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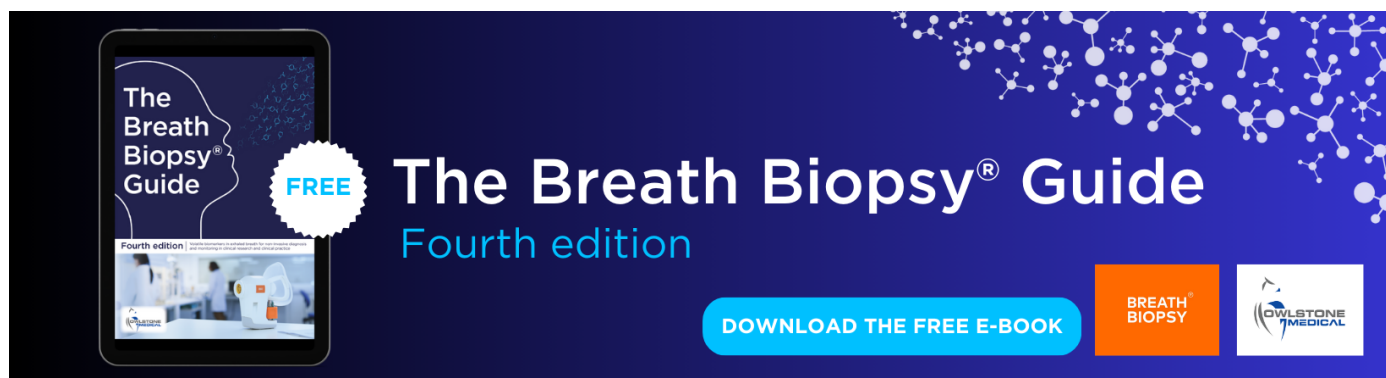
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Magnetic biocomposite scaffold based on decellularized tendon ECM and MNP-deposited halloysite nanotubes: physicochemical, thermal, rheological, mechanical and *in vitro* biological evaluations

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Abstract

The development of new three-dimensional biomaterials with advanced versatile properties is critical to the success of tissue engineering (TE) applications. Here, (a) bioactive decellularized tendon extracellular matrix (dECM) with a sol-gel transition feature at physiological temperature, (b) halloysite nanotubes (HNT) with known mechanical properties and bioactivity, and (c) magnetic nanoparticles (MNP) with superparamagnetic and osteogenic properties were combined to develop a new scaffold that could be used in prospective bone TE applications. Deposition of MNPs on HNTs resulted in magnetic nanostructures without agglomeration of MNPs. A completely cell-free, collagen- and glycosaminoglycan- rich dECM was obtained and characterized. dECM-based scaffolds incorporated with 1%, 2% and 4% MNP-HNT were analysed for their physical, chemical, and *in vitro* biological properties. Fourier-transform infrared spectroscopy, x-ray powder diffractometry and vibrating sample magnetometry analyses confirmed the presence of dECM, HNT and MNP in all scaffold types. The capacity to form apatite layer upon incubation in simulated body fluid revealed that dECM-MNP-HNT is a bioactive material. Combining dECM with MNP-HNT improved the thermal stability and compressive strength of the macroporous scaffolds upto 2% MNP-HNT. *In vitro* cytotoxicity and hemolysis experiments showed that the scaffolds were essentially biocompatible. Human bone marrow mesenchymal stem cells adhered and proliferated well on the macroporous constructs containing 1% and 2% MNP-HNT; and remained metabolically active for at least 21 d *in vitro*. Collectively, the findings support the idea that magnetic nanocomposite dECM scaffolds containing MNP-HNT could be a potential template for TE applications.

1. Introduction

Aging, trauma, infection, cancer and hereditary diseases-based factors can lead to different kinds of bone fractures and losses. Despite its exceptional self-healing ability, additional surgical procedures using biological or synthetic bone grafts are required for successful healing of large bone defects. These kinds of clinical routines include some limitations associated with healing processes and tissue

harvesting [1–3]. Tissue engineering (TE) approaches have become game changers in the reconstruction of bone defects in the last 30 years [4]. In this innovative approach, four key factors play important roles in a successful bone healing process: scaffolds, growth factors, cells and an optimum mechanical environment [5].

Most research has focused on finding suitable ways to develop engineered scaffolds with the required properties. The characteristics of the target

tissue or organ are key factors in the design of biomaterials. From this point of view, the use of composite materials developed by combining one or more of many polymers of natural origin with ceramic, clay and carbon nanotubes in order to simulate the natural environment of the bone and to provide the appropriate modeling of the *in vitro* environment comes to the fore. Such a three-dimensional (3D) porous composite biomaterial developed to promote bone formation is expected to have certain properties, including osteoinductivity, osteoconductivity, biocompatibility, and appropriate mechanical strength.

The extracellular matrix (ECM) is a complex network of multiple macromolecules such as structural proteins and polysaccharides [6] that provide the mechanical and biophysical meshwork for cells to attach, proliferate, migrate, differentiate and modulate their activity by providing tissue-specific cues [7, 8]. In this context, the use of the ECM to form scaffolds has become the core of interest in regenerative therapies to facilitate appropriate tissue remodeling. Tissue-derived acellular ECM-based biomaterials are formed by decellularization of allogeneic or xenogeneic tissue sources. The structural, biochemical and biomechanical properties of the native ECM are preserved during the decellularization process, except for living cells and cellular antigens [9]. They offer a very rich bioactive environment compared to synthetic materials and can be obtained from different tissue types. These are the main reasons why decellularized ECM (dECM) constructs derived from different tissues or organs have become central to functional tissue reconstruction [10–13]. Today, there are many products in this category that are approved by the FDA for clinical use [14]. Moreover, their intrinsic tissue-specific cues hold great promise as an inductive template to aid in the constructive remodeling of damaged tissues [15]. However, the physicochemical properties of the ECM may fall short of meeting the requirements of bone tissue engineering (BTE). The solution to this disadvantage is to strengthen the scaffold biomaterial with nanofillers to increase mechanical and biological functionality.

As a nanofiller, halloysite nanotubes (HNTs) have drawn interest for the fabrication of bone regeneration biomaterials due to their numerous favorable properties, such as good biocompatibility, tubular structure, biodegradability and nanosize, as well as to their high specific surface area, large adsorption capacity for polymer molecules, mechanical properties and availability [16–18]. HNT [$\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4 \cdot n\text{H}_2\text{O}$] is a 1:1 aluminosilicate clay mineral with a tubular morphology. Its inner surface consists of positively charged aluminol groups while the outer surface consists of negatively charged siloxanes [19]. The length, inner diameter, and outer diameter of halloysite particles are in the range of 0.4–1.5 μm , 10–20 nm, and 40–70 nm,

respectively [20–22]. Various studies have demonstrated the biocompatibility and mechanical performance of HNTs that perform very well for BTE [23–26]. On the other hand, an increasing amount of comprehensive research continues on magnetic iron oxide nanoparticles (MNPs) for their use in BTE due to their biocompatibility, superparamagnetic behavior, high antibacterial activity, osteogenic performance and chemical stability [27–33]. In addition to the advantages offered by iron oxide nanoparticles, they also have some disadvantages. For example, high surface-to-volume ratio results in high surface energy and they tend to agglomerate to reduce this energy. They easily oxidize in air due to their chemical activity. Surfactants, polymers, biomolecules, and inorganic materials are widely used to increase the stability of MNPs [34]. At this point, HNTs can be considered as an immobilization substrate for MNPs due to their properties, and can be offered as an effective and economical option for polymers, carbon nanotubes and other inorganic materials [35].

Considering the properties of HNTs, MNPs and dECM each, it was thought that a bioactive magnetic scaffold biomaterial could be developed with the use of these three components together, which could make a contribution to BTE studies. To the best of our knowledge, there is no report on the study of MNP-HNT-dECM magnetic scaffolds for BTE applications. In this study, it was aimed to develop a unique natural tendon ECM-based tissue scaffold (MNP-HNT-dECM) with increased osteogenic capacity and mechanical properties by combining iron oxide nanoparticles modified halloysite with dECM without using any chemical crosslinkers. The physical, chemical, structural, and *in vitro* biological properties of the developed biocomposite scaffolds were assessed and discussed in detail.

2. Experimental

2.1. Materials

All chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. HNTs were used as received without further purification. $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ were obtained from Merck (Rahway, NJ, USA). Sterile disposable tissue culture plasticware was obtained from Corning (Corning, NY, USA). Cell culture medium, fetal bovine serum (FBS) and other supplements were provided from Lonza (Basel, Switzerland).

2.2. Tissue decellularization

Achilles tendons of healthy adult bovine were obtained from a local veterinary controlled slaughterhouse with the approval of the supplier (Meat and Dairy Board, Ankara, Turkey). Tendons were transferred to the laboratory in cold antibiotic-containing phosphate buffered saline (PBS, pH 7.4) immediately

after slaughter. Decellularization was performed by slight modification of a previously described protocol [13]. First, tissue samples were washed with fresh PBS and then with isotonic saline solution to remove any remaining residues. Second, the cleaned samples were frozen at -80°C and were thawed at 37°C . Freeze-thaw cycles were repeated 3–5 times to promote cell lysis. In the third stage, synovial sheath around the tendons was removed with the aid of a surgical scalpel. The tendons were minced into small pieces. At the beginning of the decellularization procedure, tissue pieces were treated with hypotonic solution, hypertonic solution and PBS, respectively, on a mechanical stirrer at room temperature. The samples were then kept in 1% sodium dodecyl sulfate (SDS) for 1 d by refreshing the solution. After that, the samples were rinsed with PBS to remove any residual detergent. Finally, samples were treated with 0.1% peracetic acid (PAA)/4% ethanol for 12 h. After these steps, the samples were washed with distilled water to remove all possible residues. The resulting acellular tissue was lyophilized for 2 d after freezing at -80°C . Lyophilized samples were transferred into liquid nitrogen, frozen samples were turned into powder form with a laboratory blender and stored at $+4^{\circ}\text{C}$ for use in subsequent experiments.

2.3. Structural and chemical analyses of decellularized tendons

2.3.1. ATR-FT-IR analysis

Attenuated total reflectance-fourier transform-infrared (ATR-FT-IR) spectroscopy analysis was performed to determine possible changes in the collagen structure due to the chemical agents used and the processes applied during decellularization. The spectra of the samples were collected using a Bruker IFS 66/S model ATR-FT-IR spectrophotometer in the range of $400\text{--}4000\text{ cm}^{-1}$.

2.3.2. Morphological (SEM) evaluation

Morphological changes in lyophilized native tendon ECM and decellularized tendon ECM (dECM) were examined by scanning electron microscopy (SEM). Briefly, samples were fixed in a 2% glutaraldehyde solution in 0.1 M PBS at 4°C overnight, followed by washing with PBS, then dehydrated by passing through a graded series of ethanol (50%–95%; 30 min at each concentration). As a final step, the samples were sputter-coated with gold and viewed under a FEI Quanta 450 FEG SEM instrument (Hillsboro, OR, USA).

2.3.3. DNA content

To evaluate the efficacy of the decellularization process, the total DNA contents of decellularized and native tendon tissues were determined and compared according to a previously reported method [13]. In short, 10 mg of native and dECM tendons were weighed and digested in 20 mg ml^{-1} Proteinase K in a

buffer solution composed of 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl_2 , 0.5% Tween-20 on a heating plate at 55°C for 48 h under 1000 rpm. The homogenates were centrifuged at 3000 rpm for 15 min at 4°C and the supernatant was collected. DNA was extracted from the collected samples using a standard phenol/chloroform/isoamyl alcohol-based extraction technique. The DNA contents of the samples were determined by measuring the absorbances of the samples at 560 nm using the NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, MA, USA).

2.3.4. Total protein content

Total protein contents of native and decellularized tendon tissues were determined by Lowry's assay [36]. Briefly, a standard solution of bovine serum albumin at a concentration of 0.5 mg ml^{-1} in distilled water was made and used to prepare a set of diluted standards for a working range of $10\text{--}200\text{ }\mu\text{g protein ml}^{-1}$. The amounts of protein in standard solutions and samples were quantified by measuring their absorption spectra at 750 nm. A standard absorbance curve against microgram protein was plotted and the concentrations of the samples were calculated from the curve.

2.3.5. Turbidimetric analysis

The turbidimetric gelation kinetics of dECM was investigated as previously reported [37]. Briefly, 200 μl of samples were transferred to 96-well plates. Measurements were made at 2 min intervals for 120 min in a microplate reader (Molecular Devices) at 37°C at a wavelength of 504 nm. The normalized values were calculated using equation (1), where NA is the normalized absorbance, A is the absorbance at a given time, A_0 is the initial absorbance, and A_{max} is the maximum absorbance of the sample,

$$NA = \left[\frac{(A - A_0)}{(A_{\text{max}} - A_0)} \right] \times 100. \quad (1)$$

2.4. Synthesis of halloysite-based magnetic nanostructures

Halloysite-based magnetic nanostructures (MNP-HNT) were formed by co-precipitation as described in the literature [34]. Briefly, 0.5 g of halloysite was added to 50 ml of distilled water and stirred well under magnetic stirring overnight. The next day, the resulting suspension was sonicated for 5 min and then mixed with 100 ml of solution containing 1.165 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 0.429 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. The resulting mixture was stirred continuously for 2 h at 60°C under a nitrogen atmosphere and aqueous ammonia solution was added dropwise until the appropriate pH (pH 9–10) was reached. The black precipitate formed was separated from the solution using a neodymium magnet. The resulting black product was resuspended in distilled water and collected again.

Resuspension and collection were repeated 3–5 times until the pH was ~ 7.0 . The final wash was done with ethyl alcohol. The obtained black precipitate was incubated at 37 °C overnight and stored at room temperature in a desiccator.

2.5. Structural and chemical analyses of halloysite-based magnetic nanostructures

Transmission electron microscopy (TEM) analysis was performed to investigate the size, morphology, and distribution profile of the nanoparticles using a Jeol 2100 F model high resolution instrument (HRTEM). The magnetic properties of the samples were recorded on a vibrating sample magnetometer (VSM) (PPMS, Cryogenic Ltd, London, UK) at room temperature. The chemical structure of the nanoparticles was evaluated using the Bruker IFS 66/S model ATR-FT-IR spectrophotometer (Billerica, MA, USA) in the spectral range of 400–4000 cm^{-1} . The crystal structures of the synthesized samples were characterized using the Ultima-IV model x-ray powder diffractometer (XRD) with $\text{CuK}\alpha$ radiation (Rigaku, Tokyo, Japan). Analysis was performed at a scan rate of 2° min^{-1} within the range of 20° – $70^\circ (2\theta)$. Thermal analysis of the samples was performed on a DTA-TG instrument (Shimadzu DTG-60H, Columbia, MD, USA) under a nitrogen atmosphere in the temperature range of 25 °C–550 °C and a heating rate of $15^\circ \text{ C min}^{-1}$. Mössbauer spectra of the MNP-HNT were recorded utilizing a Wissel Mössbauer measurement system (Wissel MVC 450, Starnberg, Germany) operating in conventional constant acceleration mode. The obtained spectra were analyzed using the Win-Normos fitting program. The instrument was calibrated using the α -Fe foil spectrum at room temperature. The specific surface area of the samples was determined by the Brunauer, Emmett and Teller (BET) method of nitrogen gas adsorption at 77 K using a Quantachrome Nova 2200 model analyzer (Boynton Beach, FL, USA). Samples were degassed for 4 h at 110 °C prior to N_2 physisorption.

2.6. Preparation of dECM-based magnetic nanocomposite scaffolds

To prepare the biological nanocomposite material, the grounded dECM was digested with pepsin solution (1 mg ml^{-1} pepsin in 0.01 N HCl) on a magnetic stirrer for 48 h at room temperature. At the end of the digestion, the pH of the flowable viscous solution (pre-gel) was adjusted to pH 7.4 with ice-cold 0.1 N NaOH. Physiological salt concentration was achieved by adding 1/9 volume ratio of 10xPBS to this solution at 4 °C. In the next step, different concentrations (0%–10%) of MNP-HNT nanocomposites were added separately to the pepsin-digested and

neutralized dECM pre-gel solution, and the suspensions were mixed at 4 °C by using a mechanical stirrer with a glass rod until a homogeneous solution was obtained. In the last step, the obtained composite suspensions were poured into the wells of the multi-well culture plates and kept at 37 °C to complete gelation. Nanocomposite scaffolds were obtained by freezing and then lyophilizing the gelled samples at -80° C .

2.7. Structural and chemical analyses of magnetic nanocomposite scaffolds

2.7.1. Degradation and swelling profiles of scaffolds

The degradation behavior of the samples was determined by monitoring their mass loss in phosphate-buffered saline (PBS) (pH 7.4) for 4 weeks at 37 °C ($n = 3$ per group). Weighed (W_0) dried samples were placed in multiwell culture plates containing PBS. The buffer solution was refreshed every other day. Samples kept in the buffer were retrieved at weekly intervals, rinsed with distilled water, dried at 37 °C and weighed (W_t). The percent mass loss in the samples was calculated according to equation (2),

$$\text{Mass loss (\%)} = \left[\frac{(W_0 - W_t)}{W_0} \right] \times 100. \quad (2)$$

The swelling profiles of the samples were determined as follows: first, the samples were allowed to soak by incubation at 37 °C for 24 h in PBS (pH 7.4) and then weighed (W_d) ($n = 3$ per group). Swollen samples were removed at predetermined time intervals, the remaining solution on the surface was carefully removed with a filter paper and reweighed (W_w). The percent swelling of the samples over time was calculated using equation (3),

$$\text{Swelling ratio (\%)} = \left[\frac{(W_w - W_d)}{W_d} \right] \times 100. \quad (3)$$

2.7.2. Mechanical analysis of scaffolds

The compressive strength of the samples was measured using a Shimadzu Autograph AGS-X model universal compression tester (Kyoto, Japan) to determine the effect of incorporating MNP-HNT into the dECM matrix. The compression test was carried out with 500 N load cell by applying 80% strain at a constant speed of 2 mm min^{-1} . The test was conducted in triplicate for each sample.

2.7.3. Morphological (SEM) evaluation of scaffolds

The surface morphology of the scaffolds was analyzed using the Quanta 400 F Field Emission SEM (FEI Instruments, Hillsboro, OR, USA). All specimens were coated with a thin layer of gold before analysis.

2.7.4. Thermogravimetric analysis of scaffolds

In order to determine the effect of MNP-HNT content on the thermal stability of dECM-based

scaffolds, thermogravimetric analysis was performed using a DTG-60H model device (Shimadzu, Tokyo, Japan) at a heating rate of $10\text{ }^{\circ}\text{C min}^{-1}$ in the range of $25\text{ }^{\circ}\text{C}$ – $550\text{ }^{\circ}\text{C}$ under nitrogen atmosphere.

2.7.5. VSM analysis of scaffolds

The magnetic sensitivity of the samples was evaluated by VSM (Cryogenic Ltd.) at room temperature to determine the effect of MNP-HNT content on the magnetic properties of the scaffolds.

2.7.6. ATR-FT-IR analysis of scaffolds

Infrared spectra of the nanocomposite scaffolds were recorded with the ATR-FT-IR spectrophotometer (Bruker IFS 66/S). Spectra were collected in the region $400\text{--}4000\text{ cm}^{-1}$ at room temperature.

2.7.7. Bioactivity analysis of scaffolds

The bioactivity study of the samples was carried out at $37\text{ }^{\circ}\text{C}$ for 14 d in an artificial body fluid (Simulated Body Fluid; SBF) with an ionic concentration similar to blood plasma. The SBF solution was prepared according to the Kukoba method [38]. The SBF solution was changed daily to ensure stable ion concentration level. Apatite formations that may occur on the sample surfaces after SBF treatment were examined by SEM and energy-dispersive x-ray spectroscopy (EDS).

2.8. In vitro biological characterizations

2.8.1. Indirect in vitro cytotoxicity analysis

After the basic physicochemical characterization studies, the standard assay (ISO-EN-10993-5) described by the International Organization for Standardization was applied on the samples to determine the *in vitro* biocompatibility of nanocomposite scaffolds. At first, human dermal fibroblasts (PCS-201-012; ATCC, Manassas, VA, USA) were seeded in multi-well culture plates containing Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS and 1% (v/v) penicillin/streptomycin (standard medium; SM) and were cultured at $37\text{ }^{\circ}\text{C}$, 5% CO_2 /95% air, and 90% humidity conditions until they reached confluence.

On the other hand, both sides of the scaffolds were sterilized under a UV light source (254 nm) for 1 h. Sterilized scaffolds were then placed in the wells of another multi-well plate and kept in the serum-free standard culture medium for 24 h. Later, the medium (scaffold extract) was collected and used as the new medium (after adding FBS) for the confluent fibroblast cultures for 24 h. Cell viability based on metabolic activity was examined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Culture medium without the scaffold extract was used as negative control, and

phenol was used as positive control. The percentage of cell viability was calculated according to equation (4),

$$\text{Cell viability (\%)} = \left[\frac{OD_{\text{sample}}}{OD_{\text{negative control}}} \right] \times 100. \quad (4)$$

2.8.2. In vitro hemocompatibility analysis

In vitro hemolysis assay was performed to determine the *in vitro* blood compatibility of the scaffolds, according to the ISO 10993-4 standard. Briefly, the obtained anticoagulated blood was diluted 1:20 with PBS. The weighed samples were washed with PBS and sterilized under UV light for 40 min; the samples were placed in separate eppendorf tubes and 1 ml of diluted blood was added to each of them. PBS and distilled water were used as negative and positive controls, respectively ($n = 3$ per group). All samples were incubated at $37\text{ }^{\circ}\text{C}$ for 1 h, and then centrifuged at 1500 rpm for 10 min. The absorbance measurements of the supernatants were taken with a spectrophotometer at 545 nm. The percentage of hemolysis were calculated using equation (5),

$$\begin{aligned} \text{Hemolysis (\%)} \\ = \left[\frac{A_{\text{sample}} - A_{\text{negative control}}}{A_{\text{positive control}} - A_{\text{negative control}}} \right] \times 100. \end{aligned} \quad (5)$$

2.9. Mesenchymal stem cell culture study

Human bone marrow-derived mesenchymal stem cells (BM-MSCs) (PCS-500-012TM; ATCC, Manassas, VA, USA) were used in this study. MSCs were cultured in MSC growth medium (ATCC, PCS-500-041) containing 1% pen/strep, in an incubator set to $37\text{ }^{\circ}\text{C}$, 5% CO_2 /95% air, and 90% humidity. For this preliminary cell culture experiment, cells reaching ~80% confluence were detached by trypsinization, suspended in fresh culture medium, and seeded onto sterilized scaffolds [39]. The viability and proliferation capacity of cells on scaffolds were investigated by the MTT assay (as described in section 2.8.1). SEM assessments were performed to follow cell adhesion, proliferation, and morphological changes in cell-laden scaffolds.

2.10. Statistical analysis

All experimental data were expressed as the mean $\pm$ standard deviation (SD) of at least three experiments. Statistical analysis was performed using one-way and two-way ANOVA with the GraphPad Prism 8 program. Significance levels were set as: $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$, and $p < 0.0001^{****}$.

3. Results and discussion

3.1. Evaluation of decellularization efficiency

While a well-decellularized tissue should be free of cellular components, it should significantly preserve

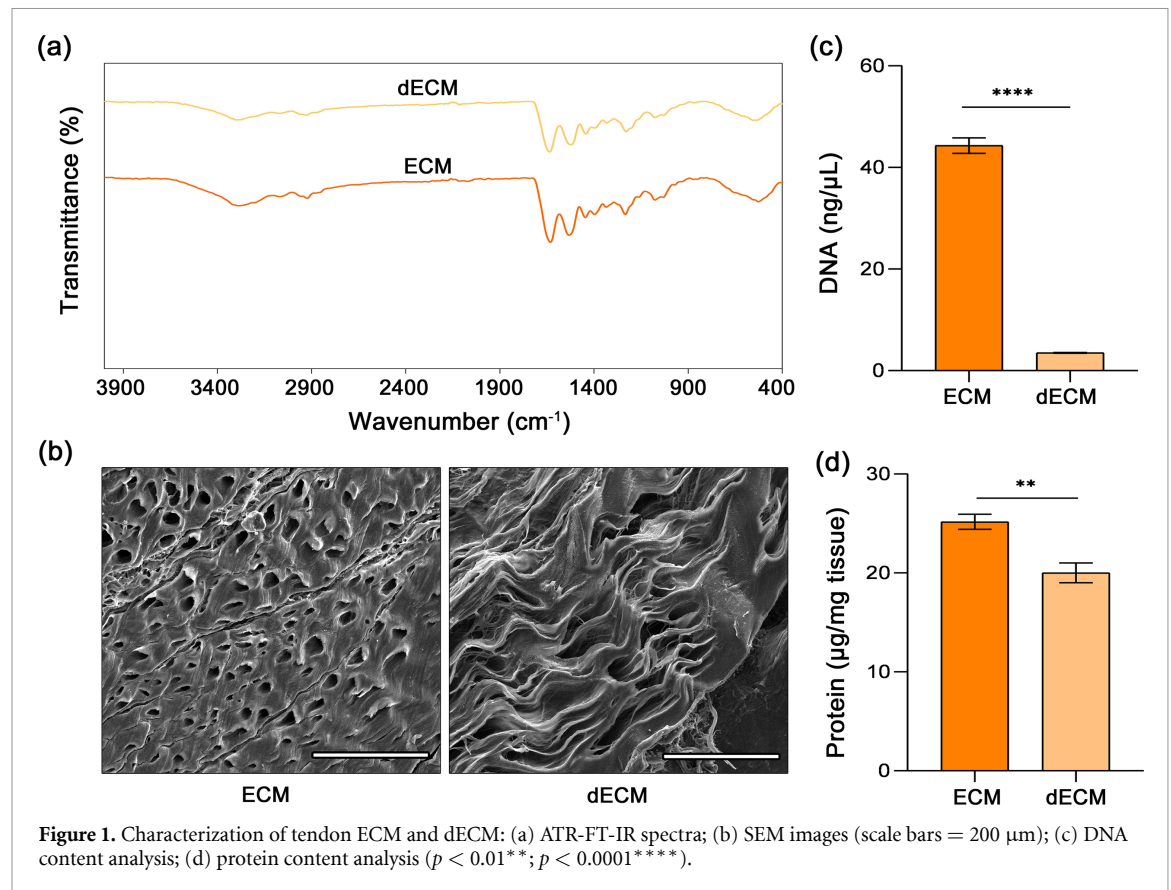


Figure 1. Characterization of tendon ECM and dECM: (a) ATR-FT-IR spectra; (b) SEM images (scale bars = 200 μm); (c) DNA content analysis; (d) protein content analysis ($p < 0.01^{**}$; $p < 0.0001^{****}$).

the structural and bioactive macromolecules of the native ECM to contribute to the remodeling process in a potential regenerative application. Therefore, the decellularization efficiency of tendon tissue was investigated by comparing native and decellularized ECM using various methods such as FT-IR, SEM, DNA and total protein analyses.

3.1.1. ATR-FT-IR analysis

Sixty percent of the tendon tissue dry mass consists of different collagen fibers. The main part of this structure consists of 95% type I collagen, while the remaining 5% consists of type III and type V collagens [40]. ATR-FT-IR analysis was performed to evaluate the possible effects of decellularization based on the use of chemicals on the ECM structure (figure 1(a)). The peaks observed in the 3289 cm^{-1} and 1920 cm^{-1} regions represent the O–H stretching band, and the C–H bending, respectively. The peaks at 1630 cm^{-1} and 1521 cm^{-1} were associated with amide-I and amide-II, respectively. The peaks at 1225 cm^{-1} and 1235 cm^{-1} correspond to sulfated glycosaminoglycans. Findings based on structural analysis revealed that native and decellularized ECM showed great similarity, except for minor differences in peak intensities. Accordingly, it was concluded that dECM is similar to native ECM, thus the decellularization method

does not cause any significant changes in collagen structure.

3.1.2. SEM evaluation

Differences in morphological structures of native tendon ECM and dECM were examined by SEM analysis (figure 1(b)). The findings showed that the tight tissue integrity in the tendon was somewhat disrupted in the dECM structure, resulting in a looser and porous structure, indicating the success of the decellularization procedure.

3.1.3. DNA content analysis

The DNA contents of native and decellularized tendon tissues were found to be $44.31 (\pm 1.52)\text{ ng }\mu\text{L}^{-1}$ and $3.51 (\pm 0.02)\text{ ng }\mu\text{L}^{-1}$, respectively (figure 1(c)). Analyses showed approximately 92% reduction in DNA content of dECM relative to native tissue following the decellularization process. Both SEM and DNA analyses indicate a successful decellularization. In another study using a similar decellularization method, the decrease in DNA content was determined to be ~80% [13].

3.1.4. Protein content analysis

Protein content analysis results of native tendon ECM and dECM are given in figure 1(d). The results show that the protein content of the tissue is preserved by ~84% after decellularization. This finding indicates

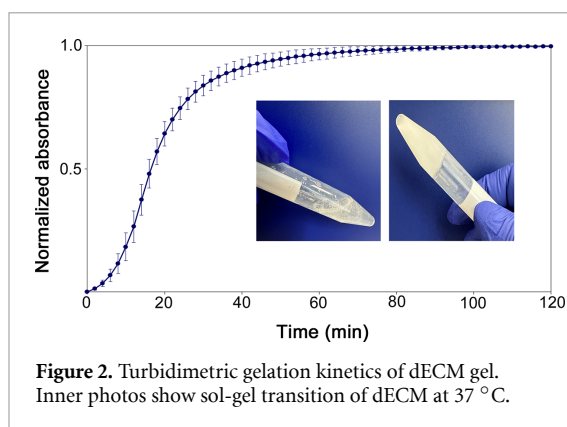


Figure 2. Turbidimetric gelation kinetics of dECM gel. Inner photos show sol-gel transition of dECM at 37 °C.

that the majority of ECM proteins, which are of great importance for the regenerative effect, remain in the dECM structural composition.

3.1.5. Turbidimetric analysis

The turbidimetric gelation analysis results of dECM are summarized in figure 2. The results reveal that the normalized absorbance value reaches a plateau within 70–75 min at 37 °C. The macroscopic images in the graph show the pregel and gel forms of dECM in the tubes.

3.2. Structural and chemical evaluation of nanocomposites

3.2.1. TEM analysis

TEM results are presented together in figure 3(a). TEM micrographs show that iron oxide-based nanoparticles are in the average size range of 12.5–19 nm. This result aligns with the findings determined according to the Scherrer equation. Furthermore, TEM analysis reveals that the synthesized MNPs exhibit a spherical morphology, and also indicate that the synthesized MNPs tend to agglomerate due to magnetic attraction forces. Consistent with the findings in the current literature, the presence of nanoparticle formations on both the surface and inner walls of HNTs indicates that MNPs are directly synthesized on the surface of halloysites. This phenomenon can be attributed to the distinctive structural features of HNT, such as large surface area, significant pore volume, and the presence of sufficient hydroxyl groups to interact [41]. Results are consistent with XRD and FT-IR results.

3.2.2. ATR-FT-IR analysis

ATR-FT-IR spectra of HNT and MNP-HNT specimens are presented in figure 3(b). The peaks at 3800–3600 cm^{-1} in the HNT spectrum are the stretching vibrations of the OH groups. The peaks at 3691 cm^{-1} and 3648 cm^{-1} were typically associated with OH groups (Al–OH) located on the inner surface, while the peak at 3628 cm^{-1} was assigned to the OH groups on the outer surface [42–44]. FT-IR peaks in the range 1200–400 cm^{-1} were assigned to the

alumina silica structure. Bands at 1090–1000 cm^{-1} belong to Si–O stretching, while bands in the range 500–400 cm^{-1} belong to Al–O–Si and Si–O–Si bending. The peak seen at 904 cm^{-1} belongs to Al–OH bending vibration, and the peak at 750–700 cm^{-1} belongs to Al–O–Si vibrations on the inner surface. The 3400 cm^{-1} and 1630 cm^{-1} bands observed in the HNT and MNP-HNT spectra are most likely due to iron oxide and water absorbed on the halloysite surface. The appearance of the absorption band of the MNP sample (Fe–O stretching vibration of Fe_3O_4 nanoparticles) at low wavenumbers (550–600 cm^{-1}) is characteristic for magnetite and maghemite [45, 46]. All samples contain certain bands of kaolin group minerals, revealing that HNT retains its original structure.

3.2.3. XRD analysis

XRD patterns of MNP, HNT and MNP-HNT are plotted in figure 3(c). The XRD patterns of the synthesized MNP-HNT nanocomposite showed 2 θ diffraction peaks at 30.3°, 35.5°, 43.4°, 57.3°, 62.8°, and 74.4°, corresponding to (220), (311), (400), (422), (511) and (533), which can be indexed as MNP, confirming the presence of Fe_3O_4 , conforming to the magnetite crystallographic model (JCPDS file #75-0033) [47, 48]. The specific peaks observed at 12.29°, 19.98°, 26.71°, 29.93° and 37.16°, corresponding to (001), (110), (100), (114), (002) and (222) reveal the existence of HNT. The presence of these peaks in the MNP-HNT nanocomposite confirms the successful formation of MNPs on the HNT. These results are consistent with observations from TEM and FT-IR analyses. The crystal size of the material was determined by the Scherrer equation [49], and the average crystal size for MNP and MNP-HNT was calculated as 13.24 nm and 15.07 nm, respectively.

3.2.4. VSM analysis

Ferromagnetic materials such as iron or magnetite are self-magnetizing. Spontaneous magnetism is a concept associated with hysteresis. Hysteresis curves are created by changing the external magnetic field applied to ferromagnetic materials [50]. The saturation magnetization of MNPs and MNP-HNTs was determined as 60–65 emu g^{-1} and 35 emu g^{-1} , respectively (figure 3(d)). It was found that the magnetization increased with the increase of the magnetic field. In another study utilizing a similar preparation, the saturation magnetization of HNT-MNP was reported as 27.91 emu g^{-1} [51]. The minimum hysteresis profile of the sample indicates its possession of superparamagnetic properties (figure S1) [52, 53]. VSM findings also indicate the presence of MNP within the HNT structure and the interaction between them.

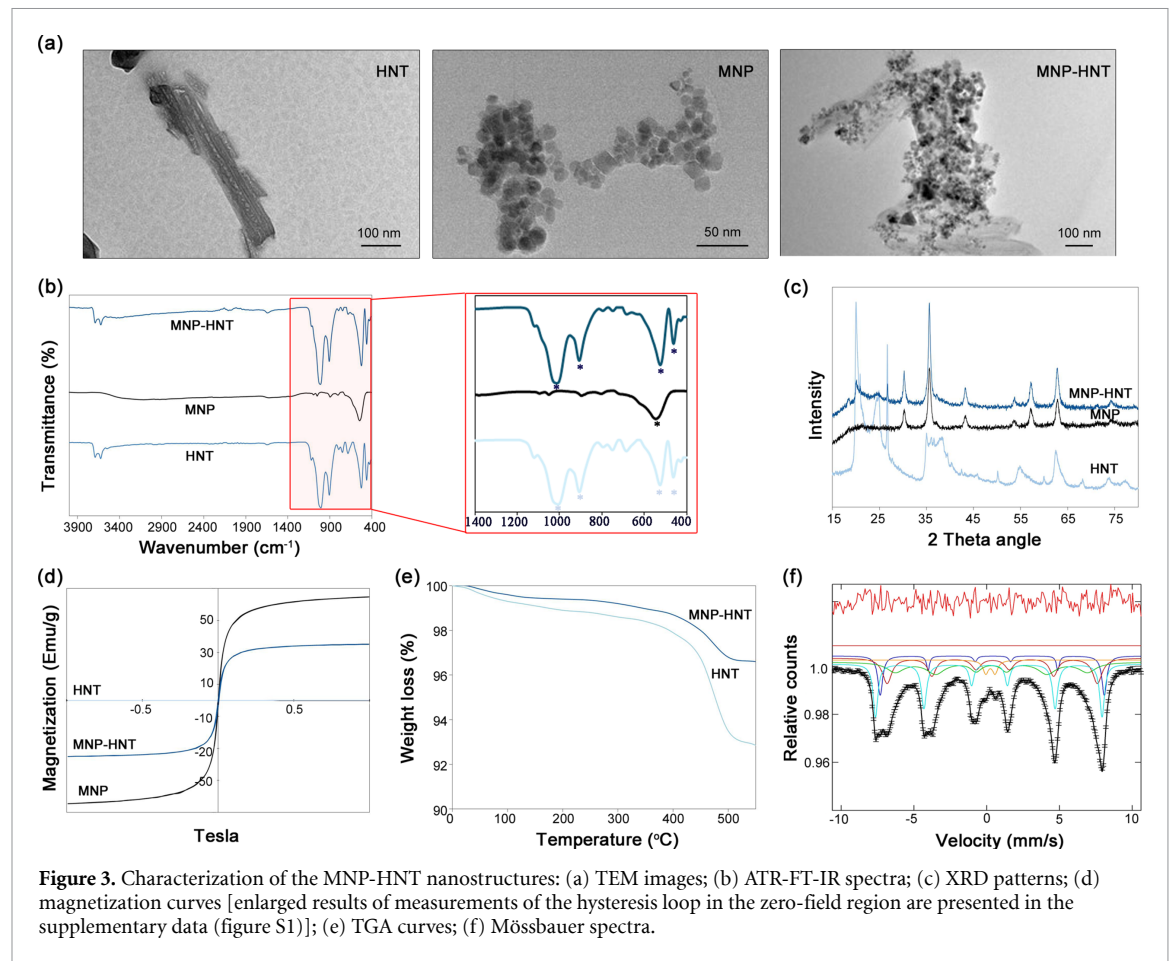


Figure 3. Characterization of the MNP-HNT nanostructures: (a) TEM images; (b) ATR-FT-IR spectra; (c) XRD patterns; (d) magnetization curves [enlarged results of measurements of the hysteresis loop in the zero-field region are presented in the supplementary data (figure S1)]; (e) TGA curves; (f) Mössbauer spectra.

3.2.5. TGA analysis

The TGA curves of HNT and MNP-HNT shown in figure 3(e) revealed that the mass loss of HNT at 0 °C–200 °C corresponded to the loss of water adsorbed on the surface and interlayer of the HNT [54]. A second phase of mass loss was observed resulting from dehydroxylation of aluminol groups at 400 °C–500 °C [55]. MNP-HNT showed a similar trend, but mass losses occurred at a lower rate. In this context, it was determined that the thermal stability of HNT increased as a result of the modification, indicating that the formation of MNPs and binding to HNTs were successfully achieved.

3.2.6. Mössbauer spectroscopy analysis

The Mössbauer spectra of the nanocomposite samples are depicted in figure 3(f) and the corresponding Mössbauer parameters calculated from the fitting of the spectra are presented in table 1. The Mössbauer spectra of the nanocomposite consist of four magnetic sextets. One of the sextets with a lower isomer shift corresponds to Fe³⁺ ions in the tetrahedral A region, while the other sextets with a higher isomer shift are related to Fe³⁺ ions in the octahedral B regions [56]. Due to the Fe³⁺ ions in the B region in different environments, three sextets were fitted in this region. The existence of these sextets

suggests that the Fe atoms in the samples exhibit magnetic ordering [57]. In addition to the ferromagnetic sextets, a doublet was formed in the Mössbauer spectra. The findings affirm that the samples contain nano-sized ferrite crystallites, which are known to be superparamagnetic [58].

3.2.7. BET analysis

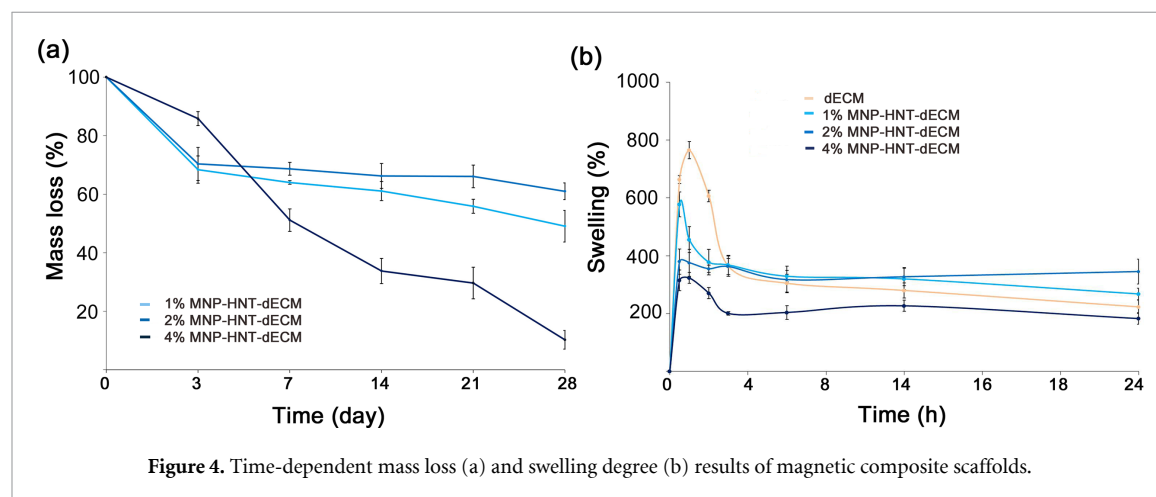
The surface area and total pore volume of HNT were found to be 9.283 m² g⁻¹ and 2.325 × 10⁻¹ cm³ g⁻¹, while the surface area and total pore volume of HNT-MNP were 12.981 m² g⁻¹ and 3.16 × 10⁻¹ cm³ g⁻¹, respectively. According to these results, it can be said that the surface area and total pore volume of HNT increases with modification. BET analyses also reveal that MNPs are located on the HNT surface. This is consistent with TEM, ATR-FT-IR, XRD and TGA analysis findings.

3.3. Structural and chemical evaluation of magnetic composite scaffolds

In order to pre-select composite scaffolds (containing 0%, 1%, 2%, 4%, 6%, 8%, and 10% of MNP-HNT) suitable for *in vitro* cell culture studies, the swelling rate and *in vitro* biodegradation behavior of the constructs were examined. The preliminary analyses revealed that the constructs containing more than 4%

Table 1. Parameters of Mössbauer spectra of the sample (B_{hf} : hyperfine magnetic field; I.S: isomer shift; Γ : line width; Q.S: quadrupole splitting; R_A : relative area).

	Site	I.S. (mm s^{-1})	Q.S. (mm s^{-1})	B_{hf} (T)	Γ (mm s^{-1})	R_A (%)
Sample	Sx-A: Fe^{+3}	0.274	−0.048	48.25	0.381	20.121
	Sx-B ₁ : Fe^{+3}	0.511	−0.046	47.736	0.295	6.811
	Sx-B ₂ : Fe^{+3}	0.406	−0.034	44.783	0.756	31.05
	Sx-B ₃ : Fe^{+3}	0.331	0.011	40.929	0.887	35.084
	Db-: Fe^{+3}	0.361	0.64	—	0.453	6.934

**Figure 4.** Time-dependent mass loss (a) and swelling degree (b) results of magnetic composite scaffolds.

MNP-HNT lost their structural integrity in a short period of 1–3 d. This was probably due to reduced intermolecular interactions between dECM proteins owing to increased MNP-HNT content. It was therefore decided to include composite scaffolds with 1%, 2% and 4% MNP-HNT in further characterization studies.

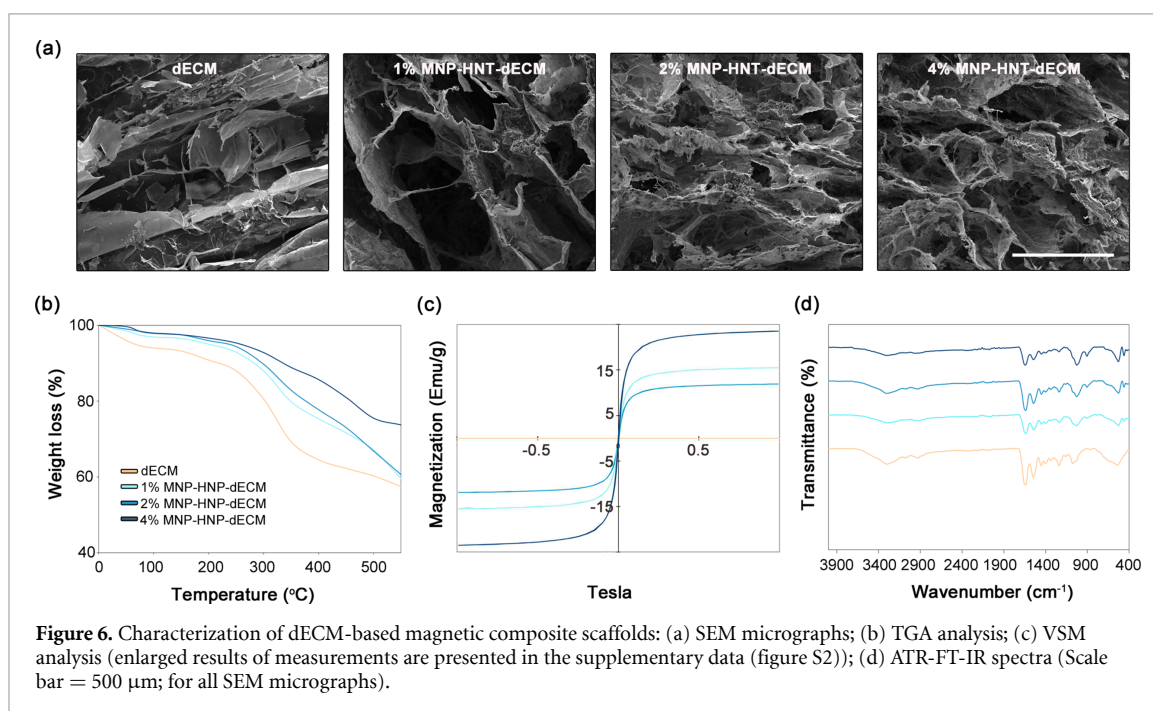
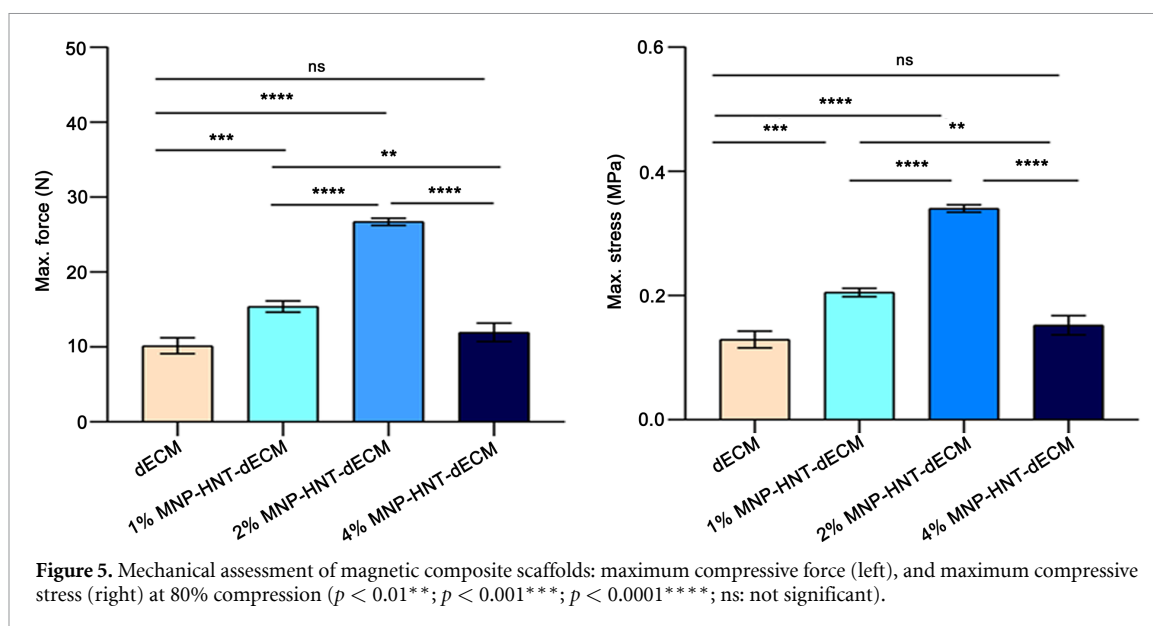
3.3.1. Degradation and swelling profiles

Evaluation of the properties of biomaterials from various aspects is important to increase the success of the application. Controllable rate of biodegradation is one of the important properties of biomaterials and should be well characterized according to clinical use needs. In this context, the degradation behavior of the constructs was evaluated and the results are summarized in figure 4(a). After 7 d, the dECM-based constructs containing 1%, 2% and 4% MNP-HNT retained 68%, 64% and 51% of their initial masses, respectively. In the following 3 weeks (day 7 to day 28), the mass loss in the 1% and 2% MNP-HNT containing dECM constructs was quite low, while a mass loss >90% occurred in the 4% MNP-HNT containing dECM, accompanied with structural erosion. Given the preliminary analyses, this finding was not surprising as increased MNP-HNT content inhibited possible hydrogen bonding between ECM proteins and eventual hydrogel matrix formation. Similar results have been reported in the literature when the nanofiller ratio is increased in ECM composites [59].

The swelling degree of biomaterials is important in terms of absorption of body fluids, diffusion of nutrients and waste metabolites into/from cells seeded on the scaffolds [60]. It depends on the chemical composition of the biomaterials. The collagen/protein content of the dECM and the number of polar groups of dECM structure have an effect on the water retention capacity [37]. The swelling test findings of composite scaffolds are presented in figure 4(b). In the early stage of the experiment (within the first 1–2 h), the swelling degree of the composites increased very sharply, after which a significant decrease in all groups was observed. Consistent with the literature, it was observed that with increasing MNP-HNT content, the swelling capacity of the samples decreased due to the hydrophobic nature of MNP [61]. 1% and 2% MNP-HNT-dECM showed a higher swelling capacity attributed to its hydrophilicity and preservation of its 3D structure. The results are in agreement with the mass loss results.

3.3.2. Mechanical analysis

Biomaterials developed for hard tissue applications such as BTE are expected to have certain mechanical properties. For this reason, compressive strength tests were applied to the developed magnetic scaffolds and the results are presented in figures 5(a) and (b). As a result, the mean maximum force values at 80% compression for dECM, 1% MNP-HNT-dECM, 2% MNP-HNT-dECM and 4% MNP-HNT-dECM were measured as 10 MPa, 15 MPa, 27 MPa and 12 MPa,



respectively. Interestingly, the mechanical strength values unexpectedly decreased when the nanocomposite content was 4%. This reduction in mechanical strength is probably due to the higher content of nano-fillers. Consistent with those in the literature [59, 62, 63], it is thought that higher nanofiller content negatively affected the gelation process of dECM by preventing the chain movement required for the process. However, the maximum stress was observed to be nearly doubled for 2% MNP-HNT-dECM compared to 1% MNP-HNT-dECM and 4% MNP-HNT-dECM. This dilemma may be the result of ion release by the MNP-HNT content. As is known, glycosaminoglycan molecules are rich in certain functional groups such as sulfate, as seen in the ATR-FT-IR results. The release of ions such as Al^{3+} , Fe^{2+} and Fe^{3+}

which exist in MNP-HNT may have induced ionic cross-linking via sulfate groups. This result can also be supported by the degradation test. The 2% MNP-HNT-dECM has a minimum % mass loss and can therefore be considered the optimum nanocomposite. Mechanical test results are in agreement with mass loss findings. Note that the compressive strength of the composite scaffolds is close to that of human trabecular bone (0.1–16 MPa), indicating the potential of the constructs for BTE [64–66].

3.3.3. Morphological (SEM) evaluation

SEM micrographs of dECM scaffolds with varying MNP-HNT contents (0%, 1%, 2%, and 4%) are displayed in figure 6(a). It is clear from the micrographs that the composite scaffolds have a

polygonal, heterogeneous macroporous structure and the MNP-HNT nanoparticles are dispersed well in the dECM matrix which introduces roughness to the pore walls of the scaffolds. It has been reported in the literature that the surface roughness and large surface area of biomaterial constructs positively influence the adhesion, spreading and proliferation of cells both in culture conditions and in the *in vivo* environment [67]. This effect is particularly associated with better adhesion of proteins that guide the growth of adherent cells to such surfaces [68–71]. It has also been shown that the surface microtopography of the substrates plays a role in the mechanical behavior of cells [72, 73]. Preliminary cell culture experiments showed a correlation between the increase in MNP-HNT content and cell proliferation. In this context, it can be stated that the findings of the study are generally parallel to the results in the literature.

3.3.4. Thermogravimetric analysis

The thermal stability of materials is an important feature in producing bioabsorbable scaffolds. Although *in vitro* and *in vivo* studies are carried out at a certain temperature, thermal degradation during a heat treatment process may result in the formation of small molecular fragments, which can alter the cytotoxicity and biocompatibility of the construct [74]. In this context, it is important to evaluate the thermal stability of the materials. The TGA curves of the tested samples and the percent mass losses depending on temperature are presented in figure 6(b). In thermograms of hydrogels, two- or three-stage mass losses usually occur due to the loss of water molecules in their structure, fragmentation of the material and the combustion of organic components [75]. The TGA data of the dECM structure is compatible with the literature. The initial mass loss around 0 °C–200 °C occurred due to evaporation of physically adsorbed and bound water molecules in the dECM. The second mass loss was observed at 200 °C–400 °C depending on the decomposition of the protein chains in the structure [76].

Table 2 summarizes the percentage weight losses for MNP-HNT-dECM composite scaffolds at 200 °C and 370 °C. Notably, there is a corresponding decrease in the weight loss of composite materials due to the increase in the amount of MNP-HNT in dECM. This observation strongly suggests that the incorporation of MNP-HNT enhances the thermal stability of the composite scaffolds, possibly through interfacial interactions between dECM and MNP-HNT within the structure.

3.3.5. VSM analysis

Figure 6(c) shows the magnetization curves of the dECM-based matrices. While magnetization was not

Table 2. Percent mass losses of dECM and composite structures at 200 °C and 370 °C.

Sample	200 °C	370 °C
dECM	9.8%	33.3%
1% MNP-HNT-dECM	3.2%	22.4%
2% MNP-HNT-dECM	3.2%	19.2%
4% MNP-HNT-dECM	3.2%	12.6%

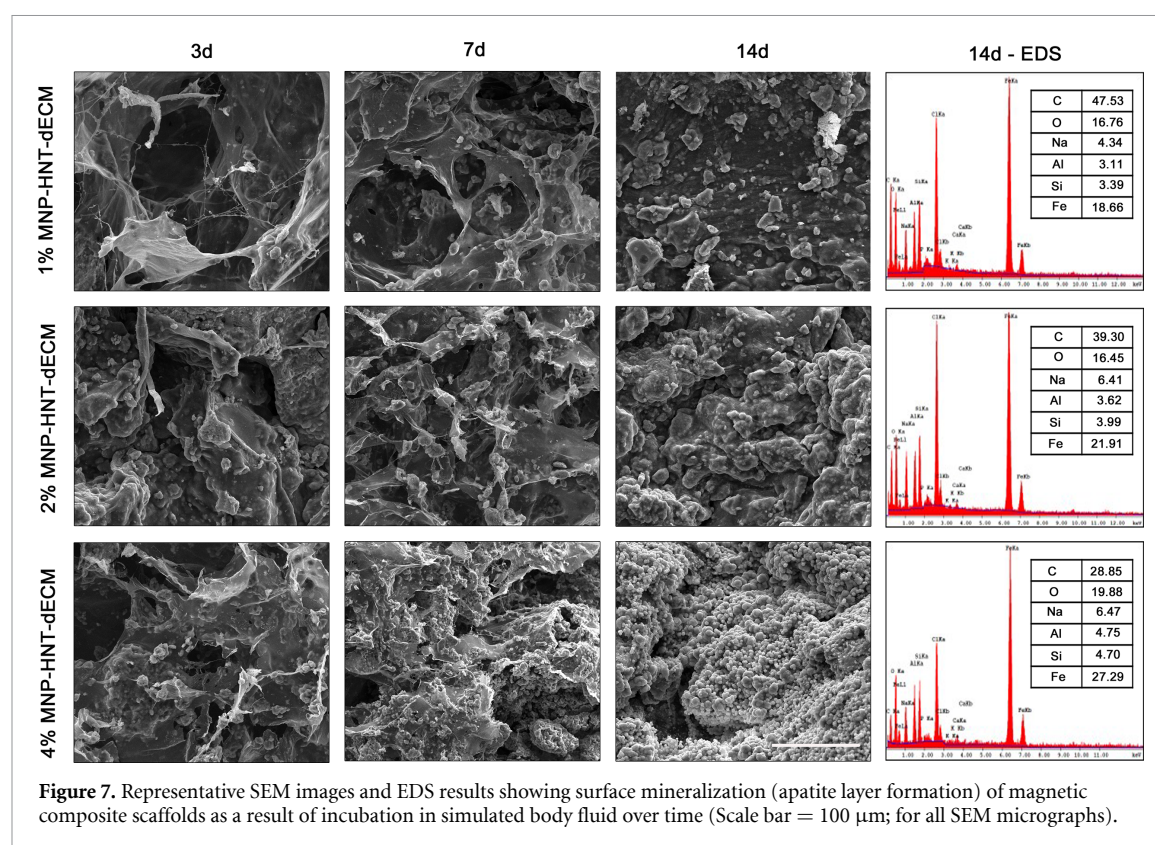
observed in dECM constructs, magnetic properties appeared in the composite scaffolds with the increase in nanofiller content (23.3 emu g⁻¹, 15.3 emu g⁻¹ and 11.7 emu g⁻¹) as expected. Note that the hysteresis loops of the samples are small and lack coercivity of remanence indicating the superparamagnetic properties of the samples (figure S2) [52].

3.3.6. ATR-FT-IR analysis

The data of ATR-FT-IR spectroscopy analysis are given in figure 6(d). The characteristic peaks of MNP-HNT and dECM were observed in all composite scaffold structures. There is an increase in peak intensities in line with the increase in MNP-HNT content. The peaks in the area of 1000–1700 cm⁻¹ and 3600–2700 cm⁻¹ are associated with MNP-HNT and dECM, respectively. The peaks in the region between 1000–430 cm⁻¹ observed in composite materials confirm the formation of MNP-HNT-dECM nanocomposites. Characteristic peaks of MNP-HNT and dECM were observed in all composite scaffold structures. There is an increase in peak intensities in parallel with the increase in MNP-HNT content. The peaks in the 1000–1700 cm⁻¹ and 3600–2700 cm⁻¹ areas are associated with MNP-HNT and dECM, respectively. The region between 1000–430 cm⁻¹ observed in composite materials confirm the formation of MNP-HNT-dECM nanocomposites.

3.3.7. Bioactivity analysis

Materials that support the formation of new tissue by interacting with body fluids and surrounding tissues after they are placed in the body are called bioactive materials. The development of materials with bioactive properties that support the formation of apatite layer with mineral content similar to natural bone tissue is an important issue, especially for BTE studies [77]. In this context, the bioactivity and apatite layer formation capacity of composite scaffolds were determined using SBF that is similar to the ion concentration of human blood plasma. Data of surface morphology (SEM) and EDS analyses of composite scaffolds were obtained for different incubation times in SBF. As the incubation time increased in SBF, an increasing amount of apatite accumulation was detected on all composite scaffold surfaces (figure 7). In addition, the presence of MNP-HNT



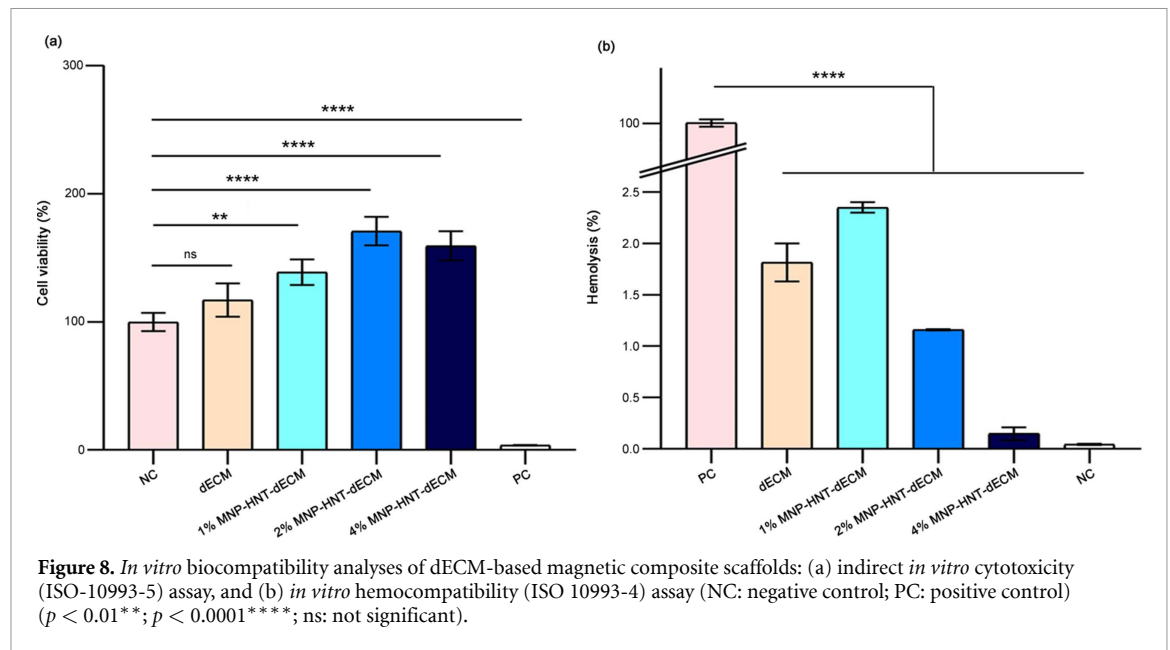
content was found to have a positive effect on increasing apatite accumulation and bioactivity. In particular, cauliflower-like hydroxyapatite layer formation on the surface of the constructs with 4% MNP-HNT content was clearly detected. The results confirm that composite scaffolds containing MNP-HNT are bioactive and induce bone-like apatite formation. EDS analysis on randomly selected areas in SEM images of the 14th day samples reveal the presence of iron, aluminum, silicon and phosphorus atoms. Note that the oxygen (O) content is higher in MNP-HNT-dECM scaffolds due to the presence of iron.

3.4. *In vitro* biological characterizations

As is known, the physical and chemical properties of materials greatly influence cell behavior; also, differences in surface texture and topography lead to notable changes in cellular response [63, 78]. Due to their various properties, the use of MNPs in bone tissue regeneration has become widespread in recent years. Nevertheless, the biological fate of MNPs remains a matter of concern [33, 79]. Extensive studies have been conducted on the biological fate of various formulations of MNPs. The findings suggest that the biological safety of MNPs is associated with competent regulation of cellular iron homeostasis. This regulation involves processes such as uptake, storage and excretion, ultimately ensuring the effective clearance of excess iron from the body [33]. On the other hand,

extensive research has also been conducted to investigate the cytotoxic properties and biological fate of HNTs. The findings from these studies indicate that HNTs can be considered a fairly safe nanomaterial and have the potential for use without causing serious side effects on living organisms, especially at low doses (including the levels used in this study) [18, 80, 81]. In addition, studies on the cytotoxic effect of MNP-HNT nanocomposites have proven the biocompatibility of this nanocomposite on non-cancerous and bacterial cells [82]. It is also stated that the biosafety of HNT and MNP depends significantly on various factors such as particle size, shape, functionalization state, concentration and cell type used [33].

For *in vitro* biological characterization of biomaterials, *in vitro* cytotoxicity and *in vitro* hemocompatibility analyses are performed with rapid and standardized methods [83]. In this study, among the cytotoxicity tests recommended by ISO 10993-5, the indirect cytotoxicity test was preferred to evaluate the potential toxic effects of the biomaterial extracts released into the culture medium [84]; the results are given in (figure 8(a)). Percent cell viabilities after 24 h of culture in the extract media were found to be 118%, 141%, 174% and 162% for dECM, 1% MNP-HNT-dECM, 2% MNP-HNT-dECM, and 4% MNP-HNT-dECM, respectively. On the other hand, percent cell viability was determined as 100% for negative control and 2% for positive control.



Indirect cytotoxicity analysis findings showed that the measured cell viability values were well above 70%, which is the lowest acceptable value according to the ISO 10993-5 standard. This suggests that the developed scaffolds are essentially non-cytotoxic *in vitro* and may be a suitable candidate for use in biomedical applications [85].

Overall, the findings indicate that HNT serves as a suitable immobilization substrate for MNPs and that the MNP-HNT nanocomposite does not adversely affect the inherent biocompatibility of dECM. Additionally, the success of decellularization was confirmed through this test. This is important as elimination of cellular antigens reduces the potential risk of severe immune response to decellularized biological matrix (dECM). Actually, the compatibility of a biomaterial candidate with the host will determine its potential for use in regenerative medicine and TE.

Hemocompatibility is another important biological property that should be studied for all types of biomaterials. The results of the *in vitro* hemocompatibility test are presented in (figure 8(b)). The findings showed that the measured hemolysis values for all developed constructs were less than the maximum acceptable value of 5%. According to ISO 10993-4:2002, this underlines that the composite scaffolds will not trigger a severe hemolysis. The percentage of hemolysis slightly increased with the increase in the MNP-HNT content, but all values demonstrated <2.5% hemolysis, confirming the compatible nature of halloysite.

3.5. Evaluation of preliminary cell culture studies

To determine whether the developed constructs are suitable substrates for mammalian cells, their interaction with human bone marrow-derived mesenchymal

stem cells were evaluated. Cell viability based metabolic activity of BM-MSCs seeded on the constructs were followed for upto 21 d, and the findings are given in figure 9(a). Cell viability was measured by using the MTT assay. The MTT analysis showed that cell-laden 2% MNP-HNT-dECM constructs had the highest cellular activity at 7 and 14 d compared to those of cell-laden 1% MNP-HNT-dECM scaffolds at the same time points. These findings revealed that scaffolds containing 2% MNP-HNT supported the metabolic activity of cells at a good level and demonstrated favorable cell biocompatibility. This can be explained by the presence of Si in the composition, the bioactive content of the scaffold, and also the increase in surface roughness [86]. There is increasing evidence that Si, as a trace element, is essential for the growth, development and healthy function of bone [87]. It takes part in bone development by stimulating type 1 collagen synthesis, also enables the activation of bone cells (osteoblasts) and inhibits bone resorption cells (osteoclasts) through various mechanisms [87]. The presence of silicon in biomaterials has been reported to contribute to increased bioactivity and osteoconductivity [88]. In this context, the findings are compatible with the literature.

SEM analysis was also performed to investigate the morphological response of mesenchymal stem cells to composite scaffolds. SEM observations of cell-laden scaffolds at different time points are given in figures 9(b) and S3. The micrographs clearly show that the cells adhere well to the surface and pore walls of the composite scaffolds, spread through their pseudopods, covering a large part of the constructs during the experimental period, thus acting as a suitable template for cell attachment and proliferation.

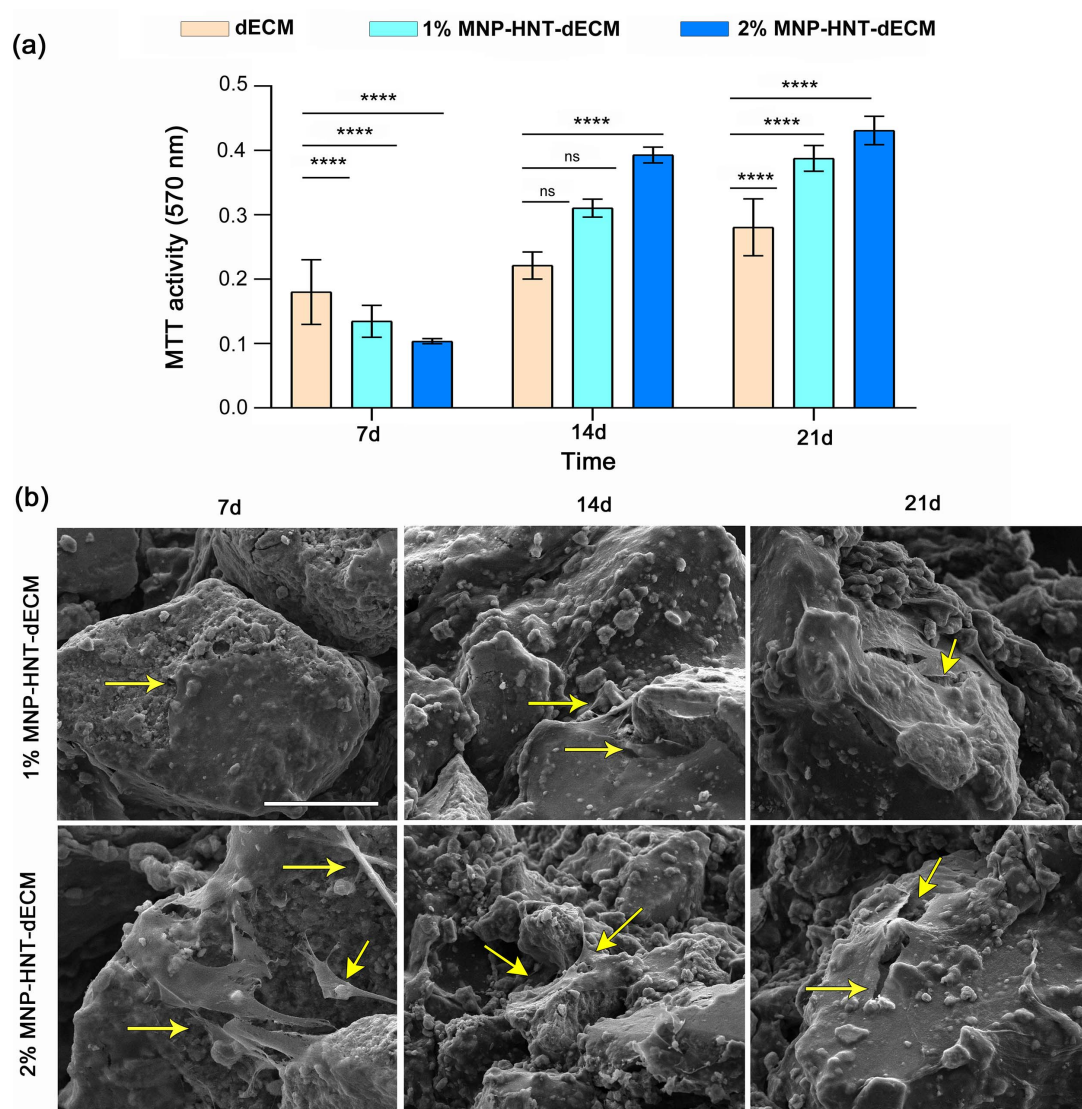


Figure 9. *In vitro* viability of BM-MSCs seeded on magnetic composite scaffolds during the culture period: (a) monitoring of metabolic activity of BM-MSCs by MTT assay; (b) SEM images showing the adhesion and proliferation of cells on the constructs. Arrows indicate extensions of cells through their pseudopods on the substrate (Scale bar = 100 μm; for all SEM micrographs). Higher magnification images are presented in the supplementary data (figure S3).

4. Conclusion

This study is on the development and characterization of new magnetic dECM-based scaffolds for potential BTE applications. dECM was combined with MNP-HNT to form a nanocomposite scaffold that can gel at physiological temperature without using any crosslinkers. Chemical and structural properties of the structures were confirmed by FT-IR and XRD, and magnetic properties were confirmed by VSM analysis. The nanocomposite construct can be defined as a bioactive material with suitable biodegradation and mechanical properties. It exhibits cytocompatibility and hemocompatibility *in vitro*, making it well-suited for biomedical applications. The interconnected macroporous structure of the constructs imbues a favorable environment for the proliferation of stem cells, especially the ones with up

to 2% MNP-HNT. Preliminary cell culture studies reveal that BM-MSCs attached to dECM scaffolds containing MNP-HNT have a higher metabolic activity, which is due to the positive effect of MNPs and HNT on cells. Overall, all characterizations conducted on the developed magnetic scaffolds suggest that they are suitable templates for cell attachment and proliferation, indicating their potential for applications in BTE. However, it is thought that the potential can be better revealed through prospective studies involving magnetic fields and subsequent *in vivo* evaluations.

Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

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Conflict of interest

Y M E is the founder and shareholder of Biovalda Health Technologies, Inc. (Ankara, Turkey). The authors declare no competing financial interests in relation to this particular article. The authors are alone responsible for the content and writing of the paper.

CRediT authorship contribution statement

- Aysel Koç-Demir: Conceptualization, Methodology, Supervision, Investigation, Visualization, Writing—original draft, Writing—review & editing.
- Ayşe Eser Elçin: Investigation, Methodology, Visualization.
- Yaşar Murat Elçin: Conceptualization, Methodology, Resources, Writing—review & editing.

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References

- [1] Qian Y Z, Zhou X F, Zhang F M, Diekwisch T G H, Luan X H and Yang J X 2019 Triple PLGA/PCL scaffold modification including silver impregnation, collagen coating, and electrospinning significantly improve biocompatibility, antimicrobial, and osteogenic properties for orofacial tissue regeneration *ACS Appl. Mater. Interfaces* **11** 37381–96
- [2] Liang Y X, Luan X H and Liu X H 2020 Recent advances in periodontal regeneration: a biomaterial perspective *Bioact. Mater.* **5** 297–308
- [3] Parenteau-Bareil R, Gauvin R and Berthod F 2010 Collagen-based biomaterials for tissue engineering applications *Materials* **3** 1863–87
- [4] Ren S S, Zhou Y, Zheng K, Xu X W, Yang J, Wang X Y, Miao L, Wei H and Xu Y 2022 Cerium oxide nanoparticles loaded nanofibrous membranes promote bone regeneration for periodontal tissue engineering *Bioact. Mater.* **7** 242–53
- [5] Andrzejewski P and Giannoudis P V 2019 The 'diamond concept' for long bone non-union management *J. Orthop. Traumatol.* **20** 21
- [6] Badylak S F 2004 Xenogeneic extracellular matrix as a scaffold for tissue reconstruction *Transpl. Immunol.* **12** 367–77
- [7] Czyz J and Wobus A 2001 Embryonic stem cell differentiation: the role of extracellular factors *Differentiation* **68** 167–74
- [8] Libby P and Lee R T 2000 Matrix matters *Circulation* **102** 1874–6
- [9] Taylor D A, Sampaio L C, Ferdous Z, Gobin A S and Taite L J 2018 Decellularized matrices in regenerative medicine *Acta Biomater.* **74** 74–89
- [10] Parmaksiz M, Dogan A, Odabas S, Elcin A E and Elcin Y M 2016 Clinical applications of decellularized extracellular matrices for tissue engineering and regenerative medicine *Biomed. Mater.* **11** 022003
- [11] Badylak S F, Freytes D O and Gilbert T W 2009 Extracellular matrix as a biological scaffold material: structure and function *Acta Biomater.* **5** 1–13
- [12] Badylak S F, Taylor D and Uygun K 2011 Whole-organ tissue engineering: decellularization and recellularization of three-dimensional matrix scaffolds *Annu. Rev. Biomed. Eng.* **13** 27–53
- [13] Ercan H, Elçin A E and Elçin Y M 2022 Assessment of dopamine-conjugated decellularized bovine tendon extracellular matrix as a bioadhesive *Mater. Today Commun.* **31** 103634
- [14] Startseva O I, Sinelnikov M E, Babayeva Y V and Trushenkova V V 2019 Decellularization of organs and tissues *Khirurgiia* **8** 59–62
- [15] Ibsirlioglu T, Elcin A E and Elcin Y M 2020 Decellularized biological scaffold and stem cells from autologous human adipose tissue for cartilage tissue engineering *Methods* **171** 97–107
- [16] Huang K, Ou Q, Xie Y, Chen X, Fang Y, Huang C, Wang Y, Gu Z and Wu J 2019 Halloysite nanotube based Scaffold for enhanced bone regeneration *ACS Biomater. Sci. Eng.* **5** 4037–47
- [17] Aguzzi C, Cerezo P, Viseras C and Caramella C 2007 Use of clays as drug delivery systems: possibilities and limitations *Appl. Clay Sci.* **36** 22–36
- [18] Dube S, Rawtani D, Khatri N and Parikh G 2022 A deep delve into the chemistry and biocompatibility of halloysite nanotubes: a new perspective on an idiosyncratic nanocarrier for delivering drugs and biologics *Adv. Colloid Interface Sci.* **309** 102776
- [19] Guimarães L, Enyashin A N, Seifert G and Duarte H A 2010 Structural, electronic, and mechanical properties of single-walled halloysite nanotube models *J. Phys. Chem. C* **114** 11358–63
- [20] Pereira I, Saleh M, Nunes C, Reis S, Veiga F and Paiva-Santos A C 2021 Preclinical developments of natural-occurring halloysite clay nanotubes in cancer therapeutics *Adv. Colloid Interface* **291** 102406
- [21] Kumar A and Han S S 2021 Enhanced mechanical, biomineralization, and cellular response of nanocomposite hydrogels by bioactive glass and halloysite nanotubes for bone tissue regeneration *Mater. Sci. Eng. C* **128** 112236
- [22] Du M, Guo B and Jia D 2010 Newly emerging applications of halloysite nanotubes: a review *Polym. Int.* **59** 574–82
- [23] Santos A C, Ferreira C, Veiga F, Ribeiro A J, Panchal A, Lvov Y and Agarwal A 2018 Halloysite clay nanotubes for life sciences applications: from drug encapsulation to bioscaffold *Adv. Colloid Interface* **257** 58–70
- [24] Bakht Khosh Hagh H and Farshi Azhar F 2019 Reinforcing materials for polymeric tissue engineering scaffolds: a review *J. Biomed. Mater. Res. B* **107** 1560–75
- [25] Vergaro V, Abdullayev E, Lvov Y M, Zeitoun A, Cingolani R, Rinaldi R and Leporatti S 2010 Cytocompatibility and uptake of halloysite clay nanotubes *Biomacromolecules* **11** 820–6
- [26] Sinha Ray S 2012 Polylactide-based bionanocomposites: a promising class of hybrid materials *Acc. Chem. Res.* **45** 1710–20
- [27] Gupta A K and Gupta M 2005 Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications *Biomaterials* **26** 3995–4021

- [28] Zong M, Zhang X, Wang Y, Huang X, Zhou J, Wang Z, De Yoreo J J, Lu X and Rosso K M 2019 Synthesis of 2D hexagonal hematite nanosheets and the crystal growth mechanism *Inorg. Chem.* **58** 16727–35
- [29] Huang X, Hou X, Zhang X, Rosso K M and Zhang L 2018 Facet-dependent contaminant removal properties of hematite nanocrystals and their environmental implications *Environ. Sci.* **5** 1790–806
- [30] Li Z, Kawashita M, Araki N, Mitsumori M, Hiraoka M and Doi M 2010 Magnetic SiO₂ gel microspheres for arterial embolization hyperthermia *Biomed. Mater.* **5** 065010
- [31] Chourpa I, Douziech-Eyrolles L, Ngaboni-Okassa L, Fouquenot J F, Cohen-Jonathan S, Souce M, Marchais H and Dubois P 2005 Molecular composition of iron oxide nanoparticles, precursors for magnetic drug targeting, as characterized by confocal Raman microspectroscopy *Analyst* **130** 1395–403
- [32] Jain T K, Morales M A, Sahoo S K, Leslie-Pelecky D L and Labhasetwar V 2005 Iron oxide nanoparticles for sustained delivery of anticancer agents *Mol. Pharm.* **2** 194–205
- [33] Santos L F, Silva A S and Mano J F 2023 Magnetic-based strategies for regenerative medicine and tissue engineering *Adv. Healthcare Mater.* **12** 2300605
- [34] Riahi-Madvaar R, Taher M A and Fazelirad H 2017 Synthesis and characterization of magnetic halloysite-iron oxide nanocomposite and its application for naphthol green B removal *Appl. Clay Sci.* **137** 101–6
- [35] Zou M, Du M, Zhang M, Yang T, Zhu H, Wang P and Bao S 2015 Synthesis and deposition of ultrafine noble metallic nanoparticles on amino-functionalized halloysite nanotubes and their catalytic application *Mater. Res. Bull.* **61** 375–82
- [36] Shen C-H 2019 Quantification and analysis of proteins *Diagnostic Molecular Biology* ed C-H Shen (Academic) Ch 8, pp 187–214
- [37] Ergun C, Parmaksiz M, Vurat M T, Elcin A E and Elcin Y M 2022 Decellularized liver ECM-based 3D scaffolds: compositional, physical, chemical, rheological, thermal, mechanical, and *in vitro* biological evaluations *Int. J. Biol. Macromol.* **200** 110–23
- [38] Kokubo T 1991 Bioactive glass ceramics: properties and applications *Biomaterials* **12** 155–63
- [39] Koç A, Emin N, Elçin A E and Elçin Y M 2008 *In vitro* osteogenic differentiation of rat mesenchymal stem cells in a microgravity bioreactor *J. Bioact. Compat. Pol.* **23** 244–61
- [40] Sharma P and Maffulli N 2006 Biology of tendon injury: healing, modeling and remodeling *J. Musculoskelet. Neuronal Interact.* **6** 181–90
- [41] Jinhua W, Xiang Z, Bing Z, Yafei Z, Rui Z, Jindun L and Rongfeng C 2010 Rapid adsorption of Cr (VI) on modified halloysite nanotubes *Desalination* **259** 22–28
- [42] Rouxhet P G, Samudacheata N, Jacobs H and Anton O 1977 Attribution of the OH stretching bands of kaolinite *Clay Miner.* **12** 171–9
- [43] Theng B K G, Russell M, Churchman G J and Parfitt R L 1982 Surface properties of allophane, halloysite, and imogolite *Clays Clay Miner.* **30** 143–9
- [44] Mo X, Wu F, Yu B, Wang W and Cai X 2020 Folate-PG modified halloysite nanotube for enhancing tumor targeting and anticancer efficacy *Appl. Clay Sci.* **193** 105664
- [45] Wang J, Meng G, Tao K, Feng M, Zhao X, Li Z, Xu H, Xia D and Lu J R 2012 Immobilization of lipases on alkyl silane modified magnetic nanoparticles: effect of alkyl chain length on enzyme activity *PLoS One* **7** e43478
- [46] Reddy K R, Lee K-P, Gopalan A I and Kang H-D 2007 Organosilane modified magnetite nanoparticles/poly(aniline-co-o/m-aminobenzenesulfonic acid) composites: synthesis and characterization *React. Funct. Polym.* **67** 943–54
- [47] Iwasaki T, Nakatsuka R, Murase K, Takata H, Nakamura H and Watano S 2013 Simple and rapid synthesis of magnetite/hydroxyapatite composites for hyperthermia treatments via a mechanochemical route *Int. J. Mol. Sci.* **14** 9365–78
- [48] Liu Z, Zhang F-S and Sasai R 2010 Arsenate removal from water using Fe₃O₄-loaded activated carbon prepared from waste biomass *Chem. Eng J.* **160** 57–62
- [49] Cai J, Miao Y Q, Yu B Z, Ma P, Li L and Fan H M 2017 Large-scale, facile transfer of oleic acid-stabilized iron oxide nanoparticles to the aqueous phase for biological applications *Langmuir* **33** 1662–9
- [50] Parkin S 2007 *Handbook Magnetism and Advanced Materials* (Wiley) (<https://doi.org/10.1002/9780470022184>)
- [51] Xie Y, Qian D, Wu D and Ma X 2011 Magnetic halloysite nanotubes/iron oxide composites for the adsorption of dyes *Chem. Eng J.* **168** 959–63
- [52] Liu S, Zhang L, Zhou J, Xiang J, Sun J and Guan J 2008 Fiberlike Fe₂O₃ macroporous nanomaterials fabricated by calcinating regenerate cellulose composite fibers *Chem. Mater.* **20** 3623–8
- [53] Nakata K, Hu Y, Uzun O, Bakr O and Stellacci F 2008 Chains of superparamagnetic nanoparticles *Adv. Mater.* **20** 4294–9
- [54] Mendioroz S, Belzunce M J and Pajares J A 1989 Thermogravimetric study of diatomites *Therm. Anal.* **35** 2097–104
- [55] Barrientos-Ramírez S, Ramos-Fernández E V, Silvestre-Albero J, Sepúlveda-Escribano A, Pastor-Blas M M and González-Montiel A 2009 Use of nanotubes of natural halloysite as catalyst support in the atom transfer radical polymerization of methyl methacrylate *Microporous Mesoporous Mater.* **120** 132–40
- [56] Justin Joseyphus R, Narayanasamy A, Shinoda K, Jeyadevan B and Tohji K 2006 Synthesis and magnetic properties of the size-controlled Mn–Zn ferrite nanoparticles by oxidation method *J. Phys. Chem. Solids* **67** 1510–7
- [57] Chae K P, Kwon W H and Lee J-G 2012 Influence of aluminum doping in Li–Co–Ti ferrite *J. Magn. Magn. Mater.* **324** 2701–5
- [58] Dönmez Güngüneş Ç, Şeker Ş, Elçin A E and Elçin Y M 2017 A comparative study on the *in vitro* cytotoxic responses of two mammalian cell types to fullerenes, carbon nanotubes and iron oxide nanoparticles *Drug Chem. Toxicol.* **40** 215–27
- [59] Parmaksiz M, Lalegül-Ülker Ö, Vurat M T, Elçin A E and Elçin Y M 2021 Magneto-sensitive decellularized bone matrix with or without low frequency-pulsed electromagnetic field exposure for the healing of a critical-size bone defect *Mater. Sci. Eng. C* **124** 112065
- [60] Ma L et al 2003 Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering *Biomaterials* **24** 4833–41
- [61] Jayakumar R, Menon D, Manzoor K, Nair S V and Tamura H 2010 Biomedical applications of chitin and chitosan based nanomaterials—a short review *Carbohydr. Polym.* **82** 227–32
- [62] Badwelan M, Alkindi M, Alghamdi O, Saeed W S, Al-Odayni A B, Alrahlah A and Aouak T 2020 Poly(delta-valerolactone)/poly(ethylene-co-vinylalcohol)/beta-tri-calcium phosphate composite as Scaffolds: preparation, properties, and *in vitro* amoxicillin release *Polymers* **13** 46
- [63] Aytekin E, Vurat M T, Elçin A E and Elçin Y M 2023 Decellularized bone matrix/45S5 bioactive glass biocomposite hydrogel-based constructs with angiogenic and osteogenic properties: ex ovo and ex vivo evaluations *Macromol. Biosci.* e2300295
- [64] Mustafafov S D, Sen F and Seydibeyoglu M O 2020 Preparation and characterization of diatomite and hydroxyapatite reinforced porous polyurethane foam biocomposites *Sci. Rep.* **10** 13308
- [65] Hernandez C J, Beaupré G S, Keller T S and Carter D R 2001 The influence of bone volume fraction and ash fraction on bone strength and modulus *Bone* **29** 74–78
- [66] Keaveny T M, Morgan E F, Niebur G L and Yeh O C 2001 Biomechanics of trabecular bone *Annu. Rev. Biomed. Eng.* **3** 307–33
- [67] de Oliveira P T, Zalzal S F, Beloti M M, Rosa A L and Nanci A 2007 Enhancement of *in vitro* osteogenesis on titanium by

- chemically produced nanotopography *J. Biomed. Mater. Res. A* **80A** 554–64
- [68] Koc A, Finkenzeller G, Elcin A E, Stark G B and Elcin Y M 2014 Evaluation of adenoviral vascular endothelial growth factor-activated chitosan/hydroxyapatite scaffold for engineering vascularized bone tissue using human osteoblasts: *in vitro* and *in vivo* studies *J. Biomater. Appl.* **29** 748–60
- [69] Majhy B, Priyadarshini P and Sen A K 2021 Effect of surface energy and roughness on cell adhesion and growth—facile surface modification for enhanced cell culture *RSC Adv.* **11** 15467–76
- [70] Huang Q, Elkhooly T A, Liu X, Zhang R, Yang X, Shen Z and Feng Q 2016 Effects of hierarchical micro/nano-topographies on the morphology, proliferation and differentiation of osteoblast-like cells *Colloids Surf. B* **145** 37–45
- [71] Xia P and Luo Y 2022 Vascularization in tissue engineering: the architecture cues of pores in scaffolds *J. Biomed. Mater. Res. B* **110** 1206–14
- [72] Zhou Z, Wu W, Fang J and Yin J 2021 Polymer-based porous microcarriers as cell delivery systems for applications in bone and cartilage tissue engineering *Int. Mater. Rev.* **66** 77–113
- [73] Hickey R J and Pelling A E 2019 Cellulose biomaterials for tissue engineering *Front. Bioeng. Biotechnol.* **7** 45
- [74] Tiaw K S, Teoh S H, Chen R and Hong M H 2007 Processing methods of ultrathin poly(ϵ -caprolactone) films for tissue engineering applications *Biomacromolecules* **8** 807–16
- [75] Vurat M T, Parmaksiz M, Elçin A E and Elçin Y M 2023 Bioactive composite hydrogels as 3D mesenchymal stem cell encapsulation environment for bone tissue engineering: *in vitro* and *in vivo* studies *J. Biomed. Mater. Res. A* **111** 261–77
- [76] Huang -C-C 2021 Characteristics and preparation of designed alginate-based composite scaffold membranes with decellularized fibrous micro-scaffold structures from porcine skin *Polymers* **13** 3464
- [77] Barrère F, Mahmood T A, de Groot K and van Blitterswijk C A 2008 Advanced biomaterials for skeletal tissue regeneration: instructive and smart functions *Mater. Sci. Eng. R* **59** 38–71
- [78] Boyan B D, Hummert T W, Dean D D and Schwartz Z 1996 Role of material surfaces in regulating bone and cartilage cell response *Biomaterials* **17** 137–46
- [79] Carvalho T S S, Torres P M C, Belo J H, Mano J and Olhero S M 2023 Bioactive magnetic materials in bone tissue engineering: a review of recent findings in CaP-based particles and 3D-printed scaffolds *Adv. Nanobiomed. Res.* **3** 2300035
- [80] Liu H-Y, Du L, Zhao Y-T and Tian W-Q 2015 *In vitro* hemocompatibility and cytotoxicity evaluation of halloysite nanotubes for biomedical application *J. Nanomater.* **2015** 685323
- [81] Lazzara G, Bruno F, Brancato D, Sturiale V, D'Amico A G, Miloto S, Pasbakhsh P, D'Agata V, Saccone S and Federico C 2023 Biocompatibility analysis of halloysite clay nanotubes *Mater. Lett.* **336** 133852
- [82] Abhinayaa R, Jeevitha G, Mangalaraj D, Ponpandian N, Vidhya K and Angayarkanni J 2018 Cytotoxic consequences of Halloysite nanotube/iron oxide nanocomposite and iron oxide nanoparticles upon interaction with bacterial, non-cancerous and cancerous cells *Colloids Surf. B* **169** 395–403
- [83] Li W, Zhou J and Xu Y 2015 Study of the *in vitro* cytotoxicity testing of medical devices *Biomed. Rep.* **3** 617–20
- [84] Donato T A G, de Almeida L H, Nogueira R A, Niemeyer T C, Grandini C R, Caram R, Schneider S G and Santos A R 2009 Cytotoxicity study of some Ti alloys used as biomaterial *Mater. Sci. Eng. C* **29** 1365–9
- [85] Chraniuk M, Panasiuk M, Hovhannisyan L, Żółędowska S, Nidzworski D, Ciolek L, Woźniak A, Jaegermann Z, Biernat M and Gromadzka B 2022 The preliminary assessment of new biomaterials necessitates a comparison of direct and indirect cytotoxicity methodological approaches *Polymers* **14** 4522
- [86] Hu Y, Liu S, Li X, Yuan T, Zou X, He Y, Dong X, Zhou W and Yang Z 2018 Facile preparation of biocompatible poly(L-lactic acid)-modified halloysite nanotubes/poly(ϵ -caprolactone) porous scaffolds by solvent evaporation of Pickering emulsion templates *J. Mater. Sci.* **53** 14774–88
- [87] Jugdaohsingh R 2007 Silicon and bone health *J. Nutr. Health Aging* **11** 99–110
- [88] Taher Mohamed S A and Emin N 2024 Effects of using collagen and aloe vera grafted fibroin scaffolds on osteogenic differentiation of rat bone marrow mesenchymal stem cells in SBF-enriched cell culture medium *Biomed. Mater.* **19** 015011