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Anti-biofilm properties of wound dressing incorporating nonrelease polycationic antimicrobials

Livnat Atar-Froyman [a, 1], Anat Sharon [b, 1], Ervin I. Weiss [a], Yael Houri-Haddad [a], Dana Kesler-Shvero [a], Abraham J. Domb [c], Raphael Pilo [d], Nurit Beyth [a, *]

[a] Department of Prosthodontics, Hebrew University-Hadassah School of Dental Medicine, P.O. Box 12272, Jerusalem 91120, Israel
[b] Department of Maxillofacial Prosthetics, Hebrew University-Hadassah School of Dental Medicine, P.O. Box 12272, Jerusalem 91120, Israel
[c] Department of Medicinal Chemistry and Natural Products, Hebrew University-Hadassah, School of Pharmacy-Faculty of Medicine, P.O. Box 12065, Jerusalem 91120, Israel
[d] Department of Prosthetic Dentistry, Goldschleger School of Dental Medicine, Tel-Aviv University, Israel

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ABSTRACT

Polycationic nanoparticles show biocompatible, broad-spectrum bactericidal properties *in vitro* and *in vivo* when incorporated in denture lining material post-maxillectomy in head and neck cancer patients. In the present study, the synthesized Crosslinked quaternary ammonium polyethylenimine nanoparticles were found to have a strong bactericidal activity against a wide variety of microorganisms rapidly killing bacterial cells when incorporated at small concentrations into soft lining materials without compromising mechanical and biocompatibility properties. This appears advantageous over conventional released antimicrobials with regard to *in vivo* efficacy and safety, and may provide a convenient platform for the development of non-released antimicrobials. This is a crucial issue when it comes to giving an answer to the serious and life-threatening problems of contaminations in immunocompromised patients such as orofacial cancer patient.

1. Introduction

Head and neck cancers encompass a diverse group of uncommon tumors that frequently are aggressive in their biological behavior. Extensive surgery that requires the removal of large oropharyngeal cancers predisposes the patients to infection with resident oral microorganisms. Moreover, post-surgery wounds are prone to life-threatening infections mainly in immune-deficient patients and in cancer patients who are at greater risk for nosocomial infections [1]. Unfortunately, despite adequate antibiotic prophylaxis, post-operative surgical site infection develops in up to 40% of cases [2].

Traditionally, a post-surgery prosthetic obturator facilitates acceptable esthetics and a reasonable level of oral function during the initial post-operative period in maxillectomy patients. The immediate surgical obturators are essential to enable retention of the surgical packing, promote healing with minimal postsurgical infection and scar contracture formation. Soft liners covering the obturator prosthesis are used for surgical wound dressing immediately after radical resection of maxillofacial tumors. One of the main disadvantages of the soft lining materials is their tendency to be easily contaminated in the oral environment and the fact that they cannot be efficiently cleaned or brushed. Microorganisms such as *Candida albicans* colonize not only the surface of the soft liners, but also penetrate those materials. This phenomenon is particularly unfavorable because it significantly reduces the possibility of efficient denture disinfection with the available surface-active agents [3]. Denture disinfectant agents such as chlorhexidine gluconate, sodium hypochlorite, hydrogen peroxide and more were reported to cause unfavorable changes to the soft liners' physical and chemical properties [4,5]. Moreover, disinfectant solutions usually increase the porosity of liner materials. Other antimicrobial agents which were incorporated in the liners, including nystatin, fluconazole, itraconazole and silver ions were reported to be highly effective [6]. Nonetheless as soon as the active compound is released, it may compromise the natural flora in the oral cavity and cause increased porosity of the liner materials. Apparently, the

* Corresponding author. Tel.: +972 2 6776142; fax: +972 2 6429683.
E-mail address: nuritb@ekmd.huji.ac.il (N. Beyth).
[1] Equal contribution.

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challenge is to formulate soft liner materials that incorporate core long-term potent antibacterial compounds that are not released into the surroundings. The absence of anti-biofilm properties in soft liners predisposes the patients to infection with resident oral microorganisms [7]. Clearly, for compromised patients it is essential to promote healing using the best infection management strategy.

Cationic polymers and in particular quaternary ammonium polyethyleneimines (QPEI) [8] are a promising class of antibacterial agents for infection management strategy devoid of potentially harmful metal ions. We previously demonstrated a reproducible sustainable synthetic route that allowed stable incorporation of QPEI in various polymer-based materials without detectable leaching into the surrounding milieu [9]. The present manuscript describes the formation of crosslinked quaternary ammonium polyethylenimine nanoparticles that show biocompatible, broad-spectrum bactericidal properties *in vitro* and *in vivo* when incorporated in denture lining material.

2. Materials and methods

2.1. Preparation of test samples

The following synthesis of QPEI nanoparticles was found to be optimal for lining materials. Synthesis of QPEI nanoparticle was as previously described [10]. Briefly, PEI (10 g, 0.23 mol monomer units) dissolved in 100 mL ethanol was reacted with dibromopentane at a 1:0.04 mol ratio (monomer units of PEI/dibromopentane) under reflux for 24 h. N-alkylation was conducted as follows: octyl halide was added at a 1:1 mol ratio (monomer units PEI/octyl halide). Alkylation was carried out under reflux for 24 h followed by neutralization with sodium hydroxide (1.25 equimolar, 0.065 mol) for an additional 24 h under the same conditions. N-methylation was conducted as follows: 43 mL of methyl iodide (0.68 mol) were added. Methylation was continued at 42 °C for 48 h followed by neutralization with sodium bicarbonate (0.23 mol, 19 g) for an additional 24 h. The supernatant obtained was decanted and precipitated in 300 mL double distilled water (DDW), washed with hexane and DDW and then freeze-dried. The purification step was repeated using additional amounts of hexane and DDW.

The test specimens were prepared by adding the synthesized QPEI nanoparticles to a soft liner material (GC soft liner, GC Europe, Leuven, Belgium). The nanoparticle powder was added at 0, 1 or 2% w/w to the soft liner material and homogeneously mixed for 30 s with a spatula.

2.2. In vitro antibacterial properties

Clinically isolated *Enterococcus faecalis*, *C. albicans*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* (isolated at the Maurice and Gabriela Goldschleger School of Dental Medicine at Tel-Aviv University, Israel), *Staphylococcus aureus* ATCC#8325-4 and *Streptococcus mutans* ATCC#27351 were used in this study. Bacteria were cultured aerobically overnight in 5 mL brain heart infusion (BHI) broth (Difco, Detroit, MI, USA), at37 °C. *C. albicans* was cultured aerobically overnight in 5 mL yeast extract (Merck, Darmstadt, Germany)-peptone-dextrose (Sigma–Aldrich, Steinheim, Germany) (YPD) broth, at 37 °C.

Antibacterial properties were determined using the direct contact (DCT) test [10]. Briefly, the sidewalls of wells in a polystyrene microtitre plate (96-well flat bottom plate, Nunclon, Nunc, Denmark) were coated with similar amounts of the tested material (surface area approximately 4 mm × 8 mm), 8 wells for each concentration. Triplicate microtiter plates were similarly prepared and tested after 1 month of material aging. During this time, each well was filled with 250 mL phosphate-buffered-saline (PBS; Sigma, St. Louis, MO., USA), which was replaced every 48 h, and the plates were incubated at 37 °C. The plates were dried before testing. The plates were positioned vertically, and a 10 μl volume of bacterial suspension was placed on the surface of each tested material. The plate was then incubated vertically for 1 h at 37 °C, the suspension liquid evaporated, and direct contact between the bacteria and the tested surfaces was ensured. Eight uncoated well walls served as control in the same microplate. The plate was then positioned horizontally and 220 μl of BHI broth were added to each well. Kinetic measurement of bacterial growth were performed using a temperature-controlled microplate spectrophotometer at 37 °C (VERSA max, Molecular Devices Corporation, CA, USA), with 5 s vortex mixing before each reading. Bacterial growth was estimated by following changes in optical density (A_{650}) in each well every 20 min for 24 h. Absorbance measurements were plotted, providing bacterial growth curves for each well in the microtiter plate, and the linear portion of the logarithmic growth phase was subjected to statistical analysis, the slope correlating with bacterial growth rate and the intercept correlating with total viable count. Calibration experiments were performed simultaneously for each plate. Triplicate wells containing 265 μl BHI were inoculated with 10 μl bacterial suspension. A fivefold dilution was repeated 7 times in triplicates. A gradual and reproducible decrease in O.D. correlated with serial dilution.

To examine the possible effect of leachable materials from the soft liner materials, the antibacterial properties of the elute from the tested materials on planctonic growth was determined quantitatively. In a separate 96-well microtiter plate, eight sidewalls were coated with each tested material and aged for one week or 4 weeks, as described above. Then, a volume of 230 mL of BHI was added to each well and the plate was incubated for 24 h at 37 °C. A 220 mL volume was transferred from each well to an adjacent set of wells and 10 μl of a bacterial inoculum, prepared as described above, were added to determine the effect of components eluted into the broth. The plate was placed in the temperature-controlled microplate spectrophotometer set at 37 °C, with 5 s mixing before each reading. Bacterial growth was assessed by following the changes in absorbance (650 nm) every 20 min for 24 h. Growth curves were analyzed as described above in the DCT.

In addition, chemical analysis was performed to trace whether any leachable materials were released into the elute collected from the tested material. For this purpose uniform, test-discs were prepared (1 mm thick, 1 cm diameter, 50 ± 2 mg). Test-discs were placed in 5 mL of DDW for 24 h, 1 and 4 weeks. At the end of the test period the water was collected, frozen in liquid nitrogen (−190 °C) and lyophilized for 24 h to collect any residues released during the incubation period of the materials. In addition, soft liner material test-discs with incorporated 0, 1 or 2% w/w nanoparticles labeled with a fluorescent dye dansyl chloride (Sigma–Aldrich) were prepared and incubated in 5 mL DDW for 1 and 4 weeks at 37 °C. At the end of the period, the DDW was collected and placed in a Luminometer device (Fluostar galaxy, BMG Labtechnologies, Offenburg, Germany) and dansyl chloride values were recorded (excitation, 335 UV; emission, 515U V).

The antibacterial effect of the novel endodontic sealer was also tested using the agar diffusion test (ADT). Test material discs were prepared as described above and evaluated. A bacterial suspension (200 μl; 10^6 CFU/mL) was spread on blood agar plates and the discs were placed on the surface. The plates were incubated for 24 h at 37 °C and the inhibition zones around each specimen were measured. The absence of an inhibition halo was scored zero. The inhibition diameter, including the disc diameter, was measured.

To characterize the interactions between the QPEI nanoparticles and the bacteria, membrane integrity was evaluated using the cytoplasmic membrane depolarization assay. Bacteria were cultured overnight in 5 mL BHI at 37 °C under aerobic conditions. Test tubes containing 9 mL of DDW with or without QPEI nanoparticles were prepared (1 mg/mL) (test group, and control, respectively). Then 1 mL of the bacterial suspension was added to each tube, followed by incubation at 37 °C in a temperature-controlled incubator for 1 h. A BacLight Bacterial Membrane Potential kit (Molecular Probes, Invitrogen) was used according to the manufacturer's instructions: 10 μl carbonyl cyanide 3-chlorophenylhydrazone (500 μm CCCP), a proton ionophore which destroys membrane potential, were added to the samples to serve as a positive (depolarized) control. Then 10 μl of the active component 3,30-diethyloxacarbocyanine iodide (3 mm DiOC2), the fluorescence of which changes from green to red with increasing membrane potential, were added to the samples. The cells were analyzed by flow cytometry (BD accuri C6) and the results were expressed as the ratio of red fluorescence to green fluorescence, mean fluorescent intensity (MFI).

2.3. Biocompatibility assays

The raw 264.7 macrophage cell line was cultured in Petri dishes in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS), 1% penicillin/streptomycin and 1% glutamine. Before the assay, the cells were seeded at a density of 60,000 cells/well in 96-well tissue culture plates (NUNC). At 24 h after plating, the cells were activated by heat-killed *Porphyromonas gingivalis* 33277 ATCC (grown at 37 °C under anaerobic conditions and heat killed at 80 °C for 10 min), and exposed to the liner material (which was prepared as described above and polymerized over a special insert that was placed in the wells). Following 24 h incubation at 37 °C in a humidified atmosphere of 5% CO_2, the plates were analyzed for cell viability.

The viability of the cells was evaluated using a colorimetric XTT assay, as described by Scudiero et al. [11]. The assay is based on the ability of metabolically active cells to reduce the tetrazolium salt XTT to orange colored compounds of formazan. In brief, following 24 h incubation, 50 mL of XTT labeling mixture were added to each well and the microplates were incubated for a further 4 h. A Vmax microplate reader (Molecular Devices Corporation) with a 450 nm optical filter and a 650 nm reference wavelength was used to measure the absorbance of each well.

The subcutaneous chamber model was used in this study as recently described [12] with some modification. Briefly, chambers (length,1.5 cm; diameter, 5.16–0.08 mm) constructed from coils of titanium wire containing a piece of soft liner material with 0, 1 or 2% w/w incorporated QPEI nanoparticles (2 × 2 mm) were subcutaneously implanted in the dorsolumbar region of each mouse. After a healing period of 1 week, the chambers were used as a biological compartment for testing the biocompatibility of the liner materials. Twenty 5- to 6-week-old female balb/c mice were divided into four groups of 5 mice each. The chambers were sampled every week for a month. The Internal Review Board of The Hadassah-Hebrew University Medical Center approved the experimental protocol.

Chamber exudates were centrifuged at 200 g for 5 min at 4 °C. The supernatants were removed and stored at −20 °C until analyzed. The pellets were immediately