## Insights into and relative effect of chitosan-krill oil, chitosan-H-aspirin, chitosan-H-krill oil-nystatin and chitosan-H-krill oil-aspirin-nystatin on dentin bond strength and functional drug delivery capacity: *In-vitro* studies

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#### ABSTRACT

Background: Restorative materials in the new era aim to be "bio-active" and long-lasting. The purpose of this study was to design and to evaluate a novel chitosan hydrogels containing krill oil (antioxidant containing material), nystatin (antifungal), aspirin (pain relieve medication and free radical scavengers) and combinations thereof (chitosan-H-krill oil, chitosan-H-krill oil-nystatin and chitosan-H-aspirin, chitosan-H-aspirin-nystatin, chitosan-H-krill oil-aspirin and chitosan-H-krill oil-aspirin-nystatin) as functional additive prototypes for further development of "dual function restorative materials," and secondly to determine their effect on the dentin bond strength of a composite. Materials and Methods: The above-mentioned hydrogels were prepared by dispersion the corresponding component in glycerol and acetic acid with the addition of chitosan gelling agent. The surface morphology (scanning electron microscope (SEM)), release behaviors (physiological pH and also in acidic conditions), stability of the therapeutic agent-antioxidant-chitosan and the effect of the hydrogels on the shear bond strength of dentin were also evaluated. Results: The release of nystatin and aspirin confer the added benefit of synergistic action of a functional therapeutic delivery when comparing the newly designed chitosan-based hydrogel restorative materials to the commercially available products alone. Neither the release of nystatin and aspirin nor the antioxidant stability was affected by storage over a 6 month period. The hydrogel formulations have a uniform distribution of drug content, homogenous texture and yellow color (SEM study). All chitosan dentin treated hydrogels gave significantly (P<0.05; non-parametric ANOVA test) higher shear bond values (P<0.05) than dentin treated or not treated with phosphoric acid. Conclusion: The added benefits of the chitosan treated hydrogels involved positive influence on the nystatin and aspirin release as well as increased dentin bond strength.

#### Key words

Chitosan, dentin bond strength, hydrogels, functional restorative materials

### INTRODUCTION

The acid-etch technique, introduced by Buonocore in 1955, was seminal and opened the doors to the possibilities of achieving a bond to natural tooth substrates with artificial acrylic-based restoratives.<sup>[1]</sup> Whilst bonding to enamel has changed a little since its inception more

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than half a century ago, bonding to dentin has proved far more elusive, undergoing enormous changes. A major advancement for achieving a sustainable bond to dentin was the introduction of the total-etch technique in the late seventies.<sup>[2]</sup> The first self-etching (SE) primer, combining an etchant and primer in a single step, was introduced in the early nineties.<sup>[3]</sup> The SE primers not only simplified bonding to dentin, but also eliminated the clinical errors associated with this exacting procedure. Bio-adhesive polymers appear to be particularly attractive for the development of alternative etch-free dentin bonding system with an added advantage of additional therapeutic delivery systems to improve the intra-dental administration of therapeutic and prophylactic agents, if necessary.<sup>[4]</sup> Chitosan, which is a biologically safe biopolymer, has been proposed as a bio-adhesive polymer and are of continuous interest due to their unique properties and flexibility in a broad range of oral applications as reported recently.<sup>[4,5]</sup>

The purpose of this study was to design and evaluate (surface morphology, stability, swelling and release behavior) novel chitosan hydrogels containing krill oil (antioxidant containing material), nystatin (antifungal), aspirin (pain relieve medication and free radical scavengers) and combinations thereof (chitosan-H-krill oil, chitosan-H-krill oil-nystatin and chitosan-H-aspirin, chitosan-H-aspirin-nystatin, chitosan-H-krill oil-aspirin and chitosan-H-krill oil-aspirin-nystatin) as functional additive prototypes for further development of "dual function restorative materials," and secondly to determine their effect on the dentin bond strength of a composite.

### 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%. Chitosan with a molecular weight  $2.5 \times 10^3$  KD was used in this study. Gelatin in powder form was purchased from Shanghai Chemical Reagent Co., (Shanghai, China) with the number-average molecular weight (Mn) of about  $8.7 \times 10^4$ . The isoelectric point is 4.0-5.0. Krill oil (Aurora Pharmaceuticals, Australia), aspirin (Safeway, Australia) and nystatin were used as received.

## Preparation of various nystatin: Additive hydrogel gels

The nystatin containing gel was prepared by dispersion of nystatin powder 0.2 g in glycerol (5% w/w) using a mortar and a pestle. A total volume of 10 ml of glacial acetic acid (3% w/w) was then added with continuous mixing and finally chitosan polymer was spread on the surface of the dispersion and mixed well to form the required

gel. The strength of the prepared gel (10 g) is 100.000 IU nystatin in each gram of the base. Nystatin gel had been prepared with three different concentrations of chitosan gelling agent (5%, 6% and 7% w/w). The summary of the newly prepared materials was presented in Table 1.

### Preparation of various aspirin containing hydrogels

The aspirin containing gel was prepared by dispersion of aspirin powder 0.3 g in glycerol (5% w/w) using a mortar and a pestle. A total volume of 10 ml of glacial acetic acid (3% w/w) was then added with continuous mixing and finally chitosan polymer was spread on the surface of the dispersion and mixed well to form the required gel. The strength of the prepared gel (10 g) is 0.3 g of aspirin in each gram of the base. Aspirin gel had been prepared with three different concentrations of chitosan gelling agent (5%, 6% and 7% w/w). The summary of the newly prepared materials is highlighted in Table 1.

#### Determination of gel pH

Prepared gel, 1 g was accurately weighed and dispersed in 10 ml of purified water. The pH of the dispersions was measured using a pH meter (HANNA instruments, HI8417, Portugal).

#### Morphology of the gels

The samples were prepared by freezing in liquid nitrogen for 10 min and then freeze-dried for 24 h. The prepared samples were fractured in liquid nitrogen using a razor blade. The fractured samples were attached to metal stubs and sputter coated with gold under vacuum for scanning electron microscope (SEM) the interior and the surface morphology were observed in (SEM, Hitachi S4800, Japan).

#### **Gel stability**

Stability of the gel formulations was also investigated. The organoleptic properties (color, odor), pH, drug content and release profiles of the gels stored at 20°C were examined on days (0, 15, 30 and 178).

Table 1: Gel formulation prepared in the study							
Gel formulation	Gel number	Chitosan (w/w%)	Aspirin (w/w%)	Krill oil (w/w%)	Nystatin	рН	
Chitosan-H	Gel-1	5	0	0	0	5.70	
Chitosan-H1	Gel-2	6	0	0	0	5.80	
Chitosan-H2	Gel-3	7	0	0	0	5.95	
Chitosan-H-A1	Gel-4	5	1	0	0	5.80	
Chitosan-H-A2	Gel-5	6	1	0	0	6.00	
Chitosan-H-A <sub>3</sub>	Gel-6	7	1	0	0	5.90	
Chitosan-H-A1N1	Gel-7	5	1	0	1	5.54	
Chitosan-H-K1	Gel-8	5	0	1	0	5.36	
Chitosan-H-K2	Gel-9	6	0	1	0	5.45	
Chitosan-H-K <sub>3</sub>	Gel-10	7	0	1	0	5.57	
Chitosan-H-K1A1	Gel-11	5	1	1	0	5.90	
Chitosan-H-K1A1N1	Gel-12	5	1	1	1	6.12	
Chitosan-H-K1N1	Gel-13	5	0	1	1	6.38	

Where K is Krill oil as additive; A is aspirin as selective additive; N is the nystatin as selective additive. Hydrogels containing chitosan in different % are synthesized and characterized

# Studies of equilibrium swelling in the alternative drug delivery systems

A known weight functionalized chitosan containing dry gels were immersed in pH 4.0, pH 9.0 buffer solutions, respectively and kept at 25°C for 48 h until equilibrium of swelling had been reached. The swollen gels were taken out and immediately weighed with microbalance after the excess of water lying on the surfaces was absorbed with a filter paper. The equilibrium swelling ratio (SR) was calculated using the following equation:

$$SR = (W_s - W_d) / W_d \times 100\%$$

Where,  $W_{\rm s}$  and  $W_{\rm d}$  are the weights of the gels at the equilibrium swelling state and at the dry state, respectively.<sup>[6]</sup> Experiments were repeated in triplicate for each gel specimen and the mean values were calculated.

### Shear bond strength tests for dentin bonding

Extracted non-carious, intact, human molars stored in water containing a few crystals of thymol at 4°C were used within 2 months. Samples were checked before use for any damage caused by their removal. The roots of the teeth were removed with a separating disc and the occlusal enamel removed by grounding wet on 60-grit silicon carbide (SiC) paper. The teeth were embedded in PVC (Consjit Tubing, SA PVC, JHB, RSA) pipe containers with cold cure acrylic resin so that the grounded occlusal surfaces projected well above the resin. The 10 mm length pipes were put on a glass surface with one end blocked by the glass and the embedding done through the open- end. Immediately after embedding the occlusal surfaces were ground wet with 180-grit followed by 600-grit SiC on a polishing machine to expose the superficial dentin. The samples were washed under a stream of tap water. A standardized jig (Ultradent ISO A2-70) with an internal diameter of 2.5 mm and height of 3 mm was used to shape the composite resin stud (SDR, Dentsply, CA, USA, Batch number 1105000609, Exp 2013-04). Two of these studs were then bonded to the polished dentin surface of each tooth through the bonding agent XP bond (Dentsply, New York, USA), as suggested by the manufacturer. The bonding agent contained: Carboxylic acid modified dimethacrylate (TCB resin), phosphoric acid modified acrylate resin, urethane dimetacrylate, triethyleneglycol dimethacrylate, 2-hydroxyethylmethacrylate, butylated benzenediol (stabilizer), ethyl-4-dimethylaminobenzoate), camphorquinone, functionalized amorphous silica, t-butanol.

In this way, 120 teeth samples (each containing two studs) were prepared and divided into 15 groups of 8 each, A-T [Table 2] and stored in artificial saliva. These groups were then treated as outlined in Table 2. After 24 h one stud of each tooth was tested for shear bond strength and the other one after 6 months. An Instron Universal Testing Machine (Griffith University, G12)

#### Table 2: Groups tested (8 teeth per groups)

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Sample (group)	Condition of bonding		
A	37% of phosphoric acid+primer+bonding immediately (negative control)		
В	Self-etching primer+bonding immediately (positive control)		
С	Gel-1+primer+bonding immediately		
D	Gel-2+primer+bonding immediately		
E	Gel-3+primer+bonding immediately		
F	Gel-4+primer+bonding immediately		
К	Gel-5+primer+bonding immediately		
L	Gel-6+primer+bonding immediately		
Μ	Gel-7+primer+bonding immediately		
Ν	Gel-8+primer+bonding immediately		
0	Gel-9+primer+bonding immediately		
Р	Gel-10+primer+bonding immediately		
R	Gel-11+primer+bonding immediately		
S	Gel-12+primer+bonding immediately		
Т	Gel-13+primer+bonding immediately		

at a crosshead speed of 0.5 mm/min was used to test the de-bonding strength. Data were analyzed using the non-parametric ANOVA test.

## Experimental methodology

### In vitro study of nystatin release profile

The release study was carried out with USP dissolution apparatus type 1, Copley U.K., slightly modified in order to overcome the small volume of the dissolution medium, by using 100 ml beakers instead of the jars. The basket of the dissolution apparatus (2.5 cm in diameter) was filled with 1 g of nystatin gel on a filter paper. The basket was immersed to about 1 cm of its surface in 50 ml of phosphate buffer pH 6.8, at 37°C ± 0.5 and 100 rpm.<sup>[7]</sup> A total sample of 2 ml were collected at 0.25,1, 2, 3, 4, 5, 6, 7 and 8 h<sup>[8]</sup> and were analyzed using a ultra violet (UV). Spectrophotometer (Cintra 5, GBC Scientific equipment, Australia) at  $\lambda$  max 306 nm.<sup>[9]</sup> Each sample was replaced by the same volume of phosphate buffer pH 6.8 to maintain its constant volume and sink condition.<sup>[10]</sup>

#### In vitro study of aspirin release profile

This release study of aspirin was exactly done as described above for nystatin except that the spectrophotometric analysis took place at  $275 \text{ nm.}^{[11]}$ 

## Cupric ions (Cu<sup>2+</sup>) reducing power and antioxidant strength assay and stability measure for microencapsulation

In order to further measure the reducing ability of negative control (35% hydrogen peroxide solution and  $CuSO_4$ ), aspirin and krill oil, the Cu<sup>2+</sup> reducing power capacity was used with slight modification.<sup>[12]</sup> Briefly, 250 µL of 37.5% hydrogen peroxide solution and  $CuSO_4$  and 250 µL CH<sub>3</sub>COONH<sub>4</sub> buffer solution (100 mmol/L, pH 7.0) were

added to a test vial containing a negative control (35% hydrogen peroxide solution and CuSO<sub>4</sub>), aspirin and krill oil sample as well as chitosan complexes of aspirin and krill oil (250 µL). Then, the total volume was adjusted with the buffer to 2 mL and mixed vigorously. Absorbance against a buffer blank was measured at 568 nm at 20 min intervals for the total time of 2 h. Increased absorbance of Cu<sup>+</sup> complex in the reaction mixture indicates increased reduction capability. Trolox (water soluble vitamin E) was used as the positive control. The results of the investigation are summarized in Graph 2. Absorbance was measured using POLARstar Omega multifunction microplate reader (BMG LABTECH, Spectral range: 220-850 nm). 24 well plates used in the investigations are Corning Incorporated Castar 3524, 24 well cell culture cluster flat bottom with lid, non-pyrogenic, polystyrene, sterile plates. (Corning Incorporated Corning, NY, 14831, USA). Further studies were conducted to evaluate and quantify the antioxidant potential of aspirin and aspirin: chitosan, krill oil and krill oil: Chitosan for the purpose of determining the stability of their activity and also correlating the micro-encapsulating influence of the chitosan on stability and efficacy of corresponding antioxidants.

## RESULTS

#### Properties of antioxidant-chitosan gels

Antioxidant content in 0.3 g of the different gel formulations from the prepared formulae was presented in Table 1. The prepared gel formulations had uniform distribution of drug content, homogenous texture and color. The pH of the formulations ranged from 5.36 to 6.38. Table 1 represents the summary of the Additive-Chitosan Gels prepared in this study.

## The characterization of additive containing-chitosan gels (Gel-1 to Gel-13)

The SEM images were obtained to characterize the microstructure of the interior of the freeze-dried additive containing composite gels and are presented in Figure 1. It could be seen that the gels displayed a homogeneous pore structure. It was thought that the micro-porous structure of the gels could lead to high internal surface areas with low diffusional resistance in the gels. The surfaces of the gels were also presented in Figure 1. The "skin" of the gels could be seen and the collapse of the surface pores might be due to the freeze-dry process.

### Studies of equilibrium swelling in the chitosan gels under investigation (Gel-1 to Gel-13)

The hydrogels remain in the cylindrical form after swelling. Compared with dry state hydrogels, the volume of the swollen state hydrogels displayed significant increases and were summarized in Figure 2.

Equilibrium SR of hydrogels exerts an influence on their

release rates. The reduction in equilibrium swelling capacity is due to the formation of a tight network structure in high content. Environmental pH value has a large effect on the swelling behavior of these gels. From Figure 2, it is clear that the SR value increases with the increase of pH. Such pH dependent properties of the hydrogels come from the polyelectrolyte nature of chitosan segments in the hydrogel network. Namely, when the pH value of the buffer solution (pH 9.0) was far higher than the isoelectric point (PI) of gel (PI 4.0-5.0), the carboxyl groups were de-protonized to carry negative charges, which made molecular chains repulsed to each other. The network became looser and it was easy for the water molecules to diffuse into the cross-linked network.

## Investigation of free radical chemistry of the *in vitro* model system

It is well-established that HO can be generated from a reaction known as the biologic Fenton reaction and this reaction requires the presence of  $H_2O_2^{[12,13]}$  which is an equivalent process occurring on the surface of the conventional restorative material under the blue light conditions. The generation of HO from the biologic Fenton reaction has been shown to be a critical factor in various reactive oxygen species induced oxidative stresses.<sup>[14,15]</sup>  $H_2O_2$  and HO might be related to apoptosis in atherosclerosis.<sup>[16]</sup> Godley *et al.* also reported that blue light induces mitochondrial DNA damage and cellular aging.<sup>[17]</sup> The reactive nature of the surface has been investigated using the SEM and comparison confirms the reactive nature of the transformation [Figures 3 and 4].

The visible surface deterioration of the surface of the composites used in the experiments indicate that the surface exposed to conventional "dentistry related chemical exposure" is significantly affected over time (24 h vs. 6 month). The chemical nature of this transformation is currently under investigation in our laboratory

#### Shear bond strengths

Figures 5 and 6 give the shear bond strength values after 24 h and 6 months respectively (MPa)

Mean shear bond strength values and difference between the groups are summarized in Figure 5 for bonding to dentin after 24 h and Figure 6 for bonding to dentin after 6 months. In general, there was an increase in bond strength of the dentin treated with the antioxidant containing hydrogels compared with the bond strength of the conventionally bonded teeth (P<0.05).

# *In vitro* release of nystatin from nystatin-chitosan gels (Gels-7, 12 and 13)

The *in vitro* release of nystatin from chitosan gels was carried out using USP dissolution apparatus type I.<sup>[18]</sup> It was found that the regression analysis of the obtained



Figure 1: Scanning electron microscope photographs of interior morphology of the selected gels under investigation: (a) Gel-4, (b) Gel-5, (c) Gel-6, (d) Gel-7, (e) Gel-8, (f) Gel-9, (g) Gel-10, (k) Gel-11, (l) Gel-12, (m) Gel-13



**Figure 2:** Water uptake degree of the gels: Gel 1-Gel 7, (n = 6, P < 0.05) % of water uptake

results from two different kinetic models (including zero order and Higuchi's model) showed that Higuchi's model gave the highest value of  $r^2$  with a significant difference (*P*<0.05) between them.<sup>[18]</sup>

Higuchi's model, where the cumulative amount of the

released drug per unit area is proportional to the square root of time, is a more suitable model to describe the release kinetics of nystatin from the gel preparations examined in the present study. The release of nystatin from chitosan gel 5% was studied with nystatin concentrations (1% w/w)in Figure 7. The principal mechanism of such interactions is the formation of hydrogen bonds involving amino group and hydroxyl groups of nystatin. It can be seen [Figure 7] that there was a somewhat lower release of nystatin from the two gels (Gel 12 and Gel 13) containing krill oil in comparison to the gel (Gel 7) without krill oil, but also containing aspirin.

## *In vitro* release of aspirin from functionalized chitosan gels (Gels-4, 5, 6 and Gel 11)

The *in vitro* release of aspirin from chitosan gels was also carried out using USP dissolution apparatus type I model<sup>[18]</sup> was also used (for the above given reasons) and showed that Higuchi's model gave the highest value of  $r^2$  with a significant difference (*P*<0.05).

The release of aspirin from chitosan Gel 5%, 6% and



Figure 3: Scanning electron microscope images of the reactive surface of the composite under experimental conditions after 24 h: (a) Gel-2, (b) Gel-3, (c) Gel-4, (d) Gel-5, (e) Gel-6, (f) Gel-7



Figure 4: Scanning electron microscope images of the reactive surface of the composite under experimental conditions after 6 month: (a) Gel-2, (b) Gel-3, (c) Gel-4, (d) Gel-5, (e) Gel-6, (f) Gel-7

7% of chitosan respectively was studied with aspirin concentrations (1% w/w) and Gels 4, 5, 6 and 11 as shown in Figure 8. The principal mechanism of such interactions is the formation of hydrogen bonds involving amino group and the carboxyl group of aspirin. Furthermore, it becomes apparent that the influence of chemical structures of an antioxidant like krill oil has significantly improved the release of aspirin from the hydrogels *P* values. The mechanism of this interaction is currently under investigations in our laboratories.

In general, the release rate of aspirin was found to be higher in the case of formulations containing higher amounts of chitosan (Gels 4, 5 and 6). However, it can also be seen that the presence of krill oil increased the release rate of aspirin even further. This phenomenon was also previously reported where the nystatin release was higher from the hydrogel than from it alone.<sup>[19,20]</sup> The higher release of Gel 11, containing both krill oil and aspirin points out toward the synergy of the antioxidant and therapeutic activity of functional materials.<sup>[21]</sup> The



**Figure 5:** Shear bond strength of hydrogels after 24 h of bonding to dentin Shear bond strength (MPa)



Figure 6: Shear bond strength of hydrogels after 6 months of bonding to dentin Shear bond strength (MPa)



**Figure 7:** Release of nystatin from Gel-7 (chitosan-aspirin-nystatin), Gel-12 (chitosan-aspirin-nystatin-krill oil) and Gel-13 (chitosan-krill oil-nystatin) in phosphate buffer at pH 6.8

more detail investigations of the interaction involved are currently being explored in our laboratories

## DISCUSSION

# Synergy of chitosan: Antioxidant system and dentin bond strength

An increase in the shear bond strength was also previously reported.<sup>[22]</sup> For chitosan-H, chitosan-propolis, chitosan-nystatin and chitosan-nystatin-propolis. Interestingly the increase in bond strength was also observed in groups of hydrogen peroxide exposed samples suggesting that additional benefits associated with chitosan: Antioxidant system need further investigations.<sup>[22]</sup>

The results of this study suggest that the optimum results for the strengthening of dentin can be achieved throughout the immediate treatment with antioxidant: Chitosan with the increase of dentin bond strength. Furthermore, impressively an almost immediately after the corresponding gel treatment and proceeding with bonding procedures is recommended with the significant increase in bond strength. The results of this study suggest that the optimum results for the increased enamel dentin bond strength can be achieved through out the immediate treatment with gels. The additional advantage of the system may suggest that, antioxidant release from chitosan gel depends upon the physical network structure (open cell like structure) as well as pH properties and flexibilities of the material. Antioxidant release occurs through the pores of the low polymer concentration while chitosan concentration increment resulted in more cross-linking of the network structure; consequently slower antioxidant release from the gel base was achieved and therefor weaker adhesive properties of materials like Gel-1 in case of groups.<sup>[23]</sup>

It was shown by others and us earlier, that the swelling properties and antioxidant release from gels were increased under acidic conditions due to the protonation of the primary amino group on chitosan.[24] Chain relaxation due to protonation of amino groups leads to a faster hydrogen bond dissociation and efficient solvent diffusion. Thus, the appreciable increase in water uptake at lower pH values can be attributed to the high porosity of the gels, which seems to govern the diffusion of the solvent in the gel matrix and thus, the release of the antioxidant from the gel.<sup>[20]</sup> 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-dentin bonds for a prolonged time (up to 6 months). It is well-documented that the hydrostatic pulpal pressure, the dentinal fluid flow and the increased dentinal wetness in vital dentin can affect the intimate interaction of certain enamel and dentin adhesives with dentinal tissue. Therefore, the newly developed chitosan: Antioxidant systems might at least be able to address the shortfalls in the current perspectives for improving bond durability through understanding factors affecting the long-term bonding performance of modern adhesives and addresses the current perspectives for improving bond durability.

Antioxidants and chitosan looking at the antioxidant strength and extended stability, could macro-encapsulation be the play a functional role Chitosan, a linear abundant polysaccharide, is selected



Figure 8: Release of aspirin from gel (5% chitosan 1% aspirin), Gel-5 (6% chitosan-1% aspirin), Gel-6 (7% chitosan-1% aspirin) and Gel-11 (5% chitosan-1% aspirin-1% krill oil) in phosphate buffer at pH 6.8



**Figure 9:** Antioxidant capacity measured at 450 nm using the previously described spectrophotometric assay to assess the hydrogels and corresponding ingredients antioxidant capacity after 24 h under storage under ambient temperature condition. Antioxidant capacity was measured during the first 2 h of exposure

as the wall material of the delivery system.<sup>[25]</sup> Due to its biodegradable, biocompatible, muco-adhesive and non-toxic nature, it has been widely used in numerous drug delivery systems. Compared with other delivery systems, chitosan nanoparticles have a special feature. They can adhere to the mucosal surface and transiently open the tight junction between epithelial cells. Some reports have indicated that chitosan can increase membrane permeability, both in vitro<sup>[26]</sup> and in vivo.[27] Microencapsulation of antioxidants have been an important area of research for several years in order to preserve the beneficial effects of antioxidants.<sup>[28]</sup> In this work, the gelation method was used to prepare the chitosan: aspirin, chitosan: krill oil and chitosan: aspirin: krill Oil microencapsulation. The stability of antioxidant-loaded chitosan complexes has been measured during storage using previously tested established methodology and results suggest there is no significant decomposition observed after 6 months storage at room temperature (24°C) as antioxidant capacity of the materials stored for 6 month have showed no diminished

capacity compare to the freshly prepared hydrogels as indicated in Figures 8 and 9.

# Investigations into stability of antioxidants in the chitosan hydrogels during storage

Stability of various conventional antioxidants in the newly designed drug delivery system during storage is an important factor to determine whether chitosan-coated nano size delivery vehicle can protect various conventional antioxidants. Hence, the stability of the microencapsulated antioxidants was measured by UV absorbance. Stabilities of microencapsulated antioxidants were compared and after 6 months of storage at 24°C, the stability of antioxidant-molecular carrier vehicle was not significantly diminished as indicated in Figures 5 and 6. This observation suggested that the antioxidant had been protected by the molecular carrier. Important to note that performance of the antioxidants such as krill oil, aspirin and a 1:1 mixture of krill oil: aspirin was enhanced by the presence of the chitosan. This is a very interesting point in itself as the synergism in increased stability and lower concentration of the active antioxidant with the same or even higher antioxidant capacity can lead to a development of the broad range to novel functional drug delivery systems and dual action restorative materials.

### CONCLUSIONS

The added benefits of the unique functionality of the hydrogels involve increased dentin adhesive bond strengths (after 24 h and after 6 month) and positive influence on the nystatin and aspirin release. Overall, there was an insignificant relapse in the shear bond strength after 6 months. This approach highlighted the importance of innovative development of functional dental restorative material with bioactive and bonding properties suitable to be used in dentin and enamel as well as show the beneficial preventative and therapeutic properties.

#### **Ethics statement**

For the purpose of this study only extracted discarded teeth were used (P<0.05).

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