Bio-Active Nano-Diamond Designer Materials and Dentures: From Design to Application

V. Tamara Perchyonok*, John Souzaa, Shengmiao Zhangb, Desigar Moodleyc and Sias Groblera

1VTPCHEM PTY LTD, Glenhuntly, Melbourne, Australia
2Department of Prosthetics, TAFE Queensland, 66 Ernest Street, South Brisbane, Australia
3School of Material Science and Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai, China
4Oral and Dental Research Institute, School of Dentistry, University of Western Cape, Cape Town, South Africa

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Introduction

The initial interaction between pathogenic bacteria and host cells is one of the critical steps leading to host colonization [1]. These first adhesion events allow host-pathogen recognition and prevent bacteria from being washed out by the host and are generally mediated by species proteins called adhesins. C. albicans binds various bacterial species, participating in polymicrobial interactions in the normally healthy host [2]. One of these is the oral commensal bacterium Streptococcus gordonii [3]. Als3 is one of eight C. albicans Als proteins (Als1-Als7, Als9), large cell-surface glycoproteins that primarily function in adhesive interactions [4].

Nano-diamond particles (NDs) are emerging as particularly well-suited for biological applications due to their biocompatibility [5-10]. It is indeed because of this feature that diamond nanoparticles should also be explored for anti-bacterial applications. While nano-diamond conjugates have shown their potential for the detection and removal of bacteria in solution, their potential for the efficient inhibition of Alsm-mediated binding phenomena has however not been fully explored [11].

Chitosan, which is a biologically safe biopolymer as well as an antioxidant, has been proposed as a bio-adhesive polymer and is of continuous interest to us due to its unique properties and flexibility in a broad range of oral applications reported by others and ourselves recently [12-15].

Lentinus edodes, known as shiitake mushroom, has received great attention due to positive health effects, including anti-tumour and hypo-cholesterolemic activity [12], related to the presence of β-glucans [13]. Propolis is a resinous substance produced by bees with antibacterial, antifungal, antiviral, and anti-inflammatory activities [14,15]. Propolis is bacteriostatic and bactericidal in high concentration [16]. Propolis has antimicrobial activity against gram-positive bacteria, e.g., S. aureus, but limited action against gram-negative bacteria and also against some fungi, e.g., C. albicans [17,18]. The copaiba tree is native to Latin America and Occidental Africa [19]. There are more than 20 species of copaiba in Brazil, and the most commonly described effects are anti-inflammatory, analgesic, antibacterial and anti-tumoral activities [20-22].

The present study aims to design functional biomaterials, evaluate the performance of chitosan: nano-diamonds based bio-active PMMA (polymethyl methacrylate) materials and investigate the potential applications of the newly developed materials for and prevention of denture stomatitis and associated conditions in denture wearers, while the performance of the material is not compromised in vitro. Our findings might be thus a step forward towards the development of alternative non antibiotic based strategies targeting bacterial infections.

Abstract

Objective: The present study aims to design functional biomaterials and evaluate performance of nano-diamond: chitosan based bio-active containing PMMA (polymethyl methacrylate) materials towards application in treatment and prevention of denture stomatitis and associated conditions in denture wearers.

Methods: The bio-active nano-diamond modified PMMA were prepared by dispersion of the corresponding component in glycerol and acetic acid with the addition of chitosan gelling agent. The release behaviors at physiological pH and also under acidic conditions and stability of the antioxidant-chitosan-nano-diamond were also evaluated. Mechanical performance such as tensile strength and compressive strength were measured as well bio-adhesive studies were investigated in order to assess the suitability of these designer materials.

Results: The bio-active nano-diamond modified PMMA materials showed a high adhesive force and they only swelled slightly in the aqueous medium. Bioactive release suggested prolonged release of the therapeutic agent from the hydrogels. The hydrogels also had significant free radical defense capability.

Conclusion: In this study we demonstrated that the newly prepared bio-active modified PMMA resins are suitable novel bio-active materials capable of comparable performance with the conventional PMMA materials with additional benefit of therapeutic bioactive release as well as potential antimicrobial properties to be demonstrated in vitro. Our findings might be thus a step forward towards the development of alternative non antibiotic based strategies targeting bacterial infections.

Materials and Methods

Propolis Brazilian (Red, Natura Nectar, USA), Copaiba Oil (Laboratorio Sao Lucas, Brazil) and Shiitake powder (Border herbal...
Preparation of nano-diamond bioactive containing PMMA materials: general protocol

The bioactive containing gel was prepared by dispersion of 0.2 g of commercially available bioactives (Propolis, Copaiba oil or Shiitake powder) and nano-diamond powder (0.08 g) in glycerol (5% w/w) (1 ml) using a mortar and a pestle following generic protocol [9-11]. 10 ml powder or shiitake mushrooms extract in each gram of the base and then mixed into a PMMA resin prior to setting.

To analyze the bio-active release (propolis, copaiba oil and shiitake mushrooms) based on the total phenolic concentration, the swelling media was analyzed after 1, 2, 24, and 96 h of immersion via UV-Visible spectrometer, from 300 to 800 nm, using polystyrene cuvettes [21]. 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.

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 and 1 mm in thickness, and tested with a gauge length of 3.5 ± 0.4 mm [24]. 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 [25].

Compressive strength

Compressive strength test samples were placed in the measuring apparatus in an appropriate manner and cross-sectional area of each sample (mm²) was determined. A compressive load (N) was applied at a crosshead speed of 1.3 mm/min [26]. The compressive strength (MPa) was measured at the sample fracture point. Mean, average, and mode in each group were calculated and normal distribution curve was evaluated. One-way ANOVA, followed by multiple comparison test (Scheffé’s test), was used for statistical analysis. Statistical significance was set at p<0.05.

Bio-adhesive investigation

Bio-adhesive studies were done using a Chatillon apparatus for force measurement. This method determines the maximum force and work needed to separate two surfaces in intimate contact [27]. The modified nano-diamond biomaterials (0.1 g) were homogeneously spread on a 1 cm² disk and then the disks were fixed to the support of the ‘oral mucosa prototype system’ structure. After a preset contact time of 1 min under contact strength of 0.5 N, the 2 surfaces were separated at a constant rate of displacement of 1 mm/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 [27].

Results

SEM Images of the newly prepared biomaterials

The SEM images were obtained for selective nano-diamond bio-active modified PMMA resins to characterize the microstructure of the freeze-dried and are presented in Figure 1. SEM observations of nano-diamond PMMA-based samples revealed a smooth surface with the formation of the valleys and crests, which could be attributed to the presence of some aqueous medium in the preparation of modified materials.

Mechanical properties investigated

Compression test: PMMA has adequate tensile and compressive strength for complete and partial dentures [28]. The compression...
behavior for composite prosthetic dentures represents the important mechanical properties specialization when using the polymer matrix materials. The compression strength values results obtained from compression tests are carried out for all bioactive prepared materials and results are summarized in Figure 2.

**Tensile strength of the bio-active functionalized materials**

The tensile behavior for composite prosthetic dentures represents the important mechanical properties specialization when using the polymer matrix materials and is summarized in Figure 3. The addition of the bioactive compounds such as shiitake extract, copaiba oil or Brazilian propolis in combination with nano-diamond powder has not influenced significantly the tensile strength of the bioactive PMMA material.

**Swelling/weight loss tests and bioactive release**

The swelling characteristics of the bio-active modified materials at pH 7.0 and pH 4 are shown in Figure 4.

The amount of bio-actives, nano-diamond chitosan (such as Brazilian propolis, shiitake mushroom extract and copaiba oil) release in swelling media was analyzed after 1, 2, 24, and 96 h of immersion (Figure 5). The bioactive phenolic release by polymeric systems usually occurs in two steps: the release of certain amounts of bioactive phenolic component in the first day of swelling as well as a prolonged release in some cases [28].

**Bio-adhesion of PMMA modified materials**

Higher adhesiveness of the modified PMMA resins is desired to maintain an intimate contact oral mucosa and the prosthetic device such as full or partial denture, therefore bio-adhesion between the newly prepared modified bio-active containing PMMA was tested against pig ear skin structure and results are summarized in Table 1.

**Discussion**

**Compressive strength of new biomaterials**

The addition of the bioactive compounds such as shiitake extract, copaiba oil or Brazilian propolis in combination with nano-diamond had significantly influenced into the compression strength of the bio-active PMMA material. Also upon incorporation of nano-diamond-chitosan-bio-active combination (10% w/w) into the PMMA material the compressive strength of the new bioactive material was significantly increased in comparison to the standard PMMA material.

**Tensile strength of bioactive PMMA materials**

Also upon incorporation of nano-diamond-chitosan-bio-active combination (10% w/w) into the PMMA material the tensile strength of the new bioactive material was significantly increased in comparison to the standard PMMA material. The slight increase of tensile strength is probably due to the potential action interference of the bio-actives and their antioxidant capacity with the polymerization rates of the PMMA presence of chitosan acts as the protective host of the excess of free radical formation and therefore some in the tensile as well as compressive strength is observed. The results are consistent with the literature [29,30] and more detailed investigations into mechanistic interaction of chitosan/bioactive/PMMA resin are currently on the way in our laboratory.
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 96 h of immersion (Figure 5).

The ND/Ch/PMMA-bioactive samples were immersed in (a) PBS and (b) Solution pH 4.0 and the bioactive (such as copaiba oil, propolis and shiitake extract delivered was quantified after regular intervals of time for 4 days.

**Figure 5:** Bioactive cumulative release profile of Nano-diamond/Chitosan/PMMA-bioactive samples.

### Table 1: Bio-adhesion table.

| Bio-materials               | Adhesive Force (N) ± SD (Skin) | Work of Adhesion (N cm) ± SD (Skin) |
|-----------------------------|--------------------------------|------------------------------------|
| Shitake/PMMA/ND             | 1.16 ± 0.320                   | 4.35 ± 0.48                        |
| Shitake/PMMA/Chitosan/ND    | 1.23 ± 0.32                    | 4.83 ± 0.42                        |
| Copaiba/PMMA/ND             | 1.26 ± 0.29                    | 4.28 ± 0.31                        |
| Copaiba/PMMA/Chitosan/ND    | 1.35 ± 0.40                    | 4.35 ± 0.65                        |
| Propolis/PMMA/ND            | 1.52 ± 0.28                    | 4.67 ± 0.42                        |
| Propolis/PMMA/Chitosan/ND   | 1.24 ± 0.22                    | 4.51 ± 0.39                        |
| PMMA                        | 1.10 ± 0.34                    | 4.41 ± 0.36                        |
| Chitosan/PMMA               | 0.97 ± 0.30                    | 4.97 ± 0.42                        |

a. Shitake/Nanodiamond/PMMA, b. Copaiba/Nanodiamond/PMMA, c. Propolis (Brazilian)/Nanodiamond/PMMA, d. Shitake/Nanodiamond/Chitosan/PMMA, e. Copaiba/Nanodiamond/Chitosan/PMMA, f. Propolis (Brazilian)/Nanodiamond/Chitosan/PMMA

**Figure 6:** Surfaces of the materials after exposure to artificial saliva and oxygen as an in vitro model for the biofilm formation after 3 weeks of storage.

**Bio-adhesion of PMMA modified materials**

Chitosan hydrogels and chitosan/nano-diamond combinations showed the highest adhesive force and the work of adhesion this can be expected because of the well-known intrinsic bioadhesive properties of chitosan as well as favorable functional surface of nano-diamond-chitosan and its favorable interaction with the bio-active components [40]. The adequate water absorption capacity together with the cationic nature which promotes binding to the negative surface of skin structure can also interpret this results. The additional benefits of the incorporation of the nano-diamonds as a highly functionalized surface which is able to interact with the surface of the skin are currently under investigation in our laboratory.

**In vitro** application of the functionalized biomaterials as protective biofilm-inhibitory surface modification.

The first step for successful colonisation of mucosal surfaces or any other surface by *C. albicans* is adhesion [41]. Although among the functions of propolis, the fungicidal property has already been shown, in this study, we aimed to verify its effectiveness in inhibiting the adhesion of *C. albicans* biofilm on a denture surface (Figure 6). It would be more desirable to develop antimicrobial material characterized by anti-adherent effect whose primary scope is the prevention, not a treatment that might cause undesirable interactions...
with host tissues. It is well documented that hydrophobic interaction could also play an important role in bacterial adherence to nanodiamond-chitosan-bioactive-PMMA surfaces because the electrical forces are minor to the hydrophobic forces, since adherence to a considerable extent occurs even in the presence of this repulsive force [41,42]. The detailed investigation into the physical nature of the process is currently under investigation in our laboratory. Our results are in accordance with the literature report that show while ND-methylulose particles are non-toxic to both pathogens, they show significant anti-biofilm activity. The presence of ND-menthol particles reduces biofilm formation more efficiently than free menthol, unmodified oxidized NDs and ampicillin, a commonly used antibiotic.

**Conclusion**

In this study we demonstrated that the newly prepared bio-active modified PMMA resins are suitable novel bio-active materials capable of comparable performance with the conventional PMMA materials with additional benefit of therapeutic bioactive release as well as potential antimicrobial properties to be demonstrated in vitro. Our findings might be thus a step forward towards the development of alternative non antibiotic based strategies targeting preventing oral candidiasis and related conditions.

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