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Insights into chitosan based gels as functional restorative biomaterials prototypes: *In vitro* approach

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ABSTRACT

Restorative materials in the new era aim to be "bio-active" and long-lasting. The purpose of the study was to design and evaluate novel chitosan hydrogels containing melatonin and/or propolis (antioxidant containing material), nystatin (antifungal), naproxen (pain relieve medication) and combinations thereof (chitosan-H-melatonin, chitosan-H-melatonin-naproxen, chitosan-H-propolis, chitosan-H-propolis-naproxen, chitosan-H-naproxen-propolis-melatonin, Chitosan/Propolis/Nystatin, Chitosan/Melatonin/Propolis/Nystatin, Chitosan/Propolis/BSA/Nystatin, Chitosan/Melatonin/BSA/Nystatin) as functional additive prototypes for further development of "dual function restorative materials", to determine their effect on the dentin bond strength of a composite, to evaluate stability of the encapsulated antioxidants as well as evaluate antimicrobial properties of the selected group of "designer" functional materials. Materials and Methods: The above mentioned hydrogels were prepared by dispersion of the corresponding component in glycerol and acetic acid with the addition of chitosan gelling agent. The surface morphology (SEM), drug-polymer solid state interaction (FT-IR spectroscopy), released behaviours (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 naproxen confers 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 naproxen or 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 colour (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 a positive influence on the naproxen release as well as increased dentin bond strength as well as demonstrating good antimicrobial properties and enhanced antioxidant stability. The therapeutic polymer approach described here has a potential to provide clinical benefit, through the use of "designer" adhesive restorative materials with the desired properties.

Keywords: Therapeutic Polymers; Adhesives; Chitosan, Hydrogels; Propolis; Melatonin; Naproxen; Antimicrobial; Dentin Bonding; Antioxidants; Bioactive

1. INTRODUCTION

The field of "functional designer dental materials for restorative treatment" is currently a hot topic in dentistry. The properties such as mechanical, physical and bonding properties have been greatly improved and modified and now represent a significant arsenal available to a front row researchers as well as dental practitioners as many of recent products on the market exhibit excellent/acceptable clinical performance [1,2]. Accordingly, it is proposed that innovation of restorative materials in the new era could be directed toward a new dimension: development of materials with "bio-active functions" to provide

therapeutic effects. The one bio-active function proposed for restorative materials, antibacterial activity can be highlighted for the restorative treatment of caries [3].

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 [4]. Whilst bonding to enamel has changed little since its inception more 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 [5,6]. 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 intra-dental administration of therapeutic and prophylactic agents, if necessary [7]. 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 [7,8].

Null Hypothesis

The purpose of the study was firstly to design and evaluate (surface morphology, stability, swelling, release behaviour) novel chitosan hydrogels containing propolis (anti-oxidant containing material and natural antimicrobial agent), melatonin (antioxidant), naproxen (pain relieve medication and free radical scavengers) and combinations thereof (chitosan-H-propolis, chitosan-H-propolis-naproxen, chitosan-H-naproxen, chitosan-H-naproxen-nystatin, chitosan-H-melatonin-naproxen and chitosan-H-melatonin) as functional additive prototypes for further development of "dual function restorative materials" such as the designer material which is able to improve the adhesion to the tooth structure as well as provide additional therapeutic property. The ability to control bacteria would be advantageous to eliminate the risk of further demineralization and cavitation, since dental caries is an infectious disease and eradication of cariogenic bacteria is the important principle. Secondly it is to determine their effect on the dentin bond strength of a composite and thirdly to evaluate some antimicrobial properties of this functional biomaterials.

2. MATERIALS AND METHODS

Propolis (Aurora Pharmaceuticals, Australia), melatonin (Now Food, Australia) nystatin, bovine serum albumin, naproxen (Sigma, USA) were used as received. Chitosan (Aldrich, Australia), glycerol (Sigma, USA), glacial acetic acid (E. Merck, Germany) were used as received. The degree of deacetylation of typical commercial chitosan used in this study is 87%. Chitosan with molecular weight 2.5×10^3 KD was used in the study. The isoelectric point is 4.0 - 5.0.

2.1. Preparation of Various Naproxen Containing Hydrogels

The naproxen containing gel was prepared by dispersion of naproxen powder 0.3 gm in glycerol (5% w/w) using a mortar and a pestle. Ten milliliters 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 gm) is 0.3 g of naproxen in each gm of the base. Naproxen 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**.

Where P is propolis as additive, Nap is naproxen as selective additive, M is melatonin as selective additive. PB = propolis: BSA (1:1), MB = melatonin: BSA (1:1) Hydrogels containing chitosan in different % are synthesized and characterized.

2.1.1. 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).

2.1.2. Morphology of the Gels

The samples were prepared by freezing in liquid nitrogen for 10 min, and then were freezing-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 scanning electron microscope (SEM, Hitachi S4800, Japan).

2.1.3. FTIR of the Gels

The samples were freeze-dried for 72 h, then the FTIR spectral of the sample was recorded on an Infrared Spectrophotometer (Nicolet 5700). The IR-spectra of the pellets were recorded from 400 - 4000 cm^{-1} taking air as a reference.

2.1.4. 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

Table 1. Gel formulation prepared in the study.

Gel formulation		Chitosan (w/w%)	Naproxen (w/w%) or Nystatin (w/w%)	Melatonin (w/w%)	Propolis (w/w%)	pH
Chitosan-H	Gel-1	5	0	0	0	5.20
Chitosan-H1	Gel-2	6	0	0	0	5.70
Chitosan-H2	Gel-3	7	0	0	0	5.17
Chitosan-H-Nap1	Gel-4	5	1	0	0	5.25
Chitosan-H-Nap2	Gel-5	6	1	0	0	5.65
Chitosan-H-Nap3	Gel-6	7	1	0	0	5.84
Chitosan-H-A1P1	Gel-7	5	1	0	1	5.24
Chitosan-H-M1N1	Gel-8	5	1	1	0	5.13
Chitosan-H-M2N2	Gel-9	5	1	1	0	5.20
Chitosan-H-M1A1N1	Gel-10	5	1	1	1	5.00
Chitosan-H-P	Gel-11	5	1 (Nystatin)	0	1	5.34
Chitosan-H-M	Gel-12	5	1 Nystatin	1	0	5.62
Chitosan-H-PBN	Gel-13	5	1 (Nystatin)	1 propolis:BSA	0	5.78
Chitosan-H-MBN	Gel-14	5	1 (Nystatin)	0	1: 1 melatonin:BSA	6.21

examined on days (0, 15, and 30).

2.1.5. Studies of Equilibrium Swelling in the Alternative Drug Delivery Systems

The known weight naproxen-containing dry gels and naproxen-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:

$$\mathrm{SR} = \left(W_s - W_d\right)/W_d \times 100\%$$

where W_s and W_d are the weights of the gels at the equilibrium swelling state and at the dry state, respectively [9]. Experiments were repeated 6 times for each gel specimen and mean value was obtained.

2.1.6. *In Vitro* Study of Naproxen 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 gm of naproxen 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˚C and 100 rpm [10]. Samples (2 ml) were collected at 0.25, 1, 2, 3, 4, 5, 6, 7 and 8 hours [11] and were analyzed spectrophotometrically by U.V. Spectrophotometer (Cintra 5, GBC Scientific equipment, Australia) at The UV-vis absorption spectrum of naproxen in water is typical of a 2-substituted naphthalene compound, presenting a three band system centred around 220 nm, 240 - 280 nm and 310 - 330 nm [12]. Each sample was replaced by the same volume of phosphate buffer pH 6.8 to maintain its constant volume and sink condition [13].

2.1.7. Cupric Ions (Cu^{2+}) 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 $CuSO_4$), melatonin, chitosan:melatonin (1:1), propolis and propolis:chitosan (1:1) the cupric ions (Cu^{2+}) reducing power capacity was used with slight modification [14]. Briefly, 250 µL of 37.5% hydrogen peroxide solution and $CuSO_4$ and 250 µL CH_3COONH_4 buffer solution (100 mmol/L, pH 7.0) were added to a test vial containing a negative control (35% hydrogen peroxide solution and $CuSO_4$), melatonin and propolis sample as well as chitosan complexes of the melatonin and propolis (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 minutes intervals for the total time of 2 hours. Increased

absorbance of Cu^{+} complex in the reaction mixture indicates increased reduction capability. Trolox (water soluble vitamin E) was used as the positive controls. The results of the investigation are summarised in the 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 melatonin and melatonin: chitosan, propolis and propolis: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.

2.1.8. *In Vitro* Antifungal Activity of the Newly Prepared Hydrogels

A type strain of *Candida albicans* strain NCPF 3153 was obtained from the Health Protection Agency Culture Collections, Salisbury, UK. The yeast was sub-cultured and maintained on Sabouraud dextrose agar. The effectiveness of the prepared nystatin gel against *Candida albicans* was measured using the standard Kirby-Bauer agar diffusion method. Five to six mm deep Muller-Hinton agar plates were inoculated by streaking a standardized inoculum suspension containing 10^{7} - 10^{8} colony forming units with a standard throat cotton swab. Hydrogels were dissolved 50 mg/100 µl in sterile distilled water and 10 µl were used to impregnated 6 mm diameter paper disks. The paper discs were placed on the Muller-Hinton agar medium and incubated at 37˚C for 24 hours. The effectiveness of the prepared gel was compared with chitosan gel containing 0% of nystatin and an antibiotic sensitivity disc (Mast Laboratories, Merseyside UL) containing 100 I.U. of nystatin per disc. The diameter of the zones of growth inhibition was measured with a caliper. Each type of the samples was tested in triplicate.

2.1.9. 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 two 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 zig (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 via 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 (PENTA), urethane dimetacrylate (UDMA), triethyleneglycol dimethacrylate (TEGDMA), 2-hydroxyethylmethacrylate (HEMA), butylated benzenediol (stabilizer), ethyl-4-dimethylaminobenzoate), camphorquinone, functionalized amorphous silica, t-butanol.

In this way were 80 teeth samples (each containing 2 studs) prepared and divided into 10 groups of 8 each, A-T (**Table 2**) and stored in a solution of artificial saliva. These groups were then treated as outlined in **Table 2**. After 24 hours one stud of each tooth was tested for shear bond strength and the other one after 3 months. An Instron Universal Testing Machine (Griffith University, G12) at a crosshead speed of 0.5 mm/minute was used to test the de-bonding strength. All data tests were analysed using the non-parametric ANOVA test.

Table 2. Groups tested (8 teeth per groups).

Group A	37% of phosphoric acid + primer + Bonding immediately (negative control)
Group B	Self-etching primer + Bonding immediately (positive control)
Group C	Gel1 + primer + Bonding immediately
Group D	Gel2 + primer + Bonding immediately
Group E	Gel3 + primer + Bonding immediately
Group F	Gel4 + primer + Bonding immediately
Group K	Gel5 + primer + Bonding immediately
Group L	Gel6 + primer + Bonding immediately
Group M	Gel7 + primer + Bonding immediately
Group N	Gel8 + primer + Bonding immediately
Group O	Gel9 + primer + Bonding immediately
Group P	Gel10 + primer + Bonding immediately

3. RESULTS

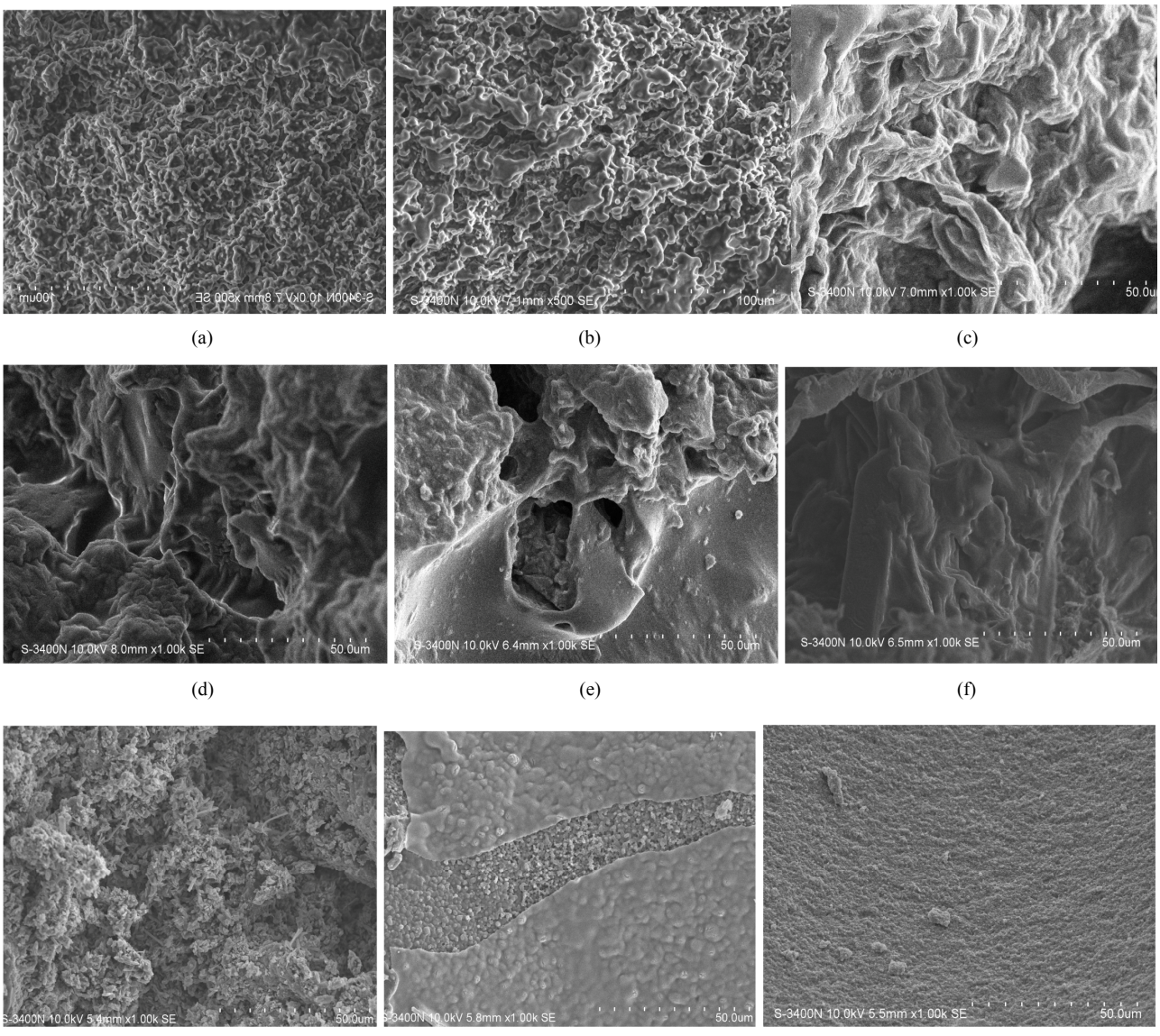
3.1. The Characterization of Naproxen-Chitosan Gels (Gel-4-Gel-10)

The SEM images were obtained to characterize the microstructure of the freeze-dried naproxen composite gels and are presented in **Figure 1**. It could be seen that the gels displayed a homogeneously 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 (**Figure 1**). The "skin" of the gels can be seen, and the collapse of the surface pores may be due to freeze-drying process.

3.2. Studies of Equilibrium Swelling in Naproxen-Chitosan Gels (Gel 1-10)

The hydrogels remain in the cylindrical form after swelling. Compared with dry state hydrogels, the swollen state hydrogel volume displays significant increases and are summarized in **Figure 2**.

Equilibrium swelling ratio (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 polyelectro-



(a) (b) (c)

(d) (e) (f)

(g) (h) (i)

Figure 1. SEM photographs of interior morphology of the selected gels under investigation for (a) Gel-4, (b) Gel-5, (c) Gel-6, (d) Gel-7, (e) Gel-8, (f) Gel-9, (g) Gel-10,(h) Gel-12, (i) Gel-13.

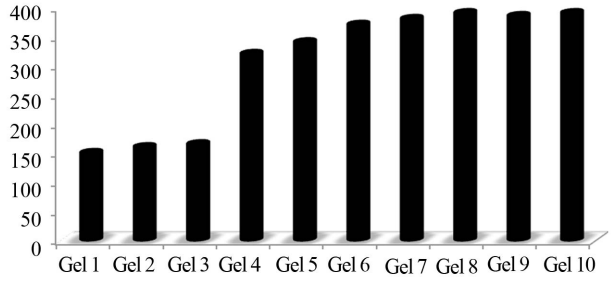


Figure 2. Water uptake degree of the gels Gel-1.

lyte 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. According to above results, we believed that the naproxen results release mechanism could result from the superposition of various effects, such as swelling property of hydrogels, the solubility of the drug and erosion property of matrix; it is not necessarily based on a single factor.

3.3. FTIR Investigations of the Gels 4-10

Drug-polymer solid state interaction was further investigated through FT-IR spectroscopy. Some representative FT-IR spectra of NAP-CS, NAP-CS and NAP-CS-Antioxidant combinations in the C=O stretching region of NAP (1800 - 1600 cm^{-1}) are shown in **Figure 3**. Spectra of the physical mixtures were the weighted average of those of the single components. No appreciable modifications in the characteristic quartet of NAP frequency bands was observed by comparing the spectra of the physical mixtures with CS with those of corresponding co-ground systems. On the contrary a reduction of intensity together with a shift towards lower frequencies for the NAP carbonyl band was observed in co-ground systems with Antioxidant, attributable to a variation in the hydrogen bond pattern due to a NAP-Antioxidant interaction [15].

3.4. Shear Bond Strengths

Figure 4 gives the shear bond strength values (MPa) after 24 hours.

Mean shear bond strength values and difference between the groups are summarized in **Figure 4** for bonding to dentin after 24 hours. In general there was an increase in bond strength of the dentin treated with the antioxidant containing hydrogels compared to the bond strength of the conventionally bonded teeth. An increase in the shear bond strength was also previously reported

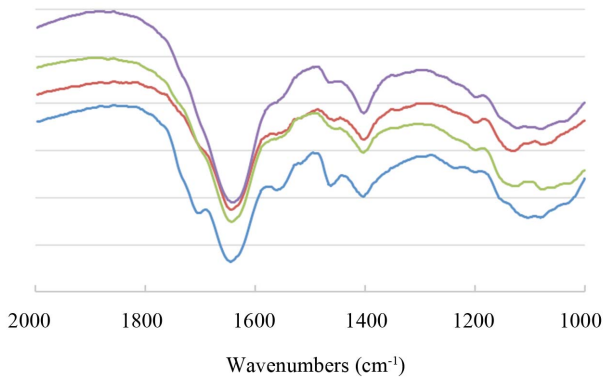


Figure 3. FT-IR spectra from the top down are: Chitosan: naproxen, Chitosan:naproxen:propolis, Chitosan:melatonin:naproxen and Chitosan:naproxen:melatonin:propolis, respectively.

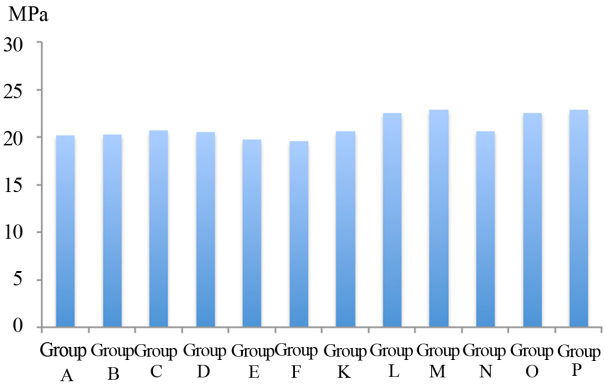


Figure 4. Shear bond strength of hydrogels after 24 hours of bonding to dentin.

[16] for chitosan-H, chitosan-propolis, chitosan-naproxen and chitosan-naproxen-propolis and chitosan:naproxen:melatonin. Interestingly the increase in bond strength was also observed in the groups of hydrogen peroxide exposed samples suggesting that there additional benefits associated with chitosan:antioxidant system are in need of further investigations [16].

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. Also, 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 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 therefor weaker adhesive properties of the materials such as Gel-1 in case of groups [17].

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 [18]. 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 [19]. The additional benefit of using chitosan:antioxidant system as a bonding/pre-bonding to enamel and dentin system lies in its ability to show favourable immediate results in terms of bonding effectiveness as well as the durability of resin-dentin bonds for a prolonged time (up to 6 months) [ref]. 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. Therefor 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.

3.5. *In Vitro* Release of Naproxen from Naproxen-Chitosan Gels (Gel-4, Gel7-Gel-10)

The *in vitro* release of naproxen from chitosan gels was carried out using USP dissolution apparatus type I as previously described [20]. The release of naproxen from chitosan gel 5% was studied with naproxen concentrations (1% w/w) and gels containing corresponding antioxidants, as shown in **Figure 5**. The principal mechanism of such interactions is the formation of hydrogen bonds involving amino group and carboxyl group of naproxen [21,22]. Also it becomes apparent that the influence of chemical structures of antioxidants such as propolis and melatonin has significantly improved the release of naproxen from the hydrogels. The mechanism of this interaction is currently under investigations in our laboratories.

3.6. Stability of Antioxidants in the Chitosan Hydrogels during Storage

Stability of various conventional antioxidants in the

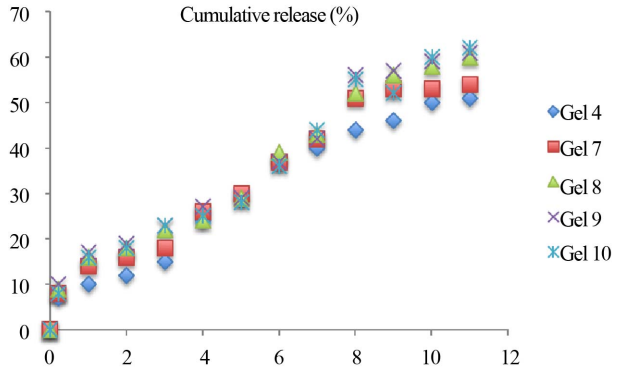


Figure 5. The effect additive such as antioxidants (propolis and/or melatonin) on naproxen release from naproxen:chitosan gels (Gel-4, Gel-7, Gel-8, Gel-9, Gel-110) in phosphate buffer pH 6.8.

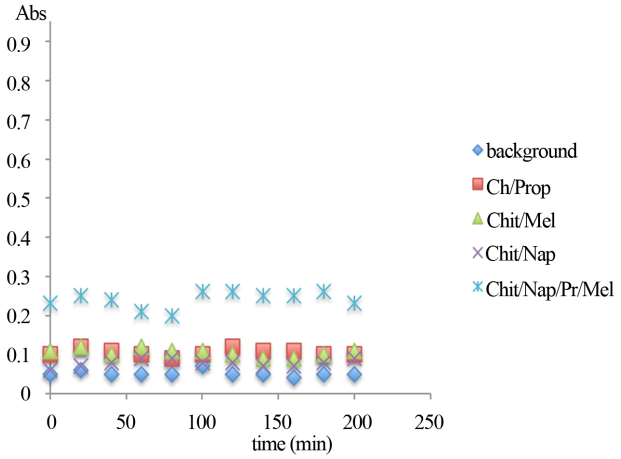


Figure 6. Antioxidant capacity measured at 450 nm using the previously described spectrophotometric assay to asses the hydrogels and corresponding ingredients antioxidant capacity after 24 hours under storage under ambient temperature condition. Antioxidant capacity was measured during the first 2 hours of exposure.

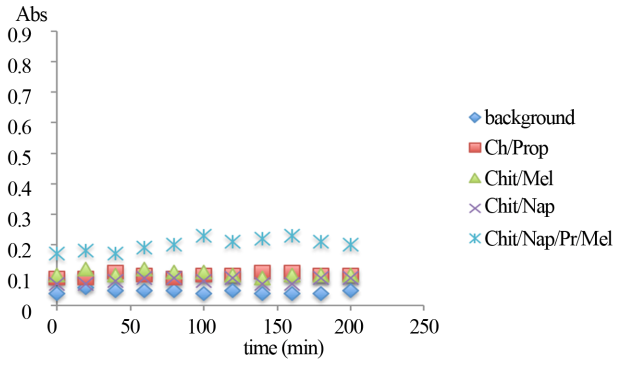


Figure 7. Antioxidant capacity measured at 450 nm using the previously described spectrophotometric assay to asses the hydrogels and corresponding ingredients antioxidant capacity after 6-month under storage under ambient temperature condition. Antioxidant capacity was measured during the first 2 hours of exposure.

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. So the stability of the microencapsulated antioxidants has been measured by UV absorbance. Stabilities of microencapsulated antioxidants have been compared after 24 hours (**Figure 6**) and after 6-month (**Figure 7**) of storage at 24˚C, the stability of antioxidant-molecular carrier vehicle was over 95%. This indicates that antioxidants have been protected by the molecular carrier via positive host:guest supra-molecular interaction. Important to note that performance of the antioxidant was enhanced by the presence of the chitosan, which 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 broad range to novel functional restorative materials.

3.7. 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. So the stability of the microencapsulated antioxidants has been measured by UV absorbance. Stabilities of microencapsulated antioxidants have been 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 suggests that the antioxidant had been protected by the molecular carrier. Important to note that performance of the antioxidants such as propolis, melatonin, chitosan:propolis, chitosan:melatonin, chitosan: propolis:melatonin:naproxen was enhanced by the presence of the chitosan, which 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 broad range to novel functional drug delivery systems and dual action restorative materials.

3.8. *In Vitro* Microbiological Study

Candida albicans was not susceptible to the blank chitosan gel containing no nystatin (no zone of inhibition was observed). The selected preparations containing Chitosan/Propolis/Nystatin, Chitosan/Melatonin/Propolis/Nystatin, Chitosan/Propolis/BSA/Nystatin, Chitosan/Melatonin/BSA/Nystatin and the Nystatin antibiotic sensitiveity disc containing 100 IU of Nystatin all give clear inhibitions zones of different diameters. The *Candida albicans* growth inhibition zones were 26.5 ± 0.5 mm (average ± STD) for the nystatin antibiotic sensitivity disc, 9.5 ± 0.8 mm for the Chitosan/Propolis/Nystatin, 8.6 ± 0.7 mm for the Chitosan/Melatonin/Propolis/Nystatin, 14.5 ± 1.3 mm for the Chitosan/Propolis/BSA/Nystatin, 10.6 ± 0.5 mm for the Chitosan/Melatonin/BSA/Nystatin and 10.0 ± 0.0 mm for the Chitosan/Nystatin formulations respectively. All the test samples give a smaller inhibittion zone than the nystatin antibiotic control disc. This indicates that release of the nystatin from the formulations were inhibited to some extent, which is in agreement with the results obtained from the nystatin release experiments where it was observed that after 8 hours only about 50% of the nystatin was released from the formulations [23]. This slow release of the nystatin from the gel will be a beneficiary effect that will enable a sustainable release over time [24].

4. CONCLUSION

We have developed and evaluated novel functionalized biomaterials, which have high loading efficiency, thus they can be used for carriers for proteinaceous drugs as well as display certain degree of defence mechanism for a free radical damage and increased antimicrobial properties of the novel functional drug delivery systems. The added benefits of the unique functionality of the hydrogels involve increased dentin adhesive bond strengths (after 24 h), positive influence on the naproxen release, additional benefits of the antioxidant stability of functional "designer" restorative material. This approach highlights the importance of innovative development of functional dental restorative material to have bioactive and bonding properties suitable to be used in dentin and enamel as well as show the beneficial preventative and therapeutic properties.

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