Efficacy of amphiphilic core-shell nanostructures encapsulating gentamicin against an in-vitro Salmonella and Listeria intracellular infection model

A. Ranjan,¹ N. Pothayee,²,³ T. P. Vadala,³ M. N. Seleem,² E. Restis,⁴ N. Sriranganathan,²,⁴* J. S. Riffle²,³ and R. Kasimanickam¹

¹Dept of Large Animal Clinical Sciences, ²Institute for Critical Technology and Applied Science, ³Macromolecules and Interfaces Institute, ⁴Dept of Biomedical Sciences and Pathobiology, Virginia Polytechnic Institute and State University, Blacksburg, VA

*Dept of Biomedical Sciences and Pathobiology, Virginia Polytechnic Institute and State University, Blacksburg, VA; Email: nathans@vt.edu; Phone number: 540-231-7171

Abstract

Core-shell nanostructures with nonionic amphiphilic shells and ionic cores encapsulating gentamicin were designed for therapy against intracellular pathogens including Salmonella and Listeria. Flow cytometry and confocal microscopy showed that their uptake into J774A.1 macrophages proceeded mainly by fluid phase endocytosis and clathrin-mediated pathways. The nanostructures were non-toxic in-vitro at doses of 50-250 µg/mL, and they significantly reduced intracellular Salmonella (0.53 log) and Listeria (3.16 log), thereby suggesting effective transport into the cells.

Gentamicin is an aminoglycoside with antimicrobial activity against Gram-positive and Gram-negative bacteria (3). Its efficacy against these bacteria is established, yet its poor cell membrane permeability limits its clinical use, particularly against intracellular bacterial infections (4). We previously reported core-shell nanostructures encapsulating gentamicin that had poly(sodium acrylate-b-ethylene oxide-b-propylene oxide-b-ethylene oxide-b-sodium acrylate) (PAA⁺Na-b-PEO-b-PPO-b-PEO-b-PAA⁺Na) with PEO-b-PPO-b-PEO amphiphilic shells and block lengths of ~3600-b-1800-b-3600 g/mole (from Pluronic F68) (8). PAA⁺Na homopolymers were also incorporated into the nanostructure cores to increase their sizes and charge, thus facilitating high loadings of the polycationic gentamicin in the cores. The stabilities of those nanostructures,
however, in the presence of phosphate salts were relatively poor. In the present study, we aimed to improve the stability of the nanostructures in physiological media to enhance delivery of gentamicin into macrophages. The strategy was to incorporate a higher molecular weight hydrophobic PPO in the shells, and more PPO relative to the hydrophilic PEO, so that enhanced hydrophobic interactions would contribute to their stabilities in physiological media. Thus, a PAA−Na-b-PEO-b-PPO-b-PEO-b-PAA−Na copolymer (nonionic portion derived from Pluronic P85) with block lengths of 2160-b-1100-b-2300-b-1100-b-2160 g/mole was blended with a hydrophilic PEO-b-PAA−Na copolymer with block lengths of 2000-b-7200 g/mole, and these were condensed with gentamicin to afford core-shell nanostructures. These were investigated in-vitro to establish transport into the macrophages and determine their activities against vacuolar Salmonella and cytoplasm-resident Listeria (1, 11).

A scheme for preparing the nanostructures is shown in Fig. 1. PAA−Na-b-PEO-b-PPO-b-PEO-b-PAA−Na (25 mg, 1.7 x 10−4 eq of carboxylates) and PEO-b-PAA−Na (25 mg, 2.7 x 10−4 eq of carboxylates) were dissolved in 25 mL of PBS. The solution was sonicated while 5 mL of gentamicin sulfate solution (13.3 mg mL−1 gentamicin sulfate equal to 40 mg gentamicin, 4.2 x 10−4 eq of cations) was added to form a turbid dispersion of nanostructures in PBS. Dynamic light scattering (DLS, Malvern Zetasizer NanoZS particle analyzer, Malvern Instruments Ltd, Malvern, UK) at 37 ± 0.1 °C, pH 7.4 in PBS showed a monomodal peak with an intensity average diameter of 548 ± 53 nm, thus indicating that a relatively homogeneous distribution of nanostructure sizes are produced with this procedure. This was in contrast to the previously-reported nanostructures prepared with less of the hydrophobic PPO component in the nanostructure shells (8) where DLS showed three peaks for the intensity average diameters centered around 11, 287 and 4373 nm. The nanostructures were collected by centrifugation at 20,000 rpm for 15 min (Optima™ L-XP Ultracentrifuge, Beckman Coulter), washed twice with sterile DI water and freeze-dried. The gentamicin concentration in the nanostructures was assayed (9), and was 25% by weight gentamicin. The nomenclature describing these nanostructures is PN (PNF when labeled with fluorescein). The methodology to synthesize PNFs was reported previously (8). For flow cytometry, 1.25 x 105 J774A.1 macrophage cells/mL were pre-incubated with inhibitors of clathrin-mediated transport (10 µg/mL of chlorpromazine), caveolar-mediated transport (5 µg/mL of filipin) or fluid phase endocytosis (450 mM of sucrose)
for 30 min (6). The cells were then incubated with 15 µg/mL of PNFs for 2 h, washed twice with HBBS buffer and the fluorescence intensities were analyzed by FACS flow cytometry (BD FACS Aria) with an excitation wavelength of 488 nm and analyzed with a 530/30 nm emission filter. Five thousand single cell events were counted to compute the geometric mean fluorescence intensities of the cells from triplicate samples. Intracellular trafficking of PNFs were also analyzed by confocal microscopy (8). Cytotoxicity was evaluated using MTS assays (Promega) by incubating J774A.1 cells with gentamicin sulfate, Pluronic P85 (precursor polymer for the nanostructure shells), the copolymers, PNs and untreated controls at a dose range of 50-250 µg/well for 24 h and absorbance at 490 nm was measured. The ability of the PNs to kill intracellular bacteria at a dose of 25 µg/mL of gentamicin (100 µg/mL of PNs) was evaluated in an intracellular infection model using J774A.1 cells infected with Salmonella typhimurium strain LT2 (6) or Listeria monocytogenes at a MOI of 10 bacteria per macrophage (5). Treatment groups were compared for differences in mean CFUs using ANOVA followed by Tukey’s procedure for multiple comparisons.

Based on the fluorescence of cells containing PNFs, flow cytometry suggested that PNF uptake was significantly reduced with chlorpromazine (~ 46%) and sucrose (~ 40%) compared to PNFs alone. It has been reported that the sucrose inhibitor of fluid phase endocytosis can also inhibit clathrin-dependent receptor internalization by blocking clathrin-coated pit formation (12). Thus, inhibition by sucrose is regarded as somewhat non-specific and could have contributed to reductions in uptake by both fluid phase and clathrin-mediated pathways (7). By contrast, filipin did not significantly inhibit particle uptake. Confocal microscopy results agreed with flow cytometry. PNFs were taken up by the cells (Fig. 2a). Macrophages were labeled positive for endosomes with Alexa fluor-488 (2b), and co-localization studies suggested that the PNFs resided in endosomes/lysosomes and also in the cytoplasm (2c). Thus, transport of the PNFs into the cells could be an interplay of delivery by fluid phase endocytosis into the cytosol or by a clathrin-mediated pathway involving endosomes as reported elsewhere (2). For the dose range studied in the MTS assays, absorbance was comparable or higher than the untreated control, indicating a lack of toxicity (Fig. 3). Higher absorbance signified higher mitochondrial activity and an enhanced metabolic state of the cells. In-vitro treatment studies showed that free
gentamicin, the copolymers alone, and the infected control did not significantly clear intracellular Salmonella or Listeria. In contrast, PNs resulted in significant reductions of intracellular Salmonella (0.53 log) and Listeria (3.16 log) (p<0.05) (Table 1). Almost quantitative reduction of Listeria suggested that their sub-cellular localization may influence the capacity for the PNs to reach their intracellular bacterial targets. Plausibly, the vacuolar-resident Salmonella may not have been exposed to a high dose of the antimicrobial due to membrane barriers around the Salmonella within the cells. In contrast, cytoplasm-resident Listeria may directly interact with gentamicin favoring efficient clearance (5). It should be noted that escape of Salmonella from the intracellular vacuoles partially depends on the duration of infection (10), and that the observed reduction in Salmonella could be due to escape of flaggelated bacilli from the vacuoles and direct exposure to gentamicin in the cytoplasm. Development of drug deliver systems which release drugs in a time-dependent manner in the cell cytosol may be highly valuable for such treatments. In summary, these studies showed that the higher hydrophobic content in the PNs improves stability in physiological solutions over previously-reported nanostructures (8), and that the efficacies of these nanostructures appear to depend on sub-cellular localization of the bacteria. Future studies aiming to target different intracellular niches of the bacterium may help develop an effective clearance strategy.

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References:


Fig. 1: Preparation of core-shell nanostructures encapsulating gentamicin
Fig 2: Confocal microscopy. (a) Uptake of green PNFs in J774A.1 cells, (b) nanostructures are shown by yellow-to-orange spots formed by green nanoparticles/dyes and red endosomes/lysosomes, showing that a majority of the Alexa-fluor appears to reside in endosomes (arrowhead), (c) PNFs are distributed throughout the cells. Colocalization of PNFs with endosomes/lysosomes after incubation for 2 h (arrowhead), and sub-cellular localization of PNFs (arrow).
Fig. 3: MTS assays showing the percentage mean absorbance at 490 nm after incubating J774A.1 cells with 50-250 µg mL$^{-1}$ of PNs along with appropriate controls. Results are expressed as means ± SD of six measurements.
Table 1: Killing of intracellular wild-type *S. typhimurium* LT2 and *Listeria monocytogenes* in J774A.1 cells incubated with free gentamicin, the copolymers alone, or the PNs at a dose of 25 µg gentamicin/mL for 6 h. Each result represents the mean of triplicate assays ± SD performed together. Asterisks represent values found to be significantly different (confidence level, \( t = 0.05 \)) from that for free gentamicin by statistical analysis (ANOVA followed by Tukey's procedure for multiple comparisons).

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<th>Groups</th>
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<tr>
<td></td>
<td>Log CFU (±SD)</td>
<td>Log CFU reduction</td>
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<td>Control</td>
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<tr>
<td>PNs</td>
<td>3.53 (± 0.20)</td>
<td>0.53**</td>
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