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Processing of Biopolymers-based, Efficiently Integrated Bilayer Membranes for Use in Guided Tissue Regeneration Procedures

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Abstract

Periodontitis is a chronic inflammatory disease affecting the tooth supporting structures - the alveolar bone in particular, to extent of eventual tooth loss. Among others, the guided tissue regeneration (GTR) procedure including placement of occlusive barrier membrane is very promising approach. Herein we develop chitosan (CHT) / gelatin (GEL) bilayer membranes via successive solvent- and freeze-casting procedures and genipin (GEN)-mediated cross-linking chemistry. By utilizing the auto-fluorescence signal from GEN cross-linking products (i.e. the secondary CHT (GEL) amines and GEN esters), the Confocal Fluorescent Microscopy (CFM) identifies the chemical as well as physical integration between layers’ interfaces, supported also by Scanning Electron Microscopy (SEM) data. The presence of non- and highly μ-porous and pore-interconnecting regions is demonstrated within cross-sections of GEL-prevalent membranes in contrast to the sheet-like organization in membrane with equal components presence. The constant processing conditions onto variable compositions did not significantly affect the pore size distributions (in 1-230 μm range), while pore wall thickness increases up to 220 μm with GEL increase, which also improves the yield stress at compression from 10 to 19 kPa and elastic modulus from 26 to 34 kPa. The further applied rapid mineralization resulted in deposition of non-regular to spherical minerals, containing nonstoichiometric carbonated apatite with Ca/P ration in 1.7-2 range, which demonstrates formation of osseointegrative interface. The intensive, composition-dependent swelling (up to 580%), as well as 67% to 100% weigh loss in 4 weeks in vitro degradation experiment imply on bilayer membranes GTR relevance.

Keywords—auto fluorescence, chitosan, gelatin, guided tissue regeneration, membrane

I. INTRODUCTION

Periodontitis is a chronic inflammatory disorder that affects ~750M people worldwide, being the fourth highest cost disease with up to 10% consumption of healthcare resources. As chronic inflammatory disease affecting the tooth supporting structures, it may destroy the alveolar bone to extent of eventual tooth loss. Further, often-complicated management of tooth loss is positioning of dental bridge or implant, the latter being often compromised by further bone loss around the tooth, i.e. by peri-implantitis. Current therapeutic strategies are placement of autographs, allografts, bioglass, β-tricalcium phosphate, hydrogels, or s.c. flap surgery in conjunction with biomaterials combinations, the use of biological factors [1], etc.

The concept of barrier membrane placement over the bone defects, which allows specific cells to repopulate the isolated spaces present the guided tissue regeneration (GTR). The cell-occlusive, space making, tissue integrative, clinically manageable and biocompatible are attributes to “ideal” membrane, for which the large diversity of (non)biodegradable, and bioactive materials have been used [2, 3]. Chronologically of the GTR membranes starts with the non-bioresorbable types (polytetrafluoroethylene, titanium, bacterial cellulose, cellulose acetate, silicon, rubber), continues with the bioresorbable (polylactic acid), poly(glycolyc acid), poly (ε-caprolactone), collagen type I, porcine skin, bacteria-derived polymers, hydroxyapatite/polyamide mixture, amniotic membrane, polyethersulfone, calcium phosphate, β- tricalcium phosphate, latex), and up-grade to bioactive types containing antibiotics (metronidazole benzoate, tetracycline hydrochloride) and growth factors (fibroblast growth factor /FGF-2/, platelet-derived growth factor /PDGF/, insulin-like growth factor /IGF-I/, bone morphogenetic protein /BMP-2/, transforming growth factor /TGF-b/). [4]-[6]
Despite diversity, their limited and random success have been reported. Among reports is also obvious lack of attention to μ-structural features as highly relevant for materials intended to interface with- and integrate to-structurally diverse tissues, such in case of periodontal tissue.

Within presented study, we attempt to develop bilayer membranes with μ-structural diversity by merging two straightforward manufacturing processes, the solvent-casting and temperature-controlled freeze-casting, and by using mixture of biocompatible polymers being well elaborated in biomedical applications, i.e. the chitosan (CHT) and gelatin (GEL). Upgraded concept in μ-structuring follow-up by means of CFM and SEM microscopies is also presented, as well as comprehensive spectroscopic evaluation of physico-chemical events, with particular attention on rapid mineralization process outcome onto the membranes.

II. METHODS AND PROCEDURES

2% w/v of CHT solution in acetic acid and 10% w/v of GEL solution in 0.1M MES (pH 5.5) buffer were prepared as stock solution, future used in membranes casting. Membranes’ library was obtained by using particular volume of each of the biopolymer solutions in following CHT/GEL weight ratios: 1/1 (A), 1/5 (B), 1/10 (C) and 1/20 (D). The 5% w/v genipin (GEN) solution was added to each of the solutions being further casted onto Teflon Petry dish and oven dry for 48h at 40 °C. The same biopolymer mixtures were subsequently applied onto the solvent-casted membranes and freeze-dried at constant temperature (-20 °C). The rapid mineralization procedure was applied on final bilayer structures, according to reported procedure [7] being modified in term of duration and final pH .

Attenuated Total Reflectance- Fourier Transform Infrared (ATR-FTIR), X-ray Diffraction (XRD) and Energy Dispersive X-ray (EDX) spectroscopy, the SEM and CFM microscopy, swelling and degradation profiles (in PBS at 37 °C) as well mechanical testing at compression in dry and wet state were assessed for all CHT/GEL combination membranes and related to processing factors.

III. RESULTS AND DISCUSSION

Physical linking between both, CHT and GEL components is assessed form ATR-FTIR data before and after GEN cross-linking and compared to both controls with typical spectral band positions (as presented within Table 1).

### Table 1. ATR-FTIR band positions for chitosan (CHT) and gelatin (GEL).

| Chitosan (CHT) | Wavenumber (cm⁻¹) | peak assignment | Gelatin (GEL) | Wavenumber (cm⁻¹) | peak assignment |
|---------------|------------------|----------------|--------------|------------------|----------------|
| 3600-3000     | OH               | 3000-3000      | OH           | 3000-3000        |
| 3237          | -NH₂ str.        | 3292           | N-H str. (amide A) |
| 2921          | -CH₂ str. in pyranose ring | 3070 | N-H str. (amide B) |
| 1635          | -C=O (amide I ) | 1630           | C=O str. (amide I) |
| 1539          | -NH₂ bend. (amide II) | 1534 | N-H bend.; C-N str. (amide II) |
| 1403          | OH, CH in the ring | 1330 | N-H bend.; C-N str. (amide III) |
| 1350-1200     | -C=N (amide III) | 1404           | C-H str.      |
| 1230          | -C=O; C-H        | 1175           | C-O str.      |
| 1152          | -C=O-C          | 1036           | C-O str.      |
| 1063, 1018    | -C=N, -C=O str. |                |               |

The H-bonding as well as ionic interactions (due to the presence of fixed charges within CHT and GEL biopolymers) are physical interaction forces, which are however not sufficient to keep the integrated and stable membrane within application scenario. The complementary use of GEN for chemical linking is straightforward approach, being applied in mixing phase while cross-linking occurs within extended period.

The Fig.1. Identifies the cross-linking related conformational changes within CHT and GEL because of structural rearrangement during formation of new bonds. According to the literature[7] two-way reaction mechanisms are possible. First reaction is characterized by the increase of C-N band at ~ 1078 cm⁻¹ on expense of C-O band at ~ 1026 cm⁻¹, combined with the decrease in intensity of protonated amine at ~ 1576 cm⁻¹, and should endows with opening of dihydropyran ring in GEN molecules, the formation of nitrogen-ridoid, the aromatic intermediates, as well as the formation of highly conjugated heterocyclic GEN-CHT (GEL) derivatives. The increase of ration between area of absorption bands at 1640-1650 cm⁻¹ and 1570-1580 cm⁻¹ for ν (C=O) and ν (N-H), respectively, in cross-linked (A, B, C and D) relative to non-cross-linked (Ac, Bc, Cc and Dc) membranes can be attributed to formation of secondary amino groups within CHT and/or GEL, and ester groups of GEN, being identified as second, slower reaction under which single bi-functional cross-link between CHT and/or GEL molecule are
formed. The presented graph inset (Fig. 1, below) imply on strong correlation between the mentioned ratio and composition, i.e. on CHT promotion of second reaction products.

Fig. 1. FTIR spectral data of CHT/GEL membranes with different (1/1-A, 1/5-B, 1/10-C and 1/20-D) w/w ration before and after cross-linking with GEN (left), with extracted data for peak area ration between carbonyl $\nu$ (C=O)/ and amino-related band $\nu$ (N-H) at ~1630 cm$^{-1}$ and ~1540 cm$^{-1}$ (right). The subscript c holds for control.

By utilizing the auto-fluorescence signal from GEN cross-linking products (i.e. the secondary CHT (GEL) amines and GEN esters), the CFM micrographs identify the chemical inter-linking as well as physical integration between layers’ interfaces. The presence of non- and highly $\mu$-porous and pore-interconnecting regions is demonstrated (Fig. 2.) within cross-sections of membranes with (by weight) prevalent GEL contribution in contrast to the sheet-like organization in membrane with equal components presence as presented in Fig. 2.

Fig. 2. CFM images from different composition (A-C) membranes (left) with respective image analysis of pore size/wall thickness distributions (right).

The constant processing conditions onto variable compositions did not significantly affect the pore size distributions (in 1-230 $\mu$m range), while pore wall thickness increases up to 220 $\mu$m with GEL increase.

In parallel to CFM, the SEM microscopy was also undertaken, and the bottom-up inspection of SEM micrographs (Fig. 3) reveal microstructurally distinct regions also varying among different membranes.

Fig. 3. SEM images from different composition (A-left and C-right) membranes in dry state.
Bottom-most section reveal the presence of dense skin (visible on membranes B and D, not presented) resulting from lyophilization process of solvent-casted layer when sharp increase in the polymer density at the film/vapor interface occurs, creating a polymer density gradient in layer thickness direction. In respect to GTR application, this layer is expected to interface with soft tissue, providing barrier function against fibroblasts penetration. Since the solvent casting was not completed to zero water content by freezing, the remaining porosity was induced also in a solvent-casted layer as seen in membranes A and C (Fig. 3) which is expected to maintain a good nutrition flow and metabolic exchange for cell proliferation and tissue growth in potential TE applications. Moreover, the pore wall thickness, as well as pore size were relatively lower in membrane A comparing to the others. This membrane significantly deviate between samples library due to specific, sheet like, anisotropic orientation parallel to the mould bottom, being assigned to typical CHT molecules arrangement. Such aligned structure combined with porous CHT was previously demonstrated to support the functional regeneration of periodontium, being demonstrated with extensive formation of mature collagen fibers perpendicular against the root surface and formation of tooth supporting mineralized tissue. On the other hand, the increment of GEL fraction increases the membrane density and at the same time reduce the structure heterogenicity, most prominently in GEL-rich membrane D.

The rapid mineralization process was successfully applied onto membranes surface. The random presence of spherical minerals (in Am and Dm) (Fig. 4) or their irregularly shaped clusters (in Bm and Cm) are visible, in higher quantity within Cm and Dm membranes. The respective EDX data (SEM images insets) reveal slight differences in Ca/P ratio among and within different regions of membranes, all in range 1.7-2, which, when supplemented with FTIR (where carbonate peak position was assigned to substitution of phosphate ion when B-type HAp is formed) and XRD findings (the broadening of 2θ at 32° due to substitution) indicates on presence on nonstoichiometric carbonated apatite minerals. The Ca/P ratio within apatite mineral phase of different tooth- or tooth surrounding tissues are summarized in literature as 2.3–2.4 for enamel, 1.63–2.01 and 1.48–1.55 for bone and pathologic bone, respectively, 1.3–1.97 for cementum, 2.1 for dentin, 1.9 for hypo mineralized dentin, and 0.9–1.7 for dental calculus. This suggest that compositions of minerals obtained by this rapid process demonstrate is chemically suitable for alveolar bone regeneration. The XRD and FTIR data (not presented), suggest on the same findings.

Fig.4. SEM micrographs with EDX (insets) of mineralization events in cross-sections of CHT/GEL membranes with different (1/1-Am, 1/5-Bm, 1/10-Cm and 1/20-Dm) w/w ration, cross-linked with 0.5 wt% GEN, after 12h incubation in 10xSBF media. The subscript m stand for mineralized membranes.

The other (GTR) relevant bulk property is mechanical stability, which is affecting the formation of tissues from proper cells phenotype and site-specific properties. On macroscopic level, GTR-intended materials must bear loads to provide stability to the tissue and to allow proper handling by surgeon handling during implantation, while microscopically, the mechanical input to cells is important clue impacting the cell growth and differentiation. Fig.5. describes the stress-strain behavior of dry (a) and fully wet (b), GEN cross-linked CHT/GEL membranes, being typical for porous biomaterials.

In both states, two major regions can be identified, first, the region of elasticity (before yield point), characterized by linear increase in stress with strain and second, the deformation (post yield point) region, where rapid increase in stress imply on pores irreversible collapse under the compression forces.
The very initial hyperbolic region which may be also identified, is excluded from these division as we assumed it as region for machine and sample adaption (including the air bubbles escape from hydrated material, as well as sample levelling to get in touch with both instrument plates between which compression occur), after which the real mechanical response curve is obtained.

![Stress-strain curve from compression testing of dry (a) and wet composites (b) with different composition (denominated as A, B, C, D).](image)

The GEL component increase (from A to D) obviously improves the yield stress at compression from 10 to 19 kPa and elastic modulus from 26 to 34 kPa, both in wet state, as more relevant for envisioned application. On the other hand, the two log higher values are measured for parameters in dry state, which however indicates its mechanical stability at manipulation (in dry). The fast and high (up to 580%), composition dependent swelling, as well as 67% to 100% weight loss in 4 weeks in vitro degradation experiment point on membranes’ relevance in GTR.

The material swelling profile as well as physiological stability, even bulk-related properties are important aspects in biomedical applications, since defect side (e.g. periodontal pocket) has limited space for membrane positioning and filler effect is desirable since provide multiple cues for efficient regeneration. Data (not presented) demonstrate fast weigh increase in first 5 min and equilibrium reached in less than 1 hour, irrespective of the composition, with increment of the swelling % at equilibrium (Figure 8b) in order from D (580%) to A (180%), i.e. from low to high
CHT content. Very convenient regression coefficient ($R^2 = 0.999$) obtained by curve fitting with second order polynomial function was obtained for CHT/GEL (w/w) ratio vs. swelling (%) at equilibrium correlation, implying straightforward regulation of swelling profile by simple composition change, i.e. ~3 times reduction of swelling % was observed by 20 times reduction of CHT mass in respect to the GEL component. Obtained data for in vitro degradation test, reveal the reverse trend (from A to D) than swelling data after 4 incubation weeks, where GEL-rich membrane (D) was 100% degraded, while membrane with (by weight) equal components contribution (A) degrade to 2/3rds of ist weight, due to CHT insolubility in physiological media. It worth mentioned that irrespective of composition, the porous part was firstly degraded while dense layer maintain its shape up to 4 weeks in case of A and B membranes.

IV. CONCLUSION

Solvent casting, followed by T/time controlled, unidirectional freeze-casting process, is demonstrated to be feasible method for constructing physiologically and mechanically stable, well integrated, and μ-structurally diverse, 3D membranes from CHT and GEL, having proved biocompatibility. Important outcome herein is utilization of auto fluorescence signal of genipin-CHT/GEL cross-linking product by CFM for demonstration of physical (by identified μ-roughness) and chemical (by developed fluorescence signal) integration between layers. Further functional (biological) testing of developed composites (i.e. penetration test, viability and proliferation, as well as antimicrobial action) as GTR membrane for potential periodontal applications are envisioned.

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