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Temporomandibular Joint Dysfunctions and Bio-Materials: Design, Free Radical Defense and Mechanism *In Vitro*

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Abstract

The aim of this investigation is to evaluate the suitability and flexibility of the bio-active containing designer materials to act as an "*in vitro*" probe to gain insights into molecular origin of TMJ. The hydroxyapatite/chitosan containing hydrogels represent a reliable bio-compatible scaffold and allowed to evaluate the defense "build in" free radical defense mechanism of the functionalized bio-scaffolds on the molecular level, compare the effectiveness of the designer materials with the earlier reported capabilities of N-acetyl cysteine and apply the newly developed materials for the evaluation of new therapeutic treatment modalities in the TMJ therapy *in vitro*.

Materials and Methods: The hydrogels were prepared by dispersion of the corresponding component in glycerol and acetic acid with the addition of chitosan: hydroxyapatite as a molecular scaffold. The surface morphology (SEM), release behaviors (physiological pH and also in acidic conditions), stability of the antioxidant-chitosan were also evaluated. Structural investigations of the reactive surface of the hydrogel are reported. Bio-adhesive studies and mechanical properties of newly prepared materials were conducted to assess the suitability of these designer materials. Ability of the materials to release the phenolic components as potential therapeutic agents was assessed.

Results: The sustained release of total phenolic component as a potential therapeutic agent confirms the added benefit of synergistic action of a functional therapeutic delivery when comparing the newly designed chitosan/HA-based hydrogel molecular scaffold. The hydrogel formulations have a uniform distribution of drug content. The bio-adhesive capacity, tensile strength, compressive strength as well as modulus of elasticity of the hydroxyapatite containing materials in the "*in vitro*" systems was tested and quantified.

Conclusion: The added benefits of the chitosan hydroxyapatite treated hydrogels involved positive influence on the phenolic component release, sustainable bio-adhesion, tensile strength, compressive strength as well as modulus of elasticity of the hydroxyapatite containing materials in the "*in vitro*" systems was tested and demonstrated *in vitro* "build in" free radical defense mechanism.

Keywords: *Hydroxyapatite; Drug release; Antimicrobial properties; Mechanical Properties; Biomolecular Scaffold*

Introduction

In the last few decades, tissue engineering has emerged as a promising multidisciplinary approach for the repair and regeneration of damaged bone tissue [1,2]. The molecular events that underline the degenerative temporomandibular joint (TMJ) diseases are poorly understood [3]. Mechanical stresses are generated during functional or para-functional movements of the jaw; adaptive mechanism of the TMJ may be exceeded by free radical accumulation leading to a dysfunctional state (i.e. disease state) [4]. Biologically relevant free radicals are very reactive and unstable molecular entities that have an unpaired electron and they can produce participate in the propagation chain reaction to form a new radical. Although oxygen free radicals participate in many physiological processes, they can be harmful

to tissue, when either their action or their generation have been left uncontrolled. The most common source of free radicals in biological systems is oxygen. The elevation of reactive oxygen species (ROS) leads to oxidative stress that causes molecular damage to the vital structures and functions [5-7].

The unsaturated fatty acids of cell membrane lipid are susceptible to peroxidative reaction. Lipid peroxidation of cell membranes has been implicated in the wide range of tissue injuries and diseases [8]. Accumulation of lipid hydro-peroxides in a membrane disrupts its function and causes it to collapse and have range of cytotoxic radicals. The most serious one is byproducts aldehydes. They may also react with transition metals like iron or cop-per to form stable aldehyde such as Malondialdehyde for example, which will damage the cell membrane. Because hemoglobin constitutes the largest iron store in the body, it is speculated to be a potential source of redox activity iron, which can catalyze the formation of free radicals that might be damaging to the joint [8]. Antioxidants can be classified as enzymatic or non-enzymatic [9]. The non-enzymatic antioxidants are further subdivided as nutrient based (such as alpha tocopherol, β- Carotene ascorbate, Glutathione, Selenium, Proanthocyanidin, Lycopene, Green tea) and non-nutrient based (such as Ceruloplasmin, Transferrin, uric acid, Peptides Camosine Anserine [9].

Antioxidants end the electron stealing reaction of free radicals by donating one of their electrons. The antioxidant does not become a free radical by donating an electron because they are stable in either form. Antioxidants act by scavenging or chain breaking, such as Vitamin E (alpha tocopherol), Vitamin C (ascorbic acid), or Vitamin A (beta carotene) and preventative antioxidants that function basically by sequestering transition metal ions and preventing fenton reactions and are therefore predominantly proteins by nature (Eg., albumin, transferring, or lactoferrin)[9].

The a etiological factors of TMJ disorders are as follows: systemic diseases (rheumatoid arthritis, psoriasis, pseudo gout, ankylosing spondylitis, etc.), secondary inflammatory component from the neighboring regions (otitis, maxillary sinusitis, tonsillitis), trauma (chronical), prevalence of dental arch defects e.g. missing of molar teeth [10], malocclusion, endocrinological disturbances, odontogenic infections (impacted third molars) [11]. Specific bacterial organisms such as *S. aureus*, *S. mitis, M. fermentas,* and *Actinobacillus actinomycetemcomitans* have been found in the synovial fluid [12]. Serum antibodies against Chlamydia species in patients with mono arthritis of the TMJ have also been reported [13].

Chitosan (CS), obtained through the alkaline deacetylation of chitin, is a copolymer of N-acetyl-D-glucosamine and D-glucosamine. CS stands out by a unique combination of favorable biological properties such as nontoxicity, biocompatibility and biodegradability, along with mucoadhesive, bacteriostatic, and wound-healing properties [14,15].

The aim of this investigation is to evaluate the suitability and flexibility of the designer materials to act as an "*in vitro*" probe to gain insights into molecular origin of TMJ and more specifically excessive damage caused by oxygen centered radicals and the defense "build in" free radical defense mechanism of the functionalized bio-scaffolds on the molecular level, compare the effectiveness of the designer materials with the earlier reported capabilities of N-acetyl cysteine and apply the newly developed materials for the evaluation of new therapeutic treatment modalities in the TMJ therapy *in vitro*.

Methods

Preparation of various hydrogels: general protocol

The bio-active 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 [14,15]. Ten milliliters of glacial acetic acid (3% w/w) was then added with continuous mixing and finally chitosan/hydroxyapaptite (10% chitosan w/w) polymer was mixed well to form the required gel. The strength of the prepared gel (10 gm) is 0.02g of bio-additive in each gram of the base.

Swelling/Weight Loss Tests and Bioactive Release

The swelling/weight loss tests were performed when triplicates of each samples composition (approximately 2 cm^2, weight normalized) were immersed in 2 mL of different fluids at 37°C for each time interval studied (1, 2, 4, 24, and 96h). Two different media were

used in accordance with the ISO 10993-9 standard. The first media was Phosphate Buffered Saline (PBS, Sigma Aldrich), intended to mimic the inorganic phase of human plasma [16]. The other media was PBS with a reduced pH which was intended to simulate the local inflammatory environment of the wounds [16,17]. This is termed Solution pH 4.0. The pH was lowered using Lactic Acid (Sigma Aldrich). The fluid absorption of each sample was calculated according to Equation 2 to obtain their swelling degree (SD). WS is the weight of the sample at each time interval (swollen weight) and WD is the dry weight before swelling [18]. After 4 days of immersion, the samples were dried and weighed in order to calculate their weight loss (WL) [Equation 3], where WD and WDS are the weight of the dried samples before and after swelling tests, respectively

$$SD = 100\,\frac{W_S - W_D}{W_D}(\%)$$

$$\mathrm{WL} = 100\,\frac{W_D - W_{DS}}{W_D}(\%)$$

To analyze the bio-active release (propolis (Brazilian or Uruguan), Copaiba oil, Oblepicha Oil) based on the total phenolic concentration, the swelling media was analyzed after 1, 2, 24, and 96h of immersion via UV-Vis spectrometer, from 300 to 800 nm, using polystyrene cuvettes [19]. For quantification of the amount of propolis released, a standard curve was created by diluting the original propolis in isopropanol resulting in several aliquots of known concentration, which were then analyzed in the same wavelength range. The area of the peak of these aliquots (of known concentration of bio-additive) was calculated and used to compare with those of the bio-additive released by the samples.

Total phenol concentration in Propolis (Brazilian and Uruguyan), Copaiba oil and Oblepicha Oil

Total phenols assay Total phenols content was estimated by a colorimetric assay based on the same procedure realized by Rocha., *et al*. [20] and described by Waterman & Mole [21] with some modifications. The samples were diluted in distilled water to obtain a concentration of 5 µg/mL of total phenols. The concentration of 5% v/v of Folin-Ciocalteu reagent and 10% v/v sodium carbonate (35% w/v) reagents were added to the samples. After the addition of the reagents, the solutions were kept in the dark at room temperature for 30 min and the absorbance was read at the wavelength of 760 nm in a spectrophotometer UV mini-1240 (Shimadzu Co., Kyoto, Japan). Gallic acid (Aldrich, Australia) was used as a standard. The analyses were performed in triplicate.

Statistical analysis

The total flavonoids, total phenolics, quercetrin and afzelin contents as well as the antioxidant activity results were statistically analyzed by the variance analysis (ANOVA) by the Prism 6 software. In all analyses, a 5% significance level was considered.

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 [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.

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 Oblepiha oil, Copaiba oil and corresponding chitosan) tested to reach required concentration as shown in results, EDTA (0-4.8 mM), $Fe(NH_4)_2(SO_4)_2$ (0-4 mM), ascorbate (4 mM) and H_2O_2 (0.2%). To chelate iron completely, 1.2 molar excess of EDTA was always use [23]. 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 [24]. The yield of OH radicals generated in the incubations was determined on the basis of degradation of deoxyribose [25]. Bityrosine formation was monitored by measuring fluorescence at 325 nm (excitation) and 415 nm (emission) according spectrophotometer [26].

Tensile strength testing of the material

Tensile testing was conducted using Instron 5565. Following American Standardized Testing Materials Standard D3039, rectangular samples were approximately 6-8 mm in length, 1 mm in width a 1 mm in thickness, and tested with a gauge length of 3.5 ± 0.4 mm. Samples were elongated at a rate of 1% of gauge length per second. The cross-sectional area of samples was evaluated using Image J image analysis software. Stress-strain curve were developed from the load-displacement curve. Young's modulus was quantified by finding the slope of the stress strain curve, and ultimate tensile strength was calculated.

Microbiology and TMJ

A type strain of *Staphylococcus aureus* (ATCC 12600), obtained from the American Type Culture Collection (Manassas, USA) was used as test bacterium for estimating the antibacterial activity of the hydrogels. The antibacterial activity of the prepared chitosan hydrogels were tested using the standard Kirby-Bauer agar disc diffusion method [27]. Five to 6 mm deep Muller-Hinton agar (Oxoid, Basingstoke, UK) plates were inoculated by streaking a standardized inoculum suspension that match a 0.5 McFarland standard and containing 10^7- 10^8 colony forming units/ml with a throat cotton swab. For each test sample 500 µg of hydrogel was applied to a 6 mm diameter paper disc. The paper discs were placed on the inoculated Muller-Hinton agar medium and incubated at 37ºC for 24 hours. The diameter of the zones of growth inhibition was measured with a caliper. Each measurement was done in triplicate and the testing of each sample was repeated 3 times. The antibacterial efficacy of the prepared gels were compared to antibiotic sensitivity discs (Mast Laboratories, Merseyside UL) containing 30 µg of tetracycline per disc.

Results and Discussion

Scanning electron microscope characterization of the bio-active restorative materials

The SEM images were obtained to characterize the microstructure of the freeze-dried gels and are presented in Figure 1. The 'skin' of the prepared modified flow able composites can be seen, and the collapse of the surface pores may be due to artifacts (freeze-drying process).

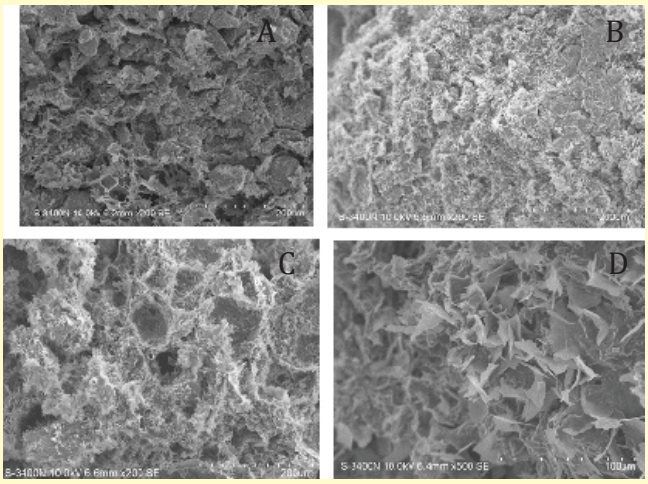

Figure 1: *SEM of the surfaces of the materials after polymerization where*
a. Hydroxyapatite/Copaiba Oil/Chitosan
b. Hydroxyapatite/Brazilian Propolis/Chitosan
c. Hydroxyapatite/Brazilian Propolis/Chitosan and
d. Hydroxyapatite/Brazilian Propolis/Chitosan

Free radical defense and bio-functional hydrogels

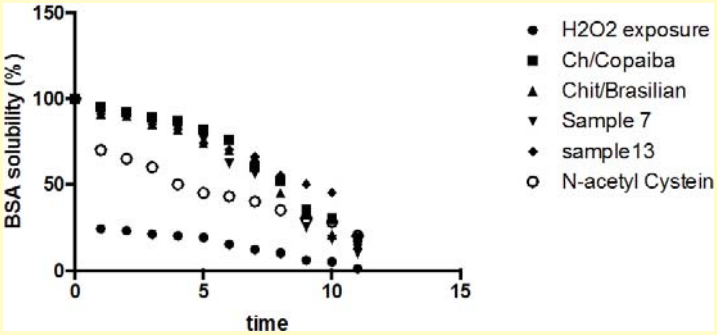

Figure 2: *Free radical defense and biomaterials as a function of the BSA solubility (%) at pH 6.7.*

Inflammation of the temporomandibular joint (TMJ) is often treated with arthrocentesis, which is thought to have an anti-inflammatory effect [28,29]. Several mechanisms elucidating the antimicrobial activity of chitosan have been postulated. The most acceptable mechanism is the interaction between positively charged chitosan molecules and negatively charged microbial cell membranes. The interaction is mediated by the electrostatic forces between the protonated NH_3+ groups of chitosan and the electronegative charges of the microbial cell surfaces. The electrostatic interaction results in the following types of interference: a) by promoting changes in the properties of the membrane wall permeability, thus provoking internal osmotic imbalance and therefore inhibiting the growth of the microorganisms and b) by the hydrolysis of the peptidoglycans in the microorganism wall, leading to the leakage of the intracellular electrolytes such as potassium ions and the low molecular weight protenaceous constituents (Eg. proteins, nucleic acid, glucose and lactate dehydrogenase) [30]. Since such a mechanism is based on electrostatic interactions, it also suggests that the greater the number of cationized groups, the higher the antimicrobial activity.

Tensile strength of the modified biomaterials

The mean and standard deviation values (MPa) for the tensile strength are summarized in Figure 3.

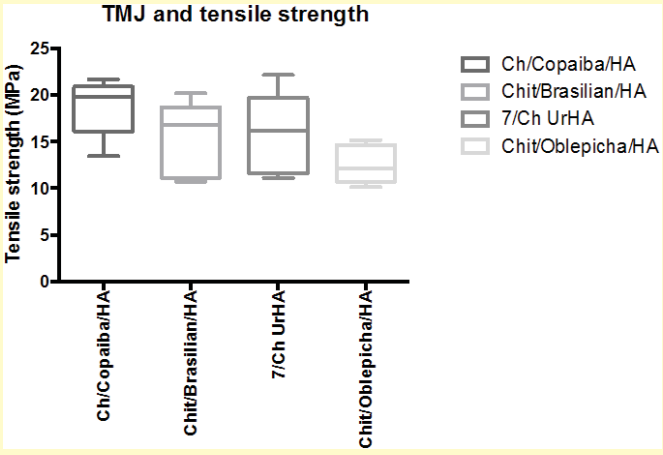

Figure 3: *Tensile strength of the modified biomaterials.*

Swelling and bio-additive release for the newly prepared hydrogels

The swelling tests in PBS and in Solution pH 4.0 for 4 days revealed that when reaching the equilibrium swelling degree (ESD), all samples swelled in accordance with (Figure 4a and 4b). Based on the ESD values, the gels can be considered as superabsorbent [33]. A peak of media uptake was observed at the beginning of all curves, and after 1 day of swelling there was a plateau-the ESD. The ESD occurs when the hydration forces (the network stretching by the initial fluid uptake) and the elastic force of the cross-linkages reach the equilibrium [34,35].

As discussed before, high amounts of bioactive led to low degree of crystallinity and low percentage of crystalline phase. When the samples swell, the amorphous chains could have more freedom to move and, if they were not cross-linked, with the help of the media, they could detach from the network to the media, increasing the weight loss [36].

The amount of bioactives (such as Brazilian propolis, Uruguyan propolis, Copaiba oil and Oblepicha oil) release in swelling media was analyzed after 1, 2, 24, and 96h of immersion (Figure 5a and 5b). The propolis release by polymeric systems usually occurs in two steps: the release of certain amounts of propolis in the first day of swelling as well as a prolonged release in some cases [37]. A trend could be observed in all curves after 4 days of immersion: there was a high bioactive release in the initial hours and the cumulative release reached constant values up to 1 day of immersion. No prolonged release was observed.

The amount of bioactive release in swelling media was analyzed after 1, 2, 24, and 96h of immersion (Figure 4b). The bioactive release by polymeric systems usually occurs in two steps: the release of certain amounts of propolis in the first day of swelling as well as a prolonged release in some cases [38]. Nonetheless, the samples released more bioactive to PBS than to Solution pH 4.0, probably indicating that the propolis release can be influenced by the media pH.

Sample	Weight loss in PBS	Weight loss in pH 4
CH/HA hydrogel	12.6 ± 5.1	12.5 ± 5.8
CH/HA/Brazilian	15.5 ± 4.8	6.78 ± 2.4
CH/HA/Uruguyan	14.3 ± 4.1	5.9 ± 2.8
CH/HA/Oblepicha	16.4 ± 5.6	6.9 ± 3.1
CH/HA/Copaiba	15.4 ± 5.4	5.9 ± 2.4

Table 1: *Total Weight Loss of the CH/HA and the CH/HA–Bioactive Samples After 4 days of Immersion in PBS and in Solution pH 4.0.*

Inhibition Zone against *Staphylococcus aureous* and bioactive hydrogels

All the test samples gave an average inhibition zone larger than the tetracycline control disc, thereby confirming the antibacterial activity of the different nano-diamond combinations against *Stapylococcus aureous* (Figure 6). Using the Student's T-test ($p < 0.01$), there was a significant difference between the rest of the samples when compared to each other and the positive control. The hydrogel with the highest antibacterial activity contained bioactive and hydroxyapatite/chitosan and the antibacterial activity was still higher than the tetracycline control disc (Figure 6).

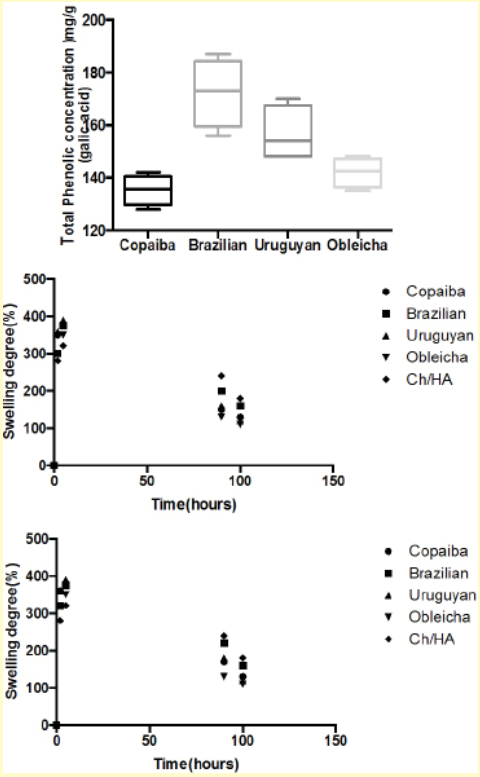

Figure 4: *Swelling degree (SD) results of Ch/HA and of Ch/HA–bioactive samples, after regular time intervals (1, 2, 4, 24, and 96 h), when immersed in (a) PBS and (b) Solution pH 4.0 for 4 days.*

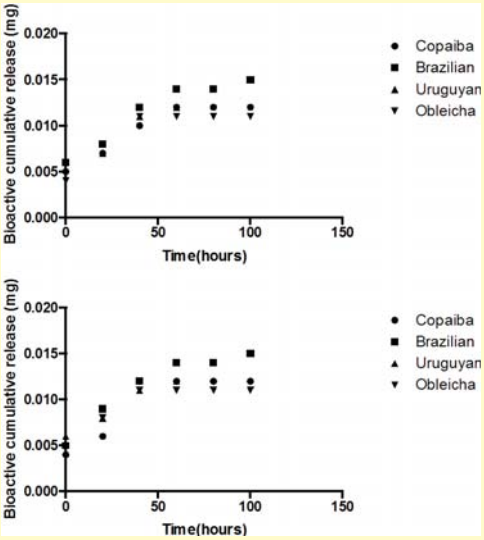

Figure 5: *Bioactive cumulative release profile of Ch/HA-bioactive samples. The Ch/HA-bioactive samples were immersed in (a) PBS and (b) Solution pH 4.0 and the propolis delivered was quantified after regular intervals of time for 4 days.*

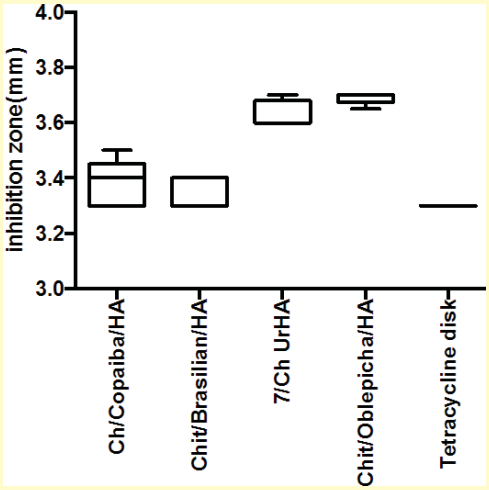

Figure 6: *Antibacterial inhibition zones produced by the different bio-active containing hydrogels against Staphylococus aureous.*

The process of inflammation plays a crucial role in wound healing. In the event of an injury, inflammatory cells such as the granulocytes, monocytes, and macrophages migrate to the injury site. Fibroblasts are activated by the growth factor that is released, and they produce matrix and new collagen fibrils [39]. But these collagen fibrils do not align with the original connective tissue. Instead, they grow at right angles to the plane of the injury. The integrity of the zone of healing is further compromised as the elastin fibers do not heal at all. Anti-inflammatory medications usually prescribed after injury allays pain and swelling but unfortunately they diminish the healing response [40]. As a result of this incomplete healing, the joint remains painful with normal or even sub normal physical activity. Such a joint may become hyper mobile and prone to re-injury due to incomplete ligament support. Newly designed hydrogels may also be beneficial in TMD management owing to its anti-microbial effect. Chlamydia, *Mycoplasma genitalium, Staphylococcus aureus, Mycoplasma fermentans, Actinobacillus actinomycetemcomitans*, and *Streptococcus mitis* have been cultured from the TMJ. The presence of *S. aureus* in the TMJ synovial fluid has been related to TMD [41].

Conclusion

In this investigation we prepared and evaluated bioactive materials containing Chitosan/Hydroxyapatite, which have proven to be suitability and flexibility of the designer materials to act as an "*in vitro*" probe to gain insights into molecular origin of TMJ and more specifically excessive damage caused by oxygen centered radicals and the defense "build in" free radical defense mechanism of the functionalized bio-scaffolds on the molecular level and apply the newly developed materials for the development of new therapeutic treatment modalities in the TMJ therapy.

Bibliography

1. Fletcher MC., *et al*. "Anatomy and patho-physiology of the temporomandibular joint. In Peterson's". Principles of Oral and Maxillofacial Surgery, edn 2. Edited by Miloro M. Hamilton (Ontario): BC Decker; (2004): 933-947.
2. Howerton DW and Zysset M. "Anatomy of the temporomandibular joint and related structures with surgical anatomic considerations". *Oral & Maxillofacial Surgery Clinics of North America* 1 (1989): 233-247.
3. Laskin DM. "Putting order into temporomandibular disorders". *Journal of Oral and Maxillofacial Surgery* 56.2 (1998): 121.
4. Laskin DM. "Diagnosis and etiology of myofascial pain and dysfunction". *Oral & Maxillofacial Surgery Clinics of North America* 7 (1995): 73-78.

5. Dworkin SF and LeResche L. "Research diagnostic criteria for temporomandibular disorders: review, criteria, examinations and specifications, critique". *Journal of Craniomandib Disorders* 6.4 (1992): 301–355.
6. Ogle OE and Hertz MB. "Myofascial pain". *Oral & Maxillofacial Surgery Clinics of North America* 12 (2000): 217-231.
7. Okeson JP., *et al*. "Fundamentals of Occlusion and Temporo-mandibular Disorders. St. Louis: C.V. Mosby; 1985.
8. Stohler CS. "Masticatory myalgias. In Oral and Maxillofacial Surgery. Temporomandibular Disorders. Edited by Fonseca RJ., *et al*. Philadelphia: WB Saunders; 2000:38-45.
9. Milam SB. "Pathophysiology of articular disk displace-ments of the temporomandibular joint. In Oral and Maxillofacial Surgery. Temporomandibular Disorders. Edited by Fonseca RJ., *et al*.: Philadelphia: WB Saunders (2000): 46-72.
10. Laskin DM. "Internal derangements". *Oral Maxillofacial Surgery Clinical North America* 5 (1994): 217-222.
11. Westesson PL., *et al*. "Reliability of a negative clinical temporomandibular joint examination: prevalence of disk displacement in asymptomatic temporo-mandibular joints". *Oral Surgery Oral Med Oral Pathology* 68 (1989): 551-554.
12. Wilkes CH. "Internal derangement of the temporomandibular joint. Pathological variations". *Arch Otolaryngology Head Neck Surgery* 115 (1989): 469-467.
13. Greene CS and Laskin DM. "Long-term status of TMJ clicking in patients with myofascial pain and dysfunction". *Journal of the American Dental Association* 117.3 (1988): 461-465.
14. Perchyonok., *et al*. "Bioinspired-Interpenetrating Network (IPNs) Hydrogel (BIOF-INPs) and TMD *in Vitro*: Bioadhesion, Drug Release and Build in Free Radical Detection and Defense, Open Journal of Stomatology" *Scientific Research* 5.3 (2015): 53-61.
15. Perchyonok., et al. "Bioactive-Functionalized Interpenetrating Network Hydrogel (BIOF-IPN): A Novel Biomaterial Transforming the Mechanism of Bio-Repair, Bio-Adhesion and Therapeutic Capability – An *In Vitro* Study". *Journal of Interdisciplinary Medicine and Dental Science* 3.1 (2015).
16. Vinklarkova L., *et al*. *BioMed Research Internationa* 1 (2014): 1.
17. Nghiep TD., *et al*. "Formation and characterization of a hydrophilic polymer hydrogel under gamma irradiation". *Journal of Radioanalytical and Nuclear Chemistry* 285.3(2010): 719-721.
18. Souza R M., *et al*. *Zeitschrift fu€r Naturforschung* (2007): 801.
19. Hung CF., *et al*. *Biological and Pharmaceutical Bulletin* 31(2008): 955.
20. Singh B., *et al*. "Influence of in situ TiB reinforcements and role of heat treatment on mechanical properties and biocompatibility of ββ Ti-alloys". *Journal of the Mechanical Behavior of Biomedical* 10 (2012): 1-12.
21. Zeighampour F., *et al*. *International Journal of Research in Medical Sciences* 16 (2014): 25.
22. Weber J and Mc Manus A. "Infection control in burn patients". *Burns* 30.8 (2004): 16-24.
23. Mirzoeva O., et al. "Antimicrobial action of propolis and some of its components: the effects on growth, membrane potential and motility of bacteria". *Microbiological Research* 152.3 (1997): 239-246.
24. Wojtyczka R., *et al*. "*In Vitro* Antimicrobial Activity of Ethanolic Extract of Polish Propolis against Biofilm Forming Staphylococcus epidermidis Strains". *Evidence-Based Complementary and Alternative Medicine* (2013).
25. Berretta A., *et al*. Journal of Evidence Based Complementary Alternative Medicine 1 (2013).
26. Mirzoeva O K., et al. "Antimicrobial action of propolis and some of its components: the effects on growth, membrane potential and motility of bacteria". *Microbiology Research* 152.3 (1997): 239-246.
27. Bauer AW., *et al*. "Antibiotic susceptibility testing by a standardized single disk method". *American Journal of Clinical Pathology* 45.4 (1966): 493-496.
28. Kalpakci KN., *et al*. "An interspecies comparison of the temporomandibular joint disc". *Journal of Dental Research* 90.2 (2011): 193-8.
29. Jones KE., *et al*. "Morphology and biomechanics of the pinniped jaw: mandibular evolution without mastication". *Anatomical record (Hoboken)* 296.7 (2013): 1049-1063
30. Perchyonok VT., *et al*. "Insights into and relative effect of chitosan-H, chitosan-H-propolis, chitosan-H-propolis-nystatin and chitosan-H-nystatin on dentine bond strength". *European Journal of Dentistry* 7.4 (2013): 412-418.

31. Nimrodh Anantha A., *et al*. "PVA and BSA stabilized silver nanoparticles based surface–enhanced plasmon resonance probes for protein detection". *Colloids and Surfaces B: Biointerfaces* 85.2 (2011): 138-144.
32. Borges J., *et al*. "Development and characterization of orally-disintegrating films for propolis delivery". *Ciência e Tecnologia de Alimentos* 33.1 (2013): 28-33.
33. Barud H., *et al*. *Evidence Based Complementary Alternative Medicine* (2013): 1.
34. Almeida E., *et al*. "The incorporation of Brazilian propolis into collagen-based dressing films improves dermal burn healing". *Journal of Ethnopharmacology* 147.2 (2013): 419.
35. Tanaka E., *et al*. "Stress relaxation behaviors of articular cartilages in porcine temporomandibular joint". *Journal of Biomechanics* 47.7 (2014): 1582-1587.
36. Arzi B., *et al*. "Osteoarthritis of the temporomandibular joint in southern sea otters (Enhydra lutris nereis)". *Journal of Comparative Pathology* 149.4 (2013): 486-494.
37. Arzi B., *et al*. "Computed tomographic findings in dogs and cats with temporomandibular joint disorders: 58 cases (2006-2011)". *Journal of the American Veterinary Medical Association* 242.1 (2013): 69-75.
38. Henry CH., *et al*. "Reactive arthritis: preliminary microbiologic analysis of the human temporomandibular joint". *Journal of Oral Maxillofacial Surgery* 58.10 (2000): 1137-1142.
39. Piecuch J and Lieblich S. "Anatomy and pathology of the temporomandibular joint". In: Peterson U. ed. Principles of Oral and Maxillofacial Surgery, Vol 3. Philadelphia: JB Lippincott (1992): 1867-1871.
40. Goldschmidt MJ., *et al*. "Streptococcal infection of the temporomandibular joint of hemato-genous origin: a case report and contemporary therapy". *Journal of Oral Maxillofacial Surgery* 60.11 (2002): 1347-1353.
41. Schurman DJ., *et al*. "Experimental *E. Coli* arthritis in the rabbit. A model of infectious and post-infectious inflammatory synovitis". *Journal of Rheumatology* 4.2 (1977): 118-128.

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