Marine Algae-PLA composites as de novo alternative to porcine derived collagen membranes

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1. Introduction

The lack of a favorable soft tissue profile at the implant recipient sites poses a great challenge in the daily medical practice. As an alternative to the autogenous connective tissue graft harvesting, which is suggested to be associated with significant disadvantages such as donor site morbidity and restrictions in size of the connective tissue graft, porcine-derived, resorbable and volume-stable collagen matrices have been preferred for clinical applications in recent years [1]. These biomaterials mainly consist of reconstituted collagen which underwent smart chemically cross-linking to improve its volume stability which maintains a good biocompatibility. It has been also proclaimed that the porous network of these materials supports angiogenesis, the formation of new connective tissue and stability of the collagen network [2]. However, despite recent advances in the decellularization processes or precautions such as microbiological screening of source animals, there is always an existing risk of transmission of infection with the cells or the tissues of the graft [3]. Additionally, considering the economic aspects, there is also a need for cost-efficient alternatives.

Carbon-based materials are promising for various tissue engineering applications thanks to their advantages of being biologically compatible, mechanically stable, and especially their abundance [4–6]. Similarly, marine algae are abundant, and they represent a vast and cheap source of potential biopolymers [7]. Owing to their proven biocompatibility and biodegradability natural and synthetic biopolymers have been utilized as graft materials [8–11]. There are various types of algae species that vary with respect to their structures and compositions. Different types of marine algae in combination with synthetic biopolymers have been shown to exhibit diverse effects such as anticancer, antibacterial and enhanced proliferation properties [12–14].

In general, marine algae powders (MAPs) are blended with polyesters including poly(caprolactone) (PCL), poly(hydroxy alkanoates) (PHA), and polylactide (PLA) to enhance their applicability in biomedical applications [15]. Among others, PLA has received the most attention due to its non-toxicity, biocompatibility, and easy

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processability [16]. PLA is also applicable to various biomedical products such as sewing needles, sutures, bone screws, braces, bandages, and wound dressings for surgical operation [14]. It has been shown that blending MAP with PLA in the composite form exhibited superior mechanical and thermal properties [17]. Furthermore, both MAP-PLA composites show better cytocompatibility than pure PLA [14]. In general, the cellular interaction on a biomaterial surface is governed by the surface chemistry and topography [18–21]. Processing methods for preparing MAP-PLA composites, such as melt casting, electrospinning, etc., lead to severe changes both in chemical characteristics and as well as the surface topography [22]. Therefore, it is not trivial to reveal neither the sole effect of the surface chemistry nor the surface topography effect on the cell adhesion and proliferation on MAP-PLA composite surfaces. The presence of thousands of different algae types, which vary with respect to their chemical composition, makes the situation more complicated.

The goal of this work was to perform a comprehensive cytocompatibility (utilizing cell proliferation, MTT, and cytotoxicity, WST assays) of different types of MAP-PLA composites blended with five different types of MAPs and PLA. The main attention was given to keep the surface topography of samples as identical as possible to reveal the sole effect of algae origin on the cellular interaction with prepared MAP-PLA surface.

2. Material and methods

2.1. Preparation of MAP

One can see the photographs of five different types of algae; Corallina elongata, Galaxaura oblongata, Cystoseria compressa, Sargassum vulgare, and Stypopodium schimperi (Fig. 1a). While the first three are red algae and the rest are known as brown algae. First marine algae were collected from two different regions: Corallina elongata (Rhodophyta) and Galaxaura oblongata (Rhodophyta) were collected nearby Antalya-Turkey and Cystoseria compressa (Phaeophyta), Sargassum vulgare (Phaeophyta), and Stypopodium schimperi (Phaeophyta) were collected nearby Iskenderun-Turkey of Mediterranean Sea. Sampling was performed in SCUBA and free dives between 0 and 40 m depth and vertical and horizontal scans underwater. During sampling, underwater photographs of macroalgae were taken with the “Olympus OM-DE-M5” camera. The identification of the materials was carried out with SZX16 stereo zoom and BX51 binocular light microscopes and immersed in 1000 ml of distilled water for 72 h to remove any water-soluble components. After filtering, the marine algae were grinded subsequently 4 times in a high-speed rotary grinder operating at 300 rpm for 10 min and vacuum-dried at 50 °C for 24 h (as schematically shown in Fig. 1b). Dried marine algae were once more grinded and then sieved (first through 200-mesh and then 400-mesh sieves) and vacuum-dried at 100 °C for 12 h. Finally, we achieved micro-scale MAPs with an absolute moisture below 3%. (Authors thank to NanoBMT Co. Ltd. for the preparation and the dispersion of MAPs.)

2.2. Preparation of composites

MAP samples as described above were washed with acetone and dried in an oven at 80 °C for 24h. Then PLA (Goodfellow, 459-898-81) and MAP were mixed in a mechanical mixer operating at 150 °C for 15 min at a rotor speed of 50 rpm [23]. After mixing, the composites were pressed into thin plates with a custom hot press at 180 °C and 8 MPa for 20 min and then placed in an oven for cooling (The glass transition temperature of algae especially for Sargassum vulgare is known around 175 °C by differential scanning calorimetry (DSC) analysis) [24]. Afterwards, thin plates were cut into 1 cm x 1 cm sample dimensions for further characterization. These samples were named as: Type I MAP-PLA (Corallina elongata), Type II MAP-PLA (Galaxaura oblongata), Type III MAP-PLA (Cystoseria compressa), Type IV MAP-PLA (Sargassum vulgare) and Type V MAP-PLA (Stypopodium schimperi).

2.3. Material characterization

Digital camera and optical microscope were used to reveal the macro morphological properties of five different PLA-MAP
composites after processing and drying steps. Afterwards, samples were sputter-coated with a thin (20–30 nm) Au layer prior to SEM analysis. Supra55VP-Carl Zeiss operating at 3 kV was used to compare morphological differences of samples at the sub-micron scale. FTIR spectra of dry samples have been acquired withBruker Vertex 80v spectrometer operating in the range of 400 cm⁻¹ and 3400 cm⁻¹. The baseline correction was done using built-in software.

2.4. Cytocompatibility analysis

Cell viability (WST) and proliferation (MTT) characteristics were comparatively evaluated by using porcine-derived collagen membrane (F, Geistlich Bioguide ®, Switzerland) as the reference and glass (G) as the control. Human fibroblasts were isolated from gingival tissues from the patients who underwent oral surgical interventions in the Department of Oral and Maxillofacial Surgery at Christian-Albrechts-Universität zu Kiel. Briefly, adherent non-gingival tissues were mechanically removed from the specimens, cut into fragments of 0.3–0.5 cm in diameter and extensive washing in phosphate-buffered solution (pH:7.4) was performed. The fragments were seeded as explants into the culture flasks and cultivated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Culture medium was Dulbecco’s modified eagle’s medium, supplemented with fetal calf serum, 10⁵ penicillin, 100 mg/L streptomycin, 2 mM L-glutamine, 100 mM dexamethasone and 1 mM L-ascorbic acid 2-phosphatase (Sigma, Deisenhofen Germany). Cells were subcultures in a second passage at a density of 3.3 x 10⁵ fibroblast-like cells in the second passage were transferred onto the matrix samples.

2.4.1. MTT assay

The MTT assay was applied to evaluate the proliferation and viability of fibroblasts on MAP-PLA composites. MTT salt is cleaved by mitochondrial dehydrogenase in the metabolic active cells and is reduced to an insoluble formazan crystal which displays a purple color. The color was detected by a Multi-scan MS spectrophotometer (Labsystems, Stockholm, Sweden). The In vitro Cell Proliferation KIT 1 (Roche, 11465007001, Mannheim, Germany) was used.

2.4.2. WST assay

At indicated time points, 20 μL of the WST-1 pre-mixed reagent (Cell Proliferation Reagent WST-1 (Roche Molecular Biochemicals, Basel, Switzerland) were added to each well and the plates were incubated at 37 °C for 1 h, as recommended by the manufacturer. Then, the plates were placed on a shaker for 1 min and the absorbance was determined in a microplate reader at 450 nm. WST assay (p < 0.05) in each time point, revealed that the overall trends of cell proliferation were increased similar in all groups without statistically significant differences.

All experiments were performed in triplicate and following the 6, 12, 24 and 72 h of incubation. The experiments were performed in triplicate, and each result is reported as the mean ± SD. Data between three or more groups were compared using the one-way analysis of variance, followed by the Dunnett’s post hoc test. P < 0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Material properties

Fig. 2 shows representative photographs of MAP-PLA samples prepared using five different algae types. There is a clear difference in the color of samples since MAPs were extracted from two different algae families; brown, and red algae. All composites exhibit a similar morphology composed of macroscale pores (which might arise due to surface tension difference between melt PLA and MAPs) and microscale pin-lock type defects (which may arise due to heating-cooling cycles). While the pore size of samples ranges from 300 μm to 750 μm, the size of pin-locks is around 30–50 μm (Fig. 2). Macro-scale pores were observed in all types of MAP-PLA composites and these might be beneficial for attachment and proliferation of cells, as well as nutrient diffusion and transport [25].

Our SEM analysis did not reveal the presence of a distinct secondary topography within and nearby macropores and all samples exhibited a typical polymer melt structure at the sub-micron scale (Fig. 2). On the other hand, we observed different wetting behavior (Fig. 3a) which may be related to the chemical composition differences. While *Corallina elongata* based Type I MAP-PLA showed the highest contact angle (CA = 109.5°), *Cystosera compressa* based
sample showed the Type III MAP-PLA lowest CA (77.3°). Although several studies reported that at the early stages the cell adhesion is more favored on hydrophilic surfaces, protein adsorption tends to occur more favorably on hydrophobic surfaces than on hydrophilic surfaces [26]. Definitely, there is a high number of publications that show that hydrophilic surfaces had a high initial rate of cell attachment [27]. On the other hand, the wettability of the surface is the cell-specific parameter for predicting cellular adhesion and proliferation [28]. For instance, Tamada et al. reported that fibroblasts adhered and proliferated at the highest rate on a relatively hydrophobic surface with CA of around 60°–90° [29]. This may explain why Type III MAP-PLA and Type IV MAP-PLA exhibited the highest growth and proliferative capacity, as seen in Fig. 4a.

FTIR spectra were recorded from all MAP-PLA composite types (Fig. 3b). In general, the chemical composition of algae varies with respect to their type and with other aspects such as climate conditions. The broadening around 1150 cm⁻¹ might be correlated with C=O–C vibrations of cellulose [30]. Here one should keep in mind that cellulose content may vary with respect to the algae type. Here, our analysis indicated that red algae seem to have higher content of cellulose. This is in accordance with our observation that Type I MAP-PLA and Type II MAP-PLA composite plates were slightly stiffer than others. Slightly visible band in the spectrum of red algae is visible at 1200–1250 cm⁻¹ which can be linked with P=O [31] and S=O [32], which are typically present in carrageenan of red algae. Chen et al. reported that carrageenan oligosaccharides showed a low cytotoxic effect at rational concentrations [33]. They showed that at relatively low concentrations, these oligosaccharides may bind basic fibroblast growth factor (bFGF) and inhibit bFGF induced cell proliferation. Peaks observed at 850–860 cm⁻¹ and 1370-1380 cm⁻¹ (PLA also exhibits a peak 1365–1385 cm⁻¹ assigned as the symmetric bending absorption of CH₃) can be attributed to sulfated groups [31]. Broad peak around 2829–2964 cm⁻¹ and relatively sharper peak at 1471 cm⁻¹ should be assigned to CH₃ stretching mode and the asymmetric bending absorption of CH₃ respectively and these are in accordance with typical bands observed in neat PLA [34]. This basically indicates an effective incorporation of MAPs with PLA. Beyond 1471 cm⁻¹ there is a clear broadening in case of Corallina elongata, and Galaxaura oblongata (both are red algae) which might correspond to N–H bending [35] and it is not present for brown algae (Cystoseria compressa, Sargassum vulgare, and Stypopodium schimperi). Broadening of an absorption peak 1620–1650 cm⁻¹ is present in all cases and this may be assigned to carboxylic groups [17]. Gep et al. reported that carboxyl groups of alginates are involved in the crosslinking of biomolecules and proteins or other bioactive compounds can directly be coupled on the alginate’s surface via these highly active groups [36]. In overall, FTIR spectra shows some distinct differences between brown algae and red algae with the literature [37].

3.2. Cytocompatibility properties

The MTT test showed that the growth and proliferative capacity of cultured fibroblasts on Type IV MAP-PLA was significantly higher than that of all groups during the whole examination period.

Fig. 3. (a) Contact angle analysis results and (b) FTIR spectra of prepared MAP-PLA composites.

Fig. 4. (a) MTT and (b) WST assays (OD:Optical density).
(p < 0.05) (Fig. 4). The increase in acceleration of proliferation in all groups could be attributed to the reduced proliferation ability regarding the increase of fibrobasts in culture flask.

The biomaterials analyzed did not show cytotoxicity, since no membrane presented lower results than the control group. Type IV MAP-PLA presented the best performance due to its higher cell viability and superior levels of proliferation. All membranes showed similar results either when compared to each other or to the control group. Cytotoxicity assays have been developed based on different parameters like metabolic activity and DNA synthesis associated with cell viability and cell proliferation [38]. MTT measures basically the mitochondrial activity of liability cells and WST-1 works similarly with MTT by reacting with the mitochondrial succinate-tetrazolium reductase forming the formazan dye [39]. Both tests are suggested to be precise methods in the evaluation of biocompatibility, especially when fibrobasts cells and cell lines were used [40]. However, the main superiority of the WST-1 test is the completion of its quantification in 0.5–4 h without an additional solubilization step, where MTT assay requires 52–72 h to complete. In the present study, both MTT and WST-1 tests were used to evaluate and confirm the effects of different compositions on the viability of fibrobasts in vitro. It has been observed that both WST-1 and MTT showed similar tendencies, but their numerical results were slightly different without statistically significant differences. This numerical discrepancy could be attributed to differences in water-solubility and storage condition of both tests. In the current study, both tests have confirmed the biocompatibility of the algae-PLA composites, since no biomaterial presented lower results than the control group. Surprisingly, WST results of the 6th hour examination revealed minimally higher values in the control group compared to the 1st, 2nd 3rd and 4th membranes, however, according to the results of the 12th, 24th and 72nd hour examinations, algae-PLA composites showed significantly higher proliferation rates, which have also testified the biocompatibility of the bio-materials tested.

In recent years, there is a growing interest on the use of algae-derived products in medicine. The main advantages of these materials were their cost efficiency and the lack of potential risk of transmission of infection with the cells or tissues of a xenograft [41]. Additionally, there is also a problematic for a growing minority of patients who may have religious or secular concerns about the use of animal-derived constituents in their care [42]. Considering this, the algae-derived membranes could be an alternative by the management of these patients.

4. Conclusion

Cell viability and proliferation assays have showed that all membranes exhibited appropriate cell viability during the whole examination period. However, Type IV MAP-PLA have showed superior proliferation characteristics compared to all other groups including commercially purchased membrane (reference membrane). Considering that, the use of algae composition which was used for the production of this biomaterial could be a feasible option in clinical practice such as guided tissue regeneration, connective tissue augmentation, and wound regeneration. However, further experimental and clinical studies are needed to clarify the exact characteristics of this composition.

Conflict of interest

There are no conflicts to declare.

References

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