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A universal biofactor-releasing scaffold enabling in vivo reloading

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Abstract

Growth factors supply to engineered tissues is essential for many physiological processes, including proper organization of the cells into functioning tissues, maintenance of their viability, vasculogenesis, proliferation and differentiation. Systems to efficiently control the release of growth factors were previously incorporated into tissue engineering scaffolds to affect cells. However, since the initial concentration of the factors in these systems is finite, their ability to provide a long-term physiological effect is limited. Here, we report on a new reloadable system, in which 3D fibrous scaffolds conjugated with an anti His-tag antibody, enable retention and controlled release of any His tag-modified proteinaceous growth factor. The scaffolds can be reloaded in vitro or in vivo with any His-tagged biomolecule at any time, according to the physiological need. We show the ability of the scaffolds to release angiogenic factors in a static cell culture or under flow in a microfluidics device and effect on endothelial cells. We also demonstrate the potential of the system to be sequentially reloaded in vivo with various factors and as a proof-of-concept we provide evidence for efficient in vivo vascularization of scaffolds after reloading with tagged VEGF.

Keywords

Tissue engineering; scaffolds; growth factors; controlled release; reloading; vascularization

Growth factors (GFs) supply to engineered tissues is essential for many physiological processes, including proper organization of the cells, maintenance of their viability, vasculogenesis, proliferation and differentiation¹. In nature, GFs are secreted by the cells, interact with sulfated GAGs and other molecules for storage, and are then presented or released into the cellular microenvironment to affect the cells in an autocrine or paracrine fashion. In tissue engineering, GFs secretion by the cells is limited and therefore, external

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supply of the factors is required¹. Systems enabling regulated supply of such factors and molecules to developing tissues are essential for effective tissue assembly.

In recent years, diverse controlled release systems were incorporated into cell supporting scaffolds to supply cues for physiological processes^{2–4}. In one approach, GFs were encapsulated within double emulsion polymeric particles, which were entrapped within the scaffold. Under physiological conditions the polymeric particles degraded, slowly releasing the GFs to the cellular microenvironment^{5–8}.

Positively-charged biomolecules, such as many GFs can be also integrated into the scaffolds by electrostatic interactions with the negatively-charged backbone of the supporting materials^{2, 9}. In this manner, the GFs are presented to the cells to affect their behavior. Other systems include physical entrapment of GFs in hydrogels or their chemical conjugation to the polymer backbone^{10–14}.

Although efficient controlled release of factors from many different systems was demonstrated, once the factors are released from the scaffolds, they either affect the cells or degraded by hydrolytic enzymes. Since their initial concentration is finite, their ability to provide long-term support to the scaffold-entrapped cells is limited³.

Here, we report for the first time on a modular 3D biomaterial scaffold, enabling the release of any biomolecule in a controlled manner. Theoretically, the scaffolds can be reloaded either in vitro or in vivo at any time, with any biomolecule, according to the physiological need. With such approach, GFs may be reloaded into the scaffold once the depot is empty, or when a different factor is needed to supply new cues according to the dynamics of tissue organization and function, or according to the needs of the entire organ (Fig. 1). Here, 3D fibrous scaffolds were chemically conjugated with an antibody specific to His-Tag (a sequence of six consecutive histidine residues, commonly used for protein purification). In this way, any biofactor, including growth factors, peptides, small molecules or any type of drug that was pre-modified with a His-Tag moiety can be specifically attached to the scaffold. Once the cells, the tissue or the entire organ consume the factors, or when a different factor is needed for triggering a different response, the scaffold can be reloaded with any type of His-Tagged biofactor.

As a proof-of-concept we chose to use electrospun albumin fibers as the scaffolding material. Such scaffolds were shown to support cell assembly into a functioning tissue^{8, 15, 16}. The scaffolds had fibers with a diameter of $2.19 \pm 0.5 \mu\text{m}$ with an average pore size of $335.4 \pm 58.85 \mu\text{m}^2$ for efficient cell infiltration and mass transfer (Fig. 2a). A mouse monoclonal anti His-Tag antibody was chemically conjugated to the carboxylic groups on the albumin fibers by carbodiimide activation. To verify proper and homogenous conjugation, the scaffolds were stained with a secondary Cy5-conjugated anti-mouse antibody. Figure 2b reveals homogeneous distribution of the anti His antibodies, ensuring uniform release of factors from the scaffolds, as well as uniform reloading of the injected biomolecules.

To evaluate the ability of the anti His-Tag scaffolds to retain biofactors and slowly release them into their microenvironment, we used the angiogenic factor VEGF. Release of VEGF

was previously shown to induce endothelial cell migration towards engineered patches and proper blood vessel formation within parenchymal tissues^{8, 17, 18}. His-tagged VEGF was loaded onto the scaffolds by a single drop. Initially, we sought to study the ability of scaffolds to specifically capture the tagged factors. As shown, significantly higher levels of His-tagged VEGF were found in the scaffolds, as compared to pristine VEGF (Fig. 2c). Next, the ability of the scaffolds to retain the factor was studied. His-tagged VEGF was introduced to anti His-Tag antibody scaffolds and to pristine scaffolds. As shown, while a burst release of the factor was detected in the pristine scaffolds, the modified scaffolds were able to retain the factor (Fig. 2d). Following, the long term release from scaffolds was studied, revealing a prolonged release only from the modified scaffolds. While 100% of the factor was released from the pristine scaffolds within the first 24 h, the modified scaffolds were able to slow the release of the factors and only 65% of the factor was released after day 7 (Fig. 2e). These results emphasize the potential of the anti His antibody-modified scaffolds for use as a controlled release system of growth factors. Additionally, the release kinetics may be tuned by selecting antibodies with different affinities¹⁹.

Next, we evaluated the reloading potential of the modified scaffolds. As reloading in the body would occur under shear flow, we initially used a microfluidic system to simulate the blood stream. Pristine or modified scaffolds were placed at the end of closed channels of a microfluidic device²⁰, and His-tagged or pristine VEGF molecules were flown (Fig. 3a). As shown, significantly higher levels of VEGF were found when His-tagged VEGF was flown over modified scaffolds, as compared to His-Tagged VEGF attachment to pristine scaffolds or to pristine VEGF attachment to the modified scaffolds (Fig. 3b). Overall, these results indicated that the modified scaffolds may be suitable for growth factor binding and for their controlled release into the cellular microenvironment under flow conditions.

Next, we sought to evaluate the physiological effect of releasing the His-tagged VEGF into the cellular microenvironment of endothelial cells. Human Umbilical Vein Endothelial Cells (HUVECs) were cultured on pristine or modified scaffolds and His-tagged or pristine VEGF molecules were supplemented once to the culture medium on day 0 of the experiment. As shown, the cells on the pristine scaffolds or those on the modified scaffolds, which received pristine VEGF were not able to grow, due to lack of available VEGF. In contrast, the His-tagged VEGF was able to attach to the modified scaffolds and was released or presented to the endothelial cells throughout the cultivation period, resulting in a more supportive environment that enabled endothelial cell growth (Fig. 3c and d).

To assess the potential of the modified scaffolds to reload biofactors in vivo we transplanted them initially onto mice omenta (Fig. 4a and b). The omentum is a fatty tissue in the abdominal cavity, which is highly vascularized²¹. We hypothesized that His-tagged factors that are systemically injected into the tail vein will circulate in the blood stream, and if they come in contact with the modified scaffold, reloading will occur. To evaluate the efficiency of factor loading into modified scaffolds, His-tagged maltose binding protein (MBP) was used as a proof-of-concept. The fluorescent dye Cy5.5 was conjugated to the protein to allow easy detection in vivo. The His-tagged, fluorescently labeled protein was injected into the tail vein of mice and allowed to circulate in the blood stream and distribute to the different organs. Twenty-four hours later, the animals were sacrificed and the lung, liver,

spleen, kidney, heart and omentum were harvested for analysis. Biodistribution analysis of the tagged Cy5.5 molecules in these organs has shown significantly higher levels of the labeled protein in the omentum, as compared to all other organs (Fig. 4c). This indicated on the ability of the anti His-Tag antibody to capture the His-tagged protein under flow in the body. Such capability would allow to reload factors into the scaffold after the initial depot had been released.

To evaluate whether factors can be in vivo reloaded into the scaffolds to replace existing factors, we performed sequential mouse tail vein injections of His-tagged MBP, conjugated with Cy5.5 or with Cy3.5 fluorescent dyes. Cy5.5-conjugated MBP was injected at $t=0$, while the Cy3.5-conjugated MBP was injected 46 h later. Accumulation was assessed by whole animal imaging just prior to the first injection and at 24 h intervals thereafter (Fig. 4d). As shown, at $t=0$, prior to reloading of the factors, no fluorescence could be detected. After 24 h, Cy5.5-conjugated His-tagged MBP molecules accumulated in the omentum, reaching the highest intensity and fading away on later time points. In contrast, Cy3.5-conjugated His-tagged MBP molecules were detected only after their injection, at $t=48$ h, reaching the highest intensity 24 h later, at $t=72$ h and were then released and probably degraded or excreted. Overall, these results suggest that sequential reloading of factors can be achieved in our system, allowing supply of the biomolecules of choice to cells and tissues. Furthermore, follow up injections of new factors enabled efficient in vivo replacement of the initially loaded factors, allowing to fit the appropriate factor to the stage of the developing tissue (Fig. 4e). Future studies should focus on assessing the limitations of the system, including the maximal times of efficient reloading and the ability to penetrate through a fibrotic tissue that may have formed around the implant.

Finally, after ensuring that the factors can be efficiently reloaded into the scaffolds in vivo, we investigated their function at the targeted site. Here, pristine or modified scaffolds were transplanted on rats' omenta, and 24 h later pristine or His-tagged VEGF molecules were injected into the tail vein. Seven-days post transplantation, the scaffolds could be identified on the omentum and blood vessels from the host could be seen entering the modified scaffolds with the His-tagged VEGF (Fig. 5a). The scaffolds and surrounding tissues were then extracted from the animals, fixed, sliced and stained with anti CD31 antibodies for assessment of blood vessels. As shown, higher levels of CD31 staining could be seen in the modified scaffolds which were injected with the His-tagged VEGF, as compared to pristine scaffolds injected with the His-tagged VEGF or modified scaffolds, injected with pristine VEGF (Fig. 5b-d). Quantification of blood vessels, revealed significantly higher density in the modified scaffolds, which were injected with the His-tagged factor (Fig. 5e). Moreover, the total area of the blood vessels in this group was also significantly higher (Fig. 5f).

Taken together, the results presented in this paper provide a proof-of-concept for the reloading strategy. Such approach may be useful for tissue regeneration when a long-term supply of factors is needed. Moreover, such efficient reloading may be useful for triggering diverse physiological processes, where a sequential supply of different factors is needed.

Methods

Electrospinning Albumin Fiber Scaffolds

Bovine serum albumin [BSA; Fraction V, MP Biomedicals, Aurora, OH; 10% (w/v)] was dissolved in tetrafluoroethylene (TFE) and distilled water (9:1), following by the addition of excess β -mercaptoethanol (Merck, Darmstadt, Germany) for overnight reaction. The solution was electrospun at room temperature using a syringe pump delivered at a rate of 2 mL/h. A high-voltage supply (Glassman High Voltage) was used to apply a 12-kV potential between the capillary tip and the grounded aluminum collector placed at a distance of 14 cm from the capillary tip. The scaffolds were dried under vacuum and were sterilized by UV light.

Fabrication of antibody-conjugated scaffolds and growth factors loading

Albumin scaffolds were activated with ethyl-3(3-dimethylamino) propyl carbodiimide (EDC; 3.6mg/mL, Sigma–Aldrich, Rehovot, Israel) in the presence of N-hydroxysuccinimide (NHS; 5.4mg/mL, Sigma–Aldrich) at a molar ratio 1:4, both EDC and NHS were dissolved in phosphate buffered saline (PBS). The scaffolds were washed with Phosphate-buffered saline containing 0.5% Tween-20 (PBST) after each step of fabrication. Following, A mouse monoclonal His-Tag-specific antibody (Abs; Abgent, San Diego, CA, 40 μ g/mL) were incubated with the activated scaffolds overnight. Then, 1M Glycine in PBS was added for 10 min to quench the chemical reaction. Finally, a blocking solution of 20% horse serum (Biological Industries, Beit Haemek, Israel) in PBS was added to the scaffolds for 1 h at 37°C or ON at 4°C. For the GFs loaded scaffolds, human VEGF, fused to a 20 a.a His-tag at the N-terminus (VEGF; Prospec, Rehovot, Israel; 1mg/mL) was loaded for 2 h at room temperature (RT).

SEM

Samples were mounted onto aluminum stubs with conductive paint and were sputter-coated with an ultrathin (150-Å) layer of gold in a Polaron E5100 coating apparatus (Quorum Technologies, Lewes, UK). The samples were viewed under a JCM-6000PLUS NeoScope Benchtop scanning electron microscope (JEOL USA, Inc.).

Immunostaining for Anti His-Tag Abs

After blocking and PBST washing, a secondary Cy5-conjugated anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA; 1:250 dilution in PBST) was added for 1 h at RT, washed again and visualized using a fluorescence microscope (Nikon Eclipse TI).

Binding under flow in a microfluidic device

To assess binding under flow we used a microfluidic system that was comprised of two layers: an upper microchannels layer and a bottom microwells layer as previously described²⁰. Abs-conjugated scaffolds and pristine scaffolds were placed in the wells of the bottom layer and the two layers were fixed. Antigen was flown in the system at a rate of 100 μ L/h. After PBST washes, the scaffolds were visualized using a fluorescence microscope (Nikon Eclipse TI).

Measurements of growth factors release

For the sustained release assay, Abs-conjugated scaffolds, pristine scaffolds and empty wells were loaded and incubated with 1 µg/mL His-tagged VEGF for 2 h at RT in non-binding 96-well plates. Then, the first sample was removed and replaced by fresh PBS solution. The scaffolds were incubated in PBS at RT for 7 days. Samples were collected after 24, 72, 120 and 168 h and stored at – 20 °C in non-binding 96-well plates for analysis. The samples were then thawed, diluted and evaluated by enzyme linked immune-sorbent assay (ELISA) for the presence of VEGF (duo-set ELISA kit for VEGF; R&D Systems®).

Cell culture, seeding and cultivation

Abs-conjugated scaffolds and pristine scaffolds were loaded with 1 µg/mL His-tagged VEGF, 1 µg/mL pristine VEGF or without growth factors. Then, GFP-expressing human umbilical vein endothelial cells (GFP-HUVECs; passage 5-9; Angio-proteomie, Boston, MA; 50,000 per scaffold) were seeded in 3 µL volume of EGM-2 (Lonza) and incubated (45 min, 37 °C, 5% CO₂). EGM-2 medium without VEGF supplement was added (100 µL per scaffold) and replaced every other day thereafter. Scaffolds were cultured under static conditions (37 °C, 5% CO₂) for 7 days and were visualized using a fluorescence microscope (Nikon Eclipse TI).

In vivo biodistribution and reloading studies

Recipient male C57BL mice (25-28 g) (Envigo Laboratories, Israel) were anesthetized using a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg) according to Tel Aviv University ethical use protocols. After a midline abdominal incision was made, the Abs-conjugated scaffolds or pristine scaffolds were stitched to the omentum. *Biodistribution:* Twenty-four hours post implantation, Cy5.5-conjugated His-tagged-MBP was injected IV. Two days later, the animals were sacrificed, the organs were harvested and imaged using Cri MaestroTM. *Reloading:* After transplantation, Cy5.5-conjugated His-tagged-MBP was injected IV and 46 h later Cy3.5-conjugated His-tagged-MBP was injected as well. The animals were imaged daily by Cri MaestroTM and the data was analyzed using ImageJ.

In vivo vascularization

Recipient Sprague Dawley male rats (150–200 g) (Envigo Laboratories) were anesthetized using a combination of ketamine (40 mg/kg) and xylazine (10 mg/kg) according to Tel Aviv University ethical use protocols. After a midline abdominal incision was made, the scaffolds were placed onto the omentum. Twenty-four hours post implantation His-VEGF (100 µg/kg; 200 µL per rat) was injected IV. Seven days after implantation, the animals were sacrificed and samples were extracted, fixed in formalin and embedded in OCT. Sections (10 µm thick) were prepared using a cryotome. The sections were stained with primary Armenian hamster anti-CD31 (1:250; Abcam, Cambridge, UK) and TritC-conjugated goat anti-Armenian hamster secondary antibody (1:500; Abcam). The sections were visualized using a fluorescence microscope (Nikon Eclipse TI).

Statistical Analysis

Data were presented as means $\pm$ SEM. Differences between samples were assessed by a Student's *t* test. All analyses were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software). $p < 0.05$ was considered significant.

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References

- Gabriel D, Dvir T, Kohane DS. *Expert Opin Drug Del.* 2012; 9(4):473–492.
- Freeman I, Cohen S. *Biomaterials.* 2009; 30(11):2122–2131. [PubMed: 19152972]
- Subbiah R, Guldberg RE. *Adv Healthc Mater.* 2018:e1801000. [PubMed: 30398700]
- Shapira A, Feiner R, Dvir T. *Int Mater Rev.* 2016; 61(1):1–19.
- Richardson TP, Peters MC, Ennett AB, Mooney DJ. *Nat Biotechnol.* 2001; 19(11):1029–34. [PubMed: 11689847]
- De la Riva B, Sanchez E, Hernandez A, Reyes R, Tamimi F, Lopez-Cabarcos E, Delgado A, Evora C. *J Control Release.* 2010; 143(1):45–52. [PubMed: 19963026]
- Perets A, Baruch Y, Weisbuch F, Shoshany G, Neufeld G, Cohen S. *J Biomed Mater Res A.* 2003; 65(4):489–97. [PubMed: 12761840]
- Fleischer S, Shapira A, Feiner R, Dvir T. *Proc Natl Acad Sci U S A.* 2017; 114(8):1898–1903. [PubMed: 28167795]
- Manning CN, Kim HM, Sakiyama-Elbert S, Galatz LM, Havlioglu N, Thomopoulos S. *J Orthop Res.* 2011; 29(7):1099–105. [PubMed: 21246611]
- Kim JK, Yoo C, Cha YH, Kim YH. *J Control Release.* 2014; 194:316–22. [PubMed: 25245775]
- Koudstaal S, Bastings MM, Feyen DA, Waring CD, van Slochteren FJ, Dankers PY, Torella D, Sluijter JP, Nadal-Ginard B, Doevendans PA, Ellison GM, et al. *J Cardiovasc Transl Res.* 2014; 7(2):232–41. [PubMed: 24395494]
- Awada HK, Johnson NR, Wang Y. *J Control Release.* 2015; 207:7–17. [PubMed: 25836592]
- Choi B, Kim S, Fan J, Kowalski T, Petrigliano F, Evseenko D, Lee M. *Biomater Sci.* 2015; 3(5):742–52. [PubMed: 26222593]
- Lorentz KM, Yang L, Frey P, Hubbell JA. *Biomaterials.* 2012; 33(2):494–503. [PubMed: 22014943]
- Feiner R, Fleischer S, Shapira A, Kalish O, Dvir T. *J Control Release.* 2018; 281:189–195. [PubMed: 29782947]
- Fleischer S, Shapira A, Regev O, Nseir N, Zussman E, Dvir T. *Biotechnology and bioengineering.* 2014; 111(6):1246–1257. [PubMed: 24420414]
- Dvir T, Kedem A, Ruvinov E, Levy O, Freeman I, Landa N, Holbova R, Feinberg MS, Dror S, Etzion Y. *Proceedings of the National Academy of Sciences.* 2009; 106(35):14990–14995.
- Borselli C, Storrie H, Benesch-Lee F, Shvartsman D, Cezar C, Lichtman JW, Vandenburgh HH, Mooney DJ. *Proc Natl Acad Sci U S A.* 2010; 107(8):3287–92. [PubMed: 19966309]
- Landry JP, Ke YH, Yu GL, Zhu XD. *J Immunol Methods.* 2015; 417:86–96. [PubMed: 25536073]
- Wertheim L, Shapira A, Amir RJ, Dvir T. *Nanotechnology.* 2018; 29(13)
- Shevach M, Soffer-Tsur N, Fleischer S, Shapira A, Dvir T. *Biofabrication.* 2014; 6(2)

Figure 1. Overview of the concept.

Electrospun albumin fiber scaffolds are conjugated with an anti His antibody. After transplantation, His-tagged biomolecules are IV injected, circulate in the blood stream and are reloaded to the scaffolds. As the conjugated antibodies are specific to His-Tag, sequential reloading can be achieved either with the same factor or a different one according to the requirements of the engineered tissue or the organ.

Figure 2. Anti His-tag antibody-modified scaffolds as a controlled release system.

a. Morphology of electrospun albumin fiber scaffolds by SEM. b. Verification of anti His antibody conjugation to the scaffold. Pristine (upper panel) or modified (lower panel) scaffolds were stained with a secondary antibody (pink). Bar= 100 μm . c. Specific binding of pristine or His-Tagged VEGF to the modified scaffolds. d. Burst release of His-Tagged VEGF from pristine or modified scaffolds. e. Long-term controlled release of His-Tagged VEGF from pristine or modified scaffolds. As shown, 100% of the factor was released from the pristine scaffolds after 24 h, while only 65% of the factor was released from the modified scaffolds after 7 days.

Figure 3. His-Tagged VEGF loading under flow and its effect on endothelial cell growth.
a. Schematics of the microfluidics system used for loading the factor. b. Attachment of pristine or His-Tagged VEGF under flow to pristine or modified scaffolds. c. GFP-Endothelial cell growth (day 7) on pristine scaffolds supplemented with His-Tagged VEGF, modified scaffolds supplemented with pristine VEGF or modified scaffolds supplemented with His-Tagged VEGF. The factors were supplemented once on day 0. Bar= 100 μm . d. Quantification of the area occupied by endothelial cells on day 7.

Figure 4. In vivo reloading of factors onto the scaffolds.

a. Schematics of the study. Modified scaffolds are transplanted onto mice omenta, followed by a systemic injection of Cy5.5 (t=0) or Cy3.5 (t=46 h) conjugated His-Tagged protein into the tail vein. The animals were either taken for biodistribution analysis or imaged to evaluate factor reloading. b. Extraction (day 7) of a modified scaffold (white arrow) stitched onto mouse omentum. c. Biodistribution of Cy5.5-conjugated His-Tagged protein molecules, 2 days after injection. d. Whole animal imaging of Cy5.5 or Cy3.5-conjugated His-Tagged proteins. Imaging was performed every 24 h. e. Quantification of reloading within the modified scaffolds.

Figure 5. The potential of the system to promote vascularization.

a. Modified scaffolds (arrow) on rat's omentum 7 days after a one-time in vivo loading with His-Tagged VEGF. Blood vessels are clearly seen infiltrating into the scaffold. b-d. Blood vessels within the scaffolds as visualized by CD31 staining (green). Bar= 100 μ m. b. Endothelial cells in modified scaffolds after systemic injection of pristine VEGF. c. Endothelial cells in pristine scaffolds after systemic injection of His-Tagged VEGF. d. Endothelial cells in modified scaffolds after systemic injection of His-Tagged VEGF. e. Blood vessel density within the different scaffolds after injection with pristine or His-Tagged VEGF. Area occupied by the vessels within the different scaffolds after injection with pristine or His-Tagged VEGF.