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REVIEW

Chitosan-Based Matrices Prepared by Gamma Irradiation for Tissue Regeneration: Structural Properties vs. Preparation Method

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Abstract In the last decade, new generations of biopolymer-based materials have attracted attention, aiming its application as scaffolds for tissue engineering. These engineered three-dimensional scaffolds are designed to improve or replace damaged, missing, or otherwise compromised tissues or organs. Despite the number of promising methods that can be used to generate 3D cell-instructive matrices, the innovative nature of the present work relies on the application of ionizing radiation technology to form and modify surfaces and matrices with advantage over more conventional technologies (room temperature reaction, absence of harmful initiators or solvents, high penetration through the bulk materials, etc.), and the possibility of preparation and sterilization in one single step. The current chapter summarizes the work done by the authors in the gamma radiation processing of biocompatible and biodegradable chitosan-based matrices for skin regeneration. Particular attention is given to the correlation between the different preparation conditions and the final polymeric matrices' properties. We therefore expect to demonstrate that instructive matrices produced and improved by radiation technology bring to the field of skin regenerative medicine a supplemental advantage over more conservative techniques.

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1 Introduction

Scaffolds are three-dimensional supports that are used as a template at the body site injury to help in guiding cell growth, regeneration, and secretion of their own extracellular matrix (ECM), thereby assisting the body in growing new, functional tissue. Scaffolds work in two ways; they either help direct cell growth or simply provide a shape for the final tissue. To be used as a scaffold in tissue engineering, a material must satisfy a number of requirements: it should not elicit severe inflammatory responses and it should degrade into non-toxic compounds within the time frame required for new tissue formation. Alongside the scaffolds should be suitable porosity for cell in-growth, a surface that balances hydrophilicity and hydrophobicity for cellular attachment, and mechanical properties that are compatible with those of the tissue as well as maintaining mechanical strength during most part of the tissue regenerating process.

Engineered scaffolds are thus designed to augment or completely replace damaged, missing, or otherwise compromised tissue or organs. These scaffolds may be permanently integrated into or bioresorbed by the body and must not only be capable of mimicking the structure and biological functions of ECM but should also provide a good environment for the cells so that they can easily attach, proliferate, and differentiate. The ECM defines the three-dimensional architecture of an organ and is engaged in a complex relationship with the cellular elements of the surrounding environment. Consequently, communication between the cell and ECM molecules influences various cellular processes, such as adhesion, proliferation, differentiation, migration, and apoptosis, as well as growth factor and cytokine modulation. Moreover, the timing of these events critically affects tissue formation and remodeling, processes that are crucial for the integration of a tissue-engineered scaffold into the surrounding environment [1]. In the case of skin, a double-layered organ whose deep-partial and full-thickness wounds pose serious threats to preserving its integrity and normal functions, it is well established that severe damaged skin requires a protective barrier for proper healing [1, 2]. Thus, the ideal skin scaffold should have high liquid absorbing capacity and be a biodegradable, biomimetic, and multilayered 3-D structure comprising dermal and epidermal equivalents. However, regardless of tissue type, a number of key considerations are important when designing or determining the suitability of a scaffold for use in tissue engineering; besides the biocompatibility, biodegradability, and mechanical properties already referred to, scaffold architecture and manufacturing technology are also determinant factors [2]. Considering this, it is not difficult to realize that the design of an ideal tissue-engineering scaffold is one of the most important challenges in regenerative medicine.

New generations of synthetic biomaterials are being developed at a rapid pace. Particularly biopolymer-based hydrogels [3], nano-scale-size fibers composed of natural and/or synthetic materials [4–6], and ceramics [7] have attracted

attention in the last decade aiming its application as scaffolds for tissue engineering. A number of methods can be used to generate instructive matrices to be employed in tissue engineering (casting [8], electrospinning [9, 10], plasma [11], etc.). However, despite the potential use of radiation technology to facilitate the development of tissue engineering, only a few studies have been reported in the preparation of instructive scaffolds and their sterilization using this technology [12–15]. The application of radiation technology for formation and functionalization of surfaces and matrices has remarkable advantages such as: room temperature reaction, absence of harmful initiators or solvents, and high penetration through the bulk materials. Additionally, radiation-synthesized scaffolds and surfaces might be simultaneously functionalized and sterilized.

Chemical formulations under study combine the use of natural and synthetic polymers in an attempt to take advantage of the biological activity of the natural materials and the hydrophilicity and mechanical strengthness of the synthetic ones. Natural materials, owing to their bioactive properties, display better interactions with the cells, and in that way enhance the cells' performance in biological systems. A good example is the frequent use of polysaccharides and proteins (like chitosan and collagen) due to their biocompatibility, biodegradability, and similarity to macromolecules recognized by the human body, which partly mimic the ECM of tissues. Inducing and stimulating the wound-healing process, these natural polymers are involved in the repair of damaged tissues and consequently in skin regeneration. Simultaneously, synthetic polymers are highly useful in the biomedical field since their properties (e.g., porosity, degradation time, and mechanical characteristics) can be tailored through gamma irradiation for specific applications. Some synthetic polymers like poly(ε-caprolactone), which is a biodegradable polyester in physiological conditions, poly(vinyl alcohol), a hydrophilic biocompatible polymer and others also exhibit wound-healing properties and enhance re-epithelialization [14–17]. Compounds with plasticizer, humectant, and/or crosslinking properties, with good human body tolerance (e.g., glycerol), have also being studied as additives to polymeric matrix formulations.

Thus, blends/copolymers with natural and synthetic materials are believed to be an effective way to develop a tissue-engineered material.

We have been working on the consolidation of the ionizing radiation techniques for the preparation of new materials for biomedical applications in C^2TN/IST aiming to establish a strong synergy between materials and biomedical studies, consolidating thus in a unique research laboratory a real bridge between these two complementary areas. In this framework, authors have been carrying out a systematic study in order to simultaneously prepare/optimize and sterilize three-dimensional biocompatible and biodegradable skin scaffolds by γ-irradiation. The main components used in the present study were chitosan, poly(vinyl alcohol) and glycerol. A brief description of the materials used in the preparation of the scaffolds discussed in this chapter is outlined next.

1.1 Chitosan

Chitosan (Chit) is a linear polysaccharide composed of poly-β(1-4)-D-glucosamine and poly-β(1-4)-D-acetylglucosamine as shown in Fig. 1a. It is a cationic polysaccharide of natural origin that is obtained by alkaline deacetylation of chitin, the main exoskeleton component in crustaceans, and one of the most abundant natural polymers. Due to an unusual combination of properties such as non-toxicity, biocompatibility, biodegradability, bioactivity, acceleration of wound healing, fat-biding capacity, etc., chitosan and its chemically modified structures have been subject of many studies for use in biomedical and pharmaceutical applications [16–22]. Chitosan bears two types of reactive groups: the C-2 amino groups on deacetylated units and the hydroxyl groups on C-3 and C-6 carbons on acetylated or deacetylated units [23]. In an acidic medium or without a catalyst, the reaction takes place at the amino group [24]. Furthermore, chitosan, being a polysaccharide, is known as a degradative-type polymer when γ-irradiated [25, 26]. One of the strategies to overcome this is the introduction of a crosslink-type polymer to the reactional system (e.g., 2-hydroxyethyl methacrylate, HEMA), which may result in a new matrix prepared/functionalized by gamma irradiation that combines the useful properties of both polymers [27]. Additionally, in order to obtain a sponge-type porous structure, blends were irradiated in a dry state.

1.2 Poly(vinyl alcohol)

Poly(vinyl alcohol), PVA (vd. Fig. 1b) is a water-soluble, white (colorless), and odorless synthetic polymer. It has a crystalline nature associated with good

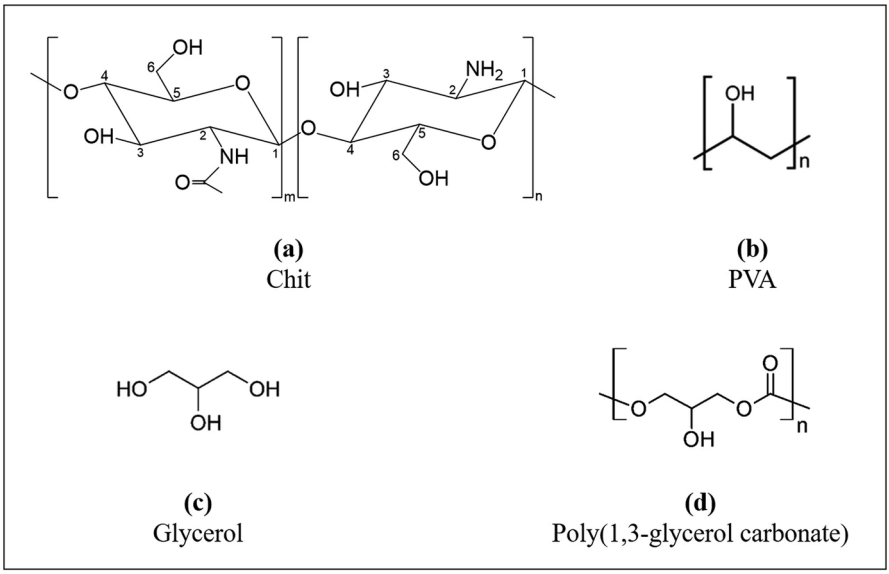


Fig. 1 Chemical structures: **a** chitosan; **b** poly(vinyl alcohol); **c** glycerol; **d** poly(1,3-glycerol carbonate)

mechanical (high tensile strength and flexibility) and barrier properties, excellent film-forming, emulsifying, and adhesive properties as well as good thermal stability. It also shows good resistance to organic solvents. However, these properties are dependent on the hydration level, since water, acting as a plasticizer agent, reduces its tensile strength, accelerating its degradation [28]. Due to its biocompatibility, nontoxicity, and the ability to easily form physically cross-linked hydrogels, the use of PVA and PVA blends has being reported successfully in several biomedical and pharmaceutical applications and still continues to be a very promising material [29]. PVA has multiple pendant alcohol groups that can work as attachment sites for biological molecules and/or cells as well as its elasticity can induce cell orientation or matrix synthesis by enhancing the transmission of mechanical stimuli to seeded cells [30].

PVA is known to be a truly biodegradable synthetic polymer since the early 1930s [31]. However, its biodegradability very much depends on the degree of polymerization, degree of hydrolysis, distribution of hydroxyl groups, stereoregularity and crystallinity. As so, the degradation rate of PVA could be controlled through these parameters. For instance, the creation of crystalline regions in PVA through physical crosslinking improves its mechanical integrity and reduces the respective water absorption capacity [32], which consequently reduces the PVA degradation rate by hydrolytic mechanisms once in contact with body tissues [33]. This behavior can be used in PVA blends with natural fast degradative polymers (e.g., chitosan) to overcome the weak physical integrity of these. It is therefore obvious that the evaluation of the biodegradability of PVA should be made in function of its polymer structure framed in the application and intended performance. In our study, the crosslinking promoted on PVA during polymeric blend irradiation for scaffold preparation results in an added mechanical stability and consequently, in a decrease in its degradation rate. This effect is desired in order to compensate the higher degradation rate of chitosan. By this way, the skin scaffold will maintain for an extended period of time their barrier and cell growth matrix properties.

1.3 Glycerol

Glycerol (vd. Fig. 1c) is a water-soluble, non-toxic, colorless, viscous sweet-tasting polyol, commonly used in the pharmaceutical, personal care, and food industries. It presents a high hydrophilicity associated with a high humectant capacity. Regarding pharmaceutical applications it is being widely used in various products such as capsules syrups, topical creams, suppositories, eye-strain reducers, etc.

In recent years, increased attention has been given to glycerol-based polymers for biomedical applications due to the multiplicity of possible formulations/compositions and molecular architectures. The presence of multiple hydroxyl groups, which can either form new hydrogen bonds with the polymer chains and/or be synthetically converted into various other functional groups, allows to improve their potential biomedical applications. Special areas of interest, involving new market opportunities, includes carriers for drug-delivery systems, sealants or coatings for tissue repair, and anti-bacterial activity agents/barriers [34].

For a better understanding, Fig. 1 presents the chemical structures of the polymers used as well as the glycerol and an example of a glycerol-based polymer [poly(1,3-glycerol carbonate)].

This chapter presents and discusses the results obtained with matrices of composition chitosan/PVA prepared by gamma irradiation by two different methods and in vitro tested as potential skin scaffolds. Results regarding matrices with other compositions are still under validation.

2 Strategy

This study aims to clarify the correlation between the different preparation conditions and the final polymeric matrices properties given their intended use as scaffolds for skin tissue engineering. With this purpose, a detailed comparative study of the properties shown by the different samples' groups was performed and divided in three main tasks:

1. Optimization of the polymeric matrices preparation (methodology, composition, polymer concentration, range of absorbed dose).
2. Evaluation of structural and functional properties of the obtained matrices.
3. In vitro biocompatibility evaluation where the cellular viability and proliferation of Human Caucasian Foetal Foreskin Fibroblast cell line was analyzed as a measure of chitosan-based matrices biocompatibility and ability to assist skin regeneration.

3 Experimental

3.1 Materials

Chitosan, Chit (medium molecular weight 190–375 kDa, 75–85% deacetylated chitin), poly(vinyl alcohol), PVA (Mw 89,000–98,000, 99%+ hydrolyzed) and glycerol (puriss p.a. ACS reagent, anhydrous, dist.) were purchased from Sigma-Aldrich and used as raw materials. All other reagents were of analytical grade and used as received.

3.2 Preparation Methodologies

In this study, chitosan-based matrices were prepared by two methods using casting and γ-irradiation procedures. The chitosan solution (2% W/V) was prepared by dissolution of the appropriate amount of chitosan in acetic acid (1% V/V) with stirring at room temperature (RT) for 24 h. A PVA solution (10% W/V) was prepared by dissolution of PVA in bi-distilled water at 80 °C until the solution was clear. Both solutions were filtered and kept at room temperature for 24 h to remove air bubbles. To the chitosan matrix-specific volumes of the PVA solution were

added under continuous stirring to get a homogeneous mixture. Chit–PVA blends with different PVA content (1, 3, 5 or 8% W/V of the final solution) were obtained. The addition of glycerol was also tested as a humectant/plasticizer agent (0.25% V/V). Depending on composition, the matrices will be ahead referred to as C2/*x*PVA or C2/*x*PVA/0.25Gly, where *x* represents the PVA content (% W/V).

The main steps of the matrices' preparation methods are depicted in Fig. 2. The first approach, Method 1, included solvent evaporation at RT of the polymeric-blend solutions in polystyrene Petri dishes until film formation (casting). Afterwards, samples were neutralized with 0.5% sodium hydroxide and washed with distilled water, followed by freeze-drying and irradiation procedures. The second method, Method 2, involved the freeze-drying of the copolymeric solutions and subsequent irradiation. Furthermore, in order to improve porosity and thus create 3D polymeric matrices with adequate features to promote cellular growth, in Method 2, immediately after the homogenization step, an additional procedure was introduced where solutions were bubbled with nitrogen before the freeze-drying process (these procedures will be mentioned ahead as Freeze (F) and Bubble freeze (BF)

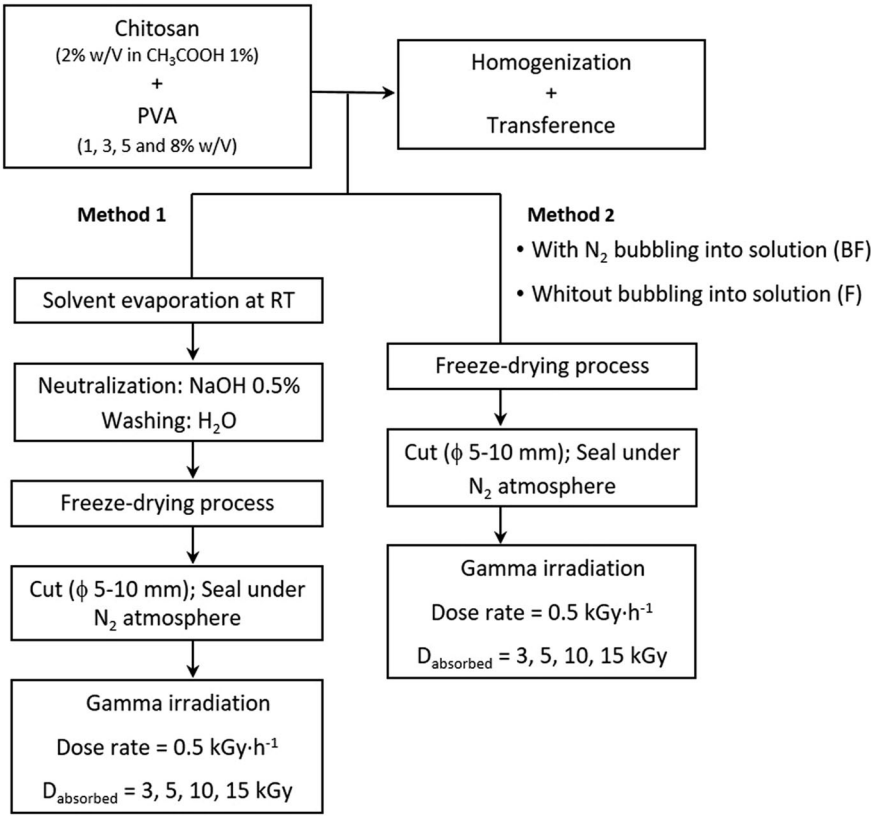


Fig. 2 Schematic representation of the experimental procedure for the preparation of chitosan-based matrices

procedures). In both tested methods, the freeze-drying step comprised the membrane freeze at −80 °C for 3 h and lyophilization for 48 h. Afterwards, small circular samples, from 5 to 10 mm in diameter, were cut with cutting hole punches, sealed under nitrogen, and irradiated. The γ-irradiation was performed in the experimental Co-60 chamber (Precisa 22) at the Ionizing Radiation Facility of Nuclear and Technological Campus of Instituto Superior Técnico (Lisbon University). A dose rate of 0.5 kGy h^{-1} was used for the purpose to achieve final doses of 3, 5, 10 and 15 kGy.

In order to give confidence and reproducibility to the irradiation processes, the validation of irradiation geometries and respective dose distribution was previously accessed by Fricke dosimetry and ionizing chamber method. Amber Perspex dosimeters (Harwell) were used to monitor the samples' absorbed dose.

3.3 Evaluation of Structural Properties

In order to clarify the correlation between the different preparation conditions (including methodology, composition, and absorbed dose) and the final polymeric matrices properties, the samples obtained by the different experimental approaches were characterized in terms of their structural properties, thermal behavior, degradation, and in vitro cells assays.

3.3.1 Structural Characterization

Structural characterization of the irradiated samples was carried out by means of attenuated total reflectance Fourier transform infrared (ATR-FTIR) using a micro-FTIR Thermo Scientific (Nicolet) i50 spectrometer equipped with an ATR slide-on diamond tip. The spectra were recorded in the 400–4000 cm^{-1} region at room temperature and with a resolution of 4 cm^{-1} (64 scans).

The microstructural characterization of previously Au-coated samples that were studied before and after degradation tests was performed by scanning electron microscopy (SEM) using a SEM instrument FEG-SEM JEOL 7001F.

3.3.2 Thermal Analysis

Thermal behavior of the samples was assessed by thermo-gravimetric analysis (TGA) using a TA instrument TGA 951. The assays were carried out under a nitrogen atmosphere from 25 to 500 °C, using a 10 °C min^{-1} heating rate.

3.3.3 Water Absorption Capacity

The water absorption capacity of the prepared samples was calculated through the following procedure: matrices' dried specimens (ϕ 8 mm) were weighted dried (W_{dry}) and immersed in distilled water at 37 °C. After having reached equilibrium ($\approx$1 h), the swollen samples were carefully removed from the water, the excess wiped off with filter paper, and weighted ($W_{swollen}$). The water absorption capacity of the samples was found through Eq. (1):

$$\text{Water absorption}(\%) = \frac{W_{\text{swollen}} - W_{\text{dry}}}{W_{\text{dry}}} \times 100. \quad (1)$$

All measurements were performed in triplicate and the result was expressed as mean value ± standard deviation (SD).

3.3.4 In Vitro Stability

In vitro stability of the scaffolds was performed in phosphate-buffered solution (PBS 1X, pH 7.4) at 37 °C based on the extent of the matrices' mass loss. Samples were dried under vacuum, weighted (W_0), and immersed in PBS solution. After 24 h of immersion, the matrices were taken out of the solution, wiped of excess fluid with filter paper, dried under vacuum, and weighted (W_{deg}). Matrices' mass loss was calculated as the percentage of weight loss before and after PBS treatment according to Eq. (2):

$$\text{Mass loss}(\%) = \frac{W_0 - W_{\text{deg}}}{W_0} \times 100. \quad (2)$$

All measurements were performed in triplicate and the result was expressed as mean value ± SD.

3.4 In Vitro Cellular Viability

The scaffolds used in this study were γ-irradiated in sealed bags at 3, 5, 10 and 15 kGy. According to previous sterilization results on similar chitosan/pHEMA matrices [22], it is possible to assure that the exposure to 4–5 kGy allows obtaining matrices microbiologically safe (i.e., the probability of obtaining a contaminated item will be much less than 1 in 10^6 items). Consequently, even if it is necessary to apply specific microbiological methodology to validate the sterilization procedure, one can say that preparation and sterilization procedures of the prepared samples occurred in one simultaneous step. Thus, no previous sterilization procedure was needed before cellular seeding.

3.4.1 Cell Culture

A Human Caucasian Foetal Foreskin Fibroblast cell line (HFFF2) was selected for the biological assays (in vitro study) in order to evaluate the effect of chitosan-based matrices on cell adhesion and viability. The HFFF2 commercial cell line was obtained from European Collection of Cell Cultures (ECACC, UK). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Glutamax), supplemented with heat-inactivated fetal bovine serum (FBS) 10% (V/V) and streptomycin and penicillin 100 U/ml (all from Gibco), and incubated at 37 °C in a humidified atmosphere with 5% of CO_2. The culture medium was changed every 2 days. After reaching 80% confluence, the cells were trypsinized and resuspended in culture medium at a concentration of 2×10^4 cell/ml medium.

3.4.2 Cell Viability Assay (alamarBlue®)

Chitosan-based matrices (ϕ 10 mm) were placed in a 48-well tissue culture plate and pre-wetted with 200 µl of culture medium to promote their expansion so that the matrices contact the walls of the well in the bottom of the chamber, avoiding floating. The fact that the matrices are soaked with culture medium enables the eventual migration of the cells inside its porous structure. After 10 min, the samples were seeded with 500 µl of the HFFF2 suspension (20,000 cells) and cultured for 1, 4, and 7 days at 37 °C. Control samples were established by culturing cells directly over the polystyrene surface of the wells.

The cellular viability was monitored with the alamarBlue® cell viability assay (Life Technologies). At days 1, 4 and 7, the supernatant of each well was replaced by 300 µl of fresh culture medium and 30 µl of alamarBlue® reagent and incubated for 2 h at 37 °C in a 5% CO_2 atmosphere. After the incubation period, the media with alamarBlue® were transferred to a 96-well plate and the optical density (OD) was read in a microplate reader (Tecan Spectra) at 570 nm with a reference wavelength of 600 nm. The measurements were made in triplicate for each treatment and time point (1, 4 and 7 days). Data were expressed as mean ± SD.

3.4.3 Cytochemistry

At 7 days of culture, the chitosan-based matrices and cytochemistry control samples (glass coverslips containing cells cultured in 24-well chambers) were fixed with paraformaldehyde, PFA 4% (in PBS), permeabilized in 0.2% TritonX-100 (Sigma-Aldrich), and stained with ToPro3 (1:500 in PBS) and Alexa488 conjugated Phalloidin (1:400 in PBS) (both from Molecular Probes), to observe cell's nuclei and actin cytoskeleton, respectively. The samples were mounted on fresh PBS on a glass slide and imaged on a Leica SPE confocal system using either a 10 × 0.3 NA or a 20 × 0.7 NA lens. Confocal images were acquired in 2–5 different fields chosen randomly at the center of scaffolds.

4 Results and Discussion

4.1 Method 1 (Casting/Freeze-Dry/Irradiation)

4.1.1 Thermal Analysis

The thermal behavior of γ-irradiated and non-irradiated chitosan-based matrices with and without PVA (prepared by Method 1) at a constant heating rate of 10 °C min^{-1} is displayed in Fig. 3. A noticeable change is observed in the relative degradation pattern of chitosan matrices when those are γ-irradiated at 5 kGy. The weight loss at 25–100 °C is generally attributed to the loss of bind/adsorbed water, however, in γ-irradiated chitosan matrices it cannot be assigned only to that. The chitosan degradation can also be responsible for this first step. When chitosan is exposed to ionizing radiation, it preferably undergoes chain scission by cleavage of

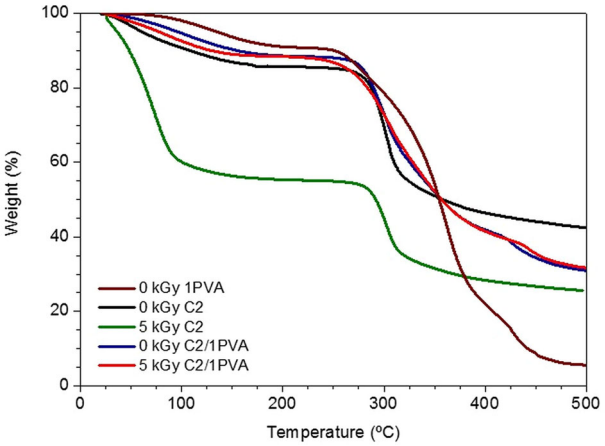


Fig. 3 Thermogravimetric curves of non-irradiated (0 kGy) and γ-irradiated at 5 kGy chitosan (C2) and chitosan-based C2/1PVA matrices

β-glucosidic linkages [35]. Moreover, as this process may include the dehydration of the saccharide ring [36], it would explain the sharpest decrease in the weight observed for the γ-irradiated chitosan matrices. The second weight loss, with an onset temperature near 255 °C for both irradiated and non-irradiated chitosan matrices, can be due to an exhaustive degradation of the saccharide structure of the molecule, including decomposition of deacetylated (and acetylated) units of chitosan. In the case of Chit/PVA matrices, Fig. 3 shows that the introduction of PVA promoted changes in the mentioned weight-loss trend of chitosan matrices. It is clear that γ-irradiated Chit/PVA matrices are more thermally stable than the chitosan-irradiated ones. This higher stability may be assigned to a Chit/PVA denser and crosslinked structure, meaning that PVA confers a stabilizing effect in the structure of irradiated chitosan matrices.

4.1.2 ATR-FTIR Characterization

ATR-FTIR spectroscopy was used to assess the polymer chemical groups and investigate the formation of crosslinked networks in chitosan-based matrices upon irradiation. Results suggest that chitosan and PVA are binded together in the obtained matrices and that peak intensity evolution is in accordance with the expected changes promoted in the structure due to γ-irradiation. Figure 4 shows the FTIR spectra of γ-irradiated and non-irradiated chitosan-based matrices with and without PVA. It can be observed that depending on composition, spectra exhibit the major peaks related to the typical chitosan and PVA patterns. The broad peak around 3350 cm^{-1} is assigned to N–H and O–H stretching from the intermolecular and intramolecular hydrogen bonds. The characteristic absorption peaks appear at about 1648 (amide I band), 1570 (amide II, N–H deformation mode), and 1322 cm^{-1} (amide III band). The absorption peaks at 1151 (anti-symmetric stretching of the C–O–C bridge), 1058 and 1028 cm^{-1} (skeletal vibrations involving

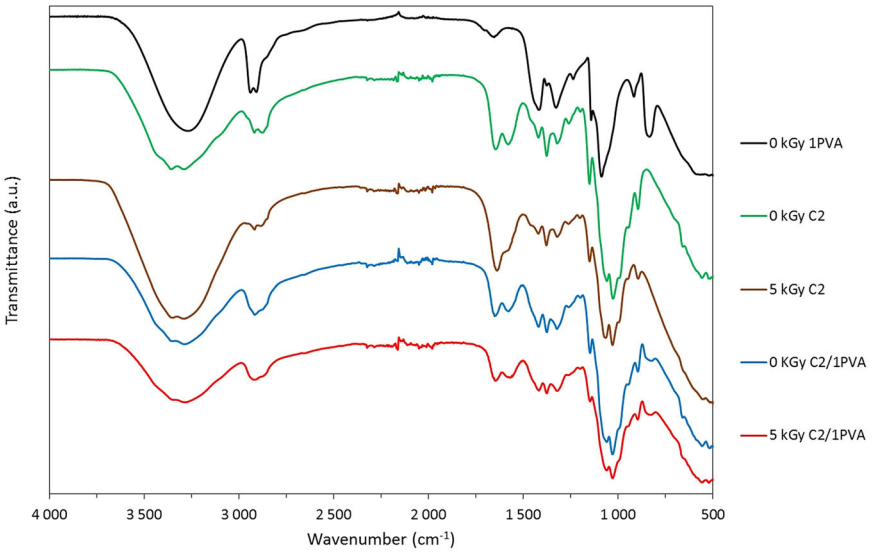


Fig. 4 FTIR spectra of non-irradiated (0 kGy) and γ-irradiated at 5 kGy chitosan (C2) and chitosan-based C2/1PVA matrices

the C–O stretching) are characteristics of saccharide structure of chitosan. The small absorption peak at 895 cm^{-1} can be used to identify the presence of the C–O–C bridge as well as β-glucosidic linkages between the sugar units in chitosan [37]. Concerning γ-irradiated chitosan matrices, it should be noted that they exhibit much similarity in the IR peaks as those of non-irradiated ones. However, a peaks' intensity decrease at 895 and 1151 cm^{-1}, which correspond to the saccharide structure, suggests some degradation by cleavage of β-glucosidic linkages due to γ-radiation exposure, which is in accordance with results obtained by TGA analysis. An intensity reduction at amine region (1570 cm^{-1}) also supports the chitosan's degradation occurrence. Regarding the spectra of Chit/PVA matrices, all major absorption peaks related to PVA are also presented, for instance, the slightly board peak at 2880 cm^{-1} refers to the C–H stretching from alkyl groups and 1415 cm^{-1} to the –C–O group. Moreover, the increase in the intensity peaks at 1415 and 1570 cm^{-1} at the FTIR Chit/PVA matrices suggests chemical crosslinking between chitosan and PVA molecules. Additionally, the boarder band at 3350 cm^{-1}, when compared with the other spectra, also seems to corroborate this.

4.1.3 Water Absorption Capacity

Due to the secretion of body fluid during skin wound healing, in the process of constructing skin engineered scaffolds it is necessary to evaluate its water absorption capacity.

According to thermogravimetric and ATR-FTIR results, the introduction of PVA into chitosan-based matrices' confers an additional structural stability to those matrices. Moreover, as γ-irradiation seems to promote some level of crosslinking

between chitosan and PVA chains and/or intermolecular bonds, it is expected that matrices' structures become more closed and stable. Water absorption capability results seem to confirm this since the percentage of absorbed water of Chit/PVA γ-irradiated matrices decreases when compared to the non-irradiated ones. Figure 5 displays the water absorption capacity of non-irradiated and γ-irradiated at 5 kGy Chit 2%/PVA 1% (W/V) matrix (referred as C2/1PVA). The images of the dry (150 μm thickness) and swollen irradiated matrices can also be observed.

4.1.4 Morphological Analysis

The morphology of chitosan-based matrices was studied through SEM. Despite showing some structural network and good surface homogeneity, the matrices prepared by Method 1 do not evidence porosity as depicted in Fig. 6 where representative matrices' images are shown.

Considering the required porous characteristics for the intended skin scaffolds application, it is clear that the chitosan-based matrices prepared by Method 1 (casting of the solutions followed by freeze-drying and irradiation) do not possess the three-dimensional interconnected porous architecture where cells could easily attach and proliferate. Consequently, aiming the improvement of cell adhesion and growth properties onto those matrices no further characterization was performed on them. Instead, a different preparation methodology was tested (Method 2).

4.2 Method 2 (Freeze-Dry/Irradiation)

4.2.1 Thermal Analysis

The thermal behavior of the γ-irradiated chitosan-based matrices obtained through Method 2, i.e., obtained through irradiation of freeze-dried polymeric blend solutions, was similar to the one observed for matrices prepared by Method 1 and

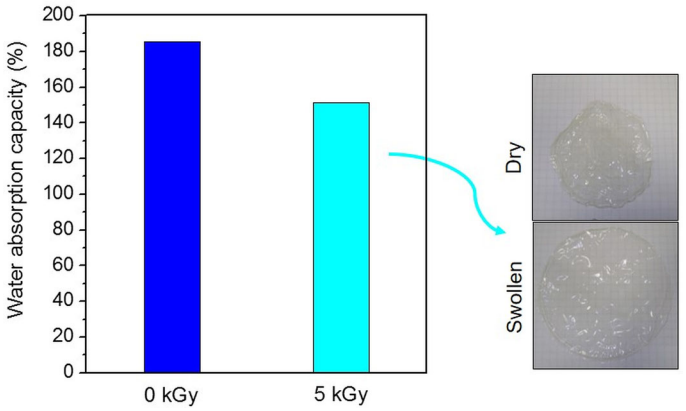


Fig. 5 Water absorption capacity (37 °C) of non-irradiated and γ-irradiated at 5 kGy C2/1PVA matrices; images of dry and swollen C2/1PVA irradiated matrix

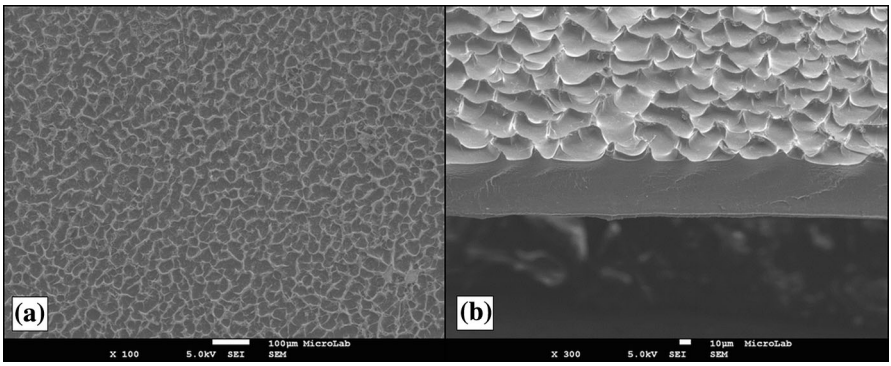


Fig. 6 SEM micrographs of C2/1PVA matrix γ-irradiated at 5 kGy: **a** surface (*scale bar* 100 µm); **b** surface and cross-section (*scale bar* 10 µm)

already reported. Figure 7a, where the thermal profile of non-irradiated and 5 kGy-irradiated C2/1PVA matrices are shown, reinforces the argument that introduction of PVA in matrices' composition can provide a shield to the chitosan backbone against radiation-induced degradation. This kind of behavior has already been reported in the literature [38]. It is also observed that the irradiation of matrices with different content in PVA promotes the same effect (Fig. 7b): the introduction of increasing amounts of PVA promotes lower initial weight losses. Thus, we may conclude that PVA confers a stabilizing effect in the matrices' molecular structure.

Comparing the dose effect on structural properties of the matrix it is possible to understand the changes in the structure (vd. Fig. 8). Up to an irradiation dose of 5 kGy the structure stability of the matrices slightly decreases while for higher irradiation doses it is possible to observe some recovery of the structural stability of the materials.

In fact, comparing the TGA curves of samples prepared through Method 1 and Method 2 with and without N_2 bubbling (vd. Fig. 9), although showing a similar thermal profile, Method 2 (freeze-drying of the solutions) leads to matrices with lower structural stability. This is understandable since the referred methodology introduces more porosity in the final matrices.

4.2.2 ATR-FTIR Characterization

The ATR-FTIR spectra of samples C2/3PVA and C2/3PVA0.25Gly irradiated at a total dose of 10 kGy depicted in Fig. 10 show the typical peaks related to chitosan, PVA, and glycerol previously mentioned. To a better understanding that information is also summarized in the figure. A better peak definition and higher intensity can be observed in the spectrum of the matrix obtained through the freeze procedure, suggesting a more organized and stable structure when compared with the one of bubble-freeze procedure. This is in accordance with TGA data.

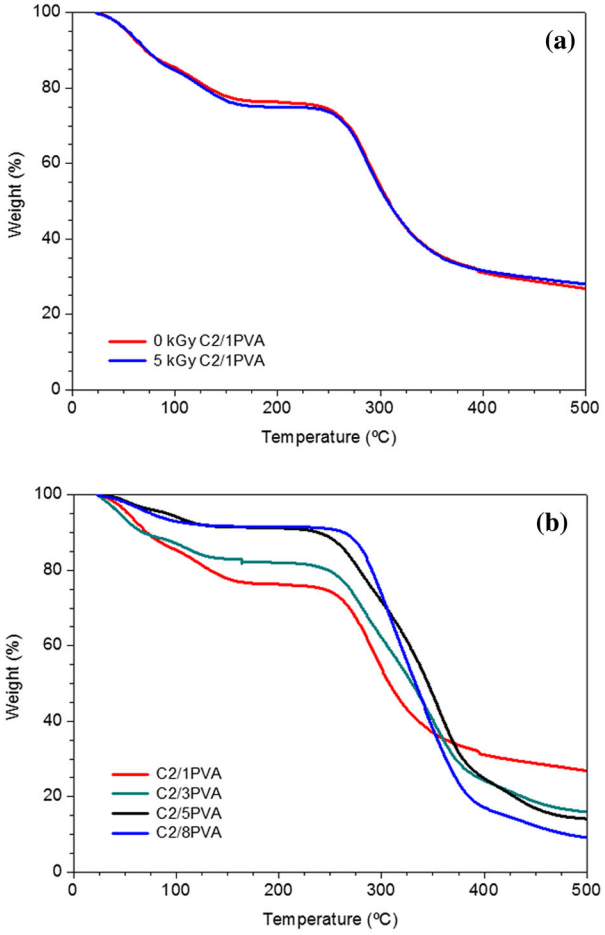


Fig. 7 Thermogravimetric curves of Chit/PVA matrices N_2 bubbled: **a** non-irradiated and γ-irradiated at 5 kGy C2/1PVA; **b** effect of PVA concentration on matrices γ-irradiated at 5 kGy

4.2.3 Water Absorption Capacity

It is well known that the water absorption and swelling capacity is one of the important issues in skin scaffold development. In fact, when the scaffolds are capable of swelling, they allow their pore size to increase, which facilitates the cells to attach and penetrate inside the inter-polymeric network. Figure 11 shows the increase of surface area and volume of bubble freeze (BF) prepared matrices from dry to swollen state. In the present study, similarly to what occurs in Method 1, results suggest that the higher the PVA content or the higher the radiation dose is (within the studied concentration and radiation dose ranges), more crosslinking between chitosan and PVA chains and/or intermolecular bonds are introduced in matrices. This confers a stabilizing effect in the structure, which became more compact and with smaller pores (the pore structures of the matrices were further

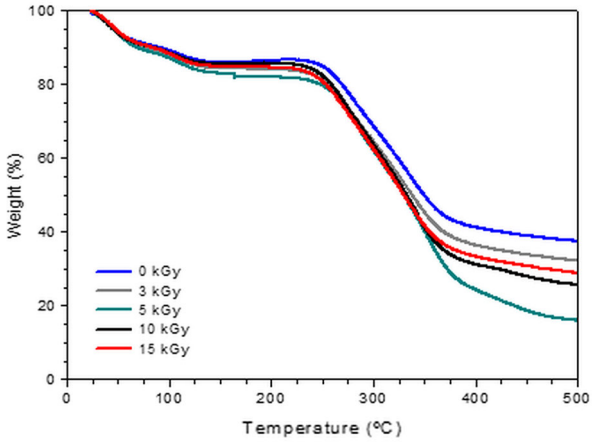


Fig. 8 Thermogravimetric curves: effect of radiation dose on C2/3PVA matrices (prepared by bubbling N_2 into the solution)

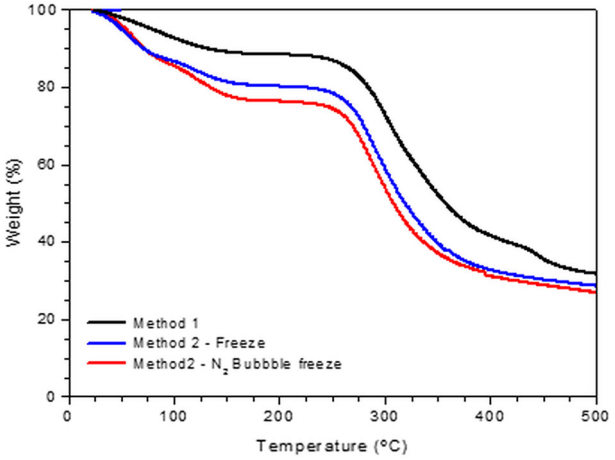


Fig. 9 Thermogravimetric curves of C2/1PVA γ-irradiated at 5 kGy. Matrices prepared by the different methods in study

confirmed by SEM). Thus, the swelling capability of these bubble freeze matrices decreases. However, for PVA contents of 8%, the increase in hydrophilic functional groups (that overcome probably from the content increase of –OH groups due to PVA linkage to chitosan C-2 amino groups) overrides the dose effect and a slight increase in the swelling capability of these matrices is observed (vd. Fig. 12). In terms of the purposed biomedical application, considering the results of water absorption capacity, chitosan-based matrices with 5% of PVA was considered to be the most promising prepared samples to be used as skin scaffold.

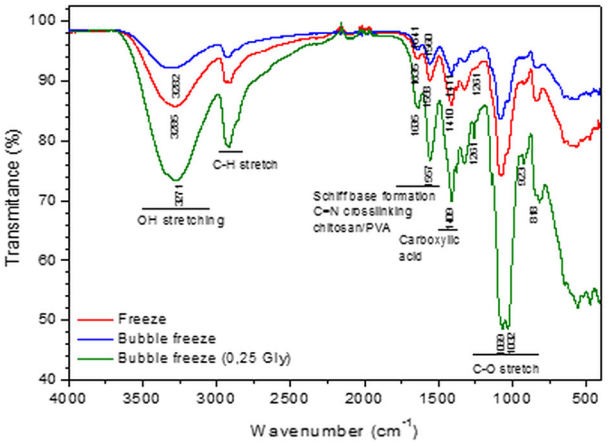


Fig. 10 ATR-FTIR spectra of C2/3PVA and C2/3PVA0.25Gly matrices irradiated at a total dose of 10 kGy

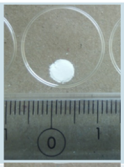
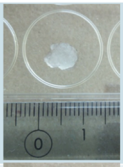
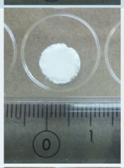
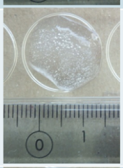
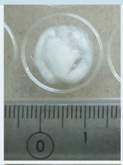
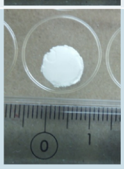
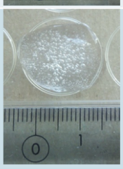
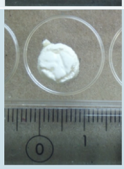
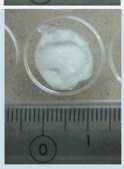
Matrice	Dry	Swollen	Matrice	Dry	Swollen
C2/3PVA 5 kGy w/ Glycerol (0.25%)			------	------	------
C2/5PVA 10 kGy			C2/8PVA 10 kGy		
C2/5PVA 15 kGy			C2/8PVA 15 kGy		

Fig. 11 BF chitosan-based matrices γ-irradiated at different radiation doses in a dry and swollen state

4.2.4 In Vitro Stability

The stability of a skin scaffold assumes an important role in the entire wound-healing process. Generally, it should have a steerable degradability and degradation rate to match the tissue regeneration. In the particular case of the obtained Chit/PVA matrices, preliminary studies of its in vitro stability were performed in phosphate-buffered solution (PBS 1X, pH 7.4) at 37 °C for 24 h. Overall results show that the higher the radiation dose, the lower the matrices' mass loss is, since more crosslinks

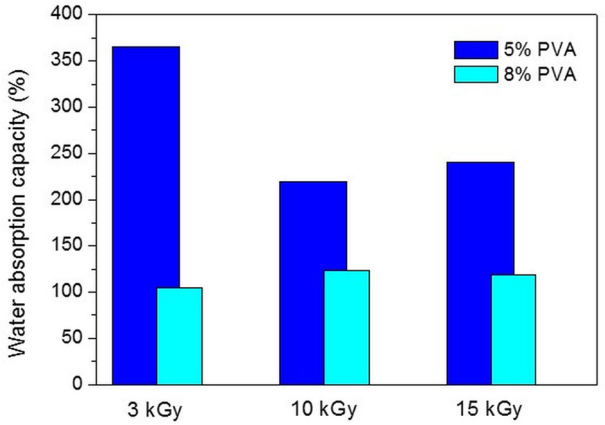


Fig. 12 Water absorption capacity of bubble freeze C2/5PVA and C2/8PVA matrices γ-irradiated at 3, 10 and 15 kGy

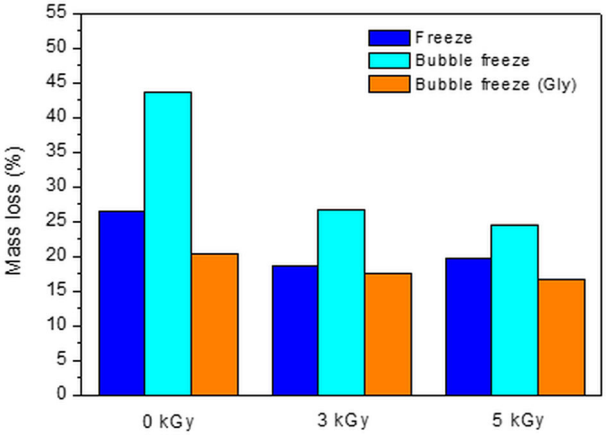


Fig. 13 Mass loss (%) after 1 day of immersion in PBS solution at 37 °C of matrices C2/8PVA non-irradiated and γ-irradiated at 3 and 5 kGy

are introduced, conferring more stability to the structure of the material. Comparing both procedures (vd. Fig. 13), freeze (without bubbling N_2) and bubble freeze (with bubbling N_2), the latter one shows higher mass loss. This is observed in all PVA contents and doses studied and is understandable since in the bubble-freeze procedure, porosity was introduced in the matrices "weakening" its structure and helping to washing out eventually free polymer chains. When glycerol is introduced in theses matrices, the presence of multiple hydroxyl groups and the manifoldness of its new bonds allows to keep the stability of the polymeric network, fulfilling its function as plasticizer agent in the blend.

4.2.5 *Morphological Analysis*

The morphology of the "bubble freeze samples" was evaluated with SEM. Figure 14 shows representative SEM micrographs of matrices' surface morphology of chitosan-based matrices with different PVA content (without glycerol). It can be observed that matrices show a good surface homogeneity and an increase in PVA percentage leads to a decrease in porosity matrices' surface. Once again, results suggest that a higher PVA content in Chit/PVA blends leads to a more crosslinked structure possibly due to more Chit-PVA bonds and OH intramolecular interactions as already mentioned. Even so, a comparison between Figs. 6 and 14 (SEM micrographs of C2/1PVA 5 kGy matrix prepared by Method 1) evidences the three-dimensional porous structure that Method 2 can promote in this type of polymeric matrix. For the same sample composition, it can be seen that the morphology of matrices' surface is also dependent on the radiation dose (vd. Fig. 15). Thus, radiation dose can be used too to tailor the morphology of the matrices. In the first irradiation stage, there seems to exist a widespread presence of crosslinking reactions that lead to a smoother surface due the increasing network density. However, for higher irradiation doses, some degradation of the terminal chains of PVA and chitosan ones can occur, promoting "surface erosion" exposing and

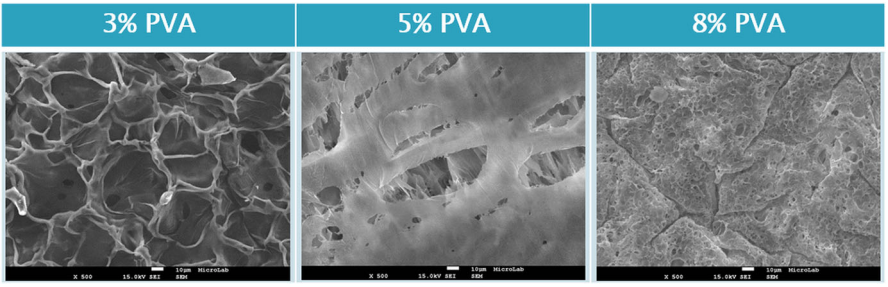


Fig. 14 SEM micrographs of the surface of bubble freeze Chit/PVA matrices irradiated at 3 kGy (*scale bar* 10 μm)

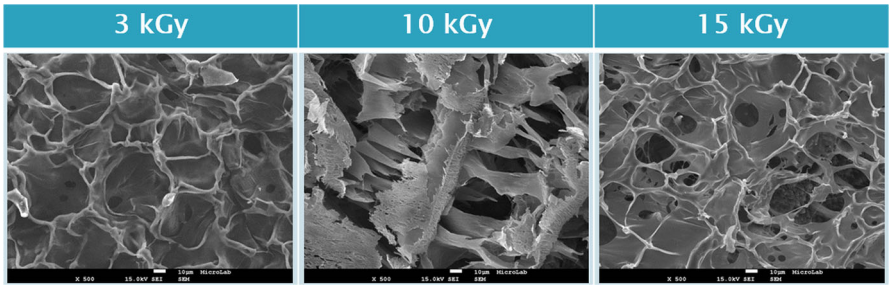


Fig. 15 SEM micrographs of the surface of bubble freeze C2/3PVA matrices when irradiated at different doses (*scale bar* 10 μm)

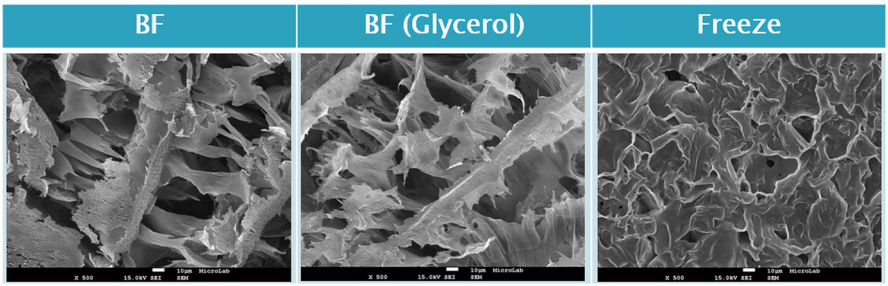


Fig. 16 SEM micrographs of C2/3PVA 10 kGy matrices' surface using different procedures: with N_2 bubbling (bubble freeze) and without (freeze) (*scale bar* 10 μm)

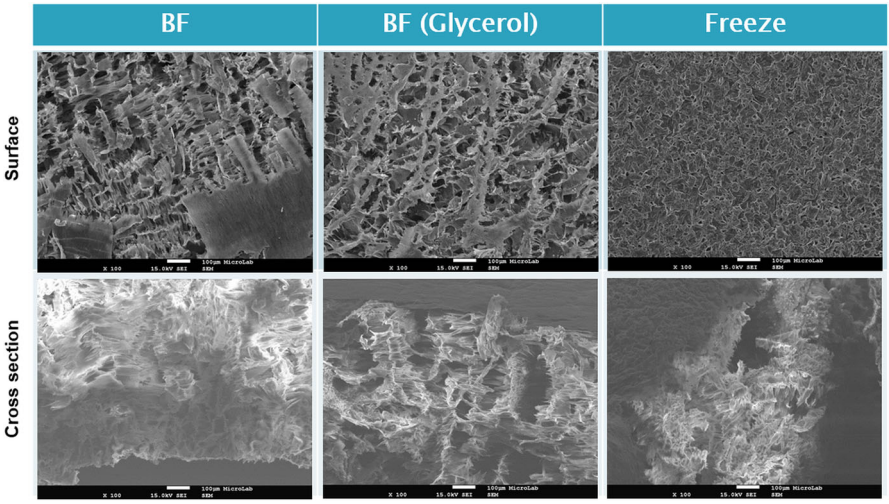


Fig. 17 Surface and cross-section SEM micrographs of C2/3PVA 10 kGy (*scale bar* 100 μm)

accentuating a rougher surface [38]. Together, these results stress out the importance of this preparation method to achieve in trimming surface properties.

Figure 16 shows the SEM micrographs of the C2/3PVA matrices irradiated at 10 kGy and prepared using the different procedures of Method 2: with N_2 bubbling (bubble freeze) and without (freeze). It can be observed that there is no significant difference on the surface morphology in the matrices prepared with and without glycerol. On the other hand, the micrographs also reveal that bubbling of N_2 leads to more open structures, showing to be a good procedure to introduce more porosity in the matrices.

Additionally, as seen in Fig. 17, despite having some differences in the surface morphology, all the matrices present a sponge-type "inner structure".

4.2.6 *In Vitro Cellular Viability*

The cellular viability of the HFFF2 fibroblasts was assessed on Chit/PVA matrices as a means to evaluate their ability to promote cell adhesion and proliferation. Tested matrices were chosen according to the best structural results previously obtained. Preliminary cell viability results on Chit/3PVA matrices (bubble freeze, BF, freeze, F, and with glycerol, Gly) irradiated at different radiation doses (Fig. 18) show that HFFF2 cells adhere well to the matrices surface but proliferate slower than control (control = HFFF2 cell cultured under the same condition but without matrices).

Results point out that only matrices γ-irradiated at 10 kGy display cellular growth on day 1. This is in accordance with SEM analysis, which has revealed that chitosan-based matrices γ-irradiated at 3 and 15 kGy lead to matrices with small surface pores hindering cells to attach. However, despite showing a good porosity, the matrix with glycerol (irradiated at 10 kGy) does not show cell growth after the first day. It appears that cells were able to attach but unable to follow this attachment with spreading. This might be due to changes in ionic equilibrium (pH) introduced by glycerol that would be intolerable to cell viability. On the other hand, still within this group of matrices γ-irradiated at 10 kGy, results at day 4 revealed that only matrices N_2 bubbled presented an increase in cell proliferation.

Given the obtained results, only matrices with 3 and 5% of PVA prepared by bubble freeze and freeze procedure were further evaluated in terms of cell viability. To comprise all the information concerning irradiation dose (*x*), PVA content (*y*), and preparation procedure [bubble freeze (BF) and freeze (F)], matrices in the figures of this section are referred to as "*x*kGy *y* BF" or "*x*kGy *y* F". The results of matrices irradiated at 5 and 10 kGy (Fig. 19) confirm the HFFF2 cell viability, i.e., cells adhesion and proliferation capability into the substrates in study. Once again, cells adhered to all matrices, which is indicative of the non-cytotoxic nature of the prepared matrices. However, cells proliferated slower than in controls with the "freeze procedure" matrices presenting a very low proliferation with the matrix C2/

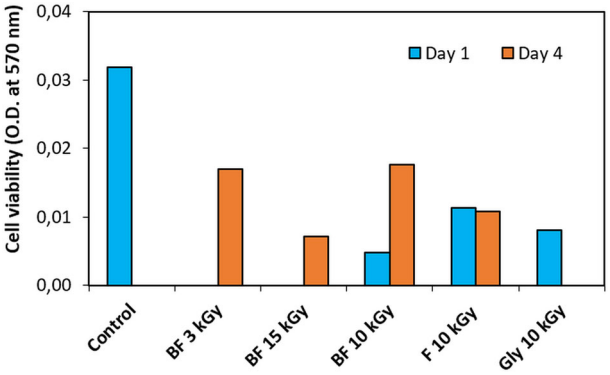


Fig. 18 Cells growing on C2/3PVP and C2/3PVP0.25Gly matrices in culture days 1 and 4. Matrices prepared by bubble freeze (BF) and freeze (F) procedures, and γ-radiation doses of 3, 10 and 15 kGy

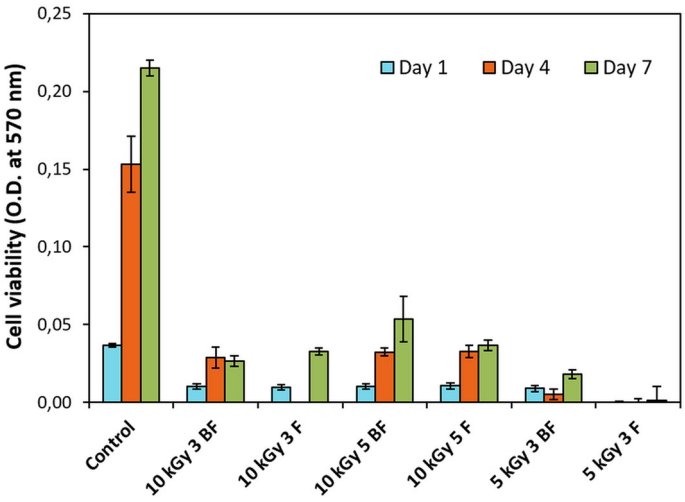


Fig. 19 Cells growing on different Chit/PVA matrices (10 and 5 kGy irradiated; 3 and 5% PVA; bubble freeze (BF) and freeze (F) preparation procedure) in culture days 1, 4 and 7 ($n = 3$; mean value ± SD)

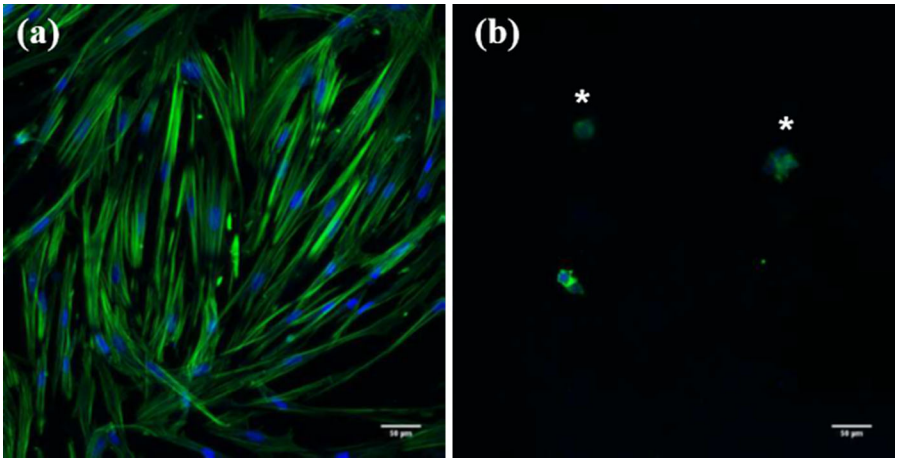


Fig. 20 HFFF2 cells growing in culture for 7 days on: **a** control cells; **b** 10 kGy 5 BF matrices (out of focus cells *asterisk*; *green* actin; *blue* DNA; *scale bar* 50 μm)

5PVA N_2 "bubble freeze" presenting the best results. Moreover, the cytochemical stainings performed showed that cell morphology at day 7 is different to glass coverslip-seeded fibroblasts (Fig. 20).

Confocal images of control samples at day 7 of culture showed HFFF2 cells with the expected morphology, a fusiform shape. In confocal images of the Chit2/5PVA BF 10 kGy γ-irradiated matrix (10 kGy 5 BF as referred on Fig. 19), we can only observe a few cells with round morphology and poor actin cytoskeletal organization as compared to control cells. This result may indicate that cells are not attaching to the substrate and growing in the best conditions but, even so, some cells were able

to invade the depth of the matrix, as can be seen by the out-of-focus cells in Fig. 20. This is consistent with the viability assay, which showed reduced cell number. Huang et al. [39] observed similar results having suggested that inhibition of cell proliferation on chitosan scaffolds is due to the loss of cell–matrix binding. This would prevent cells from recognizing and anchoring to new binding sites, and thus cells would lose some cytoskeleton integrity. Furthermore, the incorporation of proteins or peptides in the matrix that would allow the presence of multiple cell-binding ligands or the surface modification of matrix by plasma treatment that would change the electrostatic interactions may be tested in the future in order to improve cell affinity for the matrices and to achieve better morphology and behavior of the fibroblasts growing on these scaffolds.

5 Conclusions

Chitosan-based matrices for tissue regeneration were successfully prepared by gamma irradiation. Two preparation methods were tested in order to optimize the methodology. The first one involved the casting of copolymeric solutions and solvent evaporation at room temperature followed by freeze-drying and irradiation. The second approach involved the freeze-drying of copolymeric solutions followed by irradiation.

The matrices were evaluated in terms of methodology, composition, absorbed dose, structural and functional properties, and in vitro biocompatibility (cellular viability, morphology and cytochemistry). It was found that γ-irradiation and PVA content can be used to tailor the matrices' surface in terms of porosity/roughness with simultaneous sterilization. The bubbling of N_2 before freeze-dry showed to be a good procedure for introducing more porosity in the matrices although it led to higher matrices' mass loss. The swelling capability of non-irradiated matrices increases with the number of hydrophilic groups, i.e., with the PVA content. Moreover, the swelling properties are also dependent on the density of network structure.

Concerning the evaluation of cell viability, it was shown that HFFF2 cells adhered to the surface of all matrices obtained by solutions freeze-drying method, but do not reveal favorable cellular growth if glycerol is present in the composition.

Bubbling the polymer solution during the homogenization step followed by freeze-drying and a γ-irradiation dose up to 10 kGy seems to be the most promising procedure. Further modifications to introduce multiple cell-binding ligands in the matrices should be applied in order to improve cell affinity for the matrices and to achieve a better morphology and behavior of the fibroblasts growing on these scaffolds. Nevertheless, the study herein described evidence the versatility of radiation technology to tailor polymeric materials for specific applications such as skin regenerative medicine.

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work. The authors would also like to thank the Erasmus student Reda Paitian (University of Vilnius, Lithuania) for her collaboration in the preparation and characterization of chitosan-based matrices.

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