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TISSUE ENGINEERING CONSTRUCTS AND CELL SUBSTRATES

Original Research

Cell sheet biofabrication by co-administration of mesenchymal stem cells secretome and vitamin C on thermoresponsive polymer

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Abstract

Cell sheet technology aims at replacement of artificial extracellular matrix (ECM) or scaffolds, popular in tissue engineering, with natural cell derived ECM. Adipose tissue mesenchymal stem cells (ASCs) have the ability of ECM secretion and presented promising outcomes in clinical trials. As well, different studies found that secretome of ASCs could be suitable for triggering cell free regeneration induction. The aim of this study was to investigate the effect of using two bio-factors: secretome of ASCs (SE) and vitamin C (VC) for cell sheet engineering on a thermosensitive poly N-isopropyl acryl amide-Methacrylic acid (P(NIPAAm-MAA)) hydrogel. The results revealed that using thermosensitive P(NIPAAm-MAA) copolymer as matrix for cell sheet engineering lead to a rapid ON⁄OFF adhesion/deadhesion system by reducing temperature without enzymatic treatment (complete cell sheet release takes just 6 min). In addition, our study showed the potential of SE for inducing ASCs sheet formation. H&E staining exhibited the properties of a well-formed tissue layer with a dense ECM in sheets prepared by both SE and VC factors, as compared to those of VC or SE alone. Functional synergism of SE and VC exhibited statistically significant enhanced functionality regarding up-regulation of stemness genes expression, reduced β-galactosidase associated senescence, and facilitated sheet release. Additionally, alkaline phosphatase activity (ALP), mineralized deposits and osteoblast matrix around cells confirmed a better performance of ostogenic differentiation of ASCs induced by VC and SE. It was concluded that SE of ASCs and VC could be outstanding biofactors applicable for cell sheet technology.

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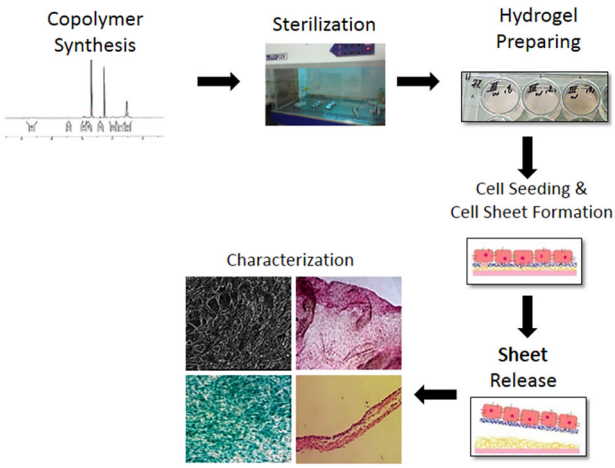
Graphical Abstract



1 Introduction

Restoring physiological function of damaged tissues requires the establishment of bio-mimetic substitutes. Current studies directed onto in vitro bioengineering of tissues provided new hopes for patients suffering from organ failure/injuries [1, 2]. In this regard, cell sheet technology has emerged as a novel method for replacement therapy. A number of cells have been used in terms of sheet fabrication studies including osteoblastic [3], epithelial [4], endolthlial/hepatocyte [5], muscle cells [6], induced pluripotent stem cells [7], and others. Mesenchymal stem cells (MSCs) from different origins or species are ideal cell types for sheet engineering, since MSCs can secret ECM in ex vivo culturing conditions [8]. Adipose tissue derived mesenchymal stem cells (ASCs) have been used as an optimal source for different cell sheet engineering projects [9, 10] regarding their availability during life, ECM secreting, pro-angiogenic, and many other desired properties.

Enzymatic digestion is a method commonly used to dissociate monolayer cell sheet to single cells, as membrane proteins and extracellular matrices are susceptible to destruct by usual enzymatic digestion [11–13]. Therefore, cell sheet technology based on temporary substrates/polymers exhibiting temperature-responsive properties seems to be promising technology in regenerative medicine [14]. After recapture from temperature-responsive polymers, cells retain their cellular structure, functions, as well as retain their ECM network [11, 12]. Temperature-responsive polymers present an inverse dissolution performance and their phase diagrams exhibited a LCST. The LCST is the lowermost cloud point of the polymer solution that is the lowest amount of the phase diagram.

The aqueous solutions of such polymers showed a LCST. The polymer aqueous solutions are homogenous at temperature below LCST and a sol to gel phase separation started when the temperature raise above LCST and exceeds a critical value called the cloud point [15]. A well-characterized themoresponsive polymer is PNIPAAm which has cloudy point at around 32 °C in water [16]. At temperatures higher than the LCST, the amphiphilic PNIPAAm chains hide the amide groups (hydrophilic part), and consequently the hydrophobic isopropyl groups are expose [17]. Below the LCST polymer shows extended hydrated form and hydrophilic interactions become dominant [4, 18]. Co-polymerization or adding salt can change the LCST of PNIPAAm. For establishing a process of sheet engineering, adhesion of cells to thermosensitive substrate is a perquisite. Surfaces representing a slight hydrophobic properties can support cell adhesion [19]. Cells can attach on PNIPAAm and PNIPAAm-based co-polymers above LCST [20] or recover with temperature lowering (by gel to sol transition), maintaining the intact deposited ECM without using proteases or enzymatic treatment [21]. Rapid detachment of cell sheets from polymer is important for maintaining viability, bio-functions and their proper in vitro assembly [11]. After designing proper polymer substrate, the desired cells for sheet engineering must be specified. Many efforts were done to polymerized PNIPAAm monomer on culture polystyrene (TCPS) dish like UV irradiation, electron beam (EB), plasma activation and plasma-polymerization. The thickness of grafted PNIPAAm on TCPS severely affected the detachment time of the cell sheet. Consequently, enhanced technologies and methods have been developed to control the thickness of PNIPAAm graft layer on TCPS [12]. Cell sheet detachment times from surfaces of TCPS grafted with PNIPAAm is slow, when cooled below LCST, often 30–60 min depending on the detachment protocols

detected [22–24]. Cell sheet detachment occurs gradually from the sheet periphery toward the interior. Thus, significant time at reduced temperature is extremely important to recover an intact cell sheet completely. Rapid detachment of cultured cell sheets is a very important factor in terms of retaining the biological functions of released cell sheets and for constructing tissue-mimicking structures, which often require the assembly of numerous cultured cell sheets. When adhered cells are kept at 20 °C or below for duration of time, the cooler environment may damage cells and their functions. Several attempts have been carried out to accelerate cell sheet detachment and recovery from TCPs to prevent damage from cooler temperatures [18]. The rate-limiting step to cell sheet recovery is the hydration of the underlying PNIPAAm grafted surface. Previous studies reported that copolymerization of NIPAAm with hydrophilic monomers such as HEMA lead to rapid cell sheet detachment due to the excellent role of PHEMA section to permit rapid re-hydration of PNIPAAm moiety of copolymer. The cell detachment from PNIPAAm-PHEMA-TCPS dish occurred at 20 °C after 13 min in comparison to PNIPAAm-TCPS dish which lasted 75 min at the same temperature [11]. In another study, P(NIPAAm-AA) copolymer was used as substrate for cell sheet study. The result indicated that the hydrophilic carboxylic group presented in the AA section of the thermos-responsive copolymer lead to accelerate surface hydration below the LCST and to accelerate cell detachment. Cells were spontaneously detached from the copolymer surfaces by reducing the temperature below the LCST (20 °C) after 30 min [12]. The introduction of polar functional groups (like OH, COOH, NH_2 and epoxy) into PNIPAAm as copolymers increases the functionality of the surface of thermoresponsive culture surface to immobilize various bioactive peptides and proteins such as synthetic cell adhesive peptides and growth factors on the surface to control cell proliferation and differentiation by temperature changes [22, 25, 26].

Because sheet engineering is based on polymers, the study of interaction of polymer/cells as well as optimizing studies is crucial. Understanding which polymer combinations could produce surfaces having appropriate properties regarding sheet detachment or functionality could facilitate substrate based functional cell sheet engineering. Besides tissue regeneration depends on controlled delivery of signaling molecules at certain concentrations and times [27]. The stem cells fate is affected by a group of external factors in particular soluble factors and signaling molecules administered in medium [28]. In this regard, the signaling supplements have been utilized in cell sheet engineering projects. Particularly, VC as a water soluble essential vitamin, has shown a positive effect on collagen expression as well as ECM biosynthesis [29]. Moreover, the study by Wei and colleagues suggested a method for MSCs sheet engineering based on VC treatment without using thermosensitive polymers [30].

Previous reports stated that the human MSC growth rates become slower as passage number increases and the cells in the first passage have unique properties close to the in-vivo situation [31]. During culturing step ASCs secret cytokines, molecules [32], exosomes, vesicles [33] and a multitude of bioactive molecules [34]. In several studies MSCs culture supernatants or SE showed different properties such as differentiation induction [35] or treatment of organ dysfunctions [34]. We hypothesized that using SE of ASCs could improve the cell sheet engineering of ASCs. Therefore, the present research was directed at design and evaluates of a rapid ON/OFF adhesion/deadhesion system by reducing temperature without enzymatic treatment.

For this purpose, thermo-responsive PNIPAAm-co-PMAA hydrogel was synthesized and investigate its behavior in terms of supporting ASCs adhesion, ECM secretion, cell sheet production and rapid releasing of ASCs sheet by a quick gel to sol transition without using enzymes. Additionally, the effect of administration of SE or VC in sheet functional properties including detachment, viability, gene expression, differentiation, senescence, molecular and histologic properties were studied.

2 Materials and methods

2.1 Fabrication of thermoresponsive co-polymer

Predetermined amount of NIPAAm (Fluka) (1.052 g) and MAA (60 µL) were dissolved in 10 mL of deionized water and the mixture was stirred under argon flow for 30 min. Then ammonium persulfate (APS) (Sigma) as initiator (0.3 mol % of all the monomers) and N,N,N′,N′-tetramethyl ethylene diamine (TEMED) (Sigma) (30 µL) as an accelerator were added to the mixture. The polymerization was performed at ambient temperature for 24 h under argon flow. Copolymer with molar ratios of NIPAAm to MAA of 93:7 was prepared. The product was purified by dialysis for 48 h utilized dialysis membrane (Cellu SepH1, with mesh of 2000 KD). The copolymer was gathered by increasing the temperature of polymer solution above its LCST. Then gained gels were vacuum dried at 40 °C for 1 day. The chemical structure of PNIPAAm-co-MAA copolymer was further characterized by ^{1}H-NMR and FT-IR.

2.2 FT-IR of co-polymer

FT-IR analyze of co-polymer was undertaken in KBr pellet using a Bruker (Tensor 27) spectrophotometer instrument.

2.3 ^{1}H-NMR of co-polymer

The chemical structure of the synthesized PNIPAAm-co-MAA copolymer was determined by ^{1}H NMR using a Bruker spectra spin 400 MHz.

2.4 Thermosensitivity test in PBS and water

The LCSTs of the PNIPAAm-co-MAA hydrogel with molar ratio of 93:7 was assessed by cloud point (turbidity) measurements. The inflection point of the turbidity curves was taken for the LCST value of the hydrogel. Different copolymer concentrations of 1.5, 3.5 and 5.5 wt% were prepared in PBS (Sigma) or water as solvent to investigate the effects of polymer concentration and solvent type on LCSTs. The optical transmittances of these solutions were measured at 500 nm wavelength using UV-vis spectrometer (UV-160, Shimadzu) with increasing solution temperature (4–37 °C, 1 °C interval). At each temperature, the samples were stabilized for 10 min before measurements. Values for the LCST of polymeric solutions were determined at the temperature with the optical transmittance close to zero.

2.5 Preparation of temporary substrate

Synthesized powder PNIPAAm-co-MAA copolymer, was sterilized under UV light for 40 min and mixed with PBS in order to make hydrogel.

The prepared hydrogel (0.5 ml) was added to each well of 24-well plates and placed in an incubator at 37°C for 24 h. Next, 2 ml of the DMEM (Gibco) medium containing 15% Fetal bovine serum (FBS, Biosera) was added to each well and incubated for 3 days in humidified atmosphere, 37 °C and 5% CO_2.

For hydrogel thickness studies, two amounts of hydrogel 1 and 0.5 ml were transferred into each well of 12 well plate as described above.

2.6 Cell isolation and culturing

Subcutaneous Adipose tissue was used for isolation of ASCs as previously described in our previous studies [2, 36]. ASCs were cultured in DMEM enriched with 10% Fetal bovine serum (FBS), 10 ng/ml basic fibroblast growth factor (bFGF, Thermoscientific), ITS (Sigma) [1, 2]. When passage 1 ASCs reached to 70% confluence, the culture growth medium was aspirated, and the cultures washed three times with DMEM-F12 (Bioidea), and then 5 times with PBS without Mg or Ca ions. DMEM-F12 containing 1% Kanamycin (Bioidea) was added to the flasks. Then the culture flasks were placed in a 37 °C humidified CO2 incubator (5%) for 24 h. Afterward, the supernatant medium of ASCs was collected, centrifuged and stored at −80 °C [37] for use as SE in this study. At 80% confluence, the cells were sub-cultured up to passage 3 and subjected to further experiments.

2.7 Cell viability assessment

Cell viability was determined by Trypan Blue dye (Sigma) exclusion assay. Breifly, trypsinized ASCs cell sheets were mixed with Trypan Blue and then counted under an inverted microscope (HundWetzlar, Germany); cells up taking blue dye were considered as non-viable. All the countings were performed in triplicates [36] and presented as percentage of viable cells.

2.8 Evaluation of cell proliferation

Passage 3 of ASCs cultures were seeded at a density of 5000 cells/well in 96-well plates. After an overnight incubation, the expansion medium was replaced with different concentrations of NIPAAm and NIPAAm for 24, 48, 72 h. The culture media was exchanged with 20 µL of MTT solution [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (5 mg/ml; Sigma-Aldrich, St Louis, MO) and incubated for 4 h. Subsequently, the MTT solution then was removed and replaced with 200 µL dimethyl sulfoxide (DMSO) (Sigma) and incubated for 20 min on a shaker. The OD values of dissolved formazan crystals in each sample were measured at a wavelength of 570 nm using an ELISA reader.

2.9 ASCs sheet fabrication

About 250,000 cells were seeded on the hydrogels in 24-well plate, and incubated at 37 °C and 5% CO_2 at a humidified atmosphere. The following day, seeded cells were divided into 4 groups including: group VC, where the cultivation medium was enriched with 50 µg/mL vitamin C (Sigma), group SE, where the cultivation medium was enriched with SE at a ratio of 1:1 SE and cultivation medium; group VC+SE, where the cultivation medium was enriched with VC and SE with the ratio of 1:1 simultaneously; and the cells cultivated in expansion medium were used as control. The media was refreshed every 3 days. After incubation for 14 days, sheet detachment was performed by cold PBS addition and temperature reduced by putting the plate on the ice for 1 min and immediately incubation on room temperature for 5 min. Hydrogel was dissolved in PBS and cell sheets were released. The sheets were transferred to a petri dish using aspiration with a sampler tip, and kept in a standard incubator until future analysis.

2.10 β-galactosidase staining

The detached sheets were fixed with a 20% formaldehyde/2% glutaraldehyde (Sigma) fixative solution for 15 min at room temperature. Following extensive washing with PBS, fixed cell sheets were incubated with 930 µl of 1X staining solution (10X solution containing 400 mM citric acid/sodium phosphate (pH 6), 1.5 M NaCl, 20 mM $MgCl_2$) (all staining reagents from Sigma), 10 µl of staining supplement and 50 µl of 20X X-gal (Sigma) prepared and pH adjusted to 5.9. After adding final solution, the cell sheets incubated over-night in dry incubator at 37°C. The percentage of β-galactosidase positive cells was counted in ten random microscopy fields (Olympus).

2.11 Histological observations

The ASC-sheets were fixed in 4% para-formaldehyde (Merck) for 4 h at room temperature and processed by paraffin embedding, sectioned, and subjected to H&E staining. Separately, the surface of fixed sheets was stained with E& H using a whole mount technique, which does not involve paraffin embedding nor sectioning.

2.12 Scanning electron microscopy (SEM)

The harvested, cell sheets were fixed by 2.5% glutaraldehyde at 4 °C for 24 h. Fixed samples were dried at room temperature. The dried samples were sputtered with gold and the imaging was performed in a SEM system (ProX, Phenom model, Holland).

2.13 Real time PCR

The gene expression analysis of Sox2, Oct-4, Nanog, and the RT were performed in released sheets prepared using VC, SE, or VC+SE. First: the isolation of the total RNA from released sheets was directed using Trizol reagent (Thermo scientific) according to manufacturer recommendations. 1 µg of total RNAs in each sample were subjected to cDNA synthesis using a kit from Thermoscientific Company (Revert Aid cDNA synthesis kit). The qRT-PCR was performed on a MicPCR system by Syber green Master mix (Themoscientific). The GAPDH was selected as housekeeping gene. The fold changes were calculated by the ΔΔCT method. Primers sequences of the genes were summarized in Table 1.

Table 1 Primer nucleotide sequences used for quantitative real time PCR

Gene	Primer sequences
Nanog	Forward: TTCTTCCACCAGTCCCAAAG
	Reverse: TCTGCTGGAGGCTGAGGTAT
Oct-4	Forward: GGTATTCAGCCAAACGACCA
	Reverse: AGCTTCCTCCACCCACTTCT
Sox-2	Forward: CAGCTCGCAGACCTACATGA
	Reverse: TGGAGTGGGAGGAAGAGGTA
TERT	Forward: CCGCCTGAGCTGTACTTTGT
	Reverse: CAGGTGAGCCACGAACTGT
GAPDH	Forward: GGGTGTGAACCATGAGAAGT
	Reverse: GGCATGGACTGTGGTCATCA

2.14 Osteogenesis in sheet

Taking into consideration that main functionality of ASCs is their potential to differentiate into osteoblast, the 6-well plates previously coated with PNIPAAm-co-MAA hydrogel (explained in part 2.5.) were seeded with ASCs. The sheet induction was conducted for 7 days with bio-factors specified in each groups (Section 2.7). Consequently, the differentiation medium was administered to the cells for 21 days. Finally, the alizarin red (Sigma) staining was performed as previously described [38] and the sheets mineralization were observed under an Invert microscope (Olympus).

2.15 Alkaline phosphatase (ALP) activity assay

The ALP activity was determined in sheet lysates using p-nitrophenyl phosphate (Sigma-aldrich). In brief: released sheets growing from 24-well plates were washed with PBS prior to addition of 1% Triton X-100 (Sigma) was added. The lysates were homogenized and incubated in the presence of substrate. Finally, the amounts of released p-nitrophenol were assessed at 405 nm using a plate reader.

2.16 Sheet shrinkage assay

ASC cell sheets were established in 6 well plates (Section 2.9). After release of the sheets by solubilizing of the thermoresponsive polymer (below LCST), the cultures diameter was measured to determine the shrinkage from the full size. Full size was defined as the diameter of one well in 6 well: 34.8 mm.

2.17 Statistical analysis

All data were reported as mean ± SD of three replicates. The ANOVA or student's t-test were applied for statistical analysis. *P*-values less than 0.05 were considered significant.

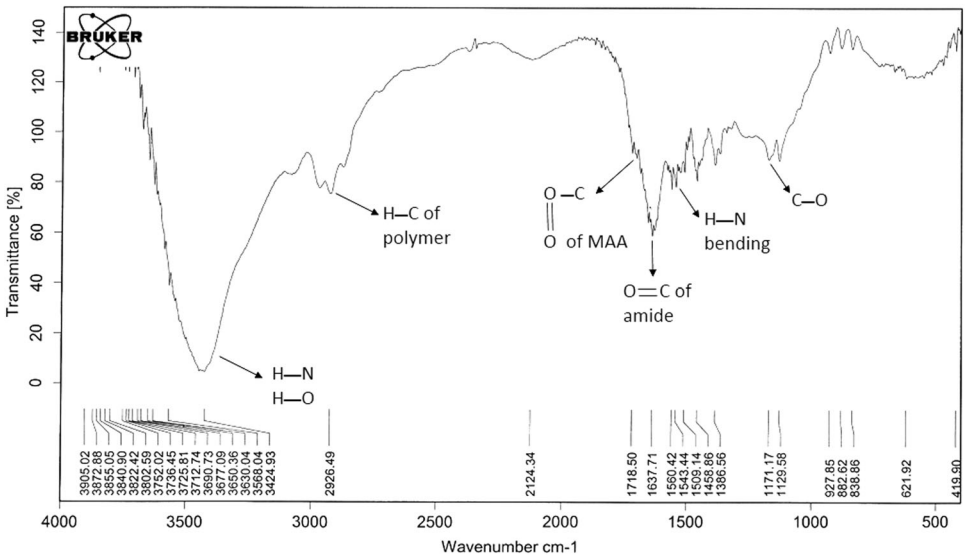


Fig. 1 FT-IR spectra of PNIPAAm-co-MAA copolymer

3 Results

3.1 FT-IR results

FT-IR spectrum showed all the PNIPAAm-co-MAA characteristic peaks which confirmed the successful copolymerization process. PNIPAAm amide group bending and stretching peaks appeared at 1550 and 1637 cm^{-1}, respectively. The aliphatic CH peak of copolymer backbone was observed at 2926 cm^{-1}. A small peak at 1716 cm^{-1} and an intense broad peak at 3600 cm^{-1} related to MAA acidic carboxylic group and OH, respectively (Fig. 1).

3.2 ^{1}H-NMR

The Fig. 2 shows the chemical structure of the copolymers assessed by ^{1}HNMR. The signals related to NIPAAm are appeared in δ (ppm) = 1.60 ($C\underline{H}_2$-CH), δ (ppm) = 1.035 (($C\underline{H}_3$)$_2$CH), δ (ppm) = 3.83((CH_3)$_2C\underline{H}$) and triplicate peak at δ(ppm) = 2.8(CH_2-$C\underline{H}$-C = O). Signals belonging to MAA are appeared in, δ (ppm) = 1.95 ($C\underline{H}_2$-C(CH_3)-COOH). The peaks at 3.3 and 2.3 were related to DMSO solvent and trace of D_2O, respectively.

3.3 LCST test

The PNIPAAm-co-MAA hydrogel thermal behavior was tested according to Fig. 3 diagram. The Fig. 4 demonstrates the effect of the concentration of the polymer solution and type of solvent on the LCSTs of the copolymers. Decreasing the co-polymer concentration resulted in increased LCST, in both water and PBS. These results showed a correlation between concentration of co-polymer and LCST point. The LCST reduces rapidly by increasing copolymers concentration from 1.5 to 5.5 wt% in water and PBS solvents. On the other hand, by comparing the LCSTs values in water and PBS showed that the presence of ions and salt can decrease LCST (Fig. 4a). The 1.5 wt% P (NIPAAm-MAA) copolymer with an LCST point at 35 °C was used for all subsequent cell sheets production in this study.

3.4 Viability study (in vitro)

Both trypan blue exclusion dye and MTT assay showed that at 72 h after incubation of the ASCs on PNIPAAm-co-MAA no cytotoxicity was detected for PNIPAAm-co-MAA hydrogel (Fig. 5). The cell viability ranged between 96 to 100% which was indistinguishable from the viability in control cells grown in cultivation medium ($p > 0.05$). The ASCs were cultured in the presence of PNIPAAm-co-MAA hydrogel for 5 days showed the same morphology and cell density of the control cells in the routine medium and on TCPS (data were not shown). These observations suggested that the PNIPAAm-co-MAA did not release any toxic compounds to the media.

3.5 ASCs sheet production

Since the cyto-compatibility of the PNIPAAm-co-MAA copolymer hydrogel was confirmed, optimized hydrogels were assessed for feasibility of producing a harvestable ASCs sheets. 14 days after sheet induction, the detachment behavior of the sheet on copolymer was studied. In this way, the lowering of temperature was performed by removing the culture plates from the incubator and placing

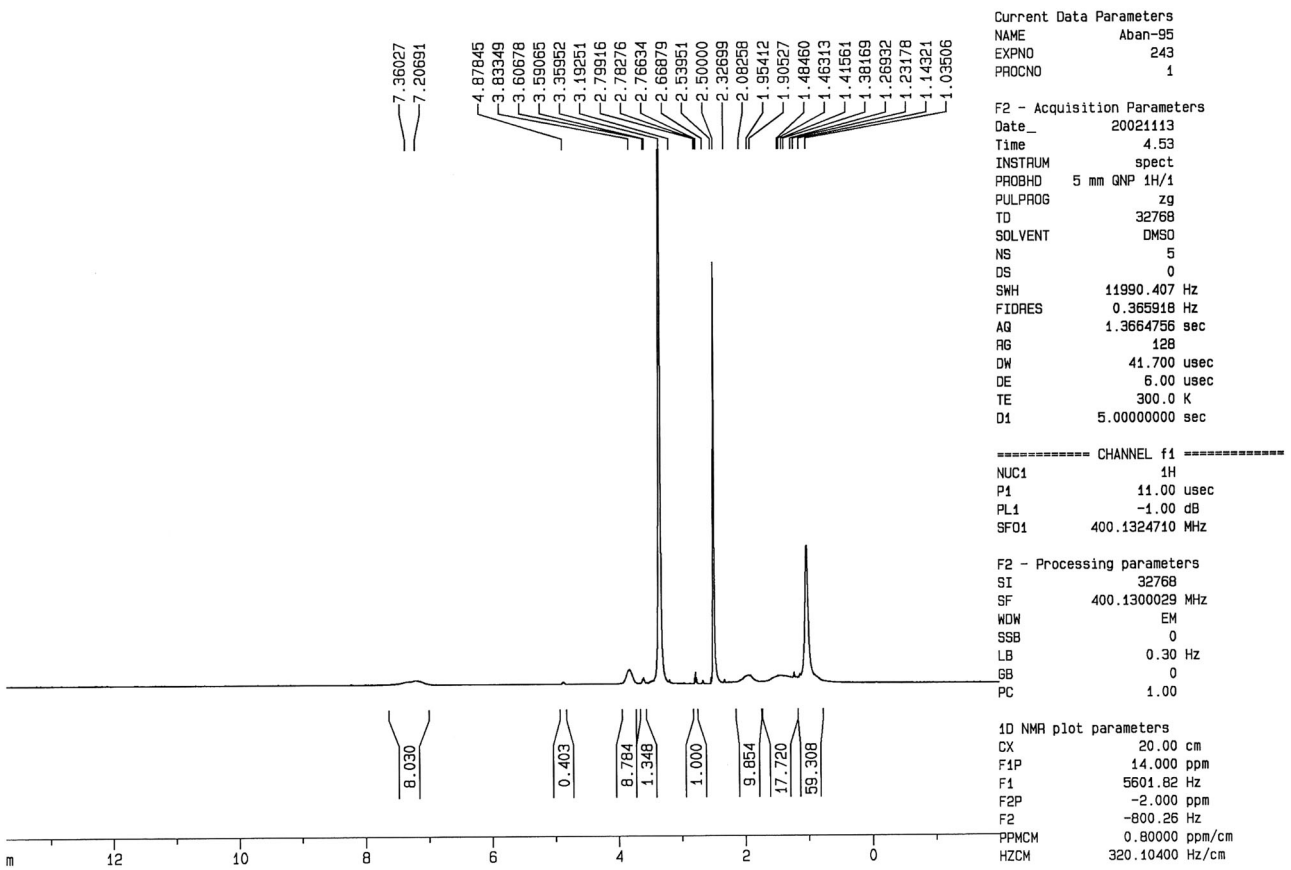


Fig. 2 H-NMR spectra of PNIPAAm-co-MAA copolymer

them at room temperature under safety cabinet, then the whole plate containing hydrogels with cells seeded on them was placed on the ice bath (4 °C) for 1 min and immediately incubated at room temperature for 5 min in order to release the sheet.

The released sheets including those prepared with just VC (Fig. 6a), SE (Fig. 6b), and both VC & SE (Fig. 6c) comprehended structured monolayers of ASCs cultures, while a confluent well of the ASCs cultivated directly on a TCPS showed only collapsed stem cells (Fig. 6d).

The full detachment of the sheet undertook ~6 min, when the sheets were free-floating in the sol form of the polymer, allowing transfer into a new sterile petri dish (Fig. 7). Due to the tight interconnection of the ASCs, the sheets were collected easily with a pipette without disrupting the cells. Interestingly, only thick hydrogel allowed the sheet formation. Sheets, which formed on thin layer (0.5 mL in 12 wells) of hydrogel, were so fragile and did not detach properly. As these sheets would not be suitable for regenerative purposes, only sheets from thick hydrogel (1 mL per well of 12 well) were used in this study detached by temperature reduction below LCST.

3.6 VC and SE improved sheet formation and properties

In the present study, ASCs were seeded onto 6-well plates previously coated with hydrogel and induced for sheet were incubated in the presence of VC or SE alone, or VC+SE for 14 days (Fig. 7). Sheets formation in cultivation media supplemented with VC or SE alone induced ASCs to reach high confluence. Albeit, the produced sheets were fragile and fragmented during releasing (Fig. 7a, b). However, when treated with SE for 14 days in cultivation medium, our ASCs become completely confluent and wrapped at the edge of wells. In addition, the entire VC+SE induced ASCs sheets were detached using the sub-LCST method (Figs. 6, 7). The morphology and integrity of the harvested whole ASCs sheets from cultures supplemented with VC+SE indicates this as an ideal supplementation for sheets formation suitable for regenerative purposes (Fig. 7c).

3.7 Hematoxylin and eosin staining

H&E staining of the ASCs sheets showed several layers of cells along with properly formed ECM in the released sheet

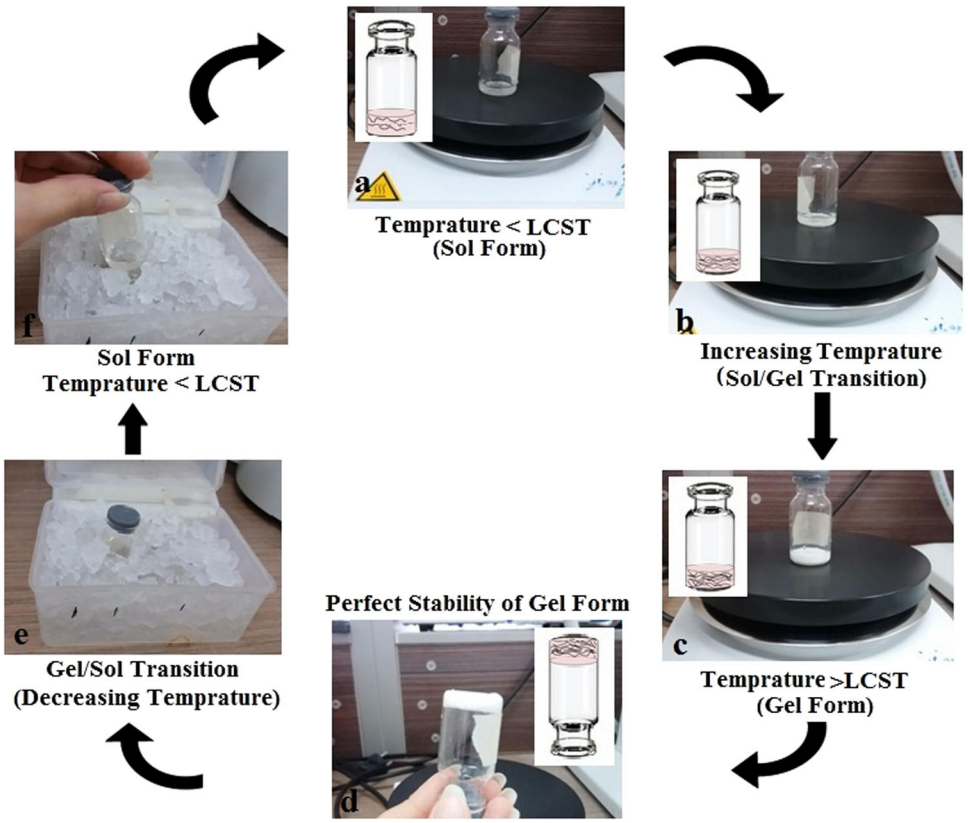


Fig. 3 Thermal behavior of PNIPAAm-co-MAA polymer. Below LCST hydrogel was seen in sol form **a**. By increasing temperature up to LCST opaque appearance was observed **b**. Finally, gel form become dominant **c**. In PNIPAAm-co-MAA hydrogel, gel form showed high stability **d**. Gel/sol transition can be achieved by reducing the temperature **e** and when the temperature lowers below the LCST sol form was observed again

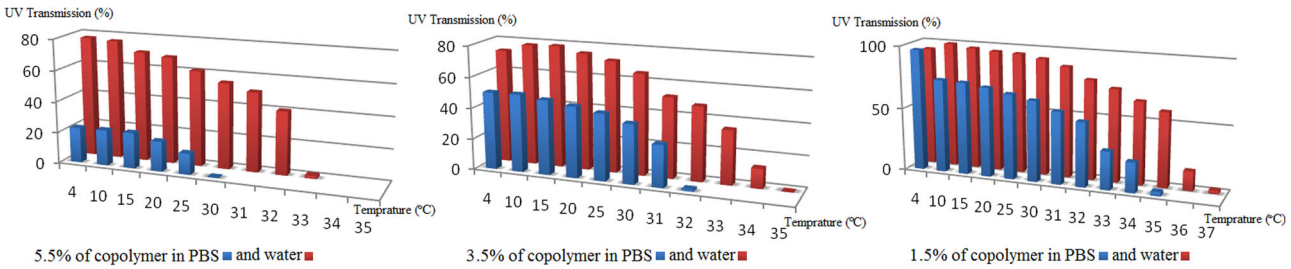


Fig. 4 Cloud point measurements of PNIPAAm-co-MAA copolymer at different temperatures (4 up to 40 °C) and concentration (5.5, 3.5 and 1.5% w/v copolymer in PBS and water). By decreasing copolymer concentration LCST point increased

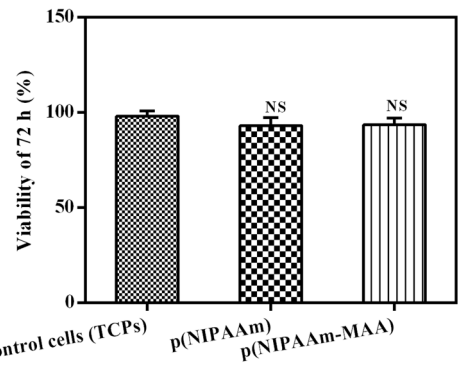


Fig. 5 The MTT assay was performed for ASCs in the presence of P (NIPAAm-MAA) and P(NIPAAm) for 72 h, without any significant effect on viability of cells (B). NS: not significant

(Fig. 8). The external layer of the sheet displayed densely deposited by ECM while the internal sections were fully comprehended by cells (Fig. 8a, b). In addition to being harvested as a whole, ASCs sheets prepared by supplementation with both VC and SE are 2 or 3-layered and their spread was uniformed similar to a 2 dimensional (2D) tissue in comparison with other sheets (Fig. 8c).

3.8 Secretome and vitamin C prevented senescent-entering of sheets

The β-galactosidase staining was performed to identify the effects of different treatments on β-galactosidase related senescent activity. The results showed that passage 10 of ASCs (Fig. 9a) has high percentage of senescent cells but

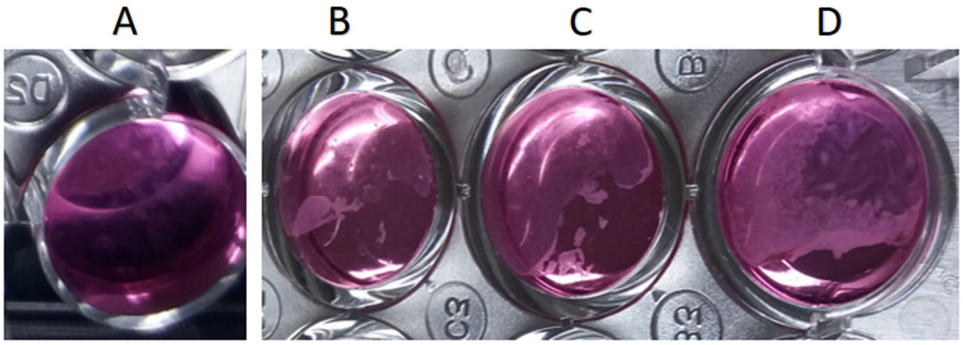


Fig. 6 Sheets in day 14 of treatments. Sheet produced by VC **a**, by SE **b** and by VC+SE treatment **c**, are compared to control ASCs in cultivation medium **d**

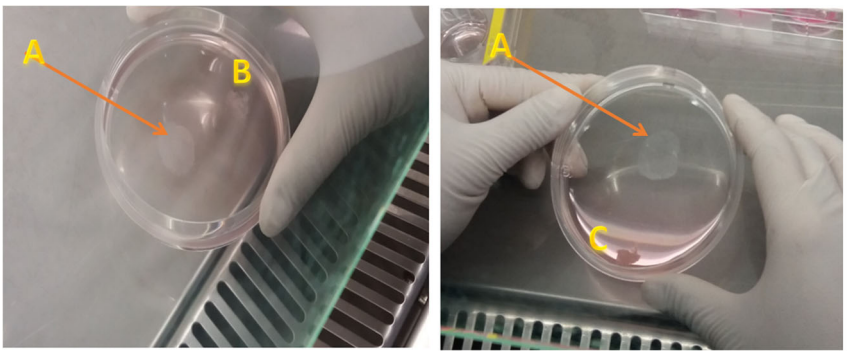


Fig. 7 Images of released sheets from plates and transferred to Petri dishes. Sheets produced by VC+SE **a**, by SE only **b** and only VC treatment **c** are displayed

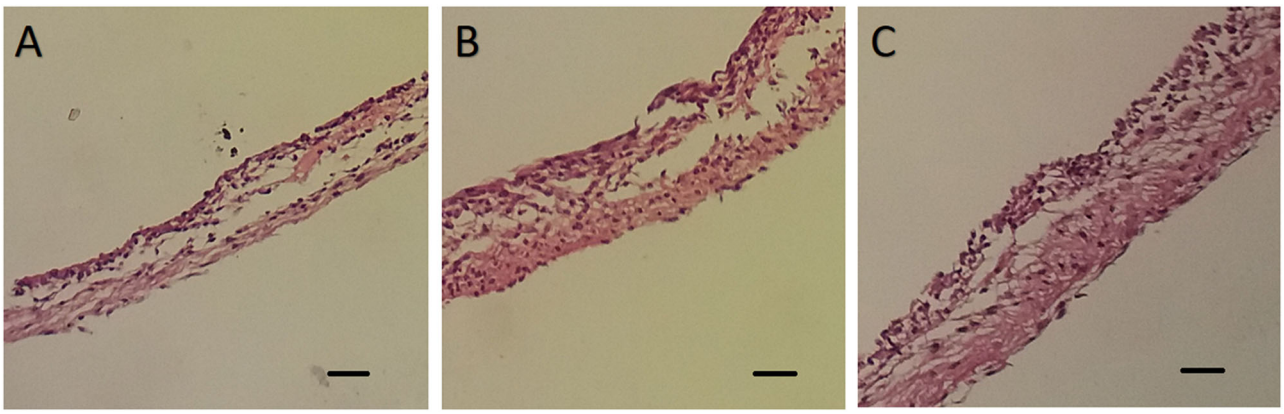


Fig. 8 H&E staining. Sheet of VC **a**, SE **b** and SE + VC **c** groups were stained with histology stains. The sheet C, produced using SE+VC, have multi-layered structure with better cross-section of the released sheet showing ~3–5 layer of cells in a sheet with extensive secreted ECM

sheets produced by VC (Fig. 9b) and SE (Fig. 9c) showed considerably less senescent cells. Interestingly, the sheets produced in the presence of both VC and SE showed a very little number of cells (Fig. 9d).

Quantitative analysis of the frequency (%) of β-galactosidase positive cells in each filed of microscopy in VC, SE, and VC+SE grown sheets (Fig. 9e) were significantly ($p < 0.001$) less than those cultured in a standard culture conditions.

3.9 Improved osteogenic potential of ASCs in sheets

The osteogenic differentiation of cells in sheets was enhanced, demonstrated by the increase in the percentage of calcium deposits in ASCs sheets cultured in the presence of both VC or SE (Fig. 10a, b). An increase of calcium deposits was observed particularly in sheet cultures with VC +SE (Fig. 10c) where the early marker of osteogenesis, ALP activity, was significantly higher ($p < 0.05$ on day 7 and $p < 0.01$ on day 14) in cultures treated with VC+SE (Fig. 10d).

3.10 SEM images after osteogenesis in sheet

To analyze cellular and subcellular structures, ASCs sheets engineered with VC, SE, VC+SE on PNIPAAm-co-MAA copolymer hydrogel. The released sheets were subjected to SEM imaging. The SEM figures revealed that all of three

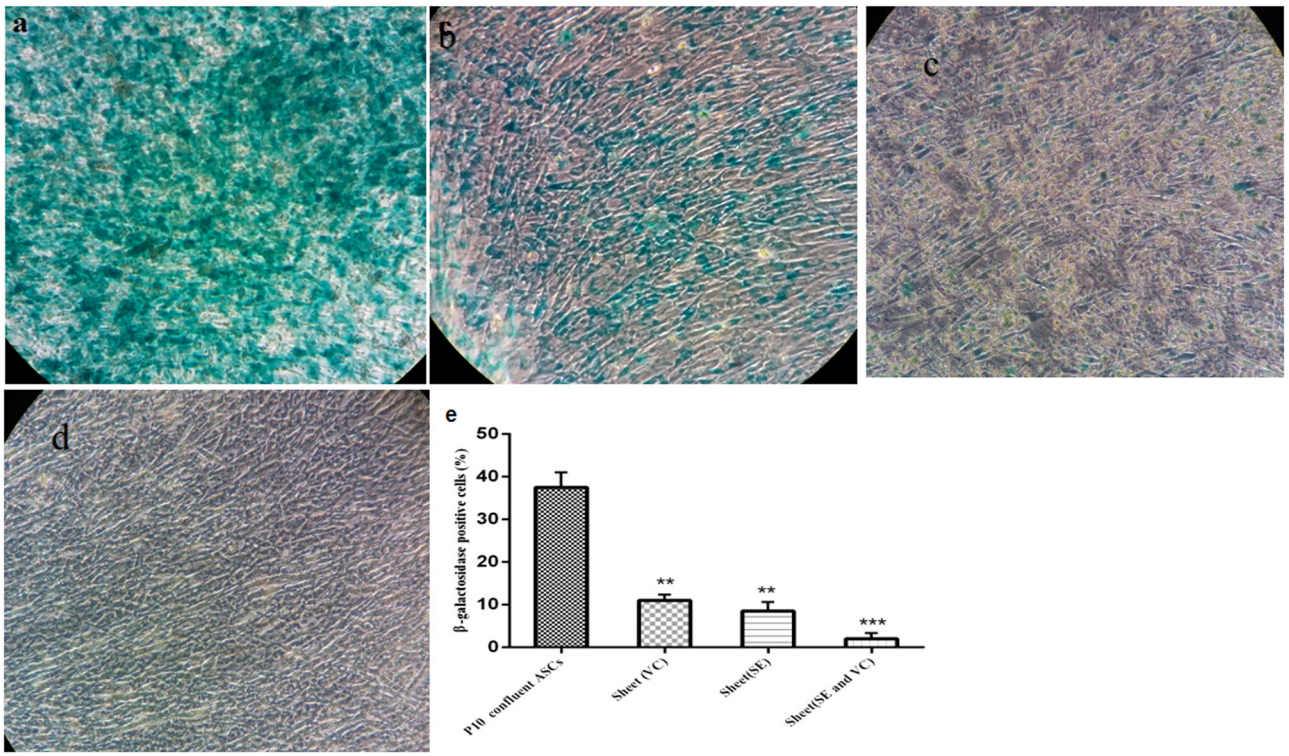


Fig. 9 β-galactosidase staining was performed on passage 10 **a** ASC standard cultures, or in sheet produced with VC **b**, SE **c**, and VC+SE **d**. Additionally, aged (β-galactosidase-positive) cells were counted in each filed of microscope and the frequency of aged cells were reported as percentages **e**. $**p < 0.01$ & $***p < 0.001$

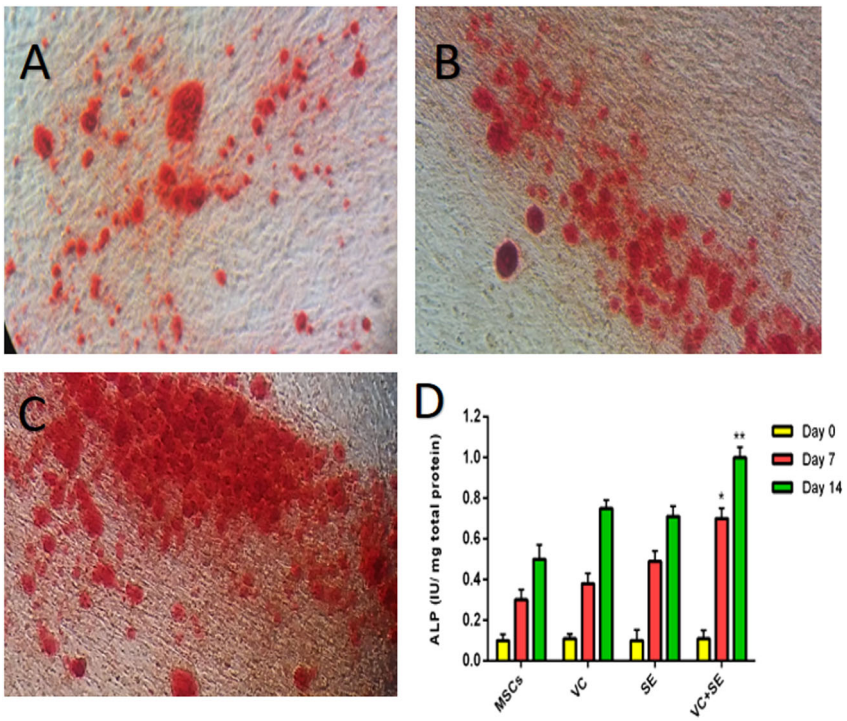


Fig. 10 Osteogenic potential of sheets produced by VC **a**, SE **b** and VC+SE **c**. The cells treated with VC+SE showed increased osteogenic potential. ALP activity of released sheet in group VC+SE was significantly higher than VC or SE grown sheets **d**. $*p < 0.05$ & $**p < 0.01$

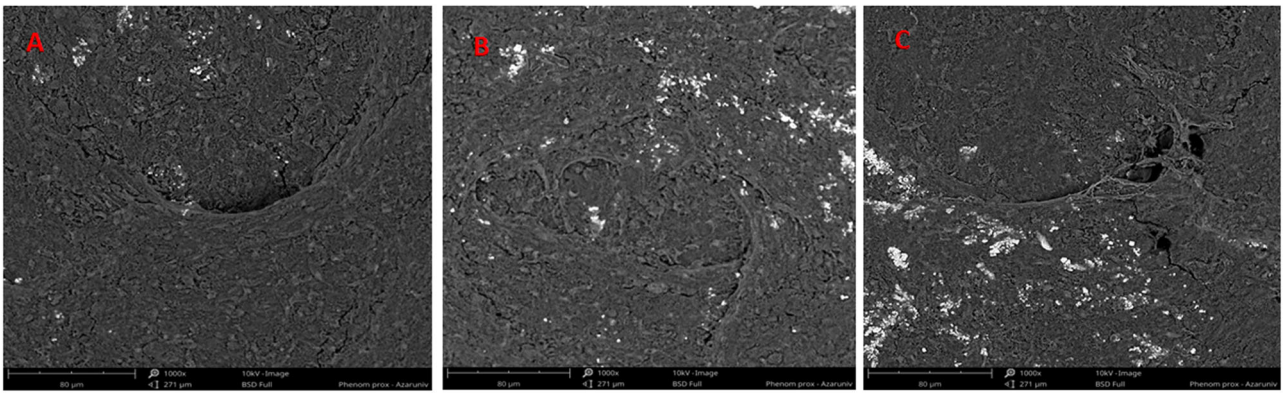


Fig. 11 SEM imaging of released sheets. The detached sheets showed flat appearance of tissues surrounded by ECM VC **a**, SE **b**, VC+SE **c**, with visible mineral deposits as expected from osteogenic cells

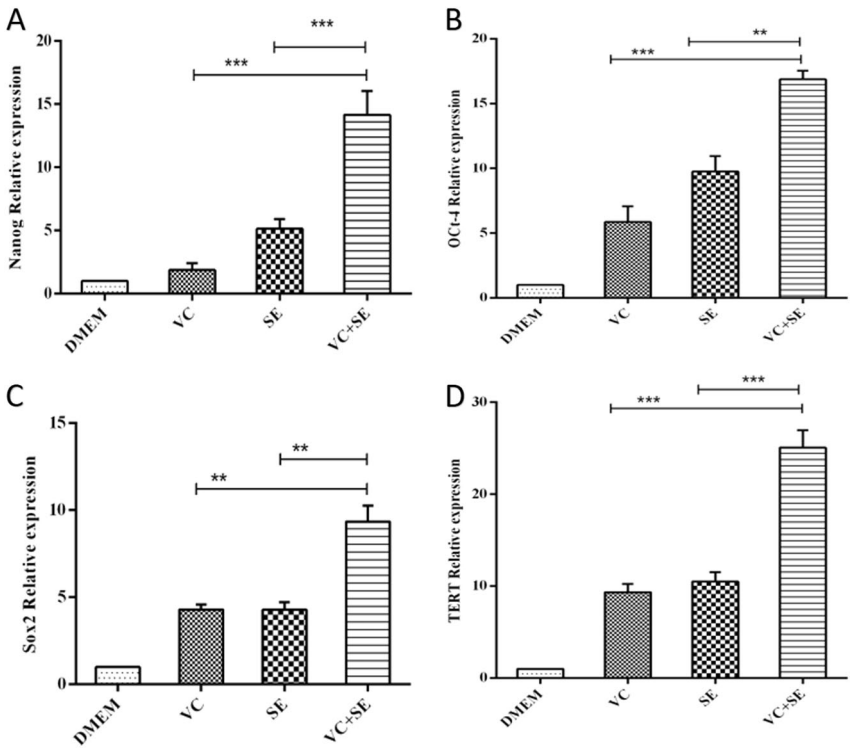


Fig. 12 The expression of stemness marker genes Nanog **a**, OCT-4 **b**. Sox-2 **c**, and TERT **d** in cells treated with VC, SE, and VC+SE. The expression of stemness genes showed an increase in expression upon treatment with both SE and VC. $^{**}p < 0.01$, $^{***}p < 0.001$

treatments of the cells have the potential to provide an ECM and the cells were embedded in their own ECM (Fig. 11). In addition, mineral like nodules were observable on the surface of the sheets, a sign of osteogenesis.

3.11 Quantitative real time PCR

The qRT-PCR results revealed that the VC treatment of ASCs on PNIPAAm-co-MAA copolymer hydrogel can increase the expression of Nanog, Oct-4, Sox-2 and TERT, which is comparable to the results, observed after SE treatment (Fig. 12). Albeit, the expression of these genes were significantly, up regulated by co-exposure of VC and SE ($p < 0.01$) as compared to either VC or SE.

3.12 Analysis of ASCs sheets shrinkage

Shrinkage of cell sheets after release from hydrogel polymers is a hinder for transplantation in regenerative medicine. To determine shrinkage in ASCs sheets released from PNIPAAm-co-MAA copolymer hydrogel the diameter of released sheets all culture conditions (VC, SE, VC+SE) were determined and compared to the standard diameter of a well in 6-well plate as control (Fig. 13). The shrinkage in

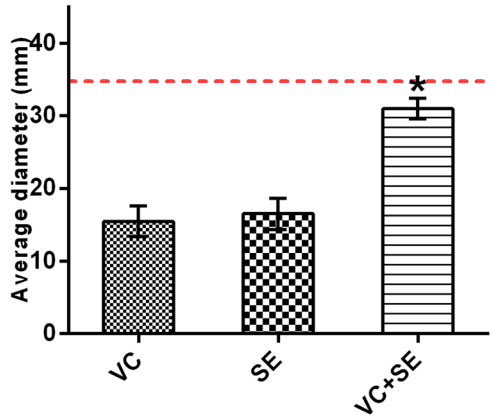


Fig. 13 Shrinkage assay by diameter measurements. Cell sheets were prepared in and released from 6-well plates. Released sheets' diameter was measured and compared to the diameter of one well (dashed red line). The sheets prepared with VC or SE showed shrinkage, while the sheets prepared in the presence of VC+SE represented excellent stability, mechanical properties, and without (or ignorable) shrinkage. *$p < 0.05$

VC and SE supplemented sheets was easily observed, while in VC+SE supplemented sheets the shrinkage is considerably reduced ($p < 0.05$). Moreover, the diameter of VC +SE released sheets was indistinguishable from the diameter of each well in 6-well plate (Fig. 13), indicating that no shrinkage is observable for ASCs sheets engineered in the presence of VC+SE after release from PNIPAAm-co-MAA copolymers.

4 Discussion

Cell sheets engineering has emerged as an innovative approach in regenerative medicine. Consequently, the thermo-responsive copolymers have been employed in order to avoid using enzymatic digestion for cell sheet release from their matrix [23, 39]. As such, engineered tissues can be produced based on cell-cells interactions, while simultaneously stimulating cells for ECM secretion and organization [39]. Because, ECM proteins are regulators of proliferation, stemness and differentiation [40]. Hence, preserving an intact ECM by avoiding neither enzymatic digestion nor employing artificial scaffolds are remarkable points in sheet mediated tissue engineering. In this regard, thermo-responsive polymers have facilitated cell sheet technology by dual hydrophilic/hydrophobic activities through manual thermal changes [15, 41]. The detachment of the cell sheet layer without trypsinization but rather with a simple gel to sol transition method through lowering the temperature below the LCST is a critical advantage of using thermos-responsive polymers. However, the cold conditions for sheet release could influence the authenticity and bioactivity of the engineered sheets. Quick detachment of cell sheets excels as a harvesting method which prevents functional damage to the cells [23]. In the present study, optimization of cell sheet engineering was directed from two aspects including using a new hydrogel copolymer PNIPAAm-co-MAA substrate and utilization of two medium enriching biofactors for treatment of ASCs.

It is of paramount importance that thermo-responsive polymers cause no cytotoxic effects. In this study, poly NIPAAm-based hydrogel and also in copolymer form PNIPAAm-co-MAA were prepared, and their biocompatibility, thermo-responsivity and structural properties of the copolymers were also investigated. Both PNIPAAm and PNIPAAm-co-MAA showed excellent biocompatibility and the ASCs grown in the presence of the hydrogels at 37°C showed nearly absolute viability (96–99%). These findings are in agreement with previous reports where PNIPAAm was characterized as cyto-compatible and has been used for drug delivery, cell sheet technology and also tissue engineering applications [42].

In several studies, PNIPAAm coated plates were used for sheet engineering [15, 23, 41, 43] (reviewed elsewhere [44]). Electron beam irradiation and plasma polymerization are the two main methods that enable covalently grafting of the PNIPAAm polymer to plates. The thickness of PNIPAAm layer is about 20 nm or even up to 1 mm [45]. However, the thermosensitive polymer coated plates are not available routinely as they are not simply produced [20, 46]. Biazar et al., used gamma radiation for Chitosan-NIPAAm polymerization [4], and NIPAAM- hydroxyethylmethacrylate (HEMA) was employed by Villa et al. using free-radical polymerization method for co-polymer, and further employed in muscle cell sheet engineering [6]. In the present study, the direct coating of plates with PNIPAAm-co-MAA hydrogel has been employed in contrast to the expensive and laborious methods previously used for production of thermos-sensitive substrates. This was possible because the hydrogel used herein provided an accessible/easy method for preparing substrate required for sheet engineering. Altomare et al., used Methylcellulose hydrogel for sheet engineering. On Methylcellulose hydrogel, cells could attach and produce sheets [47]. In this research, a PNIPAAm-co-MAA copolymer was synthesized and used for coating of TCPS plates with thickness of 5–10 mm and subsequently were seeded with ASCs and induced for sheet production. Our results showed that sheet engineering by PNIPAAm-co-MAA hydrogel is feasible. Although, according to Haraguchi et al.; employing different thicknesses of hydrogel film for cell sheet formation did not affected the sheet formation [48]. In this report, thickness was observed as important, since the sheet produced on thin gels (5 mm) showed a weak sheet formation that easily ruptured. This result could be associated with less

supportive effect of thin PNIPAAm-co-MAA copolymer for adhesion and proliferation of ASCs.

Consequently, the PNIPAAm-co-MAA copolymer hydrogel was studied for sheet engineering which previously has not been described in this field.

MSCs represent a noteworthy cell source for tissue engineering. In particular, ASCs are ideal for cell sheet engineering due to the in vitro ECM spattering and plasticity for conversion into a number of lineages [3, 15, 36]. In this study, PNIPAAm-co-MAA hydrogel was used to coat TCPS plastic prior to ASCs seeding. Subsequently, the sheet inducing media was administered. Cell sheets can be forced for spontaneous detaching by decreasing the temperature [4]. A challenging point related to existing cell sheet technology is the long time for cell detachment when sheet cooled below LCST. Quick detachment is vital for retaining the biological roles of detached cell sheets for constructing tissue-mimicking structures. When adhered cells are kept at 20 °C or below for a lengthy time, the cooler environment may damage cells and their functions. Several attempts have been carried out to accelerate cell sheet detachment to prevent damage from cooler temperatures. In a study reported by Kwon and coworkers, ~75 min, 35 min and 19 min incubation at 20 °C (below the LCST of polymer around 32 °C) was required to completely detach cell sheets from PNIPAAm-TCPS, PNIPAAm-PM (porous membrane) and PNIPAAm(PEG0.5%)-PM surfaces, respectively in a static condition. The introduction of PEG-grafted chains onto PNIPAAm grafted surfaces could lead to more rapid hydration of the cell culture surfaces due to the formation of water releasing channels with the grafted PEG chains [24, 49]. Wang et al. developed a semi-interpenetrating nanocomposite hydrogel containing polysaccharide alginate and PNIPAAm containing laponite. Rough surface textures of nanocomposite allow faster water penetration, leading to quicker fibroblast cell sheet detachment (15 to 20 min) by lowering the temperature to 10–20 °C [50]. In another study, a new thermoresponsive cell culture surfaces with terminal carboxylation of PNIPAAm chains on glass substrates was designed that cause rapid harvesting of complete smooth muscle cells (SMCs) sheets by low-temperature culturing to 20 °C after 30 min, while the SMCs were unable to be harvested as a monolithic cell sheet from PNIPAAm surfaces on glass substrates without terminal carboxy groups. A number of research groups have reported the effect of terminal groups on protein adsorption and cell adhesion onto self-assembled monolayer surfaces and concluded that carboxyl-terminated self-assembled monolayer surfaces allowed cells to adhere strongly on the surfaces [22].

Takahashi and coworkers fabricated thermoresponsive polymer brush surface of PNIPAAm immobilized glass substrates with varying chain length and PNIPAAm graft density. PNIPAAm brush surfaces were successfully optimized to recover the cell sheets of bovine carotid artery endothelial cells with their intact extracellular matrix (ECM). The results indicated that harvesting time (by lowering the temperature to 20 °C) decreased from 24 h to 30 min with increasing the polymer chain length and graft density [51]. Ebara and co-workers have co-grafted PNIPAAm and hydrophilic poly(2-carboxylisopropyl-acrylamide)) onto tissue culture polystyrene (TCPS) dishes by electron beam irradiation, and created surfaces containing carboxylic acid groups to accelerate water penetration and hydration of the PNIPAAm layer upon cooling. This surfaces facilitates rapid cell recovery (by lowering the temperature below 20 °C, and duration time of 60 min) that local influences of polar group water interaction are likely responsible for this behavior [26]. Tang et al., fabricated comb-type grafted PNIPAAm gel modified TCPS (GG-TCPS) by electron beam irradiation. This improved cell culture surface could accelerate the detachment of bovine aortic endothelial cell sheet effectively (cell detachmented was happened by lowereing the temperature to 20 °C for 25 min) [52]. Madathil and coworkers developed a novel Poly (N-Isopropylacrylamide-co-glycidylmethacrylate) (NGMA) as a thermoresponsive substrate for corneal endothelial cell sheet engineering. Cells sheets with intact ECM were harvested by lowering the temperature to 10 °C for 1 min and then at 20–23 °C for 5 min (below the LCST of NGMA (28 °C)) and by swirling movement or mechanically with the help of a sterile polyethylene terephthalate sheet [25]. In a study, Kim and coworkers grafted poly (2-hydroxyethyl methacrylate) (PHEMA) and poly (N-isopropylacrylamide) (PNIPAAm) successively onto TCPS dishes (PNIPAAm-PHEMA-TCPS dish) by electron beam irradiation. By lowering the temperature to 20 °C, ~75 min was required to completely detach cell sheets from PNIPAAm-TCPS dishes. While only 13 min to detach cell sheets from PNIPAAm-PHEMA-TCPS dishes, PHEMA layer acted as a water pool to accelerate the hydration of PNIPAAm layer due to the effective and simultaneous water supply to PNIPAAm layer, resulting in rapid hydration of grafted PNIPAAm molecules and detachment of cell sheet compare to PNIPAAm-TCPS dishes [11]. Sudo et al., fabricated poly (N-isopropylacrylamide) (PNIPAM)-immobilized Petri dishes functionalized with polar groups like carboxylic acid and dimethylamino groups by drop-casting to tune the cell adhesion and detachment properties. Successful cell sheet harvesting was demonstrated by lowering the temperature to 20 °C for 15 min [53]. In a study published by Nash and co-workers spin-coated PNIPAM into thin films on Thermanox disks, and the resulting films exhibited a rapid (mostly 5 to 10 min, in some cases 60 min, in a 4 °C medium) cell sheet detachment for various cell types [54]. Patel et al., developed a simple spin-coating technique, thermoresponsive

thin films were deposited on glass slides or silicon wafers using PNIPAAm blended with a small amount of 3-aminopropyltriethoxysilane (APTES). Cell sheet detachment happened within 2.5 min after adding cold medium. Under the same conditions, it took nearly 3 h for the cells to completely detach from the commercial thermoresponsive cell culture surface (UpCell®) [18]. In spite of using different high tech methods to produce thermoresponsive surface of TCPs, the detachment time lasted still more than 15 min in most of the studies. Therefore, in the present study we tried to address the aforementioned weak point. In this study we fabricated a thermosensitive culture surface composed of PNIPAAm-co-PMAA by simple and cost effective method and with very rapid ON/OFF adhesion/deadhesion system for cell sheet engineering lead (complete cell sheet release occurred by incubation at 4 °C for just 6 min).

Assembling functional constructs from layering of the single sheets is the ultimate goal of sheet technology, while maintaining the viability and functionality of the sheets are fundamental. Kim et al., grafted poly(2-hydroxyethyl methacrylate) (PHEMA) as well as PNIPAAm using electron beam, which accelerated hydration of those molecules consequently rapid detachment of sheets [11]. Kwon et al., used porous culture membrane grafted PNIPAAm which facilitated the water access to the polymer, and caused quick hydration of polymer [23]. In this research, a new combination of polymer PNIPAAm by adding MAA was utilized, copolymer hydrogel with higher LCST (near to 35 °C), which could maintain the viability of the released sheets via minimal temperature reduction necessary for sheet release. This bioengineering guaranteed the prevention of functional damages due to low temperature induced stress.

Interactions with soluble bio-factors and ECM define a biochemical and biomechanical niche which dynamically regulate the sense of stem cells [28], and consequently enhance their responses.

Additionally, using signaling additives in the medium could improve cell responses in terms of sheet formation. The important criteria for producing functional ASCs sheet are preserving their viability, differentiation potential, and preventing aging processes.

In previous studies, presence of VC as a inducer in media could induce cell sheet construction in different cells such as bone marrow MSC (BM-MSC), periodontal ligament stem cells (PDLSC) and umbilical cord mesenchymal stem cell [30]. Our study, confirmed the perfect inducing function of VC which resulted in overall better performance of sheet in terms of viability, anti-aging, and other functions of ASCs. Moreover, Wei et al., showed that VC can promotes MSC sheet formation by enhancing the activity of telomerase [30]. The present work is unique in the aspect that, in addition to the newly developed thermo-sensitive polymer, both VC and SE, alone or in synergism, were studied as exogenous factors on ASCs sheet engineering. It is noteworthy, that this is the first study of SE-mediated sheet engineering.

The SE of ASCs has different bio-molecules, active signaling cytokines, growth factors, exosomes and microvesicles [19]. The remarkable effects of exosomes secreted from MSCs for cell-free therapies have been reviewed by Marote et al. [55]. Additionally, studies have shown that SE of MSCs has increased regenerative effects. Preceding researches reported that SE of MSCs has therapeutic potential for treatment of heart disease [56] and liver failure [57]. Also, MSCs SE was employed for treatment of Parkinson disease in a rat model [58] with beneficial effects. The paracrine activity of MSCs is a pivotal factor for proper engineering of MSCs subjected to therapeutic purposes [59]. Likewise, it has been reported that SE of ASCs could ameliorate allergic airway inflammation and lung disease [60, 61]. In the present study, the SE of ASCs was employed for sheet engineering. Remarkably, the administration of both VC and SE enhanced the efficiency of sheet engineering observable by better ECM production and multilayered cells with well-defined histological properties.

The current study also showed reduced β-galactosidase associated senescent in ASCs sheet by a combination of SE and VC treatments. The study by Wang and coworkers showed that treatment of MSCs with a SE of Fetal MSCs significantly reduced β-galactosidase (senescence marker) expression as well as enhanced proliferation and additionally osteogenic differentiation in prolonged in vitro culturing [62]. These findinds are in agreement with the data here presented, where the SE of passage 1 ASCs produced similar positive effects on proliferation and osteogenesis potential of ASCs in sheet.

In addition, the SE of the MSCs showed less inflammatory signs with a great osteogenisis for bone regenerative medicine [63] while the present study shows positive osteogenesis enhancing potential of SE of first passage ASCs. Additionally, the study by Yu and coworkers showed enhanced ASC sheet formation, stemness and osteogenic transdifferentiation [64]. Similarly, herein is presented an up-regulation of stemness genes expression upon SE treatments. The effects of combination of VC and SE on differentiation potential of cells in sheet were further investigated, and an enhanced differentiation efficiency of ASCs sheet after the synergistic administration of SE+VC was readily observed which is in line with mentioned studies [63, 64].

The MSCs derived cell sheets prepared in different culture vessels and thermoresponsive polymers could be employed for clinical applications, however, shrinking and reduction of their size are often challenging [65–67]. Moreover, sheets prepared from cell types other than stem cells showed shrinkage problems, as for example in

harvested sheets produced from oral mucosal epithelial cells (primary cells) that showed 50% shrinkage from the original dimension [68]. Such aggregations might alter the size and the quality of the sheets. Sato et al., reported that using a novel medium mixture containing 10% fetal calf serum (FCS) and chondrogenesis differentiation medium (serum free) can prevent the shrinkage in harvested sheets and produce disc-shaped sheet [69]. Accordingly, the shrinkage should be avoided with the intention of achieving reproducible therapeutic effects. Maeda et al., were employed a cell culture insert (CCI) made of flat membrane (porosity: 12%) and defined a number of cells (18.6×10^5 MSCs/well) which enabled them to prevent shrinkage in cartilage cell sheets derived from MSCs [70]. the present study adds the finding that using a medium mixture of VC+SE improved the mechanical properties of released sheets and reduced the probability of shrinkages compared to using each of the supplements alone. Such findings could be applicable in sheet engineering for clinical purposes in regenerative medicine without the shrinkage challenges in the future.

5 Conclusion

The PNIPAAm-co-MAA copolymer was synthesized by the simple free radical polymerization method and employed effectively for rapid releasing of ASCs sheet (averagely 6 min) by a quick gel to sol transition (LCST 35 °C) without using enzymatic method. This intelligent hydrogel showed no cytotoxity, supported cell attachment, proliferation, and differentiation. Therefore, intact/functional cell sheets were harvested. Co treatment with VC and SE in the production of cell sheets from ASCs on PNIPAAm-co-MAA copolymer showed increased synergistic performance as, harvested sheets exhibited normal histological, molecular, osteogenic and functional characteristics. Hence, SE of ASCs could be an excellent factor for sheet bioengineering. The findings of this study could be applicable for engineering of intact sheet-layers with first-rate survival and therapeutic potential.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Alizadeh E, Akbarzadeh A, Eslaminejad MB, Barzegar A, Hashemzadeh S, Nejati-Koshki K, et al. Up regulation of liver-enriched transcription factors HNF4a and HNF6 and liver-specific MicroRNA (miR-122) by inhibition of Let-7b in mesenchymal stem cells. Chem Biol Drug Des. 2015;85:268–79.
2. Alizadeh E, Eslaminejad MB, Akbarzadeh A, Sadeghi Z, Abasi M, Herizchi R, et al. Upregulation of MiR-122 via trichostatin A treatments in hepatocyte-like cells derived from mesenchymal stem cells. Chem Biol Drug Des. 2016;87:296–305.
3. Akahane M, Nakamura A, Ohgushi H, Shigematsu H, Dohi Y, Takakura Y. Osteogenic matrix sheet-cell transplantation using osteoblastic cell sheet resulted in bone formation without scaffold at an ectopic site-. J Tissue Eng Regen Med. 2008;2:196–201.
4. Biazar E, Montazeri N, Pourshamsian K, Asadifard F, Ghorbana-linezhad E, Keshel SH. et al. Harvesting epithelial cell sheet based on thermo-sensitive hydrogel. J Paramed Sci. 2010;1:27–33.
5. Harimoto M, Yamato M, Hirose M, Takahashi C, Isoi Y, Kikuchi A, et al. Novel approach for achieving double-layered cell sheets co-culture: overlaying endothelial cell sheets onto monolayer hepatocytes utilizing temperature-responsive culture dishes. J Biomed Mater Res. 2002;62:464–70.
6. Villa C, Martello F, Erratico S, Tocchio A, Belicchi M, Lenardi C. et al. P (NIPAAM-co-HEMA) thermoresponsive hydrogels: an alternative approach for muscle cell sheet engineering. J Tissue Eng Regen Med. 2014;11:187–196.
7. Aasen T, Raya A, Barrero MJ, Garreta E, Consiglio A, Gonzalez F, et al. Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nat Biotech. 2008;26:1276–84.
8. Amable PR, Teixeira MVT, Carias RBV, Granjeiro JM, Borojevic R. Protein synthesis and secretion in human mesenchymal cells derived from bone marrow, adipose tissue and Wharton's jelly. Stem Cell Res & Ther. 2014;5:53.
9. Hamada M, Iwata T, Kato Y, Washio K, Morikawa S, Sakurai H, et al. Xenogeneic transplantation of human adipose-derived stem cell sheets accelerate angiogenesis and the healing of skin wounds in a Zucker Diabetic Fatty rat model of obese diabetes. Regen Ther. 2017;6:65–73.
10. Kaibuchi N, Iwata T, Yamato M, Okano T, Ando T. Multipotent mesenchymal stromal cell sheet therapy for bisphosphonate-related osteonecrosis of the jaw in a rat model. Acta Biomater. 2016;42:400–10.
11. Kim SJ, Kim WI, Yamato M, Okano T, Kikuchi A, Kwon OH. Successive grafting of PHEMA and PIPAAm onto cell culture surface enables rapid cell sheet recovery. Tissue Eng Regen Med. 2013;10:139–45.
12. Elloumi-Hannachi I, Yamato M, Okano T. Cell sheet engineering: a unique nanotechnology for scaffold-free tissue reconstruction with clinical applications in regenerative medicine. J Intern Med. 2010;267:54–70.
13. Yang M, Kang E, wook Shin J, Hong J. Surface engineering for mechanical enhancement of cell sheet by nano-coatings. Sci Rep. 2017;7:4464.
14. de las Heras Alarcón C, Pennadam S, Alexander C. Stimuli responsive polymers for biomedical applications. Chem Soc Rev. 2005;34:276–85.
15. Yamato M, Okano T. Cell sheet engineering. Mater Today. 2004;7:42–7.
16. Kubota K, Fujishige S, Ando I. Single-chain transition of poly (N-isopropylacrylamide) in water. J Phys Chem. 1990;94:5154–8.
17. Baysal BM, Karasz FE. Coil-globule collapse in flexible macromolecules. Macromol Theory Simul. 2003;12:627–46.
18. Patel NG, Cavicchia JP, Zhang G, Newby B-mZ. Rapid cell sheet detachment using spin-coated pNIPAAm films retained on surfaces by an aminopropyltriethoxysilane network. Acta Biomater. 2012;8:2559–67.
19. Shotorbani BB, Alizadeh E, Salehi R, Barzegar A. Adhesion of mesenchymal stem cells to biomimetic polymers: A review. Mater Sci Eng C. 2016;71:1192–1200.

20. Salehi R, Arsalani N, Davaran S, Entezami A. Synthesis and characterization of thermosensitive and pH-sensitive poly (N-isopropylacrylamide-acrylamide-vinylpyrrolidone) for use in controlled release of naltrexone. J Biomed Mater Res A. 2009;89:919–28.
21. Da Silva RM, Mano JF, Reis RL. Smart thermoresponsive coatings and surfaces for tissue engineering: switching cell-material boundaries. Trends Biotechnol. 2007;25:577–83.
22. Takahashi H, Matsuzaka N, Nakayama M, Kikuchi A, Yamato M, Okano T. Terminally functionalized thermoresponsive polymer brushes for simultaneously promoting cell adhesion and cell sheet harvest. Biomacromolecules. 2011;13:253–60.
23. Kwon OH, Kikuchi A, Yamato M, Sakurai Y, Okano T. Rapid cell sheet detachment from Poly (N-isopropylacrylamide)-grafted porous cell culture membranes. J Biomed Mater Res. 2000;50:82–9.
24. Kwon OH, Kikuchi A, Yamato M, Okano T. Accelerated cell sheet recovery by co-grafting of PEG with PIPAAm onto porous cell culture membranes. Biomaterials. 2003;24:1223–32.
25. Madathil BK, Kumar A, RajanAsari P, Kumary TV. N-isopropylacrylamide-co-glycidylmethacrylate as a thermo-responsive substrate for corneal endothelial cell sheet engineering. Biomed Res Int. 2014;2014:1–7.
26. Ebara M, Yamato M, Hirose M, Aoyagi T, Kikuchi A, Sakai K, et al. Copolymerization of 2-carboxyisopropylacrylamide with N-isopropylacrylamide accelerates cell detachment from grafted surfaces by reducing temperature. Biomacromolecules. 2003;4:344–9.
27. Reed S, Wu B. Sustained growth factor delivery in tissue engineering applications. Ann Biomed Eng. 2014;42:1528–36.
28. Discher DE, Mooney DJ, Zandstra PW. Growth factors, matrices, and forces combine and control stem cells. Science. 2009;324:1673–7.
29. Korkmaz A, Kolankaya D. The protective effects of ascorbic acid against renal ischemia-reperfusion injury in male rats. Ren Fail. 2009;31:36–43.
30. Wei F, Qu C, Song T, Ding G, Fan Z, Liu D, et al. Vitamin C treatment promotes mesenchymal stem cell sheet formation and tissue regeneration by elevating telomerase activity. J Cell Physiol. 2012;227:3216–24.
31. Bruder SP, Jaiswal N, Haynesworth SE. Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. J Cell Biochem. 1997;64:278–94.
32. Kilroy GE, Foster SJ, Wu X, Ruiz J, Sherwood S, Heifetz A, et al. Cytokine profile of human adipose-derived stem cells: Expression of angiogenic, hematopoietic, and pro-inflammatory factors. J Cell Physiol. 2007;212:702–9.
33. Yu B, Zhang X, Li X. Exosomes derived from mesenchymal stem cells. Int J Mol Sci. 2014;15:4142–57.
34. Klingemann H. Methods of use of culture supernatant obtained from mesenchymal stem cells from dogs and cats for treatment of organ dysfunction. Google Patents. 2014.
35. Hu G, Xu J-j, Deng Z-h, Feng J, Jin Y. Supernatant of bone marrow mesenchymal stromal cells induces peripheral blood mononuclear cells possessing mesenchymal features. Int J Biol Sci. 2011;7:364.
36. Alizadeh E, Zarghami N, Eslaminejad MB, Akbarzadeh A, Barzegar A, Mohammadi SA. The effect of dimethyl sulfoxide on hepatic differentiation of mesenchymal stem cells. Artif Cells, Nanomed, Biotechnol. 2016;44:157–64.
37. Sevivas N, Teixeira FG, Portugal R, Araújo L, Carriço LF, Ferreira N, et al. Mesenchymal stem cell secretome: a potential tool for the prevention of muscle degenerative changes associated with chronic rotator cuff tears. Am J Sports Med. 2017;45:179–88.
38. Hoseinzadeh S, Atashi A, Soleimani M, Alizadeh E, Zarghami N. MiR-221-inhibited adipose tissue-derived mesenchymal stem cells bioengineered in a nano-hydroxy apatite scaffold. Vitr Cell & Dev Biol-Anim. 2016;52:479–87.
39. Yang J, Yamato M, Nishida K, Ohki T, Kanzaki M, Sekine H, et al. Cell delivery in regenerative medicine: the cell sheet engineering approach. J Control Release. 2006;116:193–203.
40. Pokrywczynska M, Lewandowska MA, Krzyzanowska S, Jundzill A, Rasmus M, Warda K, et al. Transdifferentiation of bone marrow mesenchymal stem cells into the islet-like cells: the role of extracellular matrix proteins. Arch Immunol Ther Exp (Warsz). 2015;63:377–84.
41. Yamato M, Konno C, Utsumi M, Kikuchi A, Okano T. Thermally responsive polymer-grafted surfaces facilitate patterned cell seeding and co-culture. Biomaterials. 2002;23:561–7.
42. Fujita H, Shimizu K, Nagamori E. Application of a cell sheet–polymer film complex with temperature sensitivity for increased mechanical strength and cell alignment capability. Biotechnol Bioeng. 2009;103:370–7.
43. Canavan HE, Cheng X, Graham DJ, Ratner BD, Castner DG. A plasma-deposited surface for cell sheet engineering: advantages over mechanical dissociation of cells. Plasma Process Polym. 2006;3:516–23.
44. Chang D, Okano T. 5.530 - Medical Applications of Cell Sheet Engineering A2. In: Paul Ducheyne, editor. Comprehensive biomaterials. Oxford: Elsevier; 2011. p. 405–19.
45. Chen G, Qi Y, Niu L, Di T, Zhong J, Fang T, et al. Application of the cell sheet technique in tissue engineering (Review). Biomed Rep. 2015;3:749–57.
46. Biazar E, Khorasani M, Daliri M. Cell sheet engineering: solvent effect on nanometric grafting of poly-N-isopropylacrylamide onto polystyrene substrate under ultraviolet radiation. Int J Nanomed. 2011;6:295.
47. Altomare L, Cochis A, Carletta A, Rimondini L, Farè S. Thermoresponsive methylcellulose hydrogels as temporary substrate for cell sheet biofabrication. J Mater Sci: Mater Med. 2016;27:1–13.
48. Haraguchi K, Takehisa T, Ebato M. Control of cell cultivation and cell sheet detachment on the surface of polymer/clay nanocomposite hydrogels. Biomacromolecules. 2006;7:3267–75.
49. Kwon OH, Kikuchi A, Yamato M, Sakurai Y, Okano T. Rapid cell sheet detachment from poly (N-isopropylacrylamide)-grafted porous cell culture membranes. J Biomed Mater Res: Off J Soc Biomater Jpn Soc Biomater. 2000;50:82–9.
50. Wang T, Liu D, Lian C, Zheng S, Liu X, Wang C, et al. Rapid cell sheet detachment from alginate semi-interpenetrating nanocomposite hydrogels of PNIPAm and hectorite clay. React Funct Polym. 2011;71:447–54.
51. Takahashi H, Nakayama M, Yamato M, Okano T. Controlled chain length and graft density of thermoresponsive polymer brushes for optimizing cell sheet harvest. Biomacromolecules. 2010;11:1991–9.
52. Tang Z, Akiyama Y, Yamato M, Okano T. Comb-type grafted poly (N-isopropylacrylamide) gel modified surfaces for rapid detachment of cell sheet. Biomaterials. 2010;31:7435–43.
53. Sudo Y, Kawai R, Sakai H, Kikuchi R, Nabae Y, Hayakawa T, et al. Star-shaped thermoresponsive polymers with various functional groups for cell sheet engineering. Langmuir. 2018;34:653–62.
54. Nash ME, Carroll WM, Nikoloskya N, Yang R, Connell CO, Gorelov AV, et al. Straightforward, one-step fabrication of ultrathin thermoresponsive films from commercially available pNIPAm for cell culture and recovery. ACS Appl Mater & Interfaces. 2011;3:1980–90.
55. Marote A, Teixeira FG, Mendes-Pinheiro B, Salgado AJ. MSCs-derived exosomes: cell-secreted nanovesicles with regenerative potential. Front Pharmacol. 2016;7:231.

56. Ranganath SH, Levy O, Inamdar MS, Karp JM. Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease. Cell Stem Cell. 2012;10:244–58.
57. Parekkadan B, van Poll D, Suganuma K, Carter EA, Berthiaume F, Tilles AW, et al. Mesenchymal stem cell-derived molecules reverse fulminant hepatic failure. PLoS One. 2007;2:e941.
58. Teixeira FG, Carvalho MM, Panchalingam KM, Rodrigues AJ, Mendes-Pinheiro B, Anjo S, et al. Impact of the secretome of human mesenchymal stem cells on brain structure and animal behavior in a rat model of Parkinson's disease. Stem Cells Transl Med. 2017;6:634–46.
59. Kusuma GD, Carthew J, Lim R, Frith JE. Effect of the microenvironment on mesenchymal stem cell paracrine signaling: opportunities to engineer the therapeutic effect. Stem Cells Dev. 2017;26:617–31.
60. Yu HS, Park M-K, Kang SA, Cho K-S, Mun SJ, Roh H-J. Culture supernatant of adipose stem cells can ameliorate allergic airway inflammation via recruitment of CD4+CD25+Foxp3 T cells. Stem Cell Res & Ther. 2017;8:8.
61. Monsel A, Zhu YG, Gudapati V, Lim H, Lee JW. Mesenchymal stem cell derived secretome and extracellular vesicles for acute lung injury and other inflammatory lung diseases. Expert Opin Biol Ther. 2016;16:859–71.
62. Wang B, Lee WY, Huang B, Zhang JF, Wu T, Jiang X, et al. Secretome of human fetal mesenchymal stem cell ameliorates replicative senescen. Stem Cells Dev. 2016;25:1755–66.
63. Katagiri W, Osugi M, Kawai T, Hibi H. First-in-human study and clinical case reports of the alveolar bone regeneration with the secretome from human mesenchymal stem cells. Head Face Med. 2016;12:5.
64. Yu J, Tu Y-K, Tang Y-B, Cheng N-C. Stemness and transdifferentiation of adipose-derived stem cells using l-ascorbic acid 2-phosphate-induced cell sheet formation. Biomaterials. 2014;35:3516–26.
65. Tew SR, Murdoch AD, Rauchenberg RP, Hardingham TE. Cellular methods in cartilage research: primary human chondrocytes in culture and chondrogenesis in human bone marrow stem cells. Methods. 2008;45:2–9.
66. Wongin S, Waikakul S, Chotiyarnwong P, Siriwatwechakul W, Viravaidya-Pasuwat K. Effect of cell sheet manipulation techniques on the expression of collagen type II and stress fiber formation in human chondrocyte sheets. Tissue Eng Part A. 2018;24:469–78.
67. Tang Z, Akiyama Y, Okano T. Temperature-responsive polymer modified surface for cell sheet engineering. Polymers. 2012;4:1478.
68. Kondo M, Murakami D, Yamato M, Takagi R, Namiki H, Okano T. Serum-dependent epithelial cell sheet shrinkage upon detachment from temperature-responsive culture surfaces. FASEB J. 2009;23:468.5–.5.
69. Sato Y, Wakitani S, Takagi M. Xeno-free and shrinkage-free preparation of scaffold-free cartilage-like disc-shaped cell sheet using human bone marrow mesenchymal stem cells. J Biosci Bioeng. 2013;116:734–9.
70. Maeda S, Fujitomo T, Okabe T, Wakitani S, Takagi M. Shrinkage-free preparation of scaffold-free cartilage-like disk-shaped cell sheet using human bone marrow mesenchymal stem cells. J Biosci Bioeng. 2011;111:489–92.