Acta Biomaterialia xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Fibril growth kinetics link buffer conditions and topology of 3D collagen I networks

Liv Kalbitzer, Tilo Pompe *

Institute of Biochemistry, Universität Leipzig, Johannisallee 21-23, 04103 Leipzig, Germany

ARTICLE INFO

Article history:
Received 7 August 2017
Received in revised form 14 November 2017
Accepted 28 November 2017
Available online xxxx

Keywords:
Collagen I
3D matrices
Pore size
Fibril diameter
Turbidimetry

ABSTRACT

Three-dimensional fibrillar networks reconstituted from collagen I are widely used as biomimetic scaffolds for *in vitro* and *in vivo* cell studies. Various physicochemical parameters of buffer conditions for *in vitro* fibril formation are well known, including pH-value, ion concentrations and temperature. However, there is a lack of a detailed understanding of reconstituting well-defined 3D network topologies, which is required to mimic specific properties of the native extracellular matrix. We screened a wide range of relevant physicochemical buffer conditions and characterized the topology of the reconstituted 3D networks in terms of mean pore size and fibril diameter. A congruent analysis of fibril formation kinetics by turbidimetry revealed the adjustment of the lateral growth phase of fibrils by buffer conditions to be key in the determination of pore size and fibril diameter of the networks. Although the kinetics of nucleation and linear growth phase were affected by buffer conditions as well, network topology was independent of those two growth phases. Overall, the results of our study provide necessary insights into how to engineer 3D collagen matrices with an independent control over topology parameters, in order to mimic *in vivo* tissues in *in vitro* experiments and tissue engineering applications.

Statement of Significance

The study reports a comprehensive analysis of physicochemical conditions of buffer solutions to reconstitute defined 3D collagen I matrices. By a combined analysis of network topology, i.e., pore size and fibril diameter, and the kinetics of fibril formation we can reveal the dependence of 3D network topology on buffer conditions, such as pH-value, phosphate concentration and sodium chloride content. With those results we are now able to provide engineering strategies to independently tune the topology parameters of widely used 3D collagen scaffolds based on the buffer conditions. By that, we enable the straightforward mimicking of extracellular matrices of *in vivo* tissues for *in vitro* cell culture experiments and tissue engineering applications.

1. Introduction

In vitro cell studies are a common approach to investigate physiological and pathological processes of mammalian cells and tissues. It is well known that biochemical as well as physical parameters of the extracellular matrix (ECM), such as elasticity, topology and dimensionality, control cell behavior (e.g., differentiation, proliferation and migration) [1–4]. Nevertheless, the most abundant substrate for *in vitro* cell studies is the two-dimensional and stiff tissue culture polystyrene, which does hardly describe the native, mostly three-dimensional (3D) environment of cells. Thus, appropriate 3D matrices are needed for a relevant *in vitro* investigation of cell behavior. A requirement for such matrices is that they need to properly mimic the native ECM, which is highly diverse regarding its function and properties.

Various approaches have been used to engineer biomaterials scaffolds to modulate matrix parameters, such as composition, elasticity and topology [5–12]. In order to mimic the overall soft and fibrillar characteristics of many native ECMs, fibrillar biopolymer-based networks, e.g., reconstituted from collagen or fibrin, have been widely used. Due to their native origin, they exhibit many complex biological features, including nanostructure and specific bindings sites [13,14], and they are easily available in large amounts. Collagens are the most abundant proteins in mammals and occur in many native ECMs. Among the different collagen

* Corresponding author.
E-mail address: tilo.pompe@uni-leipzig.de (T. Pompe).

https://doi.org/10.1016/j.actbio.2017.11.051
1742-7061/

types, collagen I (Coll I) is the most prevalent one. It has been widely used and studied as a 3D cell culture scaffold material, as it can be easily reconstituted from tropocollagen into fibrillar networks by self-assembly [8,9,13].

Fibril formation of Coll I starts from self-assembly of tropocollagen, which is a semiflexible triple-helical protein of roughly 300 nm length at physiological buffer conditions. Small non-helical parts at C- and N-termini, known as telopeptides, play an important role during fibrillogenesis and fibril stabilization [15–17]. Besides the collagen type and source, the presence or absence of telopeptides, the type and size of building blocks in the reconstitution solution (differently termed in literature, like nuclei, oligomers, etc.) [17,18], other parameters, such as temperature, pH-value, concentration of specific ions and other biomolecular components, sensitively influence the kinetics of fibril formation as well as fibril morphology and network topology [19–23]. The typical D-staggered arrangement of tropocollagen in Coll I fibrils leads to the natively occurring 67 nm band pattern, but other reconstitution conditions can result into a wide range of other fibrillar forms, which are discussed elsewhere [24–26].

It was previously shown that fibril formation is a multistep process [27,28]. The first step is described as an initiation or nucleation step, which is not considered as rate-limiting for fibril formation. The second step is the formation of thin linear aggregates (termed filaments), which is then followed by a third step of lateral growth of thicker fibrils. Transmission electron microscopy studies revealed a thickness of linear filaments in the range of 4–5 nm. This finding suggested an arrangement of 5 tropocollagen molecules in cross section, which was also found in X-ray studies on early Coll I fibrillogenesis [29,30]. Based on the observation that linear growth precedes lateral growth [27,28,31], it has been suggested that either an activation step or a critical filament length of linear aggregates is required for lateral growth to be initiated [27,28]. At later stages, the consumption and final depletion of linear filaments leads to the termination of lateral growth [27]. Finally, thick and long Coll I fibrils have been formed, ranging from 20 to 800 nm in diameter and tens or hundreds of micrometers in length [32–36]. For a total Coll I concentration above a specific concentration (in the range of 0.5–1 mg/ml, depending on reconstitution conditions) such formed fibrils build up into 3D networks [37].

Although Coll I fibril formation has been extensively investigated over the last decades (see above), it is still not well understood how the physicochemical parameters during fibril formation affect the final 3D network topology, such as fibril diameter and pore size. Although several studies investigated the influence of pH-value [38,39], temperature [31,39] as well as collagen type, source and extracting method [9,18,40] on fibril formation kinetics and/or fibril diameters, a study correlating reconstitution conditions, fibril formation kinetics and 3D network topology over a large range of reconstitution parameters is not available. Additionally, it is frequently difficult to directly compare and integrate different studies that apply Coll I matrices, because of the diverse fabrication procedures, conditions during fibril formation, and the usage of different analytical techniques [9,12]. Accordingly, the knowledge on how to reconstitute well-defined 3D Coll I-based matrices of desired topology for mimicking various native ECMs is needed, which requires a better understanding of the regulation of network characteristics by the reconstitution conditions. In this context, we hypothesize a correlation between kinetics of Coll I fibril formation and network topology, which is controlled by the reconstitution conditions. Therefore, we aimed to reveal the specific dependencies by 1) a comprehensive study on the influence of varying pH-value and phosphate concentration, and the addition of sodium chloride in the reconstitution buffer in combination with 2) an analysis of the kinetics of fibril formation and the resulting topology of 3D Coll I networks over a range of Coll I concentrations. Based on our observations, we were able to reveal the influence of the reconstitution buffer on fibril formation kinetics and correlate this to the resulting network topology in terms of fibril diameter and pore size.

2. Materials & methods

2.1. Preparation & topological characterization of 3 D Coll I networks

For covalent immobilization of 3 D Coll I networks, glass coverslips (13 mm, VWR, international, Leuven, Belgium) were functionalized with poly(styrene-*alt*-maleic anhydride) (PSMA) copolymer as described elsewhere [41]. To prepare Coll I networks of different concentrations (1.0–3.0 mg/ml), Coll I stock solution (rat tail collagen I, BD Bioscience, c = 4.89 mg/ml in 0.02 M acetic acid) was prediluted in acetic acid (0.02 M). Afterwards, 2 parts of prediluted Coll I solutions were mixed with 1 part reconstitution buffer (Table 1). All preparation steps were performed on ice. For network reconstitution, a volume of 30 µl was placed on PSMA coated coverslips and samples were kept at 37 °C (95% relative humidity) for 90 min. After completion of fibril formation, networks were rinsed in phosphate buffered saline (PBS, Biochrome, Berlin, Germany) and kept in a hydrated state at 4 °C up to 5 days for further analysis.

We chose to vary the concentration ranges of the parameters shown in Table 1, because they are most frequently used to influence fibrillogenesis of Coll I *in vitro* [19–23,42]. Furthermore, we specifically observed an influence of phosphate and sodium chloride concentration on network topology in preliminary experiments. Although temperature is another frequently used parameter in similar experiments [28,32,34,42,43], we decided to conduct fibrillogenesis only at 37 °C because lower temperatures (around room temperature) frequently lead to more inhomogeneous network topologies. The range of Coll I concentrations was chosen between 1 and 3 mg/ml, as those concentrations are known to result in stable 3D networks with a pore size in the micrometer range and, thus, nicely mimic *in vivo* ECM.

Topological characterization was performed as described previously [44,45]. Briefly, networks were stained with a fluorescent dye by incubation in a freshly prepared 5-(and-6)-carboxytetramethylrhodamine succinimidyl ester (5(6)-TAMRA-SE, Biotum, Hayward, CA) solution in PBS (50 µM, 1 h, RT). Afterwards, samples were rinsed in PBS twice, incubated in PBS overnight (4 °C) and rinsed again. Imaging was performed using an inverted confocal laser scanning microscope (cLSM, LSM700, Zeiss, Jena, Germany) with a water immersion objective (40×/1.2C-Apochromat, Carl Zeiss). Stacks of 11 images at 5 µm distance (equivalent to a vertical stack size of 50 µm), 1024 × 1024 pixels in xy-resolution, 8-bit color depth and a voxel size of 0.1 × 0.1 × 5 µm (x*y*z) were taken at at least 3 positions per sample. During measurements, samples were kept in PBS in a home-made measuring cell. Pore size and fibril diameter determination were achieved using a slightly adapted imaging analysis script written in MATLAB (MathWorks, MA, USA) introduced by Franke et al. [44]. (Fibril diameter determination

Table 1
Composition, pH-value and ionic strength of the used reconstitution buffers. All buffers were tested with a Coll I concentration between 1 and 3 mg/ml.

phosphate/M	pH	NaCl/M	Ionic strength/M
0.08	7.5	–	0.23
0.08	7.5	0.07	0.30
0.04	7.5	–	0.11
0.04	7.5	0.07	0.18
0.08	7.0	–	0.20
0.08	8.0	–	0.24

Please cite this article in press as: L. Kalbitzer, T. Pompe, Fibril growth kinetics link buffer conditions and topology of 3D collagen I networks, Acta Biomater. (2017), https://doi.org/10.1016/j.actbio.2017.11.051