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SUPPLEMENTARY MATERIAL TO

Diffusion models of gentamicin released in poly(vinyl alcohol)/chitosan hydrogel

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Gentamicin release studies

High-performance liquid chromatography (HPLC) (Thermo Fisher Scientific, USA) was utilized for gentamicin components separation and the detection and quantitative analysis was done in an ion trap mass spectrometer (MS) (LCQ Advantage, Thermo Fisher Scientific). HPLC was equipped with a reverse-phase column (4.6 mm × 75 mm × 3.5 μm) Zorbax Eclipse® XDB-C18 (Agilent Technologies, USA), in front of which a precolumn (4.6 mm × 12.5 mm × 5 μm) was placed. Methanol (A), deionized water (B), and 10 % acetic acid (C) comprised the mobile phase. The optimized HPLC and MS operating parameters (mobile-phase gradient, analytes' precursor ions, fragmentation reactions used for quantification, and optimal collision energies) for the determination of gentamicin compounds were published in our previous paper.[S1,S2]

The gentamicin mass spectra were collected in the *m/z* range of 50-1000. As expected, the MS spectrum revealed the three most abundant ions since gentamicin is composed of three compounds – gentamicin C1a, C2, and C1. These ions were further chosen as the precursor ions for each compound. Their most sensitive transitions were selected for quantification purposes. The presented gentamicin concentrations represent sums of the three determined gentamicin compounds.

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Evaluation of antibacterial properties

The hydrogel samples were cut up to small pieces (~ 2 x 2 mm). Thus prepared samples were sterilized under UV light for 30 min in a laminar flow cabinet. The bacteria culture suspensions were prepared by resuspending overnight cultures in the phosphate buffer (the same buffer was used for the drug release and gel swelling studies). The initial cell numbers in the suspensions were ~10^6 CFU ml^{-1}. Approximately 2 g of each sample were added to the flasks with bacterial suspensions and incubated in the shaker water bath at 37 °C and 62 rpm. The controls were bacterial suspension in phosphate buffer, without any added samples. The cell numbers were monitored by sampling and serial dilution of the media aliquots after 15 min, 1 h, 3 h and 24 h of incubation. Nutrient agar base (1.5 wt %, 55 ^{0}C) was inoculated with 100 µl of culture medium and poured in sterile Petri dishes. The Petri dishes were incubated at 37 °C for 24 h, after which the viable cells were counted and expressed as colony forming units per ml (CFU ml^{-1}).

Cytotoxicity

Cell cultures. The cells were grown in Dulbecco's modified Eagle's medium with 4.5% glucose, supplemented with 10% fetal calf serum and antibiotic-antimycotic. The cells were sub-cultured twice a week and cell suspensions were prepared with 0.1% trypsin in EDTA. All cell lines were cultured in 25 cm^2 flasks at 37°C in atmosphere with 5% CO_2 (100% humidity). Viable cells were counted by 0.1% trypan blue exclusion, and exponentially growing cells were used for MTT assay. The viability of the cells used in the MTT assay was > 90%.

MTT assay. The viable cell cultures were seeded in 12-well plates with hydrogel samples (2 g), and control wells contained only the complete media with cells and without hydrogel samples. The incubation was conducted at 37°C for 48 hunder air flow with 5% CO_2. After incubation, the cells were separated from the hydrogel samples by trypsinization treatment. Subsequently, 100 µl of the media with cells were cultured in 96-well microtiter plates at 5x10^3 cells per well seeding density to ensure logarithmic growth rate throughout the assay. The microtiter plates were incubated for 48 h at 37°C. Three hours before the end of the incubation period, MTT solution (10 µl) was added in each well. Cells were incubated in the presence of MTT for 3 hours at 37°C, after which the medium and MTT were removed by suction.The blanks contained only media and MTT, without the cell cultures. The MTT test is based on the reduction of MTT dye to an insoluble formazan product inside the viable cells mitochondria, which causes the change of the medium color from blue to dark purple. After the media was discarded, the remaining solid precipitates with cells and purple formazan product were solubilized in 100 µl 0.04 M HCl isopropanol solution. After a few minutes at room temperature, the optical density (OD) at 540/690 nm was

measured using a Multiscan MCC340 spectrophotometer plate reader (Thermo Labsystems). Cell viability, *S*, is calculated using equation

$$S = 100 A_u / A_c \quad \text{(S1)}$$

where A_u i A_c are absorbance of tested sample and control, respectively.

DET assay. Dye exclusion test (DET) towards two fibroblast cell lines was carried out according to the previously published protocols.[S3] Viable cells were seeded in the 12-well plates (Costar) at concentration of 1×10^5/mL, whereas the control wells did not contain samples, only the seeded cells. Plates were incubated at 37 °C under air flow with 5% CO_2, during the following 48 h. After the incubation, cells were separated from samples by trypsinization method i.e. by adding 0.1 % trypsin solution. The cell number and viability were further evaluated by the trypan blue exclusion method using a formula

$$K = 100 N_s / N_k \quad \text{(S2)}$$

where K is growth inhibition expressed as a percent of control, N_k is the total number of cells (control) and N_s is the number of cells on the tested samples.

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