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FROM WASTE TO VALUE: FISH BONE DERIVED COLLAGEN TO PREPARE 3D CELL MODELS

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Biomedical Engineering

by

Inês Casais Esperança

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Resumo

Os sistemas de cultura celular tridimensionais (3D) foram desenvolvidos como modelos superiores às culturas celulares bidimensionais (2D). O uso de sistemas 3D tem demonstrado melhorar a morfologia celular, a proliferação e a sinalização, replicando a matriz extracelular (MEC) de uma forma mais precisa. Os hidrogéis são particularmente adequados para estes sistemas tridimensionais, sendo que a agarose, um polissacarídeo biocompatível, oferece suporte estrutural, enquanto o colagénio melhora a bioatividade e a adesão celular. Os hidrogéis de agarose-colagénio combinam a integridade mecânica da agarose com a biofuncionalidade do colagénio, representando uma matriz ideal para aplicações em cultura celular 3D.

Considerando o crescente foco na sustentabilidade, o colagénio derivado de ossos de peixe surge como uma alternativa promissora ao colagénio tipo I, alinhando-se com os princípios da economia circular, em que os resíduos da indústria de peixe são transformados em biomateriais valiosos.

O presente estudo avalia o colagénio extraído de espinhas de peixe através de três métodos: uma extração mais curta a uma temperatura mais elevada, produzindo péptidos mais pequenos, com alguns ossos pré tratados com ultrassons (US) e outros sem (NUS); e uma extração mais longa a uma temperatura mais baixa (4ºC) para preservar melhor a estrutura do colagénio. Foram avaliadas a biocompatibilidade, a estabilidade, as propriedades mecânicas e o potencial de crescimento de esferóides de linhas celulares de osteossarcoma humano de hidrogéis contendo os extratos de colagénio nas concentrações de 0,02% e 0,04%. O método 4°C preservou melhor a estrutura nativa do colagénio, resultando em hidrogéis com maior estabilidade e viabilidade celular. Por outro lado, a extração US produziu maiores quantidades de colagénio, mas produziu péptidos fragmentados que levaram a uma rápida libertação de colagénio e a um suporte limitado dos esferóides. A extração NUS ofereceu um meio-termo, alcançando um equilíbrio entre estabilidade e bioatividade. Além disso, concentrações mais elevadas de colagénio (0,04%) aumentaram a estabilidade e a resiliência mecânica sem melhorar o crescimento celular, enquanto concentrações mais baixas (0,02%) suportaram eficazmente a cultura de esferóides. Este estudo destaca o colagénio de espinhas de peixe como material sustentável para cultura celular 3D e valorização de resíduos de peixe na engenharia de tecidos.

Palavras-chave: Cultura de células 3D, colagénio derivado de espinhas de peixe, economia circular, hidrogel à base de agarose e colagénio, engenharia de tecidos.

Abstract

Three-dimensional (3D) cell culture systems have been developed as superior models to two-dimensional (2D) cell cultures. The use of 3D systems has been shown to improve cellular morphology, proliferation and signalling, replicating the extracellular matrix (ECM) in a more accurate manner. Hydrogels are particularly well-suited to these three-dimensional systems. Agarose, a biocompatible polysaccharide, provides structural support, while collagen enhances bioactivity and cell adhesion. Agarose-collagen hydrogels thus represent an optimal matrix for three-dimensional cell culture applications, combining the mechanical integrity of agarose with the biofunctionality of collagen.

Considering the increasing focus on sustainability, fish bone-derived collagen represents a promising alternative to common type I collagen. This approach aligns with the principles of a circular economy, whereby waste from fish processing is transformed into valuable biomaterials.

The present study evaluates collagen extracted from fish bones through three methods: a shorter extraction at a higher temperature, yielding smaller peptides, with some bones pre-treated with ultrasound (US) and others without (NUS); and a longer extraction at a lower temperature (4ºC) to better preserve the collagen structure. The biocompatibility, stability, mechanical properties and potential for spheroid growth from human osteosarcoma cell lines of hydrogels containing collagen extracts at concentrations of 0.02% and 0.04% were evaluated. The 4°C method preserved collagen's native structure best, resulting in hydrogels with greater stability and cell viability. Conversely, US extraction yielded higher collagen amounts but produced fragmented peptides that led to rapid collagen release and limited spheroid support. NUS extraction offered a middle ground, achieving a balance between stability and bioactivity. Additionally, higher collagen concentrations (0.04%) increased the stability and mechanical resilience without enhancing cellular growth, while lower concentrations (0.02%) supported spheroid culture effectively. This study highlights fish bone collagen as a sustainable material for 3D cell culture and the valorisation of fish waste in tissue engineering.

Keywords: 3D cell culture, fish bone derived collagen, circular economy, agarose-collagen-based hydrogel, tissue engineering.

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Finally, to my mom, my greatest inspiration. Thank you for your endless love, sacrifices, and unwavering belief in me. Everything I am and everything I hope to become is because of you. I could not have done it without you by my side, and I will always be thankful for your endless love and strength.

Table of Contents

Figure List

Table List

List of abbreviations

2D – Two dimensional

3D – Three dimensional

3T3 – Murine fibroblasts

4ºC – Extract of salmon bones placed in contact with a solution of $(NH_4)HCO_3$ 0.2 M at 4 ºC for 5 days

AG – Agarose

BCA – Bicinchoninic acid

CaPs – Calcium phosphates

CHCl3 – Chloroform

DMEM – Dulbecco's Modified Eagle's Medium High Glucose medium

DMSO – Dimethyl Sulfoxide

ECM – Extracellular matrix

FBS – Fetal bovine serum

MG63 – Human osteosarcoma

MTT – Tetrazolium salt thiazolyl blue

NUS – Extract of salmon bones placed in contact with a solution of $(NH_4)HCO_3$ 0.2 M at 60 ºC for 4 h

PBS – Phosphate Buffered Saline

SEM – Scanning Electron Microscopy

US – Extract of salmon bones pre-treated with ultrasound, placed in contact with a solution of $(NH_4)HCO_3$ 0.2 M at 60 ºC for 4 h

UV – Ultraviolet

Chapter 1: Introduction

1.1. Hydrogels in Tissue Engineering

1.1.1. Definition and Importance

Since the early 1900s, two-dimensional (2D) cell culture has been the standard method for culturing cells [1]. While it plays a vital role in research, this method has several limitations because 2D models do not accurately represent the complexity of tissue cells *in vitro* [2]. Alternatively, three-dimensional (3D) cell culture has shown significant improvements in studies focusing on morphology, cell number monitoring, proliferation, response to stimuli, differentiation, drug metabolism, and protein synthesis [3]. This is possible due to the ability of 3D cultures to mimic the *in vivo* environment of cells while being cultured *in vitro* [4]. At present, 3D cell culture has become increasingly popular, with applications in cancer research, stem cell research, drug discovery, and studies of other diseases [5].

Table 1 presents a comparative analysis of the advantages and disadvantages of 2D and 3D cell culture techniques, with a focus on their distinctive characteristics.

Table 1- A comparative analysis of two-dimensional (2D) and three-dimensional (3D) cell culture techniques. Adapted from Jensen and Teng (2020) [5].

Key Characteristics	2D Cell Culture	3D Cell Culture	References
Cell Morphology	Cells grow in a flat, elongated shape due to the limitation of two dimensions, which restricts them to a monolayer structure.	Cells retain their natural shape, growing in 3D aggregates or spheroids with multiple cell layers, closer to *in vivo* organization.	[2],[6]
Exposure to medium	Cells are equally exposed to nutrients and growth factors from the medium, creating uniform access but not mimicking tissue gradients.	Cells at different layers receive varying levels of nutrients and oxygen, creating natural gradients similar to those in tissues.	[2],[6],[7]

Cell Junctions	Fewer, less complex cell junctions that may not accurately represent natural cell connections, affecting cell communication.	Stronger, more complex cell junctions that resemble actual tissue, facilitating realistic cellular communication and behaviour	[2],[4],[6],[8],[9]
Differentiation	Limited ability to differentiate, often leading to simpler structures that don't mimic the organization of tissue.	Enhanced differentiation capacity, allowing cells to form structures that closely resemble actual tissue.	[2],[6],[10]
Cell Proliferation	Cells proliferate at an unregulated, rapid pace, often overestimating the growth rate compared to *in vivo* conditions.	Proliferation is more controlled, reflecting rates observed in actual tissues, contributing to a more accurate model.	[4],[6]
Response to Mechanical Stimuli	Limited response to mechanical stimuli due to the flat environment, lacking pressure or structural complexity	Cells can respond to mechanical forces more naturally, imitating the physical stresses they would encounter in tissues.	[2],[4]
Growth Environment	Cells grow in a single layer, simplifying monitoring but limiting the complexity and realism of the environment.	Cells grow in multiple layers or 3D structures, allowing for complex, tissue-like formations and interactions.	[4],[11]
Usage and Analysis	Limited use in long-term studies, as cells may lose characteristics over time and fail to replicate complex tissue responses.	Well-suited for long-term studies, maintaining cellular characteristics over time and providing more robust data for analysis.	[11]
Cost	Lower cost for large-scale studies, making 2D culture an economical option for initial screenings.	Higher cost due to the need for specialized equipment and resources to maintain 3D structures, often justifiable for more advanced studies.	[2],[4],[6]

Furthermore, 3D culture offers several methods of cell culture depending on the type of experiment being performed. Scaffold-based approaches, including hydrogels, polymeric hard materials, hydrophilic glass fibers, and non-adhesive supports , offer a range of benefits [5].

Hydrogels are particularly notable for their ability to mimic the extracellular matrix (ECM) and facilitate the movement of soluble compounds such as cytokines and growth factors throughout the tissue-like gel [5]. Thus, simpler hydrogels, even without the addition of hormones and growth factors, are able to maximize the reproducibility and offer the possibility to tune biochemical as well as mechanical properties; because of this, they have been considered for growing spheroids. Hydrogels with these features are made of natural, synthetic, or hybrid materials, such as alginate, agarose, collagen, hyaluronic acid, and polyethylene glycol [12,13].

1.2. Agarose - Collagen Hydrogels

1.2.1. Properties of Agarose

Among these materials, agarose is an interesting option due to its inert nature, low cost, and ease of availability. Agarose is a linear polysaccharide derived from red marine algae. It is composed of alternating units of D-galactose and 3,6-anhydro-α-L-galactopyranose, which are linked by α-1,3- and β-1,4-bonds, respectively. Agarose is a highly biocompatible material with optimal gelling properties and adjustable mechanical features, which makes it a popular choice for use as a biomaterial in tissue engineering scaffolds [14,15]. Furthermore, its straightforward preparation method has recently enabled its use as a non-adhesive, micromolded substrate for the growth of tumor spheroids through multicellular aggregation [16,17]. However, its potential as an ECM-mimicking material is currently limited by its low cellular bioadhesivity.

1.2.2. Properties of Collagen

Type I collagen represents the primary protein constituent of the extracellular matrix (ECM) of mammals. It features cellular binding sites, such as the "RGD" and "GxOGER" sequences (where "R" is arginine, "G" is glycine, "D" is aspartate, "O" is hydroxyproline, "E" is glutamate, and "x" is a hydrophobic amino acid), which facilitate cell adhesion, proliferation, and signalling. This bioactivity makes collagen an ideal material for the creation of biocompatible hydrogels [18-21]. Furthermore, collagen plays a crucial role in tumour

progression and invasion [22]. However, collagen has several limitations, including weak mechanical properties, limited long-term stability, and high production costs [23].

To overcome these issues, hydrogels that blend agarose and collagen are used, combining the mechanical strength of agarose and the biomimetic qualities of collagen.

1.2.3. Agarose – Collagen blended hydrogels

Composite hydrogels integrate the advantageous characteristics of each polymer, facilitating adjustable mechanical and structural features, hence enhancing ECM-like interactions that stimulate proliferation, differentiation, and matrix synthesis. Some research has been conducted on the interactions between agarose and collagen biomaterials and their effects on cells [24-30].

Batorsky et al. (2005) developed collagen-agarose microenvironments for the purpose of controlling stem cell differentiation. They observed high cell viability and improved spreading with increased collagen concentration, which facilitates tissue repair [24]. Ulrich et al. (2011) observed that agarose increased the mechanical stiffness of the environment but restricted cell motility, indicating that local stiffening facilitates cell spreading on collagen matrices [25]. Lake et al. (2011) demonstrated that the concentration of agarose affects the mechanical behaviour of co-gels under stress, influencing the alignment of collagen fibres [26]. In a study conducted by Cambria et al. (2020), agarose-collagen blends were characterised; data showed that lower collagen concentrations preserved mechanical properties, resulting in over 80% cell viability and increased expression of ECM proteins over a period of 21 days [27]. Quarta et al. (2021) demonstrated that collagen I-agarose hydrogels support the production and recovery of breast cancer spheroids, offering a reproducible biomimetic matrix [28]. López-Marcial et al. (2022) investigated the impact of collagen on chondrocyte matrix production in agarose. Their findings indicated that 2 mg/mL collagen enhanced bioactivity, whereas higher concentrations diminished mechanical properties [29]. Lastly, Zigan et al. (2024) demonstrated that agarose-collagen hydrogels could sustain mechanical characteristics while facilitating cell proliferation and glycosaminoglycan production. This highlights the potential of these hydrogels in studying cartilage mechanotransduction [30].

The research on agarose-collagen blended hydrogels indicates their potential for enhancing cell-matrix interactions, mechanical properties, and overall biofunctionality, which could be beneficial for a range of tissue engineering applications. These composite hydrogels

have demonstrated significant advantages, including improved cell viability, proliferation, and differentiation. Agarose-collagen composite hydrogels offer the structural strength of agarose and the bioactive properties of collagen, creating a more biomimetic environment for cell growth.

Considering these encouraging outcomes, the subsequent phase of investigation entails the pursuit of alternative and sustainable sources of collagen, with the objective of further enhancing the functional attributes of these hydrogels. Fish bone-derived collagen represents a valid route to convert waste into valuable biomaterials.

1.3. Fish Bone-Derived Collagen

1.3.1. Sustainability and Advantages

The increasing volume of waste is becoming a significant challenge for our society. Recent reports indicate that in 2018, the average EU resident generated 5.2 tons of waste per capita [31]. Moreover, global waste production is increasing, affecting both developed and developing countries [32]. Most of this waste is generated by industrial processes, accounting for approximately 90% of the total [31]. It is notable that waste from food manufacturing frequently contains compounds with valuable functional properties and potential technological applications. The extraction and valorisation of these compounds is consistent with the principles of a circular economy and offers societal benefits [33,34].

The fish processing sector offers significant potential for the valorisation of waste materials. Fish residues, including bones, can be employed for a variety of applications [35-37]. Fish bones have the potential to yield valuable compounds. Bones are composed of a mineral component (calcium phosphate), water, and organic material, with the proportions varying according to the species in question.

Calcium phosphates (CaPs) are a type of ceramic compound with a variety of applications in industry, particularly as bioceramics. They are the primary inorganic component of bones. These materials are employed in the creation of bone replacements due to their bioactive and non-toxic properties. Indeed, they have been demonstrated to promote cellular differentiation and encourage bone regeneration [38,39].

Despite the presence of certain lipids, the organic fraction is predominantly composed of proteins [40]. Collagen, which constitutes approximately 90% of the organic matter, represents a substantial proportion of these proteins [41]. Fish bone collagen exhibits structural similarities with collagen type I, which is predominantly composed of two chains, α1 and α2 [42] and has a comparable molecular weight (approximately 100 kDa). Additionally, it has been reported that certain fish species contain an α3 component [43].

1.3.2. Collagen-based Extraction from Fish Bones: Methods and Applications

In general, collagen derived from marine sources has minimal immunogenicity, strong water solubility, and excellent biocompatibility and biodegradability. Given their extensive range of applications, including use as a biomaterial in tissue engineering (for bone, skin and cartilage), as a wound dressing and for medication administration, it is unsurprising that these qualities have attracted scientific interest [44].

Despite the existence of multiple techniques for the extraction of collagen from fish bones [45], they all entail the treatment of the bones in an acidic solution, which results in the dissolution of the CaP phase and renders its recovery unfeasible.

In 2015, Cleland and Vashishth published an alternative technique for extracting proteins from bones, which was subsequently corroborated by other scientists [46-48]. The proposed protocol does not necessitate the utilisation of acids, as the extraction process is conducted using a saline solution comprising $(NH_4)2HPO_4/(NH_4)HCO_3$ or solely $(NH_4)HCO_3$ at a moderately basic pH. Furthermore, the method allows for the extraction of a range of proteins in their native conformation, including vimentin, haemoglobin, serum albumin, and various proteins derived from the vascular system, as well as osteocalcin, the second most abundant protein in bone tissue. This is achieved without the use of denaturing agents, which preserves the mineral portion of the bones during the protein extraction process due to the non-acidic pH.

1.4. From waste to value

The main objective of this study was to develop and characterize agarose-collagen-based hydrogels derived from fish bone collagen through different extraction methods, targeting sustainable biomaterials for 3D cell models. By repurposing fish waste for high-value applications, this research promotes sustainable practices in tissue engineering and biotechnology. Additionally, it advances 3D cell culture techniques by providing a novel hydrogel formulation that combines mechanical stability and bioactivity, supporting tumor spheroid growth in a cost-effective, environmentally conscious way. The findings could encourage broader adoption of sustainable collagen sources in biomedical research, offering a practical, scalable solution for enhanced 3D cell models.

To do this, the methodology described above was employed in the present investigation for the extraction of collagen from salmon (*Salmo salar*) bones. In particular, a basic solution of $(NH_4)HCO_3$ was utilized instead of $(NH_4)2HPO_4/(NH_4)HCO_3$ reported in the original protocol. This was done as previous work showed that protein extracts using only $(NH_4)HCO_3$ showed better functional properties, such as antioxidant activity [49].

Three different experimental conditions were assessed with the same solution. In one case, a shorter extraction time (4 hours) was performed at a higher temperature (60 ºC), primarily yielding smaller peptides; some bones in this set were pre-treated with ultrasound, while others were not. The alternative method involved a longer extraction (5 days) at a lower temperature (4 °C) to better preserve collagen structure.

The collagen extracts derived from fish by-products were used to develop hybrid agarose-collagen hydrogels, with an agarose concentration of 0.2%; this value was chosen as previous studies showed to be enough to support spheroids growth [28]. The collagen-based extract was tested at the two concentrations of 0.02% and 0.04% in the final hydrogel; the effects on mechanical, structural, and cellular properties due to the collagen addition was studied. Indeed, a comprehensive characterization of these hydrogels was performed, and their potential to support tumor spheroid growth from MG63 (human osteosarcoma) cell lines was investigated.

Chapter 2: Materials and Methods

2.1. Chemicals and reagents

The salmon bones were obtained from a local fish shop, cleaned and stored at -20 ºC prior to use.

The following reagents: (NH4)HCO3 (product number 09839, >99%), protease inhibitor cocktail (product number P8340, DMSO solution), $CHCl_3$ (product number PHR1316), Bovine serum albumin (BSA, product number A2153, lyophilized powder, ≥96% purity), Dimethyl Sulfoxide (DMSO, ACS reagent, ≥99.9% product number 472301), Dulbecco's Phosphate Buffered Saline (PBS, product number D8537), Dulbecco's Modified Eagle's Medium High Glucose medium (DMEM, product number D5671), L-glutamine (product number G7513), fetal bovine serum (FBS, product number F2442), penicillin/ streptomycin (product number P4458), Agarose, β-agarase from Pseudomonas atlantica, were purchased from Sigma-Aldrich.

The cell lines of human osteosarcoma cell line (MG63) and murine fibroblasts cell line (3T3) were obtained from American Type Culture Collection (ATCC).

Ultrapure grade water, with a conductivity of 18.2 MΩ cm, was obtained from the Ultrapure water purification system at the lab this work was developed.

2.2. Extraction from salmon bones

Three distinct extractions, following a previously reported protocol [46] with some modifications were performed to obtain the protein and mineral fractions from the salmon bones:

- The bones were pre-treated with ultrasound and successively were placed in contact with a solution of $(NH_4)HCO_3$ 0.2 M at 60 ºC for 4 h, under stirring;
- The bones were placed in contact with a solution of $(NH_4)HCO_3$ 0.2 M at 60 ºC for 4 h, under stirring;
- The bones were placed in contact with a solution of $(NH_4)HCO_3$ 0.2 M at 4ºC ◦C for 5 days, under stirring, 100 μL of a protease inhibitor solution were added to the mixture in order to prevent the degradation of the protein.

The conditions to which each sample was subjected are summarised in **Table 2**.

Table 2- Summary of experimental conditions for the extraction of each sample.

Extraction	Time	Temperature	Ultrasound pre-treatment	Protease inhibitor
US	4 hours	60 ºC	Yes	No
NUS	4 hours	60 ºC	No	No
4ºC	5 days	4 ºC	No	Yes

The solution was obtained from each extraction were subjected to dialysis against distilled water for a period of 48 h at 4 ºC (membrane Spectra/Por MWCO 600-1000 Da, total water volume of 1 L). Following this, the dialysed solutions were lyophilised, and the resulting solid was stored at 4 ºC prior to analysis.

The yield of each extraction was calculated by weight percent, according to the following equation:

$$yield\ of\ the\ extraction = \frac{weight\ of\ the\ extract\ lyophilised}{weight\ of\ the\ salmon\ bone} \times 100$$

2.2.1. Biocompatibility of the extracts

To assess the biocompatibility of both extracts, the assay of 3- [4,5- dimethylthiazol-2-yl] -2,5-diphenyl tetrazolium bromide, usually called tetrazolium salt thiazolyl blue (MTT) assay was performed first using two cell lines, namely 3T3 (murine fibroblasts) and MG63 (human osteosarcoma).

Cells were grown in DMEM medium supplemented with 2 mM glutamine, 10% of fetal bovine serum (FBS), 100 IU mL^{-1} of penicillin and 100 µg/mL of streptomycin; they were cultured in an incubator at 37 °C in a humidified atmosphere with 5% CO_2. The cells (10,000), suspended in 200 µL of culture medium were seeded into each well of a 96 multiwell plate. After 24 h incubation, the UV-sterilised extracts were added to the wells at different concentrations (0.08, 0.167, 0.33 and 0.67 mg/mL); cells were kept under incubation for 24 h. At the end of the incubation, the medium was removed, the cells were washed twice with PBS, and 200 µL of fresh serum-free medium containing 2 mg/mL MTT were added to each well. After 3 h of incubation at 37 °C, the medium was removed from the wells and 200 µL of DMSO

was added to dissolve the formazan salts. The plate was stirred for a few minutes at room temperature; then the absorbance of the solution (λ = 570 nm) was measured on a microplate reader (CLARIO star Plus, BMG Labtech, Germany).

To estimate the percentage of cell viability, the treated samples were compared to the control samples (cells without any treatment) according to the following equation:

$$Cell\ viability = \frac{absorvance\ of\ the\ treated\ sample}{absorvance\ of\ the\ control\ sample} \times 100$$

The extracts samples were subjected to a treatment with chloroform ($CHCl_3$), followed by a two-day dialysis process to remove residual solvents and lipid components prior to repeat the viability assay.

2.3. Preparation of the Hydrogel

Agarose was dissolved in sterile phosphate-buffered saline (PBS, pH 7.4) to obtain a 1% (w/v) stock solution, by heating on a hot plate. An aqueous suspension of 0.1% (w/v) of collagen-based extracted from the fish bones was obtained by dissolving the lyophilized extract in PBS; To ensure sterility and avoid contamination, the samples were then exposed to ultraviolet (UV) light for one hour.

The blended hydrogels were prepared inside the wells of a 24 well plate, and each well was loaded to a final volume of 1 mL. In detail, the warm agarose solution (at around 55°C, the gel point is at 36°C) was added at 0.2% w/v as final concentration in the hydrogel. The starting extract solution was diluted to 0.02% and 0.04% in the final blend.

The mixture in each well was gently stirred with a spatula and allowed to gel at room temperature.

2.4. Characterization of the Hydrogel

2.4.1. Stability Test

The analysis was carried out keeping the hydrogels at 4 °C in DMEM, and the samples were weighted at determined time points. The stability of the hydrogels was then calculated as residual weight percentage:

$$Residual\ weight\ \% = \frac{w - w0}{w0} \times 100$$

where w0 is the starting weight of the hydrogel at t = 0, and w is the weight at each time point.

2.4.2. Collagen Release

The hydrogels were kept in the fridge at 4 ºC and 500 µL of PBS was collected at determined time intervals to estimate the protein content, via bicinchoninic acid (BCA) assay [50]. A calibration curve was set using the protein solution included int the BCA assay kit, at known concentrations. The total quantity of collagen released was calculated using the following equation:

$$Total\ collagen\ released\ \% = \frac{c}{c0} \times 100$$

where c0 is the starting concentration of the collagen extract added to the hydrogel at t = 0, and c is the total concentration of collagen released after 5 days.

2.4.3. Mechanical Compression Test

The effect of the nature of the collagen-based extract and their concentration on Young's modulus of the hydrogels was evaluated by uniaxial compression test, represented in **Figure 1**. Briefly, the samples were incubated in PBS at 37◦C in a humidified atmosphere with 5% CO_2. Cylinders of 8 mm were punched out and loaded into the testing chamber. All tests were performed with a universal ZwickiLine (Zwick Roell, Ulm, Germany) testing machine fitted with 10 N load cell. Loaded samples were hydrated in PBS at 37°C and subjected to compression with a displacement rate of 0.01 mm/s, until 80% strain [51,52].

The compressive modulus was calculated by linear fitting between 2% and 10% of strain of the stress-strain curve. The test was performed five times for each sample type.

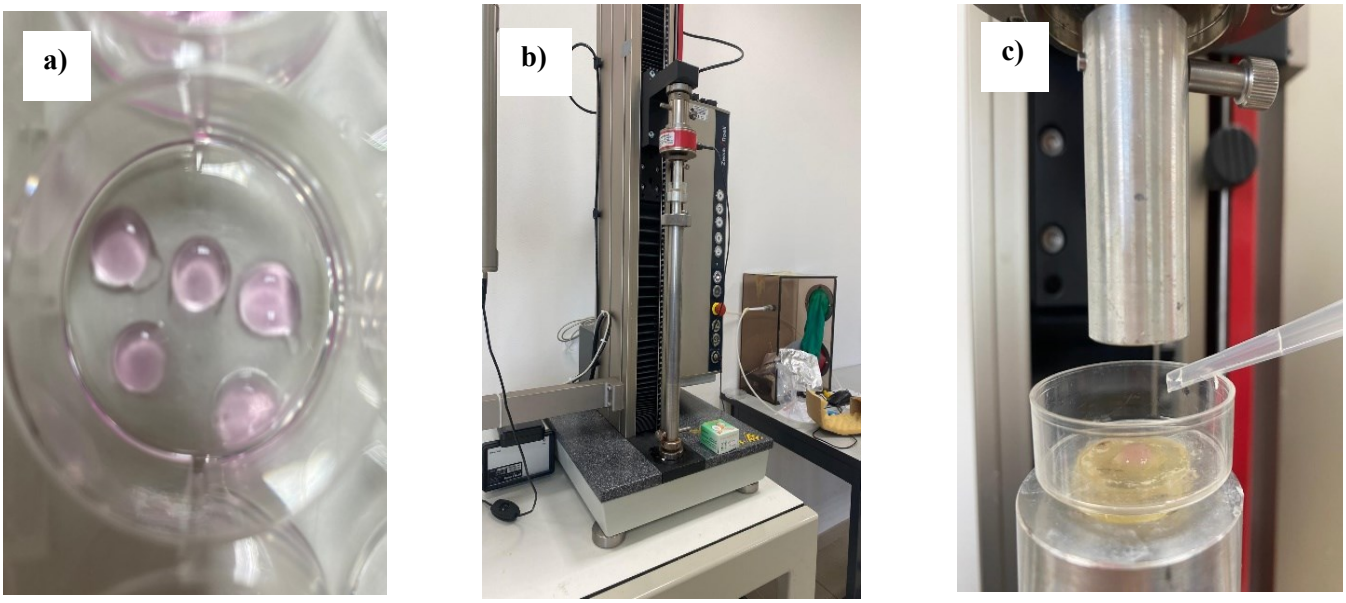

Figure 1- a) Cylinders of 8 mm; b) Universal ZwickiLine (Zwick Roell, Ulm, Germany) testing machine fitted with 10 N load cell; c) Loaded samples were hydrated in PBS at 37◦C and subjected to compression with a displacement rate of 0.01 mm/s, until 80% strain.

2.4.4. Scanning Electron Microscopy (SEM)

Surface morphology and porosity of the hydrogels were investigated by using SEM imaging techniques. SEM images were recorded with a Carl Zeiss–Merlin field emission scanning electron microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Gemini column and integrated with high efficiency in-lens and SE2 detectors, for high spatial/depth-of-field resolution secondary electrons (SE) imaging of surface structure and topography. The microscope was used in high vacuum and high-resolution acquisition mode and the images were recorded at an accelerating voltage of 5 kV and a few seconds frame-integration time, to minimize charging effects and sample damage. The hydrogels were frozen, lyophilized and cut before imaging.

2.5. Tumor Spheroid Preparation and Characterization

The human osteosarcoma cell lines, i.e., MG63 cells were grown in DMEM medium (4500 mg/L glucose) supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 ◦C in an atmosphere of 5% CO2. The cells were trypsinized, counted and added to the hydrogels at a density of 5 x 10^5 cells/mL in complete growth medium. The cell suspension containing agarose and the collagen extract was let to gel into the well plate. After gelation, 1 mL of DMEM was added over the cell-embedded hydrogels before transferring the plate into the incubator. The medium was changed every 4 days.

2.5.1. Morphological Analysis of the Tumor Spheroids

The morphological characteristics of spheroids, including their diameter and shape, were determined by optical analysis using a EVOS XL Cell Imaging System microscope (Thermo Fisher, Waltham, MA, USA). The progressively developing spheroids were observed at 24 h intervals. The mean diameter of the 3D structures was calculated by using ImageJ Software.

2.6. Cellular Assay

2.6.1. Live/dead assay

The viability of spheroids was investigated by using a live/dead assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Briefly, the activity of intracellular esterase induces non-fluorescent, cell-permeant calcein acetoxymethyl ester to become fluorescent after hydrolysis, giving a green fluorescence to the viable spheroids. On the other hand, ethidium homodimer enters and binds to nucleic acids only into damaged cells, producing a red fluorescence that indicates dead cells. The assay was performed after ten days growth. In detail, the medium was removed from the plates containing the spheroids-embedded hydrogels and the samples were washed twice with PBS. A phosphate buffer solution containing calcein and ethidium homodimer was then added, and the plate was kept in incubation at 37°C for 1 h. Finally, the solution was replaced with fresh PBS before imaging the samples under a Fluorescence Microscope (EVOS FLoid Cell Imaging Station, ThermoFisher, Waltham, MA, USA).

2.7. Statistical Analysis

The statistical analysis was conducted using the IBM® SPSS® Statistics 30 software.

To compare the means across multiple groups, a one-way ANOVA was employed when a single factor was present (type of extraction) or a two-way ANOVA was used when more than one factor was involved (type of extraction and concentration of each extract added to the hydrogel), for datasets with a normal distribution. Tukey's HSD was used for post hoc analysis.

The level of statistical significance was set at $p < 0.05$.

Chapter 3: Results

3.1. Yield of the extraction process

Three distinct extractions were employed to obtain the protein and mineral fractions from the salmon bones as described in the previous section. The yields obtained from salmon bones using different methods are illustrated in **Table 3.**

Table 3- Yield of collagen-based extract for each type of extraction.

Sample	% Yield of the extraction
US	8.90 ± 2.86% [a]
NUS	6.01 ± 0.27% [a]
4ºC	1.50 ± 0.33% [b]

[a,b] Same letters mean no statistically significant differences ($p > 0.05$)

The US method yielded the highest collagen, with an average of 8.90% ± 2.86%. The NUS method yielded a lower result of 6.01% ± 0.27%, which, while effective, was less efficient than the US but no statistically significant difference was observed between these two methods. The extraction conducted at 4°C exhibited the lowest yield, with a mere 1.50 % ± 0.33 % yield, a significantly lower value compared to the methods mentioned before.

3.2. Biocompatibility of the extracts

The biocompatibility of collagen extracts obtained using US, NUS, and 4°C extraction methods was evaluated first on 3T3 cell lines to assess the cytotoxicity levels at varying concentrations. The preliminary findings, shown on **Figure 2**, indicated that certain extracts, notably those derived via NUS method, demonstrated diminished cell viability, below 70%, the limit associated to cytotoxicity. This suggests the potential presence of cytotoxic components or residual solvents in these samples. To enhance compatibility, the extracts were subjected to a chloroform ($CHCl_3$) treatment, followed by a two-day dialysis process to remove residual solvents and lipid contaminants.

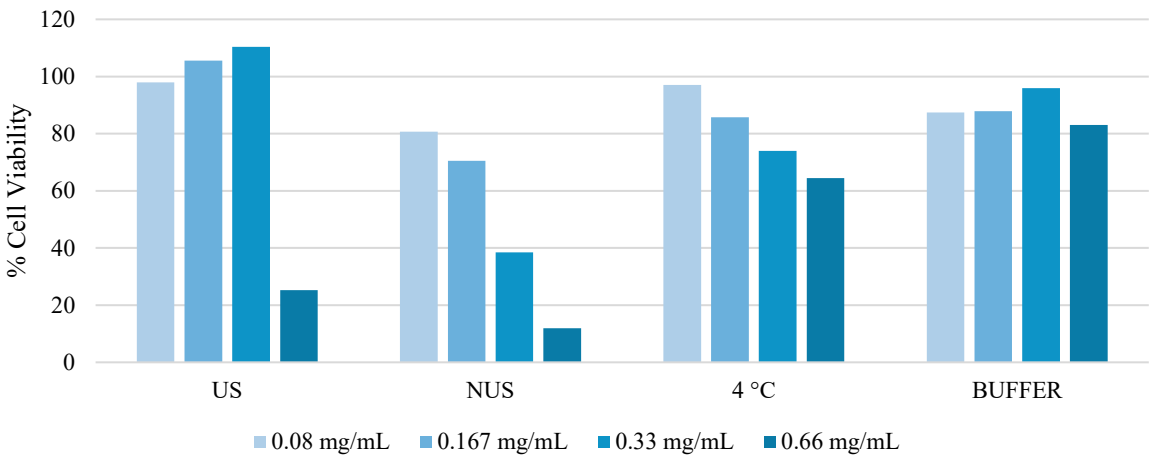

Figure 2- Biocompatibility study of US, NUS and 4ºC samples tested against 3T3 cells. For each sample 4 concentrations (0.08mg/mL, 0.167mg/mL, 0.33mg/mL, 0.66mg/mL) were tested after 24h of incubation. Results are shown as percentage over the control sample (cells without any treatment).

Following the treatment described above, the 3T3 cell line demonstrated enhanced cell viability across all extraction methods and concentrations (**Figure 3**). In fact, the viability of the US and 4ºC extract remained stable across all concentrations (0.167 mg/mL, 0.33 mg/mL, 0.66 mg/mL, and 1.32 mg/mL), always higher than 100%, thereby reinforcing its suitability for applications requiring biocompatible materials. Regarding the NUS method, the chloroform treated samples showed improved viability, where untreated extracts had previously displayed cytotoxic effects. This improvement suggests that the chloroform and dialysis process effectively removed cytotoxic components, yielding extracts that are compatible with 3T3 cells.

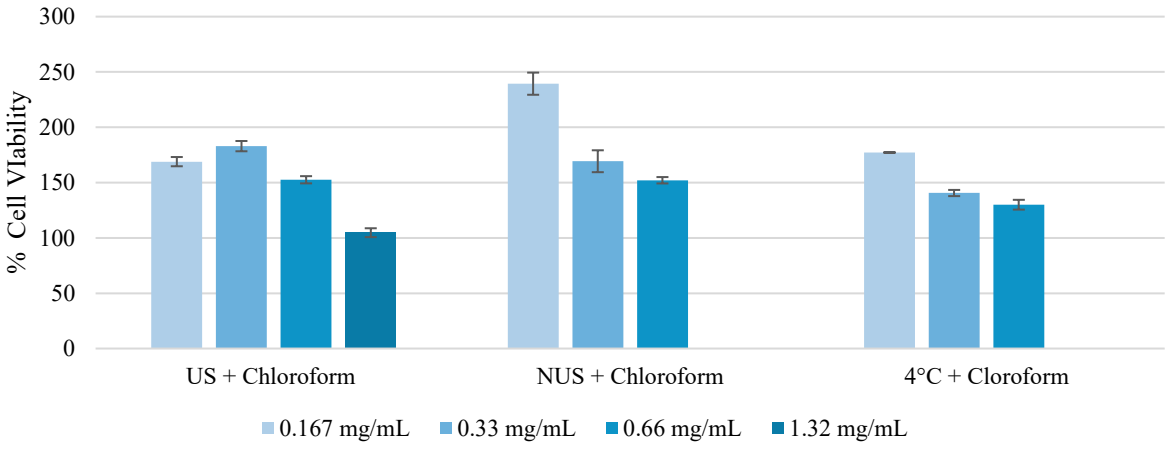

Figure 3-Biocompatibility study of US, NUS and 4ºC with chloroform treatment samples tested against 3T3 cells. For each sample 4 concentrations (0.167 mg/mL, 0.33 mg/mL, 0.66mg/mL, 1.32 mg/mL) were tested after 24 h of incubation.

The results on MG63 cell line followed a similar trend, with chloroform-treated samples showing substantially enhanced viability (**Figure 4**). US, NUS and 4ºC extracted collagen showed high viability levels across all tested concentrations (0.167 mg/mL, 0.33 mg/mL, 0.66 mg/mL, and 1.32 mg/mL), reinforcing its biocompatibility, often reaching or exceeding 100%. This outcome confirmed that the removal of residual solvents and lipids was particularly beneficial for the extracts, rendering them suitable for applications involving MG63 cells.

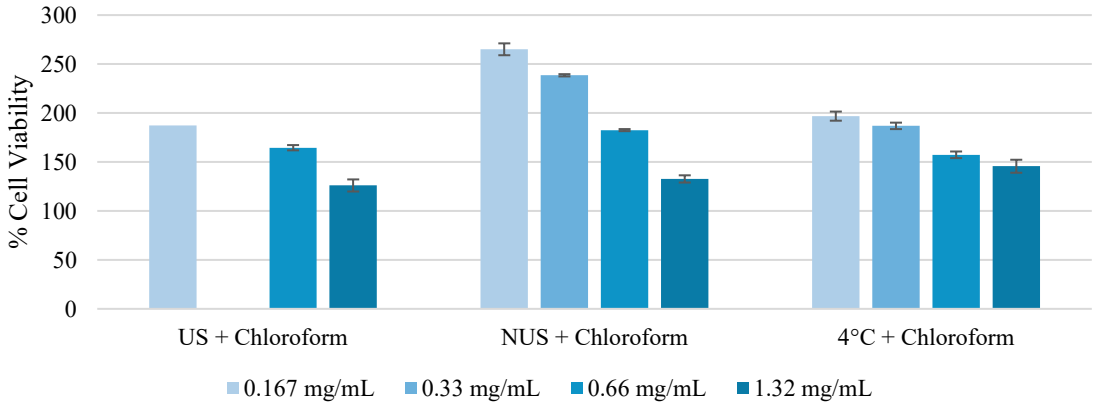

Figure 4-Biocompatibility study of US, NUS and 4ºC with chloroform treatment samples tested against MG63 cells. For each sample 4 concentrations (0.167 mg/mL, 0.33 mg/mL, 0.66mg/mL, 1.32 mg/mL) were tested after 24 h of incubation.

Overall, the chloroform treatment and subsequent dialysis were effective in improving the biocompatibility of the collagen extracts across both cell lines. This finding highlights the importance of post-extraction processing to eliminate cytotoxic residues, especially when using extraction methods that may retain unwanted solvents or lipid components. The concentrations of collagen extracts selected for testing in the formulations incorporated into the hydrogels were 0.20 mg/mL and 0.40 mg/mL. These concentrations fall within the range demonstrated to support high cell viability and will be employed to investigate the interaction between agarose--collagen-based hydrogels and MG63 cells in subsequent experiments.

3.3. Characterization of the Hydrogel

Six agarose–collagen-based hydrogels with different weight ratios were tested in this study, as two extracts concentrations were tested for three different extracts.

The goal of this study is to assess the effects of collagen extracts derived from fish bones (rather than type I collagen) on the hydrogel's biophysical characteristics and, in turn, its influence on the development of cellular spheroids; because of this, the concentrations of collagen extracts varied from 0.02% to 0.04% in the final blend. The agarose content was maintained at 0.2% in all hydrogel formulations; this value was chosen as literature showed the resulting hydrogels having the most adequate characteristics to give the embedded cells structural support, [46]. The preparation scheme is summarized in **Figure 5**.

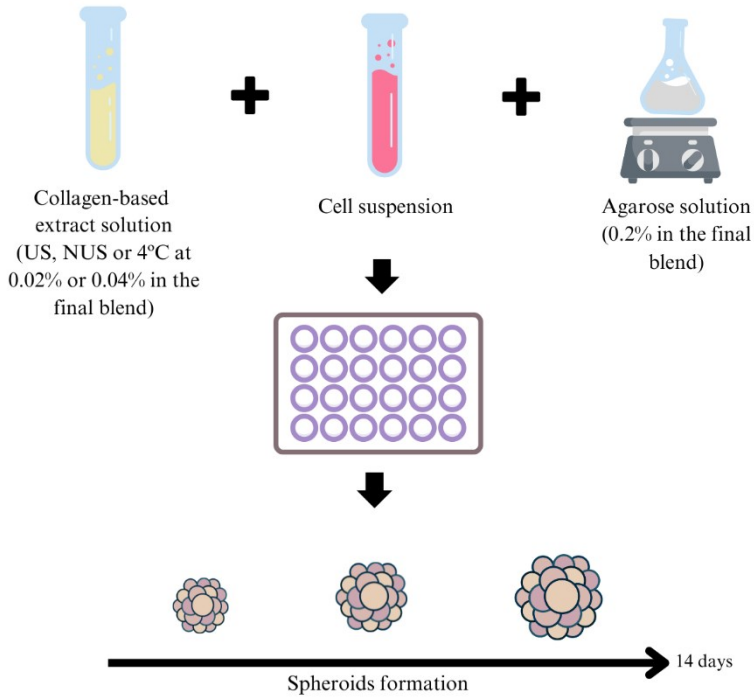

Figure 5-Schematic drawing showing the preparation of the agarose-collagen-based hydrogels containing tumour cells and subsequent spheroids formation.

3.3.1. Collagen Release

The quantity of collagen-based material potentially released was calculated through the BCA assay, as illustrated in **Figures 6.** To this aim, the hydrogels were maintained in PBS, in the fridge at 4 ºC, with the volume of solvent collected and replenished every 24 h for a period of five days. The release of collagen was observed in all hydrogel samples.

The hydrogels prepared with US collagen (**Figure 6a)**) exhibited the highest release levels at both 0.02% and 0.04% collagen concentrations, with measured values of 183.00 µg/ml and 111.57 µg/ml, respectively. These values correspond to collagen losses of 56% and 46% of the original collagen concentration present in the hydrogel after five days. The fragmentation caused by the ultrasound process likely contributed to this release, as it produces shorter collagen fragments that are more prone to diffusion from the hydrogel. The higher release at 0.02% could be attributed to a more porous network structure in comparison to the denser, more restrictive matrix formed at 0.04%.

The hydrogels prepared with NUS collagen resulted in an intermediate release profile (**Figure 6b)**), with 98.00 µg/ml at 0.02% and 185.86 µg/ml at 0.04%, corresponding to 49% and 46% losses, respectively. The method employed resulted in the production of collagen with a more cohesive structure, rather than US, which appeared to integrate more effectively into the hydrogel network, thereby reducing the overall release. As with the US samples, the denser network formed at 0.04% may impede release to a slightly greater extent than at 0.02%. However, this effect is less pronounced due to the reduced fragmentation.

The lowest release was observed for the hydrogels prepared with 4ºC collagen (**Figure 6c)**), with 48.00 µg/ml at 0.02% and 68.00 µg/ml at 0.04%, representing 24% and 17% losses, respectively. This method preserved collagen's native structure, which resulted in a stable, cohesive network within the hydrogel that minimises the diffusion of collagen. The formation of a denser matrix at the 0.04% concentration effectively reduces the release of the extracted collagen in comparison to the 0.02% concentration.

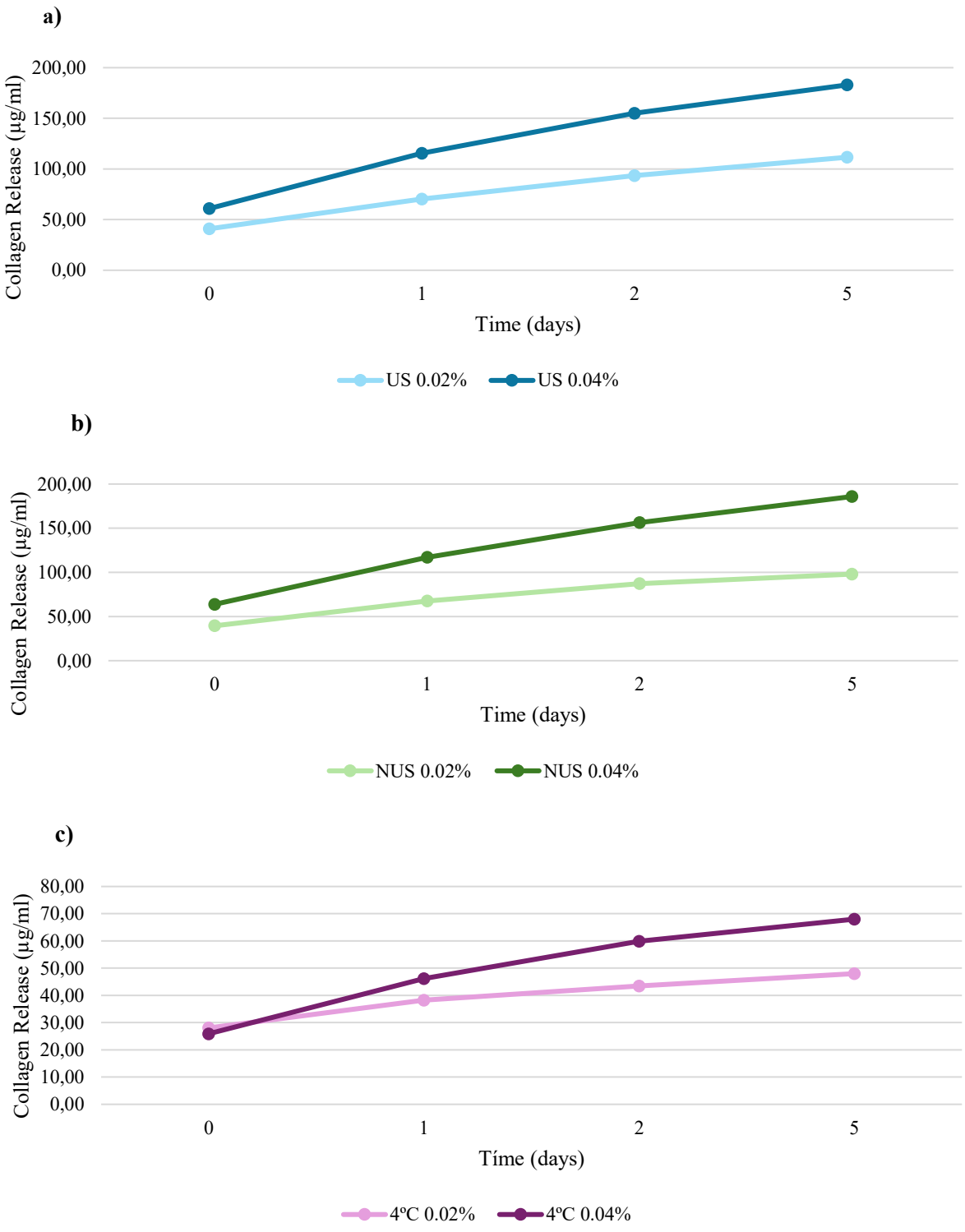

Figure 6- BCA assay performed with agarose-collagen-based hydrogels with a) US, b) NUS and c) 4ºC extract at 0.02 % 0.04 % in the final blend and at four times points up to 5 days. Time 0 corresponds to 3 h after preparation.

3.3.2. Stability Test

The stability over time of the six formulations, kept in the fridge at 4º C, was also investigated by measuring the percentage residual weight up to fourteen days and compared to the control.

The control, agarose hydrogel without collagen (AG), demonstrated the greatest stability, with a mere 15% degradation observed over the 14-day period. This minimal loss in weight indicated that the agarose matrix provided a relatively stable structure, with limited degradation under the specified experimental conditions. In contrast, the agarose-collagen-based hydrogels demonstrated a range of degradation levels.

The hydrogels containing US collagen exhibited (**Figure 7a)**) the highest values of degradation, with a loss of 30% for the 0.02% collagen concentration and 24% for the 0.04% concentration. This was consistent with the higher release rates of collagen observed, as the fragmented collagen structure resulted in a less stable matrix that was susceptible to degradation. The denser network at 0.04% exhibited moderate resistance to breakdown in comparison to 0.02%, although this effect was limited by the inherent fragmentation of the US extracted collagen.

The hydrogels comprising NUS collagen exhibited (**Figure 7b)**) moderate degradation, with a 25% reduction in weight at 0.02% and a 22% reduction at 0.04%. The more cohesive structure of NUS collagen was likely to enhance network integrity, thereby reducing degradation. The slightly higher stability observed at 0.04% percentage concentration suggests that an increased collagen concentration contributes to the formation of a more cohesive matrix.

The hydrogels comprising 4°C collagen exhibited (**Figure 7c)**) the lowest degree of degradation, with 23% and 22% weight loss at 0.02% and 0.04%, respectively. The collagen structure that was preserved by the 4°C extraction process resulted in a highly stable network, resistant to degradation at both concentrations. This stability could be attributed to the stronger integration and cohesive interactions within the hydrogel matrix, which were enabled by the more intact collagen structure.

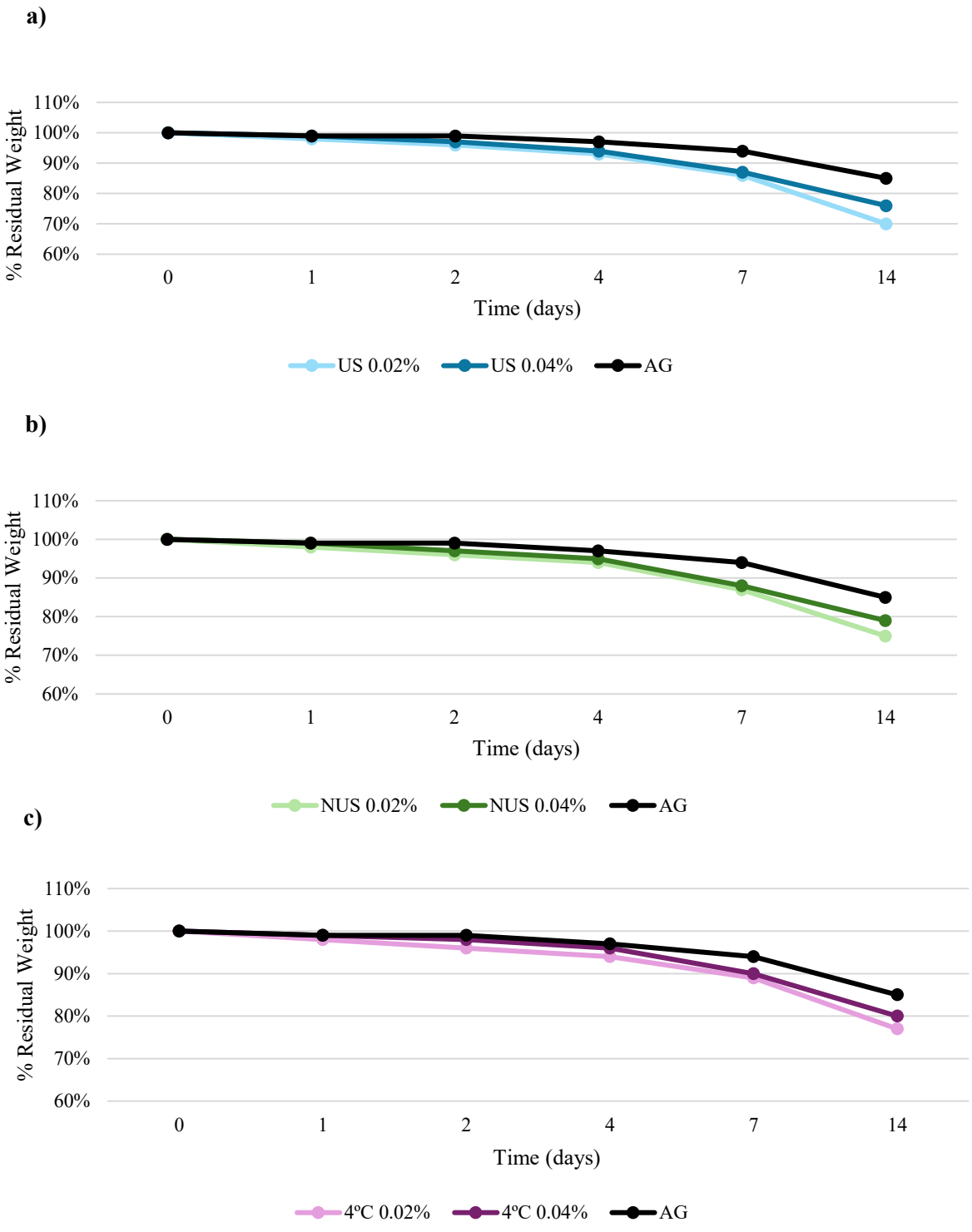

Figure 7- Degradation curves of agarose-collagen-based hydrogels with a) US, b) NUS and c) 4ºC extract at 0.02 % 0.04 % in the final blend and at four times points up to 14 days. Time 0 corresponds to 3 h after preparation.

3.3.3. Mechanical Compression Test

The structural properties of the agarose-collagen-based samples were also evaluated by mean of compression test under physiological-like conditions showing how the collagen concentration and type of extraction affected the hydrogels' mechanical properties (**Figure 8**)

There were no statistically significant differences in elasticity (E) across the extraction methods ($p > 0.05$), indicating that the extraction method alone may not significantly impact the elasticity values. While the extraction method had no effect, collagen concentration affected the elasticity; in fact, increase from 0.02% to 0.04% resulted in a significant change in elasticity ($p < 0.05$). This indicated that higher concentration leads to an improvement to the stiffness or strength in these hydrogels.

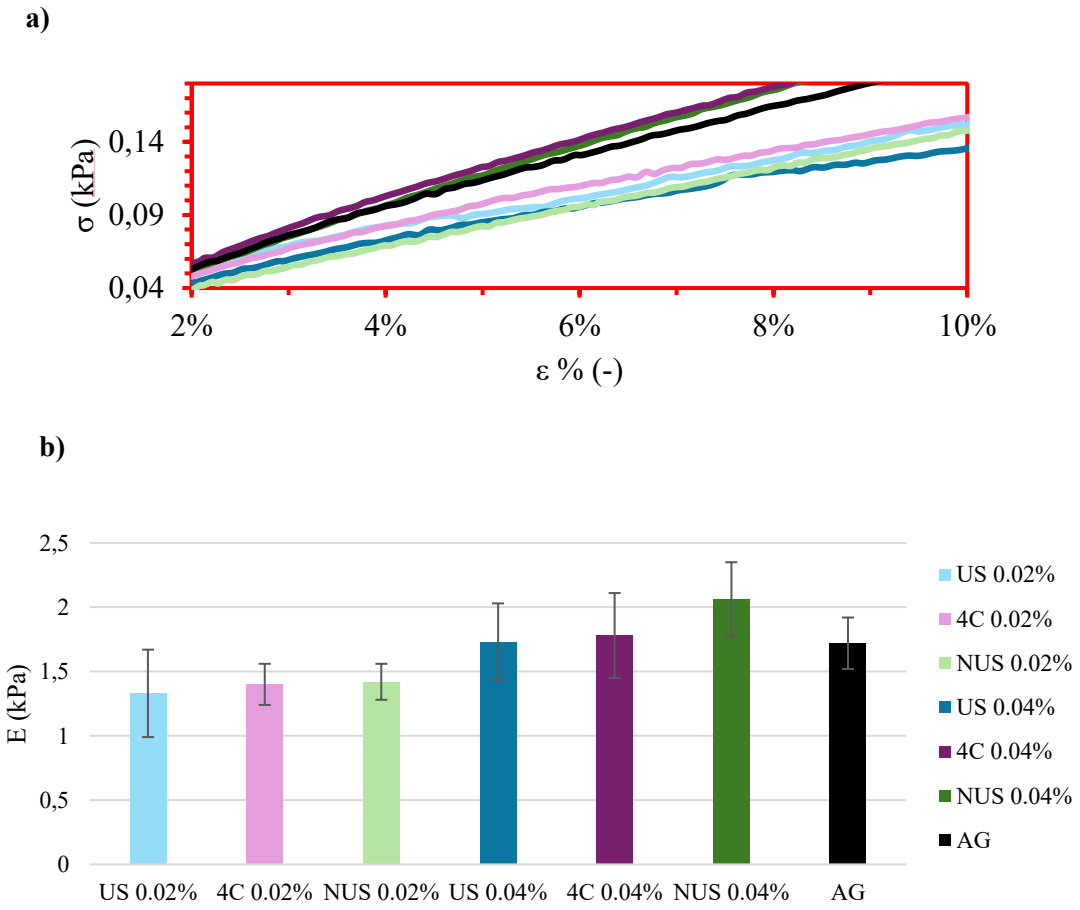

Figure 8- a) Representative stress-strain curves of the agarose-collagen-based hydrogels subjected to unconfined compression with a displacement rate of 0.01 mm/s, until 80% strain and b) Compressive moduli of the agarose-collagen-based hydrogels after incubation in PBS at 37ºC in humidified atmosphere with 5% CO_2.

In fact, an increase in the collagen concentration corresponded to an increase in the compressibility modulus. The NUS 0.04% showed the highest E modulus (2.06 ± 0.29 kPa), followed by 4ºC 0.04% (1.78 ± 0.33 kPa) and US 0.04% (1.73 ± 0.30 kPa), both higher than the control (hydrogel prepared with only agarose) with 1.72 ± 0.20 kPa. The hydrogels with the formulation at lower concentration followed the same trend, NUS 0.02% showed the highest E modulus (1.42 ± 0.14 kPa), followed by 4ºC 0.02% (1.40 ± 0.16 kPa) and US 0.04% (1.33 ± 0.34 kPa), but lower than the control.

3.3.4. Scanning Electron Microscopy (SEM)

The scanning electron microscopy (SEM) analysis of the 0.04% agarose-collagen-based hydrogels revealed that the expected pore structures were not visible; in fact, the images (**Figure 9**) showed a rough texture. This outcome is presumably attributable to the drying or preparation methodology employed prior to SEM analysis, which may have resulted in surface collapse or deformation of the hydrogel structure. Such alterations have the potential to obscure the visualization of pores and contribute to the rough appearance observed in the images. A different preparation protocol should be optimised for SEM analysis of this kind of materials.

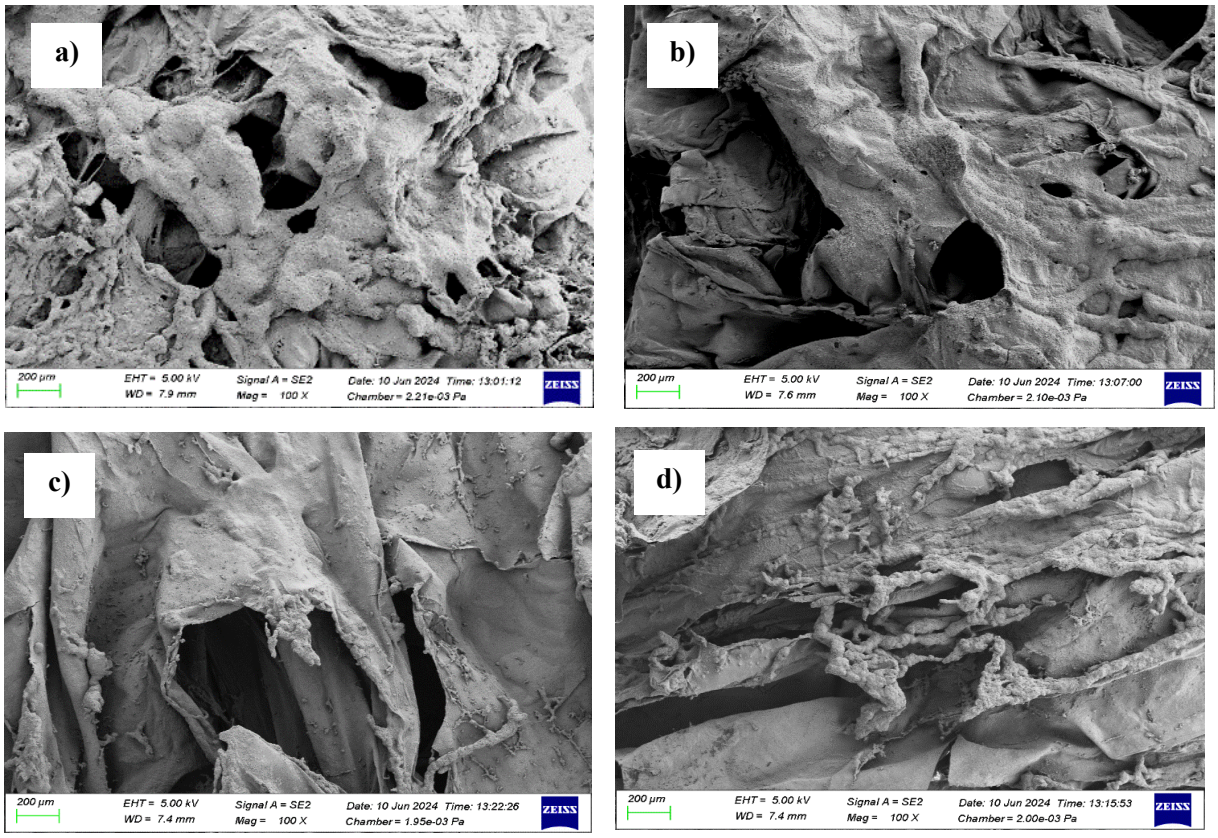

Figure 9-SEM images of the agarose-collagen-based hydrogels with a) only agarose and with 0.04% b) US, c) NUS, d) 4ºC. Scale bar is 200 µm.

3.4. Morphological Analysis of the Tumor Spheroids

One of the major advantages of hydrogels is their ability to provide more realistic 3D models for *in vitro* studies. In this study, spheroids from osteosarcoma cell line (MG63) were generated. The cells were seeded at the density of 5 x 10^5 /mL inside the six types of hydrogels (US 0.02%, US 0.04%, NUS 0.02%, NUS 0.04%, 4ºC 0.02%, 4ºC 0.04%) up to fourteen days to monitor the process of multicellular spheroid formation. Despite the formation of spheroids in all six types of hydrogels, the growth over time was not significant.

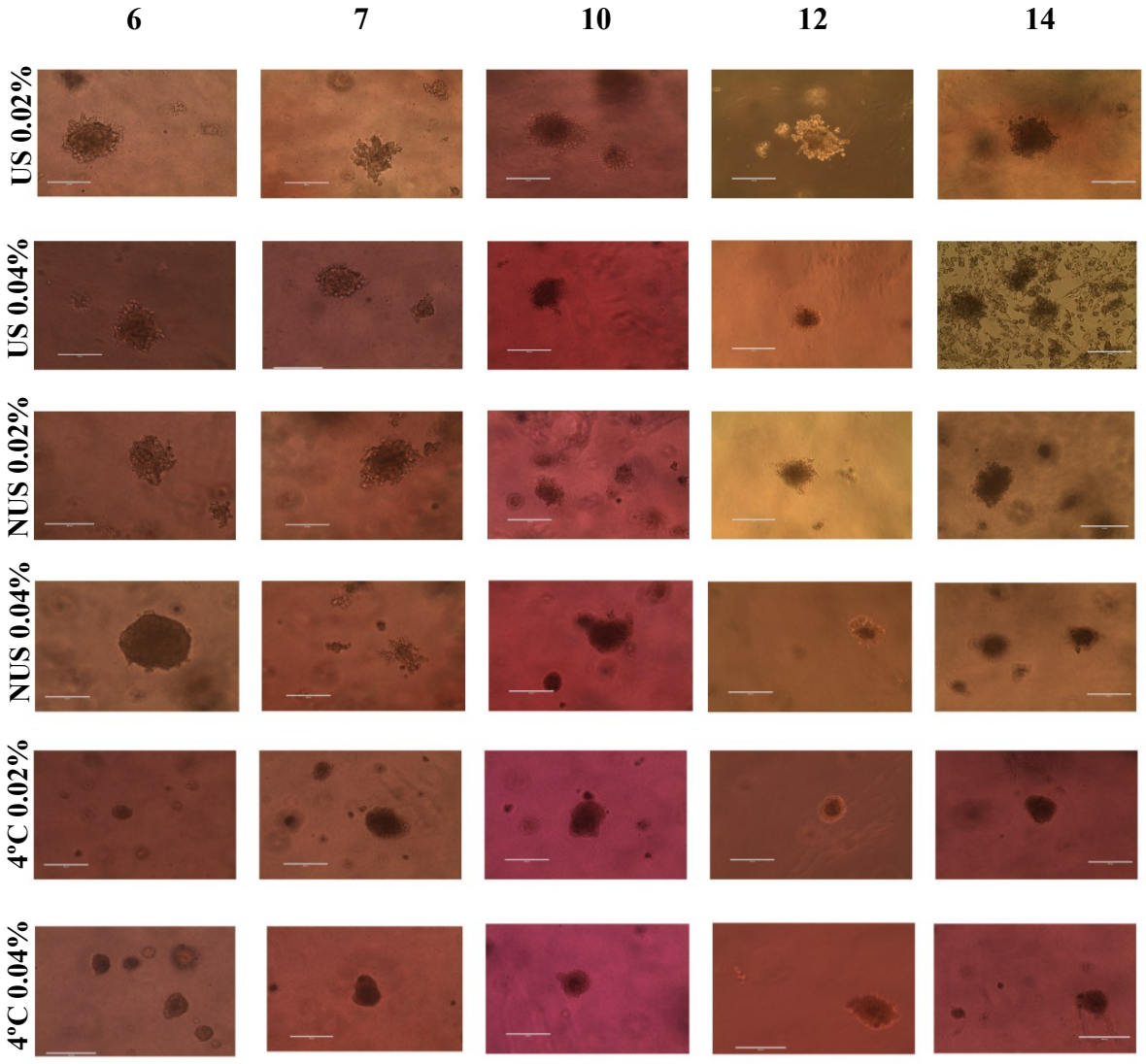

Figure 10- Optical images of the spheroids obtained with MG63 cells grown up to 14 days in US 0.02%, US 0.04%, NUS 0.02%, NUS 0.04%, 4ºC 0.02%, 4ºC 0.04% agarose-collagen-based hydrogels. Scale bar is 200 µm.

Figure 10 shows the size and morphology of the spheroids grown from day 6 to 14 in each type of hydrogel. A single image frame for each time point does not directly clarify the process through which the three-dimensional structure evolved from a single cell to a more complex aggregate. Nevertheless, they indicate that, from 1 week to 2 weeks the spheroids do not show any further size progression. This gap in understanding is also reflected in the growth curves shown in **Figures 11**.

Figure 11 a) shows that between days 6 and 14, the growth rate decreased for both US 0.02% and US 0.04%. For US 0.02%, the growth rate decreased from 224.74 ± 23.42 µm to 155.57 ± 29.00, while for US 0.04%, it dropped from 184.85± 32.63 µm to 140.90 ± 29.32 µm. This suggests that the ultrasound treatment, which often results in smaller and more fragmented collagen molecules, may have impacted the structural integrity and stability of the hydrogel. These smaller, more disrupted collagen fibers likely provide less robust support for spheroid growth, leading to a decline in size over time as the microenvironment potentially becomes less conducive for cell aggregation and proliferation.

Between days 6 and 14, **Figure 11 b)** shows that the growth of NUS 0.02% exhibited some variation but generally increased from 138.22 ± 32.53 µm to 170.32 ± 34.04 µm. This behaviour indicated that the NUS extraction process, which likely preserves longer, and more intact collagen chains compared to US extraction, provided a more stable and supportive matrix for spheroid development. The structural integrity of the collagen allowed for a better ECM-like environment, facilitating cell attachment and growth over the duration of the experiment.

A slight increase in growth is observed in **Figure 11 c)** for 4ºC formulations. For 4°C 0.02%, growth increased from 104.35 ± 30.10 µm to 136.06 ± 32.90 µm, and for 4°C 0.04%, growth increased from 111.08 ± 27.75 µm to 127.70 ± 28.41 µm. This method preserved the natural structure of the collagen the most effectively, resulting in larger collagen molecules that formed a stable hydrogel matrix. The slow and steady growth observed in these spheroids suggests that the cold extraction method produced a hydrogel environment that supports sustained, albeit gradual, spheroid formation. This controlled growth could be attributed to a well-balanced environment that mimics native tissue properties more closely.

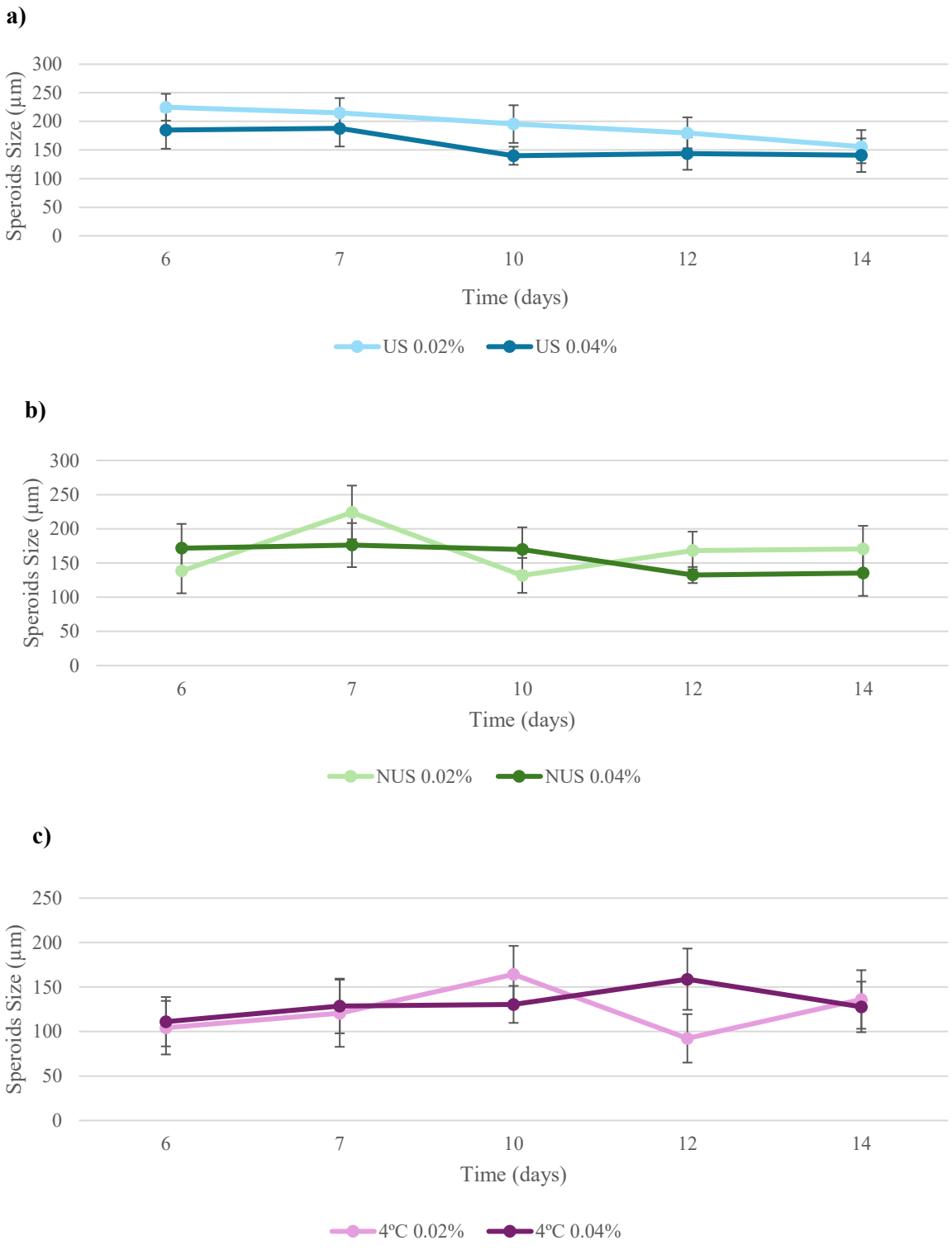

Figure 11- Average size of the spheroids obtained with MG63 cells grown up to 14 days in agarose-collagen-based hydrogels with a) US, b) NUS and c) 4ºC extract at 0.02 % 0.04 % in the final blend.

3.5. Live/dead assay

The results presented in **Figure 12** demonstrate the viability of MG63 cell line spheroids embedded in US, NUS, and 4°C 0.02% agarose-collagen-based hydrogel formulations through a live/dead fluorescence assay following a 10-day incubation period. The assay was conducted in agarose as a control, alongside the three experimental hydrogel conditions.

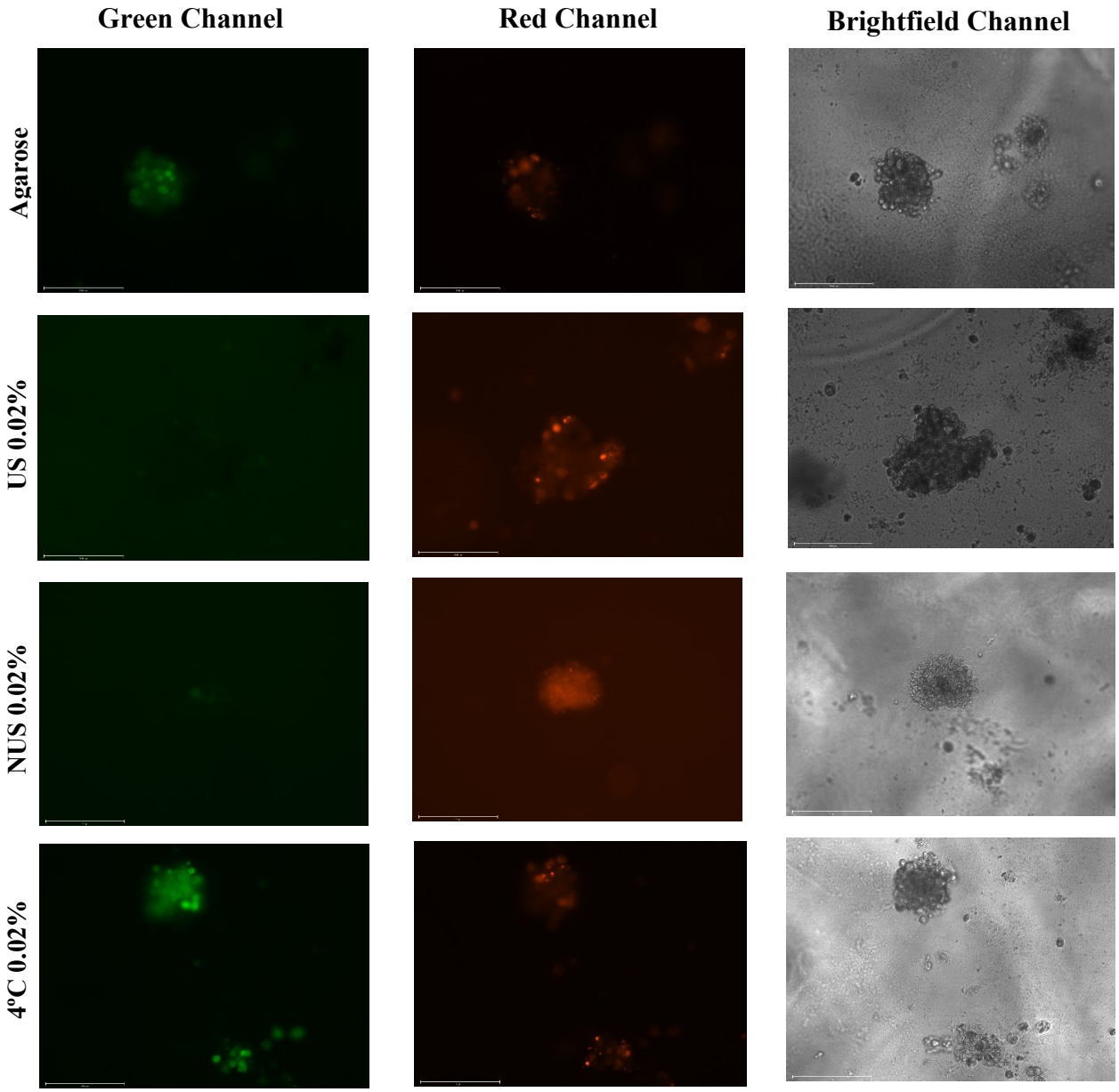

Figure 12- Live/dead assay performed with the cellular spheroids embedded into the agarose-collagen-based hydrogels at 10 days.

The Green channel that corresponds to the calcein signal, shows the presence of live cells. The control displays a substantial green fluorescence, suggesting a high proportion of viable cells. Similarly, the 4°C 0.02% hydrogel displays visible green fluorescence, indicating that it provided a supportive environment for cell viability that is comparable to that of the control. In contrast, the US 0.02% and NUS 0.02% hydrogels display markedly diminished green fluorescence, indicating a reduction in cell viability under these conditions.

The Red Channel that corresponds to propidium iodide signal, is associated to dead cells which are permeable to the nuclei staining. As shown in **Figure 12**, the agarose control exhibits minimal red fluorescence, suggesting that cell death is relatively low. However, the US 0.02% and NUS 0.02% hydrogels display a notable increase in red fluorescence, which is indicative of elevated levels of cell death in these conditions. The 4°C 0.02% hydrogel displays an intermediate level of red fluorescence, indicating a moderate degree of cell death in comparison to the control and the other experimental hydrogels.

The US extraction resulted in a hydrogel with less favourable properties for cell support due to fragmented collagen. NUS offered a moderate balance but still showed some degradation effects. 4°C preserved collagen's natural structure the most effectively, leading to a hydrogel that supports higher cell viability and reduced cell death. This preservation translates into a more biocompatible environment that aligns closely with the native ECM, justifying the observed differences in cell outcomes across the hydrogel formulations.

The results demonstrate that compared to the control, the 4°C 0.02% hydrogel formulation provide more favourable conditions for cell survival, whereas the US 0.02% and NUS 0.02% hydrogels appear less supportive, resulting in increased cell death.

Chapter 4: Discussion

In this work, blended hydrogels, composed of agarose (fixed at 0.2% weight) and collagen derived from three different extraction methods (US, NUS, 4°C) at two concentrations (0.02% or 0.04%), were prepared as enabling matrices for the growth of 3D cellular structures. These hydrogels combine the biomechanical properties of agarose, the bioadhesivity of collagen, and the sustainable nature of the extracts to create biocompatible hydrogels, showing significant potential for applications in 3D cell culture models.

Agarose and collagen have been previously used to prepare hydrogels and represent valuable candidates for creating viable hydrogels due to their biocompatibility and low cost. In this work, the use of collagen derived from fish bone extracts enhances the sustainability of these hydrogels.

This study evaluated three collagen extraction methods and examined how each technique and concentration influenced the biocompatibility, stability, mechanical properties, and cell viability of the hydrogels formulated.

The yield of collagen was highest with the US extraction method and lowest with the 4°C method. The combination of high temperature and ultrasound treatment in the US method effectively maximized collagen yield, whereas the more conservative 4°C conditions prioritized maintaining the collagen's structure, resulting in a lower yield. However, a higher yield did not necessarily translate to enhanced biocompatibility.

Biocompatibility testing underscored the importance of collagen purity and structural integrity. Initial tests indicated cytotoxicity in NUS samples, necessitating further processing with chloroform treatment and dialysis to remove residual solvents or contaminants. The improved viability across all extracts post-treatment highlighted the need for rigorous post-extraction purification, particularly for methods that might retain cytotoxic residues. After chloroform and dialysis treatment, the biocompatibility of the 4°C and US extracts increased, with cell viability exceeding 100% at all concentrations.

After improving the biocompatibility of the extracts, the hydrogels were prepared and characterized.

The collagen release rates in hydrogels prepared with different extraction methods revealed a trend tied to the structural integrity of the collagen molecules. Hydrogels containing US-extracted collagen showed the highest release rates due to the more fragmented nature of

the collagen molecules created by ultrasound processing. This finding is consistent with studies demonstrating that ultrasound treatment disrupts the collagen matrix, resulting in smaller peptides with greater solubility but less cohesive network formation within hydrogels [53,54]. By contrast, hydrogels prepared with 4°C collagen exhibited the lowest release rates, confirming that the cold extraction technique better preserved the native collagen structure, resulting in a more integrated, stable hydrogel matrix. Literature supports the conclusion that low-temperature extraction enhances collagen stability within hydrogels, retaining bioactivity and matrix integrity over time, which is critical for applications requiring sustained structural support, such as tissue engineering scaffolds [55].

These findings align with the observed stability results. Hydrogels with US collagen exhibited the highest weight loss, attributed to the loose network structure formed by fragmented collagen, making them more susceptible to degradation. On the other hand, hydrogels prepared with 4°C collagen demonstrated the lowest degradation, suggesting that the preserved, native collagen structure leads to a more stable matrix.

Compression testing illustrated the impact of collagen concentration and extraction method on hydrogel elasticity, with significant variations in the compressive modulus across formulations. Higher collagen concentrations (0.04%) consistently improved the elasticity of the hydrogels, with NUS hydrogels showing the highest compressive modulus. This enhancement in stiffness reflects findings by Xu et al. (2021), who observed that the collagen component in composite hydrogels contributes to mechanical resilience and stiffness, enhancing the hydrogel's ability to mimic the ECM [56].

Relating collagen release to stability and mechanical compression tests, the results indicate that higher collagen concentrations (0.04%) generally yield better outcomes in terms of both stability and mechanical properties. This trend suggests that the denser, more cohesive matrix formed at higher collagen concentrations provides not only better resistance to degradation but also greater structural integrity. The tighter network formed by collagen at 0.04% limits collagen release while contributing to improved mechanical properties.

A significant outcome of this study was the formation of tumor spheroids in the 3D hydrogels, particularly in the 4°C formulation. Hydrogels prepared with 4°C-extracted collagen facilitated the growth of larger, more structurally cohesive spheroids over 14 days, with optimal results at 0.02%. Moreover, the Live/Dead assay confirmed higher cell viability in the 4°C hydrogels at 0.02%. The 4°C hydrogel displayed a more favourable environment for cell

viability compared to US and NUS samples, as evidenced by higher green fluorescence, indicating live cells. This result reinforces that a stable collagen matrix, as seen with 4°C extraction, provides a more supportive microenvironment conducive to sustained cell viability and aggregation. The limited support observed in US hydrogels could be attributed to excessive collagen release, creating a less stable scaffold that compromises long-term cell aggregation and spheroid formation. Notably, while lower collagen concentrations (0.02%) produced hydrogels with reduced mechanical strength compared to those with higher concentrations, these matrices still supported effective spheroid culture. This finding suggests that lower collagen content may provide an optimal balance between structural support and flexibility, making them particularly advantageous for certain 3D cell culture applications where a less rigid environment is beneficial.

Chapter 5: Conclusions

This research illustrated the potential of fish bone-derived collagen as a sustainable and effective material for three-dimensional cell culture applications, particularly in the development of agarose-collagen hydrogels to support tumour spheroid formation. By converting fish waste into a high-value biomaterial, this study contributes to the development of circular economy practices in biotechnology, with the dual benefit of reducing the environmental impact and advancing tissue engineering techniques.

The comparative evaluation of three collagen extraction methods (US, NUS, 4°C) demonstrated that the extraction process exerts a direct influence on the structural integrity, biocompatibility, and performance of the collagen within hydrogels. The 4°C extraction method, while yielding less collagen, preserved the structural integrity needed for stable, biocompatible hydrogels that support long-term cell growth and spheroid formation. In contrast, US extraction, though efficient in yield, results in a fragmented collagen structure, leading to increased collagen release and reduced stability and cell support. The NUS method offered a compromise, yielding hydrogels with balanced mechanical properties and cell viability, making it a versatile option for applications requiring both stability and bioactivity.

The concentration of fish bone-derived collagen within agarose-collagen hydrogels influenced the physical characteristics of the 3D matrices, particularly their compressive strength. Higher collagen concentrations (0.04%) contributed to increased stability and mechanical resilience, suggesting that a denser collagen network supports structural integrity. However, this concentration did not yield additional benefits in terms of cellular morphology or growth. Lower collagen concentrations (0.02%) produced hydrogels with reduced mechanical strength compared to those with higher concentrations, yet these matrices remained effective for spheroid culture. This implies that a lower collagen content might provide a suitable balance between structural support and adaptability for certain 3D cell culture applications.

Overall, these findings underline the significance of selecting appropriate collagen extraction methods to tailor biomaterials for desired properties, with low-temperature extraction being most effective in preserving collagen’s bioactive qualities for tissue engineering applications.

Chapter 6: Future Prospects

Further studies could investigate the optimisation of extraction methods with a view to achieving an equilibrium between mechanical strength and bioactivity. For instance, a hybrid approach combining the mildness of low-temperature extraction with ultrasound could result in collagen with enhanced bioactivity and structural integrity.

Moreover, the application of these hydrogels beyond tumour models to include wound healing and regenerative medicine would facilitate a deeper understanding of the versatility of fish bone-derived collagen. It would also be beneficial to investigate the long-term stability and functionality of these hydrogels in dynamic bioreactor systems, which are capable of more accurately simulating physiological conditions than static cultures. This could facilitate the evaluation of their viability as scaffolds for tissue regeneration or as drug delivery vehicles in clinical settings.

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