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# Collagen matrix deposition is dramatically enhanced *in vitro* when crowded with charged macromolecules: The biological relevance of the excluded volume effect

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**Abstract The excluded volume effect (EVE) rules all life processes. It is created by macromolecules that occupy a given volume thereby confining other molecules to the remaining space with large consequences on reaction kinetics and molecular assembly. Implementing EVE in fibroblast culture accelerated conversion of procollagen to collagen by procollagen C-proteinase (PCP/BMP-1) and proteolytic modification of its allosteric regulator, PCOLCE1. This led to a 20–30- and 3–6-fold increased collagen deposition in two- and three-dimensional cultures, respectively, and creation of crosslinked collagen footprints beneath cells. Important parameters correlating with accelerated deposition were hydrodynamic radius of macromolecules and their negative charge density.**


*Keywords:* Excluded volume effect; Macromolecular crowding; Extracellular matrix; Collagen deposition; Procollagen C-proteinase

## 1. Introduction

Tissue engineering combines cell biology and materials science to provide therapeutic strategies for the generation of tissues *in vitro* and/or *in vivo* when situations result in complete organ failure or ineffectual natural or augmented repair processes. As with every cell culture, be it two- or three-dimensional, cells are isolated from preexisting tissue. They are either terminally differentiated or show various degrees of "stemness" e.g. adult bone marrow-derived and embryos. The isolation and seeding process removes the harvested cells from a context of highly complex and dense arrays of macromolecules, the extracellular matrix (ECM), and places them abruptly onto a naked or thinly-coated tissue culture plastic while being bathed in large volumes of non-crowded aqueous medium. This situation is far from physiological given the fact that the total concentration of protein and RNA inside pro- and eukaryotic cells is in the range of 300–400 g/l [1–3] and that the extracellular space is usually dominated by dense arrays of ECM macromolecules. Even blood has a solute concentration of 80 g/l [4]. The addition of fetal calf serum in routine culture media, however, fails to create a crowded environment [5]. As the cells are confronted with an environment devoid of ECM they start to rebuild their environment by producing their own ECM. While fibronectin is deposited rapidly *in vitro* [6], the deposition of a collagen matrix, the primary structural biological material in all tissues and organs, is enzymatically rate-limited. As the current culturing practices are characterized by a lack of macromolecular crowding and hence excluded volume effect (EVE), the procollagen conversion and as a consequence, collagen matrix deposition, is notoriously slow *in vitro* [5]. Many weeks are needed to create cohesive tissue sheets that contain sufficient ECM [7]. This represents a major bottle-neck in tissue engineering and impedes the studies of ECM formation *in vitro*. In order to overcome this obstacle we tested a series of polymeric macromolecules for their ability to exclude volume and speed-up specific enzymatic steps required for collagen deposition *in vitro*.

## 2. Materials and methods

### 2.1. Tissue culture

Low passage (3–8) normal primary embryonic lung fibroblasts (WI-38; American Tissue Culture Collection, VA, USA) were routinely cultured as outlined in Lareu et al. [5]. Fibroblasts were seeded on 24-well plates at 50 000/well and the following day the media was

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*Abbreviations:* EVE, excluded volume effect; PCP, procollagen C-proteinase; ECM, extracellular matrix; DxS, dextran sulfate; PSS, polysodium-4-styrene sulfonate; Fc, Ficoll™; PLLA, poly L-lactic acid; PCOLCE1, PCP enhancer protein 1; DAPI, 4′,6-diamidino-2-phenylindole; DLS, dynamic light scattering; MMP2, matrix metalloproteinase 2

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replaced with 0.5% FBS, 100 μM of L-ascorbic acid with or without macromolecules. The macromolecules employed were 500 kDa dextran sulfate (DxS) and 10 kDa DxS at 100 μg/ml (pK Chemicals A/S, Koge, Denmark), 200 kDa polysodium-4-styrene sulfonate (PSS) at 100 μg/ml (Sigma–Aldrich, Singapore), 400 kDa Ficoll™ (Fc) and 70 kDa Fc at 50 mg/ml (Amersham Pharmacia, Uppsala, Sweden). The medium and cell layer fractions were harvested separately. Collagen from medium, cell layer and footprints was extracted with pepsin under acidic conditions [5]. For footprint analysis, cell layers were washed twice with HBSS and solublized three times with 0.5% sodium deoxycholate (Sigma–Aldrich) on ice for 10 min as outlined in [8].

### 2.2. Three-dimensional (D) culture and bioreactors

250 000 WI-38 fibroblasts were seeded onto either Biobrane® (3.5 cm diameter) (Bertek Pharmaceuticals Inc, WV, USA) or poly L-lactic acid (25 × 50 × 3 mm) (PLLA, 0410-2 × 45; Transome Inc., FL, USA) felt scaffolds in 6 well plates and cultured routinely in 10% FBS for 1 and 2 days, respectively. For static treatment, ascorbic acid and with or without 100 μg/ml of DxS 500 kDa were supplemented and incubated for 3 and 5 days, respectively. The PLLA scaffolds were placed in modular parallel-plate bioreactors design according to Gemmiti and Guldberg [9] and incubated initially as for static culture following transfer to the bioreactor modules for 5 days. Treatments were as for static culture. Bioreactor flow rate was 0.2 ml/min. After culture treatments, the scaffolds were removed form the bioreactor modules and pepsin-treated for deposited collagen as above.

### 2.3. Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein samples were separated under non-reducing conditions either using pre-cast gradient NuPage 3–8% Tris-acetate polyacrylamide gels (Invitrogen, Singapore) or 5% resolving/3% stacking polyacrylamide gels as outlined in [10]. Protein standards used were the Precision Plus Dual Color and Prestained Broad Range (Bio-Rad, Singapore), and collagen type I (Koken Co., Tokyo, Japan). Protein bands were stained with the SilverQuest™ kit (Invitrogen) and densitometric analysis was performed on the collagen α-bands with a GS-800™ Calibrated Densitometer (Bio-Rad).

### 2.4. Western immunoblotting

Proteins were extracted from the cell layer, subjected to reducing-SDS–PAGE (NuPage 3–8% Tris-acetate gels) and immunoblotted [5]. Primary antibodies for procollagen C-proteinase (PCP; rabbit anti-human BMP-1, AB81031; Chemicon International, CA, USA) and PCP enhancer protein 1 (PCOLCE1; rabbit anti-human PCOLCE1, CL1PCOLE1; Cedarlane Laboratories Ltd, Ontario, Canada) were used at 1/2500 dilutions. The signal was detected with chemilumines-

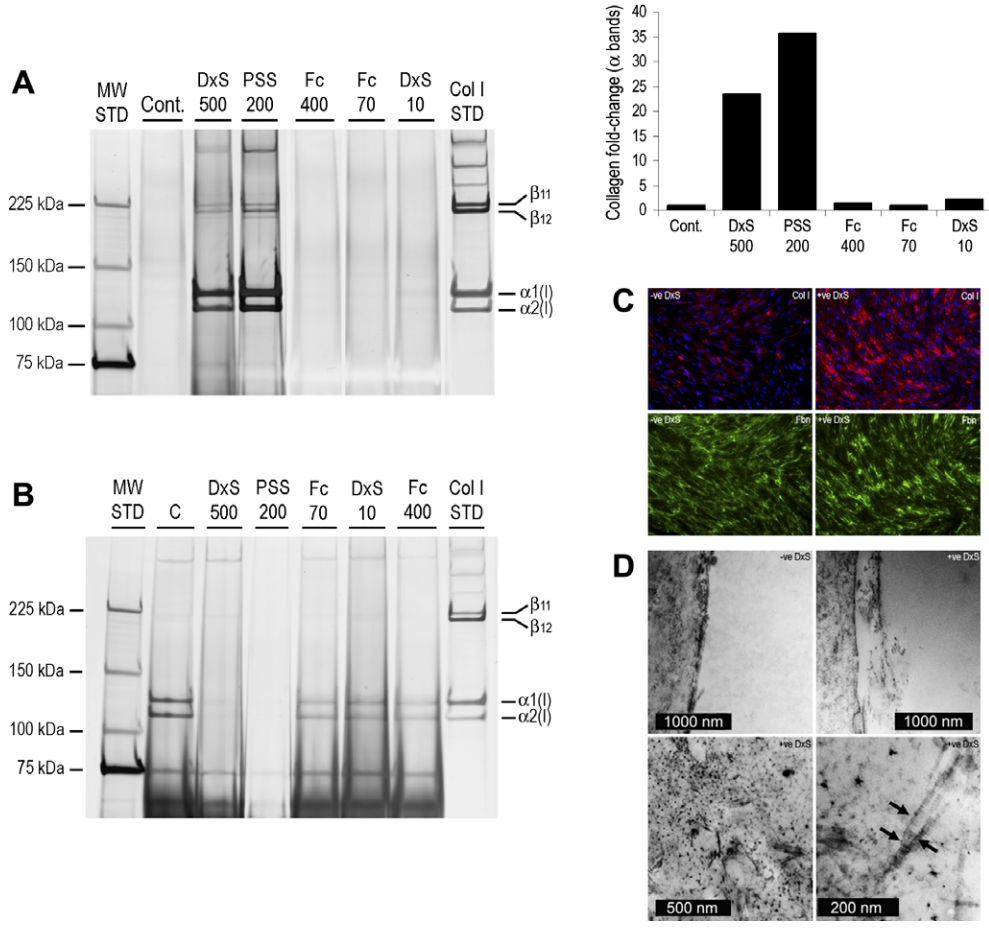


Fig. 1. EVE enhanced collagen deposition. (A) SDS–PAGE (NuPage) and densitometric analysis of collagen deposition (fold-change relative to Cnt) onto the cell layer after 48 h in absence and presence of different macromolecules. (B) SDS–PAGE (NuPage) analysis of collagen from the medium fraction of the above treatments. Each lane contains three pooled individual samples. (C) Double immunofluorescence staining of collagen (red; nuclei counterstained with DAPI) and fibronectin (green) in the ECM in the absence and presence of DxS 500 kDa (100 μg/ml). All magnifications at 10×. (D) Transmission electron micrograph of the pericellular matrix in the absence and presence of DxS 500 kDa (100 μg/ml). Arrows indicate collagen-typical fibrillar formations in the presence of EVE. *Abbreviations*: Col I, collagen type 1; Cnt, control; Fbn, fibronectin; MW STD, molecular weight standards.

cence (Super Signal® West Dura kit; Pierce Biotechnology Inc., IL, USA) and captured with a VersaDoc Imaging System model 5000 (Bio-Rad).

### 2.5. Immunocytochemistry

Cell layers or footprints were fixed with 2% paraformaldehyde (Sigma–Aldrich) and double-immunofluorescence was carried-out in PBS with 3% BSA. Primary antibodies used were mouse anti-human collagen I at 1:100 (AB745; Chemicon International) and rabbit anti-human fibronectin at 1:200 (F7387; Sigma–Aldrich). Secondary antibodies were goat anti-mouse AlexaFluor594 (Molecular Probes, OR, USA) and chicken anti-rabbit AlexaFluor488 at 1/400 dilutions, respectively. Cell nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole; Molecular Probes). Images were captured with an Eclipse TE2000-E inverted epifluorescence microscope (Nikon, Singapore). All digital images were background-subtracted based on conjugate control.

### 2.6. Transmission electron microscopy

3.4 million fibroblasts were seeded in 35 mm dishes and were allowed to attach for 16 h after which they were treated with ascorbate in the presence or absence of DxS for 48 h as described above. Cultures were then washed in PBS and fixed in 2.5% glutaraldehyde in PBS, pH 7.4, for 16 h at 4 °C. Cell layers were scraped, pelleted and embedded in 0.5% agarose. Agarose cylinders containing cell pellets were then processed for electron microscopy (osmication, dehydration, infiltration, araldite embedding). Thin sections were viewed in a JEOL 1220 (Tokyo, Japan) electron microscope. Digital images were taken with a ES 500 DDC camera (Gatan GmbH, Munich, Germany) using Digital Micrograph software.

### 2.7. Biophysical measurements

Dynamic light scattering (DLS) measurements on macromolecules were performed in HBSS, pH 7.4, and corrected for viscosities according to Harve et al. [11]. For zeta (ζ)-potential measurements, macromolecules were dissolved in water and measured using a ZetaPlus analyzer (Brookhaven Instruments Corporation, NY, USA) at 25 °C. ζ-potential was expressed as the mean of 10 readings and tabulated with the standard error of mean.

## 3. Results and discussion

### 3.1. EVE enhances collagen deposition

Only the large negatively charged polymeric macromolecules induced collagen deposition above control levels (Fig. 1A). As described previously [5], the benchmarked polymer DxS 500 kDa resulted in enhanced collagen deposition (23-fold). More remarkably, PSS (200 kDa), a sulfonated anionic polymer, caused an even greater collagen deposition (36-fold). PSS enabled virtually the complete conversion of procollagen into collagen. This was apparent by its complete absence from the medium fraction (Fig. 1B). Neither DxS 10 kDa nor the Fc range caused substantial collagen deposition above control. Immunochemistry of extracellular collagen deposition in the presence of DxS (500 kDa) corroborated the quantitative biochemical data above (Fig. 1C). In contrast, the fibronectin pattern and staining intensity were not significantly enhanced. In the absence of EVE, collagen staining was wispier and strictly colocalized with fibronectin, whereas under EVE both the collagen and fibronectin deposition patterns become more granular and not always showed complete colocalization. Furthermore, preliminary ultrastructural studies confirmed the presence of fibrillar pericellular matrix in crowded versus not crowded cultures (Fig. 1D). On several occasions, aggregates of a width of 25–80 nm with collagen-typical cross-striation were observed.

### 3.2. Biophysical characterization of macromolecules

Biophysical characterization of the macromolecules was studied to establish correlates with their ability to create effective EVE and thus enhance collagen fibrillogenesis through enzymatic processes (see below). Molecular size in solution and net surface charge density were the key parameters. The hydrodynamic radius describes the effective size of a macromolecule in a physiological, aqueous environment. It combines the physical size with the space its hydration shell(s) occupies. Although anionic DxS (500 kDa) and neutral Fc 400 have comparable molecular weights, DxS had a larger hydrodynamic radius, and hence greater percentage fraction volume occupancy (Fig. 2A). Astonishingly, this was at 500 times lower concentration. These data corroborate the findings in [5], where comparison between anionic and neutral dextrans (500 and 670 kDa, respectively) demonstrated a greater-than 2-fold hydrodynamic radius for the smaller but negatively-charged dextran, enabling dramatically greater procollagen conversion *in vitro*. An investigation into the surface charge density of

**A**

| | DxS 500 | PSS 200 | Fc 400 | Fc 70 | DxS 10 |
|---|---|---|---|---|---|
| Net charge: | -ve | -ve | N | N | -ve |
| Hydro. Radius (nm): | 46 | 22 | 4.5 | 3 | <1* |
| % fract. vol. occ.: | 5.2 | 1.2 | 2 | 3 | <0.01 |

**B**

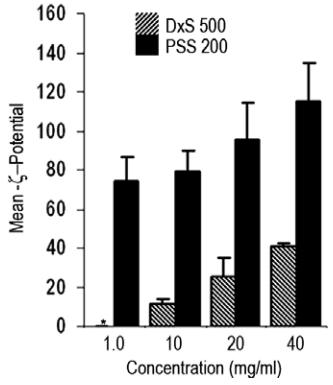


Fig. 2. Biophysical properties of the macromolecules. (A) Net charge, hydrodynamic (Hydro.) radius and percentage fraction volume occupancy (% fract. vol. occ.) are shown for the macromolecules. (B) ζ-potential values of DxS 500 kDa and PSS 200 kDa at various concentrations measuring surface charge density. ∗ indicates below sensitivity of instrument. *Abbreviations*: N, neutral; -ve, negative.

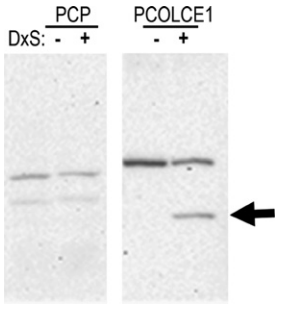


Fig. 3. Western immunoblot of PCP and PCOLCE1 proteins after 48 h of culturing in 10% FBS in the absence or presence of DxS 500 kDa (100 μg/ml). Arrow indicates the 34–36 kDa C-terminus activated form of PCOLCE1.

DxS and PSS revealed that PSS had a 3–4 times higher ζ-potential (Fig. 2B). This would account for the more potent volume exclusion with respect to collagen deposition for PSS even though the percentage fraction volume occupancy for DxS (500 kDa) was 4.3-fold greater at the same concentration. Procollagen, a negatively charged macromolecule (p*I* of 5.2 at physiological pH of 7.4) would have additional volume exclusion due to electrostatic repulsion, which is a key parameter that influences EVE [12]. Therefore, the potency of EVE in our system, with negatively charged macromolecules, is the combined effect of steric and electrostatic repulsion. The latter property, which is appreciably stronger for PSS, would explain the enhanced procollagen conversion of PSS over DxS 500 kDa.

### 3.3. Enhanced extracellular enzymatic activity

Although increased procollagen conversion and subsequent collagen deposition due to the establishment of EVE in tissue culture is a definitive functional assay for PCP activity, Western immunoblotting for the PCP protein did not detect a quantitative difference in the presence of EVE (DxS 500 kDa, 100 μg/ml) (Fig. 3). However, although PCP has many extracellular target proteins, an allosteric enhancer of PCP-procollagen-specific activity, PCOLCE1, has been shown

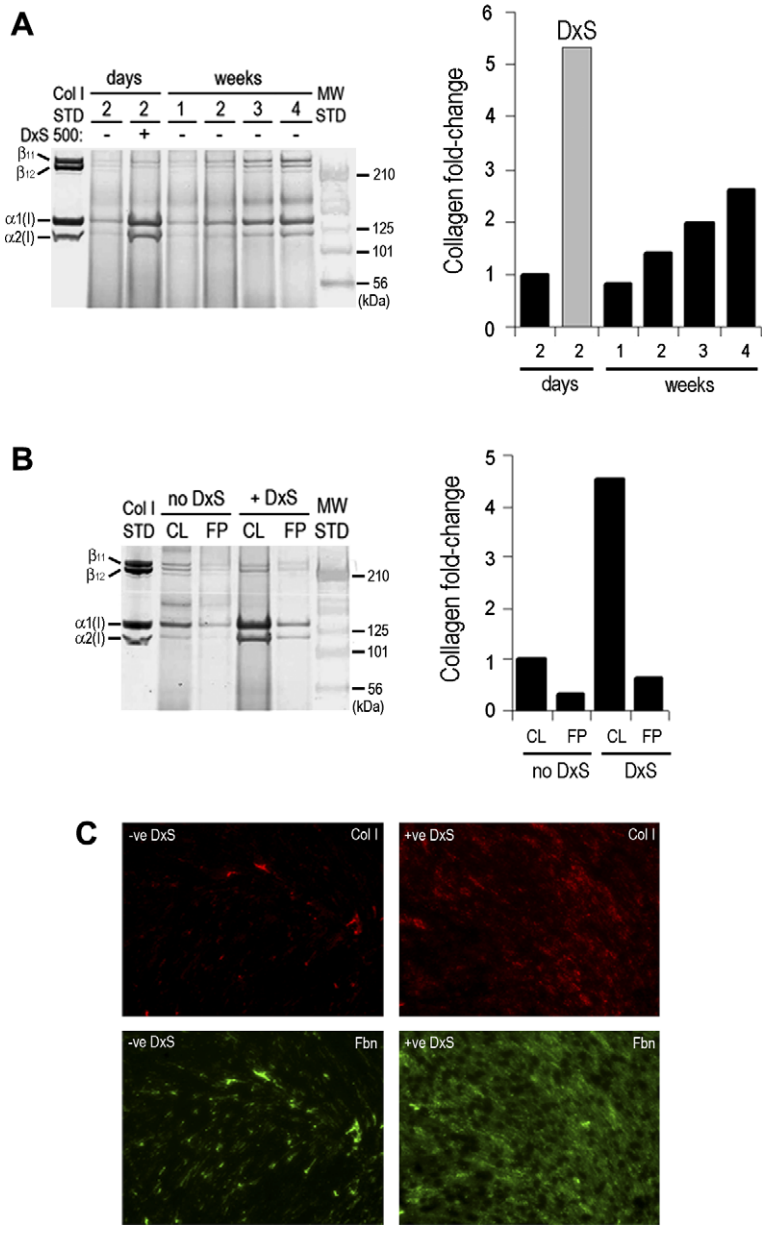


Fig. 4. EVE enhanced the rate of collagen deposition on the supporting material. (A) SDS–PAGE (NuPage) and densitometric analysis of collagen deposition after 2 days culturing in the presence of DxS 500 kDa (100 μg/ml; grey column) and in its absence for 2 days and up to 4 weeks. Fold-change values are relative to 2 days culturing in the absence of DxS. (B) SDS–PAGE (NuPage) and densitometric analysis of 2 days for total collagen deposition (CL) and footprints (FP; after cell solublization with detergent) in the absence (reference) and presence of DxS 500 kDa (100 μg/ml). (C) Double immunofluorescence staining of collagen (red) and fibronectin (green) of the footprints in the absence and presence of DxS 500 kDa. All magnifications at 10×. All lanes contain three pooled individual samples.

to enhance PCP activity 10-fold [13–15]. Only in the presence of crowding did we detect a 36 kDa active form of this PCP enhancer protein (Fig. 3). Furthermore, the PCOLCE1 C-terminus, a fragment with an apparent MW of 16.5 kDa, has been shown to inhibit matrix metalloproteinase 2 (MMP2) with greater efficiency than tissue inhibitor of MMP2 [16]. Therefore, by introducing EVE, we not only accelerated PCP enzymatic activity but also the proteolytic conversion of PCOLCE1 into a PCP-enhancing element and an additional strong metalloproteinase inhibitor. This would complement the matrix deposition process by inhibiting proteins that destroy the ECM. Finally, the ECM forming process is augmented under EVE with an increased lysyl oxidase activity resulting in an enhanced presence of collagen beta crosslinks [5].

### 3.4. EVE enhances collagen deposition onto the supporting material

Although several studies have been continuously hinting at the potential of EVE [17,18] it has not been exploited for applications in biological *in vitro* systems. We were able to dramatically enhance collagen deposition quantitatively and temporally in the presence of DxS 500 kDa. In the presence of EVE, 48 h of culturing resulted in greater-than 5- and 2-fold collagen deposition compared to the control cultures (without EVE) for 1 and 4 weeks, respectively (Fig. 4A). In addition, the amount of ECM, with respect to collagen and fibronectin, was also enhanced on the tissue culture plastic. Both quantitative (SDS–PAGE; Fig. 4B) and immunostaining (Fig. 4C) of ECM footprints after detergent removal of the cellular layer revealed substantial amounts of collagen and fibronectin deposited directly on the culture plate when cultured in the presence of DxS. There was an almost complete fine granular confluent coverage of the supporting surface, which was contrasted by the small amounts of patchy coverage in its absence. Therefore, the application of EVE is an important tool in creating greater and more confluent ECM coverage of the supporting material. This is particularly important for the 3-D bioreactor setting, where flow rates and scaffold design can generate strong local shear forces which detach cells [19,20]. To attain suitable seeding rates onto scaffolds, incubations are usually first performed under static culture conditions for several days [21,22]. Therefore, speeding up the matrix formation within scaffolds would greatly enhance cell adhesion, survival, migration and biological functionality. We investigated the enhanced deposition of collagen onto 3-D scaffolds through the addition of DxS 500 kDa (100 μg/ml) in the media. Under static culture, the presence of DxS resulted in more than 6-fold increased collagen incorporation into both Biobrane (Fig. 5A) and PLLA scaffolds (Fig. 5B). Under bioreactor conditions, there was a 3-fold increase in collagen matrix formation on the PLLA scaffold compared to the control (Fig. 5B). Of note, although the bioreactor experiments were neither optimized for cell seeding density or flow rate, the beneficial effects of EVE were immediately evident.

### 3.5. Mechanism of EVE

The successful application of EVE in our *in vitro* system can be explained by a spectrum of thermodynamic effects [17] such as driving optimal folding of proteins enhancing their function [23], enhancing enzyme catalytic activity [24], specifically creating substrate–enzyme complexes with longer half-lives [25],

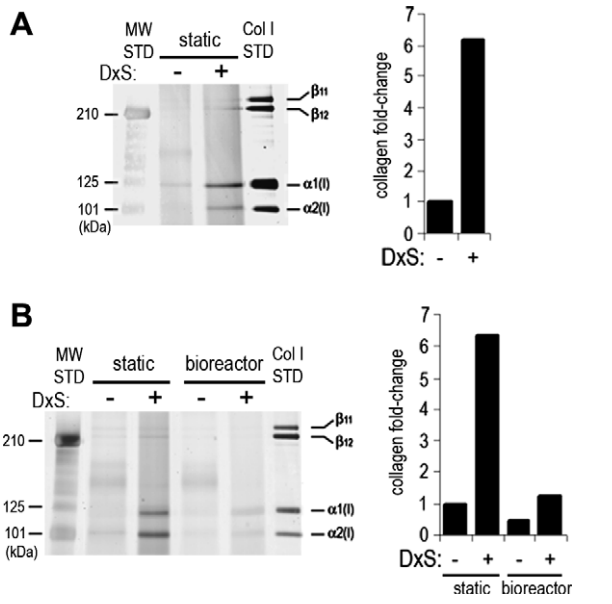


Fig. 5. EVE enhanced matrix deposition onto 3-D scaffolds. (A) Biobrane and (B) PLLA 3-D scaffolds analyzed by SDS–PAGE (in-house) and densitometry of collagen deposition in the absence and presence of DxS 500 kDa (100 μg/m). Biobrane was cultured for 3 days under static conditions whereas PLLA was used under both static (5 days) and bioreactor (5 days) conditions. Fold-change values are relative to static cultures in the absence of DxS.

and enhancing protein aggregation and specific polymerization of monomers into greater order structures [26]. Accordingly, we propose that in our system EVE (1) shifted the enzymatic reaction equilibrium towards procollagen conversion by stabilizing the PCP-procollagen transition complex, (2) caused a tighter or more frequent binding of the enhancer protein (PCOLCE1) to PCP increasing its activity by allosteric regulation, (3) induced proteolytic conversion of PCOLCE1 into a PCP-enhancing fragment and a MMP inhibiting fragment, and (4) promoted self-assembly of the collagen triplehelices into insoluble fibers. Supporting evidence for point 4 comes from groups that have shown that collagen in solution self-assembles faster in the presence of macromolecules [27–29]. However, further work needs to be done to address these issues in more detail.

In conclusion, EVE is an indispensable component of intra- and extracellular biochemistry and can be regarded, in its various forms, as a creator and keeper of organic life through biochemistry. Authorities in the field state that many estimates of reaction rates and equilibria made with uncrowded solutions in the test tube differ by orders of magnitude from those of the same reactions operating under crowded conditions within cells or the extracellular compartment [1,30]. Along this line, our data show that when EVE is applied to a biological system with living cells, its effects are striking and from a biotechnological point of view, highly promising for providing solutions to advanced tissue engineering and certainly any other biological processes that are emulated *in vitro*.

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