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Chitosan whiskers from shrimp shells incorporated into dimethacrylate-based dental resin sealant

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A resin-based sealant containing chitosan whiskers was developed for use as a pit and fissure sealer. Chitosan whiskers were synthesized and then characterized using fourier transform infrared spectrometry and transmission electron microscopy. The whiskers were next incorporated into dimethacrylate monomer at various ratios by weight and subsequently analyzed for their antimicrobial and physical properties. The dimethacrylate-based sealant containing chitosan whiskers had a greater antimicrobial activity than control sealant and they were comparable with antimicrobial commercial resin sealants. The inclusion of the whiskers did not reduce the curing depth or degree of double bond conversion and the reduction in hardness was minimal. In conclusion, a resin-based sealant containing chitosan whiskers can be considered an effective antimicrobial pit and fissure sealant.

Keywords: Dimethacrylate, Chitosan whisker, Antimicrobial activity, Physical test

INTRODUCTION

Dental caries is one of the most prevalent oral health concerns worldwide. The occlusal surface, especially pits and fissures, is vulnerable to attack by the oral microflora and their metabolic byproducts. The sealing of pits and fissures by dental sealants has been found to protect teeth from decay. The principle sealing protocol is the creation of a physical barrier to protect the fissure from the ingress of bacteria and food remnants through the use of flowable sealant materials, which are allowed to harden. Resin-based sealants have been shown to have an advantage over other materials because of their good retention and superior sealing characteristics[1]. Many commercial resin-based sealants are composed of dimethacrylate-based monomers, such as 2,2-Bis[4(2-hydroxy-3-methacryloyloxy-propoxy)phenyl]propane (Bis-GMA) or triethyleneglycol dimethacrylate (TEGDMA) with camphorquinone (CQ) as the most commonly used photoinitiator. These monomers harden and seal the pits and fissures which have first been treated with an inorganic acid etching solution which enables the sealant to adhere to the enamel surface and ensures successful retention for years[2]. Although these resin-based sealants have retention rates which are clinically acceptable, some laboratory studies have shown marginal leakage occurs around sealants after thermo-cycling process[3]. This leakage could permit the adhesion of cariogenic bacteria around the sealant in clinical condition and lead to catastrophic failure in caries prevention. Some clinical study showed evidence of caries development in the pit and fissure even though the sealant was retained[4]. Antimicrobial substances are expected to reduce bacterial infection underneath the sealant materials, thus reducing initiation of secondary caries. Indeed, some studies have shown lower antimicrobial activity from commonly used dimethacrylate-based sealants[5]. In addition, several synthetic disinfectants such as fluoride[6,7], triclosan[8], and chlorhexidine[9] have been proposed for resin-based sealant to reduce both bacterial load and adhesion. However, to the best of our knowledge, no natural antimicrobial substances have been incorporated into dimethacrylate-based resin monomers for pit and fissure sealing purposes.

Chitosan [β-(1, 4)-2-amino-2-deoxy-D-glucose] is a natural polysaccharide derived from the deacetylation of chitin. Specific functional groups of chitosan-chitin copolymers and their derivatives have been shown to exhibit many biological phenomena, including antimicrobial activity. An antimicrobial mechanism has been proposed in which the interaction of positively charged chitosan oligomers with negatively charged microbial cell membranes causes the leakage of intracellular contents and damage on bacterial cells[10]. A study on hydroxyapatite indicated an alteration in adsorption of *Streptococcus mutans* (*S. mutans*) by chitosan thus preventing the subsequent colonization of additional bacteria on the tooth surface[11]. Chitosan supplementation in dental products such as chitosan chewing gum[12] and chitosan conjugated chlorhexidine mouthwash[13] have shown satisfactory results in oral bacteria adhesion and inhibition experiments. A previous study on whisker-form chitosan, a small fibrous structure, in alginate-chitosan yarn indicated antibacterial activity and improved physical properties[14].

The purpose of this study was to evaluate the antimicrobial effect of nano-sized chitosan whiskers

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impregnated into resin sealant against *Streptococcus mutans*. In addition, the physical properties of this chitosan-resin sealant were evaluated for the depth of cure, hardness and degree of double bond conversion.

MATERIALS AND METHODS

Preparation of chitosan whiskers

Shells of *Litopeneous vannamei* shrimp were decalcified by 1N HCl (Carlo Erba, Italy) for 48 h, and subsequently deproteinized with 4%w/w NaOH (Carlo Erba, Milan, Italy) at 80°C for 4 h to obtain chitin flakes[15]. Chitin whiskers were prepared from the obtained chitin flakes, as previously described[16], by hydrolyzing the chitin flakes in 3N HCl at 104°C for 6 h under vigorous stirring. The preparation ratio of chitin flakes to HCl aqueous solution was 1 g/30 mL. The acid hydrolyzed suspension was immediately diluted with distilled water, followed by centrifugation (10,000 rpm for 10 min) to separate the chitin solid fraction from the aqueous medium. The centrifugation process was repeated three times. The obtained suspension was dialyzed against distilled water at room temperature overnight to remove excess HCl. The chitin whiskers in suspension were then deacetylated in 50%w/v NaOH aqueous solution (Tai Seng Long, Bangkok, Thailand) with 0.5%w/w $NaBH_4$ (Sigma-Aldrich, Singapore) as a reducing agent against extensive depolymerization of chitosan to obtain the final chitosan whiskers preparation. The ratio of chitin whiskers to NaOH aqueous solution was 1 g/10 mL. This procedure was performed at 105°C for 5 min and repeated three times. The final product was diluted and dialyzed against distilled water until the pH reached 6.3. The suspension was kept at 4°C and ultrasonicated for 5 min prior to using to ensure homogeneity[14].

Characterization of chitosan whiskers

Fourier transform infrared spectrometry (FTIR; Thermo Nicolet NEXUS 670, MN, USA) was conducted to investigate chemical structure of the chitosan whiskers. The chitosan whiskers from the as-prepared chitosan whisker suspension were dried, mixed with KBr powder and pressed into a pellet. The scanning range was 4,000–400 cm^{-1} with 64 scans at a resolution of 4 cm^{-1}. The degree of deacetylation (DD) was observed from FTIR spectra peak height of A_{1655} and A_{3450}, which are the absorbance bands of the C=O (1,655 cm^{-1}) and O-H (3,450 cm^{-1}) groups, respectively. The ratio was calculated based on the following equation[17]:

$$\%DD=100-[(A_{1,655}/A_{3,450})\times 115]$$

Morphology of the as-prepared chitosan whiskers was observed by a transmission electron microscope (TEM, JEM-2100, JEOL Ltd., Tokyo, Japan). Samples for TEM observations were prepared by dropping a dilute chitosan whisker suspension on formvar grids and left to dry prior the observation. The dimensions of the whiskers were determined from selected TEM images, from which at least 60 individual whiskers were measured for their lengths and widths using SemAfore 4.0 image-analytical software.

Preparation of freeze-dried chitosan whiskers and chitosan whiskers incorporated resin sealant

The freshly chitosan whisker suspension was freeze-dried using a lyophilizer (Flexi-Dry™, Stone Ridge, New York, USA) at −50°C for 12 h. The experimental resin-based sealant was a mixture of 57% Bis-GMA (PolySciences, PA, USA; Lot.No.605207), 41.9% TEGDMA (Sigma-Aldrich, Singapore; batch No.01612MH), 0.86% 2-dimethylaminoethyl methacrylate (DMAEMA; PolySciences, USA, Lot.No.52900) and 0.24% CQ (Sigma-Aldrich, Singapore, Lot.No.550396) by weight. Freeze-dried chitosan whiskers were proportionally incorporated in four experimental resin monomer groups (1, 1.5, 2, and 2.5% by weight) with a hand homogenizer (S8N-8G, Ultra-turrax, Königswinter, Germany) for 10 min. The resin group without chitosan whiskers was used as control.

Antimicrobial property of chitosan whiskers incorporated resin sealant

The specimen was prepared in a 5 mm-diameter and 2-mm height round plastic mold and subsequently activated by a light curing unit (Optilux™ 501, KERR, USA) for 40 s. Resin sealant without chitosan whiskers was used as a control group. Three commercial resin-based sealants: no antimicrobial resin sealant (Delton®, Dentsply, USA), fluoride-containing resin sealant (Teethmate™F-1, Kuraray Medical, Kusashiki, Japan) and triclosan-containing protective sealant (Seal&Protect™, Dentsply, IL, USA) were used for comparison. Clear shade was used for all commercial sealants. The antimicrobial property was carried out against cariogenic gram-positive bacteria, *S. mutans* (UA 159), using two methods.

Agar diffusion test

Three samples from each experimental group were gas sterilized and left for 48 h before use. The sample discs were placed on *Streptococcus mutans*-cultured agar plates and incubated at 37°C, 5% CO_2 for 24 h. The zone of bacterial inhibition (the halo around the disc specimen after being placed) of each group was observed. The diameters of the inhibition zones were measured in millimeters through the centers of the disc specimens and repeated 10 times, rotating 36° each time on each specimen. When there was no inhibition zone, a diameter of 5 mm was recorded[18].

Colony forming units count test (CFU count)

Samples were prepared and sterilized as the same manner. *Streptococcus mutans* suspension grown in mid-log phase was pelleted and resuspended in phosphate buffered saline. One milliliter of bacterial suspension was divided into 8 test groups. The bacteria count was performed. A sample from each group was seeded into a test tube and incubated at 37°C with 5% CO_2 with shaking. Bacteria were counted after 12 h of

incubation. The experiments were performed in triplicate. The bacterial reduction rate (BRR) was calculated according to the following equation[19]:

Bacteria reduction rate (BRR)=$[(N_1-N_2)/N_1]\times100$
Where N_1 and N_2=viable count at 0 and 12 h.

Morphology of chitosan whiskers in resin-based sealant
The whiskers-resin samples were prepared in a 5 mm diameter and 2 mm height round plastic mold and subsequently light activation as describe above. The sample was embedded in epoxy resin and thin sectioned by ultramicrotome (LKB, Ultrotome V, Stockholm, Sweden). The specimens were left dry on a formvar copper grid and then observed by TEM.

Physical properties of chitosan whiskers incorporated resin-based sealant
The lowest amount by weight of chitosan whiskers demonstrated antimicrobial activity against *S.mutans* by visual inspection and significantly reduced in bacterial load was considered as the most effective chitosan-resin group. This group was enrolled in this part of test to compare with other commercial resin sealants.

Depth of cure
The assay was performed according to ISO 6874:2005, which specified the requirements and test methods for polymer-based materials intended for pits and fissures sealants. Five samples from each group were prepared in a 4 mm diameter and 6 mm height metallic mold. The liquid resin was poured in the mold, which was placed between two glass cover slips, and subsequently activated by a light curing unit for 40 s. The specimens were removed and cleaned by tissue paper in order to remove any uncured resin. The depth of cure was determined by digital vernier caliper (Mitutoyo, Kawasaki, Japan).

Vickers hardness
The specimens were prepared as described above but in 3 mm diameter and 2 mm height metallic molds and kept at room temperature in 50±10% relative humidity for 24 h before testing. Ten specimens were used for each group. The specimen was consecutively loaded with 10 imprints by 500 grams of load force for 15 s. Vickers hardness number (VHN) was determined by a microhardness tester (FM-700e, Future-TECH, Tokyo, Japan).

Degree of double bond conversion
For uncured liquid specimen, a potassium bromide liquid cell was coated with thin film by a drop of 100 microliters of uncured liquid resin. To prepare a cured specimen, a 5 mg sample was prepared in 3 mm diameter and 2 mm height plastic mold, covered with a glass slide, and light activated for 400 mW/cm^2 for 40 s. The sample was finely ground with potassium bromide powder and remolded in 13 mm diameter and 0.5 mm height flat round disc. Specimens were prepared as 5 paired sets of materials and analyzed by FTIR spectrometer. The degree of conversion (DC) was obtained from cured and uncured specimens and calculated from the ratio of the height of the absorbance peak of the aliphatic C=C bond (1,638 cm^{-1}) relative to that of the aromatic C=C bond (1,580 cm^{-1}), used as internal standard, by means of the following equation[20]:

DC=$100\times[1-(R_{cured})/(R_{uncured})]$
Where R=ratio of peak height at 1,638 cm^{-1} and 1,580 cm^{-1}

Statistical analysis
The BRR and physical properties data were analyzed by one-way ANOVA test. Post hoc multiple comparison test was performed to determine differences between groups. A confidence level of 95% ($p<0.05$) was considered significant.

RESULTS

The characteristic absorption band of the chitosan whiskers (Fig. 1b) was observed at 1,658 cm^{-1} (C=O, amide I band) and 1,604 cm^{-1} (N-H bending, amine and amide II). For chitin whiskers (Fig. 1a), the functional group peak at 1,658 cm^{-1} was also observed with the occurrence of the peak shifting from 1,604 cm^{-1} to the lower wavenumber (*i.e.* 1,560 cm^{-1} corresponding to N-H, amide II band) and an additional peak at 3,113 cm^{-1} (N-H stretching, amide II band). Figure 2 shows the size distribution of the chitosan whiskers. The length (L) of these whiskers ranged from 328 to 965 nm (with an average of approximately 648 nm), while the width (d) ranged from 20 to 87 nm (with an average of approximately 59 nm). The aspect ratio (L/d) of these whiskers was approximately 11.

According to the agar diffusion assay, no growth

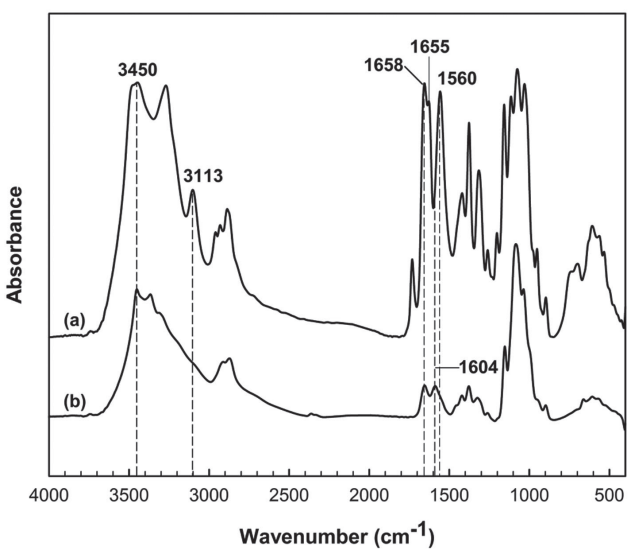

Fig. 1 FTIR spectra of (a) chitin whiskers and (b) chitosan whiskers.

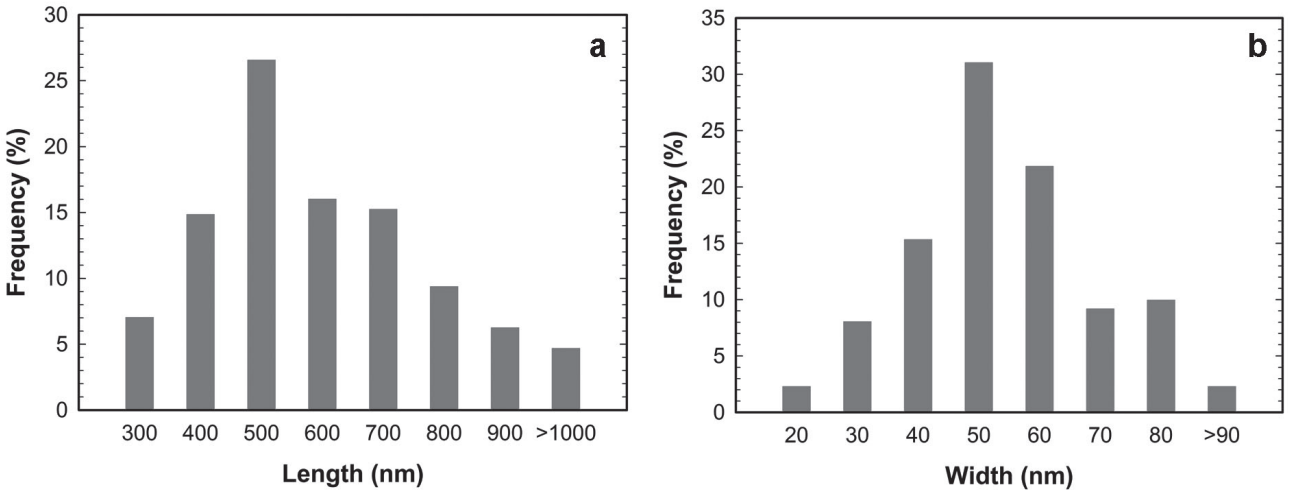

Fig. 2 The length (a) and width (b) distribution of chitosan whiskers.

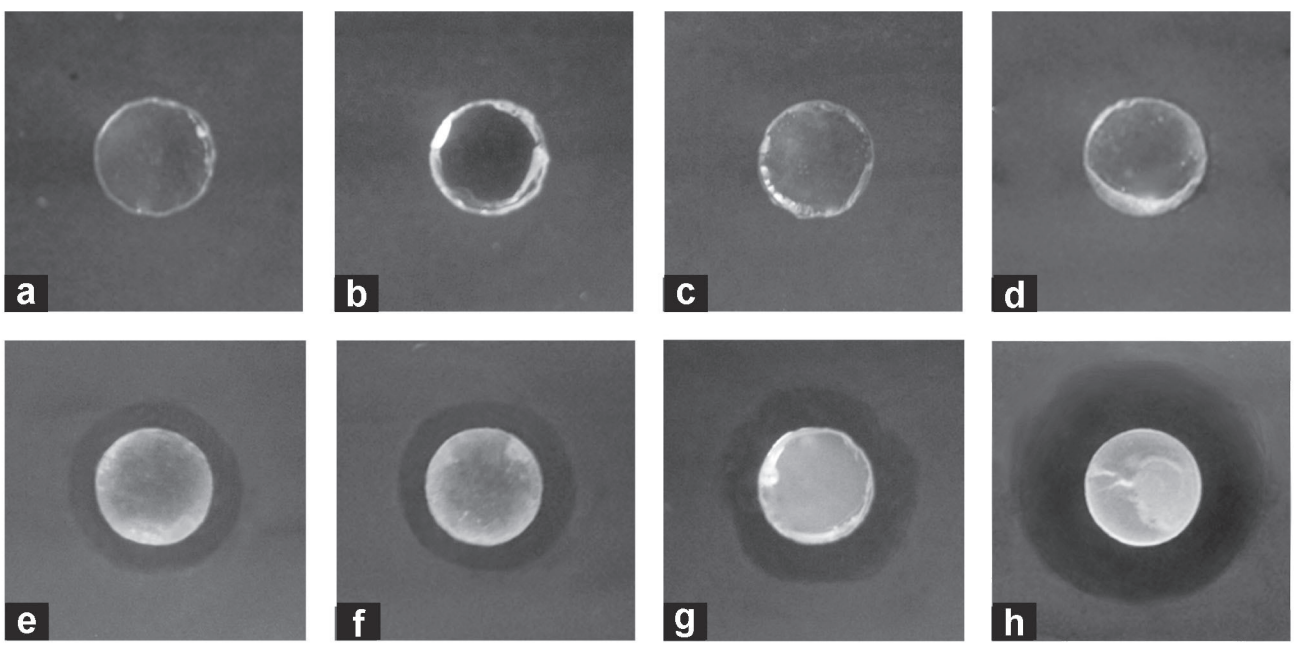

Fig. 3 The zone of inhibition induced by chitosan whisker resin-based sealant.
(a) Control, (b) Delton®, (c) 1% by wt., (d) 1.5% by wt., (e) 2% by wt., (f) 2.5% by wt. chitosan whisker resin-based sealant, (g) Teethmate™F-1 and (h) Seal&Protect™.

inhibition was noted for the control, Delton®, 1.0%, or 1.5% (Fig. 3a–d) whisker containing resins. However, a clear and distinct zone of inhibition was seen around the 2.0%, 2.5% chitosan-resin discs (Fig. 3e, f), Teethmate™F-1 disc (Fig. 3g) and Seal&Protect™ disc (Fig. 3h) which the greatest width of inhibition zone was observed (Table 1). The results of the colony forming unit count test seen in Table 1 were similar to those of the visible inhibition test. The control samples resulted in a lowest bacterial reduction rate compared with the others. The Delton, 1.0% and 1.5% chitosan containing resin samples caused significantly lower reduction rates than the 2%, 2.5% chitosan-resin group, Teethmate™ F-1 and Seal&Protect™ (Table 1). Based on the antimicrobial assay results, 2% by wt. chitosan whiskers incorporated resin-based sealant was selected to analyze by TEM and further test for physical characteristics. A TEM image of 2% by wt. chitosan-resin group demonstrated a scattered fiber arrangement with some areas of aggregation (Fig. 4).

Table 1 shows the assayed physical properties of the chitosan-resin group compared to commercial products

Table 1 Antimicrobial and Physical properties of the chitosan whisker incorporated resin-based pit and fissure sealant

	Width of inhibition zone (mm)	Bacterial reduction (%)	Depth of Cure (mm)	Vickers hardness (VHN)	Degree of conversion (%)
n	9	9	10	10	5
Seal&Protect™	15.2(0.2)	83.1(0.7)[a]	N/A*	2.8(0.6)[c]	40.1(1.8)[d]
Teethmate™ F-1	11.4(0.2)	76.9(0.3)[a]	4.48(0.18)[b]	16.5(0.3)[b]	46.6(1.5)[c]
2.5% whiskers	10.7(0.3)	75.9(0.6)[a,b]	5.72(0.07)[a]	15.2(0.4)[b]	59.3(1.4)[a]
2% whiskers	10.1(0.2)	72.2(0.6)[b]	5.83(0.09)[a]	15.4(0.4)[b]	57.6(1.7)[a]
1.5% whiskers	5.0(0)	39.2(3.7)[c]	5.78(0.12)[a]	15.5(0.4)[b]	58.2(1.3)[a]
1% whiskers	5.0(0)	31.2(4.7)[c,d]	5.84(0.13)[a]	15.7(0.5)[b]	59.6(0.8)[a]
Delton®	5.0(0)	25.9(3.8)[c,d]	3.84(0.10)[c]	16.2(0.4)[b]	71.9(2.2)[b]
Control (no whiskers)	5.0(0)	13.7(2.9)[e]	5.92(0.06)[a]	18.3(0.5)[a]	60.3(1.8)[a]

Values were shown by mean(SD).
N/A : not applicable due to casting failure in stainless steel mold according to ISO 6874:2005
Different alphabetic superscription represents the significant differences between experimental groups; $p<0.05$)

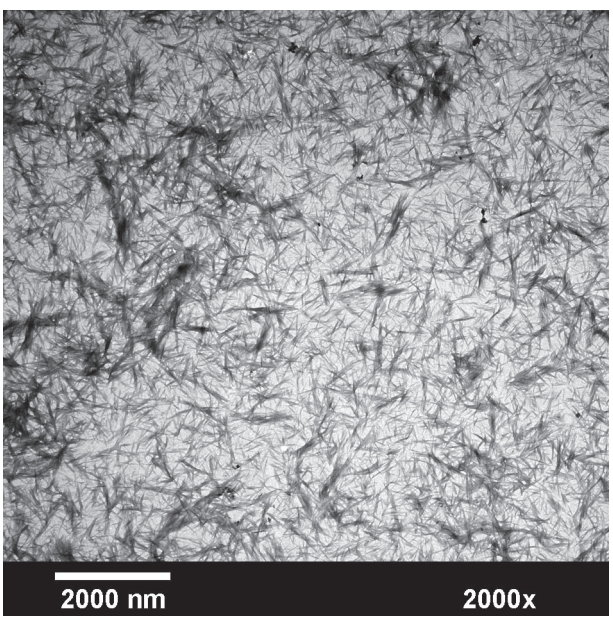

Fig. 4 A TEM image of the chitosan whiskers distributed in resin monomer matrix of sealant containing 2% by wt. chitosan.

and control. In this part of study, the physical properties of the Seal&Protect™ group could not be assayed because of its brittleness and specimen casting failure. The whisker free control produced the greatest depth of cure, which was not statistically different with the 2% whisker containing resin. Delton® and Teethmate™F-1 had lower cure depths which were significantly less than those of the control and test samples. We found the control samples had the highest hardness values at 18.3±0.5, which was significantly higher than the other materials. While the chitosan whisker samples showed the lower hardness, however there was no significant difference compared with the commercial products. The control and the chitosan whisker samples were statistically similar in double bond conversion degree, but significantly lower than Delton®. Teethmate™F-1 had the lowest conversion degree.

DISCUSSION

The FTIR spectra were shown in order to explain the ongoing deacetylation process from chitin to chitosan. Compared with chitin whiskers, chitosan whiskers had lower peak heights of both C=O and N-H of amide II band (the functional group present in the chitin structure). An absence of the chitosan whiskers peak at 3,113 cm^{-1} indicates the loss of the amide II functional group. These results confirm the depletion of *N*-acetyl groups ($-NHCOCH_3$) after the deacetylation process, a reaction of turning chitin from shrimp shells to chitosan oligomers in the copolymer backbone. According to the method of Baxter *et al*. (1992), the DD of Chitosan calculated from the FTIR spectra was 74%[17]. The DD of chitosan affects the antimicrobial activity and mostly depends on the nature of the chitosan source or conditions used during the deacetylation reaction such as pH and temperature. The chitosan used in this study was subjected to a *N*-deacetylation process under an alkaline condition and heating. Higher temperature can produce chitosan with a more potent bacteriostatic and bactericidal effect[21]. Although a longer reaction time can provide a higher DD, which exhibits more potent antibacterial effect[21], a greater DD could not be attained in this study. When attempting to create smaller sized whiskers and increase deacetylation time, the intense reaction damaged the whisker's configuration and induced whisker aggregation prior to combination with the resin sealant.

Seal&Protect™ and Teethmate™F-1 samples induced a zone of inhibition while Delton® and resin sealant without chitosan whisker group resulted in no

observed change. This result was similar to an earlier study on Teethmate™F-1, which had demonstrated an antibacterial activity against *S. mutans* when compared with other commercial resin sealants containing fluoride[6]. In our study, the inhibition zone was also distinctly observed in the Seal&Protect™ group. This is likely from the strong antimicrobial effect of triclosan on *S.mutans*, which has been previously reported[22]. Both triclosan and fluoride are well recognized for their antibacterial effects and have a long history of use in dental products.

Two percent by weight of chitosan whiskers was the minimum amount of the whiskers in resin sealant visibly demonstrating antimicrobial activity against *S. mutans*. The antimicrobial effect of whiskers in the sealant may result from direct contact between the bacteria and chitosan whiskers present or released from the resin sealant surface[14]. A TEM image of 2% chitosan by wt. impregnated resin displays the well distributed and condensed fibrous structure of chitosan whiskers in the resin matrix. This phenomenon is probably derived from the amino group protonation (NH_3^+) on the chitin-chitosan copolymer, which has a direct relationship with a high degree of deacetylation. This study provides DD values as high as 74, which is likely to result in segregation of individual whiskers. Most of the whiskers were homogeneously distributed in the colloidal suspension although some aggregated whiskers were observed. However, clusters of aggregated whiskers could be seen, which may be the result of interactions between positive-charge chitosan and the alkali reactant as described above[23] or the mixing process of freeze-drying whiskers into resin. In this study, careful attention was paid the synthesis of the chitosan in form of whiskers and to intentionally incorporate them into the resin as segregated as possible. In our previous pilot study, after being impregnated with nano-sized chitosan whisker the resin monomer showed a high transparency, nearly equal to the nanometer wavelength of light transmission. The amount of chitosan whiskers in the resin sealants was limited to 2.5% by weight or less because of the thickening and precipitation observed at higher amounts in pilot studies.

The chitosan whisker sealants showed a similar curing depth to that of the control group and were significantly greater than the commercial resin sealants. All test groups passed the minimum requirement of at least 1.5 mm curing depth corresponding to ISO 6874:2005. The curing depth obtained for the experimental sample may have resulted from the nano-sized whiskers in the resin monomers, which are capable of transmitting light throughout the sealant. The gross appearance of chitosan whisker resin sealant group remained clear and was similar to the control group. The nano-sized of the whiskers appears to be advantageous for the curing light to penetrate into the deepest area of tooth pit and fissures in clinical situations.

Although the hardness value of the chitosan whiskers group showed the lowest number, it was not significantly different from the other dental resin sealant brands. The aggregation of whiskers, as seen in TEM image, may affect the hardness value due to contact between whiskers and the loading tip during testing. The chitosan whiskers, being a biopolymer, are considered low rigid materials and not as capable of force loading as cured methacrylate polymer. Clinically, however, the pit and fissure area is functionally considered as a non-load-bearing area. Thus, a lower surface hardness value should be clinically acceptable. However, how stress is distributed is a more crucial concern. Morphologically, the chitosan whiskers have a rod-like pattern and are considered as high aspect ratio fibers, which allows for force absorbance and stress delivery along the rod. The whiskers could function in the dental resin-based sealant by converting it into a more flexible material. Greater flexibility is important for resin sealants to be able to remain in the pits and fissures where force is not directly loaded but absorbed over the long term in everyday use.

Degree of double bond conversion was used to indicate the conversion of carbon double bonds to single bonds in the dimethacrylate-polymeric chain. Rueggeberg *et al.* (1999) determined both peak area and peak height using 10 different methods and concluded using the peak height of absorption at 1,638 and 1,580 cm^{-1} provides the best fit to the Beer-Lambert law and demonstrates smaller variation in conversion values[20]. To calculate the double bond conversion, uncured liquid and cured solid specimens were tested in comparison. In our results, the double bond conversion degree of chitosan whisker-resin group was not significantly different from the control group. This suggests the chitosan whiskers are inert and do not affect the conversion rate of the dimethacrylate-resin monomer system. The degree of conversion refers to the completeness of monomer reaction by light activation. The degree values of these groups were similar to each other and within the range of the degree of conversion of dimethacrylate monomer, which is 50–60%[20]. This is likely due to the common components in the dimethacrylate-based monomer of both groups, Bis-GMA and TEGDMA, although differences in quantity exist. However, both control and test sample values were different from comparative commercial sealants, which, as proprietary formulations, might contain different kinds of monomer and additives.

Future studies should investigate whether whisker-form chitosan in resin sealant has a long-term effect. A previous study of alginate/chitosan whiskers nanocomposite yarn showed rapidly release of whiskers within the first 2 h with a very gradual increase to the maximum value 30 h after immersion[14]. The present study on chitosan whiskers followed the same procedure as Watthanapanit *et al.*[14] and selected the 12-h test for initial antimicrobial effect. It is critical that a study on whiskers release be conducted regarding the lasting effects on antimicrobial activity by chitosan resin sealant. The release of whiskers out of the material might have some drawbacks on the gross sealant's quality. Because of its natural origin, it is possible that the hydrolytic degradation could attack the chitosan

structure and deteriorate the fibrous form. The need for other physical tests such as solubility and wear test is apparent as well. The whiskers should be certified as to their stability in moisture conditions and their degradation from the resin sealant over time to determine the validity of the use of whisker-form chitosan impregnate resin over the long term.

CONCLUSION

An innovative chitosan whiskers incorporated into dimethacrylate-based resin sealant has been developed. In this study, the chitosan whiskers in resin sealant exhibited antibacterial property against *S. mutans* (UA159). The morphological data of incorporated chitosan whiskers indicated a mostly scattered distribution of small-size fibrous figures. The gross appearance of the sealant is clear and transparent. The two percent by weight of distributed chitosan whiskers showed acceptable physical properties compared with control, including greater depth of cure and double bond conversion degree. While the incorporation of fibrous whiskers moderately reduced the hardness value, the high aspect ratio in absorbing and spreading out the biting force could prove beneficial. In conclusion, the chitosan whiskers from shrimp shells can be used in dimethacrylate-based sealant as an alternative antimicrobial pit and fissure sealant.

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