Towards Bio-active fissure sealants for Preventive Dentistry: *in vitro approach*

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Abstract:

Background: For the prevention of dental caries, fissure sealants application is recommended if pits and fissures are very deep and narrow, creating a physical barrier for the plaque accumulation, in the specific anatomical areas of the tooth.

Aim: We aimed to report the synthesis, characterization and application of the newly developed chitosan-fucoidan bio-composites flowable fissure sealant materials. The detailed investigation into physical and chemical properties of these materials is reported here.

Results: The results of this study suggests that the optimum results for the strengthening of enamel can be achieved throughout the immediate treatment with bioactive:chitosan:nanodiamond with the increase of dentin bond strength. Also, impressively an almost immediately after the corresponding modified flowable material treatment and proceeding with bonding procedures is recommended with the significant increase in bond strength. The additional benefit of using chitosan:antioxidant system as a bonding/pre-bonding to enamel and dentin system lies in its ability to show favorable immediate results in terms of bonding effectiveness as well as the durability of resin-enamel bonds for a prolonged time (up to 6 months). The release and uptake of phosphorous after 5 cycles of de-remineralization, according to the chemical analysis of the solutions. Therefore, the treatment with chitosan seems to act more on the demineralization of tooth enamel with little effect on the remineralization process. Regarding the net phosphorus loss (net P loss), it can be seen that net demineralization occurs in all cases. However, the net

amount of phosphorous released by the control group samples was significantly higher than those groups treated chitosan. The net P loss for the control group was 475 mg of P, whereas the groups containing chitosan had a net P loss in the range of 30–182 mg.

Higher adhesiveness of the gels is desired to maintain an intimate contact with skin or tooth. The Modulus of Elasticity (MOE) of biomaterial treated enamel increased with time. A rapid significant increase in MOE was observed after 20 min treatment with premise material.

Conclusion: The materials were tested using effective in-vitro free radical generation model as functional additive prototypes for further development of ”dual function restorative flowable materials”. We quantified the effects of functional designer biomaterials on the enamel bond strength of a composite The added benefits of the chitosan or fucoidan (host:guest complex) treated hydrogels involved positive influence on increased dentin bond strength in the “prime free” technique as well as demonstrated in vitro “build in” free radical defense mechanism and there for acting as a “proof of concept” for the functional multi-dimensional restorative materials with the build in free radical defense mechanism.

Clinical significance: Fissure sealants are recommended to be applied soon after the tooth eruption, mainly at the level of the first permanent molars. The additional benefits of the application of bioactive fissure sealant materials lies in the build-in functionality of these materials to chemical attack in oral cavity as well as additional antibacterial action.

Introduction

The term pit and fissure sealant is used to describe a material that is introduced into the occlusal pits and fissures of caries–susceptible teeth, thus forming a micromechanical-bonded, protective layer cutting access of caries-producing bacteria from their source of nutrients. Field of bio-materials has established itself as an integral part of biomedical research development through the focus on designing "smart molecular machines" capable to contain a build in functionality to address specific limitations of the conventional clinical protocols at the molecular level.

Chitosan has been used as a wound dressing material due to its superior tissue- or mucoadhesive property, hemostatic activity, low toxicity, relevant biodegradability and anti-infection activity.[Kim 2008] Chitosan is a cationic polysaccharide and its adhesive properties are mainly based on ionic interactions with tissues or mucus layers.

Fucoidan is a sulfated polysaccharide that contains L-fucose and sulfate. It is commonly found in marine brown seaweeds.[Adhikari, 2006. Adhikari, 2006] Fucoidan can increase the level of alkaline phosphatase (ALP), type-1 collagen expression, osteocalcin and BMP-2 and even helps in mineral deposition associated with bone mineralization. [Adhikari, 2006]

Nanodiamonds were first synthesized by Soviet scientists in 1962 through the detonation of trinitrotoluene (TNT) with hexogen (RDX) in a closed chamber.[Mitura, 2007.] Today it can be prepared at room temperature at low cost. There are different types of nanodiamonds [Krüger 2006] namely: single-walled carbon nanotubes, multi-walled carbon nanotubes, carbon black and those with a single-particle size of 2-10 nm. Nanodiamonds (NDs) are carbon nanoparticles with a diamond like octahedral structure of about 2 to 8 nm in diameter [Krüger 2006]. Like diamonds it is chemically stable, stiff, strong and extremely hard. Like nanomaterials, it has a small size, large surface area, and high adsorption capacity. Thus NDs have superior physical and chemical properties compared to conventional materials and therefore render the ND particles ideal additives to formulation and improvement of conventional dental composites. Considering that the application of nanoparticles as fillers in polymeric matrices has shown encouraging results in the strengthening of the materials [Krüger 2006] it could be expected that the incorporation of ND nanoparticles into dental polymeric materials could have an enhancing effect on the mechanical properties of the resulting nanocomposites.

As our continuous interest in combining favorable bio-active properties of biological scaffolds such as chitosan and fucoindan with conventional dental materials, in order to enchance the properties, functionality and biological compatibility we aimed to report the synthesis, characterization and application of the newly developed chitosan-fucoidan bio-composites flowable fissure sealant materials. The detailed investigation into physical and chemical properties of these materials is reported here.

Materials and Methods:

Chitosan (Aldrich, Australia), glycerol (Sigma, USA), glacial acetic acid (E. Merck, Germany) were used as received. The degree of de-acetylation of typical commercial chitosan used in this study is 87%. Fucoidan (Doctor's Best Science Based Nutrition, 70%, USA). Chitosan with molecular weight 2.5x 10^3 KD was used in the study. The isoelectric point is 4.0–5.0.

Remineralization/demineralization prototype: pH cycling

The procedure for the model was adapted from the earlier work of Diniz *et. al*.[Diniz, 2010)Two demineralization solutions were prepared , DE 4.0 and DE 4.8 pocessing pH 4.0 and 4.8, respectively , with the following composition pH 4.0 and 4.9, with the following composition: 2.0mM $Ca(NO_3)_2$, 2.0mM Na_2HPO_4 and 75mM acetate buffer. The acidity was adjusted with HCl. Remineralization solution RE with pH 7.4 was prepared with the following composition: 1.5mM $Ca(NO_3)_2$, 0.9mM Na_2HPO_4 and 150mM and 20mM cacodylate buffer. Each tooth sample was submerged individually for 3 h, on 20 mL (4 mL/mm of exposed enamel) of demineralizing solution (DE4.0 or DE4.8). The sample was then removed from the solution, washed with distilled and deionized water for 10 s and dried with paper towels. Subsequently, the samples were immersed individually for 21 h in 10 mL (2 mL/mm of exposed enamel) of re-mineralizing solution (RE). This sequence constitutes a cycle and was repeated for five consecutive days, starting with fresh DE and RE solutions after each cycle. According to Tagliaferro et al. this cycling procedure induces a typical subsurface caries-like lesion approximately 50–60 mm deep.[Tagliferro 2007] Phosphorus chemical analysis was carried out by a spectroscopic method described elsewhere.[Murphy 1962].

Assay design

A random experimental design with 5 groups (Premise, 10%CNO, 10%CNB, 10%CNU and 10%CNC) containing 3 pieces each was used, and all groups were submitted to the pH cycling. One group (blank) was prepared without exposed dental enamel. This was achieved by completely coating the piece with acid resistant varnish. The blank was added to the experiment in order to verify that the materials employed in the pieces did not promote release or absorption of calcium and phosphorus, therefore, having no effect in quantitative analyses of these ions. Two other groups (control) did not contain chitosan in the samples, and were cycled in solutions DE4.0 and DE4.8, respectively. Bioactive modified nano-diamond chitosan containing flowable composites were applied on enamel by means of a small brush and cured for 20 seconds. After a certain period (time of chitosan action), the sample was submitted to pH cycling. The results were analyzed using the software Prism. Averages were compared by the Student test in the 95% confidence level.

The demineralization and the remineralization processes were followed separately by phosphorous chemical analysis. The amount of phosphorous released (DE) or absorbed by the dental specimen was calculated cycle by cycle. The sum of 5 cycles are represented in the figure, separately for the demineralization and the remineralization process for the different group of specimens studied. The figure shows also the net phosphorous loss (net P loss=DE-RE).

Shear bond strength etch and no etch prototype

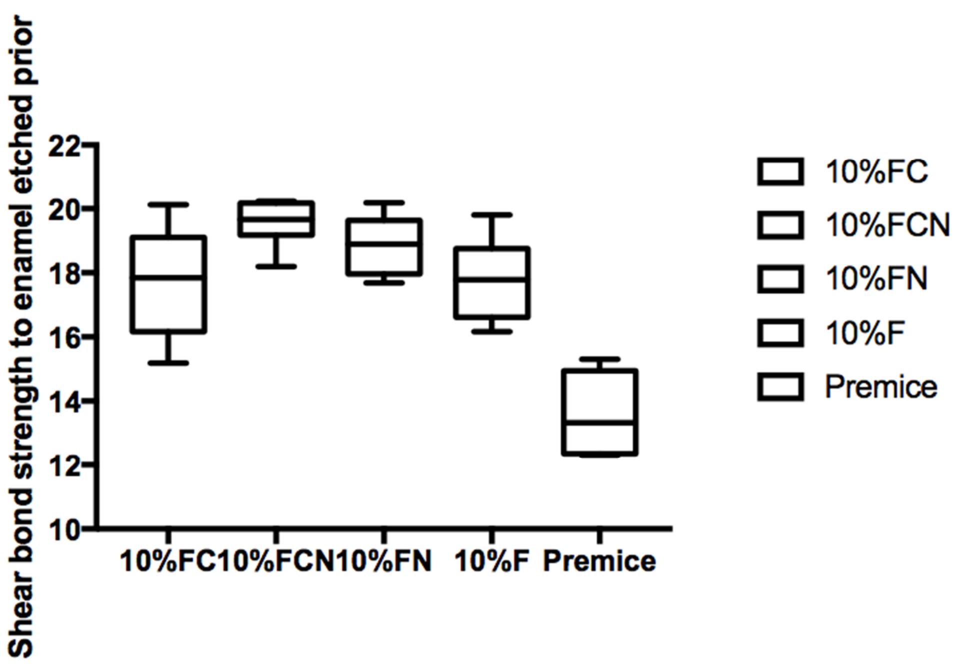
Extracted human molars were used within 2 months of storage in water containing thymol crystals. Only undamaged teeth were selected. The roots of the teeth were removed and all the occlusal enamel exposed. The teeth were embedded in 10 mm length PVC (Consjit Tubing, SA PVC, JHB, RSA) pipes with cold cure acrylic resin so that the exposed enamel is projected well above the acrylic and the dentin then thoroughly washed under tap water. Two modified flowable materials composite studs each with an internal diameter of 2.6 mm and height of 1 mm, were bonded to the enamel surface of each tooth via etching with orthophosphoric acid (37%) prior to applying and curing with modified flowable material or no etching used prior to applying the modified flowable material.

In this way, 30 tooth samples (each containing two studs) were prepared and divided at random into 5 groups of 8 each. The teeth were stored in a solution of artificial saliva. After 24 h, the shear bond strength of one stud of each tooth was tested for failure (Zwick Universal Testing Machine, Germany) by means of a knife-edged rod at a crosshead speed of 0.5 mm/min. The other stud was tested after 6 months. All data were analyzed using the non-parametric ANOVA test.

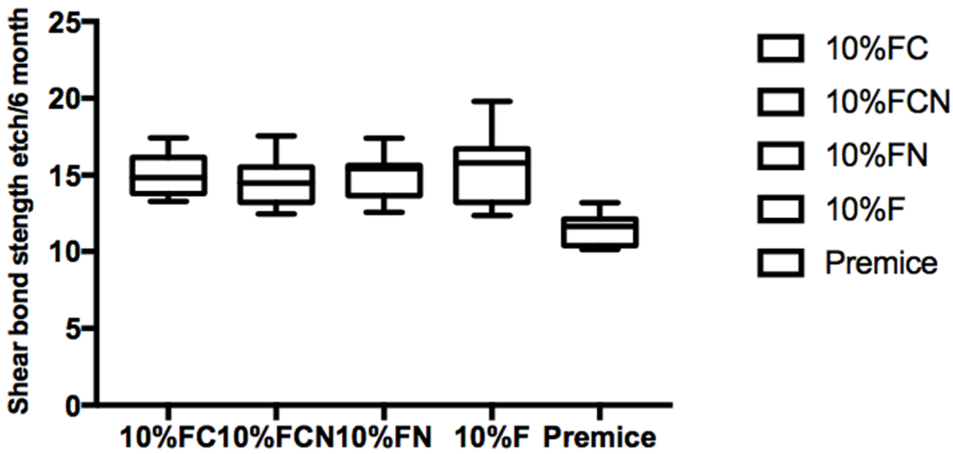
Bioadhesive study:

Bioadhesion studies were done using Chatillon apparatus for force measurement.[Thumma S, Majumdar S, ElSohly MA, Gul W, Repka MA. Chemical stability and bioadhesive properties of an ester prodrug of 9-tetrahydrocannabinol in poly(ethylene oxide) matrices: Effect of formulation additives. Int J Pharm. 2008;362:7] This method determines the maximum force and work needed to separate two surfaces in intimate contact.[Repka MA, McGinity JW. Physical-mechanical, moisture absorption and bioadhesive properties of hydroxypropylcellulose hot-melt extruded films. Biomaterials. 2000;21(14):1509–17.] The hydrogels (0.1g) were homogeneously spread on a $1cm^2$ glass disk and then the disks were fixed to the support of the tensile strength tester using double side adhesive. The gel was brought into contact with the contact with slice of enamel was established in order to imitate adhesion of the gel to the tooth structure, after a preset contact time (1 min) under contact strength (0.5N) the 2 surfaces were separated at a constant rate of displacement (1mm/s). The strength was recorded as a function of the displacement, which allowed to determine the maximal detachment force, Fmax, and the work of adhesion, W, which was calculated from the area under the strength-displacement curve.

Results and discussion:



a


Shear bond stength etch/6 month
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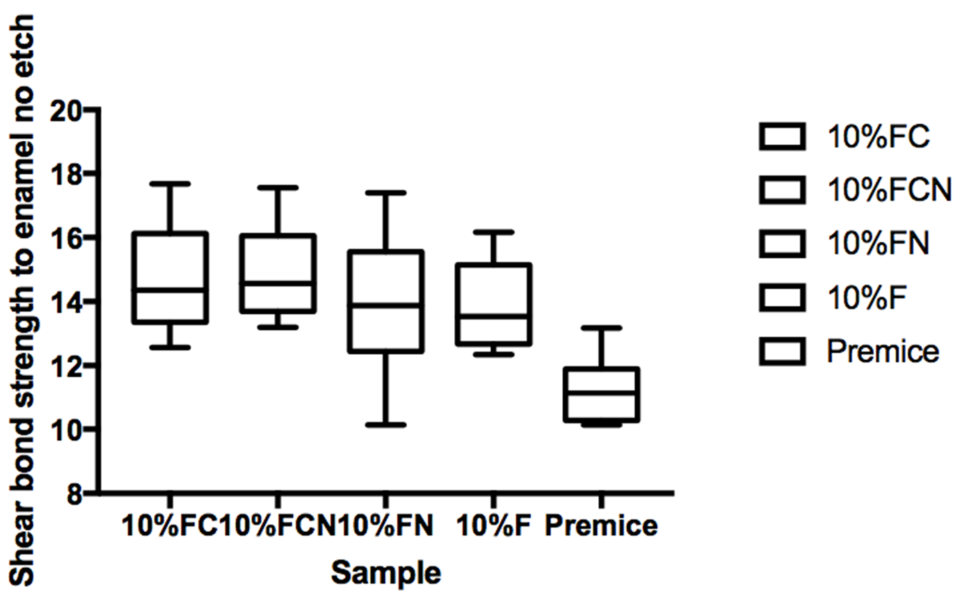
b.


Shear bond strength to enamel no etch
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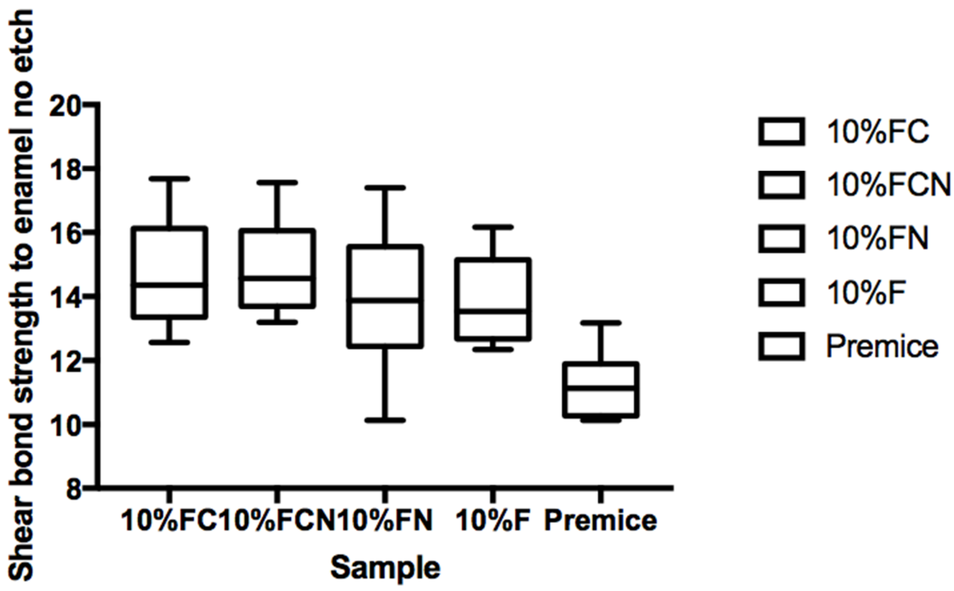

d.

Figure 1a, b, c and d Shear bond strength in mPa a.enamel bonding with etchin after 24 hours debonding. enamel bonding with etching after 6 month debonding. c. shear bond strength to enamel bonding with no etching after 24 hours debonding. d. shear bond strength to enamel bonding with no etching after 6 month debonding,

Figure 1a and Figure 1b gives the shear bond strength values (MPa) after 24 hours and after 6 month of storage of samples in artificial saliva, respectively, using conventional fissure sealant protocol discussed in the experimental section. In general there was an increase in bond strength of the enamel treated with the modified Premise containing nanodiamond:chitosan materials compared to the bond strength of the conventionally bonded teeth.

An increase in the shear bond strength was also previously reported [Perchyonok, 2015] for chitosan containing hydrogels. Interestingly the increase in bond strength was also observed in the groups of hydrogen peroxide exposed samples suggesting that there additional benefits associated with nanodiamond:chitosan:bioactive system are in need of further investigations [Perchyonok 2014].

The results of this study suggests that the optimum results for the strengthening of enamel can be achieved throughout the immediate treatment with bioactive:chitosan:nanodiamond with the increase of dentin bond strength. Also, impressively an almost immediately after the corresponding modified flowable material treatment and proceeding with bonding procedures is recommended with the significant increase in bond strength. The additional advantage of the system may suggest that, chitosan:bioactive and nano-diamond interaction with crystalline hydroxyapatite structure of the enamel layer increases the dentin bond strength observed especially in the case of the direct bonding between the hydrogel and the enamel interface. The additional benefit of using chitosan:antioxidant system as a bonding/pre-bonding to enamel and dentin system lies in its ability to show favorable immediate results in terms of bonding effectiveness as well as the durability of resin-enamel bonds for a prolonged time (up to 6 months) [Perchyonok 2015].

Remineralization/de-mineralization results and total P loss: Effects of chitosan on de-remineralization of dental enamel

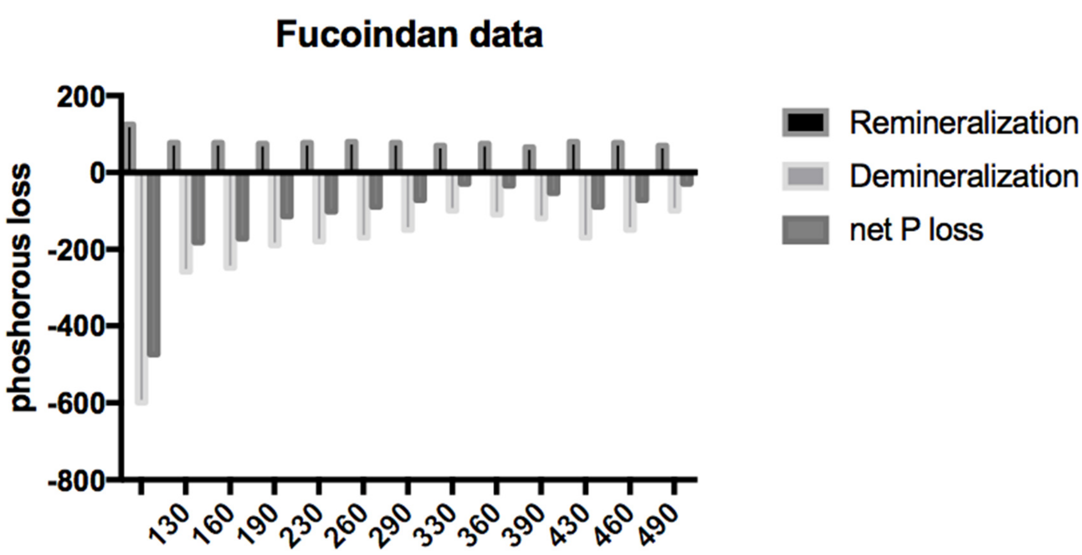

Figure 2: Cumulative phosphorous content of demineralization (expressed as negative number) and remineralization solution and their difference (net P loss) after 5 cycles for each group of samples investigate.

The influence of chitosan can also clearly be seen in Fig. 2. This figure shows the release and uptake of phosphorous after 5 cycles of de-remineralization, according to the chemical analysis of the solutions. It is seen that release of phosphorus into the demineralizing solution (i.e., loss of phosphorus from the samples) showed larger amplitude (from 600.2mg to 101.3 mg) than the uptake of phosphorus by the samples from the remineralizing solution (from 125.2 mg to 66.1 mg). Therefore, the treatment with chitosan seems to act more on the demineralization of tooth enamel with little effect on the remineralization process. Regarding the net phosphorus loss (net P loss), it can be seen that net demineralization occurs in all cases. However, the net amount of phosphorous released by the control group samples was significantly higher than those groups treated with chitosan. The net P loss for the control group was 475 mg of P, whereas the groups containing chitosan had a net P loss in the range of 30–182 mg.

Bio-adhesion

Higher adhesiveness of the gels is desired to maintain an intimate contact with skin or tooth structure and results are summarized in Table 1. Functionalized hydrogels showed the highest adhesive force, and this result can be expected because of the well-known intrinsic bio-adhesive properties of chitosan [Vasir 2002]

Table 1: Adhesive strength in enamel

Modified flowable compound	Adhesive Force(N) ± SD (enamel)	Work of Adhesion(N.cm) ± SD (wnamel)
Fucoindan/Chitosan/Premise	1.87± 0.36	6.11 ± 0.25
Chitosan/Premise	1.12± 0.19	3.45± 0.15
Fucoindan/Premise	1.18± 0.12	3.54± 0.18
Fucoidan/Nano/Premise	1.85± 0.41	6.25 ± 0.30
Fucoidan/Nano/Chitosan/Premise	1.92± 0.32	6.75 ± 0.32
Premise	1.38± 0.21	4.34± 0.25

Modulus of elasticity

The mean and standard deviations of MOE of de-mineralized enamel treated with modified Premise at different time periods are shown in Figure 3. The results of two-way ANOVA showed that both factors, “material treatment’’ and ‘‘treatment duration’’, had a significant effect on the MOE of de-mineralised dentine ($p < 0.001$). Interaction of the two factors was also significant ($p < 0.001$). The MOE of biomaterial treated enamel increased with time. A rapid significant increase in MOE was observed after 20 min treatment with premise material.

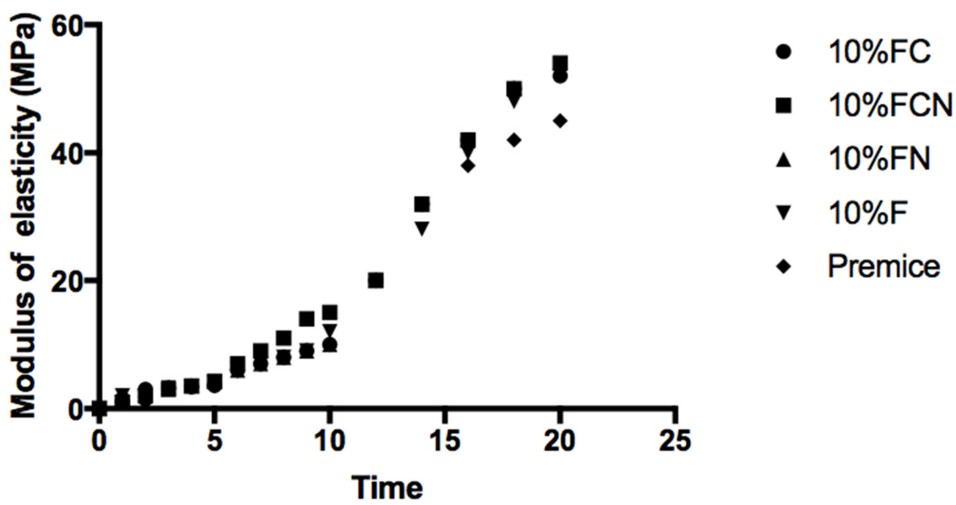

Figure 3: Effects of the time duration on the modulus of elasticity following the hydrogel treatment

Discussion

Enamel shear bond strength

The results of this study suggest that optimum bond strengthening of dentin can be achieved throughout the immediate or conventional adhesive treatment with bio-functional hydrogels. Initial results have proven that this significant increase in bond strength and the durability of resin-dentin bond lasts for a prolonged time (up to 3 months). Although the correlation between the force and work of adhesion was noticeable for all samples. This performance can be expected because of the well-known intrinsic bio-adhesive properties of this material. Also ionic vs covalent bonding of the chitosan: therapeutic agent complex may depend on the pH of the environment as the -COOH groups in substituents of nano-diamonds ionize at alkaline pH and form covalent "amide" linkage at low pH. The adequate water absorption capacity, together with its cationic nature, which promotes binding to the negative surface of skin or dentin structure, can also explain these results. Hydration of the polymer causes mobilization of the polymer chains and hence influences polymeric adhesion.[Perchyonok 2015 Appropriate swelling is important to guarantee bio-adhesion; however, over-hydration can form slippery non-adhesive hydrogels

Chitosan and fucoidan are potent antioxidants with multiple free hydroxyl groups [Perchyonok, 2015]. This study has shown that newly developed hydrogels are capable of improving the modulus of elasticity of de-mineralised enamel.

Bio-adhesion

Higher adhesiveness of the gels is desired to maintain an intimate contact with skin or tooth structure and results are summarized in Table 1. Bioactive flowable composites showed the highest adhesive force, and this result can be expected because of the well-known intrinsic bio-adhesive properties of chitosan, fucoidan, nanodamonds [Vasir, J.K., Tambwekar, K., Garg, S., 2003. Bioadhesive micro- spheres as a controlled drug delivery system. Int. J. Pharm. 255, 13–32. Wang, F.J., Wang, C.H., 2002. Sustained release of etanidazole from spray dried microspheres prepared by non-halogenated solvents. J. Control Release 81, 263–280.].

Modulus of elasticity

The mean and standard deviations of MOE of de-mineralized dentine treated with flavonoids at different time periods are shown in Figure 3. The results of two-way ANOVA showed that both factors, “bioactive flowable composite treatment’ and ‘‘treatment duration’’ had a significant effect on the MOE of demineralised dentine ($p < 0.001$). Interaction of the two factors was also significant ($p < 0.001$). The MOE of bioactive flowable composites treated enamel increased with time. A rapid significant increase in MOE was observed after 15 min treatment with the bio-active containing restorative material.

The important aspect of any newly designed/ developed restorative material is cytotoxicity as Grobler *et. al* [Grobler, 2015] investigated the cytotoxic effect of nanodiamonds and also the effect of the incorporation in a dental material (Premise), who found a higher shear bond strength ($p < 5\%$) after 3 months of Premise treated with nanodiamonds, chitosan, cyclodextrin (CD) and combinations thereof than for the control Premise. The sequence for the Vickers hardness was: CD (32.5) < nano (34.8) < CD Nano (38.8) < Premise (39) < Chitosan Nano (42.2). Nanodiamonds (92%) and the combination of chitosan + nanodiamonds (93%) showed little cytotoxicity. The shrinkage was lower for all the additions than for Premise alone.

Free radical defense mechanism and antioxidant capacity of bio-active fissure sealants:

Dental caries is one of the most common infectious diseases worldwide. Saliva has many functions in the oral cavity and is the first line defense against dental caries. Oxidative stress can affect initiation and progression of many inflammatory and infectious diseases such as dental caries. Therefore, we needed to evaluate the potential capability of our bio-active flowable composites to fight excess of free radicals in our previously reported in-vitro model.(Figure 4)

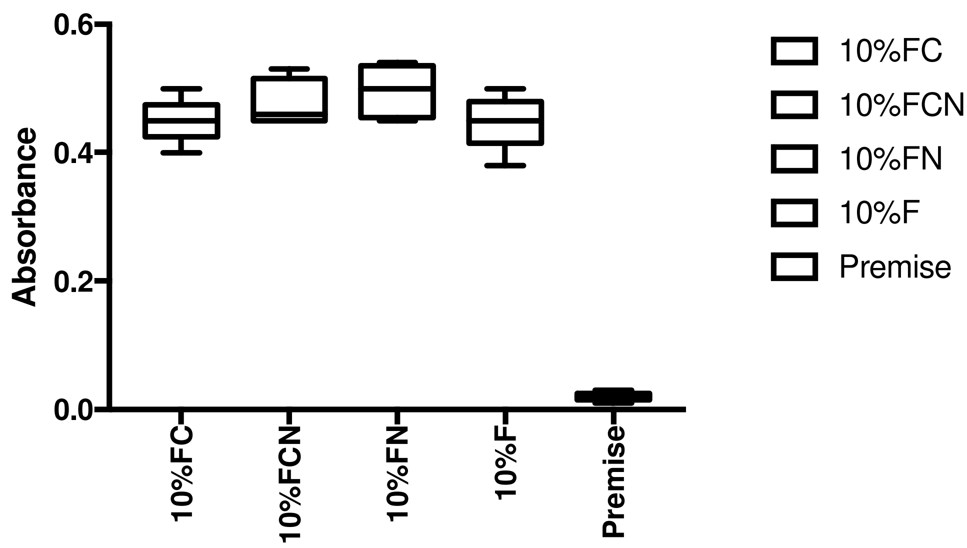

Figure 4: Antioxidant capacity measured at 450nm using the previously described spectrophotometric assay to assess the hydrogels and corresponding ingredients antioxidant capacity after 24 hours under storage under ambient temperature condition. Antioxidant capacity was measured during the first 2 hours of exposure

The stability of bio-active modified restorative materials has been measured during storage and no significant decomposition observed after 6 month storage at room temperature (24^{o}C).

Nanodiamonds as well as chitosan and fucoindan have demonstrated remarkable free radical defense properties as it has been previously reported. Nanodiamond as well as chitosan and fucoindan particle is very active molecule in contact with living organism.[Grabarczyk 2009] Two molecules can be weakly attracted to one another through intermolecular forces. These forces might include van der Waals interactions and hydrogen bonding

[Grabarczyk, 2009]. Further investigation on establishing the link between the molecular relevance of excess of free radical formation and development of caries lesion model prediction is currently on the way in our laboratory.

Conclusion

The materials were tested using effective in-vitro free radical generation model as functional additive prototypes for further development of ”dual function restorative flowable materials”. We quantified the effects of functional designer biomaterials on the enamel bond strength of a composite The added benefits of the chitosan or fucoidan (host:guest complex) treated hydrogels involved positive influence on increased dentin bond strength in the “prime free” technique as well as demonstrated in vitro “build in” free radical defense mechanism and there for acting as a “proof of concept” for the functional multi-dimensional restorative materials with the build in free radical defense mechanism.

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