Published in final edited form as:

Biomaterials. 2012 June ; 33(16): 4100–4109. doi:10.1016/j.biomaterials.2012.02.037.

The promotion of *in vitro* vessel-like organization of endothelial cells in magnetically responsive alginate scaffolds

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Abstract

One of the major challenges in engineering thick, complex tissues such as cardiac muscle, is the need to pre-vascularize the engineered tissue *in vitro* to enable its efficient integration with host tissue upon implantation. Herein, we explored new magnetic alginate composite scaffolds to provide means of physical stimulation to cells. Magnetite-impregnated alginate scaffolds seeded with aortic endothelial cells stimulated during the first 7 days out of a total 14 day experimental course showed significantly elevated metabolic activity during the stimulation period. Expression of proliferating cell nuclear antigen (PCNA) indicated that magnetically stimulated cells had a lower proliferation index as compared to the non-stimulated cells. This suggests that the elevated metabolic activity could instead be related to cell migration and re-organization. Immunostaining and confocal microscopy analyses supported this observation showing that on day 14 in magnetically stimulated scaffolds without supplementation of any growth factors, cellular vessel-like (loop) structures, known as indicators of vasculogenesis and angiogenesis were formed as compared to cell sheets or aggregates observed in the non-stimulated (control) scaffolds. This work is the first step in our understanding of how to accurately control cellular organization to form tissue engineered constructs, which together with additional molecular signals could lead to a creation of an efficient pre-vascularized tissue construct with potential applicability for transplantation.

Keywords

Alginate scaffold; Magnetic nanoparticles; Magneto-mechanical cell stimulation; Endothelial cell organization; Vessel-like structures

1. Introduction

Tissue engineering and regenerative medicine can offer solutions to a number of compelling clinical problems that have not been adequately addressed through the use of permanent replacement devices. While encouraging results have been achieved with thin-walled tissues (e.g., engineered urinary bladder [1]), tissue engineering of dense tissues such as the heart, that depend on vascular supply, remains a challenge. One of the major obstacles in engineering thick, complex tissues such as muscle is the need to vascularize the tissue *in vitro*. Vascularization *in vitro* is important for maintaining cell viability during tissue growth, inducing structural organization and promoting vascularization upon implantation [2].

It has already been shown that apart from molecular signals (e.g. growth factors), additional physical cues, such as electrical signaling, mechanical stimulation, and medium perfusion are required in tissue engineering [3–6]. Bioreactors developed to apply mechanical forces *via* piston/compression systems, substrate bending, hydrodynamic compression and fluid shear (relevant for vasculature homeostasis) [7–10], require direct contact with the cell culture and have limited degrees of freedom in which these forces can be applied. In addition, it would be desirable to be able to apply the same physical cues *in vivo* as *in vitro*.

Hydrogel nanocomposites impregnated with magnetic particles are attractive materials for achieving remote actuation with adjustable direction and possibility of *in vivo* applicability. The magnetic field can be coupled to the particle to actuate a process within a target cell regardless of whether there are intervening structures, such as tissue. Stress parameters can also be varied dynamically simply by varying the properties (e.g. strength, frequency) of the applied field. Polymer and hydrogel nanocomposites laden with magnetic particles have already been demonstrated as potential candidates for pulsatile drug delivery and soft actuator applications [11–15].

The feasibility of mechanical stimulation induced by magnetic forces has been shown by binding of magnetic particles to membranes of mesenchymal stem cells (MSCs) to stimulate calcium stores, change membrane potential, upregulate genes related to bone and cartilage formation and promote bone matrix mineralization *in vitro* [16–21]. However, to the best of our knowledge this approach has not been fully realized in 3D cell cultures within polymeric scaffolds.

In the present paper, we explore whether impregnation of alginate scaffolds with magnetically responsive nanoparticles (MNP) and further exposure to an alternating magnetic field would exert a direct effect on endothelial cell activity. We hypothesized that exposure of a magnetic scaffold to an alternating magnetic field would enable the mechanical stimulation of endothelial cells and influence their organization into early capillary-like structures *in vitro*. This work is the first step forward in our understanding of how to accurately control cellular organization into tissue engineered constructs, which together with additional molecular signals could lead to a creation of an efficient pre-vascularized tissue construct ready for transplantation.

2. Materials and methods

2.1. Materials

Sodium alginate (LVG, 100 kDa, >65% guluronic acid) was obtained from NovaMatrix FMC Biopolymers (Drammen, Norway). Ferric chloride hexahydrate, ferrous chloride tetrahydrate, D-gluconic acid, hemicalcium salt and sodium hydroxide were obtained from Sigma–Aldrich (St. Louis, MO). Phosphate buffered solution (PBS) and Dulbecco's

modified eagle's medium (DMEM) supplemented with L-Glutamine, 4.5 g/L Glucose and Sodium Pyruvate were obtained from MediaTech (Manassas, VA), BenchMark™ heat inactivated fetal bovine serum (FBS) was obtained from Gemini Bio-Products (West Sacramento, CA). Double Deionized Water (DDW) used in all experimental procedures was obtained using a Milli-Q water purification system. Alginate was modified with functional peptides RGD and heparin binding peptide (HBP) as previously described [22].

2.2. Magnetite preparation

Magnetite was obtained from ferric chloride hexahydrate and ferrous chloride tetrahydrate (at a molar ratio of 2:1, weighing 2.08 g and 0.768 g respectively dissolved in 24.8 mL of DDW) by alkaline precipitation with aqueous sodium hydroxide (a 15.2 mL of 2 N sodium hydroxide). Magnetite was magnetically separated and washed twice with 20 mL of water [23,24]. Magnetite (total theoretical weight of 890 mg) was dispersed in 11 mL of 1.2% (w/v) alginate solution by heating in a water bath (90 °C) for 20 min with periodic mixing. Then the suspension was cooled down to room temperature and sonicated at maximum power for 5 min (550 Sonic Dis-membrator, Fisher Scientific, Waltham, MA) keeping the tube chilled on ice-water bath. The heating and sonication cycles were repeated twice resulting in a stable ferro-fluid with a final magnetite concentration of 8% (w/v). Particle size measurements were determined by Photon Correlation Spectroscopy (PCS) using a Delsa™ Nano C particle size analyzer equipped with two laser diodes (658 nm, 30 mW, Beckman-Coulter, CA).

2.3. Fabrication of macroporous magnetic alginate scaffolds

Macroporous scaffolds (5 mm diameter; 2 mm thickness) were fabricated by a freeze-dry technique [25]. In brief, the alginate was dissolved in DDW to obtain a 1.2% (w/v) solution, and then cross-linked by adding D-gluconic acid, hemicalcium salt (1.08% (w/v)), while stirring the mixture for achieving uniform cross-linking of the alginate. Magnetically responsive alginate scaffolds were fabricated using the same method from a mixture of alginate stabilized ferro-fluid and alginate to obtain theoretical 1.2% (w/v) final concentration of magnetite post-crosslinking. The cross-linked alginate solution was poured into 96-well plates (100 µL/well), chilled to 2–8 °C overnight, frozen at –20 °C for 24 h, and lyophilized. Scaffolds were sterilized by exposure to UV light for 20 min.

2.4. Morphology characterization

Scaffold internal morphology was characterized by scanning electron microscopy (SEM; model Supra 50VP, Carl Zeiss, Germany). The dry scaffolds were attached to sample stubs with conductive paint and sputter-coated with an ultrathin (100 Å) layer of carbon using a Polaron E 5100 coating apparatus. The samples were viewed under SEM at an accelerating voltage of 12 kV.

2.5. Magnetic characterization and iron content

The magnetic properties of the scaffolds were obtained from hysteresis curves measured by an alternating gradient magnetometer (Princeton Instruments Corporation, Princeton, NJ). The iron content of scaffolds was determined spectro-photometrically using a Synergy™ 4 multimode plate reader (BioTek, Winooski, VT). The scaffolds were dissolved in 1 mL sodium citrate (4% (w/v) in PBS) at 37 °C. Then the magnetite was isolated by centrifugation at 15,000 rpm for 30 min at room temperature (RT) dissolved in 1 N hydrochloric acid and iron content was determined at 335 nm against standard dilutions of iron salts.

2.6. Rheological and mechanical strength characterization

The dynamic viscoelastic behavior of wetted scaffolds (diameter 10 mm, thickness 2 mm) was determined using a stress-control Bohlin CS-10 rheometer (Malvern Instruments, Westborough, MA), operated in the plate–plate mode with 8 mm diameter. The scaffolds were wetted with culture medium for 2 days in a humidified atmosphere of 5% CO₂ and 95% air, at 37 °C. The elastic (G') and loss modulus (G'') measurements were conducted at a frequency sweep range of 0.1–2 Hz, at 37 °C. Shear elastic/storage modulus (G'), as a measure of scaffold stiffness, was also compared in all test groups at 0.5 Hz.

2.7. Cell culture

Bovine aortic endothelial cells, passage 7 (a gift from Dr. Robert Levy at Children's Hospital of Philadelphia, PA) were cultured on cell culture flasks (275-cm²) in 10 mL high glucose DMEM, supplemented with 1% Pen-Strep, 2% L-glutamine and 10% fetal bovine serum (FBS) for 7 days, then removed from the flasks by 0.25% trypsin/EDTA and counted using the trypan blue exclusion assay.

The cells were seeded at an initial cell density of $2.23 \times 10^7/\text{cm}^3$, by adding 15 μL of the suspended cells onto the scaffolds, placed in 96-well plates, followed by 2-min centrifugation (800 rpm, 4 °C) and a short (15 min) incubation (37 °C, 5% CO₂) in 200 μL of culture medium. The cell constructs were then transferred to 48-well plates supplemented with 1 mL of culture medium and incubated under standard conditions.

2.8. Magnetic stimulation setup

Two 48-well plates containing 24 scaffolds each were placed in a 45 mm gap between two Helmholtz coils (HHS 5201-98, Schwarzbeck Mess-Elektronik, Schöna, Germany). An AC magnetic field of 10–15 Gauss was produced by passing an electrical current through serially connected coils at frequency of 40 Hz. An electrical current of 1.37 A was generated by an Associated Power Technologies 5000 series power supply (Diamond Bar, CA) by applying 1.3 V. Magnetic-field strength was calibrated by a hand-held Lake Shore 410 magnetometer (Lake Shore Cryotronics, Inc., Westerville, OH).

2.9. Cell viability and DNA content

Cell viability was evaluated by AlamarBlue™ assay (AbD Serotek, Kidlington, UK) [26]. The constructs ($n = 3$ –5) were transferred into new wells and incubated with 1 mL of 10% AlamarBlue™ working solution in DMEM medium with no additives for 2 h. Aliquots of AlamarBlue containing medium (300 μL) were placed in a 96-well plate and the fluorescence was measured (excitation 540 nm, emission 590 nm) using a plate reader Synergy™ 4 (BioTek, Winooski, VT). Medium-wetted cell-free scaffolds were subjected to the same processes for use as blank.

DNA content was analyzed using Quant-iT™ PicoGreen® dsDNA reagent (Life Technologies™, Carlsbad, CA) [27]. The constructs ($n = 2$ –3) were dissolved in 200 μL sodium citrate (4% (w/v) in PBS) to free the cells, followed by centrifugation (6000 rpm, 10 min, at RT). The cell pellet was suspended in 100 μL lysis buffer (SDS 0.02% (v/v) in sodium citrate (0.015 M)-saline, pH 7) and incubated for 1 h at 37 °C. Then, 100 μL of Quant-iT™ PicoGreen® dsDNA assay solution (1:200) was added, followed by 10-min incubation at RT. Aliquots (200 μL) were placed in a 96-well plate and the fluorescence was measured (excitation 480 nm, emission 520 nm) using a plate reader. Medium-wetted cell-free scaffolds were subjected to the same processes for use as blank.

2.10. Immunostaining and confocal imaging

Cell constructs were fixed in 4% (v/v) warm methanol-free formaldehyde in DMEM (Thermo-Fisher Scientific Inc.) for 7 min, washed twice in DMEM buffer ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1.8 mM), KCl (5.36 mM), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.81 mM), NaCl (0.1 M), NaHCO_3 (0.44 mM), NaH_2PO_4 (0.9 mM), at pH 7.4, and permeabilized using Triton-X 100 (0.2% (v/v) in DMEM) for 10 min at RT. The constructs were washed 3 times, blocked for 1 h at room temperature in DMEM containing 5% BSA (Millipore, Bedford, MA) and then washed. The samples were incubated for 4 h and labeled with Alexa-Fluor 546-conjugated phalloidin (1:300), which stains F-actin for cell visualization (Life Technologies, Carlsbad, CA). For nuclei detection, the cells were incubated for 5 min with DAPI reagent (1:30000; Life Technologies). Imaging of the cell constructs was performed with an Olympus Fluoview FV1000 (Melville, NY) laser scanning confocal microscope.

2.11. Protein analysis by Western blotting

Cell constructs were dissolved in citrate buffer (6% (w/v), pH 7.4) to free the cells. The mixture was centrifuged, and the cell pellets suspended in RIPA lysis buffer (Thermo-Fisher Scientific Inc.) supplemented with complete protease inhibitor (Roche, Indianapolis MN), then centrifuged. The total extracted protein was measured using BCA reagent assay (Thermo-Fisher Scientific Inc.). Equal amounts of the samples were size-fractionated by 10% (w/v) sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) then electro-transferred for 1 h at 100 V to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA) and blocked in 5% BSA solutions. Membranes were probed using anti-PCNA (1:800; Sigma-Aldrich, St. Louis, MO) and anti- GAPDH (1:1000, Cell Signaling Technology, Danvers, MA). Fluorescent (680 nm and 800 nm) secondary antibodies (at a dilution of 1:5000) were used against the rabbit and/or mouse primary antibodies, respectively, on an Odyssey near-infrared imager model 9201-01 (LI-COR, Lincoln, NE). Densitometry analysis was carried out using ImageJ software.

2.12. Statistics

Statistical analyses were performed with GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA). All variables are expressed as mean $\pm$ SEM from at least three independent experiments. A linear 2-way ANOVA model was used for comparisons of multiple groups. The model included the effects of treatment, time, and treatment-by time interaction. The Bonferroni's correction was used to assess the significance of predefined comparisons at specific time points. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Characterization of the scaffolds

Magnetite stabilized with 1.2% (w/v) alginate resulted in the appearance of aggregates displaying normal distribution with a mean size of 776 ± 416 nm and a polydispersity index of 0.225. The actual magnetic nanoparticle (MNP) loading within one scaffold (5 mm diameter and 2 mm thickness) was determined to be 1.36 ± 0.02 mg/scaffold. This mass of the MNPs resulted in a (w/w) MNP to alginate ratio of ~ 1.2 and a total of $\sim 1.2\%$ (w/v) MNP concentration in the fully hydrated scaffold. We also tested the behavior of scaffolds loaded with 5-fold lower and 2-fold greater concentrations of MNPs. The MNP concentration selected in this study was found optimal based on the mechanical properties of the scaffolds and the cellular responses to stimulation.

3.1.1. Morphology and wetting properties—On the microscopic level (by SEM, Fig. 1A and B), pore size and the gross porous morphology of scaffolds was not affected by the MNP loading. Pore sizes did not differ significantly between the MNP-alginate and the non-magnetic alginate, and were 115 ± 26.5 and 99.9 ± 27.2 μm , respectively. This is in accordance with the fact that pore size in the macroporous alginate scaffold is generally controlled by the freezing regime during scaffold preparation [28]. MNPs appeared to be embedded within the scaffold walls and were shown to be homogeneously distributed throughout the matrix. The non-magnetic alginate scaffold displayed a smooth polymer surface in contrast to the rough coarse-grained like surface in the case of the MNP-scaffolds (Fig. 1A vs. B). Clumps or aggregates of MNPs not associated with scaffold's walls were not observed. These observations indicate that the alginate-stabilized MNPs interact well with the alginate matrix forming a homogeneous composite material.

Morphologically on a macroscopic level, the MNP-alginate scaffolds (*i.e.*, MNP-scaffold) are comparable to the non-magnetic alginate scaffolds (*i.e.*, alginate scaffolds) in terms of wetting rates and preservation of the geometrical shape upon complete hydration (24 h) with the cell culture medium (Fig. 1C).

3.1.2. Magnetization—The magnetic properties of scaffolds were obtained from the hysteresis curves measured by an alternating gradient force magnetometer (AGFM, Fig. 2A). MNP-scaffolds exhibited a near superparamagnetic behavior showing a slight hysteresis (Fig. 2A, inset). The magnetic moment dependence on the magnetic field was nearly linear up to 1 kOe, reaching 65–70% of the saturation magnetization value. A comparatively low increment in magnetization was observed upon further increasing the field to 5 kOe. The average normalized values of MNP-scaffold magnetizations calculated at 15 Oe (experimental) and 5 kOe (saturation magnetization) were found to be 1.25 and 24.0 emu/g composite or 2.13 and 40.8 emu/g magnetite, respectively. A slight hysteresis, which is an indicative of slow magnetic relaxation process, resulted in a remnant magnetization (remanence in magnetic literature) magnetization of 0.40 emu/g of composite or 0.68 emu/g of magnetite, measured at 0 Oe over time scale smaller than about 1 s.

3.1.3. Rheology—The mechanical spectra of wetted MNP- or alginate-scaffolds, revealed similar loss and storage moduli profiles over the tested frequency range, 0.1–2 Hz, indicating that impregnation with magnetic particles does not affect hydrogel-like behavior of the resulting scaffold (Fig. 2B). However, quantitative comparison of the elastic/storage modulus (G') at 0.5 Hz, indicative of the scaffold stiffness and elasticity (elastic resistance), revealed differences in scaffold properties. MNP-scaffolds were found to be more elastic than the alginate scaffolds, with G' values of 5530 ± 204 and 3380 ± 134 Pa, respectively.

3.2. Effects of magnetic stimulation on scaffold-seeded ECs

The effect of an externally applied alternating magnetic field at a frequency of 40 Hz and strength of 10–15 Oe was investigated over the 14-day cultivation period. After seeding into MNP-scaffold or alginate scaffold, the endothelial cells (ECs) were allowed to properly adhere and recover for 24 h. The cell constructs were then exposed to an alternating magnetic field (magnetically stimulated) for 7 days, while the control cell constructs were cultivated in a similar incubator without exposure to a magnetic field. After 7 days of stimulation, the magnetic field was turned off and both “stimulated” and “non-stimulated” groups were cultivated for an additional 7 days at zero field conditions. The experimental design is summarized in Fig. 3A.

3.2.1. Effects of magnetic stimulation on EC viability and proliferation—Over a two-week period, we investigated whether magnetic stimulation affects endothelial cell

metabolic activity and cell count within magnetically responsive (MNP) and non-magnetic (alginate) scaffolds. Results are presented as metabolic activity per cell (i.e., normalized to DNA content in the construct relative to day 0). Fig. 3B reveals that cell metabolic activity is significantly elevated in magnetically stimulated MNP-impregnated scaffolds compared to the non-stimulated constructs on both day 3 and day 7 of cultivation. In the non-magnetic alginate scaffold, however, significantly enhanced metabolic activity was noted in the stimulated group only on day 7 of stimulation, albeit not to the same extent found in the MNP-scaffolds. Interestingly, after peaking on day 7 of stimulation, cell metabolic activity in all test groups decreased to its initial value by day 14. These results suggest that magnetic stimulation alone without MNP impregnation into the scaffold has a direct effect on cell activity, while MNP integration into the walls of the constructs (in close proximity to stimulated cells) significantly further enhances this effect.

To further substantiate whether the differences in cell activity are a direct result of cell proliferation, the expression of proliferative cell nuclear antigen (PCNA) was quantified during cultivation. PCNA expression was decreased in magnetically stimulated constructs compared to non-stimulated constructs in both MNP- and alginate-scaffolds during cultivation (Fig. 4A and B). Although statistical differences were not seen at all time-points, the trend remained consistent over the entire cultivation period. These results indicate that the increase in cell metabolic activity upon magnetic stimulation is probably not due to cell proliferation.

3.2.2. Effects of magnetic stimulation on EC organization within the scaffold—

Changes in endothelial cell morphology in the MNP- and alginate-scaffolds were followed by immuno-fluorescent staining against F-actin and DAPI. By day 7, endothelial cells cultivated within MNP- and alginate-scaffolds presented well organized, interconnected, mesh-like structures. Zooming into the created structures enabled us to distinguish different cell organization patterns between the two scaffold types, exposed and non-exposed to a magnetic field (Fig. 5). Non-stimulated MNP-scaffolds exhibited thinner structures, as compared to magnetically stimulated MNP-constructs, where several vessel-like structures (loops) could be detected at this time point. All other experimental groups displayed “tissue-like structures” which were much thicker and interconnected.

The overall cell organization in culture changed morphologically by day 14 (Fig. 5). Magnetically stimulated cells were mostly found in separated loop-like structures, instead of massive interconnected structures. Non-stimulated cells displayed mostly interconnected structures with nearly no loop-like structures. The greatest extent of well-organized loop-like structures was found in MNP-scaffolds exposed to a magnetic field, as confirmed by loop quantification assay (Fig. 6). In the MNP-scaffolds, loop-like organization was present also in the control (non-stimulated) group, though at a much lower extent and mostly as part of a bigger cell sheet or a large cell aggregate. In non-magnetic alginate scaffolds, the cells were still mostly organized as interconnected tissue-like structures. Loop-like structures were found to a much lower extent in the magnetically stimulated non-magnetic alginate group, while in the non-stimulated group such organization was barely detectable.

4. Discussion

The present study provides evidence that impregnation of alginate scaffolds with magnetically responsive nanoparticles (MNP) and subsequent exposure to an alternating magnetic field creates an appropriate microenvironment for endothelial cell activity and growth, thus leading to their pronounced organization into capillary (loop)-like structures *in vitro*.

To obtain magnetically responsive alginate scaffolds, magnetic nanoparticles should be blended with alginate and further cross-linked and dried. However, magnetite crystals obtained by an alkaline precipitation (the Massart method [29]), ranging between 5 and 20 nm in diameter, tend to aggregate due to strong surface forces and high surface area. Precipitated magnetite particles can be stabilized (peptized) in water or non-polar liquids by the addition of surfactants with a proper hydrophobic/hydrophilic balance [30] or by using alginate [31]. Since our scaffold is comprised of alginate, it seemed very reasonable to stabilize the magnetite with alginate to allow good blending between the main matrix component and the magnetic particles, and to keep scaffold composition simple. Because alginate is a high molecular weight macromolecule with a uniform distribution of charged carboxylic groups, fine stabilization (due to charge or steric repulsions) of individual magnetite crystals is most likely impeded. For this reason, larger magnetite clumps of a submicron size of 776 ± 416 nm and high polydispersity index of 0.225 were obtained after stabilization with alginate. Yet, visually the alginate-stabilized ferro-fluid appeared to be homogeneous and was easily miscible with alginate, resulting in uniform distribution of MNP aggregates in alginate solution and in the fabricated scaffold. Scanning Electron Microscopy revealed homogeneous distribution of MNP aggregates of 300–900 nm throughout the matrix and the two types of scaffolds (*i.e.*, with and without inclusion of magnetically responsive nanoparticles) resembled each other both on macro- and microscopic levels, namely shape, wetting properties and pore size. However, closer examination of the scaffold's physical properties revealed differences between magnetic and non-magnetic scaffolds in magnetization, roughness of the pore surface wall and elasticity.

Magnetite as a bulk material is ferrimagnetic (*i.e.*, it has a remanent magnetic moment observable over long time scale such as months in the absence of magnetic field), however individual magnetite crystals smaller than 25–30 nm in diameter are super-paramagnetic (*i.e.*, magnetize in a magnetic field and lose their average magnetization within a short time after the field is removed [32]). Our magnetic scaffolds behaved almost as a super-paramagnetic material showing only slight hysteresis (inset graph, Fig. 2A) and remanent magnetization of 0.40 emu/g of composite on the time scale of measurement (less than a second) comparing to the diamagnetic (*i.e.* magnetize in the direction opposing the external field, namely non-magnetic) properties of plain alginate scaffolds. The low remanent magnetization observed in the magnetic scaffolds for this short period of time is unlikely to influence cell organization.

In addition to the scaffold magnetic properties, MNP aggregate inclusion changed the surface roughness of the scaffold pore walls and scaffold stiffness. The MNP-scaffolds were found to be significantly more elastic compared to the non-loaded ones. The elastic response of the alginate scaffold is greatly influenced by the extent of calcium crosslinking, which restricts chain movement and reduces elasticity. Steric hindrance created by incorporation of MNP between polysaccharide chains may be a possible reason for the increased elasticity of the magnetic scaffolds. Magnetite crystal aggregates are significantly larger than single alginate molecules and interfere with the “egg-box” [33] formation created between the polymer chains. The resulting material is more branched and more elastic compared to the control, non-loaded scaffold.

Investigation of cellular behavior in the studied scaffolds indicated that impregnation of alginate scaffolds with MNPs does not lead to toxicity effects, meaning that the magnetic scaffolds are compatible with cells and suitable for cell cultivation. On the contrary, cells cultured in both magnetic and non-magnetic scaffolds and exposed to an AC external magnetic field showed significantly higher metabolic activity compared to cells not exposed to a magnetic field during the first week of cultivation. The effect of increased cell metabolic activity was more pronounced in the MNP-scaffold exposed to a magnetic field.

This significant difference in the cell metabolic activity was seen already on day 3 of cultivation in magnetic scaffolds; while in the non-magnetic alginate scaffold increased cell metabolic activity was observed only on day 7. These results show that the magnetic field itself has a positive impact on cell metabolic activity; however this effect is clearly enhanced by the presence of MNPs in the nanocomposite scaffold. Elevated cell metabolic activity usually indicates possible activation of various biological processes such as proliferation, organization or differentiation. Proliferation cell nuclear antigen (PCNA) expression levels (indicative of cell proliferation) exhibited a similar trend in all groups. PCNA expression increased on day 7 of cultivation and decreased on day 14 in all cultivation groups, indicating that the proliferation was transient and probably associated with intensive tissue regeneration. However, PCNA expression levels were consistently lower in stimulated cells cultured in both magnetic and non-magnetic scaffolds compared to those in the control matrices (not exposed to a magnetic field). It is thus clear that the increased metabolic activity in magnetically stimulated constructs was not an indication of enhanced cell proliferation, but rather of induction of other cellular processes such as cell organization.

Immunostaining and further confocal examination of the constructs showed that by day 7, endothelial cells seeded in the MNP-scaffolds and exposed to an externally applied magnetic field had organized into vessel-like (loop) structures. No such arrangement was found in any other constructs at this time point; instead cells were found to display interconnected mesh-like structures. By day 14 the overall cell organization had morphologically changed. Vessel-like structures were found in all constructs, however the highest extent of well-organized loops was found in the MNP-scaffolds exposed to a magnetic field. These results were further confirmed by loop quantification (Fig. 6).

Morphology, adhesion properties and overall behavior of the endothelial cells may be influenced by any number of scaffold properties. While the impact of surface roughness and low temporary scaffold magnetization on cells is mainly speculative and according to our knowledge has not extensively been investigated and described in literature, the effect of substrate elasticity on cell behavior has been well-studied for various cell types, including endothelial cells [22,34–38]. However all reported studies differ from the 3D cultivation system described here in the type of scaffold, investigated cell types, cultivation system, and magnitude of the examined moduli, making a direct comparison difficult. Our results show no statistical difference in cellular behavior in both scaffold types when not exposed to a magnetic field. However it is of note that lack of measured effects does not necessarily indicate lack of influence on studied cells. For this reason, further detailed research is needed in order to fully understand the effects of the nanocomposite material properties on cellular behavior.

The results of this study point out the clear role of magnetic stimulation on cell viability and organization. The experimental data show that neither magnetic field nor magnetic scaffold alone significantly affected endothelial cell organization into vascular-like structures. However, the magnetic scaffold exposed to an alternating magnetic field enabled enhanced stimulation of endothelial cells and organization into early capillary-like structures *in vitro*. Although the mechanism of endothelial cell stimulation in magnetically responsive alginate scaffolds is yet to be investigated, we can postulate a few possible causes. First, because MNPs and their aggregates are anisotropic in terms of their geometrical shape (i.e. not of ideally spherical geometry) they might generate a vibrating local scaffold deformation of small amplitude when exposed to an alternating magnetic field. This local vibration can apply direct forces on cells adhered on the MNP decorated scaffold surface and result in a local magneto-mechanical effect on cells. Alternatively, it is possible that the scaffold undergoes a general deformation, creating bending/stretching forces that exert a mechanical effect on the cells. In the reported experiments, possible effects of mechanical stimuli due

the presence of the MNPs cannot be separated from possible direct effect of the magnetic field on cells. A magnetic field has been reported to affect various aspects of cell behavior. However, the mechanism for the direct magnetic field influence on cells remains obscure. Our observations could possibly be attributed to synergy of the mechanical and direct magnetic field stimuli since we did not separate the two in our experiments. Overall, our results indicate that magnetically responsive alginate scaffolds exposed to an alternating magnetic field provide physical cues to seeded endothelial cells, driving their organization into early vessel-like structures. This strategy combined with chemical signals (i.e. growth factors) can potentially lead to the creation of an efficient pre-vascularized tissue construct *in vitro* which can be transplanted and efficiently integrated *in vivo* with the host tissue.

5. Conclusions

The present study describes the creation of a stimulating microenvironment suitable for promoting endothelial cell organization into capillary-like structures *in vitro*. The microenvironment was constructed from a macroporous alginate scaffold impregnated with magnetically responsive nanoparticles. The inclusion of magnetic particles had no significant effect on the porosity, stability and wetting properties of the composite scaffolds making them appropriate for cellular support and cultivation. The added magnetic properties of composite scaffolds in combination with an externally applied alternating field led to an induced capillary-like organization of endothelial cells. The scaffold, whose development is described herein, has potential to serve as a useful tool for *in vitro* tissue engineering with proven ability to induce organization of endothelial cells into capillary-like structures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Yulia Sapir gratefully acknowledges the generous fellowship from the late Mr. Daniel Falkner and his daughter Ms. Ann Berger and thanks the Azrieli Foundation for the award of an Azrieli Fellowship supporting her PhD program. This study was partially supported by The Louis and Bessie Stein Family foundation through the Drexel University College of Medicine (BP, YS, SC), American Associates of the Ben-Gurion University of the Negev, Israel (YS), the European Union FWP7 (INELPY) (SC) and USA Award Number 5R01HL107771 from the National Heart, Lung and Blood Institute (BP). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Heart, Lung and Blood Institute or the National Institutes of Health. Authors thank Olga Kryukov from Ben-Gurion University for preparing the peptide-modified alginate and Richard Sensenig from the Department of Surgery, Drexel University College of Medicine for reviewing the manuscript and providing constructive suggestions. Prof. Cohen holds the Claire and Harold Oshry Professor Chair in Biotechnology.

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2012.02.037.

Fig. 1. Scaffold morphology: Scanning electron microscopy (SEM) images of the (A) 1.2% (w/v) MNP-alginate and (B) non-magnetic alginate scaffolds. (C) Macroscopic view of the (a) dry (b) pre-wetted for 30 min and (c) hydrated with culture medium for 24 h scaffolds. The black scaffold is magnetic-alginate composite. The white is control alginate scaffold without inclusion of MNPs.

Fig. 2. (A) Magnetization curves of MNP-scaffold and Alginate-scaffolds; $M_r(0 \text{ Oe}) = 0.4 \text{ emu/g}$ (remanent magnetization); $M_f(15 \text{ Oe}) = 1.25 \text{ emu/g}$ (magnetization at the experimental field condition); $M_s(5 \text{ kOe}) = 24 \text{ emu/g}$ (saturation magnetization). Inset graph depicts magnified region near the center of coordinates. (B) Mechanical spectra of the medium-wetted scaffolds. G' -elastic modulus; G'' -viscous modulus.

Fig. 3. (A) Experimental time scale. (B) Metabolic activity per cell in the different constructs. Data ($n = 3-5$ constructs per tested group) presented are normalized to the DNA content in the construct, relative to day 0. Asterisks denote significant difference (by two-way ANOVA) $*p < 0.05$, $**p < 0.01$ and $***p < 0.005$ (Bonferroni's pos-hoc test was used for comparison between the groups).

Fig. 5. Endothelial cell organization in MNP-alginate and alginate constructs, on days 7 and 14 post cell seeding. The cells are stained for F-actin (red) and nuclei (blue) (bar: 30 μ m). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6.

Average loop number per image field counted on day 14 post cell seeding. A total of 25 randomly selected fields were analyzed per each group. Asterisks denote significant difference (by 2-way ANOVA), * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$ (Bonferroni's post-hoc test was used for comparison between the groups).