Preparation of TGF-beta1/affinity-bound alginate macroporous scaffolds

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Abstract

Spatio-temporal presentation of growth factors is one of the key attributes of the cell's microenvironment. The design of macroporous alginate scaffolds, wherein TGF-β1 or BMP-4 is electrostatically bound to affinity binding sites of alginate sulfate, mimicking their presentation by the extracellular matrix (ECM), was previously shown to enable sustained presentation and release of each factor, thus increasing their biological activity. Specifically, TGF-β1/affinity-bound scaffolds induced the chondrogenic differentiation of human mesenchymal stem cells (hMSCs) seeded within these scaffolds. The prolonged activity of the affinity-bound TGF-β1 enabled efficient induction of signaling pathways leading to chondrogenesis, up to the appearance of committed chondrocytes. Similarly, BMP-4 affinity-bound to the macroporous alginate scaffold enabled efficient induction of osteogenic differentiation in hMSC constructs. Subsequent construction of a multicompartment inductive system, spatially-presenting TGF-β1 and BMP-4 in two distinct layers, enabled complete differentiation of hMSC to chondrocytes and osteoblasts, depending on the type of factor in use in the respective layer.

This paper describes in detail the preparation method of the TGF-β1 or BMP4/ affinity-bound alginate scaffolds, and the set of analyses performed to characterize the resultant scaffolds, including release profile study, released factor bioactivity, and functionality of the scaffolds as hMSC-inductive scaffolds.

Keywords: *alginate; alginate-sulfate; macroporous scaffold; TGF-β1; affinity binding; protocol*.

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1. INTRODUCTION

A synthetic cell microenvironment design should consider the key external factors affecting cellular fate. For example, growth factors are most frequently required for inducing cell signaling pathways, leading to specific cellular processes. Usually, growth factors are supplemented to the external medium of cultivation systems as soluble molecules, not simulating the natural mode of action by which growth factors are presented to cells. Such inappropriate factor presentation to the cells, as well as the diffusion limitations existing in 3D cultivation systems, may explain the limited effect of these supplemented factors on cell differentiation *in vitro* [1].

In an ideal cell microenvironment, specific growth factor administration and its unique presentation and release to the cultured cells should be carefully assessed. There are several strategies which focus on ways to spatially and temporally control the availability of bioactive growth factors. These strategies include incorporation of factor-loaded microparticles into the scaffolds [2,3], and covalent binding of the factors to the matrix [1,4,5]. Both these strategies, however, do not mimic the natural presentation of the factors by extracellular matrix (ECM), and their application raises issues such as the possible effects of the microsphere presence [6,7], and the effect of covalent binding on factor conformation and activity [8,9].

Naturally in tissues, the soluble growth factors are typically bound to the ECM via electrostatic interactions with heparin/heparan sulfate glycosaminoglycans (GAGs) present in the matrix [10,11]. In the native cellular microenvironment, heparin and heparan sulfate bind many growth factors, chemokines and cell adhesion molecules, collectively known as heparin-binding peptides, via high affinity and specific electrostatic interactions [10,11]. Such interactions are mediated by low- and high-sulfated sequences in these GAG chains [12,13]. These interactions play a critical role in assembling protein-protein complexes, such as growth factor-receptor or enzyme-inhibitor, on the cell surface and in the ECM, that are directly involved in initiating cell signalling events or inhibiting biochemical path-

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ways [14,15]. Numerous works have explored the addition of heparin into the matrix to capture the factors [16-20]. However, heparin is a multi-faceted molecule with several biological functions other than binding growth factors, such as anticoagulation, and its delivery may induce distinguishable blood-thinning effects [16] and other biological responses [21].

To overcome these limitations and to mimic the natural growth factor spatio-temporal presentation, a novel bio-inspired macroporous alginate scaffold was designed, wherein the growth factor is electrostatically bound to affinity binding sites of alginate-sulfate.

Alginate-sulfate, produced by sulfation of the uronic acid monomers in the algae-derived polysaccharide alginate [22], was designed to mimic the natural interactions of heparin-binding proteins with heparin/heparan sulfate GAGs. Sulfated alginate was shown to bind heparin binding proteins with equilibrium binding constants comparable to those obtained between the proteins and heparin [22,23]. Such interactions with alginate-sulfate were found to enhance protein stability against enzymatic proteolysis induced by trypsin [24,25].

Scaffolds composed of combinations of alginate-sulfate with unmodified pristine alginate have been shown to provide a unique type of an affinity-binding system, which has been shown to maintain prolonged release of multiple proteins, while retaining the properties and characteristics of alginate as a supportive cell vehicle [23,25,26].

The fabrication of macroporous alginate scaffolds, wherein the factor is affinity-bound to alginate sulfate, created a unique inductive matrix easy for cell seeding and cultivation, which triggers specific and desired signaling pathways, followed by proper cellular processes. Specifically, the affinity binding of chondro-inductive factor TGF-β1, and the osteo-inductive factor, BMP-4, to macroporous alginate/alginate-sulfate scaffolds was shown to enable their sustained and local presentation, thus increasing their activity as chondrogenic and osteogenic inducers respectively [27,28]. The prolonged activity of the affinity-bound TGF-β1 enabled the efficient induction of signalling pathways in human mesenchymal stem cells (hMSCs), leading to chondrogenesis, up to the appearance of committed chondrocytes of the hyaline cartilage type. Similarly, BMP-4 as affinity-bound to the macroporous alginate scaffold enabled efficient induction of hMSC osteogenic differentiation.

The presentation of the inducible factors as affinity-bound to the matrix, mimicking their presentation by the ECM, enables the construction of complex hierarchical structures, spatially-presenting different factors in separate and distinct compartments. For example, for osteochondral defect repair, where simultaneous regeneration of cartilage and bone are required, a bi-layered system was constructed, spatially-presenting the chondro-inductive TGF-β1 and the osteo-inductive BMP-4 in two distinct layers. The prolonged presentation and activity of the factors induced the complete differentiation of hMSCs to chondrocytes and osteoblasts, depending on the type of inducible factor in use in the specific layer [28].

Herein, we provide a detailed protocol for the synthesis of TGF-β1 or BMP-4/ affinity bound alginate scaffolds, the set of analyses performed to characterize the resultant scaffolds, including release profile studies, released factor bioactivity, and functionality of the scaffolds as an inductive platform for guided cell differentiation.

2. MATERIALS

Specifications of all chemicals used are quoted in the procedure as a reference to the reader. All chemicals, unless specified otherwise, were from Sigma Aldrich (Israel) and were of analytical grade.

Specific equipment

For scaffold fabrication: homogenizer, lyophilizer, orbital shaker, sterile low protein binding (LB) Eppendorfs of two different volumes (0.5, 1.5 ml) (Eppendorf, Germany, 0030 108.094 0030 108.116)

For protein release study: plate reader for Enzyme-Linked Immuno-Sorbent Assay (ELISA) detection

For histology/immunohistochemistry: paraffin-embedding instrument and microtome

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a resistivity of 18.2 MΩ cm at 25 °C), and analytical-grade reagents. Prepare and store all reagents at room temperature (RT), unless indicated otherwise.

2.1. Materials and solutions for the preparation of TGF-β1/affinity bound scaffolds

1. **Proteins:** Recombinant human TGF-β1, (CHO cell-derived, Peprotech, Rocky Hill, NJ), recombinant human BMP-4, HeLa cell-derived, Peprotech), or other heparin-binding protein of interest.
2. **Alginate solution**: Dissolve sodium alginate (Pronova™ UP LVG, viscosity of ~54 [mPa·s], M.W. ~ 100 kDa, >65 % guluronic acid monomer content, Drammen, Norway) in water to a final concentration of 1.2 % (w/v). The suspension is dissolved by stirring for 2 h until a clear solution is achieved. Filter (0.22 μm) the solution into a sterile 50 mL Falcon tube and store at 4 °C for up to 1 month.

Note: Store alginate solutions only in plastic containers. Glass containers contain calcium ions that may leach out and gradually cross-link the alginate.

3. **Cross-linker solution for alginate gelation**: Dissolve D-Gluconic acid/ hemi-calcium salt (Sigma Aldrich, Cat. No. G4625) in water to a final concentration of 1.44 % (w/v). The suspension is dissolved by stirring for 1–2 h until a clear solution is achieved. Filter (0.22 μm) the solution into a sterile Falcon tube and store at 4 °C for up to 1 month.
4. **Alginate-sulfate** solution: The sulfation of sodium alginate (Pronova™ VLVG, viscosity of ~10 [mPa·s], M.W. ~30-50 kDa, >65 % guluronic acid monomer content, NovaMatrix FMC Biopolymers, Drammen, Norway) is consisted of two main steps; first, conversion of the sodium salt of alginate to a tertiary amine salt and second, O-sulfation with carbodiimide and sulfuric acid [22]. The detailed protocol for alginate sulfation is published in [29].

Note: Surface Plasmon Resonance (SPR) analysis may be carried out to evaluate binding of various heparin-binding proteins to alginate-sulfate, and compared to non-modified alginate or other materials [22,23]. Alginate-sulfate may be characterized by FTIR to reveal the changes in spectrum profile relative to unmodified sodium alginate. The IR spectrum of alginate-sulfate shows a new major peak at ~1260 cm^{-1} and a minor peak at ~800 cm^{-1}. The peak at ~1260 cm^{-1} is assigned to S=O symmetric stretching, and the one at ~800 cm^{-1} to S-O-C stretching [22]. Prepare a fresh stock solution by dissolving dry alginate-sulfate in sterile water to a final concentration of 2.5 % (w/v).

5. **Bovine Serum Albumin (BSA) solution for well-plate pretreatment**: Dissolve BSA (Sigma Aldrich) in Phosphate Buffered Saline (PBS, Biological Industries, Israel) to a final concentration of 1 % (w/v). The solution is intended to prevent non-specific adsorption of the protein to the plastic surfaces.
6. **BSA-pretreated plates and Eppendorfs:** Incubate 96-well plates with BSA solution for 2 h at 4 °C. Discard and wash with PBS. Dry in a biological cabinet. Sterilize by UV for 20 min.
7. **Protein solution**: Centrifuge protein vial before opening.

Reconstitute the protein according to manufacturer's instructions, without addition of carrier protein (*e.g.*, BSA). Distribute to aliquots and immediately store at -80 °C.

2.2. Materials and solutions for *in vitro* release study from TGF-β1/affinity-bound scaffolds

1. **Enzyme-Linked Immuno-sorbent Assay (ELISA)** kit for released factor quantification: human recombinant TGF-β1 or BMP-4 ELISA kit from R&D Systems (Minneapolis, MN), or equivalent.
2. Low protein binding (LB) Eppendorfs.
3. **Solution for scaffold dissolution**: 6 % (w/v) sodium citrate (trisodium dihydrate, Sigma Aldrich) in PBS.
4. **BSA-pretreated plates**: Incubate 48-well plates with BSA solution for 2 h at 4 °C. Discard and wash with PBS. Dry in a biological cabinet. Sterilize by UV for 20 min.
5. 1 % PS/DMEM: High-glucose Dulbecco's modified Eagle's medium (DMEM, Biological Industries), supplemented with 1 % (v/v) penicillin–streptomycin (PS, Biological Industries).

2.3. Materials for the released TGF-β1 bioactivity assay

1. 1 % PS/DMEM: High-glucose DMEM, supplemented with 1 % (v/v) penicillin–streptomycin (PS).
2. Low protein binding (LB) Eppendorfs.

2.4 Materials for cell culture and analyses of cell constructs

1. **Basal medium for hMSC chondrogenic induction** in TGF-β1/affinity bound scaffolds: high-glucose DMEM, supplemented with 50 μg/mL ascorbate-2-phosphate (Sigma Aldrich), 100 μg/mL sodium pyruvate (Sigma Aldrich), 40 μg/mL proline (Biological Industries), 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.1 mg/mL Neomycin , 1 % (Pen-Strep Neomycin, Biological Industries), ITS + Premix [6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 μg/mL, 6.25 ng/mL selenious acid, 1.25 mg/mL BSA, 5.35 mg/mL linoleic acid, Biological Industries] and 100 nM dexamethasone (Sigma Aldrich).

Note: The chemically defined medium for cell culture depends on cell type and on the desired cellular process to be induced. For example, for osteogenic induction of hMSCs in BMP-4 affinity-bound scaffolds, 5 mM of β-glycerophosphate (Sigma Aldrich) may be added to the basal medium. The medium should be serum-deprived, if the effect of the affinity bound protein is to be investigated.

2. For immunofluorescence: DMEM-based buffer ($CaCl_2 \cdot 2H_2O$ dihydrate, 1.8 mM; KCl, 5.36 mM; $MgSO_4 \cdot 5H_2O$, 0.81 mM; NaCl, 0.1 M; $NaHCO_3$, 0.44 mM; NaH_2PO_4, 0.9 mM; pH 7.4). All salts are from Sigma Aldrich.

Note: Requirement for DMEM-based buffer - alginate scaffolds are sensitive to most commonly used buffers since these may disturb the calcium cross-linking, leading to scaffold dissociation. DMEM-based buffer is appropriate for all staining procedures.

3. Blocking buffer: DMEM-based buffer, containing 5 % (v/v) fetal bovine serum (FBS) or 1 % BSA (w/v).

4. Methanol solution, cold, stored at -20°C.
5. For histology: ethanol solutions, 70 %, 90 %, 100 % (v/v), xylene, and paraffin-embedding instrument.

3. EXPERIMENTAL PROCEDURES

3.1. Procedure for fabrication of TGF-β1/affinity-bound alginate scaffolds

The procedure below is described for preparation of ~100 scaffolds. The general scheme of preparation is depicted in Figure 1.

1. Place desired amount of alginate solution 1.25% (w/v) in a sterile glass vial
2. Add D-gluconic acid/hemi-calcium salt (cross-linker) into alginate solution by slow drop-by-drop addition of Ca^{2+}-gluconate to alginate with extensive homogenization (26,000 rpm, 5 min), and homogenize for another 3 minutes after last Ca^{2+}-gluconate addition.
   Amount of cross-linker should be calculated to reach 0.2 % (w/v) and 1.08 % (v/v) cross-linker and alginate concentrations, respectively. Recommended amounts, for preparation in one 20 mL vial: combine 8 mL of alginate solution 1.25 % (v/v) with 1.25 mL of cross-linker 1.44 % (v/v).
   Shake gently on orbital shaker for 15 min, at RT (to remove air bubbles).
3. Bioconjugate (growth factor and alginate-sulfate) preparation: Mix protein with alginate-sulfate solution (2.5 %, w/v) in LB Eppendorf by pipetting. Incubate for 1.5 h, at 37 °C. **For protein-free control**: use sterile water instead.
4. Add bioconjugate solution to cross-linked alginate solution and mix by pipetting for 1 min. The final mixture is consisted of 0.1 % alginate-sulfate (w/v), 1 % alginate (w/v) and 0.18 % (w/v) D-gluconic acid/hemicalcium salt. Control scaffolds (without protein): final component concentrations in cross-linked solution are 1.0 % and 0.2 % (w/v) for the polymer and cross-linker, respectively.
   Quantities, initial and final concentrations of compounds in the affinity-bound scaffolds are summarized in Table 1.
5. Pour 100 µL per well of the final solution into BSA-pretreated 96-well plates (Corning, tissue culture plates).
6. Shake gently on an orbital shaker for 5 min, at RT (to remove air bubbles).
7. Cool the plates at 4 °C, for 1 h.
8. Freeze the plates at -20 °C overnight.
9. Lyophilize (−56 °C, 0.05–0.08 mbar) the scaffolds for at least 48 h. After freeze-drying, use scaffolds immediately, or store frozen in a desiccator.

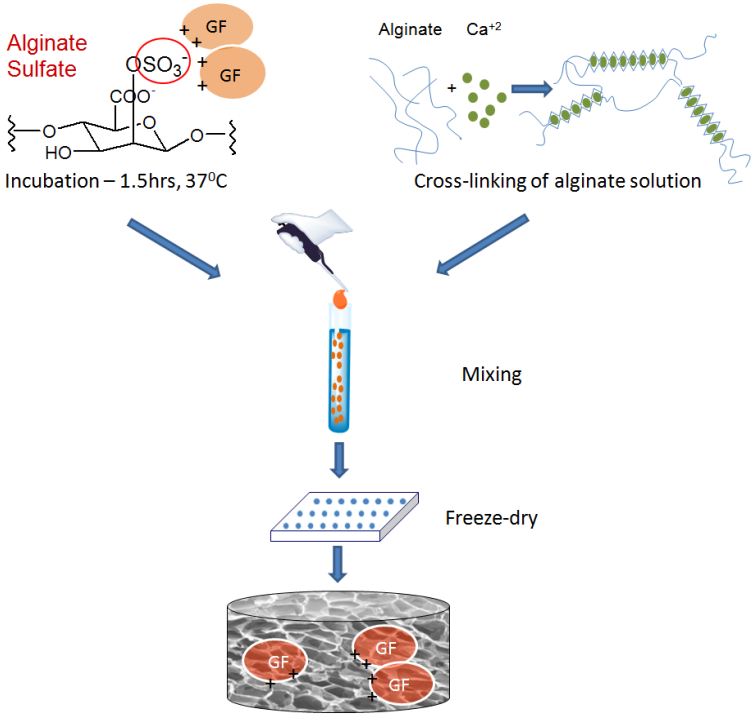

Figure 1. A scheme describing the fabrication of alginate scaffolds with affinity-bound growth factor. Adopted with permission from [27].

Table 1. Quantities, initial and final concentrations of compounds in the affinity-bound scaffolds.

	Initial concentration, w/v	Volume, µl	Final concentration, w/v
LVG-Alginate	1.25 %	80.00	1 %
Calcium	1.44 %	12.50	0.18 %
Alginate-Sulfate	2.50 %	4	0.10 %
Protein	50 ng / µl	4	200 ng / scaffold
Total:		100.50	

3.2. Procedure for *in vitro* release study from TGF-β1/affinity-bound scaffolds

1. Sterilize dry scaffolds under UV for 30 min
2. **Optional step** – determination of initial protein amount in the scaffolds (scaffolds at t=0) – dissociate dry scaffolds with 500 µl 6 % sodium citrate in PBS in LB Eppenforfs.
3. Transfer the scaffolds to the 48-well BSA-pretreated release plates.
4. Wet the scaffolds by addition of 50 µl of 1 % PS/DMEM (release medium). After initial wetting is complete, add 450 µl medium to each well.
5. Protein release study:
   - 5.1 Incubate the release plate at 37 °C on orbital shaker, under gentle shaking.
   - 5.2 Scaffolds at *t* = final – collect the medium (last time point), dissociate the scaffolds with 6 % sodium citrate in PBS in LB Eppenforfs, for determining the residual entrapped protein amount. Immediately snap-freeze separately the collected medium and the dissociated scaffold solution in liquid nitrogen and store at -80°C.
   - 5.3 Collect and replace daily total medium for 7 days (suggested time points: 2 h, 5 h, 1 day, 3 days and 7 days later) in LB Eppendorfs. Immediately snap-freeze the collected release medium, and store fractions at -80 °C for ELISA.
   - 5.4 Thaw samples and determine protein amount in samples by ELISA, according to the manufacturer's instructions. Run protein-free (control) scaffold samples in parallel, to determine and normalize possible background readings of empty scaffolds.
   - 5.5 Calculate the percentage of cumulative released growth factor (GF) as: (sum of released GF/ total amount of loaded GF) ×100 %; the total amount of loaded GF is the sum of released protein fractions + the residual non-released GF extracted from the scaffold at *t* = final.
   - 5.6 Typical results are depicted in Figure 2.

3.3. Procedure for the released TGF-β1 bioactivity assay

Note: Collagen quantification by Sircol colorimetric assay was used to assess released TGF-β1 bioactivity, and was also shown to be applicable for the released BMP-4. For other growth factors of choice, other bioassays may be adopted.

1. Seed primary cardiac fibroblasts into 24-well plate at ~1.5 x 10^5 cells / well, in 1 % PS /DMEM.

Note: The assay was originally performed with mouse and rat primary cardio-fibroblasts, but other fibroblasts may be investigated for the assay.

2. Thaw release medium collected from the 5 h and /or 24h time-points of the release study, with at least 20 ng / mL of protein (confirmed by ELISA).
3. Dilute to final concentration of 10 ng / mL with fresh 1 % PS/ DMEM.

The control (protein-free) experimental group should be taken from release medium of empty scaffolds diluted in the same manner. An additional control of fresh 1 % PS/ DMEM could be used.

4. One hour after cell seeding, replace the medium by diluted release medium.
5. Cultivate for an additional 3 days.
6. Perform collagen quantification using Sircol colorimetric assay (R&D Systems), as previously described [30]. Anticipated results: biologically active TGF-β1 is known to enhance collagen production in primary cardiac fibroblasts [31], see Figure 3.

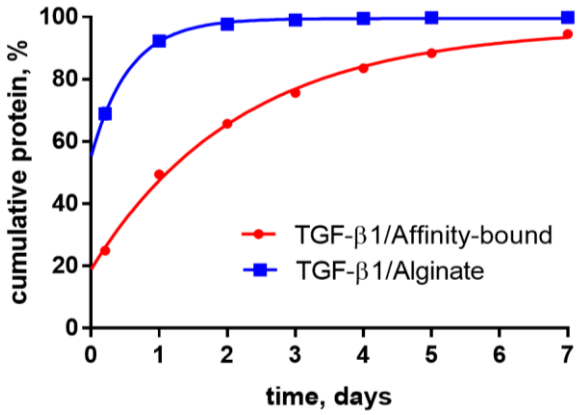

Figure 2: In vitro release study of TGF-β1 from alginate scaffolds fabricated with or without alginate-sulfate, analyzed by ELISA, pointing out a sustained release profile from the affinity-binding scaffolds. The data represent the mean ± SEM of four scaffolds per time point. Goodness of fit (R^2) for nonlinear regression of first-order kinetics: alginate-sulfate/alginate – 0.9919, alginate – 0.9628. P (interaction, 2-way repeated measures ANOVA) <0.0001; significant differences in all time points. Reprinted with permission from [27].

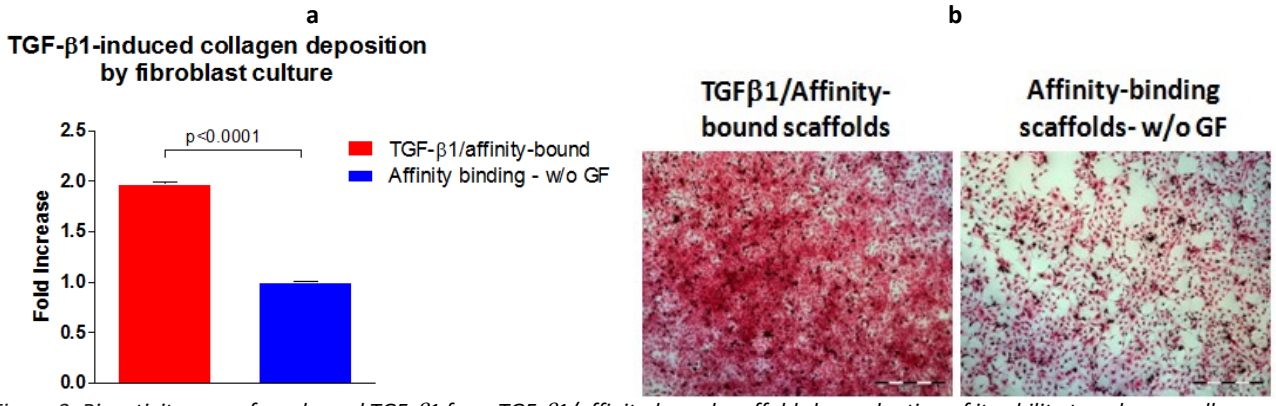

Figure 3: Bioactivity assay for released TGF-β1 from TGF-β1/affinity-bound scaffolds by evaluation of its ability to enhance collagen deposition in rat monolayer cultures of primary cardiac fibroblasts. The degree of collagen deposition was determined after 3 days, using the Sircol colorimetric assay. ***a.*** *Quantification of collagen deposition in a fibroblast culture. Values are normalized to the degree of collagen deposition in fibroblasts cultured with release medium from the empty scaffolds * - p <0.0001 (two-tailed unpaired t test).* ***b.*** *Representative micrographs of collagen deposition, stained by Sircol assay, in rat cardiac fibroblasts cultured with released TGF-β1 from TGF-β1/affinity-bound scaffold (left) or with release medium from empty scaffolds (right) (bar: 500 μm). Reprinted with permission from [27].*

3.4. Cell seeding and culture in affinity-binding scaffolds:

1. Seed cells (for example, human mesenchymal stem cells (hMSCs)), at an initial cell density of 2-5×10^5 cells/scaffold by dropping 15 μL of the cell suspension in culture medium onto the scaffold placed in a 96-well plate.
   Note: The readers are encouraged to test the system with additional cell types.
2. Centrifuge the well plate with cell constructs for 2 min at 100 × g to achieve homogenous cell distribution within the scaffold.
3. Add 50 μL/well of basal medium, and incubate for 30 min (37 °C, 5 % CO_2).
4. Gently transfer the cell constructs with small forceps to 12-well plates supplemented with 2 mL of culture medium.

3.5. Cell construct analysis:

1. Transfer the scaffolds to a clean Eppendorf tube, and dissolve by adding 200 μL (per scaffold) of 6 % (w/v) sodium citrate in PBS. Centrifuge at 2,400 *g* for 10 min at RT. Discard the liquid, and use the cell pellet for Western immunoblotting or other subsequent analyses of choice, or immediately store at -80°C for future analyses (See Figure 4).
   Note: Optimization of scaffold number per sample (*e.g.*, pooling) could be required to get optimal results. In general, use at least 1–2 × 10^6 cells per sample for Western immunoblotting.
2. The cell constructs in the scaffolds could be subjected to DNA content analysis. In case of chondrogenic induction, cell pellets should be dissolved in 0.5 mL papain solution (5 mM EDTA, L-cysteine, 155 mM NaCl, pH 6.8) and incubated at 65 °C overnight. Store the supernatant at -80°C until analysis for DNA content using bisbenzimidazole Hoechst 33258 dye (Sigma Aldrich).
3. Alkaline phosphatase (ALP) activity could be measured using the ALP colorimetric assay kit, according to the manufacturer's instructions (MD Biosciences, MN or AnaSpec, Fremont, CA). ALP activity should be normalized to DNA content (See Figure 5).
4. The cell constructs in the scaffolds could be subjected to qPCR, and other downstream analyses.
5. Cell viability and/or metabolic activity could be measured using various metabolic assays (*e.g.*, Fluorescein Diacetate (FDA), see in [32], XTT, PrestoBlue™ and many others) by direct incubation of the scaffolds in working reagent solution and following the manufacturer's instructions for measurement.

3.6. Histology and Imaging

1. Soak scaffolds in alcohol solutions: 70 %, 30 min; 70 % 30 min; 90 % 30 min, for gradual dehydration.
2. Transfer to Xylene, 1 h RT, twice.
3. Continue as for other histological samples: transfer to warm paraffin for 2 h embedding and block preparation.
4. Cut into 5-μm-thick sections using Microtome and mount on slides.
5. Slides may be stained with hematoxylin & eosin (H&E) to visualize nuclei and cytoplasm, respectively, or Masson's trichrome staining for nuclei, cytoplasm, and collagen (See Figure 6). To detect phosphate salt deposits, typical during osteogenic differentiation (mineralization), use von Kossa reagents (silver nitrate and sodium thiosulfate, Sigma Aldrich or commercial kits) [33] and Nuclear Fast Red solution for counter-staining (see Figure 7).

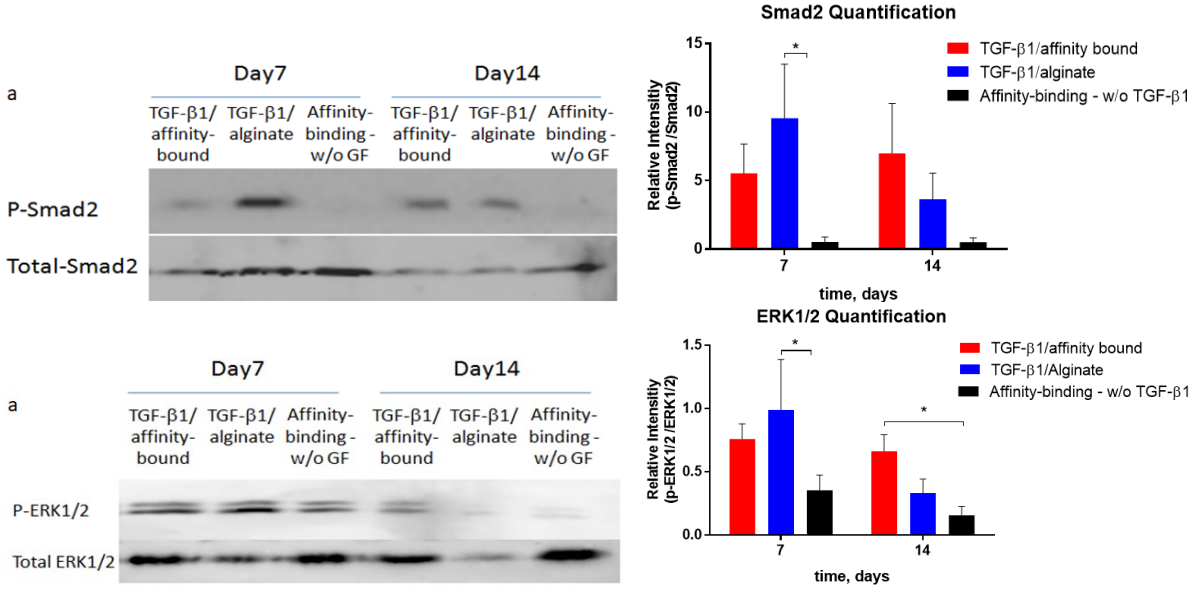

Figure 4: *Western Blot (WB) analysis for hMSC constructs, cultivated in TGF-β1/affinity-bound alginate scaffolds, indicating prolonged TGF-β1-induced activation of signaling pathways. Results represent a pool of cells extracted from 5-7 constructs per time point.* ***A.*** *Smad2 Western blots for TGF-β1/Smad signaling pathway. Antibodies: primary rabbit antibody against human phosphorylated Smad2 and total Smad2 (Cell Signaling, Danvers, MA) and anti-rabbit peroxidase (HRP) – conjugated secondary antibody (Pierce Biotechnology, Rockford, IL).*
a. *Representative Smad2 Western blots.* ***b.*** *Densitometric analysis of Smad2 Western blots. Levels of Smad2 phosphorylation were quantified by the band intensity of phospho-Smad2 relative to total Smad2. The relative intensity was normalized to the expression level in control of affinity-binding scaffolds w/o TGF-β1, on day 2. P (1-way ANOVA) day 7 and 14=0.0199, 0.133, respectively. * - p<0.05 (Bonferroni's post-test).* ***B.*** *ERK1/2 Western blots for TGFß1/MAPK signaling pathway.* ***a.*** *Representative ERK1/2 Western blots.* ***b.*** *Densitometric analysis of ERK1/2 Western blots. Levels of phosphorylation were quantified by the band intensity of phospho-ERK1/2 relative to total ERK1/2. The relative intensity was normalized to the expression level in control of affinity-binding scaffolds w/o TGF-β1, on day 2. P (1-way ANOVA) day 7 and 14=0.0004, 0.0164, respectively. * - p <0.05 (Dunnett's post-test), when compared to BMP-4/affinity-bound scaffolds. Antibodies: Mouse anti-phospho-Thr202/Tyr204 ERK1/2 antibody and rabbit Anti-total ERK1/2 antibody (Cell signaling), anti-mouse peroxidase (HRP) conjugate secondary antibody (Sigma Aldrich), and anti-rabbit peroxidase (HRP) – conjugated secondary antibody (Pierce Biotechnology). Reprinted with permission from [27].*

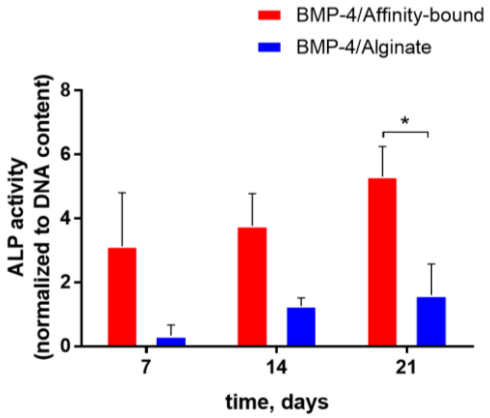

Figure 5. Alkaline phosphatase (ALP) activity levels in hMSC constructs, dissociated from BMP-4/affinity-bound alginate scaffolds. ALP activity levels in hMSC constructs, cultivated in BMP-4/affinity-bound scaffolds, were significantly higher compared to those in BMP-4/alginate scaffolds, after 3 weeks (- p (Bonferroni's post-test, 2-way ANOVA) <0.05). Reprinted with permission from [28].*

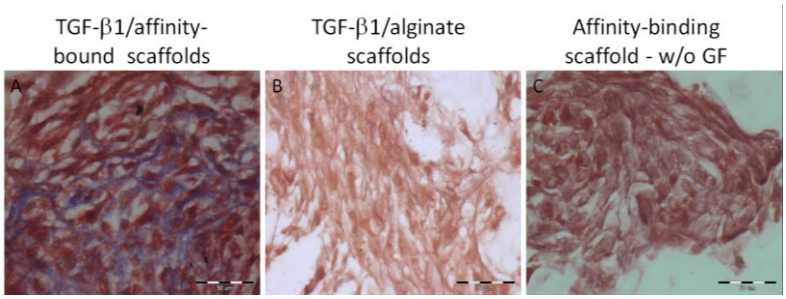

Figure 6. Masson's trichrome staining kit (Bio-optica, Milan, Italy) for collagen staining (blue) of hMSCs constructs within different scaffold types, after 14 days in culture, demonstrating induced cartilage ECM deposition in hMSC cell constructs in TGF-ß1/affinity bound scaffolds (bar: 50 μm). Reprinted with permission from [27].

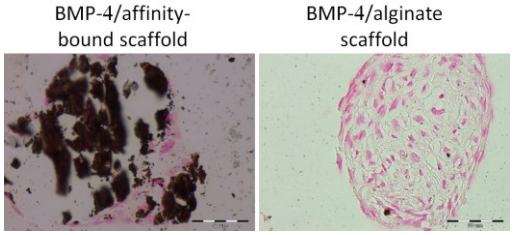

Figure 7. Von Kossa staining for mineralized bone matrix in thin sections (5 μm) in 3 week-old hMSC constructs within BMP-4/affinity bound scaffolds, demonstrating induced hMSC osteogenic differentiation (bar: 50 μm). Reprinted with permission from [28].

3.7. Immunofluorescent imaging of cell constructs:

Whole cell constructs within alginate scaffolds may be visualized by Laser Scanning Confocal Microscopy, for detection of cell morphology, cellular organization and specific markers.

Note: This general protocol is suited for most primary antibodies. Refer to specific manufacturer's instructions for each antibody, and modify the protocol as necessary.

1. Fixation and permeabilization of cell constructs:
   Transfer scaffolds to cold methanol solution for cell fixation and permeabilization.
2. Blocking: Transfer the scaffolds to DMEM-based buffer, containing 5 % (v/v) FBS for 1 h at RT, and wash with DMEM-based buffer once for 1 min.
3. Add primary antibody in blocking solution, and incubate overnight at 4 °C.
4. Wash with DMEM-based buffer three times, 15 min each wash.
5. Add secondary antibody in blocking solution, and incubate for 4 h at RT. Protect from light from now on.
6. Wash with DMEM-based buffer three times, 15 min each wash.
7. Add nuclear stain of choice (e.g. propidium iodide (PI, Molecular Probes, Invitrogen, Karlsruhe, Germany), DAPI, *etc.*); incubate for 5 min at RT.
8. Wash with DMEM-based buffer three times, 15 min each wash.
9. Discard the liquid, but maintain the cell construct wet.
10. Visualize the cell constructs using laser scanning confocal imaging system mounted on an inverted microscope.

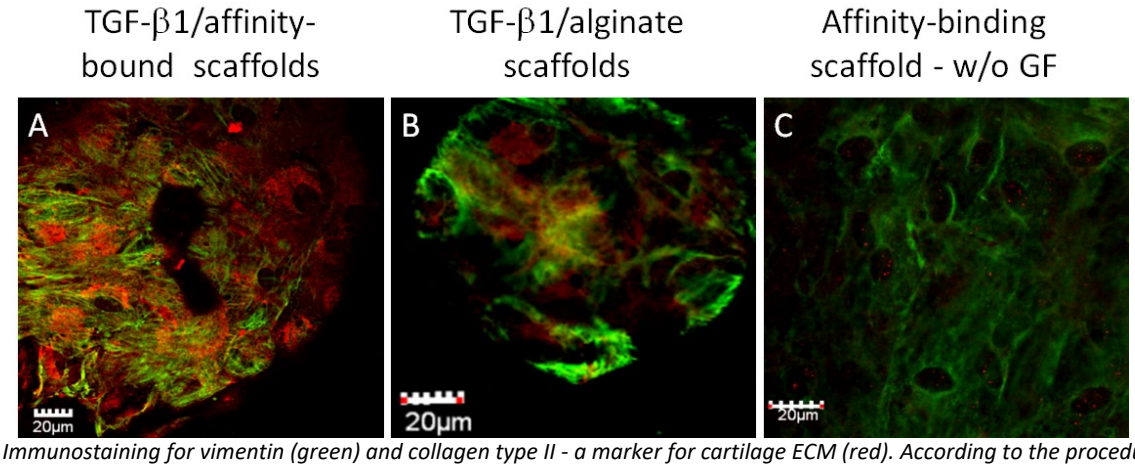

Figure 8: *Immunostaining for vimentin (green) and collagen type II - a marker for cartilage ECM (red). According to the procedure above, the scaffolds were incubated overnight with anti-collagen type II (Novus Biologicals, Littleton, CO) and anti-vimentin (Zymed, San Francisco, CA) antibodies, followed by incubation with Cy3-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Baltimore Pike, PA) and goat anti-mouse Alexa 488-conjugated antibody (Molecular Probes), respectively (bar: 20 μm) Reprinted with permission from [27].*

4. SUMMARY

The protocol above refers to TGF-β1 and BMP-4, which are well-established examples. However, depending on the cellular system, the protein of interest could be any heparin-binding protein. The affinity-binding scaffold may be a good platform for investigating other cell types and other cellular processes. Moreover, subsequent complex systems may be constructed, spatially-presenting various heparin-binding proteins to achieve hierarchical tissue structures.

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SAŽETAK

Procedura za dobijanje alginatnih makroporoznih nosača sa afinitetno vezanim faktorom rasta TGF-beta1

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Prostorno-vremenska raspodela faktora rasta je jedna od ključnih karakteristika ćelijskog mikro-okruženja. U ranijim istraživanjima su dobijeni makroporozni alginatni nosači sa elektrostatički vezanim faktorima rasta TGF-β1 ili BMP-4 za afinitetna vezivna mesta alginat-sulfata, imitirajući na taj način, njihovu raspodelu u ekstracelularnom matriksu. Pokazano je da su ovi nosači omogućili neprekidno prisustvo i otpuštanje svakog od faktora rasta povećavajući tako i njihovu biološku aktivnost. Posebno, nosači sa vezanim faktorom rasta TGF-β1 su podstakli hondrogenu diferencijaciju humanih mezenhimskih matičnih ćelija (engl. human mesenchymal stem cells, hMSC) gajenih unutar nosača. Produženo dejstvo vezanog faktora rasta je omogućilo efikasnu aktivaciju signalnih puteva koji dovode do hondrogeneze, sve do pojave zrelih diferentovanih hondrocita. Na sličan način su makroporozni alginatni nosači sa vezanim faktorom rasta BMP-4 omogućili diferentovanje istih matičnih ćelija hMSC u pravcu osteogeneze. Najzad, u višefaznom sistemu koji je sadržao TGF-β1 i BMP-4 u dva odvojena sloja omogućeno je potpuno diferentovanje ispitivanih matičnih ćelija hMSC u hondrocite odnosno osteoblaste u zavisnosti of prisustva faktora rasta u datom sloju. U ovom radu, detaljno je opisan metod pripreme alginatnih nosača sa vezanim faktorima rasta TGF-β1 odnosno BMP-4, kao i serija analiza za karakterizaciju dobijenih nosača uključujući ispitivanja kinetike otpuštanja, bioaktivnosti otpuštenih faktora rasta i funkcionalnosti nosača za podsticanje diferentovanja humanih mezenhimskih matičnih ćelija.

Ključne reči: alginat, alginat-sulfat, makroporozni nosač, TGF-β1, afinitetno vezivanje, procedura.