

Insights into and relative effect of chitosan-H, chitosan-H-propolis, chitosan-H-propolis-nystatin and chitosan-H-nystatin on dentine bond strength

Victoria Tamara Perchyonok^{1,2}, Shengmiao Zhang³, Sias R. Grobler⁴, Theunis G. Oberholzer²

¹ Department of Research and Innovations, VTPCHEM Pty. Ltd., Glenhuntly, Melbourne, 3163, Australia,
² School of Dentistry and Oral Health, Griffith University, Gold Coast, Southport, 4215, QLD, Australia,
³ School of Material Science and Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai, 200237, China,
⁴ Oral and Dental Research Institute, Faculty of Dentistry, University of the Western Cape, Private Bag X1, Tygerberg 7505, Cape Town, South Africa

Correspondence: Dr. Victoria Tamara Perchyonok
 Email: tamaraperchyonok@gmail.com

ABSTRACT

Objective: The purpose of the study was to design and evaluate novel functional chitosan hydrogels (chitosan-H-propolis, chitosan-H-propolis-nystatin and chitosan-H-nystatin) by using the chitosan-H polymer as “dual function restorative materials.”
Materials and Methods: The nystatin/antioxidant carrier gel was prepared by dispersion of the corresponding component in glycerol and 3% acetic acid with 5% chitosan gelling agent was then added to the dispersion with continuous mixing. The natural bio-adhesive functionalized chitosan hydrogels were combined with built in drug delivery system and bio-actives such as propolis in order to increase the dentin bond strength capacity and maintain therapeutic properties of the alternative drug delivery system. The surface morphology, release behaviors (physiological pH and also in acidic conditions), stability of nystatin:antioxidant:chitosan and the effect of the hydrogels on the shear bond strength of dentin were also evaluated. **Statistical Analysis Used:** Non-parametric ANOVA test was used to assess significance of higher shear bond values than dentine treated or not treated with phosphoric acid. **Results:** The release of both nystatin and propolis confer the added benefit of dual action of a functional therapeutic delivery when comparing the newly designed chitosan-based hydrogel restorative materials to commercially available nystatin alone. Neither the release of nystatin nor the antioxidant stability was affected by storage. Chitosan-H, chitosan-propolis, chitosan-nystatin and chitosan-nystatin-propolis treated dentine gives significantly ($P < 0.05$) higher shear bond values ($P < 0.05$) than dentine treated or not treated with phosphoric acid. **Conclusion:** The added benefits of their unique functionality involve increased dentin adhesive bond strengths (after 24 h and after 6 months) and positive influence on the nystatin release. Nystatin was a model therapeutic agent, evaluating the concept of using functional materials as carriers for pro-drugs as well as displaying a certain degree of defence mechanism for free radical damage of the novel functional drug delivery. Overall, there was an insignificant relapse in the shear bond strength after 6 months.

Key words: Antioxidant-chitosan hydrogels, bond strength, functional restorative materials, scanning electron microscopy

INTRODUCTION

The human mouth is a unique organ that facilitates oral intake and speech production.^[1] It is a site of multiple

pathologies including dental caries, periodontitis, dentin hypersensitivity, aphthous stomatitis (cancer score), oral mucositis and vesiculo-erosive oral mucosal disease.^[2-6]

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In the last decades, there has been an increasing interest in the role that reactive oxygen species (ROS) and antioxidants play in the aging process and in the development of diseases associated with old age. The proposed involvement of oxidative damage in aging has prompted studies to examine the expected beneficial effects of antioxidant supplementation.^[7-10]

Chitosan, produced commercially by deacetylation of chitin, is a natural polysaccharide composed of randomly distributed β -1, 4-linked D-glucosamine and N-acetyl- α -glucosamine. Because of its non-toxic, biocompatible, biodegradable and mucoadhesive properties, chitosan is widely used in the pharmaceutical field.^[11] Chitosan has been used as a carrier system for hormones,^[11] proteins,^[12] enzymes,^[13] and genes.^[14] Because of its apparent pKa = 5.6,^[11] the application of chitosan in oral administration of protein and peptide drugs is restricted by its easy dissolution in a low pH environment.

Propolis has been used in the treatment of inflammatory conditions, with both local and systemic applications.^[15] The antioxidant property of propolis could be attributed to its radical scavenging ability,^[16] which was better than the antioxidant property of vitamin C.^[17] The anti-inflammatory property of propolis is probably due to the presence of caffeic acid phenethyl ester.^[18]

Nystatin is an antifungal compound aimed at the treatment of *Candida* species. It is approved for topical use due to the very low absorption through the gut and skin membranes. It is used as a prophylactic agent in immunosuppressed patients and patients receiving chemotherapy to control topical *Candida* infection.^[19-21]

The purpose of the study was to design and evaluate novel functional chitosan hydrogels (chitosan-H-propolis, chitosan-H-propolis-nystatin and chitosan-H-nystatin) by using the chitosan-H polymer as "dual function restorative materials." The natural bio-adhesive functionalized chitosan hydrogels were combined with built in drug delivery system and bio-actives such as propolis in order to increase the dentin bond strength capacity and maintain therapeutic properties of the alternative drug delivery system. The surface morphology, release behaviors (physiological pH and also in acidic conditions), stability of nystatin: Antioxidant: Chitosan and the effect of the hydrogels on the shear bond strength of dentin were also evaluated.

MATERIALS AND METHODS

Propolis (Aurora Pharmaceuticals Pty Ltd., Underwood, QLD and Australia), Chitosan (Aldrich, Castle Hill, NSW, Australia), glycerol (Aldrich, Castle Hill, NSW, Australia) and glacial acetic acid (E. Merck, Darmstadt, Germany) were used as received. The degree of deacetylation of typical commercial chitosan used in this study is 87%. Chitosan (Aldrich, Castle Hill, NSW, Australia) with a molecular weight 2.5×10^3 KD was used in the study. Gelatin in powder form was purchased from Shanghai Chemical Reagent Co., (China [Mainland], Yangpu District, Shanghai, China) with the number-average molecular weight of about 8.7×10^4 . The isoelectric point (PI) is 4.0-5.0.

Preparation of various nystatin gels

Chitosan hydrogels were prepared using methodology previously described elsewhere.^[22] The corresponding antioxidant was incorporated into the mixture and the summary of the newly prepared materials is highlighted in Table 1.

Determination of gel pH

One gram of the prepared gels was accurately weighed and dispersed in 10 ml of purified water. The pH of the dispersions was measured using pH meter (HANNA instruments, HI8417, Portugal).^[22]

Morphology of the gels (scanning electron microscopy [SEM] study)

The samples were prepared by freezing in liquid nitrogen for 10 min and then were 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 SEM. The interior and the surface morphology were observed in a SEM (SEM, Hitachi S4800 and 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 after the following time periods (0 h, 24 h, 6 months).

Table 1: Gel formulation prepared in the study

Gel formulation	Gel number	Chitosan (w/w%)	Nystatin (w/w%)	Antioxidant	pH
Chitosan-H	Gel-1	5	0	0	5.10
Chitosan-H-P	Gel-2	5	0	1 (propolis)	5.34
Chitosan-H-PN	Gel-3	5	1	1 (propolis)	5.43
Chitosan-H-N	Gel-4	5	1	0	4.78

P: Propolis, N: Nystatin, PN: Propolis plus nystatin

Studies of equilibrium swelling in the alternative drug delivery systems

Known weights of nystatin-containing dry gels were immersed in pH 4.0 and 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 removed, the excess of water on the surfaces absorbed with a filter paper and the gels immediately weighed on a microbalance. The equilibrium-swelling ratio (SR) was calculated using the following equation:

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

Where W_s and W_d are the weights of the gels at equilibrium swelling state and at dry state, respectively.^[23]

Experiments were repeated in triplicate for each gel specimen, and the mean value was obtained.

In vitro study of nystatin release profile

The release study was carried out with United States Pharmacopeia (USP) dissolution apparatus type 1 (Copley UK), 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 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 at 100 rpm.^[24] Samples (2 ml) were collected at 0.25, 1, 2, 3, 4, 5, 6, 7 and 8 h^[25] and were analyzed by a ultraviolet-visible spectrophotometer (Cintra 5, GBC Scientific Equipment, Australia) at a wavelength (λ) of max 306 nm.^[26] Each sample was replaced by the same volume of phosphate buffer at a pH of 6.8 to maintain its constant volume and sink condition.^[27]

Shear bond strength tests for dentine bonding

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 ground away (at a 90° angle by using a jig) to expose the underlying dentin. The teeth were embedded in 10 mm length PVC (Consjit Tubing, SA PVC, JHB, RSA) pipes with cold cure acrylic resin so that the grounded dentin projected well above the acrylic and the dentin then thoroughly washed under tap water. Two composite studs (SDR, Dentsply, New York, USA, Batch No. 1105000609), each with an internal diameter of 2.5 mm and height of 3 mm, were bonded

to the polished dentine surface (up to 600-grit fineness) of each tooth via a bonding agent, XP bond (Dentsply, New York, USA) as suggested by the manufacturer. The bonding agent contained: Carboxylic acid modified dimethacrylate (tetracarboxylic acid resin), phosphoric acid modified acrylate resin, urethane dimethacrylate, triethyleneglycol dimethacrylate, 2-hydroxyethylmethacrylate, butylated benzenediol (stabilizer), ethyl-4-dimethylaminobenzoate, camphorquinone, functionalized amorphous silica and *t*-butanol.

In this way, 48 tooth samples (each containing two studs) were prepared and divided at random into six groups of 8 each (A, B, C, D, E, F). The teeth were stored in a solution of artificial saliva.^[28] These groups were then treated as outlined in Table 2. After 24 h, the shear bond strength of one stud of each tooth was tested for failure (Zwick Universal Testing Machine, Materialprufung, 1446, 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.

RESULTS

Properties of antioxidant-nystatin-chitosan gels

The compositions of the different gel formulations are provided in Table 1. The prepared gel formulations had uniform distribution of drug content, homogenous texture and yellow color. The pH of the formulations ranged between 4.78 and 5.43 [Table 1].

Figure 1 shows a graph of the degree of water uptake by the gels at equilibrium (Gel-1 to Gel-4).

SEM images were obtained [Figure 2] to characterize the microstructure of the interior of Gels 1-4.

Figure 3 shows SEM images of the reactive surface of the composites under experimental conditions after 24 h: (a) Gel-1, (b) Gel-2, (c) Gel-3, (d) Gel-4 and Figure 4 the reactive surface of the composite after 6 months.

Table 2: Groups tested (8 teeth per group)

Samples	Testing conditions
Group A	37% phosphoric acid+primer+bonding immediately (negative control)
Group B	Self-etching primer+bonding immediately (positive control)
Group C	Gel-1+primer+bonding immediately
Group D	Gel-2+primer+bonding immediately
Group E	Gel-3+primer+bonding immediately
Group F	Gel-4+primer+bonding immediately

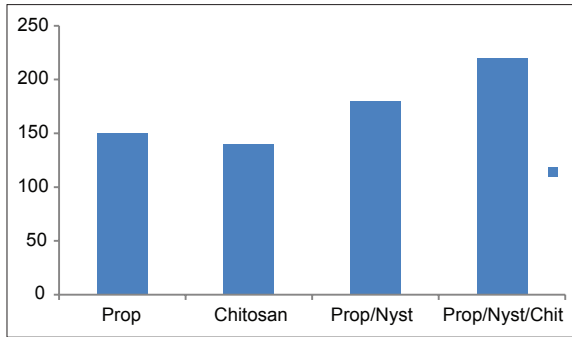


Figure 1: Water uptake degree of the gels: Gel-1 to Gel-4 ($n = 6, P < 0.05$), where y -axis is swelling ratio (%)

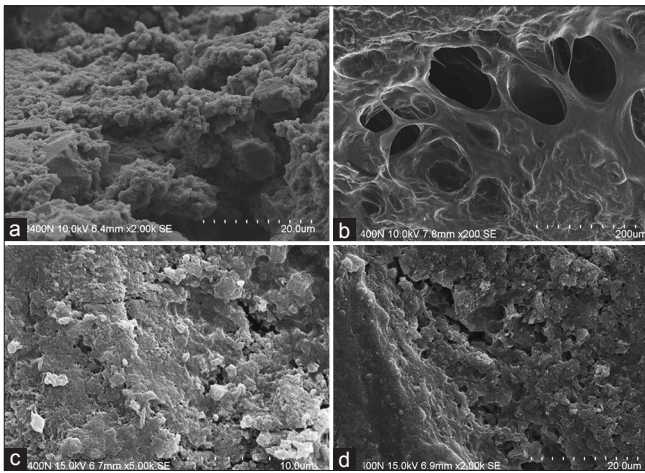


Figure 3: Scanning electron microscopy images of the reactive surface of the composite under experimental conditions after 24 h: (a) Gel-1, (b) Gel-2, (c) Gel-3, (d) Gel-4

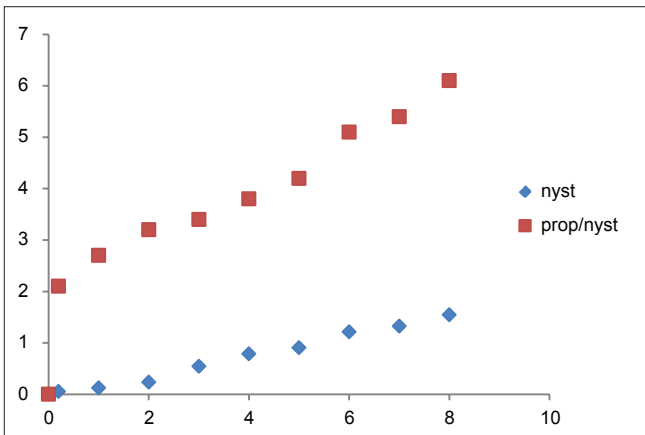


Figure 5: The release of nystatin from chitosan-nystatin (Gel-4) and nystatin from chitosan-propolis-nystatin (Gel-3) from 5% w/w chitosan gels in phosphate buffer pH 6.8

Figure 5 shows the release of nystatin from chitosan-nystatin (Gel 4) and nystatin from chitosan-propolis-nystatin (Gel 3) from 5% w/w chitosan gels in phosphate buffer pH 6.8.

The mean shear bond strength values of the groups are summarized in Figure 6 for bonding

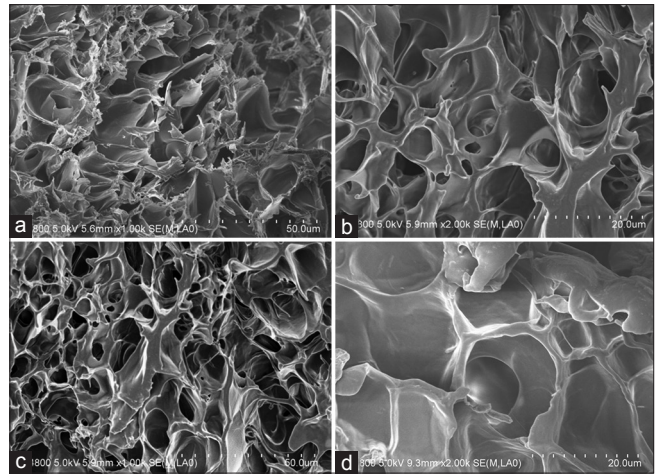


Figure 2: Scanning electron microscopy photographs of interior morphology of the selected gels under investigation for (a) Gel-1, (b) Gel-2, (c) Gel-3, (d) Gel-4

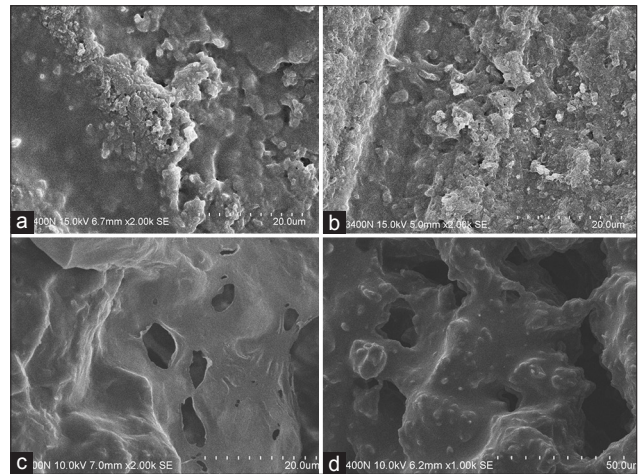


Figure 4: Scanning electron microscopy images of the reactive surface of the composite under experimental conditions after 6 month: (a) Gel-1, (b) Gel-2, (c) Gel-3, (d) Gel-4

to dentin after 24 h and Figure 7 for bonding to dentine after 6 months. It was found that chitosan-H, chitosan-H-propolis, chitosan-H-propolis-nystatin and chitosan-H-nystatin treated dentine gave significantly higher shear bond values ($P < 0.05$; non-parametric ANOVA test) after 24 h than dentine treated or not treated with phosphoric acid. Overall, there was no significant relapse in the shear bond strength after 6 months.

DISCUSSION

Chitosan has the unique ability to prolong adhesion of the therapeutic agent to the oral mucosa as well as inhibit the adhesion of *Candida albicans* cells to the human buccal cells,^[29] preventing the development of mucositis and therefore warranting further developing of the functional materials.

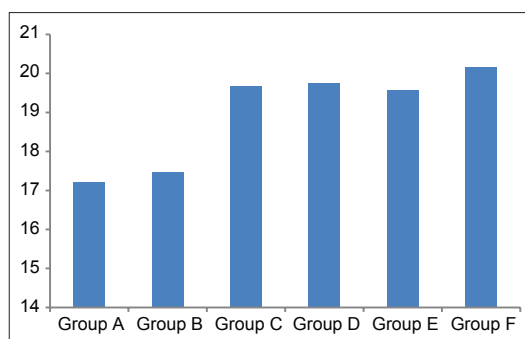


Figure 6: Shear bond strength (MPa) of the six groups after 24 h of bonding to dentine

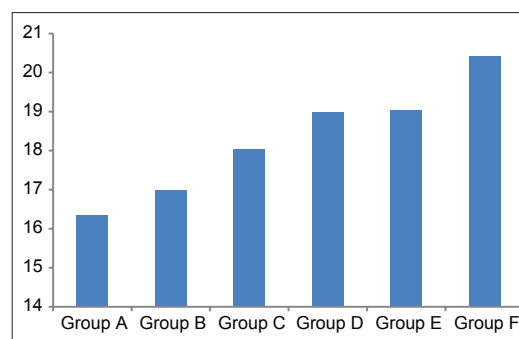


Figure 7: Shear bond strength (MPa) of the six groups after 6 months of bonding to dentine

Propolis was found to be very effective against Gram-positive bacteria,^[30] especially against *Staphylococcus aureus*^[31] and Gram-negative bacteria, *Salmonella*.^[32] The rapid decrease in nystatin antifungal activity observed with acid preparation was confirmed in published data.^[33] The ranges of pH values recommended to optimize nystatin stability and antifungal efficacy are 5.0-7.0 and 6.0-8.0 respectively.^[33] Moreover, the presence of acid must be prescribed because it facilitates candidiasis growth.^[33] However, in our study, the pH ranges did not seem to alter nystatin stability nor maintain its antifungal activity. This observation suggesting the crucial role of chitosan as an additional antifungal agent and highlighting its additional functional features (including: Positive charge density; molecular weight; concentration; hydrophilic/hydrophobic characteristic and chelating capacity; physical state, namely water-soluble and solid state of chitosan; environmental factors, involving ionic strength in medium, pH, temperature and reactive time).^[34] Table 1 represents the summary of the nystatin-antioxidant-chitosan gels prepared in this study.

The gels used displayed homogeneously porous 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 [Figure 1]. The “skin” of the gels can be seen and the collapse of the surface pores may be due to the freeze-drying process.

The hydrogels remained in cylindrical form after swelling. Compared with dry state hydrogels, the swollen state hydrogel volume displayed significant increases [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 PI of gel (PI: 4.0-5.0), the carboxyl groups were de-protonized to carry negative charges, which made molecular chains repulse 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 H₂O₂.^[35]

The generation of HO from the biologic Fenton reaction has been shown to be a critical factor in various ROS-induced oxidative stresses.^[36,37] H₂O₂ and HO might be related to apoptosis in atherosclerosis.^[38] Godley *et al.* also reported that blue light induces mitochondrial deoxyribonucleic acid damage and cellular aging.^[39]

The reactive nature of the surface was investigated using the SEM and comparison confirms the reactive nature of the transformation [Figures 4 and 5].

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

In vitro release of nystatin from chitosan gels

The *in vitro* release of nystatin from chitosan gels was carried out using USP dissolution apparatus type 1. The regression analysis of the obtained results for two

kinetic models including zero order and Higushi's model showed that Higushi's model gave the highest value of r^2 with a significant difference ($P < 0.05$).

Higushi's model, where the cumulative amount of the released drug/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) and gels containing corresponding antioxidant as shown in Figure 5. The principal mechanism of such interactions is the formation of hydrogen bonds involving amino group and hydroxyl groups of nystatin. The nystatin release was significantly higher than the nystatin alone, suggesting that the positive synergy observed, combined the antifungal and antioxidant capacity of synthetic and natural biomaterials into a functional biocompatible gel as well as providing the additional benefit of nystatin as supported by Figure 5.

There was a statistically significant increase in the shear bond strength of dentine treated with the four gels compared with the bond strength of the conventionally bonded dentine after 24 h [Figure 6]. However, the gel containing only chitosan [Group 6, Figure 7] gave a significant relapse in the shear bond strength after 6 months in comparison with the other three gels containing a combination of chitosan/nystatin/propolis [Figure 7]. Interestingly, the increase in bond strength was also observed in groups of hydrogen peroxide exposed samples (Groups C-F compared to Group B as a standard) suggesting that there are additional benefits associated with the chitosan: Antioxidant system that are in need of further investigations.

The results of this study suggest that the optimum results for the strengthening of dentine bonding can be achieved through the immediate treatment of dentin with chitosan-H, chitosan-H-propolis, chitosan-H-propolis-nystatin and chitosan-H-nystatin with an increase in the dentine bond strength. The additional advantage of the system may suggest that antioxidant release from chitosan gel depends on 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 therefore weaker adhesive properties of materials such as gel-1 in the case of groups.^[39]

Earlier findings by us and others showed 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.^[40] 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.^[40] The additional benefit of using chitosan: Antioxidant system as a bonding/pre-bonding to the 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 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 address the current perspectives for improving bond durability.

CONCLUSION

We have developed and evaluated several dual-functional chitosan hydrogels with several targets as therapeutic restorative materials. The added benefits of their unique functionality involve increased dentin adhesive bond strengths (after 24 h and after 6 months) and positive influence on the nystatin release. Nystatin was a model therapeutic agent, evaluating the concept of using functional materials as carriers for pro-drugs as well as displaying a certain degree of defence mechanism for free radical damage of the novel functional drug delivery systems.

Ethics statement

For the purpose of this study only extracted teeth were used. The teeth were discarded specimens collected from the Department of Maxillofacial and Oral Surgery, Faculty of Dentistry, UWC.

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