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Original Article

Synthesis and incorporation of rod-like nano-hydroxyapatite into type I collagen matrix: A hybrid formulation for 3D printing of bone scaffolds

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ABSTRACT

Over the recent years, nanometric hydroxyapatite (HA) has gained interest as constituent of hybrid systems for bone scaffold fabrication, due to its biomimicry and biocompatibility. In this study, rod-like nano-HA particles were introduced in a type I collagen matrix to create a composite mimicking the bone composition. HA nanorods (40 − 60 nm × 20 nm) were synthesised by hydrothermal method involving the use of an ammonium-based dispersing agent (Darvan 821-A) and fully characterised. The homogeneous dispersion of HA nanoparticles throughout the final hybrid formulation was achieved through their suspension in a collagen solution in presence of Darvan 821-A. The resulting homogeneous collagen/nano-HA suspension proved to be suitable for extrusion printing applications, showing shear thinning and sol-gel transition upon simil-physiological conditions. Furthermore, mesh-like structures were printed in a gelatine-supporting bath by means of a commercial bioprinter further demonstrating the potential of the designed hybrid system for the fabrication of 3D bone-like scaffolds.

1. Introduction

Nanometric hydroxyapatite (HA) has gained increasing interest in the biomedical field thanks to its similarity to the inorganic phase of human bones. In fact, 65–70 % of bone mass is constituted by non-stoichiometric HA in form of nanometric crystals with plate-like morphology (50 nm in length, 25 nm in width and 2 − 3 nm in thickness). These crystals nucleate and grow in the gaps formed by type I collagen fibrils constituting the bone matrix, orienting their crystallographic c-axis along the fibril longitudinal axis [1].

The mimicry of the composition and the structure of bone mineral phase proved to be a successful strategy to confer superior biological properties to synthesised HA particles, such as enhanced resorbability, higher bone cell adhesion, proliferation and activity. These properties are strongly related to the superior features of nanometric HA, such as high exposed surface area and roughness [2]. Therefore, considered the key role played by structural parameters on the biological behaviour of nanometric HA, many efforts have been dedicated to strictly control the synthesis parameters in order to obtain particles with defined morphology, size and stoichiometry. The hydrothermal-assisted method is one of the most adopted procedures for the synthesis of plate- and rod-like HA particles. According to this method, the aqueous solution containing the HA precursors is treated in specific reactors allowing the growth of HA particles at high pressures and temperatures. In particular, the final size and morphology of the resulting HA particles are mainly influenced by the duration and the temperature of the hydrothermal treatment [3].

A common drawback that hamper the achievement of nanometric HA is the occurrence to a great extent of aggregation among the formed primary particles caused by an entropy-driven tendency to reduce their surface area [4]. For this reason, several dispersing agents have been used in HA synthesis with the aim to increase the repulsive force among particles and reduce their aggregation tendency. At the same time, they have also proved to provide a beneficial confining effect during the growth of HA grains, resulting in final particles with nanometric size. Among the others, Darvan 821-A is an ammonium polyacrylate mainly used as dispersing agent for the production of homogeneous and highly concentrated HA colloidal suspensions (up to 54 vol%) for near-net-shape object production [5]. Cunniffe and co-workers reported the use of this dispersing agent in the synthesis of non-agglomerated HA particles for biomedical purposes, identifying 0.1–0.5 vol% as the optimal concentration range to prevent aggregation while producing particles with controlled nanometric dimension [6]. In this work we combined for the first time the use of Darvan 821-A with the hydrothermal synthesis to produce non-agglomerated nanometric HA particles with rod-like morphology (HA_D).

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One of the most common applications of nano-HA in the biomedical field is its use as reinforcing phase within polymeric matrices to develop composite materials for bone scaffold fabrication [7–9]. In this context, type I collagen, the main organic phase of bone, is considered one of the most suitable matrices for bone regeneration applications due to its exceptional biocompatibility and biological properties deriving from the presence in its structure of cell recognition sites [10,11]. Furthermore, collagen-nanoHA composites are highly biomimetic systems able to combine the flexibility and toughness associated with collagenous matrix with the high compressive strength of the inorganic phase [12].

Despite these attracting and promising features, the fabrication of collagen-based scaffolds results heavily hindered by the low stability of collagen molecules, which does not allow the involvement of high temperatures and severe process conditions. For this reason, most of the scaffolds based on the combination of type I collagen and HA particles reported in literature are realised by freeze-drying the hybrid formulations, leading to highly porous structures [13–16]. Even if the pore size distribution can be tailored through the selection of the freezing temperature and the regulation of the lyophilisation process, the achievement of fine geometrical features can be more difficult to control. With the aim to design and fabricate scaffolds with advanced biomimetic properties, 3D printing technologies can be exploited to realise more complex microarchitectures, enabling higher control over the final shape and architecture compared to the conventional manufacturing techniques [17–19]. The introduction of the extrusion-based 3D printing in bone tissue engineering has increased the precision and the repeatability of scaffold manufacturing, in addition to the overall process biocompatibility [17,18,20]. However, these techniques require specific rheological properties of the processed materials such as shear thinning, yield stress and fast shear recovery in order to ensure printability and high printing fidelity [17,21,22].

Given the high potentialities of additive manufacturing techniques, the ERC BOOST project aims to exploit the 3D extrusion printing to produce biomimetic scaffolds, which can reproduce both the chemical composition and the microarchitecture of the natural bone in order to stimulate bone regeneration. In this context, in order to pave the way for the fabrication of the final biomimetic scaffold, the present contribution specifically aims at the design of a homogeneous hybrid Collagen/nano-HA formulation, which mimics the composition of bone tissue and shows suitable properties for 3D extrusion printing.

In particular, rod-like nano-HA particles (HA_D) were produced by an optimised hydrothermal method combined with the use of Darvan 821-A, as size controlling agent. In parallel, the dispersing action of Darvan 821-A was investigated and exploited to avoid the agglomeration of HA particles and to obtain highly homogeneous collagen/nanoHA_D hybrid formulations, respecting the ratio between organic and inorganic phases present in natural bone tissue. According to authors' knowledge, this is the first work reporting the dual use of Darvan 821-A as size controlling agent during HA synthesis and as dispersing agent to prevent the formation of particle aggregates throughout the collagenous matrix, as schematically illustrated in Fig. 1. The rheological behaviour of the developed Collagen-nanoHA_D formulations was assessed in order to investigate their potential printability through an extrusion-based bioprinter and, finally, mesh-like structures were successfully printed exploiting a gelatin-supporting bath, showing the feasibility of the proposed approach and laying the ground for the reproduction of more complex bone-like architectures.

2. Materials and methods

2.1. Materials

Chemicals used for HA particle synthesis, i.e. potassium phosphate dibasic trihydrate, calcium chloride and sodium hydroxide and gelatin powders derived from porcine skin were purchased from Sigma Aldrich. Darvan 821-A was provided by Vanderbilt Minerals. Type I collagen powders derived from bovine Achilles tendon were purchased from Blafar (Dublin, Ireland).

2.2. Synthesis of rod-like nanosized HA

5.48 g of potassium phosphate dibasic trihydrate were dissolved in 100 ml of doubled distilled water. Successively, 0.2 vol% of Darvan 821-A was added to the solution and the pH was set to 10.5 through the addition of 1 M sodium hydroxide solution. In order to have a Ca/P molar ratio of 1.67, a second solution obtained by dissolving 4.44 g of calcium chloride in 60 ml of doubled distilled water was prepared and, after 1 h of stirring, added dropwise into the solution containing the phosphate precursor while constantly maintaining the pH at 10.5 through the addition of sodium hydroxide. The resulting solution was kept under stirring for 3 h, constantly maintaining the pH at 10.5. After an overnight ageing step performed at atmospheric pressure and room temperature, the supernatant was removed, and the remaining slurry was poured in a 250 ml Teflon-lined hydrothermal reactor and placed in an oven at 100 °C for 4 h. After cooling, the supernatant was removed, and the resulting slurry was centrifuged in order to separate HA particles. The latter were washed three times with distilled water and once with pure ethanol. In the end, HA_D particles were collected in a Petri dish and dried in an oven at 100 °C for 24 h.

A control sample was prepared following the same synthesis procedure but without the addition of Darvan 821-A in order to demonstrate the effect of this agent on particle features.

2.3. Incorporation of rod-like nano-HA_D particles into collagen-based solution

In order to mimic the ratio between the organic and the inorganic phase present in natural bone tissue, the hybrid Collagen/nano-HA_D formulation was characterised by 65 wt% of nano-HA_D and 35 wt% of type I collagen [23,24]. In details, nano-HA_D particles were suspended in 1 M NaOH containing Darvan 821-A by sonicating for 1 h using an ultrasonic bath (Digitec DT 103H, Bandelin, Berlin, Germany) and subsequently stirring overnight to promote the proper dispersion of HA_D particles. Different concentrations (0 vol%, 0.5 vol% and 3 vol%) of Darvan 821-A were tested in order to define the most appropriate in terms of particle dispersion and final composite properties. The resulting HA_D suspension was subsequently combined with the type I collagen solution, previously obtained by dissolving collagen in 0.5 M acetic acid under stirring overnight at 4 °C. To promote the physiological reconstitution of collagen fibres, the pH of the suspension was adjusted to 7.4 by adding the proper amount of NaOH solution (1 M) and reaching a final collagen concentration of 1.5 wt%. The overall procedure was performed at 4 °C to avoid premature collagen cross-linking.

2.4. Characterisation of nano-HA particles

The morphology of nano-HA particles was characterised through Field-Emission Scanned Electron Microscopy (FESEM) using a Zeiss Merlin instrument. Prior to analysis, HA particles were homogenously suspended in isopropanol through a 5-minutes ultrasonic treatment with an ultrasonic bath (Digitec DT 103H, Bandelin). A drop of the suspension was poured on a carbon-coated copper grid (Taab Laboratory Equipment Ltd) which in turn was fixed on a microscopy metallic stub and allowed to dry. A platinum layer was deposited on HA powders before FESEM analyses in order to enhance the sample conductivity.

The Ca/P ratio of HA particles was investigated through Energy Dispersive Spectroscopy (EDS) measurements using the AZtec software (Oxford Instruments).

Wide-angle X-Ray Diffraction measurements (XRD) were performed employing a CuKα radiation emitted at 40 kV and 40 mA. The

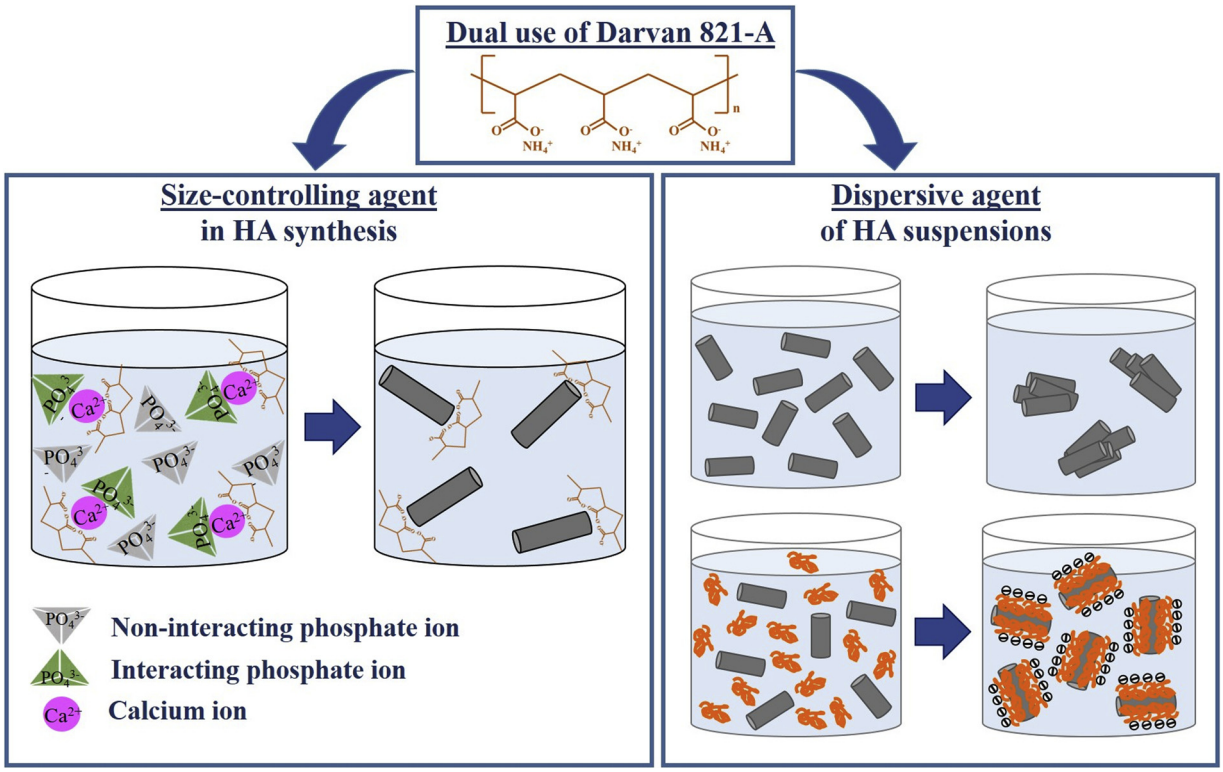

Fig. 1. Schematic representation of the dual use of Darvan 821-A in this work: as size-controlling agent during HA synthesis (left) and as dispersive agent of HA suspensions (right).

investigated angular range was set from 10 to 80 2θ degrees, with a step size of 0.013 2θ degrees and a scan step time of 45 s. XRD spectrum analysis for phase identification was performed through the X'Pert HighScore software. In addition, the mean crystallite dimension was calculated through the application of the Scherrer's equation on the XRD peak placed at 25.8 2θ degrees, representing the crystallite dimension along the c-axis.

2.5. Characterisation of HA_D/Darvan 821-A suspensions

In order to investigate the dispersive effect of Darvan 821-A, aqueous suspensions of HA_D were prepared considering different concentrations of the dispersing agent (0 vol%, 0.5 vol%, and 3 vol%). The resulting suspensions were sonicated for 1 h using an ultrasonic bath (Digitec DT 103H, Bandelin, Berlin, Germany), stirred overnight and finally analysed through Dynamic Light Scattering employing a Zetasizer ZS instrument (Malvern) in order to measure the particle size distribution and the particle zeta potential values.

2.6. Morphological analyses of Collagen/nano-HA_D bulk samples

To investigate the dispersion of the rod-like nano-HA_D into the collagenous matrix and the homogeneity of the resulting composite system, morphological analyses were performed on sample cross-sections of Collagen/nano-HA_D produced with different DARVAN 821-A concentrations (0 vol%, 0.5 vol%, and 3 vol%). Bulk samples of the hybrid formulations were realised by exploiting the sol-gel transition of collagen at 37 °C. In details, 400 µl of Collagen/nano-HA_D suspension were poured into a PDMS mould and subsequently incubated at 37 °C for 3 h to create samples 10 mm in diameter and 5 mm in thickness. After removal from the mould, the samples were frozen at – 20 °C and lyophilized for 24 h using a Lyovapor L-200 freeze-dryer (Büchi, Switzerland) under vacuum (< 0.1 mbar). Cross-sections of lyophilized samples were coated with a 7 nm thin platinum layer and analysed by Field-Emission Scanning Electron Microscopy (FESEM) using a ZEISS MERLIN instrument.

2.7. Printability of the Collagen/nano-HA_D formulation

The study of the visco-elastic properties of the hybrid system was carried out by means of rheological tests in order to evaluate their suitability for 3D printing of high-resolution constructs. The material printability was further investigated using a commercially available 3D Bioprinter (BIOX, Cellink).

2.7.1. Rheological tests

All the rheological tests were performed using a DHR-2 controlled stress rotational Rheometer (TA Instruments, Waters) equipped with a parallel plate geometry with a diameter of 20 mm and a Peltier plate system to constantly control the system temperature. Flow ramp tests at 4 °C were conducted to investigate the variation in the suspension viscosity over a wide range of shear rates (0.01–1000 s^{-1}). Furthermore, the sol-gel transition of the systems was observed by means of a time sweep analysis carried out at 37 °C under 1 % strain for 60 min.

2.7.2. Preliminary printing tests

Mesh-like structures (10 × 10 mm surface, 1 and 5 mm thickness) were produced using a commercial bioprinter empowered with a temperature controlled pneumatic print-head designed to fit 3 ml syringes equipped with 30 G (120 µm inner diameter) needles and constantly kept at 4 °C to avoid the premature collagen sol-gel transition. The deposition of the Collagen/nano-HA_D suspension was supported by a gelatin slurry, prepared following the protocol reported by Hinton et al. [19] and kept at 20 °C during the entire printing process.

In brief, to create the gelatin slurry, porcine gelatin powders (Type A, Sigma Aldrich) were dissolved in 150 ml 11 mM $CaCl_2$ at a concentration of 4.5 wt%. The gelatin solution was poured in 500 ml jar

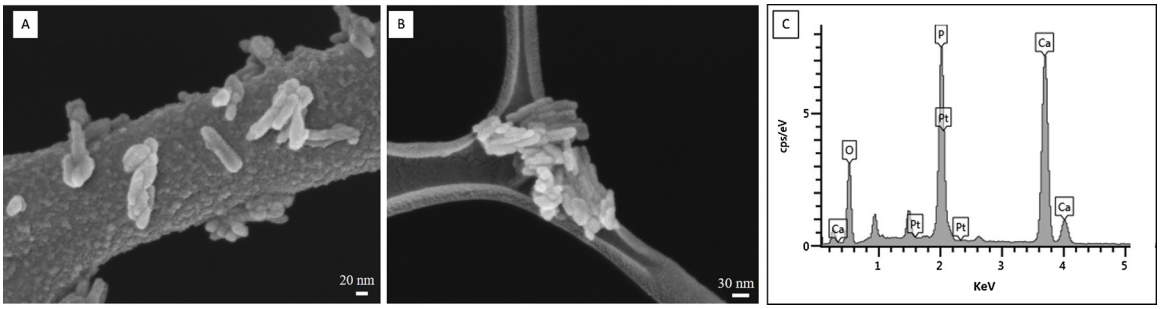

Fig. 2. FESEM images of nano-HA particles synthesised through hydrothermal reaction A) without Darvan 821-A, B) using 0.2 vol% of Darvan 821-A. C) EDS spectrum of HA_D particles.

and gelled at 4 °C overnight. Then, 350 ml of 11 mM $CaCl_2$ at 4 °C were added to the jar and the solid gelatin was blended for 120 s. The resulting suspension was subsequently transferred into 50 ml conical tubes and multiple centrifugation treatments (4200 rpm, 2 min) were exploited to collect the slurry particles after accurately removing the supernatant. The gelatin slurry was then stored at 4 °C until use.

The printing parameters such as printing speed, air pressure and layer height were optimized with the aim to obtain the highest resolution according to the selected 3D mesh-like structure.

After printing, the 3D constructs were incubated at 37 °C for 3 h to liquefy and remove the supporting bath while promoting the sol-gel transition of the hybrid systems.

3. Results

FESEM micrographs of HA produced through hydrothermal treatment without Darvan 821-A revealed particles with an elongated morphology, but with highly heterogeneous lengths ranging from 20 to 80 nm (visible in Fig. 2A). At variance, the introduction of 0.2 vol% of Darvan 821-A (HA_D) led to the production of more uniform-sized rods with length of 40 − 60 nm and a width of 20 nm (visible in Fig. 2B), similar to those of HA in natural bones. EDS analysis of HA_D particles revealed a sub-stoichiometric composition, with a Ca/P ratio equal to 1.5, close to that of the inorganic phase of bones. The unmarked peaks at 0.93 and 1.486 keV in the EDS spectrum correspond to the Copper Lα and to Aluminium Kα signals, respectively, belonging to the mesh grid used for FESEM sample preparation.

The XRD spectrum of the synthesised particles shows a perfect correspondence with the hydroxyapatite reference pattern (Ref. Code 00-024-0033) confirming the absence of any secondary phases, as visible in Fig. 3. The crystallite size calculated through the Scherrer's equation on the peak at 25.8 2θ degrees resulted to be 38.8 nm, thus confirming the nanometric size of the primary HA_D particles.

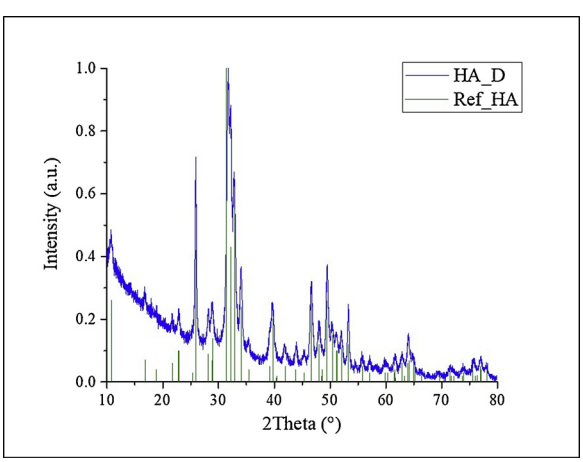

Fig. 3. XRD spectrum of HA_D particles compared with the hydroxyapatite reference pattern (Ref. Code 00-024-0033).

The HA_D particles were successively dispersed in water in presence of different concentration of Darvan 821-A (0 vol%, 0.5 vol% and 3 vol %) in order to investigate its dispersive ability and the creation of a homogeneous HA suspension for the subsequent incorporation into type I collagen matrix. DLS measurements performed on HA_D suspensions confirmed the stabilising effect of Darvan 821-A, clearly visible in Fig. 4. In fact, particle size distribution measurements of the non-containing Darvan HA_D suspension revealed the formation of micrometric HA_D aggregates. On the contrary, the principal peaks of size distributions related to HA_D suspensions containing 0.5 vol% and 3 vol% of Darvan 821-A were centred at the mean value of 40 nm and 68 nm, respectively, in accordance with the particle size revealed by means of morphological assessment.

The limited stability of the non-containing Darvan HA_D suspension was further confirmed by zeta potential measurements which revealed a value of -17 mV, inferior to the theoretical threshold of ± 30 mV associated to stable suspensions [25]. On the contrary, in presence of Darvan 821-A, the zeta potential increased to more negative values, measuring -43.5 mV and -49.7 mV for 0.5 vol% and 3 vol% Darvan respectively.

Rod-like HA nanoparticles (HA_D) dispersed in Darvan 821-A basic solution (1 M NaOH) were further incorporated into a concentrated collagen solution (1.5 % w/v). To this aim, nano-HA_D suspensions containing 0 vol%, 0.5 vol% and 3 vol% Darvan 821-A were tested in order to investigate the most suitable concentration to obtain a homogenous dispersion of HA_D into the collagenous phase while preserving the strength and stability of the final hybrid system. The resulting hybrid formulations were investigated by means of both morphological and rheological tests, particularly observing the potential beneficial effects derived from the use of Darvan 821-A. As evidenced by the morphological assessment performed on previously lyophilised Collagen/nano-HA_D samples, the inorganic particles were successfully dispersed and properly embedded in the organic collagenous matrix in presence of the dispersing agent (Fig. 5B, C). The presence of HA_D and the use of Darvan 821-A did not hinder the reconstitution of the fibrous collagen structure naturally occurring at pH 7.4 and 37 °C (physiological pH and temperature) as clearly evidenced by Fig. 5D. No significant agglomeration was observed for nano-sized HA_D particles, except for the presence of few particle clusters less than 1 μm in size. On the contrary, the hybrid system realised without the use of Darvan 821-A (Fig. 5A) showed the evident formation of agglomerates, where HA_D particles were also less embedded in the collagen matrix.

The hybrid system was further assessed in terms of rheological properties in order to investigate its potential as biomaterial for extrusion-based printing technologies. In particular, the variation of the suspension viscosity at 4 °C was analysed in a wide range of shear rates comprised between 10^{-2} and 10^{3} s^{-1}.

The flow ramps reported in Fig. 6 show the shear thinning

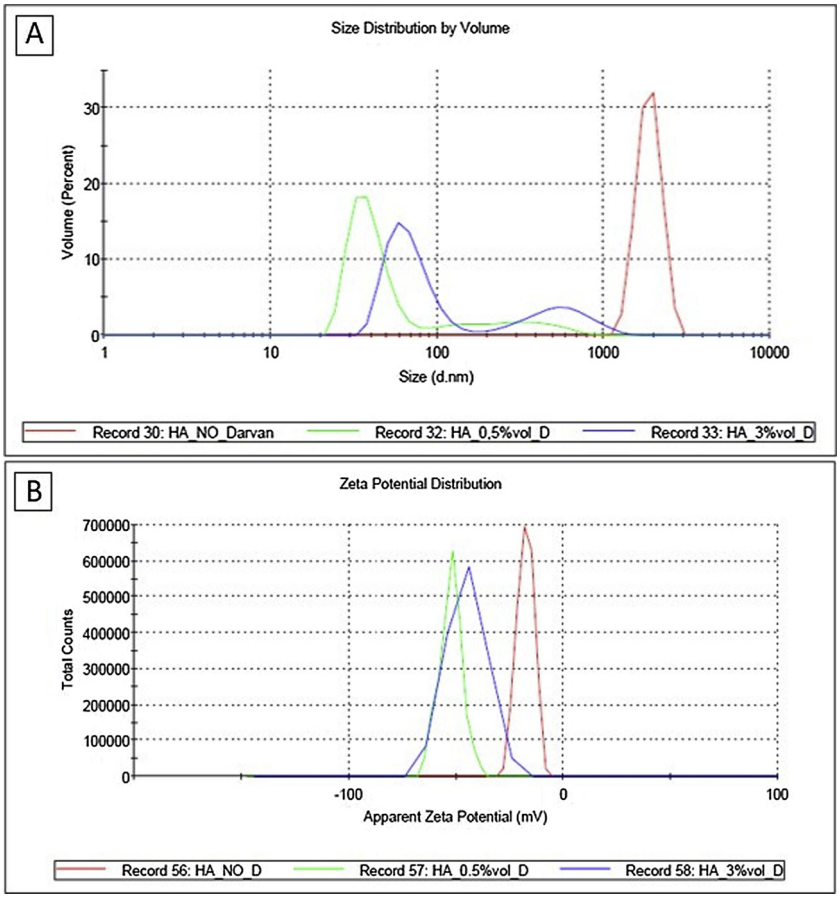

Fig. 4. DLS measurements of particle size (A) and Z-potential (B) of HA_D suspensions containing 0 vol%, 0.5 vol% and 3 vol% of Darvan 821-A.

behaviour of Collagen/nano-HA_D suspensions containing 0 vol% (Fig. 6A), 0.5 vol% (Fig. 6B) and 3 vol% Darvan 821-A (Fig. 6C) at 4 °C clearly evidenced by a sharp decrease of the viscosity for increasing values of shear rates and shear stresses applied. Compared to analogue system without the dispersing agent, Darvan-containing formulations (0.5 vol% and 3 vol%) led to higher values of viscosities at low shear rates, about 302 Pa.s and 212 Pa.s respectively, at variance with Collagen/nano-HA_D system without Darvan which showed a value of about 75 Pa.s. All the suspensions (with and w/o Darvan) showed similar values ranging between 0.14 and 0.2 Pa.s at the highest shear rate

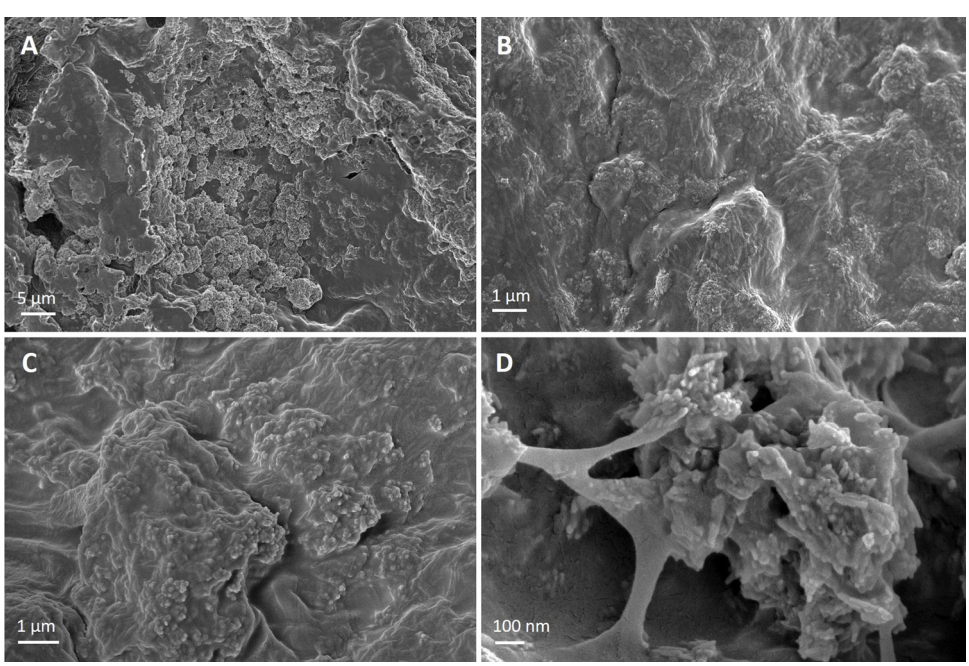

Fig. 5. FESEM images of Collagen/nano-HA_D systems containing 0 vol% (A), 0.5 vol% (B) and 3 vol% (C, D) Darvan 821-A.

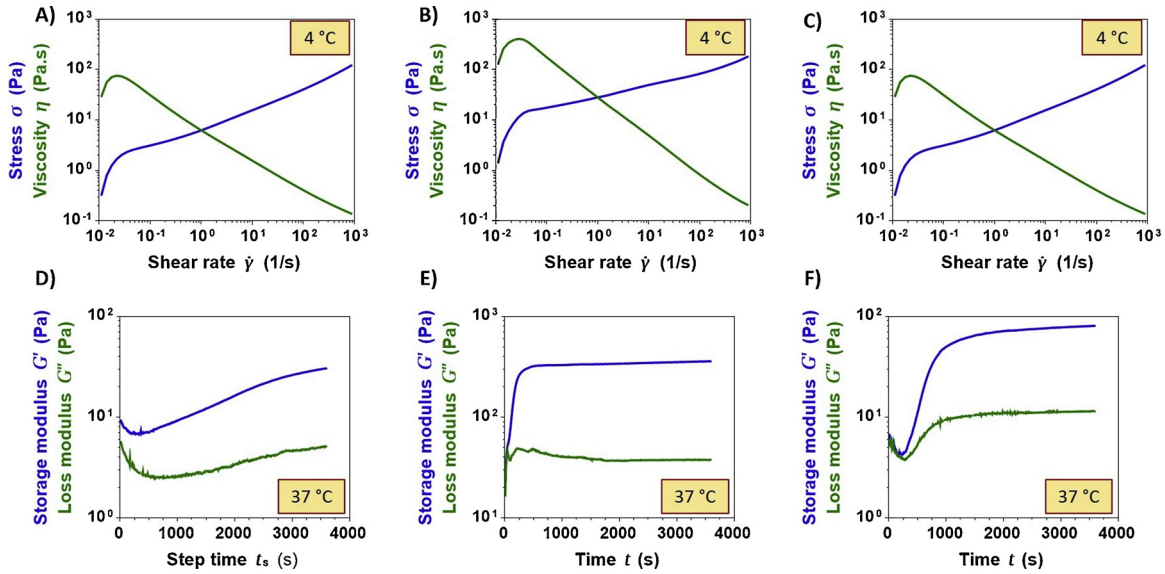

Fig. 6. Rheological analyses showing shear thinning at 4 °C and sol-gel transition at 37 °C of the Collagen/nano-HA_D suspension without Darvan 821-A (A, D) Collagen/nano-HA_D suspension with 0.5 vol% (B, E) and 3 vol% (C, F) Darvan 821-A.

applied.

The results related to time sweep tests performed applying an oscillatory stress at constant 1 % strain and 1 Hz clearly showed the sol-gel transition of the material at 37 °C. However, a stable gel was formed after about 250 s only for the systems containing Darvan 821-A, where a significant gap between the storage (G') and the loss (G'') modulus was observed. In addition, a slightly increase of the overall complex modulus (G*) of the material was registered along the duration time of the test reaching a final value of G' and G'' of about 358.3 Pa and 37.7 Pa for the Darvan-containing system at 0.5 vol% and 80.3 Pa and 11.4 Pa for the formulation containing 3 vol%, as shown in Fig. 6E and F respectively. On the contrary, the hybrid system without Darvan 821-A showed a poor reconstitution of the gel at 37 °C, showing 30.7 Pa and 5.2 Pa of storage and loss modulus respectively.

The rheological properties of the hybrid Collagen/nano-HA_D formulation were considered as guiding reference to optimise the fabrication of 3D mesh-like structures by extrusion-based printing process.

With this perspective, preliminary printing tests were performed by means of an extrusion-based system equipped with 30 G needles (120 μm internal diameter) in order to achieve 3D constructs with high final resolution. Firstly, the Collagen/nano-HA_D suspension was loaded into a 3 ml cartridge and kept at 4 °C to avoid premature collagen gelation. As shown in Fig. 7, the deposition of the suspension was supported by a gelatin-based extrusion bath to avoid the structure collapsing, reproducing the method already reported by Hinton et al. [19] and defined as freeform reversible embedding of suspended hydrogels. Prior to printing, the gelatin slurry was poured in the multi-well system to create a 7 mm thick layer and kept at 20 °C in order to preserve its visco-elastic behaviour during the entire process. Pressure and printhead speed were regulated to be 30 kPa and 7 mm/s, respectively, to enable the extrusion of filaments with size reproducing the inner needle diameter and arranged in mesh-like structures (10 × 10 mm surface, 1- and 5-mm thickness). At this stage, the printed constructs were incubated at 37 °C for 3 h to liquefy and remove the gelatin extrusion bath while promoting the sol-gel transition of the hybrid systems. Due to the continuous needle clogging, the extrusion of Collagen/nano-HA_D suspension realised without the use of the dispersing agent was not possible and thus not reported in the results.

4. Discussion

The synthesis of HA particles reproducing the morphological and structural features of physiologic HA represents an attracting strategy to fabricate advanced scaffold for bone regeneration with peculiar biological properties. Therefore, many research groups have focused their efforts to develop synthesis methods to produce HA particles with rod-like morphology and nanometric size, similarly to HA crystals present in bone tissue.

The hydrothermal treatment plays a key role in favouring the formation of HA rod-like structures, as the involved high pressure and temperature promote the growth along a preferential direction of the HA crystal nuclei [2]. Furthermore, by controlling the temperature and the duration of the hydrothermal treatment is possible to tailor the rod length [3]. In this work, the first authors' aim was to optimize an effective and reproducible method for the production of bone-like HA, able to mimic the morphology and the size of HA crystals present in bones (50 × 25 × 2 nm) [26]. In particular, the selected hydrothermal treatment was conducted at relatively lower temperature and shorter reaction time (100 °C for 4 h) compared to those reported in the literature, even longer than 20 h [27–29].

However, particles obtained through the hydrothermal method have usually a broad size distribution that can be narrowed through the use of a surfactant during the synthesis. For long time, surfactants as cetyltrimethyl ammonium bromide have been used as size and shape directing agents, but due their high toxicity, the complete surfactant removal through a post-synthesis calcination of HA particles was required. However, this additional thermal treatment can lead to a modification of particle crystallinity and to the formation of secondary phases [30]. For this reason, in recent years several researchers focused their attention on the identification of safer and more environmental-friendly agents, easily removable through simple washing steps. Among the alternative agents showing improved characteristics, Darvan 821-A is an anionic surfactant, which is expected to interact with calcium ions and to regulate its availability at the surface of the growing HA crystals, as schematically represented in Fig. 1 (left). A systematic investigation carried out by Cunniffe and collaborators identified the value of 0.5 vol % as the maximum Darvan concentration to achieve a satisfactory particle yield, while for concentration lower than 0.1 vol% they did not observe a significant size-controlling action [6]. For this reason, the authors chose 0.2 vol% as the optimal Darvan concentration for the starting HA solution successively subjected to the hydrothermal treatment. In the present work, the size-controlling action of Darvan was further confirmed, as HA particles prepared without the surfactant demonstrated more heterogeneous rod lengths, suggesting a less

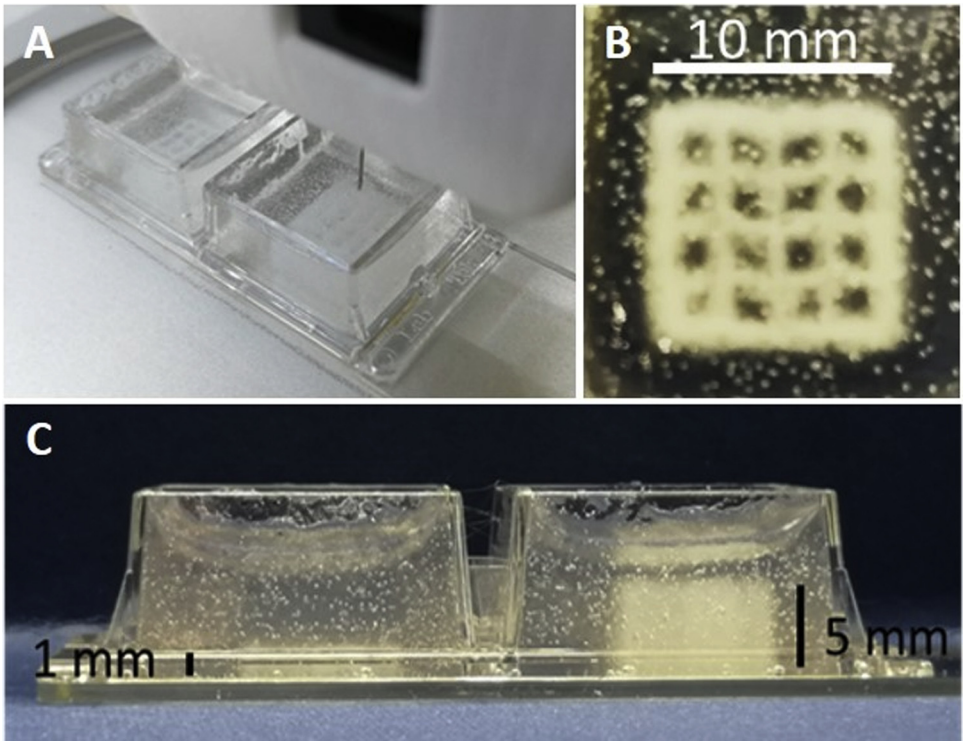

Fig. 7. Preliminary printing tests. Images show the successful printing of the Darvan-containing Collagen/nano-HA_D hybrid system in a gelatin-based supporting bath by means of a commercial BIOX 3D printer. Mesh-like structures (10 × 5 mm; 10 × 1 mm) were realised using 30 G needles. In detail, the pictures represent the manufacturing process (A), the transversal section (B) and the height (C) of the 3D printed structures.

controlled crystal growth.

Even if the use of HA particles alone has been widely reported for bone engineering field, their combination with a polymeric resorbable phase has been proved to widen the spectrum of regenerative applications. For instance, the introduction of nano-HA into collagenous matrices enables the development of biomimetic hybrid systems better reproducing the natural composition and mechanical properties of bone tissue. The poor resorbability and the brittleness associated to the use of HA nanoparticles alone can be dimmed by the presence of collagenous matrix, thus increasing the final biocompatibility and biodegradability of the material [31,32].

However, one of the main limitations in the fabrication of collagen/HA scaffolds is the achievement of a homogeneous distribution of HA particles throughout the embedding polymeric matrix. For this reason, the use of biocompatible polymeric dispersants such as chitosan or, in alternative, the *in-situ* mineralisation of collagenous scaffolds during the fabrication process have been proposed as promising approaches to avoid the HA aggregation in the resulting hybrid system [33,34]. However, the *in-situ* formation of HA crystals hinders the control over the final purity and crystallinity obtained as well as the ratio between organic and inorganic phase. Furthermore, the introduction of additional polymers acting as dispersing agents in the material formulation alters the original biomimetic peculiar composition of collagen/HA systems.

In the present work, as an alternative approach, Darvan 821-A was exploited to pre-disperse rod-like nano-HA_D particles before their combination with the collagen solution, without altering the final composition and volume ratio between type I collagen and inorganic particles [23]. The use of a dispersing agent was essential in order to reduce the aggregation tendency of HA particles in aqueous-based environments. In fact, HA particles are strongly inclined to aggregate due to their thermodynamic-driven tendency of reducing their entropy through the decrease of their surface energy [4]. As expected, DLS measurements of HA_D suspensions showed significant formation of micrometric aggregates in the absence of the dispersing agent. At variance, thanks to the introduction of Darvan, which adsorbs on particle surface leading to higher negative values of zeta potential, the intra-particles repulsive forces raise and the resulting suspensions are significantly more stable [5].

Different concentrations of Darvan 821-A (0, 0.5 and 3 vol%) were investigated in order to detect the most appropriate to obtain a homogenous hybrid system, whose rheological properties are suitable for the extrusion printing process.

The morphological assessment conducted on Collagen/nano-HA_D formulations, after the sol-gel transition and the further freeze-drying treatment, confirmed the formation of homogeneous hybrid systems with a satisfactory distribution of rod-like HA_D particles into the collagenous phase. The previous dispersion of rod-like HA_D in 1 M NaOH both at 0.5 % and 3 % v/v of Darvan 821-A proved its beneficial effects in preventing HA_D agglomeration, leading to the formation of nanoparticle clusters less than 1 μm in size. In addition, the presence of the anionic surfactant did not hinder the reconstitution of collagen fibrils and the formation of a final fibrous structure triggered by simil-physiological conditions. On the contrary, the absence of the dispersing agent led to the detection of big clusters of HA_D in addition to a poorer reconstitution of the fibrous collagenous matrix.

Rheological characterisation demonstrated the peculiar sol-gel transition at pH 7.4 and at 37 °C of the designed homogeneous Collagen/nano-HA_D suspensions. The rapid increase of the storage modulus (G') of the hybrid material observed at 37 °C is indicative of the formation of collagen fibrils due to the physical crosslinking of collagen molecules and the resultant reconstitution of a solid matrix [35]. However, the significant contribution of the loss modulus (G'') due to the presence of high amount of water and the weak nature of the intermolecular bonds between collagen molecules suggest the need for a following chemical crosslinking treatments in order to increase the mechanical strength of the final system.

In addition, the reported curves showed how lower concentrations of Darvan 821-A seem to promote the reconstitution of a stronger matrix indicated by greater values of G'. These results may suggest a strong interaction of the dispersing agent not only with the inorganic phase but also with the collagen molecules, partially hindering the fibril self-assembly for increasing concentrations. At the same time, the formation of significant HA_D agglomerates in absence of Darvan 821-A

led to the poor sol-gel transition of the hybrid system, identified by lower and similar values of storage (G') and loss (G'') moduli.

Along with the rapid sol-gel transition at 37 °C, the rheological assessment highlighted the strong dependence of the suspension viscosity on the variation in the shear stresses and shear rates applied to the material at 4 °C. In particular, the viscosity of the suspensions significantly decreased up to 0.16 – 0.2 Pa with increasing values of shear rates and stresses, suggesting a typical pseudoplastic behaviour of the material alternatively defined as *shear thinning* behaviour [21,35]. The higher value of viscosity detected in case of the Collagen/nano-HA_D system at 0.5 vol% Darvan 821-A further suggests that lower concentrations of the dispersing agent promote the development of systems with rheological properties able to better support the printing process.

As previously reported, the detected rheological features are particularly promising in case of the design of systems suitable for 3D extrusion printing applications. In detail, the *shear thinning* behaviour is normally required to enable the extrusion of the material ink especially through very thin needles associated to high shear stresses, while the transition to a solid state may be exploited to stabilise the extruded structure [19,21,36].

The potential use of the designed hybrid system for 3D printing technologies represents an additional significant value for the fabrication of biomimetic bone-like scaffolds.

As reported in the literature, most of the scaffolds based on type I collagen and HA for bone tissue engineering are realised by freeze-drying the hybrid system [33,37,38] or in alternative the by the soaking of lyophilised collagen matrices in HA suspensions [31,39] leading to a limited control over the final shape and porosity. At the same time, Collagen/HA systems developed for 3D printing applications consider low concentration of inorganic phase in order to promote the proper extrusion of the material, where greater needle diameters are normally preferred to prevent clogging during the process [40,41].

In this work, we conducted preliminary printing tests with the Collagen/nano-HA_D hybrid system to prove its potential as biomaterial ink for the fabrication of high-resolution 3D printed bone-like scaffolds.

To increase the final printing fidelity while avoiding the printed structure collapse due to the insufficient values of viscosity and yield stress in stationary conditions, the freeform reversible embedding of suspended hydrogels (FRESH method) was exploited [19]. This innovative technique exploits a thermo-reversible biocompatible gelatin support bath to enable the deposition of complex biological structures in order to improve the printing fidelity. Once the structure is printed, the gelatin bath is melted by raising the temperature to 37 °C, thus enabling the post processing of the printed construct. After the proper optimisation of the printing parameters, mesh-like structures with a size of 10 × 10 mm and a thickness of 1 mm and 5 mm were successfully obtained with Darvan-containing Collagen/nano-HA_D systems by using 30 G needles having an internal diameter of about 120 μm. The use of Darvan 821-A proved to be essential in the realisation of a homogeneous and properly dispersed system suitable for the 3D printing of high-resolution structures, whereas the formulation realised without the use of the dispersing agent was not properly extruded because of the continuous needle clogging.

Therefore, the preliminary printing tests and the reported results proved the suitability of the Darvan-containing Collagen/nano-HA_D system for 3D printing technologies and encourage further improvements targeted to 3D printing of high-resolution complex constructs, closely resembling the native structure of bone tissue.

The use of Darvan 821-A in the development of hybrid Collagen/nano-HA_D formulations proved its beneficial effects on both particle dispersion and the rheological properties of the final material especially for lower concentration of dispersing agent.

However, the low mechanical strength of the reconstituted matrix evidenced by G' and G'' values in the rheological analyses requires further investigation concerning the use of proper crosslinking methods able to enhance the mechanical properties and stability of the final 3D printed scaffolds.

5. Conclusions

In this work, a hybrid formulation for 3D printing based on rod-like nanometric HA_D particles embedded into a type I collagen matrix was successfully obtained. In particular, HA nanorods were synthesised through hydrothermal method combined with the use of Darvan 821-A as size/shape controlling agent. A homogeneous hybrid material was achieved through the combination of a collagen solution with a stabilised aqueous suspension of HA_D, containing 0.5 and 3 vol% of Darvan 821-A as dispersing agent. The rheological analyses performed on the Collagen-HA_D hybrid systems highlighted a shear thinning behaviour in addition to the peculiar sol-gel transition of the material at 37 °C. In order to demonstrate the suitability of the developed hybrid formulation for the fabrication of 3D scaffolds, mesh-like structures were printed in a gelatin-supporting bath, easily removed at the end of the printing process after incubation at 37 °C, which also led to the sol-gel transition of the collagen matrix and the consequent stabilisation of the printed structure. On the contrary, the hybrid system realised without the use of Darvan 821-A showed the formation of HA_D aggregates and worse rheological properties, hindering the proper extrusion of the material through the use of very thin needles.

In conclusion, the results obtained in this work proved the potentiality of the Collagen/nano-HA_D hybrid systems as valid material for printing 3D applications. In particular, future works will consider the design of biomimetic scaffolds able to reproduce the architecture of natural bone exploiting the combination of 3D printing technologies with. stl models extracted from micro-computed tomographic analyses of human bone. Due to the higher complexity of the potential structures and the poor mechanical properties of physically crosslinked collagen matrices, alternative chemical crosslinkers will be tested in order to increase the stability of the 3D printed scaffolds, promoting the formation of covalent bonds between collagen moieties. Finally, in view of the final applications of developed biomimetic constructs, their ability in promoting cell adhesion, proliferation and differentiation will be fully investigated.

Summary of novel conclusions

High efficiency of Darvan-821-A as size-controlling agent in physiologic-like hydroxyapatite synthesis and successful development of homogeneous Collagen/nano-hydroxyapatite suspensions with controlled rheological behaviour suitable for 3D printing of bone-like scaffolds.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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