

Inhibition of Biofilm Formation on Ventilation Tubes by Surface Modification

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Abstract. *Aim: The purpose of this study was to modify the surface characteristics of a ventilation tube (VT) with polyethylene glycol (PEG) coating and to evaluate the effect on biofilm formation. Materials and Methods: VTs made of polyethylene were coated with PEG. Streptococcus pneumoniae R6 strain was used and a crystal violet assay was carried out to measure the in vitro and in vivo biofilm formation of rats bearing VTs. Results: In the in vitro experiment, the optical density of the uncoated VT was 0.34 ± 0.09 and the optical density of the PEG-grafted VT was 0.22 ± 0.06 ($p < 0.05$). In the in vivo experiment, the optical density of the uncoated VT was 0.54 ± 0.12 and that of the PEG-grafted VT was 0.32 ± 0.13 ($p < 0.05$). Scanning electron microscopy showed that surface modification, roughness and hydrophilic characteristics improved and biofilm formation decreased. Conclusion: The reduced biofilm formation on the VT may be explained by the alteration of surface tension and roughness induced by PEG coating.*

Otitis media (OM) is caused by bacterial infection of the middle-ear cavity and is the most common reason for pediatric patients to visit a physician and to undergo ventilation tube (VT) insertion (1). Postoperative otorrhea is a common complication following VT insertion and is thought to be caused by pathogenic bacteria present in acute OM (2). Most cases are curable with otic drops, however, in severe cases, the VT should be removed.

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Bacterial biofilm is found attached to surfaces, and co-exists with an extracellular exopolysaccharide matrix. The bacteria present have greatly-reduced metabolic and divisional rates (3) and this often leads to failure of diagnosis by conventional culture techniques and eradication by antibiotic treatment in cases of biofilm infection (4).

Bacterial biofilm formation has been implicated in high rates of persistent otorrhea after VT insertion (5). It has been shown that coating medical implants with antimicrobial agents may effectively prevent the initial adherence of staphylococcal biofilm to the implants (6, 7).

Factors affecting biofilm formation include surface characteristics such as roughness (8). There were reports that surface roughness affects biofilm formation of various pathogens (9, 10). Van der Mei reported that prevention of bacterial adhesion can be achieved by coating the surface with a hydrophilic polymer (11).

Thus, we hypothesized that by performing a surface modification of polyethylene VT, using a hydrophilic polyethylene glycol (PEG) coating, biofilm formation can be inhibited. The purpose of this study was to test this hypothesis by evaluating the preventive effect of PEG coating on biofilm formation of *Streptococcus pneumoniae* R6.

Materials and Methods

Materials. Polyethylene film from NamilEnpla (Hwaseong, Korea) was used for the surface tension analysis and morphological analysis. VTs made of polyethylene (Tecfen, Santa Barbara, CA, USA) were used for the biofilm formation analysis. PEG-diacrylate and 2,2-dimethoxy-2-phenyl acetophenone (DMPA), were purchased from Sigma (St. Louis, MO, USA).

Oxygen plasma treatment and grafting of PEG. After loading the polyethylene film and VT in a low-pressure plasma reactor (PTS-0031DT; IDT-ENG, Seoul, Korea), oxygen gas was supplied at a flow rate of 100 standard cubic centimeters per minute (sccm). After

purging the plasma chamber with oxygen gas, surface modification of polyethylene film and VT was performed. Gas concentrations, pressure, treatment time, and power to chemical modifications were controlled. Both 100 W and 100 sccm flow rates were employed for surface modification of the polyethylene film and VT.

Grafting of PEG-diacrylate onto the plasma-treated polyethylene film and VT was performed by irradiation. The plasma-treated polyethylene film and VT, coated with a PEG-diacrylate solution which contained 6% PEG-diacrylate and 0.3% DMPA initiators in benzene, was irradiated for 5 min with ultraviolet light. The PEG-diacrylated PE tubes were dried in a vacuum oven overnight, and the stability of the PEG-grafted polyethylene film and VT was tested by washing with 30 ml of benzene for 1 h with shaking at 70 rpm.

Contact angle measurement. Drops of distilled water (0.025 ml) were placed on non-coated film, polyethylene plasma-treated film and PEG-grafted film. Static contact angles were measured using a contact angle meter (G-1; Erma Inc., Tokyo, Japan).

Bacterial strain and growth conditions. *S. pneumoniae* R6 strain (ATCC BAA-255) used in this study was obtained from the American Type Culture Collection (Manassas, VA, USA). Bacteria were grown on a blood agar plate with 5% sheep's blood. A fresh colony was transferred in trypticase soy broth (TSB) and grown at 37°C for 12 h in 5% CO₂.

In vitro biofilm formation experiment. Biofilm formation of PEG-grafted VTs (n=7) was compared with that of uncoated ones (n=7). For quantitative analysis of *in vitro* biofilm formation, crystal violet treatment was performed using a modification of a previously reported protocol (12, 13). Briefly, *S. pneumoniae* was grown up to the mid-logarithmic phase and a solution containing 1×10⁸ (CFU)/ml was diluted 1:100 with fresh sterile medium, and 200 µl of this solution was inoculated into 96-well microplates. The VTs were submerged in the wells and incubated in stationary mode at 37°C for 18 h in 5% CO₂. After incubation, the tubes were removed and gently washed three times with 200 µl sterile phosphate-buffered saline (PBS). Thereafter, the tubes were air-dried, and stained with 50 µl of 0.2% crystal violet for 15 min. Excess stain was decanted-off and the tubes were washed three times with sterile distilled water. Adherent crystal violet was dissolved in 200 µl of 95% ethanol and the optical density (OD) was measured at 570 nm in an automatic spectrophotometer (SpectraMax plus 384 microplate reader; Molecular Devices, Sunnyvale, CA, USA).

In vivo biofilm formation experiment. The experimental protocol was reviewed and approved by the Animal Research and Care Committee at Dongguk University Ilsan Hospital (Gyeonggi, Korea). Sixteen specific pathogen-free, male, Sprague Dawley rats weighing 150-200 g (Orient Bio, Gyeonggi, Korea) were assigned to the study. Animals were examined by otomicroscopy in order to document abnormal middle-ear status. Rats were assigned randomly to groups that received either a bacterial inoculation (experimental groups with uncoated VT or PEG-coated VT, n=8) or no procedure (control group, n=8). For the experimental groups, 50 µl of *S. pneumoniae* cell suspension containing 3×10⁷ CFU was injected into the right middle-ear cavity and a VT was inserted. Animals were sacrificed two weeks after bacterial inoculation, and the VTs were acquired. Biofilm formation was analyzed using crystal violet assay, as described above.

Analysis by scanning electron microscopy (SEM). For morphological analysis of biofilm formation, samples from the *in vivo* experiment were observed with SEM. Samples were pre-fixed by immersion in 2% glutaraldehyde in 0.1 M phosphate buffer, and post-fixed for 2 h in 1% osmic acid dissolved in PBS. Samples were treated in a graded series of ethanol and *t*-butyl alcohol, dried in a freeze dryer (ES-2030; Hitachi, Tokyo, Japan), platinum-coated using an ion coater (IB-5; Eiko, Kanagawa, Japan), and observed under an FE-SEM (S-4700; Hitachi, Tokyo, Japan).

Statistical analysis. Data are expressed as the mean±SD. The comparisons were performed using the Mann-Whitney test in SPSS for Windows (Ver. 12.0, SPSS Inc., Chicago, IL, USA). Differences were considered significant when *p*-values were less than 0.05.

Results

Contact angle analysis. Surface tension of the grafted polyethylene film was analyzed by measuring water contact angles. A lower contact angle indicates higher surface interaction between water and sample. Plasma treatment changed the tube surface from hydrophobic to hydrophilic, *i.e.*, while the control polyethylene samples had a contact angle of 71°, the plasma-treated ones had 18°. The PEG-coated samples demonstrated complete water spreading on their surfaces.

Morphological analysis. Surface morphologies of VTs were investigated with SEM by comparing the features in non-coated VTs with the plasma-treated and PEG-grafted ones. We observed decreased surface roughness in the plasma-treated and PEG-grafted VTs. The plasma treatment (Figure 1B and E) and PEG grafting (Figure 1C and F) seemed to induce surface smoothness, compared with the untreated controls (Figure 1A and D).

In vitro biofilm formation experiment. A quantitative analysis of *in vitro* biofilm formation by *S. pneumoniae* on VTs was performed. The mean OD measured for uncoated VTs was 0.34±0.09 and that from PEG-grafted VTs was 0.22±0.06; the difference was significant.

In vivo biofilm formation experiment. A quantitative analysis of *in vivo* biofilm formation by *S. pneumoniae* on VTs was performed. The mean OD measured for the uncoated VTs was 0.54±0.12 and that from PEG-grafted VTs was 0.32±0.13; the difference was significant.

Discussion

A biofilm results from a unique phenotype of microorganism which is attached to surfaces, and co-exists with an extracellular exopolysaccharide matrix. Biofilm is the counterpart of planktonic (free-floating) bacterial form and displays a different phenotype from that of the planktonic form. It has greatly-reduced metabolic and divisional rates (3). It is known that

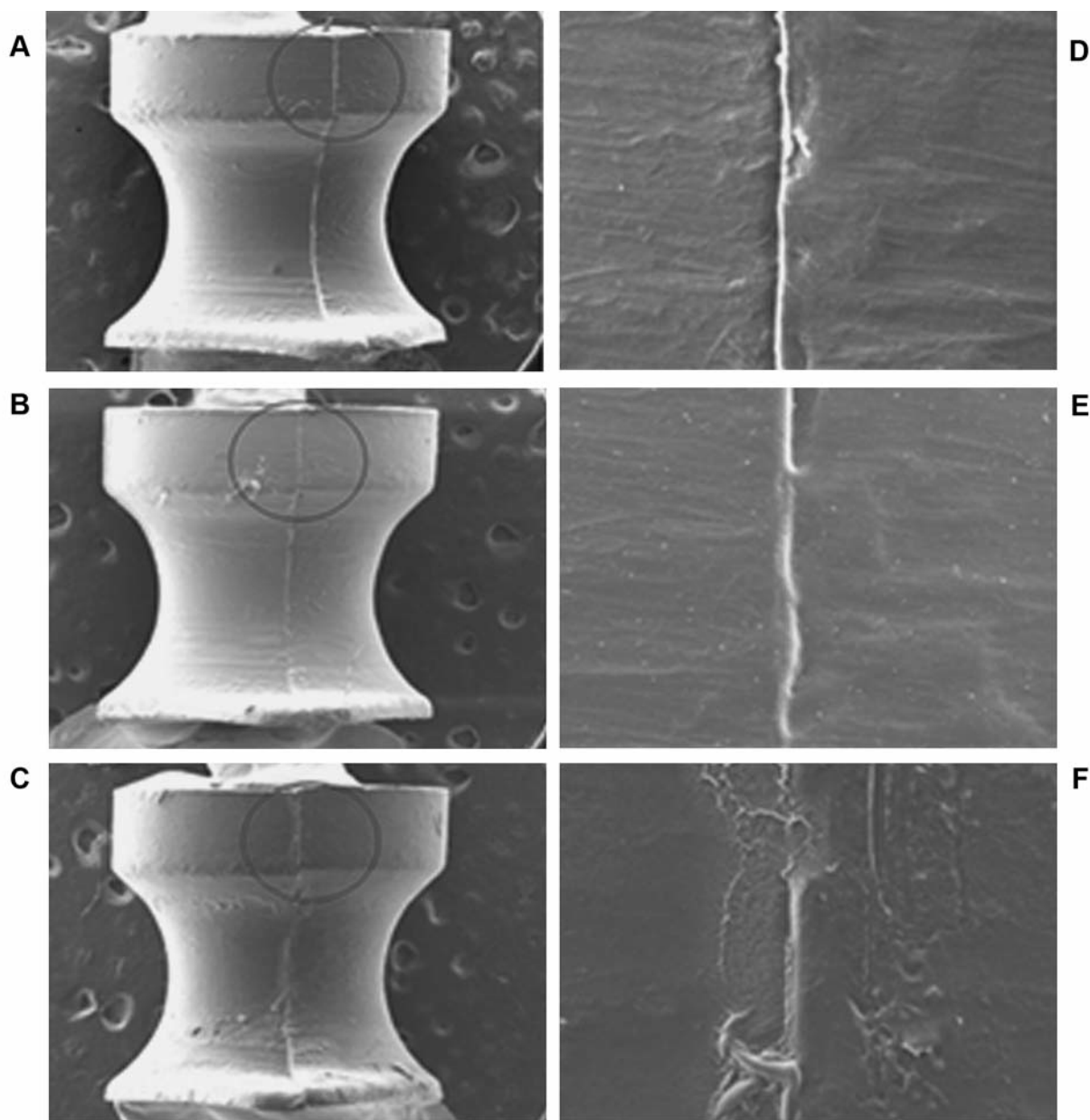


Figure 1. Morphologies of ventilation tube by scanning electron microscopy with low ($\times 30$) and high ($\times 400$) magnifications; non-coated ventilation tube (A and D), oxygen plasma-treated ventilation tube (B and E), and polyethylene glycol-graft ventilation tube (C and F). The area of high magnification is represented by a red circle.

diagnosis of biofilm by conventional culture techniques and eradication of biofilm with antibiotics is very difficult because of the reduced metabolic and divisional rates (4).

OM is known to be caused by infection with bacteria such as *S. pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. However, culturability of the middle-ear fluid from patients with OM has been variable (14). Because of the variable culturability and antibiotic resistance, there has been controversy over the pathological nature of OM (15).

Recently, studies have shown evidence of biofilm involvement in the pathophysiology of OM (16, 17). Ehrlich *et al.* established a biofilm formation model of chinchilla by transtympanic inoculation of *H. influenzae* (16). Post *et al.* showed that after transbular injection of *H. influenzae*, biofilm developed on the middle-ear mucosa of chinchillas (18). In humans, mucosal biofilms were visualized by confocal laser scanning microscopy on 92% of middle-ear mucosae from children with OM (19).

One of the treatment modalities for OM is VT insertion, but postoperative otorrhea is one of the most common complications of such treatment (6). A total of 13% of cases of postoperative otorrhea lasted more than 30 days, and 3.5% required parenteral antibiotics or VT removal (20). There have been many reports that show that bacterial attachment to implanted medical devices can become a source of chronic infection (21, 22). It was reported that bacterial biofilm formation plays an important role in postoperative otorrhea (6). Bacteria identified from infected VTs are known to be similar to these found in acute OM (2).

Biedlingmaier *et al.* showed the development of bacterial biofilms on VTs composed of silicone, fluoroplastic and silver-oxide impregnated silicone (23). Jang *et al.* reported biofilm formation on the VT of a patient with ciprofloxacin-resistant *Pseudomonas otorrhea* (24).

Several methods have been introduced to prevent biofilm formation on VTs (6, 25). Coating the surface with antibiotics is known to be an effective method; however, there is a possibility of the development of resistance and potential complications. Hydrophilic polymers, such as hyaluronic acid and poly-N-vinylpyrrolidone, are also known to be effective in inhibiting bacterial adhesion to surfaces (26). Heparin is known to prevent microbial adhesion and colonization by making the surface negatively-charged and preventing microbial colonization of catheters (27).

In this experiment, we modified surface characteristics of polyethylene VTs by PEG coating. We have shown that by surface modification, roughness was reduced, hydrophilic characteristics improved, and biofilm formation decreased. We believe that surface modification by PEG coating may be a promising modality for the prevention of biofilm formation on various medical devices.

The reduced bacterial adherence and biofilm formation on VT caused by PEG coating may be explained by the alteration of surface tension and roughness induced. However, the exact mechanism is still to be elucidated.

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References

- 1 Younis RT, Lazar RH and Long TE: Ventilation tubes and prophylactic antibiotic eardrops. *Otolaryngol Head Neck Surg* 106: 193-195, 1992.
- 2 Bluestone CD: Otitis media in children: To treat or not to treat? *N Engl J Med* 306: 1399-1404, 1982.
- 3 Mah TF and O'Toole GA: Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* 9: 34-39, 2001.
- 4 Fitzgerald G and Williams LS: Modified penicillin enrichment procedure for the selection of bacterial mutants. *J Bacteriol* 122: 345-346, 1975.
- 5 Gander S: Bacterial biofilms: Resistance to antimicrobial agents. *J Antimicrob Chemother* 37: 1047-1050, 1996.
- 6 Jang CH, Park H, Cho YB and Choi CH: Effect of vancomycin-coated tympanostomy tubes on methicillin-resistant *S. aureus* biofilm formation: *In vitro* study. *J Laryngol Otol* 124: 594-598, 2010.
- 7 Malaty J and Antonelli PJ: Effect of blood and mucus on tympanostomy tube biofilm formation. *Laryngoscope* 118: 867-870, 2008.
- 8 Quirynen M, van der Mei HC, Bollen CM, Schotte A, Marechal M, Doornbusch GI, Naert I, Busscher HJ, van Steenberghe D: An *in vivo* study of the influence of the surface roughness of implants on the microbiology of supra- and subgingival plaque. *J Dent Res* 72: 1304-1309, 1993.
- 9 Rodriguez A, Autio WR and McLandsborough LA: Effect of surface roughness and stainless steel finish on *Listeria monocytogenes* attachment and biofilm formation. *J Food Prot* 71: 170-175, 2008.
- 10 Morgan TD and Wilson M: The effects of surface roughness and type of denture acrylic on biofilm formation by *Streptococcus oralis* in a constant-depth film fermentor. *J Appl Microbiol* 91: 47-53, 2001.
- 11 van der Mei HC, Leonard AJ, Weerkamp AH, Rouxhet PG and Busscher HJ: Surface properties of *Streptococcus salivarius* HB and nonfibrillar mutants: Measurement of zeta potential and elemental composition with x-ray photoelectron spectroscopy. *J Bacteriol* 170: 2462-2466, 1988.
- 12 Oggioni MR, Trappetti C, Kadioglu A, Cassone M, Iannelli F, Ricci S, Andrew PW and Pozzi G: Switch from planktonic to sessile life: A major event in pneumococcal pathogenesis. *Mol Microbiol* 61: 1196-1210, 2006.
- 13 Baldassarri L, Creti R, Recchia S, Imperi M, Facinelli B, Giovanetti E, Pataracchia M, Alfarone G and Orefici G: Therapeutic failures of antibiotics used to treat macrolide-susceptible *Streptococcus pyogenes* infections may be due to biofilm formation. *J Clin Microbiol* 44: 2721-2727, 2006.
- 14 Diamond C, Sisson PR, Kearns AM and Ingham HR: Bacteriology of chronic otitis media with effusion. *J Laryngol Otol* 103: 369-371, 1989.
- 15 Rayner MG, Zhang Y, Gorry MC, Chen Y, Post JC and Ehrlich GD: Evidence of bacterial metabolic activity in culture-negative otitis media with effusion. *JAMA* 279: 296-299, 1998.
- 16 Ehrlich GD, Veeh R, Wang X, Costerton JW, Hayes JD, Hu FZ, Daigle BJ, Ehrlich MD and Post JC: Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. *JAMA* 287: 1710-1715, 2002.
- 17 Dohar JE, Hebda PA, Veeh R, Awad M, Costerton JW, Hayes J and Ehrlich GD: Mucosal biofilm formation on middle-ear mucosa in a nonhuman primate model of chronic suppurative otitis media. *Laryngoscope* 115: 1469-1472, 2005.
- 18 Post JC, Hiller NL, Nistico L, Stoodley P and Ehrlich GD: The role of biofilms in otolaryngologic infections: Update 2007. *Curr Opin Otolaryngol Head Neck Surg* 15: 347-351, 2007.
- 19 Hall-Stoodley L, Hu FZ, Gieseke A, Nistico L, Nguyen D, Hayes J, Forbes M, Greenberg DP, Dice B, Burrows A, Wackym PA, Stoodley P, Post JC, Ehrlich GD and Kerschner JE: Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. *JAMA* 296: 202-211, 2006.

- 20 Ah-Tye C, Paradise JL and Colborn DK: Otorrhea in young children after tympanostomy-tube placement for persistent middle-ear effusion: Prevalence, incidence, and duration. *Pediatrics* 107: 1251-1258, 2001.
- 21 Slusher MM, Myrvik QN, Lewis JC and Gristina AG: Extended-wear lenses, biofilm, and bacterial adhesion. *Arch Ophthalmol* 105: 110-115, 1987.
- 22 Everaert EP, Mahieu HF, van de Belt-Gritter B, Peeters AJ, Verkerke GJ, van der Mei HC and Busscher HJ: Biofilm formation *in vivo* on perfluoro-alkylsiloxane-modified voice prostheses. *Arch Otolaryngol Head Neck Surg* 125: 1329-1332, 1999.
- 23 Biedlingmaier JF, Samaranyake R and Whelan P: Resistance to biofilm formation on otologic implant materials. *Otolaryngol Head Neck Surg* 118: 444-451, 1998.
- 24 Jang CH, Cho YB and Choi CH: Structural features of tympanostomy tube biofilm formation in ciprofloxacin-resistant *Pseudomonas otorrhea*. *Int J Pediatr Otorhinolaryngol* 71: 591-595, 2007.
- 25 Park H, Jang CH, Cho YB and Choi CH: Antibacterial effect of tea-tree oil on methicillin-resistant *Staphylococcus aureus* biofilm formation of the tympanostomy tube: An *in vitro* study. *In Vivo* 21: 1027-1030, 2007.
- 26 Cassinelli C, Morra M, Pavesio A and Renier D: Evaluation of interfacial properties of hyaluronan coated poly(methylmethacrylate) intraocular lenses. *J Biomater Sci Polym Ed* 11: 961-977, 2000.
- 27 Russell PB, Kline J, Yoder MC and Polin RA: Staphylococcal adherence to polyvinyl chloride and heparin-bonded polyurethane catheters is species dependent and enhanced by fibronectin. *J Clin Microbiol* 25: 1083-1087, 1987.

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