Self-Assembled Collagen Microparticles by Aerosol as a Versatile Platform for Injectable Anisotropic Materials

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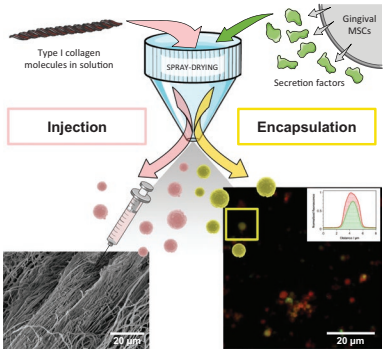

An innovative spray-processing approach enables fast and cheap production of anisotropic microparticles containing >90 wt% type I collagen. Their injection in a biological medium results in the formation of fibrils retaining innate molecular characteristics while forming ordered, organized structures at higher length scales. The encapsulation of temperature-sensitive stem-cell products demonstrates the versatility of the approach for tissue regeneration applications.

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Extracellular matrices (ECM) rich in type I collagen exhibit characteristic anisotropic ultrastructures. Nevertheless, working in vitro with this biomacromolecule remains challenging. When processed, denaturation of the collagen molecule is easily induced in vitro avoiding proper fibril self-assembly and further hierarchical order. Here, an innovative approach enables the production of highly concentrated injectable collagen microparticles, based on collagen molecules self-assembly, thanks to the use of spray-drying process. The versatility of the process is shown by performing encapsulation of secretion products of gingival mesenchymal stem cells (gMSCs), which are chosen as a bioactive therapeutic product for their potential efficiency in stimulating the regeneration of a damaged ECM. The injection of collagen microparticles in a cell culture medium results in a locally organized fibrillar matrix. The efficiency of this approach for making easily handleable collagen microparticles for encapsulation and injection opens perspectives in active tissue regeneration and 3D bioprinted scaffolds.

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Tissue repair is a priority need of any living tissue or organ for recovering functional properties after injury. The repair process is a complex phenomenon that involves soluble mediators, blood cells and components of the extracellular matrix (ECM), especially the cells from the tissue itself such as fibroblasts. Collagen is the major insoluble fibrous protein in the ECM of connective tissue. Type I collagen being one of the most abundant, many methods have been proposed as scaffold for tissue engineering[1] and for its formulation in an attempt to control the release of active molecules.[2] Formulations of beads are mainly based on techniques of emulsion in a water/oil[3] or water/organic solvent mixture[4] under conditions in which proteins are not denatured. However, traces of oil or solvents are associated with the beads formed.[5] An alternative technique is the thermally induced phase separation (TIPS), in which the drops of the solution are introduced into a bath of liquid nitrogen followed by a lyophilization step.[6] Complex coacervation can also be used to produce collagen beads in a controlled manner[7,8] by taking advantage of the polyelectrolyte nature of the collagen triple helix. In the cited cases, the fragility of collagen beads requires a crosslinking step to reinforce their mechanical properties, by chemical agents (such as aldehydes or carbodiimides) bringing potential toxicity. More recently, the use of microfluidic devices,[9] 3D printers[10] and even soft robotics[11] enabled the production of uncrosslinked collagen beads for encapsulation. However, the inner structure of these collagen microparticles remains porous unlike that of the ECM. Taking into account that autologous tissue grafts remain the gold standard as they possess all the properties required for new tissue growth (including composition, ultrastructure, and mechanical properties), the use of native collagen molecules to reach 3D biological-like ultrastructure appears as an important attribute to build efficient biomaterials for tissue engineering.[1,12]

Type I collagen solutions are classically obtained in vitro from living tissues (tendons, dermis) placed in acidic media. Dilute dispersions of collagen triple stranded molecules at low concentrations (≈1 mg mL^{-1}) can be further stabilized in vitro to form hydrated gels used in interaction with cells or tissues.[1] However these loose matrices neglect the fundamental

structure-function relationship of connective tissues. Indeed, these conditions lead to a large volume of extrafibrillar space while type I collagen-based ECM are most often dense and organized, forming complex scaffolds providing specific function to the tissue. It was shown that beyond a critical concentration, molecules in solution spontaneously self-organize to form ordered liquid crystalline phases.[13] A sol–gel transition is induced by a rise in pH, stabilizes the mesophase geometries, ending in biomimetic fibrillar organizations.[14] Mechanical properties of these biomaterials allow their use without requiring any additive, rendering their application non-toxic. Besides, mimicking the 3D ECM organization could promote a more physiological response of mesenchymal stem cells (MSCs);[15] such behavior was demonstrated in particular for osteoblasts[16] and fibroblasts.[1] This shows the interest of working at higher collagen concentrations.[1]

Aside the scaffold, another strategy for tissue regeneration is the on-site delivery of bioactive products such as MSCs secretion factors.[17] The secretion of a broad range of bioactive molecules by MSCs, such as growth factors, cytokines and chemokines, constitutes their most biologically significant role under injury conditions. The production of active secretion products suited to their therapeutic use, and their delivery implies to minimize the extent of cell death in the vicinity of injured areas but also the preservation of their phenotype against possible mutations or differentiation process. Hence, conditioned medium (CM) composed of the secretion products of MSCs from gingival connective tissue (gMSCs),[18] instead of the whole cells was used as an alternative. CM of gMSCs was previously shown to have a similar effect as gMSCs on cell differentiation.[19] Gingival tissue is known to heal rapidly and without scarring; CM of gMSCs thus appears as a good candidate to promote wound healing.

Based on the cited knowledge, an approach combining both strategies appears as a promising new therapeutic way for efficient tissue regeneration. Herein, we present a one-pot aerosol-assisted processing strategy leading to the easy, cheap and fast continuous production of highly concentrated (>90 wt%) type I collagen microparticles, encapsulating (or not) temperature-sensitive bioactive molecules. The spray-processing approach adopted consists in atomizing a diluted acid-soluble collagen solution in order to form a mist of very thin droplets, immediately dried by evaporation of their solvent in a controlled atmosphere (thanks to the high solution/air interface area of the droplets). The concentration in the collagen drops is high enough to induce the self-assembly of collagen molecules and a subsequent liquid crystal order, i.e., nematic oriented domains. This strategy allows obtaining within seconds highly concentrated collagen microparticles circumventing the high increase of viscosity of type I collagen solutions that usually prevents fast processing of this protein, and consequently its use at biological concentration. A fine control of both formulation and processing parameters (such as the temperature) to prevent denaturation of the biomolecules ensure the encapsulation of gMSC secretion products for further injection, and provide evidence to consider whole-cell encapsulation. The features of the injected material resemble that of living dermis tissue, with local anisotropy and fibrils organized in bundles. The design of such new functional cell-free capsules broadens the range of strategies available for biotherapy and opens perspectives for building through collagen injection[20] anisotropic 3D scaffolds with hierarchical order from the fibril, to bundles, to the material scale.

Collagen Microparticles Preparation: The spray-drying device available in the laboratory (see the Experimental Section) is commonly used for producing organic, inorganic or hybrid (organic/inorganic) microparticles.[21] The spray-drying set up (**Figure 1**a) was first optimized to obtain dense type I collagen microparticles; the main drawback being the risk of irreversible thermal denaturation of collagen into gelatin as mentioned above. For this purpose the following parameters: solution flow, air flow, aspiration and temperature were chosen as follows. The spraying step was conducted using initial concentrations of acid soluble collagen between 0.7 and 5 mg mL^{-1} to limit the solution viscosity and promote a steady flow. Indeed a high concentration of collagen in solution can even lead to the formation of a physical gel[22] that could hinder both flow and formation of droplets. The drying step is strongly dependent on the solvent evaporation time, which is in turn related to both the temperature of dry air injected in the chamber and the aspiration rate. The dry air and nozzle temperature were fixed at 35 ± 1 °C. This temperature range is low enough to prevent protein denaturation but high enough to induce the solvent (acetic acid in water) evaporation in the droplets and reach the cyclone particle collector in dry form. Overall, the drying process takes no more than 4 s. Figure 1b,c illustrates the collected collagen microparticles obtained from different initial concentrations of type I collagen solutions as observed by scanning electron microscopy (SEM) (see also Figure S1, Supporting Information). The observation shows that the selected processing conditions lead to the precipitation of micron-sized collagen particles. Each droplet of solution leading to one dry microparticle, the initial concentration of the collagen solution appears to influence the size of the microparticles and the polydispersity, both increasing at higher concentrations. This trend was confirmed by performing laser granulometry (Figure 1d). The diameter distribution reveals two main populations in counts for the lowest and highest investigated concentrations, the first one at 0.6 ± 0.3 and 0.6 ± 0.1 µm, respectively, and the second one at 0.9 ± 0.2 and 1.3 ± 0.6 µm, respectively. A third population appears at 5.2 ± 0.5 µm for the highest concentration only. This range of diameters is consistent with works in the literature where microparticles were obtained by spray-drying chitosan.[23] We identified the importance of the initial collagen concentration, emphasizing the versatility of the process for targeting different uses where the bead size may play an important role in adjusting release kinetics, as in drug or biomolecules controlled release systems.

Ultrastructure of the Collagen Beads: Type I collagen possess lyotropic properties in vitro in acidic conditions characterized by the occurrence of mesophases above 40 mg mL^{-1}.[24] As a consequence of the collagen concentration during solvent evaporation, self-organization of the molecules may occur in the droplets during the drying step. Polarized light microscopy (PLM) observations (Figure 1e and Figure S2, Supporting Information) show optical birefringence of the collagen microparticles suggesting a local ordering of the collagen molecules or fibrils. The resolution of the PLM being in the same order

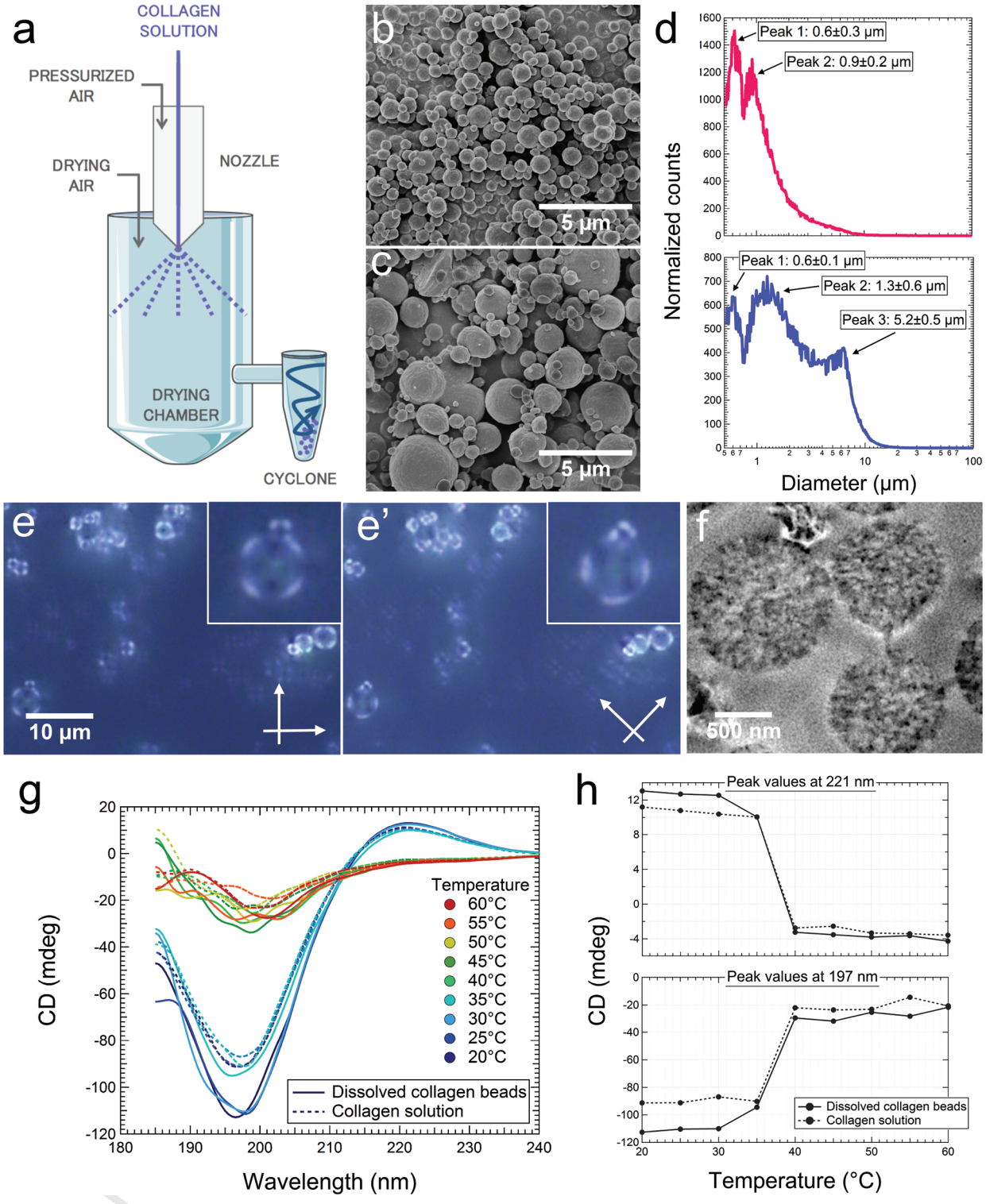

Figure 1. a) Schematics of the spray-processing of a collagen solution. b,c) Scanning electron microscopy images of pure collagen beads from spray-dried collagen solutions at 0.7 and 5.0 mg mL^{-1}, respectively. d) Normalized diameter distribution in counts of collagen microparticles from spray-dried collagen solutions at 0.7 mg mL^{-1} (upper graph) and 5.0 mg mL^{-1} (lower graph). e) Under crossed polarizers, collagen microparticles display birefringence and dark domains (along the direction of the polarizers: 0 and 90°) inside and on their surface e') that turn to bright after a 45° rotation of polarizers. f) Ultrathin stained transmission electron microscopy image of pure collagen beads. g) Circular dichroism of a collagen solution (dashed line) and of a solution made of dissolved collagen beads (solid line) at the same concentration and in the same solvent (acetic acid at 0.5 M). h) Average ellipticity at 221 nm (upper graph) and 197 nm (lower graph). The difference between the peak values of the two solutions at low temperature can be attributed to slight differences in pH.[36]

of magnitude than that of the diameter of the microparticles, it is difficult to identify a specific birefringence texture. Nevertheless, after a 45° rotation of the polarizers (Figure 1e'), extinction of light occurs in areas where the collagen molecules lie parallel to the direction of the polarizers indicating a probable nematic alignment all over the surface of the largest microparticles (see the inset). SEM observations at higher magnification provided no evidence of fibrillogenesis in vitro (Figure S3a, Supporting Information). Thus we investigated transmission electron microscopy (TEM) (Figure 1f) to conclude on the fibrillar nature of the microparticles and on their possible local organization. Local order at such scale is considered as possible since it was previously reported for spray-dried chitin-silica particles where chitin possesses as well liquid crystal properties.[25] In our case, fibrils are not observed (Figure 1f and Figure S3b, Supporting Information), even though the high collagen concentration in the microparticles can induce the formation of few collagen fibrils.[26] However, their observation is only possible if the main axis of the fibril is oriented along the section plane. As collagen molecules are evenly distributed all throughout the microparticles (no detection of hollow microparticles), the presence of some small fibrils cannot be totally excluded. Finally, although it remains difficult to assess precisely the local molecular order in the microparticles (diameter of the beads versus resolution of the optical microscope), it seems that the molecules assembly, preferentially driven by thermodynamics, is limited by the fast drying kinetics. Indeed, in addition to the size of the domains (restricted by the dimensions of droplets), the fast solvent evaporation may prevent the formation of higher ordered mesophases (such as cholesteric phase).

Physico-Chemical Characterization of the Collagen Microparticles: To assess the water content of the pure collagen beads, we performed dynamic vapor sorption (DVS) experiments. A typical isothermal curve[27] was obtained with reduced hysteresis (Figure S4, Supporting Information). At ambient temperature and humidity conditions, 25 °C and 40%RH respectively, the microparticles contain less than 10 wt% water (thus more than 90 wt% type I collagen). Differential scanning calorimetry (DSC) experiments performed on the initial collagen solution and on the dissolved collagen microparticles in acetic acid at 500×10^{-3} M show the same endothermic peak at 40 °C (Figure S5, Supporting Information). At this temperature, collagen denaturation into gelatin occurs through the irreversible unfolding of the triple helix.[28] In addition, circular dichroism (CD) experiments (Figure 1g,h) show typical sigmoidal-shaped curves[29] with a large negative peak around 221 nm and a smaller positive peak around 200 nm characteristic of triple-helix conformation.[30] These results confirm that the non-fibrillar collagen microparticles observed by TEM (Figure 1f) is indeed made of collagen molecules and not gelatin, which is also in agreement with the observation of birefringence by PLM (Figure 1e,e').

Overall, these results prove that the process is compatible with the use of thermo-sensitive biomolecules. The suitability of dense collagen microparticles for encapsulation was then investigated.

Encapsulation of gMSCs Secretion Factors in Collagen Microbeads: In order to minimize the number of synthesis steps, we adapted our preparation strategy to obtain simultaneous aerosolization of collagen solution and CM composed of the secretion products of gMSCs. A first limitation is related to the risk to induce the thermal denaturation of the CM components during spray-drying. However, given the gMSCs culture conditions (i.e., 37 °C) it is reasonable to assume that the secretome will not be denatured since the previously established collagen processing conditions lie below this temperature. In addition to the temperature, a limitation was the difference in pH of the CM (pH ≈ 7) with the collagen solution (pH ≈ 3.5). It is worth mentioning that such a low pH should not interfere with the biomolecules activity since it is described to be involved in the promotion of wound healing.[31] Yet, in order to limit the pH stress for the secretome, we adapted the atomization process by using a three-fluid nozzle (able to mix two solutions at the extreme tip of the nozzle and to atomize it at the same time) to limit the contact time between the two solutions (**Figure 2**a,a' and see the Experimental Section). The use of a coaxial nozzle to produce particles from two liquids simultaneously allows the control of the average composition of the produced particles. However, we needed to ensure that each particle was composed of both components; the CM could be found only at the surface of the collagen particle (which would limit the intended effect) or not interacting at all with collagen. Microparticles issued from two different collagen:CM volume ratios were produced (1:1 and 1:3) (**Table 1**). Observations by SEM (Figure 2b,c) show that the particles display the same morphology as those previously obtained from collagen only. When imaged under the confocal microscope, collagen:CM particles present extensive colocalization of signal intensity of both fluorescence channels, which confirms the effective encapsulation of CM within the collagen particles (Figure 2d–g). The profile integration (Figure 2e'–g') of three individual particles, chosen according to the individual channel intensity confirm the presence of both fluorophores in each of the analyzed individual particle. The shape of the profile does not indicate any particular segregation between the two components, suggesting the CM to be evenly distributed within each microparticle. This indicates that i) there is no macroscopic phase segregation between collagen and the CM and ii) the encapsulated medium does not destabilize the collagen packing.

Therapeutic applications through cell-free administration of secretome remain challenging.[15] In order to assess the capacity of the loaded microparticles to release bioactive factors in a physiological environment, the microparticles were suspended in a phosphate buffer at 37 °C and collected at different timespans. Two molecules of interest were targeted for their potential efficiency in ECM regeneration: HGF (hepatocyte growth factor) and VEGF (vascular endothelial growth factor).[32] The kinetics of the release was characterized by performing an ELISA test, and shows an increasing release rate until 6 h for both HGF (Figure 2h) and VEGF (Figure 2i) and a decrease until 24 h, with the complete release of VEGF within the explored timespan. Such kinetics could help better tissue repair by recruiting and activating host cells during the healing process. The coupling of collagen microparticles and gMSCs secretion products, both with central roles in the healing process, was successful. This led us to investigate the possibility to use them as an injectable suspension since administration

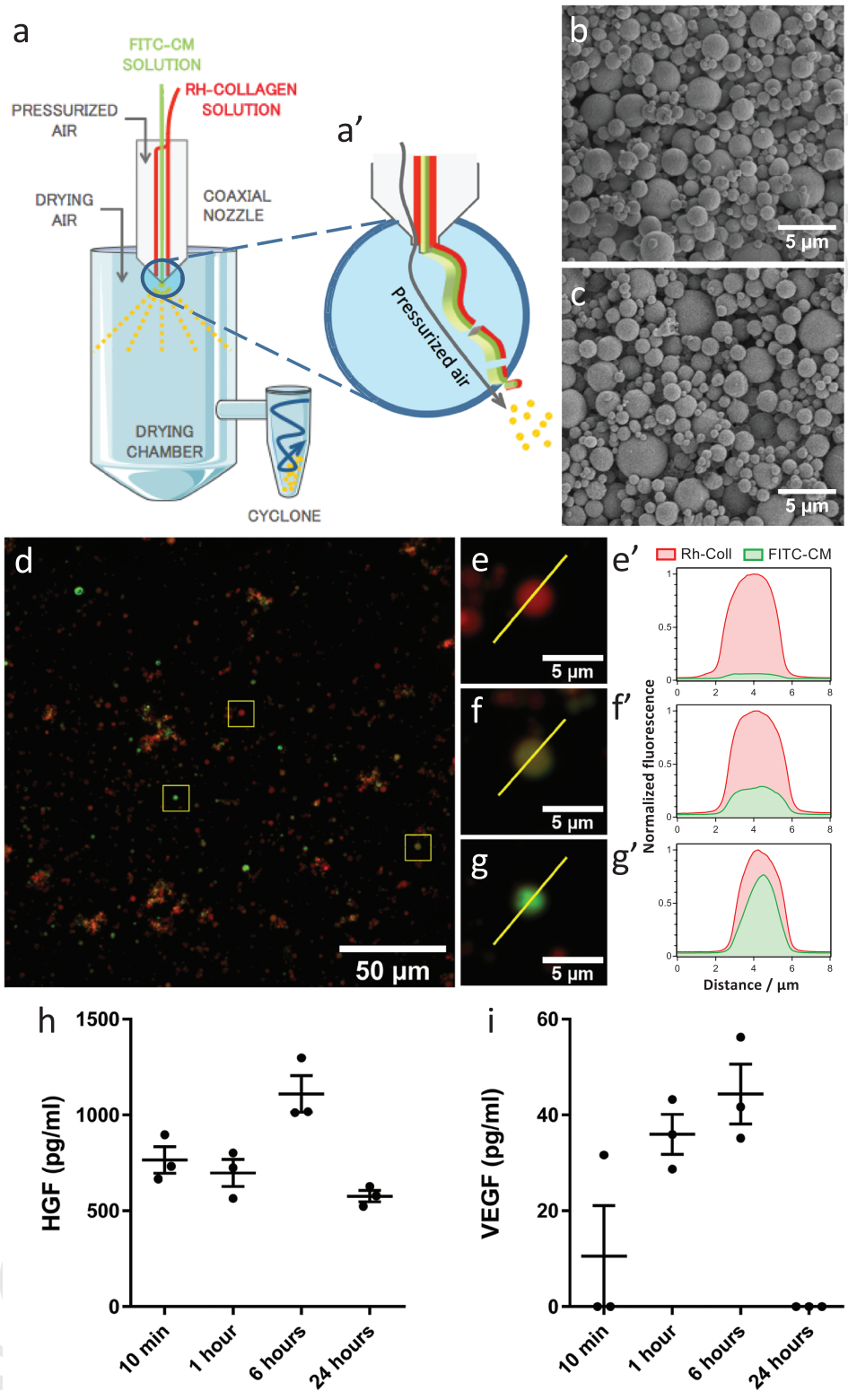

Figure 2. a) Coaxial nozzle spray-drying of acid soluble RITC-tagged collagen solution (Rh-Coll) and gMSC derived fluorescein-tagged conditioned medium (FITC-CM). a') Solutions are mixed upon air shearing at the tip of the nozzle. b,c) SEM images of Coll/CM beads obtained at 1:1 and 3:1 volume ratio, respectively. d) Confocal microscopy images of Rh-Coll/FITC-CM particles with sum z-projection of red and green channels of 52 image planes. e–g) Detail of selected particles and profile line analyzed. e'–g') Red and green channel plot profiles of particles depicted in (e)–(g), respectively. (h) and (i) release kinetics of growth factors of interest from encapsulated conditioned medium in collagen microparticles (respectively, HGF and VEGF) in a phosphate buffer after 10 min, 1, 6, or 24 h at 37 °C.

Table 1. Spray drying conditions of Coll/CM mixtures.

Volume ratios	[Rh-Coll] [mg mL^{-1}]	[FITC-CM] [mg mL^{-1}]	T [°C]	Q_{Coll}/Q_{CM}	m_{Coll}/m_{CM}	CM [wt%]
1:1	1	5.6×10^{-3}	34–36	1	178	0.56
3:1	1	5.6×10^{-3}	35–37	3	535	0.18

and dissemination of cell therapy derived products in sites of interest are determinant for efficient therapeutic applications.

Injectable Collagen Suspension: To assess to which extent these systems could be used to fabricate organized 3D shapes, collagen:CM particles were directly suspended in DMEM to mimic the biological environment of the ECM. The pH of DMEM being close to physiological pH, suspension of collagen microparticles in such medium will simultaneously solvate the collagen molecules and induce their self-assembly into a fibrillar collagen gel. Indeed, a dense fibrillar matrix is obtained (Figure S6, Supporting Information), indicating that the fibrillogenesis process of collagen is not destabilized by the encapsulated secretion products. In an attempt to reach higher hierarchical order (i.e., 2D alignment), collagen microbeads were suspended in aqueous solvent and immediately injected in phenol-red free DMEM (**Figure 3**a, and Video S1, Supporting Information). A final high collagen concentration of 80 mg mL^{-1} was successfully injected through a 26G needle, which is comparable to the needles used for fillers. The injection produced strands of fibrillar collagen as characterized by SEM (Figure 3b) and confirmed by TEM (inset in Figure 3b). A drop of collagen hydrogel hanging at the needle end after injection is depicted in Figure S7 (Supporting Information). Collagen fibrils appear more organized on the outer part of the strand than in the inner part, which may be a consequence of the extrusion process (i.e., shearing) as showed by PLM (Figure 3c,c''). Birefringent textures are seen and follow the extrusion direction (orange domains) after inducing a first order retardation with a gamma plate (Figure 3c'''). Noticeably, our material is processed in aqueous solvents which induces a swelling phenomenon of the collagen strands (from 260 µm diameter at the tip of the needle to about 400 µm after one week in DMEM). As observed by SEM, fibrils diameter appears homogeneous in size. This characteristic together with the fibrils alignment reminds those of biological dermis tissue (Figure 3d) where the collagen bundles are locally oriented in the same direction under PLM (nematic birefringence texture shown in Figure 3d') as observed in our material at higher magnification (Figure 3e). Such biological features differ from other 2D oriented collagen materials found in the literature, using larger diameter needles for extrusion[33] or electrospinning,[34] enlarging the field of applications for injectable anisotropic collagen-based materials.

The advantages of the spray-drying approach for the formation of dense anisotropic collagen microparticles include synthesis in sterile conditions, continuous processing and easy scalability to mass production. The versatility and the robustness of the encapsulation process by aerosol open ways for encapsulating a broad range of biomolecules whose activity requires further evaluation by in vitro and in vivo models for regenerative tissue repair. Injection of the microparticles leads to materials made of 2D oriented collagen fibrils with tunable structure over several length-scales and composition (e.g., hierarchical organic/inorganic scaffold). The fact that the size of the needle can be adapted to the size of the defect and to the type of tissue without altering the patient's comfort opens perspectives in collagen-based biomaterials. One step further, these 2D building blocks may serve in 3D-bioprinting to produce oriented collagen fibrils over several centimeters forming biomimetic collagen scaffolds with desired shape.

Experimental Section

Processing of Pure Collagen Microparticles: Collagen solutions were prepared following a procedure described elsewhere[35] and the concentration of the sprayed collagen solutions was adjusted to 0.7, 2.1, 3.6, or 5.0 mg mL^{-1} by dilution with 0.5 M acetic acid. The beads were formed by using a Büchi 290 mini spray-dryer. Solution flows between 0.3 and 1.5 mL min^{-1} were used to reach different final beads sizes. Air flow was set at 414 L h^{-1} and aspiration rate at 20 m^3 h^{-1}. The inlet temperature was set at 30 °C, measured inlet temperature at 35 ± 1 °C and measured outlet temperature at 21 ± 3 °C. The resulting material consists of a white powder which was collected in a flask at the bottom of the cyclone for characterization.

Production of CM: The CM was composed of the secretion products of gMSCs. The gMSCs were isolated from gingiva (surgical stomatology residues collected from healthy subjects). This study was conducted in accordance with ethical principles stated in the declaration of Helsinki. An informed, written consent was obtained from the donors, prior approval by an Institutional Review Board was not required. gMSCs were isolated and cultivated as previously described.[18] Briefly, primary explant cultures were amplified in DMEM, fetal calf serum (20%), penicilin (100 µg mL^{-1}), streptomycin (100 µg mL^{-1}), amphotericin B (2 ng mL^{-1}) until they reached confluence. gMSCs were cultivated until 60% confluence, washed three times in PBS and incubated in serum- and antibiotic-free medium for 48 h and frozen at −80 °C. Gingival MSCs' CM was dialyzed against carbonate buffer (Spectrum© dialysis membrane, 3 kDa) and tagged with FITC before being equilibrated in PBS. For ELISA test, a second dialysis with a sterile solution of polyethylene glycol (PEG, 35 kDa, Fluka) dissolved in milliQ water was performed (see Material and Methods section, Supporting Information). Collagen was tagged using RITC in slightly basic pH and subsequently dissolved in 0.5 M acetic acid. Two different collagen:CM volume ratios were produced (1:1 and 3:1).

Processing of Collagen Microparticles Containing Bioactive Molecules: The same spray-drier was used with slight differences as follows. The collagen concentration was kept at 1 wt% and the CM concentration in the final solid particles varied between 0.18 and 0.56 wt% (see Table 1 for details). To limit the pH stress for the secretome we have recurred to a three fluid nozzle (two liquids + air) enabling the contact of the collagen solution and the secretome only at the end of concentric channels at the nozzle tip.

To assess the release kinetics of bioactive factors, 2–3 mg of the resulting powder was mixed with 600 µL of sterile PBS 1X. The resulting suspension was placed at 37 °C in order to mimic a physiological environment. The supernatants were analyzed by ELISA (R&D Systems) at different time points (10 min, 1, 6, and 24 h).

Injection of Pure Collagen Microparticles in Cell Culture Medium: Pure collagen beads made from a collagen solution (3.6 mg mL^{-1}) were mixed with an aqueous solvent (200 µL) to reach a final concentration of 80 mg mL^{-1}. The mixture was quickly transferred to a 1 mL syringe equipped with a 26G1/2" needle. The mixture was injected right after in phenol red-free DMEM in a transparent 5 mL UV cuvette at ambient temperature. After injection the cuvette was hermetically covered with

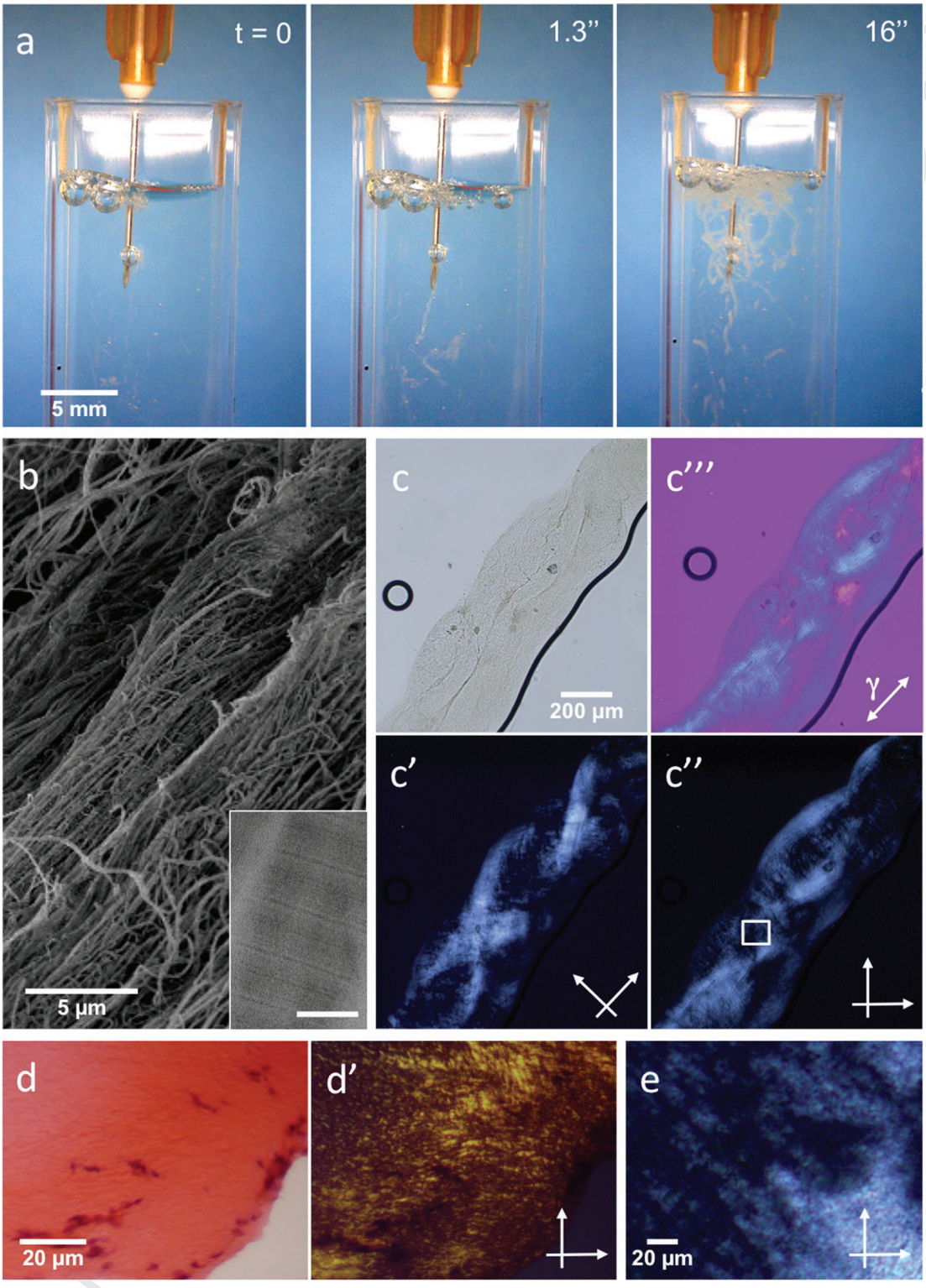

Figure 3. a) Sequence of still images taken from the video of collagen beads (80 mg mL^{-1}) suspended in aqueous solvent being injected in DMEM medium (phenol red free). b) SEM microscopy image of the final collagen matrix displaying fibrillar organizations and local alignments with the typical cross-striated pattern of type I collagen fibrils as evidenced by TEM (in inset, scale bar 50 nm). c–c’’) PLM images of injected fibrillar collagen strands in hydrated state containing birefringent patterns on the surface evidenced by rotating the polarizers from 45°–135° (c’) to 0–90° (c’’), typical of alignment domains. c’’’) PLM image after inducing a first-order retardation with a gamma plate with polarizers at 0–90°. d) Histology section of rat dermis as observed under the Microscope and d’) displaying nematic birefringent textures when observed under crossed polarizers. e) High magnification of the inset in (c’’).

parafilm and kept for a week at 4 °C before analysis of the injected material.

Characterization of Collagen Materials: More information about Differential scanning calorimetry, laser granulometry, scanning electron microscopy, circular dichroism, dynamic vapor sorption, confocal microscopy, TEM samples preparation and observations, release kinetics of HGF and VEGF can be found in the Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

biomaterials, collagen, encapsulation, self-assembly, spray-drying

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