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Towards Bio-active Restorative Materials with Copaiba Oil and Oblepicha Oil: *In vitro*

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Abstract

We developed and evaluated chitosan - fucoidan bio-composites with additional bioactive components of oblepicha oil and copaiba oil for bioactive restorative material as intra-dental and wound healing applications such as bioadhesion to soft and hard tissue in vitro, dentin bond strength and free radical defense mechanism for the compounds in the oral environment.

Keywords: Free radicals; Dental restorative materials; Fucoidan; Chitosan; Bioactives

Introduction

The 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. In designing such a bio-material the following properties are required to be demonstrated by the material: response to the cellular environment around the material, to improve and promote device integration, tissue regeneration, changes in pH or cell associated enzymatic activity for example [1,2].

Functionalized biomaterials are ideal candidates to address problems associated with adhesion to biological tissues (soft and hard), due to the unique ability to build in suitable physical properties (elasticity, tensile and adhesive strength), biocompatibility and biodegradability in contact with physiological fluids [3,4].

The process of wound healing involves a series of cellular and cytokine-mediated events results in the generation of Reactive Oxygen Species (ROS), which have been found to play a deleterious role [5,6]. The surface application of substances with free radical scavenging properties has shown to significantly improve wound healing and protect tissues from oxidative damage [7,8].

Copaiba oleoresin is widely used as a popular medicine, through topical and oral administration [8,9]. It has various ethnopharmacological indications, including but not limited to wounds, asthma, as an antiseptic for wounds, skin ulcers, aching joints, general inflammations, as a tonic and to treat ulcers and other digestive diseases, and cancer [10,11].

Oblepicha oil has been used in traditional Chinese medicine since the Tang Dynasty, going back more than 1000 years [12,13]. This plant has been used extensively in the oriental traditional system of medicine for treatment of asthma, skin diseases, gastric ulcers and lung disorder [14,15]. A wide spectrum of pharmacological effects of oblepicha have been recently reported, including antioxidant, immunomodulatory, anti-atherogenic, anti-stress, hepatoprotective, radioprotective and tissue repair [16,17].

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 [18-22]. Chitosan is a cationic polysaccharide and its adhesive properties are mainly based on ionic interactions with tissues or mucus layers. Low-molecular-weight chitosan is particularly known to facilitate closer interaction with the surface of the epithelial cells. Despite the advantages, the rigid crystalline structure of chitosan makes it hard to dissolve in water, and this has partially retarded its potential for such application. Modification of the chitosan with PEG can enhance the water solubility of chitosan and permit the formation of chitosan-based hydrogels by crosslinking of the PEG [23].

Fucoidan is a sulfated polysaccharide that contains L-fucose and sulfate. It is commonly found in marine brown seaweeds [24]. 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 [25].

As part of our continuous interest in developing functionalized biomaterials we report the synthesis, characterization and

application of the newly developed chitosan-fucoidan bio-composites with additional bioactive components of oblepicha oil and copaiba oil as intra-dental and wound healing applications such as bio-adhesion, dentin bond strength and free radical defense mechanism for the compounds in the oral environment.

Materials and Methods

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%. Fucoidan (Doctor's Best Science Based Nutrition, 70%, USA) is used. The sulfate degree of fucoidan is 70%. Chitosan with molecular weight 2.5x 10^3 KD was used in the study. The isoelectric point is 4.0–5.0. Oblepicha Oil (Diveevo, Russia), Oleo De Copaiba (Sao Lucas, Carvalho Leite, Medicamentos Ltda, Farmaceuticos Industrials) were used as received.

Preparation of various hydrogels: general protocol

The therapeutic agent such as oblepicha oil or copaiba oil containing gel was prepared by dispersion of 0.2 gm in glycerol (5% w/w) (1 ml) using a mortar and a pestle following the earlier reported generic protocol [6]. Ten milliliters of glacial acetic acid (3% w/w) was then added with continuous mixing and finally chitosan or fucoidan (10% chitosan w/w) polymer was mixed well to form the required gel. The strength of the prepared gel (10 gm) was 0.2 gm of oblepicha oil or copaiba oil in each gram of the base. The summary of the newly prepared materials is presented in (Table 1).

Bio-adhesive study

Bio-adhesion studies were done using Chatillon apparatus for force measurement [22]. This method determines the maximum force and work needed to separate two surfaces in intimate contact [22]. The hydrogels (0.1gm)were homogeneously spread on a 1cm^2 glass disks 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 dentin 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.

Table 1: Gel formulations prepared in the study.

Gel formulation		Bio-scaffold Chitosan or Fucoidan (% w/w)	Oblepiha oil	Copaiba oil	pH
Chitosan	Gel-1	5	0	0	6.14
chitosan/ oblepicha oil	Gel-2	5	1	0	6.35
Fucoidan	Gel-3	5	0	0	6.01
fucoidan/ oblepicha oil	Gel-4	5	1	0	6.15
chitosan/copaiba oil	Gel-5	5	0	1	6.23
fucoidan/ copaiba oil	Gel-6	5	0	1	6.43

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 $CuSO_4$), Oblepicha oil, Copaiba oil the cupric ions (Cu^{2+}) reducing power capacity was used with slight modification [19]. 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 $CuSO_4$), Oblepicha oil, Copaiba oil sample as well as chitosan and fucoidan complexes of the Oblepicha oil, Copaiba 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 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. 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 Costar 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 Oblepicha oil, Copaiba oil and combination with chitosan and fucoidan 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.

Protein cross-linking as a model for detection of free radical activity and activation of "molecular defense forces": Bovine Serum Albumin (BSA), a completely water-soluble protein, was polymerized by hydroxyl radicals generated by the Fenton reaction system of Fe^{2+}/EDTA/ H_2O_2/ascorbate [20]. As a result, the protein loses its water-solubility and the polymerized product precipitates. The decrease in the concentration of the water-soluble protein can easily be detected.

The in vitro incubation mixtures contained reagents, added in the sequence as follows, at the final concentrations: bovine serum albumin (0.8 mg/ml), phosphate buffer, pH 7.4 (10 mM), water to reach 2.5 ml total volume, antioxidant (such as Oblepicha oil, Copaiba oil and corresponding chitosan and fucoidan complexes) tested to reach required concentration as shown in results, EDTA (0-4.8mM), $Fe(NH_4)_2(SO_4)_2$ (0-4mM), ascorbate (4 mM) and H2O2 (0.2%). To chelate iron completely, 1.2 molar excess of EDTA was always used [21]. The reaction mixture was incubated for 20 min at ambient temperature. The supernatant was precipitated with an equal volume of Trichloroacetic Acid (10%) at 0 degrees Celsius. The precipitate thus obtained was re-dissolved in 1 ml of Na_2CO_3 (10%) in NaOH (0.5 M) and the final volume made

up to 2.5 ml by water. An aliquot of the solution was used for protein determination [22]. The yield of OH radicals generated in the incubations was determined on the basis of degradation of deoxyribose [23]. Dityrosine formation was monitored by measuring fluorescence at 325 nm (excitation) and 415 nm (emission) according to the spectrophotometer [24].

Modulus of elasticity (MOE)

Modulus of elasticity was measured using 3-point bend test (50 gm load cell, cross-speed 0.5 ml/min) following the methodology previous reported [6] and detailed protocol described in Appendix 1. Load/displacement curves were converted to stress-strain curves and the apparent MOE was calculated at 3% strain. Displacement (D) during compression was displayed in millimeters and calculated at a maximum strain of 3% using the formula D = eL2/ 6T, where e, strain; L, support span length and T, the thickness of the specimen. The MOE of the specimens was expressed in MP (megapascals) and calculated using the following formula E = PL3/4DbT, where P, the maximum load; L, the support span length; D, the displacement; b, width of the specimen, and T, the thickness of the specimen.

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 a pH meter (HANNA instruments, HI 8417,Portugal).

Morphology of the gels: 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 scanning electron microscope (SEM, Hitachi S-4800, 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).

Studies of equilibrium swelling of the prepared hydrogels

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:

$$SR = (W_{s} - W_{d}) / W_{d} * 100\%$$

where, W_s and W_d are the weights of the gels at the equilibrium swelling state and at the dry state, respectively [18]. Experiments were repeated in triplicate for each gel specimen and the mean value was 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 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) using the following methodology: Groups C-M: Gels (1-4) (0.005 gm, 10 seconds) + primer + Bonding immediately) or Groups L-T Gels (1-4) (0.005 gm, 10 seconds) + Bonding immediately). The bonding agent contained: carboxylic acid modified dimethacrylate (TCB resin), phosphoric acid modified acrylate resin (PENTA), urethane dimethacrylate (UDMA), triethyleneglycol dimethacrylate (TEGDMA),2-hydroxyethylmethacrylate (HEMA), butylatedbenzenediol (stabilizer), ethyl-4-dimethylaminobenzoate), camphorquinone, functionalized amorphous silica, t-butanol.

In this way were 90 teeth samples (each containing 2 studs) prepared and divided into 18 groups of 8 each, A-T (Table 2) and stored in a solution of artificial saliva. These groups was then

Table 2: Groups tested (5 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	Oblepicha oil (0.005g)+ primer+ Bonding immediately
Group D	Copaiba oil (0.005g)+ primer+ Bonding immediately
Group E	Gel1+primer+ Bonding immediately
Group F	Gel2+primer+ Bonding immediately
Group J	Gel3+primer+ Bonding immediately
Group K	Gel 4 + primer +Bonding immediately
Group L	Gel 5 + primer +Bonding immediately
Group M	Gel 6 + primer +Bonding immediately
Group L	Oblepicha oil (0.005g)+ Bonding immediately
Group M	Copaiba oil (0.005g)+ Bonding immediately
Group N	Gel1+ Bonding immediately
Group O	Gel2+ Bonding immediately
Group P	Gel3+ Bonding immediately
Group R	Gel4 + Bonding immediately
Group S	Gel5 + Bonding immediately
Group T	Gel6 + Bonding immediately

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 at a crosshead speed of 0.5 mm/minute was used to test the debonding strength. All data tests were analyzed using the non-parametric ANOVA test.

Results

The characterization of functionalized hydrogels (Gel 1-6)

The SEM images were obtained to characterize the microstructure of the freeze-dried propolis containing fucoidan and chitosan gels and are presented in Figure 1. It could be seen that the gels displayed a homogeneously pore structure. It was thought that the microporous 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.

Dentin shear bond strength

Mean shear-bond strength values and difference between the groups are summarized in Figure 2A for bonding to dentin after 24 hours. In general, there was an increase in bond strength of the dentin treated with the functionalized hydrogels compared to the bond strength of the conventionally bonded teeth. An increase in the shear bond strength was also previously reported for groups of hydrogen peroxide exposed samples [6], suggesting that additional benefits associated with chitosan: antioxidant systems are in need of further investigations [18-22]. In general after 3 months the bond strength (Figure 2B) was somewhat lower than at the beginning. The non-parametric ANOVA test was used to test for significant differences amongst the 8 mentioned groups and the corresponding control groups A and B (significant level $p < 0.05$ in both the cases.

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 3a and 3b. Functionalized hydrogels showed the highest adhesive force, and this result can be expected because of the well-known intrinsic bio-adhesive properties of chitosan [18-22].

Modulus of elasticity (MOE)

The mean and standard deviations of MOE of demineralized dentin treated with hydrogels at different time periods are shown in Figure 3. The results of two-way ANOVA showed that both factors, "hydrogel treatment" and "treatment duration", had a significant effect on the MOE of demineralised dentin ($p < 0.001$). Interaction of the two factors was also significant ($p < 0.001$). The MOE of BIOF-IPNs treated dentin increased with time. A rapid significant increase in MOE was observed after 10 min treatment with Gels 1-6.

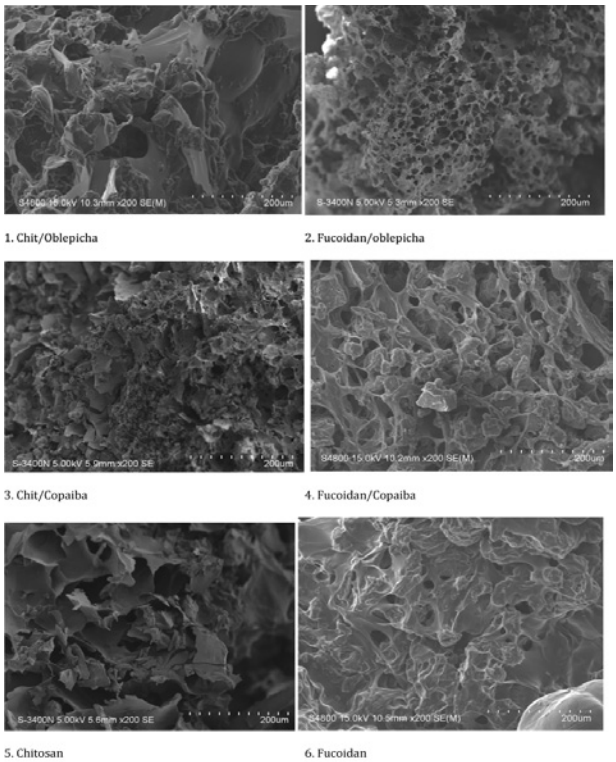

Figure 1: SEM photographs of interior morphology of the selected gels 1-6 under investigations.

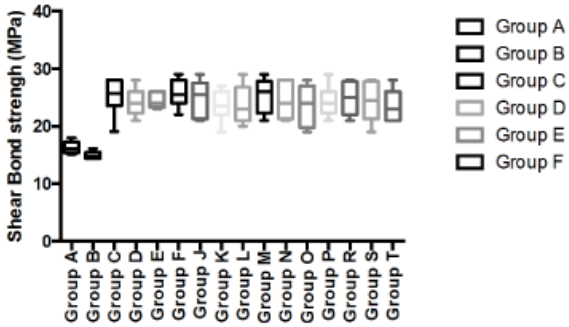

Figure 2a: Dentin shear bond strength after 24 hours.

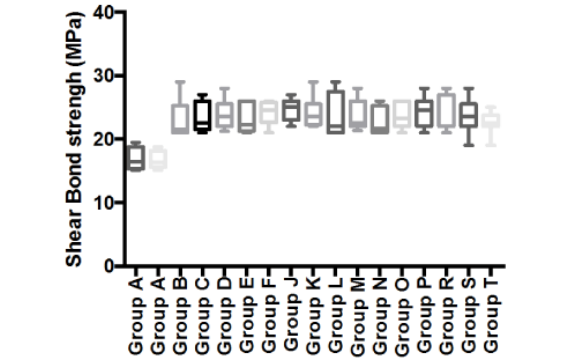

Figure 2b: Dentin shear bond strength after 3 month.

Table 3a: Adhesive strength in dentin.		
Hydrogel	**Adhesive Force(N) ± SD (Dentin)**	**Work of Adhesion(N. cm) ±SD (Dentin)**
chitosan/oblepicha	1.78± 0.41	6.01 ± 0.25
Chitosan	1.12± 0.19	3.45± 0.15
Fucoidan	1.18± 0.12	3.54± 0.18
Oblepicha oil	1.61± 0.11	2.95± 0.16
Copaiba oil	1.58± 0.16	2.45± 0.14
Fucoidan/oblepicha	1.85± 0.41	6.25 ± 0.30
chitosan/copaiba	1.92± 0.32	6.75 ± 0.32
Fucoidan/Copaiba	2.10± 0.41	6.98 ± 0.31

Table 3b: Adhesive strength in pig ear skin.		
Hydrogel	**Adhesive Force(N) ± SD (Skin)**	**Work of Adhesion(N. cm) ± SD (Skin)**
chitosan/oblepicha	3.78± 0.41	16.01 ± 0.45
Func/oblepicha	2.85± 0.41	18.25 ± 0.50
Chitosan	1.22± 0.13	3.75± 0.15
Fucoidan	1.28± 0.12	3.64± 0.16
Oblepicha oil	1.81± 0.14	3.25± 0.14
Copaiba oil	1.58± 0.16	2.65± 0.16
chitosan/copaiba	3.92± 0.32	15.75 ± 0.62
Fucoidan/Copaiba	4.10± 0.41	18.98 ± 0.61

Discussion

Dentin 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). 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 BIOF-IPNs systems might be able to address the shortfalls in the current perspectives in improving bond durability.

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 oblepicha or copaiba oils 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 [18-22]. Appropriate swelling is important to guarantee bio-adhesion; however, over-hydration can form slippery nonadhesive hydrogels [18-22].

Chitosan and fucoidan are potent antioxidants with multiple free hydroxyl groups [18-24]. These hydroxyl groups can form bridge-type hydrogen bonds within the side chains of hydroxyl, carboxyl, amino or amide groups of the collagen molecules [18-24]. The formation of these hydrogen bonds is the reason of the stability of chitosan-collagen or fucoidan: collagen interaction [24,25]. By positioning itself between collagen molecules, host: guest complex formed by chitosan: bioactive agent (oblepicha or copaiba oils and its active in) can potentially also form ionic bonds, as well as covalent bonds with collagen fibrils [24,25]. Furthermore, in the process of formation of hydrogen bonds host: guest complex, molecules can replace the water molecules bound to collagen in the extra-complex compartments [24,25]. This study has shown that newly developed hydrogels are capable of improving the modulus of elasticity of demineralised dentine.

SEM images of the dentin surface exposed to BIOF-IPNs Gels 1-4 for 2 weeks in artificial saliva. The surface of the dentin has been significantly influenced by the treatment with bio-active containing gels, illustrating previously reported unique properties of the chitosan and fucoidan in promoting the formation of the hydroxyapatite crystals as demonstrated in figures 4A-4D.

Interestingly, the surface of the dentin has been significantly influenced by the treatment with bio-active containing gels, confirming previously reported unique properties of the chitosan in promoting a formation of the hydroxyapatite crystals, as well as prevent demineralization of enamel and dentin, which has been reported previously (Figure 4) [18-22]. The potential applications of chitosan and fucoidan in dentistry include strengthening of the collagen matrix, increasing resin–dentine bond strength, inactivation of collagen-bound proteases and remineralization of root caries [18-22]. The detailed investigation of the potential mechanism is currently being researched in our laboratory.

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 3. BIOF-INPs showed the highest adhesive

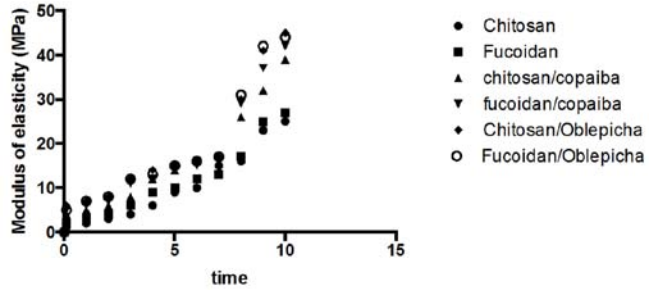

Figure 3: Effects of the time duration on the modulus of elasticity following the hydrogel treatment.

force, and this result can be expected because of the well-known intrinsic bio-adhesive properties of chitosan, fucoidan, oblepicha oil as well as copaiba oil [24,25].

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, "hydrogel treatment" and "treatment duration" had a significant effect on the MOE of demineralised dentin ($p < 0.001$). Interaction of the two factors was also significant ($p < 0.001$). The MOE of BIOF-IPNs treated dentine increased with time. A rapid significant increase in MOE was observed after 10 min treatment with the bio-active containing gels.

Free radical defense mechanism and antioxidant capacity of bio-active hydrogels

The nutraceutical carriers are required to have the ability to protect the active compound against chemical degradation by the surrounding dispersion medium and control the release rate of the incorporated compound [19,20]. As the material of the carrier, it is also required to be non-toxic, biocompatible and biodegradable [21,22] Chitosan and fucoidan are linear abundant polysaccharide, selected as the wall material of the delivery system due to their biodegradable, biocompatible, mucoadhesive and non-toxic nature [18-22], They can adhere to the mucosal surface and transiently open the tight junction between epithelial cells. Some reports have indicated that chitosan and fucoidan can increase (Figure 5) membrane permeability, both *in vitro* [24, 25] and *in vivo* [25].

The stability of bio-active oil-loaded chitosan complexes has been measured during storage and no significant decomposition observed after 6 month storage at room temperature (24°C).

Evaluation of bio-active hydrogels for free radical quenching capacity

When a wound occurs, it is generally accompanied by classical symptoms of inflammation, such as pain, redness and edema. In the inflammation stage, the main aim is the removal of debris, damage tissue, and bacteria by neutrophils and macrophages, which have a role in antimicrobial defense and debridement of devitalized tissue by production of proteolytic enzyme and reactive oxygen species [18-22]. The amount of uncontrolled ROS is the main cause of the inability of the healing process to continue as efficiently as possible. Therefore, it would be ideal to utilize the antioxidant capacity of the "designer hydrogels" to detect and to "fight the free radical excess". This has been assessed using previously described model using well-established that HO radical can be generated from a reaction known as the biologic Fenton reaction and this reaction requires the presence of H_2O_2 [18-22].

We reported earlier that protein cross-linking as a model for detection of free radical activity and activation of "molecular defense forces". Bovine Serum Albumin (BSA), a completely water-soluble protein, was polymerized by hydroxyl radicals

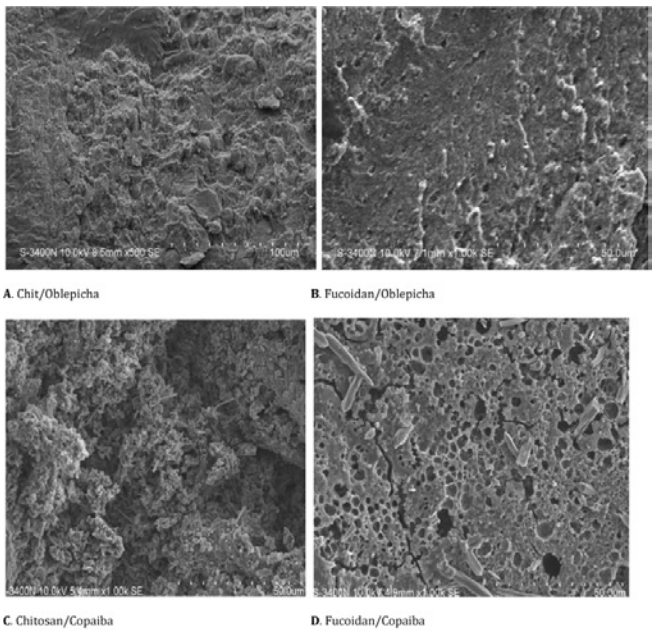

Figure 4: A. Dentin surface exposed to Chit/Oblepicha in artificial saliva. B. Dentin surface exposed to Fucoidan/Oblepicha in artificial saliva. C. Dentin surface exposed to Chitosan/Copaiba in artificial saliva. D. Dentin surface exposed to Fucoidan/Copaiba in artificial saliva.

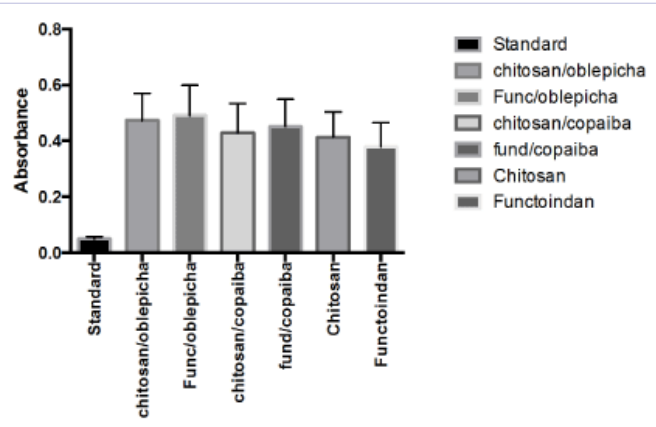

Figure 5: Antioxidant capacity measured at 450nm using the previously described spectrophotometric assay to assess the hydrogels and corresponding ingredients antioxidant capacity after 24 hours under storage under ambient temperature condition. Antioxidant capacity was measured during the first 2 hours of exposure.

generated by the Fenton reaction system of Fe^{2+}/EDTA/ H_2O_2/ ascorbate [18-22]. As a result, the protein loses its water-solubility and the polymerized product precipitates. The decrease in the concentration of the water-soluble protein can easily be detected.

We adopted the method for recording changes in water solubility of the model protein Bovine Serum Albumin (BSA) exposed to free radicals generated by an inorganic chemical system. As clearly demonstrated by the Figure 6, upon exposure to standard H_2O_2 in the form of Fe^{2+}/EDTA/ H_2O_2/ascorbate used as a control, upon incorporation of the chitosan substituted

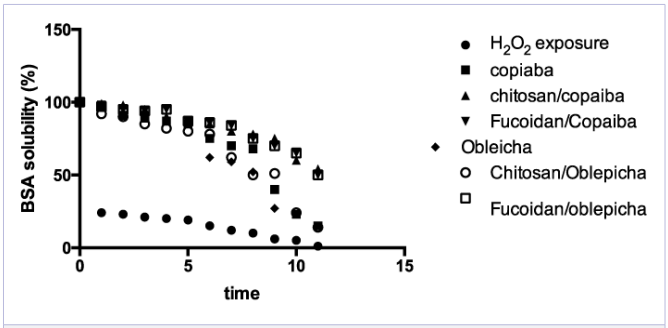

Figure 6: Affects of the various antioxidant on the solubility of BSA protein in the drug delivery system.

hydrogels, the build in antioxidant capacity and therefore free radical defense of the in-vitro model has been activated and are of significant value to take notice. This model represents the practical approach of in-situ monitoring and tests the amount of free radical production and synergistic antioxidant defense of the system. Further investigations and fine-tuning of the system are currently on the way in our laboratory (Figure 6).

The present results demonstrate the capability of the newly designed hydrogel system to play an important role as a "build in" free radical defense mechanism, and acting as a "proof of concept" for the functional multi-dimensional restorative repair materials. Further biological evaluations of the above materials such as cytotoxicity and antimicrobial properties are currently being evaluated in our laboratories.

Conclusion

We quantified the effects of functional designer chitosan and fucoidan biomaterials on the dentin bond strength of a composite and evaluate the bio-adhesive capacity of the materials in the 2 separate "*in vitro*" systems. The added benefits of the chitosan or fucoidan as oblepicha oil or copaiba oil (host: guest complex) treated hydrogels involved positive influence on increased dentin bond strength in the "prime free" technique as well as in vitro "build in" free radical defense mechanism and acts as a "proof of concept" for the functional multi-dimensional restorative wound healing materials.

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