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Leading Opinion

Electro-spinning of pure collagen nano-fibres – Just an expensive way to make gelatin?☆

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Abstract

Scaffolds manufactured from biological materials promise better clinical functionality, providing that characteristic features are preserved. Collagen, a prominent biopolymer, is used extensively for tissue engineering applications, because its signature biological and physico-chemical properties are retained in *in vitro* preparations. We show here for the first time that the very properties that have established collagen as the leading natural biomaterial are lost when it is electro-spun into nano-fibres out of fluoroalcohols such as 1,1,1,3,3,3-hexafluoro-2-propanol or 2,2,2-trifluoroethanol. We further identify the use of fluoroalcohols as the major culprit in the process. The resultant nano-scaffolds lack the unique ultra-structural axial periodicity that confirms quarter-staggered supramolecular assemblies and the capacity to generate second harmonic signals, representing the typical crystalline triple-helical structure. They were also characterised by low denaturation temperatures, similar to those obtained from gelatin preparations ($p > 0.05$). Likewise, circular dichroism spectra revealed extensive denaturation of the electro-spun collagen. Using pepsin digestion in combination with quantitative SDS-PAGE, we corroborate great losses of up to 99% of triple-helical collagen. In conclusion, electro-spinning of collagen out of fluoroalcohols effectively denatures this biopolymer, and thus appears to defeat its purpose, namely to create biomimetic scaffolds emulating the collagen structure and function of the extracellular matrix.

Keywords: Collagen denaturation; Gelatin; Denaturation temperature; Second harmonic generation; Transmission electron microscopy; Circular dichroism

☆ *Editor's Note:* Leading Opinions: This paper is one of a newly instituted series of scientific articles that provide evidence-based scientific opinions on topical and important issues in biomaterials science. They have some features of an invited editorial but are based on scientific facts, and some features of a review paper, without attempting to be comprehensive. These papers have been commissioned by the Editor-in-Chief and reviewed for factual, scientific content by referees.

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1. Introduction

Collagen type I accounts for up to 70–90% of the collagen found in the body and it is present in the form of elongated fibres in various tissues. Individual fibrils can be greater than 500 μm in length and 500 nm in diameter [1,2]. These building blocks are rod-like triple helices that are stabilised by intra-molecular hydrogen bonds between Gly and Hyp in adjacent chains [3–6]. Tissues rich in fibrous collagen such as skin and tendon are generally used to extract collagen. Dilute acidic solvents are used to break intermolecular cross-links of the aldimine type, whilst proteolytic enzymes, such as pepsin, are used to cleave the more stable cross-links of the keto-imine type. Pepsin cleaves only the non-triple-helical *C*- and *N*-telopeptides, leaving the triple-helical molecule intact [7–9]. Extracted collagen from either of the above preparations is favoured for biomedical applications, since *in vitro*, under appropriate conditions, will spontaneously self-assemble to form biodegradable and biocompatible insoluble fibrils of high mechanical strength, low immunogenicity and with a *D*-periodicity indistinguishable from that of native fibres [10–14].

Electro-spinning has been recently introduced as the most promising technique to manufacture *in vitro* fibrous scaffolds for tissue engineering application with fibre diameter ranging from a few microns to less than 100 nm. Such materials aim to mimic extracellular matrix components, such as collagen fibrils whose diameter *in vivo* range from 20 nm to 40 μm [15–17]. Currently, the most widely adopted method involves the electro-spinning of pure collagen or collagen-poly(ε-caprolactone) blends out of highly volatile fluoroalcohols such as 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) [18–25] or 2,2,2-trifluoroethanol (TFE) [24,26]. However, it has been shown earlier with non-collagenous proteins that fluoroalcohols not only denaturate the native structure, but also lower the denaturation temperature [27–29]. Moreover, in a recent publication, it was shown that 45% of collagen was apparently lost during electro-spinning [30]. Additionally, electro-spinning of collagen using either HFP or TFE has been reported repeatedly to yield collagen nano-fibres that do not swell when in aqueous media like other collagenous structures [31–33], but instead are readily soluble in water, tissue fluids or blood [20,22,24,34–36]. Since gelatin is the water-soluble degradation product of the originally water-insoluble collagen fibril [37], the observed water solubility of the electro-spun collagen scaffolds might point to an extensive conformational change. Given, the above, our hypothesis is that through the electro-spinning process, denaturation of collagen takes place and gelatin is created.

To verify our hypothesis, we conducted a series of specific experiments that distinguish collagen from gelatin. Collagen is a crystalline [38–41] (second harmonic generation experimentation), triple-helical molecule [2–6,31] (circular dichroism experimentation), whilst gelatin is characterised by destroyed α-chains, disrupted triple-helical and fibrillar structure and lacking internal structure or configurational order [37]. Moreover, the collagen fibrils possess a high degree of axial alignment and exhibit a characteristic *D* banding (the finger print of fibrous collagens), which results from alternating overlap and gap zones, produced by the specific packing arrangement of the 300 nm long and 1.5 nm wide collagen molecules. This produces an average periodicity of 67 nm in the native hydrated state [1,3,4,12,14,31,33,42–45], although dehydration and shrinkage during conventional sample preparation for transmission electron microscopy results in lower values of around 55–65 nm [1,44] (transmission electron microscopy experimentation). Furthermore, the denaturation temperature of collagen is higher than the denaturation temperature of gelatin [31,46–52] (differential Scanning calorimetry experimentation). Most importantly, the tight triple-helical structure of the collagen molecule makes it resistant to pepsin or trypsin, unless its folding is locally compromised by either point mutations or heat denaturation [53] (pepsin digestion and SDS-PAGE experimentation). Such molecules are unstable at physiological temperatures and they are degraded intra-cellularly [14]. Based on all the above, we demonstrate for first time that the electro-spun collagen scaffolds are not crystalline; are not triple-helical; are not quarter-staggered arranged; have denaturation temperature lower than or similar to gelatin; and are pepsin susceptible. Freeze-dried collagen dissolved in HFP and freeze-dried again (HFP-recovered collagen) also exhibited similar properties with those obtained from gelatin preparations, clearly indicating that fluoroalcohols are the major cause of denaturation. Taken together, this builds up the strongest evidence that electro-spinning of collagen or co-spinning of collagen-synthetic polymers out of fluoroalcohols results in the creation of gelatin, a protein derived from denatured collagen and is characterised by destroyed α-chains, disrupted triple-helical and fibrillar structure and lacking internal structure or configurational order [37].

2. Materials and methods

2.1. Materials

Porcine skin type A and bovine type B gelatin were obtained from Sigma–Aldrich (Singapore). Purified type I freeze-dried bovine dermal atelocollagens were obtained from Koken Co. (Japan) and Symatese Biomateriaux (France). In-house type I atelocollagen from porcine Achilles tendon was extracted as has been described previously [54]. Medical grade poly(ε-caprolactone) (mPCL) was purchased from Birmingham Polymers Inc (USA). Rat-tail tendons and normal human skin were used as representatives of native assemblies. Unless noted otherwise, all chemicals and reagents were purchased from Sigma–Aldrich (Singapore).

2.2. Nano-fibre fabrication through electro-spinning

Typical protocols for the electro-spinning were used based on previous publications [18,19,21,24]. The following preparations were investigated: (a) in-house, Koken and Symatese collagens and Sigma gelatin type A and B dissolved in HFP at 50 mg/ml concentration; (b) Koken collagen dissolved in HFP and TFE at 180 mg/ml concentration; and (c) in order to investigate whether blending and consequent co-spinning of collagen with mPCL could prohibit or restrict the denaturation of collagen mPCL–Symatese collagen and mPCL–Sigma gelatin type A and B blends (5:1 ratio) and mPCL dissolved in HFP at 125 mg/ml concentration. Either of the above solutions was loaded into a syringe pump (KD-Scientific, USA), which was set at 0.75–1.2 ml/h. Upon application of high voltage (10–15 kV; applied current was below 1 μA) (Gamma High Voltage Research, USA) between the syringe