Cariogenic biofilms can develop secondary caries within a week time in an undisturbed condition in vitro indicating high caries risk

Fahimeh Hayati, DDS,[a] Ayako Okada, DDS, PhD,[a,b] Junji Tagami, DDS, PhD,[a,c,d] and Khairul Matin, BDS, PhD[a,d]

[a]Cariology and Operative Dentistry, Department of Restorative Sciences, Graduate School, Tokyo Medical and Dental University, [b]Department of Translational Research, School of Dental Medicine, Tsurumi University, Yokohama, [c]Global Center of Excellence Program; International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo Medical and Dental University, and [d]Support Program for Improving Graduate School Education, Tokyo Medical and Dental University, Tokyo, Japan

Purpose: This in vitro study assessed the potency of cariogenic biofilms in developing secondary caries in human extracted teeth.
Materials and Methods: Cavities ($3 \times 2 \times 2$ mm^3) were prepared in the blocks of mid-buccal human extracted molars. Cavities were filled with Clearfil AP-X composite with (SE-Bond) or without (No-bond) application of an adhesive. Biofilms were formed on both groups of specimens using three species of cariogenic bacteria inside an oral biofilm reactor for 20 hours and specimens were further incubated separately for 7 days or 30 days dividing into two groups. Developed secondary caries lesions were examined using fluorescence microscope and scanning electron microscope. Sizes of the lesions were measured by using image analysis software. Data were statistically analyzed by one-way ANOVA and Turkey's HSD methods.
Results: The secondary caries were visible at resin-enamel interfaces in all cases including the 7-day specimens. Photomicrographs and data on image analysis clearly showed that the lesion size was smaller in SE-Bond sample compared to No-bond samples, which was statistically significant ($p < 0.05$).
Conclusion: It is suggested that sucrose supplemented biofilms of mutans streptococci have the potency in developing secondary caries lesion within a week time indicating high caries risks in case plaque-biofilms left undisturbed in the oral cavity. **(Int Chin J Dent 2009; 9: 61-68.)**
Key Words: cariogenic biofilm, mutans streptococci, resin composite, secondary caries.

Introduction

Human dental plaque biofilm is a very complex microbial community that forms on the hard tooth tissues as well as the restorative materials.[1-3] Caries development is a dynamic process between demineralization of dental hard tissue that eventually results in cavitations and this development depends upon acids and enzymes produced by bacteria present in dental plaque biofilms.[4] Dental plaque biofilms may harbor many bacteria that are involved in the development of disease conditions such as secondary caries and demineralization process of marginal enamel and dentin.[5,6] *Streptococcus gordonii* is regarded as an early colonizer of tooth surface, while *Streptococcus mutans* and *Streptococcus* are considered to be involved in latter stages of the dental biofilm colonization.[7] Bacterial adhesion to the surface of composite resins and other dental restorative materials plays an important role of secondary caries formation.[8]

Secondary caries is the same as a primary caries located at the margin of a restoration, also known as the recurrent dental caries. It is reported that most suspected cariogenic groups of bacteria such as mutans streptococci were widely present and could have an important role in the development of secondary caries around amalgam restorations.[9] Another possibility of secondary caries development has been mentioned as adhesive systems are not capable of eliminating microbes when they colonize in the micro-space between the tooth and the restorative material.[10]

Artificial mouth models for biofilm formation can benefit in vitro experiments on primary and secondary

caries with regard to the microbial and antimicrobial properties of restorative materials or problems resulting from insufficient marginal adaptation of restorations.[11] Information is scarce on the bacterial community formed on resin composites that may produce secondary caries and how much time the pathogens may take to produce such a lesion, are important issues to investigate. With a view to obtain more information on these subjects, an oral biofilm reactor (OBR) was used in this study to grow multi-species cariogenic (mutans streptococci) biofilms.[12-14] Therefore, the purpose of this study was to assess the potency of cariogenic biofilms formed inside an OBR on developing secondary caries in human extracted teeth.

Materials and Methods

Tooth specimens

   Forty caries-free human maxillary third molar teeth were used in this in vitro study.   Tooth blocks (approximately $5 \times 4 \times 4$ mm^3) were prepared from the mid-buccal portion of human molars by cutting with a low speed diamond saw (Isomet, Buehler, Lake Bluff, IL, USA) under running water as coolant. The convex enamel surfaces on the outermost buccal slices were reduced up to 0.5 mm by gently polishing on an 800-grit silicone carbide paper under running water to prepare a flat enamel surface and then polished with water based diamond paste of 0.25 μm. Finally, the blocks were cleaned with deionized water (Milli-Q water, Japan Millipore Corp., Tokyo, Japan) ultrasonically cleaned for 5 minutes to remove the remaining grit left during polishing.   The blocks were rinsed with Milli-Q water three times for two minutes each dried accordingly and enamel surfaces were coated with nail varnish (Fig. 1).
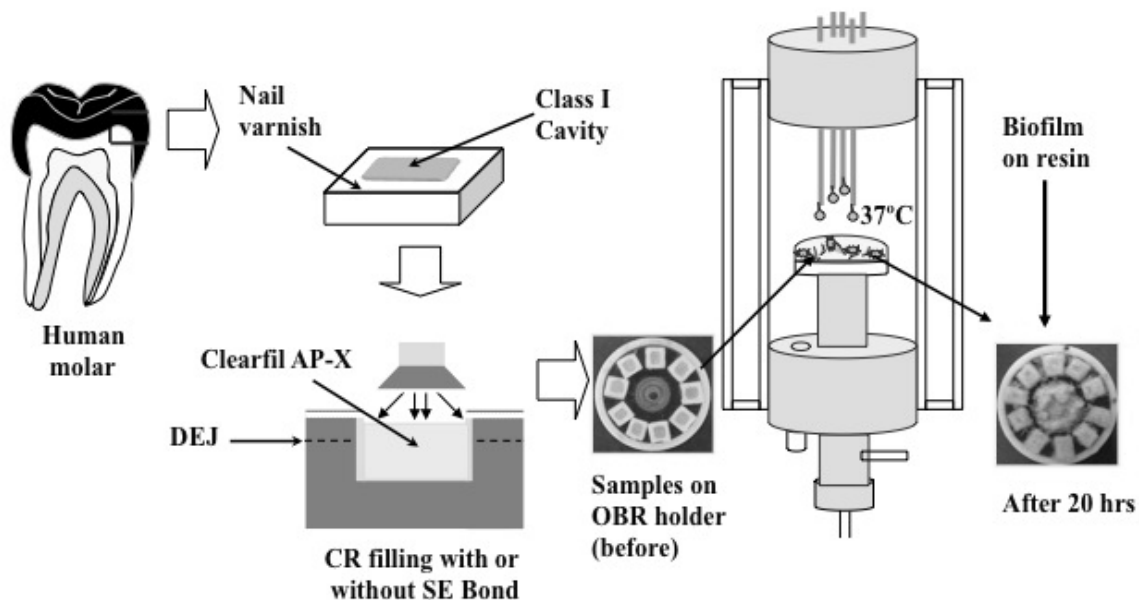

Fig. 1.   Schematic diagrams on the left sequentially show the specimen preparation, cavity preparation and the procedure of restoration. Diagram on the right represents one of the chambers of the oral biofilm reactor (OBR) in which the artificial biofilms had been formed in situ on the surface of the specimens. Digital photographs represent tooth specimens before and 20 hours after biofilm formation.

Restorative materials

   A two-step self-etching adhesive materials (Clearfil SE Bond) and a hybrid composite (Clearfil AP-X) were used in this study. The lot numbers and chemical compositions of the materials are listed in Table 1 according to

the information provided by the manufacturer (Kuraray Medical, Tokyo, Japan).

Cavity preparation and composite restoration

Class 1 cavities (3 mm long, 2 mm wide, 2 mm deep) were prepared in specimens using a regular-grit tapered bar (ISO #170, Shofu, Kyoto, Japan), diameter 0.8-1.0 mm that mounted in the milling machine (Cendres & Metaux SA CH-2501, Biel-Bienne, Switzerland) and cavity surfaces were finished with a straight bar (ISO #109, Shofu) in fixed in the milling machine under water coolant.

The prepared specimens of human teeth were randomly divided into two groups according to filling conditions; with application of adhesive (SE-Bond) and without any applying any bond (No-bond). Cavities of the specimens were filled with a two-step self-etching primer/adhesive system and a-light-cured hybrid composite (Clearfil AP-X). The cavities were not filled completely, keeping about 0.8 mm deep unfilled space for biofilm accumulation at the top, and light-cured for 10 s using a conventional halogen light curing unit (XL3000, 3M-ESPE, Minneapolis, MN, USA). The cured adhesive surface was then carefully filled with a hybrid restorative (Clearfil AP-X, shade A3) and light-cured for 40 s. In the second group, the other twenty cavities were also filled with AP-X without application of any bond that remained as No-bond group (Fig. 1).

Table 1. Materials used in the present study.

Material	Batch number	Composition		Procedure
Clearfil SE Bond	007214	Primer;	HEMA, MDP, Hydrophilic dimethacrylates Photoinitiator, Water	Apply 20 s, Gentle air-blow
	0103A	Bond;	Bis-GMA, MDP, HEMA, Photoinitiator, Filler	Apply 15 s, Mild air-blow, and light-cured 20 s
Clearfil AP-X	1079AA	Bis-GMA, TEGDMA, Photoinitiator, Barium glass, Silanated colloidal silica (filler content 85.0 wt%)		Light-cured 40 s

Manufacturer; Kuraray Medical Inc, Tokyo, Japan: HEMA, 2-hydroxyethyl methacrylate; MDP: 10-methacryloyloxydecyl dihydrogen phosphate; Bis-GMA: bisphenol-A diglycidyl methacrylate; TEGDMA: triethylene glycol dimethacrylate.

Preparation of bacteria suspensions

Suspensions of *Streptococcus mutans MT8148* (*S. mutans*)*, Streptococcus sobrinus 6715* (*S. sobrinus*) and *Streptococcus gordonii ATCC10558* (*S. gordonii*) in phosphate buffered saline (PBS) at an optical density 500 nm (OD_{500}) of 2.5 were prepared from a 16-hour fresh cultures in brain heart infusion broth (Becton Dickinson, Sparks, MD) after washing three times with PBS. The suspensions were stored at 4°C with continuous gentle stirring until use.

Growing biofilm on the specimens inside OBR

Artificial *S. mutans*, *S. sobrinus*, and *S. gordonii* biofilms were grown on the surface of class-1 cavity with/without adhesive material inside two identical water jacket-encircled chambers of the OBR. Four specimens from each group were placed on a Teflon holder around a flat bulb pH electrode of the OBR by using red utility wax (GC, Tokyo, Japan) keeping the top of each cavity open for biofilm accumulation. The Teflon holder bearing the specimens set through the bottom opening of the chamber by a silicon plug. Pooled sterile saliva was then poured on the specimen surfaces and incubated for 30 minutes to obtain a coating of salivary pellicle. The chamber encircled by a water jacket was sealed with another silicon plug fitted with five stainless steel tubes (21gauge) so that the chamber itself served as an incubator with a 37ºC inner temperature. The other ends of the five stainless steel tubes were connected to silicon tubes passing through peristatic pumps regulated by a computer operated controller (Eyela EPC-2000, Tokyo Rika, Tokyo, Japan). One of them was used to collect the mixed suspension of *S. mutans, S. sobrinus*, and *S. gordonii*, two to collect sucrose added HI and the

other two to collect PBS from the prepared stock as described above. All of these liquids form water domes which are mixed by the force of gravity exerted from the falling liquid drops on the holder and are diffusely distributed over all of the specimens.

After 20 hours incubation of the biofilm in the OBR chamber, each specimen with artificial biofilms was removed from the Teflon holder in the OBR. The specimens with the undisturbed biofilms were then separated into two groups for further 7 days or 30 days inoculation to observe the progression of the secondary caries lesions. A 24-well culture plate (Corning Inc., New York, NY, USA) was used to keep all the specimens in separate wells to incubate at 37ºC and sucrose added HI was supplemented by changing on every alternate day.

Fluorescence microscopy (FM)

Morphology of the lesions developed at resin-enamel interface was investigated by using a fluorescence microscope (FM) followed by image analysis of the lesions. In order to detect and measurement of the secondary caries lesions, after the storage period, the samples were rinsed with PBS buffer and fixed in 4% paraformaldehyde with 1% glutaraldehyde in PBS for 1 hour. The samples were rinsed with PBS three times for 2 minutes each, and finally rinsed with Milli-Q water three times for 20 minutes each and dried. Following these procedures, the samples were embedded in a self-curing epoxy resin (Epon 815, Nissin EM, Tokyo, Japan), subsequently ground to expose in the longitudinal direction with 800-grit silicon carbide paper under running water and finished with diamond pastes down to 0.25 μm particle sizes. The samples were cleaned with Milli-Q water applying mild ultrasonication in between each step for 10 minutes and finally dried.

Secondary caries lesions at the interfaces were evaluated with an inverted fluorescent microscope (Olympus CKX41, Olympus Imaging Corp., Tokyo, Japan) and imaged using a DP70 system with the aid of DP Manager Software (Olympus). In order to observe blue fluorescent by using BP460-490C filter, that produced green micrographic pictures on the PC. Images were taken at ×40 and ×100 magnifications after setting a scale bar each time. By using the DP70 system and Manager, maximum length and maximum width were directly measured. Later, area of the lesions was measured using ImageJ V.1.34 software[15] utilizing the scale bar on each image as reference for calibration.

Scanning electron microscopy (SEM)

The morphology of lesions and structural changes at the interface of enamel and resin were investigated by using an SEM (S-3400NX, Hitachi, Tokyo, Japan). The polished specimens were dried accordingly and sputter-coated with 300 nm of gold using a SC-701AT (Elionix, Tokyo, Japan) and observed with a SEM. The experiments were repeated three times in order to establish reproducibility.

Statistics analysis

All numerical data was analyzed using the Statistical Package for the Medical Science (SPSS Ver.11 for Windows, SPSS Inc., Chicago, IL, USA) for statistical procedures. The number of specimens was four in each group (n=4) and the experiments were repeated three times under the same conditions to ensure reproducibility. The data for maximum width, maximum length and area of secondary caries lesions were analyzed by one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test with a confidence level of 95%.

Results

FM observations and image analysis

FM images clearly displayed development of secondary caries in all groups with remarkable morphological patterns loosing green fluorescence and appearing brighter (Fig. 2). As expected, in FM images and data on image analysis (Table 2) it appeared that the lesion size was smaller in SE-Bond treated specimens compared to untreated No-bond specimens. Although, at 7-day that difference was very marginal gap formation between enamel and resin was very difficult to detect in case of SE-Bond (Fig. 2a), but that could randomly be detected in No-bond specimens (Fig. 2b). The widths of both 7-day and 30-day SE-Bond specimens were significant smaller compared to 30-day No-bond specimens ($p<0.05$). Demineralization along the cavity wall extended more (length of the lesion) in No-bond specimens compared to SE-Bond in human teeth that penetrated deep significantly in 30 days ($p<0.05$). The area of the demineralized enamel lesion was smallest in 7-day SE-Bond treated specimens. In reverse, lesion area was largest in 30-day No-bond treated specimens that showed significant differences when the data were compared ($p<0.05$). All these data indicate that damage was less in SE-Bond specimens and was severe in No-bond specimens. The three cariogenic species microcosm plaque biofilms demonstrated strong acidogenic and acid-tolerating potency by keeping low pH around the tooth surface for relatively long period of time.
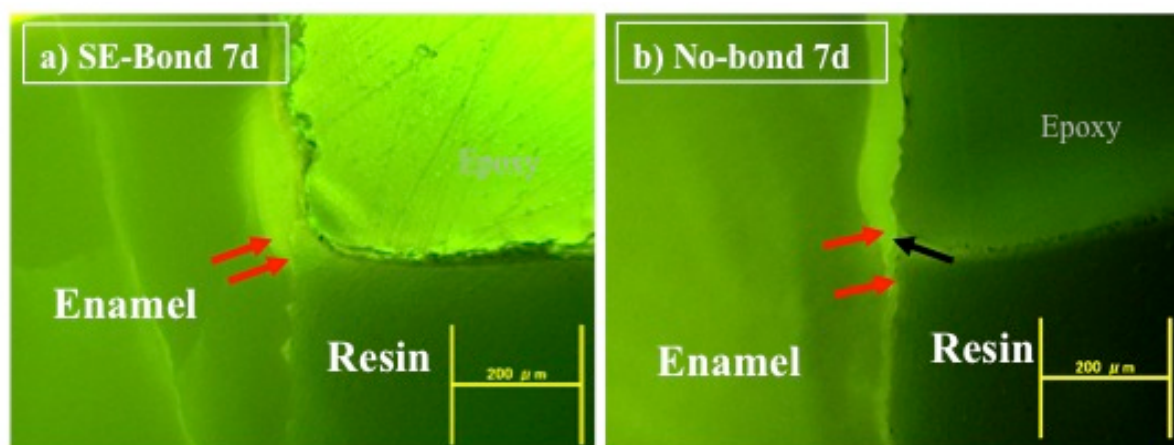

Fig. 2. FM photographs of specimens with a) and without b) adhesive material after 7 days (original magnification ×100). Red arrows indicate secondary caries lesions in both photographs. A clear lesion mark of enamel demineralization can be seen in SE-Bond specimen, but almost no gap between enamel and composite resin can be detected a). Enamel demineralization extended deep along the enamel wall in No-bond specimen with a gap between enamel and composite resin indicated by black arrow b).

Table 2. Average sizes (maximum width, maximum length, and area) of secondary caries lesions.

Bond	SE-Bond		No-bond	
Incubation period	7 d	30 d	7 d	30 d
Maximum width (μm)	(79±22)[a]	(86±10)[b]	(110±43)	(163±61)[a,b]
Maximum length (μm)	(178±70)[a]	(199±42)	(202±36)	(296±70)[a]
Area (μm²)	(9.9E+03± 5.4E+03)[a]	(1.3E+04± 2.7E+03)	(1.6E+04± 2.5E+03)	(4.2E+04± 8.1E+03)[a]

All data indicate (Average±SD). Same superscript letters are significantly different according to one-way ANOVA and Tukey's HSD test ($p<0.05$), n=4 in each group.

SEM observations

Presence of secondary caries lesions due to demineralization of enamel particles, adhesive material remain sandwiched at enamel-resin interface in SE-Bond groups and gaps in No-bond groups were observed on SEM inspection (Fig. 3). SE-Bond remained bonded on to resin but lost attachment with the enamel by inevitable acid

attacks from the biofilm (Fig. 3a). On the other hand, gap formation between enamel and resin were remarkably wide in case of No-bond samples. For example approximately gap size in case of human sample of No-bond was 9.2 μm (Fig. 3b). Also, in 7 day specimens similar demineralization pattern was observed, although lesion size very small in case of SE-Bond group.
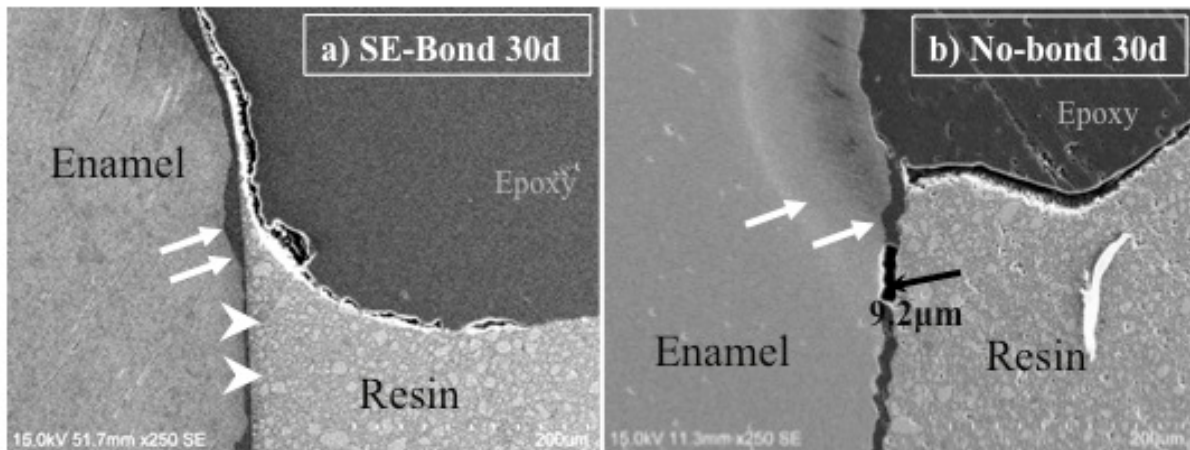

Fig. 3. SEM photographs of specimens with a) and without b) adhesive material after 30 days (Magnification, ×250). Enamel demineralization was small in SE-Bond specimens indicated by white arrows and almost no gap between enamel and composite resin can be detected, arrowheads indicated bonding layer between enamel and resin a). Damage was large in No-bond specimen shown by white arrows with a visible wide gap indicated by black arrow b).

Discussion

The development of secondary caries around restorative materials is determined by the physico-chemical properties of the materials (e.g., shrinkage, corrosion, solubility, fluoride content, and permeability) and their clinical performance; that is, cavity-sealing ability, microleakage of bacteria, fluids, and acidic products and cavity preparation. The multi-factorial etiology of secondary caries includes age, oral hygiene, bacterial colonization, salivary composition, fluoride bioavailability, and most of all the quality of restoration including the properties of restorative materials. It was expressed that extensive experimental and clinical researches are needed to develop suitable prevention approaches for secondary caries expression and to know the complex interaction among the restoration, the oral environment and the tooth surface.[16] The development of an OBR made it possible to create an in vitro secondary caries model induced by cariogenic streptococci on the enamel wall at composite resin interface. In the present study, all specimens presented carious lesion formation on the exposed tooth surfaces around the composite resin restorations. Restorative materials may harbor bacteria to initiate plaque accumulation and secondary caries formation in the oral cavity and organic acids can be trapped within the glucan barrier of the mature plaque produced by these bacteria, resulting in a prolonged low pH around the tooth surface.[17] During the experiment, when there is active biofilm formation in almost uninterrupted condition the bacteria secreted acids, reduced pH and dissolved minerals of the enamel in similar caries process like in the oral cavity within one week time. Among several characteristics of *S. mutans* include a proclivity for adhesion to the tooth surface;[18] synthesizing WIG with sucrose as a substrate, resulting in subsequent formation of plaque[19] and reduction of the pH by producing acid,[20] which causes demineralization of the tooth and leads to the carious lesion.

S. mutans is the leading pathogen responsible for the development of dental caries. When sucrose intake is

frequent, *S. mutans* synthesizes water insoluble glucans catalyzed by glucosyltransferases from dietary sucrose. Glucans are important in the adhesive interactions of *S. mutans* with the tooth surface as well as contributing to the formation of the matrix of cariogenic biofilms. The later strongly adheres to tooth surfaces and aids in preventing diffusion of bacterial organic acids which in turn leads to decalcification of tooth tissues enamel (dental caries).[21] Combination of *S. gordonii* and *S. sobrinus* with *S. mutans* the caries inducing artificial biofilm appeared to be more natural as they can build up a multispecies microbial community that is much more stable and resistant to external inhibiting factors. In the oral cavity, *S. sobrinus* seems to be more acidogenic than the other species of mutans streptococci.[22,23]

The caries lesions were clearly visible at the interface of restorations by FM in all specimens due to the loss of green fluorescence in both enamel and dentin. For instance, no detectable lesion was visible in a control specimen without biofilm formation (data not shown), compared to that a typical enamel lesion developed by the cariogenic biofilm in almost all experimental specimens could easily be detected, which appeared as bright-green rather than pure fluorescent green from the normal enamel. In order to permit accurate quantitative analyses of the secondary caries size, we measured maximum width and maximum length of the outer lesions using the FM images at low magnification (×40).

In vitro caries model allows the simultaneous production of primary and secondary caries-like enamel lesions in a considerable number of specimens, and facilitates the possibility to manipulate and transfer plaque and tooth without necessarily terminating the experiment.[24] During restorative treatment when SE-Bond is applied it makes a compact interface or bond between enamel and resin inhibiting direct penetration of acid. Also, SE-Bond forms coating layers on enamel surface of the unfilled cavity that protected the enamel to some extent. It was clearly understood within one week time that the use of Clearfil SE-Bond systems for bonding of composite resin to enamel contributes to inhibit secondary caries compared to without adhesive application (No-bond). In all groups the length of the lesion was larger than the width indicating easier acid penetration along the enamel-resin interface rather than widely spreading into the enamel structure.

In the present study, visible marginal gaps between the restorative material and tooth structure observed by SEM. Results also showed that larger lesions were associated with wider gaps when incubation period was longer (30 days) and that occurred mainly in all No-bond specimens that findings are in agreement with previous in vitro studies[25] demonstrating that secondary caries lesions develop next to marginal gaps. Expectedly, the size of secondary caries lesions in bonded (SE-Bond) groups was smaller than that of un-bonded (No-bond) groups. That might be because SE-Bond can build up a satisfactory adhesion between composite resin and enamel inhibiting penetration of acids and can minimize gap formation.

Therefore, it can be suggested that cariogenic biofilm of *mutans streptococci* can develop secondary caries in human teeth within a week time and may cause extensive damage to the tooth structures if left unnoticed which is an indication of high caries risk for clinical conditions. Further studies on biofilm attachment on restorative materials and secondary caries development would be beneficial.

References

1. Siegrist BE, Brecx MC, Gusberti FA, Joss A, Lang NP. In vivo early human dental plaque formation on different supporting substances. Clin Oral Implants Res 1991; 2: 38-46.
2. Hannig M. Transmission electron microscopic study of in vivo pellicle formation on dental restorative materials. Eur J Oral Sci 1997; 105: 422-33.
3. Hannig M. Transmission electron microscopy of early plaque formation on dental materials in vivo. Eur J Oral Sci 1999; 107: 55-64.
4. Abrahamson M, Lewandowski Z, Geesey G, Skjak-Braek G, Strand W, Christensen BE. Development of an artificial biofilm to study the effects of a single microcolony on mass transport. J Micobiol Met 1996; 26:161-9.
5. Wallman C, Krasse B. Mutans streptococci in margins of fillings and crowns. J Dent 1992; 20: 163-6.
6. Daneshmehr L, Matin K, Nikaido T, Tagami J. Effects of root dentin surface coating with all-in-one adhesive materials on biofilm adherence. J Dent 2008; 36: 33-41.
7. Marsh P, Bradshaw DJ. Microbial community of dental plaque. In: Dental Plaque Revisited: Oral Biofilm in Health and Disease, Newman HN, Wilson Eds, Bioline: Cardiff; 1999, p. 237-53.
8. Montanaro L, Campoccia D, Rizzi S, et al. Evaluation of bacterial adhesion of *streptococcus mutans* on dental materials. Biomaterials 2004; 25: 4457-63.
9. Gonzalez-Cabezas C, Li Y, Gregory RL, Stookey GK. Distribution of three cariogenic bacteria in secondary carious lesions around amalgam restorations. Caries Res 1999; 33: 357-65.
10. Pashley DH, Carvalho RM. Dentine permeability and dentine adhesion. J Dent 1997; 25: 355-72.
11. Tang G, Yip HK, Cutress TW, Samaranayake LP. Artificial mouth model systems and their contribution to caries research: a review. J Dent 2003; 31: 161-71.
12. Ono M, Nikaido T, Ikeda M, et al. Surface properties of resin composite materials relative to biofilm formation. Dent Mater J 2007; 25: 613-22.
13. Gyo M, Nikaido T, Okada K, Yamauchi J, Tagami J, Matin K. Surface response of fluorine polymer-incorporated resin composites to cariogenic biofilm adherence. Appl Environ Microbiol 2008; 74: 1428-35.
14. Okada A, Nikaido T, Ikeda M, et al. Inhibiting of biofilm formation using newly developed coating materials with self-cleaning properties. Dent Mater J 2008; 27: 565-72.
15. Abramoff MD, Magelhaes PJ, Ram SJ. Image processing with ImageJ. Biophotonics International 2004; 11: 36-42.
16. Tsuchiya S, Nikaido T, Sonoda H, Foxton RM, Tagami J. Ultrastructure of the dentin-adhesive interface after acid-base challenge. J Adhes Dent 2004; 6: 183-90.
17. Van Houte J, Russo J, Prostak KS. Increase pH lowering ability of streptococcus mutans cell masses associated with extracellular glucans-rich matrix material and the mechanisms involved. J Dent Res 1989; 68: 451-9.
18. Clark WB, Gibbons RJ. Influence of salivary components and exteracellular polysaccharide synthesis from sucrose on the attachment of *streptococcus mutans* 6715 to hydroxyapatite surfaces. Infect Immun 1977; 18: 514-23.
19. Hamada S, Slade HD. Biology, immunology, and cariogenicity of *streptococcus mutans*. Microbiol Rev 1980; 44: 331-84.
20. Clark WB, Bammann LL, Gibbons RJ. Comparative estimates of bacterial affinities and adsorption sites on hydroxyapatite surfaces. Infect Immun 1978; 19: 846-53.
21. Senpuku H, Miyauchi T, Hanada, N, Nisizawa T. An antigenic peptide inducing cross-reacting anti-bodies inhibiting the intraction of streptoccus mutans Pac with human salivary components. Infect Immun 1995; 63: 4695-703.
22. de Soet JJ, Toors FA, de Graaff J. Acidogenesis by oral streptococci at different pH values. Caries Res 1989; 23: 14-7.
23. Kohler B, Birkhed D, Olsson S. Acid production by human strains of *streptococcus mutans* and *streptococcus sobrinus*. Caries Res 1995; 29: 402-6.
24. Seemann R, Bizhang M, Kluck I, Loth J, Roulet JF. A novel in vitro microbial-based model for studying caries formation--development and initial testing. Caries Res 2005; 39: 185-90.
25. Totiam P, Gonzalez-Cabezas C, Fontana MR, Zero DT. A new in vitro model to study the relationship of gap size and secondary caries. Caries Res 2007; 41: 467-73.

Correspondence to:

Dr. Khairul Matin
Cariology and Operative Dentistry, Dept of Restorative Sciences,
Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University
1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan
Fax: +81-3-5803-0195 E-mail: matin.ope@tmd.ac.jp