Electroconductive nanoengineered biomimetic hybrid fibers for cardiac tissue engineering†

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We report for the first time the preparation of a fibrous material composed of surface grafted spherical nanosilver and collagen using one-step electrospinning. The resulting composite showed comparable morphology to the control without nanosilver, but had improved electrical conductivity. Under electrical stimulation, fibrous materials containing nanosilver increased connexin-43 expression and proliferation of neonatal cardiomyocytes. Furthermore, composites containing nanosilver prevented biofilm formation but did not activate macrophages.

The rational design of composites for regenerative medicine must balance the safety of the resulting material while retaining the nanostructure activity once incorporated within the composite.¹ The development of hybrid materials where nanostructures are linked to a biomolecule presents numerous advantages over synthetic polymers, including the reduction of immune responses and improved biointegration. Due to the limited regenerative capacity of cardiac muscle, massive tissue loss in the heart can occur following ischemic injury,² leading to complications in affected patients. The field of tissue regeneration has not been left out of the ‘fever’ of using nanoparticles for regenerative medicine,³ and including cardiac tissue,⁴–⁶ where gold,⁷–ⁱ⁰ has been used for boosting the electrical properties of non-conductive polymers,¹¹ as a strategy to stimulate functional muscle regeneration.

Most studies completed to date have used non-fibrous matrices combined with nanoparticles, added to create a functional structure. For example, metal nanoparticles and carbon nanotubes have been shown to increase the conductivity of biomaterial scaffolds.¹²,⁶,⁸ Other authors have indicated that inherent anti-oxidant properties of some nanoparticles could be beneficial for cell survival under conditions of oxidative stress within the damaged cardiac muscle.¹² Other approaches have used micro-sized ‘cable’-like structures of silver for improving the electrical conductivity of a synthetic polymer.¹³ However, all of these technologies do not provide a biodegradable material with a composition similar to the natural extracellular matrix found in the myocardium, i.e. fibrous collagen structures. Further, the implantation of biomaterials for cardiac tissue engineering needs to consider bacterial colonization¹⁴,¹⁵ and should not induce an inflammatory response.¹⁶

In the present contribution, we have developed a multi-functional, biomimetic, collagen-based fibrous material containing spherical nanosilver (AgNPs),¹⁷ a better electrical conductor than gold, which have also displayed non- or anti-inflammatory,¹⁸,¹⁹ and antibiofilm/antimicrobial properties.²⁰–²³ Our composite material, in comparison to other hybrid fibrous materials,²⁴ was prepared in a single step using collagen protected-spherical nanosilver that was crosslinked in situ and electro-aligned to form stable and active fibrous structures. Further, our composite material presents a new paradigm in the development of electroconductive structures for cardiac tissue engineering, where only micromolar concentrations of nanosilver produced an increment in the macroscopic properties of the composite.

Fig. 1a shows a schematic representation of collagen fiber preparation using a custom-made electrospinning device. Note that collagen capped AgNPs were freshly prepared, and freeze-dried prior to spinning. Mixtures of pure collagen and collagen capped AgNPs were dissolved in 2,2,2-trifluoroethanol and carbodimide crosslinkers added (EDC = 320 mM and NHS = 260 mM). The resulting mixture was delivered at a rate of 3.33 µL min⁻¹ and fibers were collected on a rotary collector operating at 400 rpm. This protocol produces stable fibers that did not require additional chemical treatment after formation to prevent their melting in phosphate buffer at physiological temperature (Fig. S1, ESI†). A representative scanning electron microscopy (SEM) image for...
The effect of increasing concentrations of collagen capped AgNPs on the width of the generated fibers was determined (Fig. 1b, bottom). Concentrations of AgNPs > 7.0 μM reduced the fiber width by ≈50%, which could be attributed to changes in the collagen fiber assembly due to electrostatic repulsion secondary to increasing concentration of AgNPs, which are positively charged.25 Interestingly, no difference in width was observed between the fibers with 7.0 μM AgNPs compared to those with no AgNPs (p < 0.05). Therefore, to isolate the electroconductive effects, for the rest of this study we focused on the AgNPs concentration that did not affect the width of the native collagen fibers and/or the denaturation temperature once assembled (≈190–195 °C, Fig. S2, ESI†). However, type I collagenase degradation of the materials containing nanosilver was reduced by half (2.5 ± 0.41 vs. 1.2 ± 0.14% min⁻¹, respectively).

The presence of nanosilver within the collagen structure was also assessed by high-resolution SEM imaging. Samples were coated with a 5.0 nm carbon layer before imaging (Fig. 1c, top left). Backscattering images indicate the presence of the metallic nanoparticles (Fig. 1c, top right), which corresponds to silver, as shown by energy-dispersive spectroscopy (EDS; Fig. S3, ESI†). During SEM imaging of the fibers containing AgNPs, we observed different focal planes for the AgNPs, which suggests that AgNPs are distributed within the various layers of the bundled collagen fibers, and not exclusively on the fiber surface. In terms of size, AgNPs had an average diameter of 20 ± 8.0 nm, which is within the range measured for AgNPs resuspended in TFE (10 ± 5.0 nm; Fig. 1c, bottom). This indicates that our protocol for preparing the collagen fibers containing AgNPs does not alter the nanoparticle size distribution.

Upon incorporating AgNPs within the collagen fibers, the characteristic surface plasmon band from spherical nanosilver particles was observed (≈410 nm max; Fig. 1d, left). The intensity of this color has been used as an indirect measurement of nanoparticle stability and surface composition.22,25

![Figure 1](image-url)
Fig. 2  (a) Schematic representation of the main steps used to prepare collagen fibers for cell culture. (i) Image of the fiber collector. White arrows indicate the areas where fibers are collected forming a 'bridge' between them. (ii) Representative image of the collagen fibers prepared. (iii) Image of fibers being collected. (iv) Collagen fibers layered onto a glass slide (left) compared to a clean glass section (right). (b) Left: Simplified scheme for cardiomyocyte isolation from 2 day old rats. Middle: Bottom, microscopic image shows freshly seeded cells onto collagen fibers; and at top, an image of the same field after 12 h incubation. Red arrows indicate examples of elongated cardiomyocytes attached to the fibers. Right: Shows the electrical stimulation device used for culturing the attached cells for 24 h. The bottom image shows a bottom view of the plate where the two carbon electrodes that provided the voltage are seen. Cell pacing provided an electrical stimulation equivalent to ≈ 300 beats per min. (c) Confocal fluorescence microscopy images for neonatal cardiomyocytes seeded onto collagen fibers with and without AgNPs. Cells were submitted to electrical stimulation for 24 h (1 V, 5 ms pulse duration and 5 Hz frequency). After fixation, cells were stained for nuclei (DAPI), alpha-sarcomeric actinin (α-SA) and connexin-43 (Cx43). Images of the merged channels are also provided. Scale bar = 50 μm. (d) Left: Number of Cx43 + foci per cell for cells exposed to CPACE or non-electrical stimulation. In all cases > 50 cells were counted from different areas and from at least four slides, from different experiments of the same group. Error bars correspond to standard deviations. P-values were calculated from t-tests for 2 groups with unequal variance. Control groups were cells seeded onto cell culture treated glass slides. Right: Average Cx43 intensity/cell for neonatal cardiomyocytes analyzed by flow cytometry. Error bars represent standard deviation (n = 3), obtained from three independent samples. P-values were calculated from t-tests for 2 groups with unequal variance.
Overall, AgNPs within the fibers remained stable (~30% decrease) in PBS buffer at 37 °C (Fig. 1d, left inset). Regarding the secondary protein structure within the assembled fiber, the presence of amide I (~1623 cm⁻¹, C=O stretching), amide II (~1550 cm⁻¹, N–H stretching), amide III (~1200 cm⁻¹, C–H stretching), amide A (~3300 cm⁻¹, free N–H stretching) and amide B (~2960 cm⁻¹, asymmetrical CH₂ stretching), are indicators that the collagen fiber structure is persevered upon incorporation of AgNPs.27,28 Further, Raman spectra for the collagen fibers showed all the features below 1000 cm⁻¹ representative of collagen fibers.29 However, the presence of AgNPs enhances the signals at 3030 cm⁻¹ (CH-stretching), 1606 cm⁻¹ (COO-asymmetric vibration), and 1348 cm⁻¹ (CH-stretching). This enhancement can be attributed to surface enhancement of the Raman scattering signal, a.k.a. SERS, elicited by the near proximity of the nanosilver within the collagen fibers.30 This observation is also an evidence of the structural incorporation of the nanoparticles within the collagen fibers.

Atomic force microscopy (AFM) of the collagen fibers was also performed to gain further insight into the nanomechanical properties of the materials with and without AgNPs. Fig. S4a (ESI†) shows characteristic stiffness curves measured by AFM for the samples with and without AgNPs, a representative AFM image for pure collagen fibers is shown in Fig. S4b (ESI†). Stiffness values calculated for the fibers containing AgNPs were ~15% higher than the control group (0.0018 ± 1 × 10⁻⁵ and 0.0021 ± 1 × 10⁻⁵ μN nm⁻¹ for control and AgNPs samples, respectively). However, such differences are within the experimental error of the technique, and we cannot therefore conclude that the addition of Ag impacted the nanomechanical properties of the composite. Our physical characterization also included measuring electrical conductivity for the fibers using a 4-electrode system. Fig. 1e (top) shows a representative image of the fibers with and without AgNPs that were used for measuring electrical conductivity. These images clearly show the yellow color provided by AgNPs when incorporated within the collagen fibers. The addition of AgNPs increased the electrical conductivity of the fibers by eight times (Fig. 1e, bottom).

In vitro cell compatibility for the materials was assessed using freshly isolated neonatal rat cardiomyocytes31 (see ESI† for further details). Fig. 2a summarizes the main steps involved in preparing fibers for in vitro compatibility testing. Freshly isolated cardiomyocytes were then seeded onto the collagen fibers at a density of 40 000 cells per cm² and incubated for 12 h. After the 12 h incubation with no pacing, we observed that cells were attached to the fibers and had become elongated (red arrows in Fig. 2b). Cells were then placed in the C-PACE EP device and submitted to electrical stimulation (5 Hz at 1 V) for 24 h.

Regarding the anti-bacterial capabilities, collagen fibers containing silver nanoparticles prevented the formation of Pseudomonas aeruginosa biofilms (Fig. S6, ESI†). Immunologically, when comparing macrophage polarization between the collagen fibers without AgNP to those with the AgNPs, no differences were observed in the proportion of M1 and M2 macrophages (Fig. S7, ESI†).

Conclusions

In summary, collagen-fibers containing spherical nanosilver prepared using electrospinning had improved electrical conductivity that enhanced connexin-43 and proliferation of neonatal rat cardiomyocytes under electrical stimulation. Our materials also prevented biofilm formation, compared to fibers without nanosilver. Although further experimentation is still required, including an in vivo tests and assessment of the effect of nanosilver size and shape on the composite conductivity, our materials are the first generation of biomimetic collagen fibers where micromolar concentrations of nanosilver suffice for improving the biophysical properties of collagen fibers for better supporting cardiomyocytes and preventing bacterial infection.

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## References


