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# Anti-biofilm properties of wound dressing incorporating nonrelease polycationic antimicrobials

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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

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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).

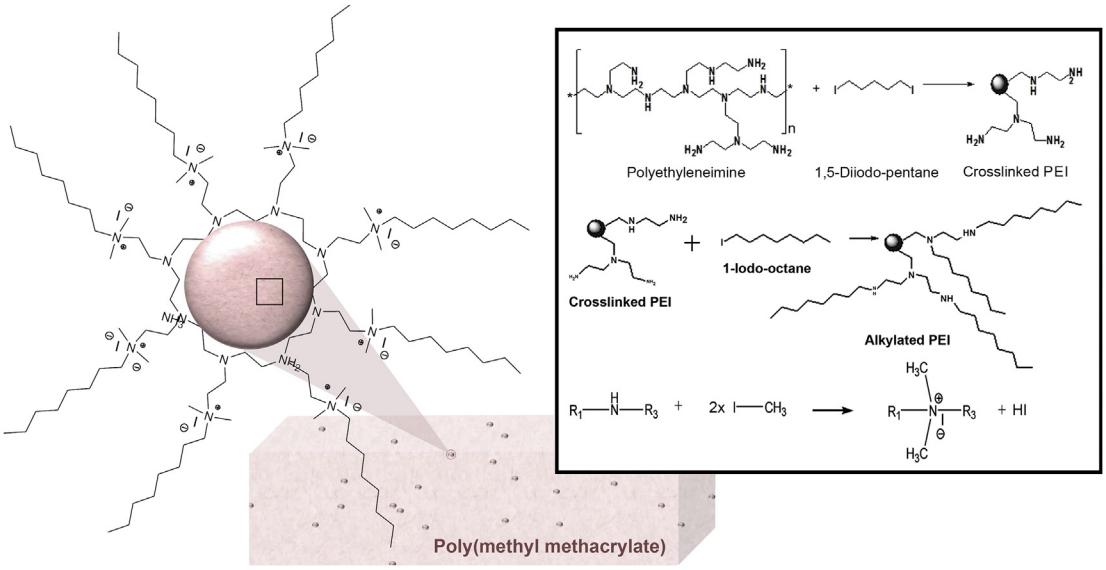
### 2.3. Biocompatibility assays

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




| Yield | wt/wt 96% |
|---|---|
| FT-IR (KBr) | 3400 $cm^{-1}$ (N-H), 2928 $cm^{-1}$ and 2856 $cm^{-1}$ (C-H), 1620 $cm^{-1}$ (N-$H_2$), 962 $cm^{-1}$ quaternary nitrogen. |
| H-NMR (DMSO) | 0.845 ppm (t, 3H, $CH_3$, octane hydrogens), 1.24 ppm (m, 10H, -$CH_2$-, octane hydrogens) 1.65 ppm (m, 2H, CH, octane hydrogens), 3.2-3.6 ppm (m, $CH_3$ of quaternary amine, 4H of PEI and 2H of the octane chain). |
| Particle size (ALV,R) | 53±2 nm |
| Elemental analysis | %C = 33.6, %H = 6.83, %N = 6.83, %I = 47.6 |
| **Zeta potential (mV)[e]** | 60.9±2.1 |

**Fig. 1.** Schematic showing soft liner material incorporating QPEI nanoparticles (for nanoparticle characterization, see table).

resuspended in 200 mL PBS, and the total cell count was calculated using a hemocytometer. The presence of TNFα and IL-10 in the chamber supernatants was determined by a two-site enzyme-linked immunosorbent assay (ELISA).

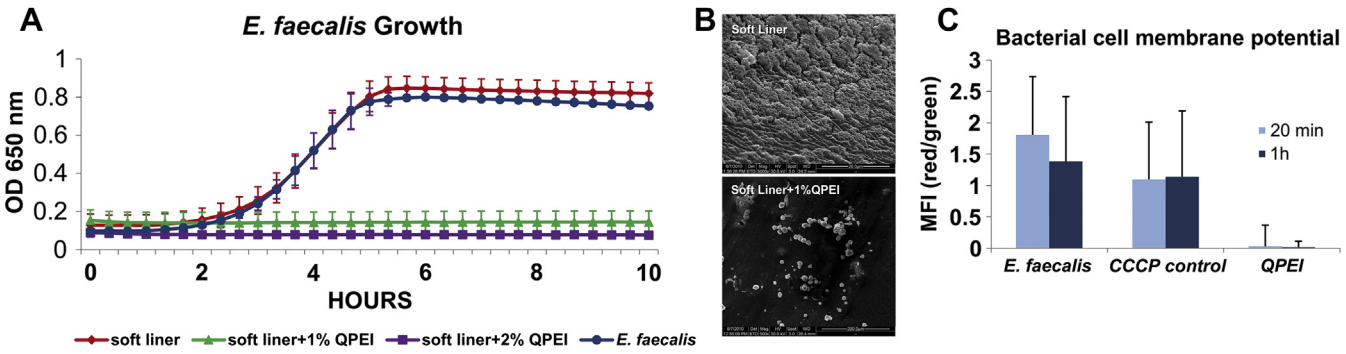
The presence of cytokines was determined by two-site ELISA [13]. The TNFα assay was based on commercially available antibody pairs (Pharmingen, San Diego, CA, USA). The 96-well ELISA plates were coated with 1 mg/mL anti-mouse cytokine monoclonal antibodies, and blocked with 3% bovine serum albumin (BSA). After addition of the samples, a secondary biotinylated antibody that served as the detecting antibody was added, followed by a streptavidin–horseradish peroxidase conjugate (Jackson Immunoresearch Laboratories, West Grove, PA, USA). The substrate used was o-phenylenediamine (Zymed, San Francisco, CA, USA). The reaction was terminated by the addition of 4 N sulfuric acid, and the optical density was read with the aid of a Vmax microplate reader (Molecular Devices Corporation) at 490–650 nm.

### 2.4. Physical properties

Tensile properties (strength and stiffness) of the soft liner material incorporating QPEI nanoparticles were tested on standard samples (80mm × 6mm × 2 mm) of GC Soft-Liner with 0, 1 or 2% w/w incorporated QPEI nanoparticles (n = 15 for each test group), using a loading machine – Instron 4502 (Instron Corp., Canton MA, USA) 100 N load cell, cross head speed – 50 mm/min, force – 10 N, and maximal displacement – 200 mm. Additionally, the adhesion failure point between the soft liner materials and the obturator base resin was evaluated using a loading machine, Instron 4502. All specimens were placed under tension until failure in a universal testing machine at a crosshead speed of 5 mm/min. The point of failure was recorded.

### 2.5. In vivo antibacterial properties

The protocol of the study was approved by the institutional Helsinki Committee for Human Clinical Trials (ClinicalTrials.Gov Identifier: NCT3261). Three adult post first maxillectomy surgery with no history of radiology or chemical treatment and with no other systemic diseases, volunteered to participate in the study. Obturators in 3 post-surgery maxillofacial patients were lined with a soft liner material (soft GC line, GC Europe, Leuven, Belgium) with and without QPEI nanoparticles. Liner specimens were collected from the obturators after one week, followed by full replacement of the liner. Specimens were dyed using a live/dead kit (Live/Dead *Bac*Light viability kit, Molecular Probes, OR, USA). The stained bacteria were examined using a confocal laser scanning microscope (CLSM); the fluorescence emission of the samples was detected using a Zeiss LSM 410 confocal laser microscope (Carl Zeiss Microscope, Germany). Red fluorescence was measured at 630 nm and green fluorescence was measured at 520 nm; objective lenses: ×60/oil, 1.4 numerical aperture. Horizontal plane (x–y axes) optical sections were made at 5-μm intervals from the surface outward and images were displayed individually. The biofilm was quantified by measuring the area occupied by the microorganisms in each individual layer in relation to the tested area. The bacterial index was determined with the aid of Image Pro 4.5 software (Media Cybernetics, USA).




| | Direct contact test (log) | | | | Agar diffusion test (mm) | | |
|---|---|---|---|---|---|---|---|
| | Control | 0%wt/wt | 1%wt/wt | 2%wt/wt | 0%wt/wt | 1%wt/wt | 2%wt/wt |
| *Candida albicans* | 6 | 5.8±0.003 | 0±0.000018 | 0±0.0003 | 0 | 0 | 0 |
| *Enterococcus faecalis* | 6 | 4.9±0.0012 | 0±0.00006 | 0±0.00006 | 0 | 0 | 0 |
| *Streptococcus mutans* | 6 | 6.6±0.00036 | 0±0.0003 | 0±0.00012 | 0 | 0 | 0 |
| *Staphylococcus aureus* | 6 | 5.1±0.00048 | 0±0.00024 | 0±0.00006 | 0 | 0 | 0 |
| *Pseudomonas aeruginosa* | 6 | 5±0.0018 | 0.66±0.0024 | 0.48±0.0012 | 0 | 0 | 0 |
| *Staphylococcus epidermidis* | 6 | 5±0.0018 | 0.66±0.0024 | 0.24±0.0012 | 0 | 0 | 0 |

**Fig. 2.** Soft liner material incorporating polycationic antimicrobial nanoparticles inhibits bacterial growth. (A) Antibacterial activity of modified liner incorporating QPEI nanoparticles *in vitro*. Representative outgrowth curve of the Gram-positive *E. faecalis* after direct contact with 1 month-aged samples incorporating QPEI, (B) corresponding SEM micrographs, (C) cytoplasmic depolarization, expressed as linear mean fluorescence index (MFI) of the red *vs*. green ratio, a higher ratio indicating a higher membrane potential (carbonyl cyanide 3-chlorophenylhydrazone (CCCP)-positive control). The table presents the outgrowth (log ± SD), and agar diffusion inhibition zone (diameter; mm) adjacent to the disc samples of the 6 tested microorganisms.

Soft liner specimens removed from the volunteers' obturators were pressed, using sterile tweezers, against an agar plate, leaving an imprint on the nutrient agar (Tryptic Soy Blood Agar (TSBA) (Difco) plate. Six consecutive imprints were made for each sample on the same agar plate. The plates were then incubated for 24 h at 37 °C and bacterial growth was recorded as growth or non-growth.

Intra-oral odor levels were determined by means of a portable sulfide monitor (Halimeter sulfide monitor, Interscan Corp., CA., USA) as previously described [14]. The volunteers were asked to refrain from talking for 5 min prior to measurement. The monitor was zeroed on ambient air, and the measurements were performed by insertion of a disposable one-fourth inch plastic straw approximately 5 cm into the partially opened oral cavity. Participants were asked to breathe through their noses during the measurements. The results were recorded as peak ppb hydrogen sulfide equivalents.

## 3. Results

### 3.1. Characterization of QPEI nanoparticles

A schematic view of the incorporated nanoparticles in the obturator base resin material and characteristics of the synthesized quaternized alkylated PEI-based nanoparticles appear in Fig. 1.

### 3.2. In vitro antimicrobial properties

Total bacterial growth inhibition was observed in all the tested microorganisms after direct contact with the soft liner materials incorporating 2% wt/wt QPEI nanoparticles (Fig. 2). Based on the linear portion of the logarithmic growth in the calibration curves, the number of residual viable bacteria on each tested specimen was determined and analyzed. In samples with incorporated QPEI nanoparticles, the number of residual viable bacteria decreased by 5–6 logs ($p < 0.0001$) *vs.* the number in liners without nanoparticles. SEM images revealed that most of the observed bacteria on the liner incorporating QPEI nanoparticles underwent changes in morphology and dispersion (Fig. 2). Biofilm formation with intact membranes and dividing cells was observed on the non-modified soft liner (Fig. 2).

A cytoplasmic membrane depolarization assay after 20 min and 1 h in the control group of untreated bacteria showed a red/green fluorescence ratio of 1.8 and 1.39, respectively. When the bacteria were exposed to a low concentration of QPEI nanoparticles, the ratio decreased to 0.017 after 20 min, and 0.03 after 1 h expose (Fig. 2).

The antibacterial properties of the eluted components released from the tested materials were evaluated. All the microorganism growth curves were similar to those of the appropriate control for the 0, 1 or 2% w/w added nanoparticles (data not shown). In these tests, the elute from the specimens did not show any organic molecules or polymers, and bacterial outgrowth was similar to that seen in the elute of the non-modified lining material. The latter correlated with the ADT where, no inhibitory halo was observed (Fig. 2).

### 3.3. Biocompatibility

The biocompatibility of the soft liner material incorporating QPEI-based nanoparticles was tested both *in vitro* and *in vivo*. *In vitro* examination showed that the QPEI nanoparticles did not affect the cytotoxicity of the base liner. The viability of the macrophages was similar to that of the control (Fig. 3). Further *in vivo* investigation using the chamber model in mice revealed that the migration of leukocytes was not amplified and the levels of the secreted inflammatory cytokine TNFα in the chamber remained as in the control (Fig. 3).

### 3.4. Physical properties

The tensile properties tested (strength and stiffness) revealed no compromise in mechanical properties following incorporation of

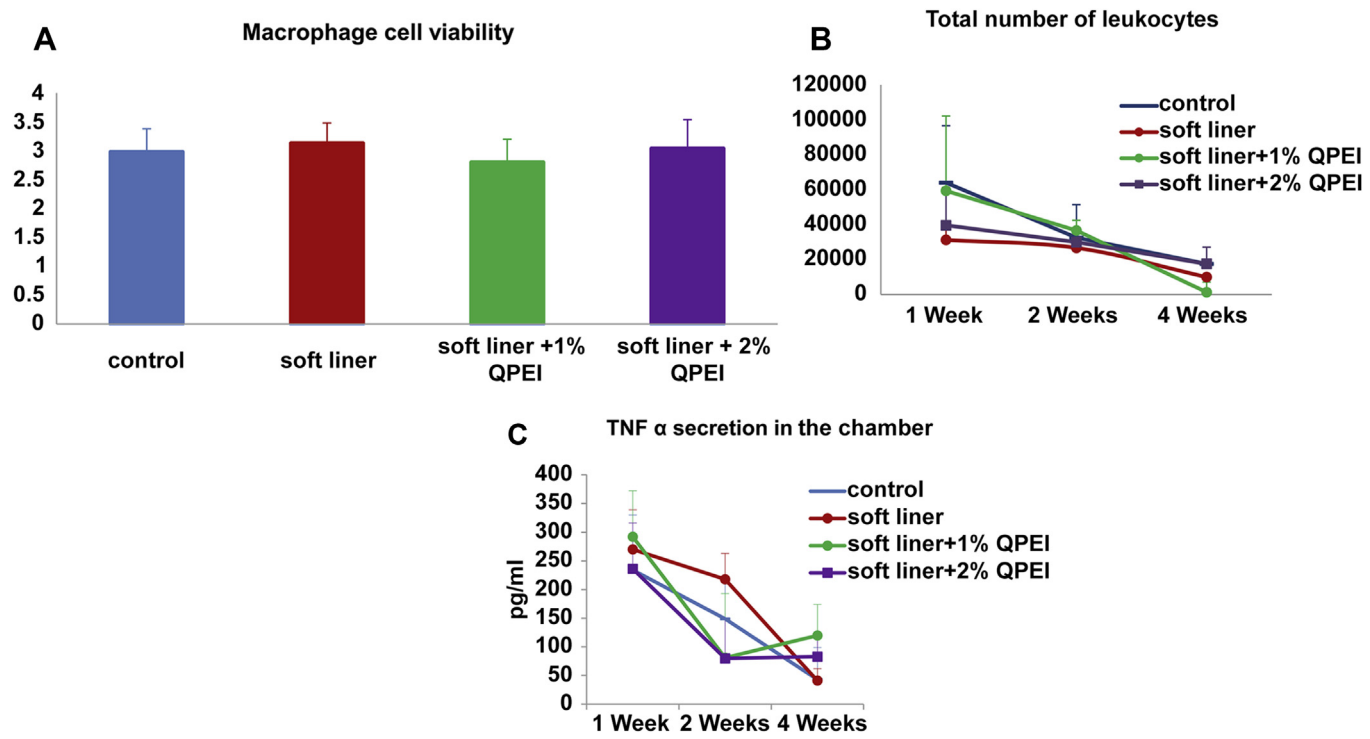



**Fig. 3.** Biocompatibility assessment. The cytotoxicity of the modified liners incorporating QPEI was evaluated using (A) XTT macrophage RAW *in vitro*, (B) *in vivo* migration of leukocytes and (C) secreted inflammatory cytokine TNFα was tested in mice using the chamber. No statistical differences were found *vs*. the control.

QPEI nanoparticles (Table 1). The failure point test showed no evident effect of QPEI addition to the liner on obturator-soft liner attachment (Table 1).

### 3.5. *In vivo* antibacterial properties

Specimens were subjected to CLSM examination. Reduced bacterial viability was depicted in the one-week-old biofilms formed on the liner incorporating QPEI nanoparticles. Agar imprint of the liner specimens showed bacterial growth only in samples without QPEI nanoparticles (Fig. 4).

No odor difference was recorded between soft liner specimens with or without QPEI nanoparticles in the same patient. A qualitative evaluation by a laboratory technician resulted in reports of a strong offensive odor that was evident only in the soft liner specimens without QPEI nanoparticles.

**Table 1**
Tensile properties(strength and stiffness) of the soft liner material incorporating QPEI nanoparticles.

| Physical properties | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Strength (Mpa)[a] | Stiffness ($K = F/\delta$)[b] | Failure location[c] | | | Dissolution | |
| | | | Mid | Interface | Margins | UV | g/ml |
| 0% QPEI | 0.1955 | 0.07 | 47% | 20% | 33% | – | – |
| 1% QPEI | 0.1839 | 0.05 | 60% | 13% | 27% | – | – |
| 2% QPEI | 0.1496 | 0.04 | 93% | 0% | 7% | – | – |

[a] Strength. Insignificant differences were observed between the 1% group and the control group. In the 2% group the decrease in strength was significant *vs*. the other groups, but within standard range (ANSI/ADA ISO10139).

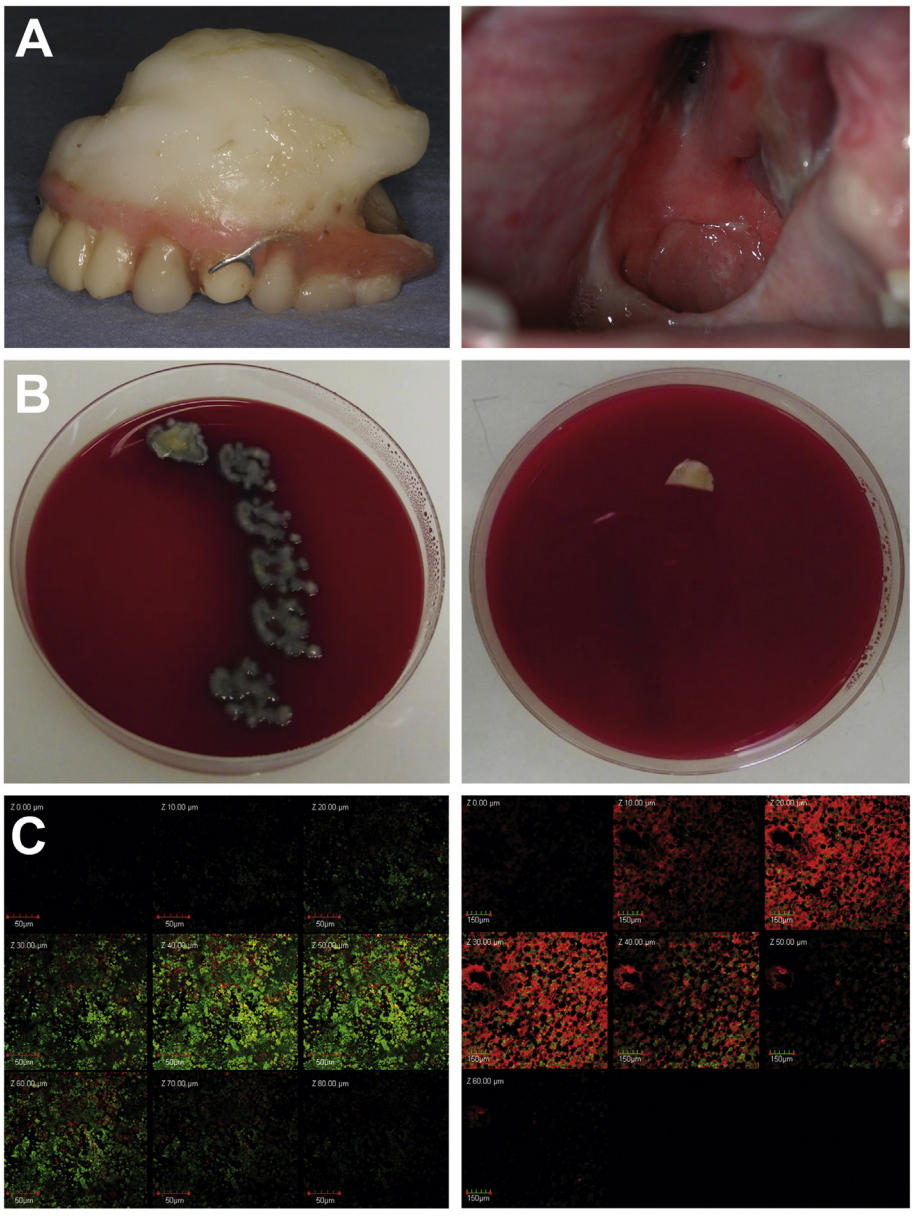
[b] Stiffness. Force *vs*. displacement: the amount of force needed to exert unit elongation. No statistical difference was observed in the presence of absence of the nanoparticles ($p < 0.001$).

[c] Failure point test. Three failure locations were evaluated: (*i*) mid-point of the sample, (*ii*) margins of the sample and (*iii*) acryl-soft liner interface. Modification of the soft lining material to achieve antibacterial properties using QPEI showed no increase at acryl-soft liner failure point.

## 4. Discussion

We modified a commercially available polymethyl methacrylate (PMMA)-based soft liner by incorporating low concentrations of QPEI nanoparticles during its polymerization. When incorporated in resin-based dental materials [10], the most potent and long-lasting antibacterial effect was obtained using crosslinked poly(-ethyleneimine) N-alkylated with octyl halide, followed by quaternization of the amino groups with methyl iodide (Fig. 1). Additionally, QPEI nanoparticles and modified soft liner properties were determined (zeta potential, particle size and chemical composition were determined for the particles and particle distribution was determined following liner modification). Physico-chemical parameter evaluation, including size, charge, and hydrophobicity, have emphasized the importance of the degree of alkylation on activity [15]. We previously synthesized and tested a pool of polycationic nanoparticle derivatives consisting of alternating polyamines and alkyl halides, using various synthetic methods. Comparative analysis of numerous QPEI samples showed that their antimicrobial potency is related to alkyl chain length, extent of cross-linking, N-methylation and particle size, revealing a reproducible sustainable synthetic route.

We determined the minimal concentration needed for total bacterial growth inhibition of six test microorganisms by the QPEI nanoparticles incorporated in the soft liner material (Fig. 2). After one month aging, modified soft liner incorporating a low percentage of QPEI nanoparticles completely inhibited proliferation of viable *E. faecalis*, *S. mutans*, *C. albicans*, *S. aureus*, *P. aeruginosa* and *S. epidermidis* (Fig. 2), indicating that the antibacterial activity emerges when the microorganism comes in direct contact with the




| | Soft liner | Soft Liner + QPEI |
|---|---|---|
| Viable cells% | 75.1 ± 17.17 | 32.38 ± 17.26 |
| Safety | No tissue irritation | No tissue irritation |
| Odor | Highly offensive odor | No odor |

**Fig. 4.** Soft liner materials incorporating polycationic antimicrobial nanoparticles inhibit bacterial growth in post-surgery maxillofacial patients. (A) Representative image of obturator prosthesis and excision site (right and left panels, respectively). Circles represent sample sites (5 mm diameter). Representative results of biofilms formed on liner material after 1 week in patient's mouth (B) agar imprint of non-modified (left panel) and liner material incorporating QPEI (right panel) and (C) confocal laser scanning microscopy of biofilm outgrowth stained using BacLight LIVE/DEAD viability stain. Non-modified liner samples (left panel) show mainly live cells (stained green), the liner samples incorporating QPEI nanoparticles (right panel) show mainly dead cells (stained red). The table summarizes the average number of viable bacteria (percentage ± SD), tissue irritation and mouth odor descriptions of all patients.

modified material surface [10,16]. Incorporation of QPEI-based nanoparticles (at 1 or 2% wt/wt), in a soft liner material displayed potent growth inhibitory activity against all the Gram-negative bacterial strains tested including clinically challenging species such as *E. faecalis* and *Pseudomonas* spp. Bacterial death correlated with membrane depolarization in the presence of QPEI (Fig. 2). Membrane potential is an important marker of membrane integrity. A reduction in the magnitude of the membrane potential is referred to as electrical depolarization. When the membrane potential is reduced to zero this indicates that the membrane is

completely ruptured [17]. The decrease in the mean fluorescent index value indicates that the bacterial membrane had a lower membrane potential value, i.e. lower cytosolic concentrations, a finding that may suggest that the nanoparticles caused membrane damage and changes in cell permeability. Interestingly, a less effective outcome was obtained with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) proton ionophores, a known highly depolarizing compound material. This suggests that QPEI nanoparticles have a similar mechanism of action involving destabilization of the cytoplasmic membrane. These results concur with previous findings that suggest that although the cationic polymer mode of action is not well understood, these polymers are believed to have strong bactericidal activity, including cell membrane disruption and lysis [15,18].

Because soft liner materials are replaced every other week, and are easily damaged by disinfectant solutions, we tested their physical properties and antibacterial effect after 1 month aging. The non-modified inactive sealers became active when QPEI nanoparticles were incorporated, displaying a strong antibacterial effect against all the tested microorganisms (Fig. 2). The activity of most antibacterial agents used in soft liners is dependent on their release from the set material and elution into the surrounding milieu. Consequently, incorporation of such components into the liner results in continuous dissolution and eventual material degradation over time. It follows that antibacterial properties are likely to diminish within a short period of time, especially in the oral aqueous environment. Materials containing released antibacterial compounds will become less stable and, therefore, will not fulfill the basic requirement of remaining stable over time. Liner materials should, if possible, act by direct contact and be water-insoluble. These properties should ensure the stable, non-degradable properties of the set material.

As soft liner materials are often used in intimate contact with the mucosa, our next step was to test the biocompatibility of the modified soft liner material incorporating QPEI nanoparticles. *In vitro* examination showed that the QPEI nanoparticles did not affect the cytotoxicity of the base liner (Fig. 3). Further *in vivo* investigation using the chamber model in mice revealed that the migration of leukocytes was not amplified by the QPEI nanoparticles and the levels of the secreted inflammatory cytokine TNF$\alpha$ in the chamber remained as in the control (Fig. 3).

Having attained potent antibacterial activity and adequate biocompatibility of the modified soft liner, we tested the physical properties essential for proper soft liner function in the oral cavity. The tensile properties tested (strength and stiffness) revealed no compromise in mechanical properties following incorporation of QPEI nanoparticles (Table 1). Adhesion failure between soft liner materials and the obturator base resin is commonly encountered in clinical practice. This adhesion failure may result in localized unhygienic conditions at the debonded regions, increasing the potential for contamination and functional failure of the prosthesis. Thus, we further determined the failure point at which the soft liner material detaches from the obturator base material and found no evident effect of QPEI addition to the liner on acryl-soft liner attachment. Moreover, nanoparticles were not eluted from the modified liner (Table 1).

On the basis of the screening described above, an *in vivo* clinical proof of concept trial was performed. Obturators in 3 post-surgery maxillofacial patients were lined with a soft liner material with and without QPEI nanoparticles (Fig. 4). Liner specimens were collected from the obturators after one week followed by full replacement of the liner. Clinical examination of *in situ* bacterial contamination and the bacterial reaction to the antibacterial compound is indeed to a large extent a more precise means of examining the antibacterial properties as compared with *in vitro* assays. Confocal scanning laser microscopy (CSLM) is a powerful tool for studying the distribution of dead and live bacteria in a formed biofilm [19]. Confocal laser scanning microscopy (CLSM) allowed us to explore the vitality of bacteria in the different layers of the biofilm following treatment. Bacterial viability in the one-week-old biofilms formed on the liner was reduced dramatically in specimens incorporating QPEI nanoparticles (Fig. 4). No abnormal tissue irritation was observed or reported by the patients when the soft liner was used with or without QPEI nanoparticles. As post-maxillectomy patients have a typically offensive mouth odor, which frequently occurs due to post-operative infection, odor was also tested. The dental technician participating in this trial consistently reported a highly offensive odor when removing the inner aspect of the surgical obturator in the sessions that included QPEI-absent liner. These results are coincident with our previous clinical findings showing that QPEI compounds are highly potent antibacterial agents [20]. Taken together, these results suggest that QPEI nanoparticles offer the potential to prevent contamination in orofacial cancer patients.

## 5. Conclusion

QPEI nanoparticles when incorporated in wound dressings such as denture lining materials appear to offer several advantages, including (i) a potent, broad spectrum and long-lasting rapid antibacterial effect, (ii) an anti-biofilm effect *in vivo*, (iii) active ingredients that do not include metal ions and are not released into the surroundings, (iv) physical and biocompatible properties similar to those of a conventional dressing, (v) the ability to overcome the disadvantages of common antiseptics or antimicrobials, with respect to bioavailability and/or toxicity and (vi) possible prevention of persistent infections associated with antibiotics resistant bacteria. Finally, this wound dressing strategy proffers an additional tool for management of infection in life-threatening contaminations, especially in immune-compromised patients.

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