Antibody-based Quantification of Gentamicin Release from Orthopedic Implants

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Disclosures: Markus A. Wimmer (3C - Endolab; 5 - Corin USA, Liposphere; 8 - Elsevier; 9 – ISTA).

INTRODUCTION: Localized drug delivery systems (DDSs) are increasingly used to deliver antibiotics directly to the site of infection. Among DDSs, titanium (Ti) oxide nanotubes (TNTs) created on the surface of a Kirschner wire and coating the TNT surface through electrophoretic deposition (EPD) of gentamicin and chitosan represent a promising approach to prevent and eradicate periprosthetic joint infection (PJI) (1). The accurate quantification of gentamicin released from TNTs is indispensable to prevent local toxicity. A traditional method of gentamicin quantification is the ninhydrin reaction (2). However, since detection by ninhydrin is based on the presence of amine groups, gentamicin quantification in the presence of other amine-containing substances such as chitosan, can be challenging. The goal of this project was to optimize the quantification of gentamicin by a competitive enzyme-linked immunosorbent assay (ELISA) (3) and to compare its accuracy to the ninhydrin reaction when both gentamicin sulphate and chitosan are deposited in TNTs.

METHODS: First, an indirect, competitive ELISA method was optimized for the detection of gentamicin using gentamicin solutions with known concentrations. Briefly, gentamicin sulphate was coupled to bovine serum albumin (BSA) with a coupling agent 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). The coupled gentamicin sample was analyzed by Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis, followed by semi-dry transfer and Western blot to confirm successful coupling. For the ELISA assay, 96-well microtiter plates were coated with the BSA-gentamicin complex overnight. The following day, the wells were blocked with 1% BSA for 1 hour. Consequently, 50 µl of gentamicin solution and 50 µl of mouse monoclonal antibody to gentamicin was added simultaneously to the wells for 1 hour. A second blocking step followed for 1 hour, then an HRP-conjugated secondary antibody was applied. Tetramethylbenzidine (TMB) substrate was added to the wells in the dark, and the optical density was determined with a spectrophotometer at 450 nm. Different amounts of gentamicin-BSA coating and different sample and antibody dilutions were tested for optimal signal/background intensity. Second, TNTs with EPD of chitosan or EPD of gentamicin plus chitosan were prepared. Release solutions were prepared by fractional volume sampling (PBS, pH 7.4, 37 C) and analyzed with the competitive ELISA method and the ninhydrin reaction. Data analysis was performed by GraphPad v10.1.0 statistical software, $p<0.05$ was considered statistically significant.

RESULTS: We successfully optimized a competitive ELISA protocol for the quantification of gentamicin in *in vitro* release solutions. A standard curve created by plotting gentamicin concentration and reciprocal optical density (1/OD) yielded $y = 12.17x2 - 46.965x + 54.362$ with $R^2=0.998$ **(Figure 1)** (representative of n=3 experiments, inter-assay variability 16.41%, intra-assay variability 6.68%). Analysis of release solutions from TNTs and EPD of chitosan by ELISA resulted in significantly lower optical density (and estimated gentamicin concentration) compared to analysis with the ninhydrin reaction **(Figure 2)** (n=3 TNTs with EPD of chitosan, $p<0.0001$ ELISA vs ninhydrin, two-way ANOVA and Sidak`s multiple comparison). Analysis of release solutions from TNTs and EPD of 50 µg gentamicin and chitosan resulted in a trend of lower estimated gentamicin concentration by ELISA compared to the ninhydrin reaction **(Figure 3)** (n=2 TNTs with EPD of gentamicin and chitosan, $p>0.05$, two-way ANOVA and Sidak`s multiple comparison)**,** indicating that when chitosan is present in the release solution, the ninhydrin reaction results in an overestimated gentamicin concentration due to an interference of amine groups from chitosan.

DISCUSSION: The optimized ELISA assay demonstrated high sensitivity and specificity compared to the ninhydrin assay in detecting and quantifying gentamicin sulphate when other amines from chitosan were present in the release solutions. Therefore, we conclude that the ELISA assay is more accurate than the ninhydrin reaction for gentamicin detection in release samples from complex coatings. The release data showed an initial burst release of gentamicin followed by a sustained release period, further confirming previously demonstrated release kinetics from TNTs and EPD of gentamicin and chitosan. A limitation of our study is that additional methods for quantifying drug release, such as mass spectrometry were not employed for comparison. Future studies will evaluate the accuracy of the ELISA method in quantifying gentamicin concentration in biological fluid, such as mouse serum.

SIGNIFICANCE/CLINICAL RELEVANCE: The ability to accurately assess drug release from localized and complex DDSs is crucial for monitoring and evaluating the performance of antimicrobial coatings and for the prevention of local toxicity.

REFERENCES: **1.** Della Fara G, et al. Electrophoretic deposition of gentamicin and chitosan into titanium nanotubes to target periprosthetic joint infection. J Biomed Mater Res B Appl Biomater. 2023 Sep;111(9):1697-1704. **2.** Frutos P, et al. A validated quantitative colorimetric assay for gentamicin. J Pharm Biomed Anal. 2000 Jan;21(6):1149-59. **3.** Odekerken JC, et al. ELISA-based detection of gentamicin and vancomycin in protein-containing samples. Springerplus. 2015 Oct 15;4:614.

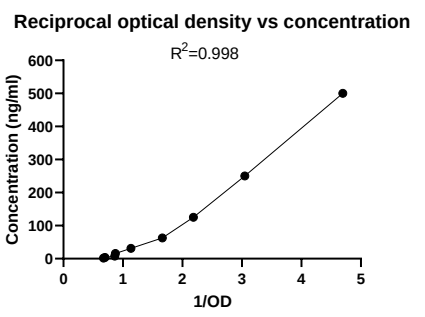

Figure 1. Gentamicin standard curve obtained by the ELISA assay (representative of n=3 experiments).

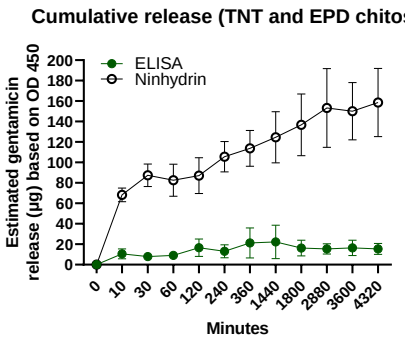

Figure 2. Estimated gentamicin release from TNTs and EPD of chitosan, determined by ELISA and ninhydrin assay.

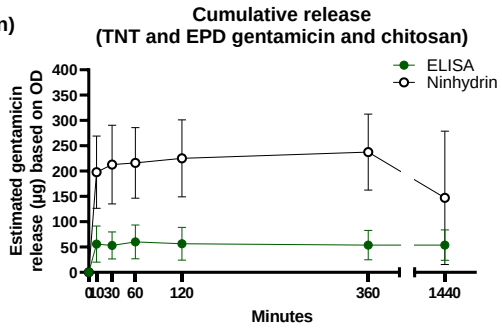

Figure 3. Estimated gentamicin release from TNTs and EPD of gentamicin and chitosan, determined by ELISA and ninhydrin assay.