Natural polymer-based bioinks exploiting internal gelation mechanism for cardiac tissue engineering

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***Abstract* – 3D bioprinting techniques are rapidly increasing their relevance in the field of tissue engineering as potential tools for the fabrication of complex 3D structures that could find applications as therapeutic patches or *in vitro* models. In this work, we investigated the use of alginate and gelatin based bioink for cardiac tissue engineering. The hydrogels were developed through an internal gelation mechanism, allowing 3D printing of uniform self-supporting fibres without the need for post-crosslinking or the use of support materials. Varying gelatin content, bioinks with tuneable rheological properties were developed. Biological validation showed that the bioinks were biocompatible and promoted cell adhesion.**

Keywords - Alginate dialdehyde, gelatin, bioink, internal gelation

I. INTRODUCTION

Cardiovascular diseases (CVDs) represent a social and economic worldwide burden, causing about 18 million deaths per year [1]. New resolutive therapies are highly demanded as heart transplantation still remains the only effective therapeutic option in case of end stage heart failure.

3D bioprinting techniques are gaining tremendous attention in tissue engineering and regenerative medicine as the precise and controlled deposition of bioinks can be exploited for the fabrication of complex 3D structures [2]. In cardiac tissue engineering, bioinks can be employed to develop scalable and reproducible bioprinted constructs as therapeutic patches for cardiac regeneration or *in vitro* models for preclinical validation of new advanced approaches.

Alginate (Alg)-based bioinks have been widely investigated thanks to their tuneable properties and cost-effectiveness [3]. Alg-based materials are currently being investigated in clinical trials for cardiac regeneration. Algisyl-LVR™ is an Alg-based injectable hydrogel designed to confer mechanical support to the left ventricle to prevent or reverse the progression of heart failure [4].

Alg-based hydrogels are usually obtained through physical crosslinking of Alg chains with polyvalent cations (e.g., calcium ions, Ca^{2+}). The sol/gel transition of Alg occurs under physiological conditions and almost instantly [5].

Alg 3D bioprinting usually exploits an external gelation mechanism, by the extrusion of an Alg sol phase into Ca^{2+} enriched supporting bath [6]. The crosslinking is fast and non-homogeneous, localized at the interface between the printed filament and the coagulation bath [7]. A thermosensitive supporting bath containing calcium ions derived from dissociations of salts, such as calcium chloride ($CaCl_2$), has been proposed to achieve higher 3D spatial resolution and shape fidelity, in the so-called "FRESH" method [8], [9].

Recently, internal gelation of Alg chains has been investigated to develop 3D self-standing constructs without the use of a supporting bath [10]. In this case, a water-insoluble calcium salt (e.g., $CaCO_3$) is dispersed within the Alg sol phase, and Alg crosslinking is triggered by pH-induced progressive release of Ca^{2+} promoted by the addition of an acidifying compound (e.g., glucono-d-lactone - GDL). [7] Internal gelation results in homogeneous gel and allows the 3D printing of uniform self-supporting fibers without the need for post-crosslinking or the use of supporting materials or bath. 3D printing based on this crosslinking mechanism has been limited to Alg-based bioinks. Even though Alg is biocompatible, non-immunogenic and non-toxic, its applications are restricted due to the lack of cell-adhesive motives and poor *in vivo* degradation [4]. To overcome such limitations, herein we investigated the use of oxidized alginate or alginate dialdehyde (ADA) to improve materials degradability, and its combination with gelatin (Gel) to promote cell adhesion and enhance biological properties. In this work, novel Alg-ADA-Gel hydrogels based on internal gelation mechanism were optimized for applications as bioinks for prospective applications in *in vitro* cardiac tissue engineering.

II. MATERIALS AND METHODS

ADA was produced through partial oxidation of sodium alginate via sodium metaperiodate following the protocol by Sarker *et al.* [11]. The oxidation degree was calculated through a starch-iodine method, measuring the unconsumed sodium metaperiodate as described by *Sarker et al.* [11]. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and ^{13}C CPMAS (cross polarization/magic angle spinning) NMR of alginate powder and ADA lyophilized samples were carried out to investigate the successful formation of aldehyde groups in the alginate chains. Molecular weight of ADA and Alg were determined through viscometric analysis using an Ubbelohde viscometer.

Alg-ADA-Gel hydrogels were optimized maintaining constant ADA, $CaCO_3$ and GDL concentration and varying the polymer weight ratios of Alg:Gel (% w/w) from 100:0 to 50:50 keeping

the final content of Alg + Gel equal to 3% w/v. Rheological characterizations were performed at 37°C using MCR 302 rheometer (Anton Paar) equipped with a parallel plate geometry with a diameter of 25 mm.

In vitro water absorption and dissolution tests were carried out by immersing bulk hydrogels in phosphate buffered saline (PBS) at 37°C to investigate their stability as a function of time. At each time point, the weight of the samples was measured both in wet state and, then, in dry state after freeze-drying. Water absorption degree was calculated as the percentage of water absorbed by hydrogel samples *versus* time. Dissolution degree was calculated as the percentage of dry sample weight loss *versus* time.

Hydrogel printability was assessed using RegenHU - 3D Discovery^{TM} bioprinter by optimizing printing parameters. Model filaments and grid geometries were printed. To investigate changes in printability over time, grid structures were printed at selected time points (from 10 to 90 min). Images were collected with Nikon Eclipse Ti2 spinning disk and NIS-Elements software (Nikon) and the data were analysed using ImageJ software.

In vitro cytocompatibility studies on printed cells-laden Alg-ADA-Gel hydrogels were performed using adult human cardiac fibroblasts (AHCFs), according to ISO 10993-5. Cell viability of AHCFs embedded in printed hydrogel filaments was assessed by Live/Dead assay. Briefly, AHCFs were dispersed in PBS and incorporated in the bioinks with the dual syringe mixing method. 3D bioprinted grid structures were obtained on a 12-well plate, maintained in a humified incubator at 37 $^{\circ}$C, 5% CO_2 and images were collected using Nikon Eclipse Ti2 fluorescence microscope equipped with a spinning disk.

III. Results and Discussions

Alginate dialdehyde was successfully prepared through partial oxidation of alginate via sodium metaperiodate with an average production yield of 75% and an average oxidation degree of 25%. ATR-FTIR and ^{13}C CPMAS NMR spectra further confirmed aldehyde groups formation and successful ADA production. Furthermore, 93% decrease in the viscosity average molecular weight of ADA respect to Alg was measured. Aldehyde groups of ADA are expected to undergo Schiff base reaction with primary amino groups in Gel. On the other hand, ADA carboxyl groups may electrostatically interact with calcium ions.

Initially, the effect of the addition of ADA into Alg-based hydrogels was investigated. The relative amount of the polymers in Alg-ADA hydrogels was optimized to achieve a stiffness value similar to that of Algysil and close to that of cardiac tissue (i.e., G': 1-3 kPa) [12]. The presence of ADA in the selected Alg-ADA hydrogel compositions caused an increase in the hydrogel dissolution rate compared to initial Alg-hydrogels: after 21 days of incubation in PBS, a weight loss of 42% *vs* 25% was measured in bulk Alg-ADA vs. Alg hydrogels, respectively.

Then, Alg-ADA-Gel hydrogels were prepared by optimizing Gel amount to confer cell-adhesive properties to the samples. Alg-ADA-Gel hydrogels preserved cardiac tissue-like viscoelastic properties by properly tuning Gel concentration.

Rheological tests showed a decrease in storage modulus G' as a function of Gel content. Moreover, printability studies of Alg-ADA-Gel hydrogels, moreover, evidenced that all the compositions possessed time-dependent shear thinning properties suitable for 3D bioprinting and influenced by Gel content. Alg-ADA bioinks alone (without Gel) underwent a rapid crosslinking, forming self-standing scaffolds with a printability window of up to 30 min. For bioinks containing Gel, cross-linking time increased, hence the printability interval increased consistently as a function of the amount of Gel in the bioinks, reaching 90 min for samples with 50:50 of Alg:Gel ratio. Within the printability windows, optimal printability times were identified for each hydrogel composition, depending on crosslinking time. Exemplary images of printed grid structures are reported in Fig.1.

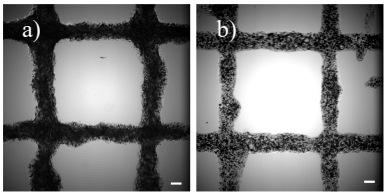

Fig. 1: Representative optical microscopy images of printed grid-shaped structures using: (a) Alg-ADA ink and (b) Alg-ADA-Gel ink with 50:50 Alg:Gel weight ratio. Structures were printed at the optimal printability times for each composition. The scale bar is equal to 250 um

Furthermore, *in vitro* tests showed that viability of AHCFs in contact with the analyzed samples was high (ISO-10993-5) and an increase in Gel content also improved cell adhesive properties of hydrogels.

Finally, the production of cell-laden constructs was investigated using a bioink with the highest content of Gel (1.5% w/v) tested in this work. As shown in Fig. 2, Live and Dead staining performed on a bioprinted cellularized hydrogel grid demonstrated the absence of death cells suggesting the biocompatibility of the bioinks and biofabrication conditions.

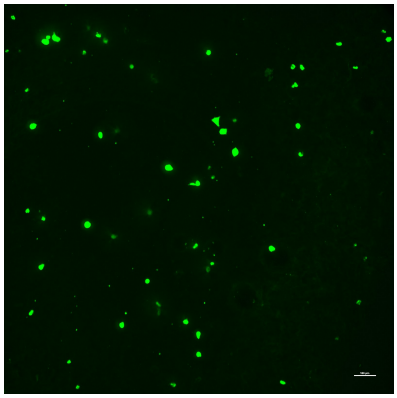

Fig. 2: Live/Dead stained AHCFs in 3D bioprinted constructs visualized after an incubation time of 1h Scale bar equal to 100 um.

IV. Conclusion

In this work, novel Alg-ADA-Gel inks based on internal gelation mechanism were developed to provide suitable properties for bioprinting in cardiac tissue engineering. Specifically, the incorporation of Gel through ADA was investigated to improve the biological features of Alg-based hydrogels and to study the influence of Gel on the cross-linking mechanism. Shear thinning inks with biomimetic viscoelastic properties, cell adhesion ability and tunable stability in physiological conditions were obtained by varying ADA and Gel contents. Printability studies showed that increasing Gel content delayed the cross-linking time of the hydrogels widening the printability window and increasing the optimal printing time. *In vitro* cell tests demonstrated the biocompatibility of Alg-ADA-Gel bioinks. Finally, 3D cell-laden constructs were printed using a selected Alg-ADA-Gel bioink containing AHCFs. In the future such bioink could be exploited to design *in vitro* models of cardiac fibrotic tissue. Alternatively, by changing the types of encapsulated cells, the bioink could be used for the biofabrication of patches for cardiac tissue regeneration.

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