previously described [3]. The high expression of GFP in the bacteria for *in vitro* experiments was induced by 2 mM IPTG for 3.5 h, before encapsulation (*E. coli* GFPI). *E. coli* strain DH5 was used as a nontransfected GFP control.

SCID mice. Severe Combined Immune Deficiency (SCID) mice, were about 2 months old, 20 g body weight. The animals were on a special diet supplied with vitamins, received acidified water *ad libitum*, and were bred in a sterile compartment. The protocol for animal experiments was approved by the Local Ethical Committee.

Procedures

Diffusive permeability of modified membrane for microbeads of diameter 0.2 µm. The original or surface-modified HFs were filled up with about a 20-µl volume of 1% suspension of FluoroSpheres, 0.2 µm microbeads in physiological saline. The encapsulated FluoroSpheres were incubated for 24 h in 1 ml of physiological saline and then the sample of the saline from the outer medium was tested for the presence of the beads. As a positive control, 20 µl of not encapsulated microbeads suspension was added and incubated in 1 ml of physiological saline. The physiological saline alone was incubated as a negative control. The samples of the FluoroSpheres were evaluated in a flow cytometer.

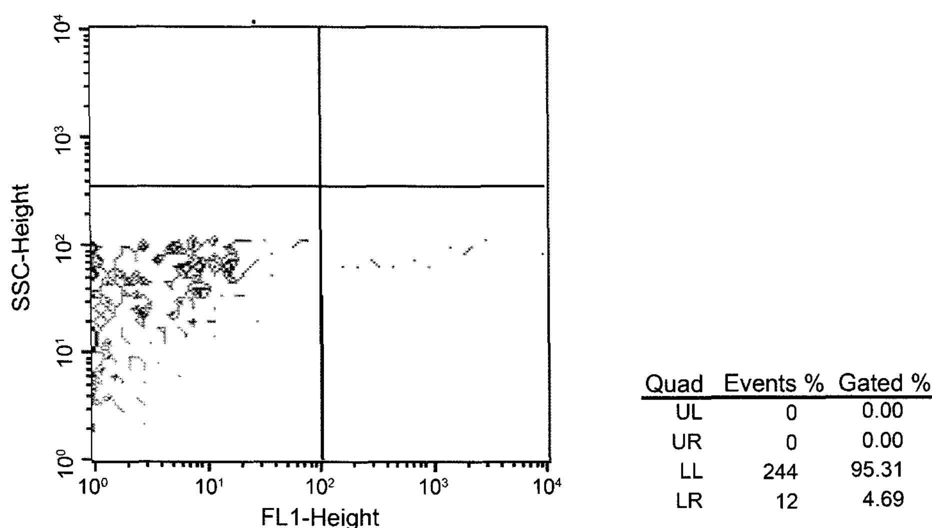
Antibiotic impact on *E. coli in vitro*. 1) The *E. coli* GFPI at the concentration of about 1.5×10^8 bacteria/ml was incubated for 2 h in 1 ml of LB culture medium with addition of tetracycline at a concentration of 1 mg/ml (35°C). The initial concentration of bacteria was set by spectrophotometry at 550 nm to an absorbance of 0.125. As a control the *E. coli* GFPI was incubated in LB medium devoid of antibiotic. After incubation, the samples of bacteria suspension were evaluated in a cytochemical reaction with PI in a flow cytometer.

2) The 48-h culture in 1-ml LB medium of *E. coli* GFPI encapsulated in HF at the concentration 8.5×10^5/HF was incubated with tetracycline at a concentration of 1 mg/ml (35°C), and a control HF-encapsulated *E. coli* GFPI was incubated in LB medium devoid of antibiotic.

The HF content was analyzed after 1, 2, 24, and 48 h from application of the tetracycline. The bacteria were analyzed in a flow cytometer after cytochemical reaction with PI. The presence of *E. coli* GFPI fluorescence as well as PI fluorescence of the bacteria was assessed. The samples of the outside culture medium in which the HF-encapsulated *E. coli* GFPI were cultured were analyzed for the presence of bacteria as well.

Evaluation of the antibiotic impact on *E. coli in vivo*. The suspension of *E. coli* GFP in LB/RPMI at the concentration of about 1.5×10^8 bacteria/ml was encapsulated in HF for implantation to the animal. The HFs of 2 cm length containing bacteria were implanted subcutaneously (s.c.) (3–4 HF/mouse) into SCID mice under barbiturate anesthesia. After 2 days, each mouse was subcutaneously treated with 1 ml of tetracycline solution at a concentration of 1 mg/ml. The site of tetracycline injection was far from the implanted HF. At a predetermined time, retro-orbital peripheral blood samples of about 0.5-ml volume were taken from the mice in barbiturate narcosis, to assay the possible presence of bacteria in the peripheral blood. Then, the animal was sacrificed and HFs were explanted. The content of the explanted HFs was washed out with 0.5 ml of sterile physiological saline and analyzed in a FACSCalibur flow cytometer for the presence of GFP and PI fluorescence of microorganisms. As the negative control, SCID mice with HF- encapsulated *E. coli* GFP implanted received 1 ml of physiological saline. A further procedure was as described above. Each experiment *in vitro* and *in vivo* was repeated at least 3 times.

Flow cytometry. The presence of microorganisms was assessed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytochemistry Systems, U.S.A.) equipped with the argon ion (488 nm) laser. The results were processed by the CellQuest software system (Becton Dickinson, U.S.A.). Microorganisms were separated from other events



Quad	Events %	Gated %
UL	0	0.00
UR	0	0.00
LL	244	95.31
LR	12	4.69

Fig. 1. Microbeads (yellow-green fluorescence) are not transported to the supernatant (quadrant lower right, LR) over modified hollow fibers within 24-h incubation.
The microbeads have dimensions similar to *E. coli*.

on light scatter characteristics (the gate of FSC and SSC) with the proper threshold.

RESULTS

Permeability Evaluation of Modified Membrane for 0.2-μm Microbeads, Comparable in Size with Bacteria

The FluoroSphere microbeads of dimensions similar to the bacteria encapsulated in modified HF were incubated for 24 h in physiological saline. The modified HF appeared to safely enclose FluoroSphere microbeads 0.2 μm in diameter for a 24-h period (Fig. 1).

Evaluation of Antibiotic-induced Changes in *E. coli* GFPI

The *E. coli* GFPI culture was treated with tetracycline and then tested with PI. The percent number of populations GFP^+PI^+ (dead) organisms were analyzed by cytometry. It was observed, that the percent of PI^+ population with retained GFP (GFP^+) or with partially lost GFP ($GFP^{+/-}$) increased after 2 h treatment with tetracycline and was 20.7±4.5% as compared with a negative control (0.4±0.1%), demonstrating the antibiotic impact on the dying cells.

The *E. coli* GFPI culture encapsulated in modified HF was treated with tetracycline and then was tested with PI. For the test, bacteria were washed out (released) from the HF and the percent number of populations GFP^+ and PI^+ organisms were assayed by flow cytometry.

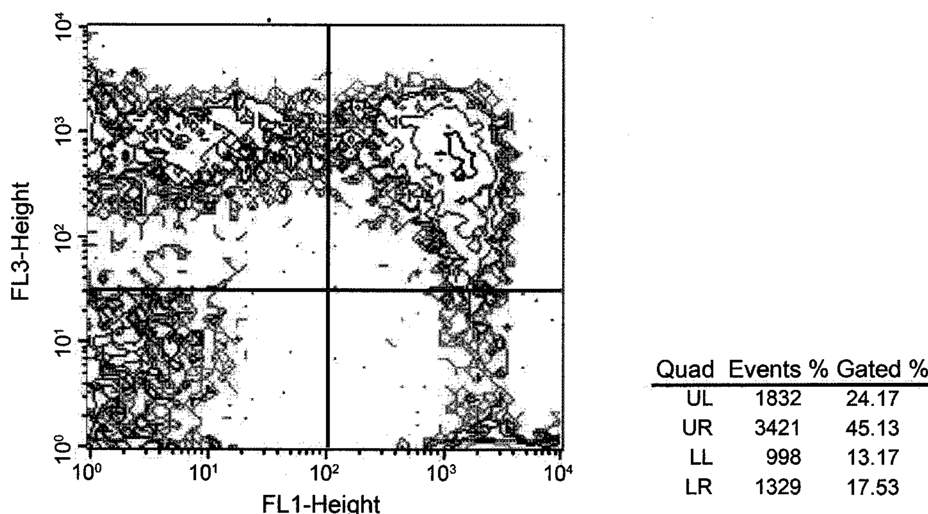
The flow cytometric assessment of encapsulated *E. coli* GFPI during culture with addition of tetracycline is presented in Fig. 2. As compared with the negative control (Fig. 3), the *E. coli* GFPI living cells localized as GFP positive and PI negative (lower right, LR quadrant) are recognizable. Numerous bacteria containing GFP with cell membrane permeable for PI (upper right, UR quadrant) are considered as dying.

Some of the PI positive (PI^+) bacteria lost their GFP from the cytosol ($GFP^{+/-}$) (upper left, UL quadrant) with a different fraction of GFP protein remaining within the cell.

It was observed that the percent of PI^+ population with retained GFP (GFP^+) or with partially lost GFP ($GFP^{+/-}$) increased about 19 times after 2-h treatment with tetracycline and was 18.2±2.2% as compared with a negative control of 0.95±0.78% (Fig. 4). These proportions changed after 24-h culture in the presence of tetracycline to 61.7±21.2% and 7.1±2.1% in the negative control, respectively, and after 48-h culture were 85.8±12.7% and 11.6±5.2%, respectively. The percent of $GFP^{+/-}/PI^+$ cells changed with the time of tetracycline treatment. There were no GFP-positive microorganisms observed within the culture medium outside the HF loaded with *E. coli*.

Evaluation of the Antibiotic Impact on *E. coli in vivo*

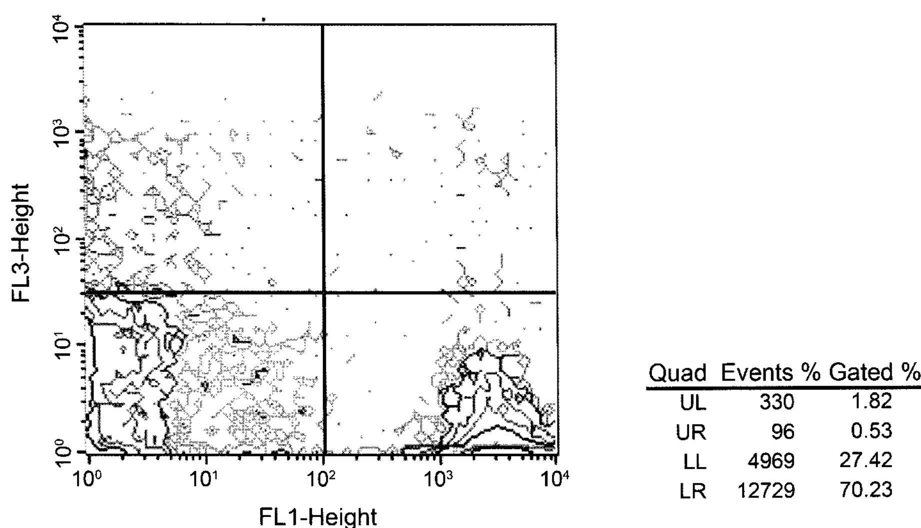
The mice could not effectively treated with IPTG to induce a rise of GFP fluorescence within *E. coli* GFP. However, the *E. coli* GFP bacteria had a constitutive, low expression of GFP fluorescence, which was about 18 times higher than in nontransfected *E. coli* strain DH5 (Fig. 5). The SCID mice were used as a host for encapsulated bacteria. No release of bacteria from HF, which would induce sepsis in the peripheral blood of the animal, was observed on the 2nd day after s.c. implantation of encapsulated bacteria.



Quad	Events	% Gated %
UL	1832	24.17
UR	3421	45.13
LL	998	13.17
LR	1329	17.53

Fig. 2. *E. coli* GFPI encapsulated in modified hollow fiber after 24-h culture, and then treated with tetracycline for 2 h at a concentration of 1 mg/ml, 35°C.

FL-1 and FL-3 fluorescence is from GFP and PI, respectively. There are about 18% of GFP^+PI^- bacteria (living cells; quadrant lower right, LR), 45% of GFP^+PI^+ bacteria (cells permeable to PI, still no activation of proteases; quadrant upper right, UR), and 24% $GFP^{+/-}PI^+$ bacteria in quadrant upper left (cells losing fragments of GFP, but with unfragmented DNA; UL). Within this quadrant some cells losing DNA (translocating down on FL3 axis) may be noted as well.



Quad	Events	% Gated %
UL	330	1.82
UR	96	0.53
LL	4969	27.42
LR	12729	70.23

Fig. 3. *E. coli* GFPI encapsulated in modified hollow fibers after 24+2-h culture in culture medium, 35°C (negative control).
FL-1 and FL-3 fluorescence are as in Fig. 2. Most cells (70%) remain in the $GFP^{+}PI^{-}$quadrant (living cells; quadrant lower right, LR), and about 2% of cells is in the fraction losing nucleic acids ($GFP^{+/-}PI^{+/-}$) (quadrant upper left, UL). GFP+ and PI+ bacteria (damaged cells permeable to PI) are present as 0.5% of cells only in the experiment.

The analysis of *E. coli* GFP encapsulated in HF was performed on the 2nd day from implantation and 6–8 and 24 h after tetracycline injection.

The explanted and washed out from HF *E. coli* living GFP^{+}/PI^{-} or damaged $GFP^{+/-}/PI^{+}$ cells were at 6 h tetracycline treatment equal to 76.7±8.8% and 23.3±8.8%, respectively; at 8 h of treatment equal to 33.3±7.1% and 66.7±7.1%; and at 24 h from start of treatment equal to 78.3±7.8% and 21.8±7.8%, respectively. The negative control animals were treated with physiological saline injection, and the GFP^{+}/PI^{-} or damaged $GFP^{+/-}/PI^{+}$ values at 24 h for the subpopulations of bacteria were equal to 58.2±18.0% and 41.8±18.0%, respectively.

The ratio of GFP^{+}/PI^{-} to $GFP^{+/-}/PI^{+}$ (living to damaged cells) was 3.3 at 6 h, 0.5 at 8 h and 3.6 at 24 h, respectively, whereas for tetracycline untreated control it was 1.4. The most substantial necrotic bacteria share induced by single subcutaneous tetracycline treatment was therefore observed after 8 h. The obtained ratio values of living to damaged

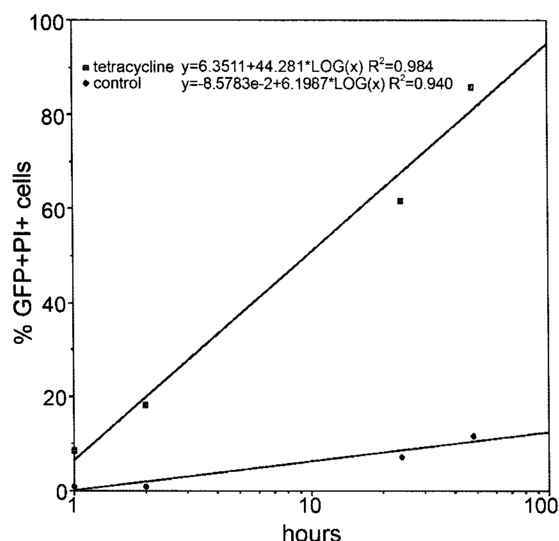


Fig. 4. The percent of $GFP^{+/-}PI^{+}$ *E. coli* GFPI encapsulated in HF during incubation with tetracycline for different periods.
Samples were taken after 1, 2, 24, and 48 h from addition of 1 mg/ml tetracycline. The control was encapsulated *E. coli* GFPI incubated without tetracycline. Simple, straight line alignments for both versions are presented.

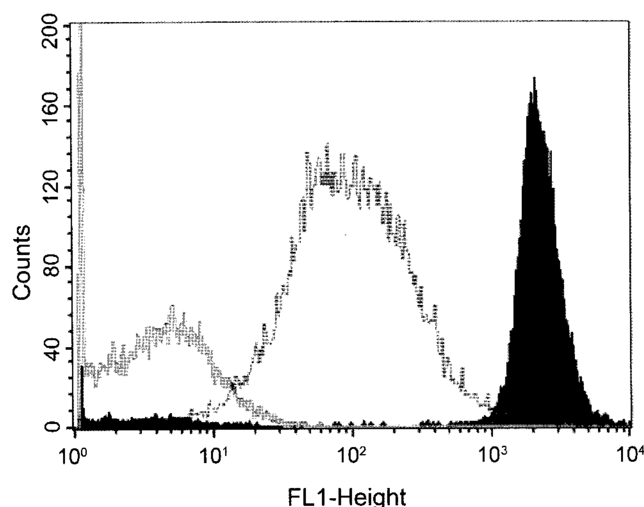


Fig. 5. The histogram of green fluorescent protein expression values for different *E. coli*.
Median fluorescence intensity (MFI) units for *E. coli* DH5 strain is 5 units, for *E. coli* GFP untreated with IPTG (constitutive GFP expression) utilized in the *in vivo* experiments is 90 units, and for *E. coli* GFPI after treatment *in vitro* with IPTG is 1,963 units.

cells decreased after 6 and 8 h as compared with the control ratio values in the untreated animals, but they returned to the control ratio value at 24 h from start of the tetracycline treatment. This may suggest the repopulation of the surviving bacteria within the HF. The calculated tetracycline concentration applied in the mice was about 20-times lower as compared with our *in vitro* experiment.

Discussion

The following sequence of events may be recognized with flow cytometry within the process of tetracycline effect on *E. coli* GFP. First, in the dying bacteria, the cell membrane permeability increases for propidium iodide (PI), which binds to intracellular nucleic acids DNA and RNA. The binding process enriches the maximal fluorescence value (MIF) when all PI-available domains are occupied, whereas the GFP fluorescence does not change. The next stage involves initiation of GFP fragments loss from the cells, probably due to protease(s) activation within the PI-permeable cells. Probably the GFP aggregates, the inclusion bodies, are degraded by proteases [6, 11], some with substrate recognition tags located in the interior of the primary sequence [4]. Thus, larger protein fragments may appear, diffuse out of damaged cells and from the HF to the host tissues, where they may be recognized by the immunological system.

This process of protein fragmentation within the bacteria progresses without significant loss of cellular DNA and RNA, as propidium iodide binding does not change. Finally, when most GFP fluorescence disappears from the cells owing to loss of proteolytic fragments of GFP from the bacteria, the DNA and RNA degradation (fragmentation) is observed. The DNA fragmentation must be a fast process as compared with the GFP degradation process, as only few DNA-losing cells are noted. This clearly recognizable sequence of the bacterial death process was not previously described, but the model of *E. coli* GFP process may be similar in the other prokaryotic organisms. In our opinion, the HF-enclosed prokaryotic cells treated with antibiotic may have an application for an antigenic peptide release (*e.g.*, in a vaccination program). There is, for instance, a constructed recombinant intergenus multidomain chimeric protein expressed in *E. coli* for simultaneous expression of hemolysin BL of *Bacillus cereus*, listeriolysin O of *Listeria monocytogenes*, and enterotoxin B of *Staphylococcus aureus* [5]. The construct contains the antigenic determinants of the mentioned toxins encoded by the transfected gene in the bacteria, and possible may release antigenic fragments when treated with an antibiotic, being a polyvalent vaccine in eukaryotic organism.

Acknowledgments

This study was partly supported by grant 501-1-1-24-06/06 and grant CMKP 2006.

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