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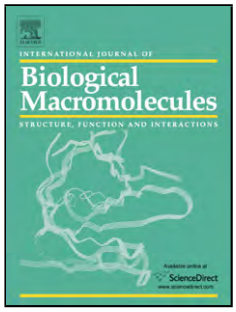

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Tunable injectable alginate-based hydrogel for cell therapy in Type 1 Diabetes Mellitus

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ABSTRACT

Islet transplantation has the potential of reestablishing naturally-regulated insulin production in Type 1 diabetic patients. Nevertheless, this procedure is limited due to the

low islet survival after transplantation and the lifelong immunosuppression to avoid rejection. Islet embedding within a biocompatible matrix provides mechanical protection and a physical barrier against the immune system thus, increasing islet survival. Alginate is the preferred biomaterial used for embedding insulin-producing cells because of its biocompatibility, low toxicity and ease of gelation. However, alginate gelation is poorly controlled, affecting its physicochemical properties as an injectable biomaterial. Including different concentrations of the phosphate salt Na_2HPO_4 in alginate hydrogels, we can modulate their gelation time, tuning their physicochemical properties like stiffness and porosity while maintaining an appropriate injectability. Moreover, these hydrogels showed good biocompatibility when embedding a rat insulinoma cell line, especially at low Na_2HPO_4 concentrations, indicating that these hydrogels have potential as injectable biomaterials for Type 1 Diabetes Mellitus treatment.

KEYWORDS: alginate, hydrogel, insulin, diabetes

1. INTRODUCTION

Type 1 Diabetes Mellitus (T1DM) is a metabolic disorder characterized by an autoimmune response that promotes the destruction of beta-cells within the pancreatic islets, resulting in a lifelong inadequate insulin secretion [1]. The most used therapy is the subcutaneous administration of exogenous insulin. Although maintaining physiologic blood glucose levels is the key in T1DM treatment, exogenous insulin injections fail to provide constant metabolic control leading to hypoglycaemia and diabetic complications [2]. Alternatively, pancreatic islet transplantation has the potential of reestablishing naturally-regulated insulin production thus, restoring the physiologic metabolic glucose control in T1DM patients. Nevertheless, there are some issues that make this treatment strategy difficult such as the low islet survival after transplantation and the lifelong immunosupression to avoid rejection [2 3]. One of the strategies developed to overcome this bottleneck is the islet embedding within a biocompatible matrix [4]. The matrix provides mechanical protection and also acts as a physical barrier keeping high molecular weight immune system components out, while

allowing the diffusion of oxygen, nutrients and therapeutic factors like insulin. In this way, islets survival is increased and the required number of pancreatic islets per patient can be optimized [3 5].

Hydrogels are three-dimensional networks composed of cross-linked polymers that possess many interesting properties for biomedical applications such as high water content, biocompatibility and mechanical properties mimicking the structural and mechanical properties of extracellular matrices [6]. Furthermore, a great advantage of hydrogel-based cell therapies is that they allow a minimally invasive cell delivery by means of hydrogel injection in the transplant site [7]. All these properties have converted hydrogels into a biomaterial extensively used in tissue engineering applications [8-12]. One of the most used materials is alginate [10]. This is a natural polymer isolated from brown algae that can form hydrogels. Besides, it shows great properties like biocompatibility, low toxicity and ease of gelation [13]. Among all its biomedical applications, alginate has been commonly used in pancreatic islets embedding [14 15].

The most commonly used method for alginate hydrogel preparation is the ionic cross-linking, where the aqueous alginate solution is combined with ionic cross-linking agents such as divalent cations. The modification of the internal hydrogel structure leads to changes both on the swelling behavior and the mechanical properties and, therefore, its stiffness, which has been described as an important conditioner for the differentiation of stem cells towards mature cells [16-18]. For example, in alginate capsules with stiffness lower than 10 KPa, human Embryonic Stem Cells are able to grow and promote pancreatic differentiation, while in capsules in the range of 22-73KPa of stiffness cell proliferation is restricted and pancreatic progenitors induction is strongly suppressed [19]. However, gelation is usually poorly controlled which limits effectiveness as an injectable biomaterial for tissue engineering applications [20 21]. The ideal gelation process of a therapeutically useful hydrogel should be quite fast, in the order of seconds to minutes and, at the same time, the hydrogel should remain in a viscous state long enough to facilitate its manipulation and injection [20-22]. On this regard, alginate gelation process has been modulated by modifying the alginate and/or the Ca^{2+} ions source, achieving, therefore, different ranges of physicochemical properties [20 23]. The modulation of alginate has also been described by adding cholic acid from bile acids

improving the physicochemical properties, the stability of the alginate hydrogels and the viability of the embedded cells [24 25]. The lack of injectability forces to make the hydrogel in a mold outside the body and implanting the final gelled product by invasive surgical procedure instead of by simple injection. Thus, the practical use of hydrogel-based therapies in the clinic is significantly restricted [22]. Alternatively, retarding agents can be added slowing down the alginate gelling reaction and achieving a better control over the gelation rate and a wider working time [13]. Phosphate salts act as retarding agents due to the ability of phosphate groups to interact with the Ca^{2+} source producing calcium phosphate. This prevents Ca^{2+} ions from reacting with sodium alginate to form the alginate hydrogel. Once the phosphate compound is depleted, alginate can form the hydrogel [13 21]. These properties of alginate have been shown, for example, in dental material impressions and orthodontic models [26], where sodium phosphate is added to delay the gelation time of the hydrogel, providing longer working times when loading in alginate impressions [21 27] as well as their biocompatibility with the rat insulinoma cell line INS1E. Hydrogels that gellify too fast, force clinicians to manage this technology very quick under stressful daily work conditions. Seeing that fast alginate gelation supposes a restriction when it is used as a in injectable biomaterial, we have explored the effect of a phosphate salt Na2HPO4 as a retardant agent in order to improve its gelation for cell therapy application in T1DM treatment. To that end, we have characterized the physicochemical properties of the different alginate hydrogels containing Na2HPO4. The novelty of this research resides in the alginate hydrogel gelation delay without affecting their injectability, helping, therefore, to their translation from bench to the clinic. On this regard, hydrogels with a delayed gelation time would add more flexibility to the application of these scaffolds in hospitals.

2. MATERIALS AND METHODS

2.1. Materials

Ultrapure sodium alginate with molecular weight of 75-200KDa and Guluronate/Mannuronate ratio ≥1.5 was purchased from FMC Biopolymer. Calcium sulphate dihydrate ($CaSO_4 \cdot 2H_2O$), FITC apoptosis Detection Kit, Cell Counter Kit-8 (CCK-8) and the *In Vitro* Toxicology LDH based Assay were purchased from Sigma-

Aldrich. Di-Sodium Hydrogen Phosphate dyhydrate ($Na_2HPO_4 \cdot 2H_2O$) was purchased from Panreac. LIVE/DEAD® Viability/Cytotoxicity Kit was purchased from Life Technologies, Rat Insulin ELISA kit from Mercodia and the Pierce® BCA Protein Assay from Thermo Scientific. In this study, a rat insulinoma cell line called INS1E [28] which has been provided by the University of Geneva Medical Center has been used.

2.2. Characterization of alginate hydrogels containing Na_2HPO_4

2.2.1. Alginate hydrogel preparation

Ultrapure sodium alginate (FMC Biopolymer), was dissolved in 1% D-mannitol (Sigma-Aldrich) at a concentration of 1.5%. Then, it was filtered through a 0.22µm pore Minisart Syringe Filter (Sartorius). For gelation, 2.7mL of 1.5% alginate were mixed with 60µL of 1.22M $CaSO_4 \cdot 2H_2O$ (Sigma-Aldrich) diluted in 240µL of distilled water through two LuerLock syringe (BS Syringe) connected with a Fluid Dispensing Connector (Braun). Alginate and CaSO4·2H2O were mixed 15 times until complete homogenization. For retardation gelation time, 60µL of $Na_2HPO_4 \cdot 2H_2O$ (Panreac) were added in the cross-linking reaction from the following solutions 0.1M, 0.3M, 0.5M, 0.6M and 0.9M. Hydrogels were molded between two glass plates with 2 mm spacers, obtaining 6, 10 or 14mm diameter discs with a circular punch (Fig 1A).

2.2.2. Rheology and injectability

Rheological properties of all hydrogels were measured on the rheometer AR1000 (TA instruments. New Castle, United States) with flat plate geometry and compared to alginate hydrogels formed without Na_2HPO_4. Oscillatory shear measurements were conducted at 20°C to obtain the gelation time, the elastic modulus (G') and the viscous modulus (G''). These were determined via time sweep, by dosing 4 drops of 100µL of 1.5% alginate solution and 8 smaller drops of 5 µL of $CaSO_4/Na_2HPO_4$ mixture on the rheometer platform. Next, all conditions were set with a gap at 400µm, a delay time of 3 seconds, a displacement of $1e^{-3}$ rad and an angular frequency of 1Hz. Then a pre-shear of 20001/s was applied to initiate alginate gelation and immediately G' and G'' moduli measurements were performed as a function of time and gelation time was considered

as the value of the G' modulus plateau (Fig. 1B). Three independent experiments, with three replicates each one, were conducted.

Injectability of hydrogels was assessed by passing the hydrogel through a syringe at 25ºC with gauges from 25 to 30. Three independent experiments, with three replicates each one, were conducted.

2.2.3. Homogeneity

To evaluate homogeneity of all different alginate hydrogels, five discs of 10mm in diameter of each hydrogel were weighted (wet weight). Discs were dried at 70ºC in a drying oven (J.P. Selecta. Abrera, Spain) for 1 hour and weighed again (dry weight). Dry/wet ratios were calculated in three independent experiments with three replicates each one.

2.2.4. Swelling and water content

To evaluate swelling behavior and water content, discs of 10mm in diameter were punched and weighted (wet weight: Ws). Then, all discs were lyophilized (Telstar cryodos Freeze Dryer. Terrassa, Spain) and reweighed (dried weight: Wd). Water content (Wc) was calculated as: Wc=Ws-Wd. Afterwards, the dried alginate discs were placed in DPBS with Ca^{2+} and Mg^{2+} to estimate their swelling capacity. At selected time points, discs were removed from DPBS and wiped using filter paper, and then weighed and returned to DPBS until the swelling ratio reached the equilibrium. Swelling ratio (SR) was determined in every time point using the following formulae: SR=(Ws-Wd)/Wd. Three independent experiments, with three replicates each one, were conducted.

2.2.5. Compressive properties

Uniaxial unconfined and confined compression studies were performed to measure the compressive properties, Young's (Es) and aggregated (Ha) moduli and Poisson coefficient of all different alginate hydrogels. Young's modulus is a measurement of the elasticity of a material that has been subjected to opposite forces along an axis and the aggregated modulus defines the stiffness of a material. Discs of 6mm in diameter were punched from each hydrogel and evaluated following the protocol described by Acosta Santamaría [29]. An Instron MicroTester 5548 machine (Instron. Massachusetts, United

States) was used with a precision of 0.0001 N and 0.001 mm in force and displacement, respectively. A monotonic ramp at 1mm/min cross-head velocity was carried out with a 50 N load cell. From the unconfined compression test data, Es modulus was obtained from the slope of the linear region in the stress–strain curve using the initial cross-section area. From the confined compression test data, the Ha modulus was obtained following the same procedure. Poisson coefficient is a constant of a material describing the lateral expansion during axial compression, and is defined as the ratio of lateral and axial strains and directly deduced from Es and Ha. Three independent experiments, with six replicates each one, were conducted.

2.2.6. Pore morphology

Scanning electron microscopy (SEM) images of all different alginate hydrogels were acquired. Samples were frozen in liquid nitrogen and subsequently lyophilized (Telstar cryodos Freeze Dryer. Terrassa, Spain) for at least 24 hours. Finally, samples were coated with a Gold/Palladium thin film and examined with an SEM Inspect™ F50 (FEI Company. Hillsboro, United States).

2.3. Biocompatibility of alginate hydrogels formed with Na_2HPO_4 containing INS1E

2.3.1. Cell culture conditions in alginate hydrogels

Rat insulinoma INS1-E cells were cultured in complete medium (RPMI 1640 supplemented with 10% Fetal Bovine Serum (Gibco), 1% penicillin/streptomycin/glutamine (Invitrogen), 1% sodium pyruvate 100mM (Sigma), 1M HEPES (Lonza) and 0.1% mercaptoethanol (Sigma). 1.5% alginate was mixed with $5x10^6$ cells/mL and hydrogels were formed following the procedure mentioned above. Once alginate had gelled, 14mm flat discs were punched and cultured in a 24 well-plate with complete medium in a humidified incubator at 37ºC and 5% CO_2. The entire procedure was performed under sterile conditions.

2.3.2 Flow cytometry viability and apoptosis assays

Viability and apoptosis of INS1E cells embedded within all different alginate hydrogels were evaluated. At selected time points, medium was removed and hydrogels were dissolved in 1% trisodium citrate dihydrate. Then, cells were collected and stained using

the LIVE/DEAD® Viability/Cytotoxicity Kit (Life Technologies), and the Annexin-V-FITC apoptosis Detection Kit (Sigma-Aldrich). All samples were analyzed using the BD FACS Calibur flow cytometer (BD Company. Franklin Lakes, United States). Unstained cells and cells stained with calcein or ethidium were established as controls in the cell viability assay. Unstained cells and cells stained with annexin or propidium iodide were established as controls in the apoptosis assay. Three independent experiments, with three replicates each one, were conducted.

2.3.3 Metabolic activity and cell membrane activity assay

These assays were determined using the Cell Counter Kit-8 (CCK-8) (Sigma) and the *In Vitro* Toxicology LDH based Assay (Sigma-Aldrich) respectively. In the CCK-8 assay the absorbance was recorded using the Infinite M200 microplate reader (TECAN Trading AG. Männedorf, Switzerland) at 450nm with reference wavelength set at 650nm. In the LDH assay the absorbance was read at 490nm, with 690nm measurement as background. Membrane damage values from the samples were relativized to hydrogels formed without the retardant agent. Three independent experiments were conducted with three replicates each.

2.3.4 Glucose-Stimulated Insulin Secretion (GSIS) assay

To assess the INS1-E cells glucose response, GSIS assay was performed 7 days after cell embedding within all different alginate hydrogels. Discs were washed and incubated with Krebs-Ringer bicarbonate (KRB) for 30 minutes. Next, KRB was replaced with KRB containing 3.3mM glucose and incubated for 2 hours. Then, supernatants were collected and discs were washed and incubated for 2 hours in KRB containing 16.6mM glucose. Final supernatants were collected. The insulin content of collected supernatants was quantified with the Rat Insulin ELISA (Mercodia). Insulin concentration was normalized to total protein content determined with the Pierce® BCA Protein Assay (Thermo Scientific). Three independent experiments, with three replicates each one, were conducted.

2.3.5 Statistical analysis

Statistical analysis was performed with SPSS software, version 21.00.1. Data were expressed as means ± standard deviation and differences were considered significant, for comparison of groups using ANOVA, Tukey's Post Hoc Test when $p<0.05$.

3. RESULTS

3.1 Characterization of alginate hydrogels containing Na_2HPO_4

3.1.1 Na_2HPO_4 effect on hydrogels rheological and injectability properties

The influence of Na_2HPO_4 on the alginate hydrogels formation was studied by oscillatory shear measurements. Elastic modulus (G') and viscous modulus (G'') were measured as a time function in order to characterize the gelation process. G' modulus values were higher than G'' modulus values among alginate hydrogels (Table 1), indicating an elastic solid-like behavior.

G' values of hydrogels with 0.1M and 0.3M Na_2HPO_4 were significantly higher than controls ($p<0.05$), while in alginate hydrogels with higher Na_2HPO_4 concentrations, the final G' modulus diminished significantly ($p<0.05$), demonstrating lower elastic properties. G" moduli were all statistically different than hydrogel without Na_2HPO_4 ($p<0.05$), except for the hydrogel containing 0.1M Na_2HPO_4. G' modulus stabilized when hydrogels solidified, establishing the gelation time when the G' modulus reached the plateau. The obtained gelation times differed considerably among the different conditions tested (Table 1). The gelation of alginate hydrogels without Na_2HPO_4 was around 4 minutes (4.2±0.2 minutes), while the presence of higher concentrations of Na_2HPO_4 (0.5M, 0.6M and 0.9M) slowed down the gelation process significantly ($p<0.01$). Finally, when injectability was assessed, all hydrogels passed through all needles in less than a minute (data not shown).

3.1.2 Hydrogel homogeneity

The homogeneity was determined by comparing the hydratation degree of five punched discs equidistantly distributed in each alginate hydrogel. Discs were weighted in the swollen state (Ws), dried and reweighted (Wd). Homogeneity was calculated as the average of the Wd/Ws ratios (Table 2) of the different discs of each hydrogel containing

different concentrations of Na_2HPO_4. No statistical differences among hydrogels were detected, indicating that Na_2HPO_4 does not affect the homogeneity of hydrogels.

3.1.3 Swelling analysis

The influence of different concentrations of Na_2HPO_4 on water content and swelling properties of alginate hydrogels was assessed. All the hydrogels contained a high percentage of water (around 97%) with no significant differences among them (Table 2). Next, all lyophilized alginate discs were immersed in DPBS in order to calculate the swelling ratio at different time points. Water uptake by the hydrogels increased over the time until they reached the equilibrium (Fig. 2). Hydrogels containing 0.5M, 0.6M and 0.9M Na_2HPO_4 reached the equilibrium within 100-200 minutes, while the rest of the hydrogels needed 2 days, indicating that the Na_2HPO_4 content affects the water uptake rate. The final stable swelling ratio of all hydrogels was similar (Fig. 2).

3.1.4 Compressive properties

Compressive properties of hydrogels by Young (Es) and Aggregate (Ha) moduli were determined by Uniaxial unconfined and confined compression respectively, allowing the calculation of Poisson coefficient. All hydrogels followed a similar tendency with higher Ha than Es values which was in accordance with the need of applying a higher force to deform the hydrogel, because in the unconfined compression test, the hydrogel can generate a lateral deformation when a load is applied; while in the confined compression test the lateral hydrogel deformation is constrained because the discs are placed in a confined space [30]. No statistical differences among hydrogels without Na_2HPO_4 and 0.1 and 0.3M Na_2HPO_4 hydrogels were appreciated (Table 3). In contrast, the presence of 0.5M, 0.6M and 0.9M Na_2HPO_4 showed lower Es and Ha values than hydrogel without Na_2HPO_4 ($p<0.001$). Poisson coefficient values did not change among all the hydrogels (Table 3).

3.1.5 Pore morphology

The effect of Na_2HPO_4 on the internal structure of all hydrogels was examined by SEM. Hydrogels showed a microporous internal structure and the degree of alginate cross-linking and pore size were similar among hydrogels without Na_2HPO_4 and with 0.1 and

0.3M Na_2HPO_4 (Fig. 3A-C). However, hydrogels with 0.5M, 0.6M and 0.9M Na_2HPO_4 showed lower degree of cross-linking leading to an increase of their pore size (Fig. 3D-F).

3.2. Biocompatibility of alginate hydrogels containing Na_2HPO_4 with INS1E

After physicochemical characterization, the biocompatibility of alginate hydrogels containing Na_2HPO_4 was assessed.

3.2.1 INS1E cell viability and apoptosis

We quantified the viability of INS1E cells within the different hydrogels by flow cytometry. At day 1 after cell embedding, the percentage of dead cells in all the hydrogels was higher than at the rest of the time points (Fig. 4A). Hydrogels with 0.5M, 0.6M and 0.9M Na_2HPO_4 at day 1 showed significantly higher dead cell percentages than the control hydrogel ($p<0.05$). 7 days after hydrogel formation cell death percentages decreased drastically, keeping below 1% until the end of the assay with no statistical differences among hydrogels. Hence, solely the highest concentrations of Na_2HPO_4 (over 0.5M) affected cell viability at day 1 after hydrogel formation.

We also quantified the percentage of apoptotic cells within the hydrogels. Apoptosis correlated with cell viability with a dramatical reduction of apoptotic cell percentages at day 7 in all the hydrogels, and no statistical differences were detected among the samples at any time point (Fig. 4B). Hence, different Na_2HPO_4 concentrations neither promote nor reduce the early apoptotic percentage of INS1E cells within alginate hydrogels.

3.2.2 INS1E cell metabolic activity and membrane integrity

Next, the effect of Na_2HPO_4 on the cell metabolic activity over three weeks was quantified. The highest metabolic activity of embedded INS1E cells was achieved a week after hydrogel formation and, afterwards, it remained stable in alginate hydrogels with low concentrations of Na_2HPO_4. The different Na_2HPO_4 concentrations did not affect the metabolic activity of the embedded cells at day 1, except at 0.9M concentration that showed significantly higher metabolic activity than the control ($p<0.001$) (Fig. 5A). At day 7, only hydrogels with 0.6M and 0.9M Na_2HPO_4 showed a significant reduction on the INS1E cell metabolic activity compared to control ($p<0.001$). At day 21, only hydrogels formed with 0.1 and 0.3M Na_2HPO_4 remained

with similar metabolic activity levels than control. The rest of the hydrogels showed a significant metabolic activity reduction which was more notorious when the amount of Na_2HPO_4 was higher ($p<0.001$). The effect of Na_2HPO_4 on INS1E cell membrane integrity was also assessed. No significant differences over three weeks were detected among all the hydrogels, except for hydrogels with 0.9M Na_2HPO_4, which demonstrated significantly higher membrane damage comparing to the control at day 1 ($p<0.05$), day 7 and 21 (both, $p<0.01$) (Fig. 5B).

3.2.3 INS1E cell glucose response

We finally evaluated the insulin secretory response to different concentration of glucose to test the application of these hydrogels in the treatment of T1DM. At day 7, when embedded cells showed the highest viability, INS1E cells produced and released insulin (Fig. 6), responding to glucose stimuli in all the studied alginate hydrogels. There were no significant differences in glucose-stimulated insulin secretion assay among hydrogels, except for the hydrogel with 0.9M Na_2HPO_4 that showed significantly lower insulin response after exposure to 16.6mM glucose ($p<0.001$).

4. DISCUSSION

4.1 Characterization of alginate hydrogels containing Na_2HPO_4

We have characterized and compared distinct alginate hydrogels formed with varying concentrations of Na_2HPO_4, which allows modulating the gelation time and tuning the physicochemical properties of the resultant hydrogels. We have been able to establish the most adequate physicochemical properties of an injectable biomaterial that could be used as a scaffold for insulin-secreting cells.

The rheological results showed a stronger elastic behavior, which is characteristic of a predominantly solid-like behavior, an important factor for the attachment of therapeutic cells [31]. A higher final elastic modulus was observed in hydrogels with 0.1 and 0.3M Na_2HPO_4 than in the control hydrogel, due to the slower release of Ca^{2+} in the presence of HPO_4^{2-}, resulting in a more uniform dispersion of calcium throughout the hydrogel before the cross-linking occurs [20]. However, when the Na_2HPO_4 concentration increased, the elastic properties of the resultant hydrogels diminished significantly. We hypothesize that at high Na_2HPO_4 concentrations, a part of Ca^{2+} ions are retained as $CaHPO_4$, resulting in a lower degree of cross-linking between Ca^{2+} and sodium alginate, leading to a reduction of the elastic properties. This hypothesis based on the Ca^{2+} ions

availability is also reflected on the gelation time, since hydrogels with higher elastic properties (low Na_2HPO_4 concentrations) showed shorter gelation times, while hydrogels with lower elastic properties (high Na_2HPO_4 concentrations) demonstrated longer gelation times. Hence, based on their gelation characteristics, alginate hydrogels containing low Na_2HPO_4 concentrations, in the order of 0.1-0.3M, could be good candidates as injectable biomaterials.

Swelling properties are also very useful for studying hydrogels behavior, since it depends on the inner morphological structure of the scaffold, and it is related to the elastic and mechanical properties of the hydrogel [32-34]. Our results showed that Na_2HPO_4 affects the water uptake rate of alginate hydrogels. Hydrogels containing high Na_2HPO_4 concentrations showed faster water uptake rate which inversely correlated with their elastic properties, as hydrogels with faster water uptake rates demonstrated lower elastic modulus. Regarding the compressive properties, the tendency of both Es and Ha moduli also correlated with the swelling behavior and the elastic properties. Alginate hydrogels containing 0.1 and 0.3M Na_2HPO_4 demonstrated the highest stiffness, with Es and Ha values around 9-11KPa, while hydrogels with higher Na_2HPO_4 concentrations were softer, around 5-7KPa. Importantly, all tested alginate hydrogels ranged within the described native soft tissues Es values, validating the studied alginate hydrogels as 3D matrixes able to mimic the characteristics of native soft tissues (0.1KPa to 40KPa) [35 36].

SEM analysis provided visual proof of the changes promoted by Na_2HPO_4 on the internal hydrogel structure. Hydrogels with absence of Na_2HPO_4 or with 0.1 and 0.3M Na_2HPO_4 showed a similar degree of cross-linking as well as similar small pore size, which can explain their similar mechanical properties and slower water uptake rate. On the contrary, alginate hydrogels containing higher Na_2HPO_4 concentrations demonstrated lower degree of cross-linking and, consequently, a higher pore size. These results confirm the hypothesis that Ca^{2+} ions remain as $CaHPO_4$ providing a lower cross-linking degree. Moreover, hydrogels with a bigger pore size showed softer mechanical properties and an increased permeability reflected on the higher water uptake rate. Similarly, poly-L-lactic acid hydrogels with big pores have shown an enhancement of their permeability and a decrease of their mechanical properties [30].

4.2. Biocompatibility of alginate hydrogels containing Na_2HPO_4 with INS1E

Cell viability and apoptosis assays showed correlation at day 1, when the percentage of dead cells and apoptosis were as high as almost 10% and 60% respectively, as a consequence of the embedding process itself that generates a huge stress on cells [37]. Cell viability was higher in hydrogels containing low Na_2HPO_4 concentrations (0.1M and 0.3M) due to their physicochemical properties. In fact, although the porosity of these hydrogels was lower, it did not compromise the nutrient and oxygen diffusion through the hydrogel. Also, these scaffolds provided appropriate mechanical signals to promote cell proliferation and functionality [30]. Importantly, at day 7, dead cells percentages were significantly reduced, cell metabolic activity was the highest and the membrane damage level was the lowest over the whole study.

Focusing on the effect of mechanical properties and porosity over cell behavior, it has been described that INSE cells within stiffer hydrogels are not able to proliferate [37]. It can be explained by the high alginate concentration that increases the stiffness of the scaffold and reduces the pore size causing higher mechanical cell constrain and nutrient diffusion problems [38]. All our hydrogels contain 1.5% of alginate, and, consequently, their stiffness is lower and show higher porosity than 4% alginate hydrogels. Thus, the mechanical stimuli on the cells and nutrient diffusion capacity are different. This fact would explain the differences of INS1E cells behavior between 1.5% and 4% alginate hydrogels with higher cell viability and metabolic activity in our scaffolds.

Finally, cells within alginate hydrogels were able to secrete insulin after low and high concentrations of glucose stimulation, similarly to INS1E cells in polyacrylamide gels with stiffness around 13.4KPa [39]. Cells within all hydrogels demonstrated similar secreted insulin levels, except hydrogels containing 0.9M Na_2HPO_4, which showed lower secreted insulin in accordance to its lower metabolic activity. Insulin diffusion through the scaffolds was not affected by the smaller pore size of alginate hydrogels with low Na_2HPO_4 (0.1M and 0.3M) as the hydrogel with higher pore size (0.9M Na_2HPO_4) showed lower amount of secreted insulin. Hence, based on our data, it can be concluded that 1.5% alginate hydrogels containing 0.1 and 0.3M Na_2HPO_4, besides having great injectability properties and an adequate gelation time, provide the best mechanical properties and porosity for INS1E cell support and recovery after hydrogel formation.

5. CONCLUSIONS

The present study widely deepens on the modulation of alginate gels properties by the inclusion of Na_2HPO_4 as a retardant agent, demonstrating that its addition in the alginate hydrogel forming reaction slows down its gelation time, changes its mechanical properties as well as its porosity, which are very important parameters for cell survival, proliferation and functionality. Hydrogels containing 0.1 and 0.3M Na_2HPO_4 showed the ideal injectable properties for their application in the clinic, as well as a good biocompatibility with the preservation of the functionality of INS1E cells. Hence, these scaffolds are excellent candidates to be used as injectable biomaterials.

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CAPTIONS

Fig. 1. A) Detailed procedure of alginate hydrogels preparation. 1.- 1.5% Alginate solution and $CaSO_4$-Na_2HPO_4 placed in independent syringes. 2.- Syringes connected and content mixed. 3.- The resultant mixture spread between two glasses with 2mm spacer. 4.- Disk punched from the hydrogel. B) Elastic modulus (G') through time and gelation time determination from rheological measurements.

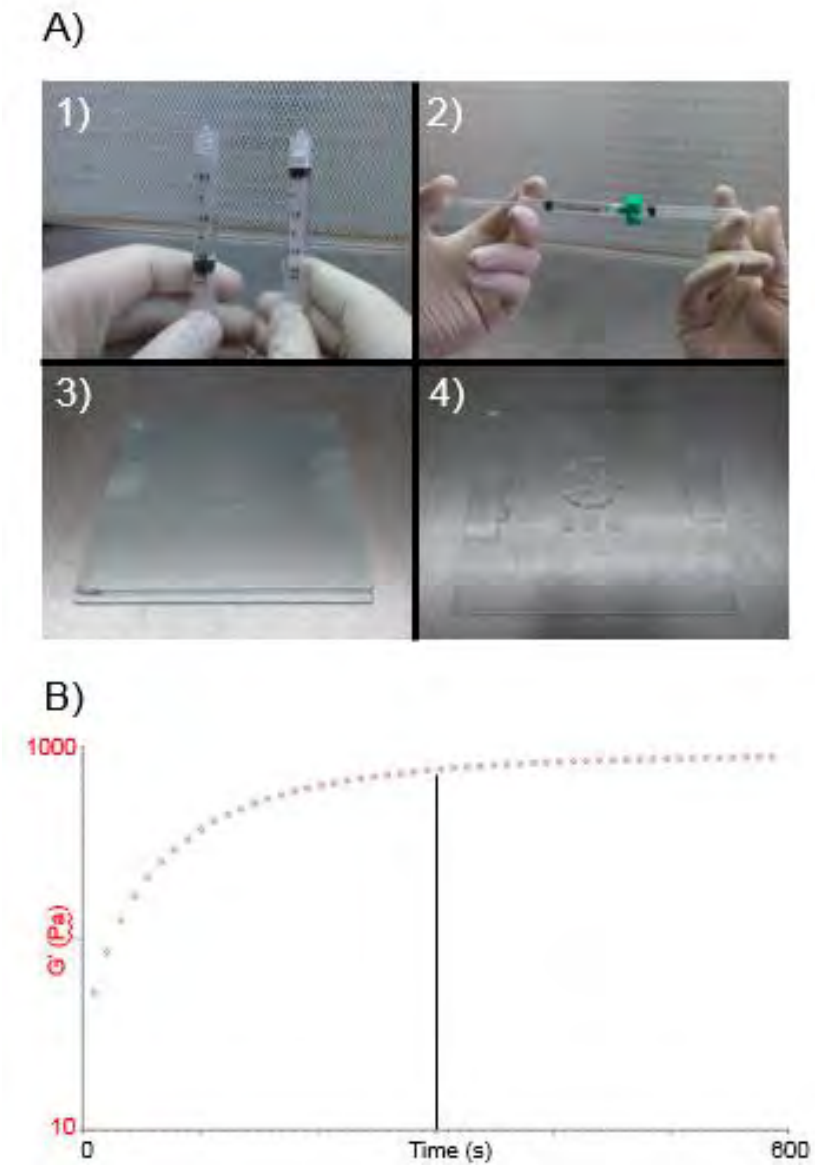
Fig. 2. Swelling ratio profile of alginate hydrogels containing different concentrations of Na_2HPO_4 and control without Na_2HPO_4.

Fig. 3. Scanning electron microcopy (SEM) images from the cross-section of alginate hydrogels. A) Control hydrogel without Na_2HPO_4. Alginate hydrogels containing different Na_2HPO_4 concentrations: B) 0,1M, C) 0,3M, D) 0,5M, E) 0,6M and F) 0,9M.

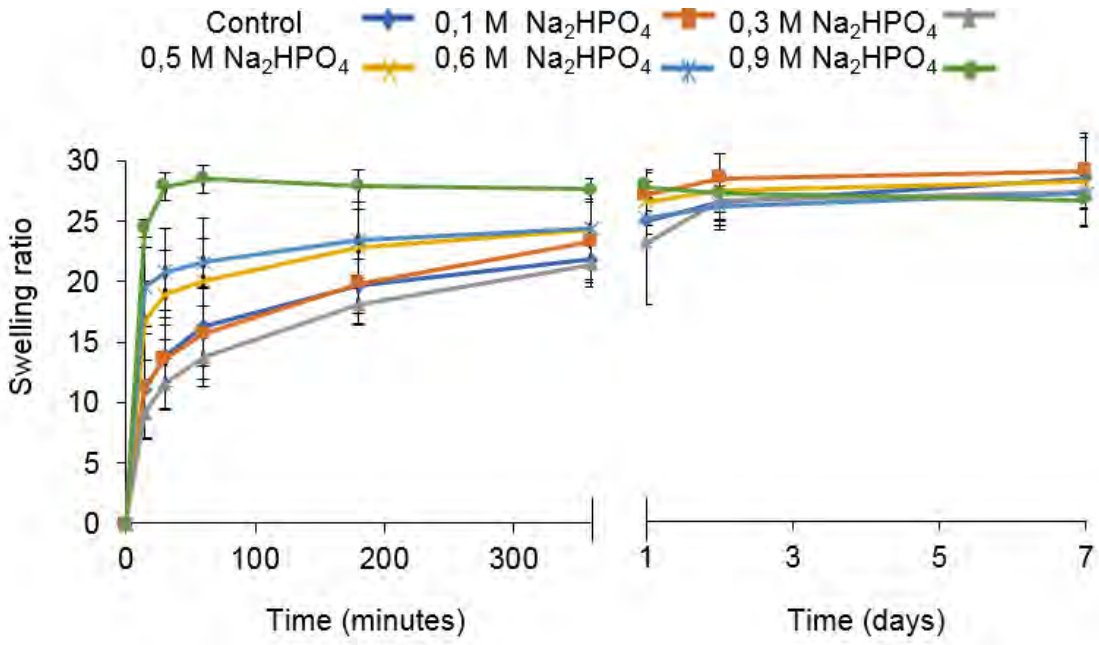
Fig. 4. Effect of different Na_2HPO_4 concentrations on the viability of INS1E embedded within different alginate hydrogels compared to control without Na_2HPO_4. (A) Live/dead analysis and (B) early apoptosis analysis, assessed by flow cytometry. Values represent mean±SD. *: $p<0.05$ compared to control hydrogel.

Fig. 5. Effect of Na_2HPO_4 on the metabolic activity and membrane damage of INS1E embedded within different alginate hydrogels compared to control without Na_2HPO_4. (A) Metabolic activity and (B) membrane damage analysis. Values represent mean±SD. *: $p<0.05$ compared to control hydrogel.

Fig. 6. Effect of Na_2HPO_4 on the insulin secretion of INS1E embedded within alginate matrices compared to control without Na_2HPO_4 by glucose-stimulated insulin secretion assay. Values represent mean±SD. *: $p<0.05$ compared to control hydrogel.



International Journal of Biological Macromolecules, Albert Espona-Noguera, Fig 1.



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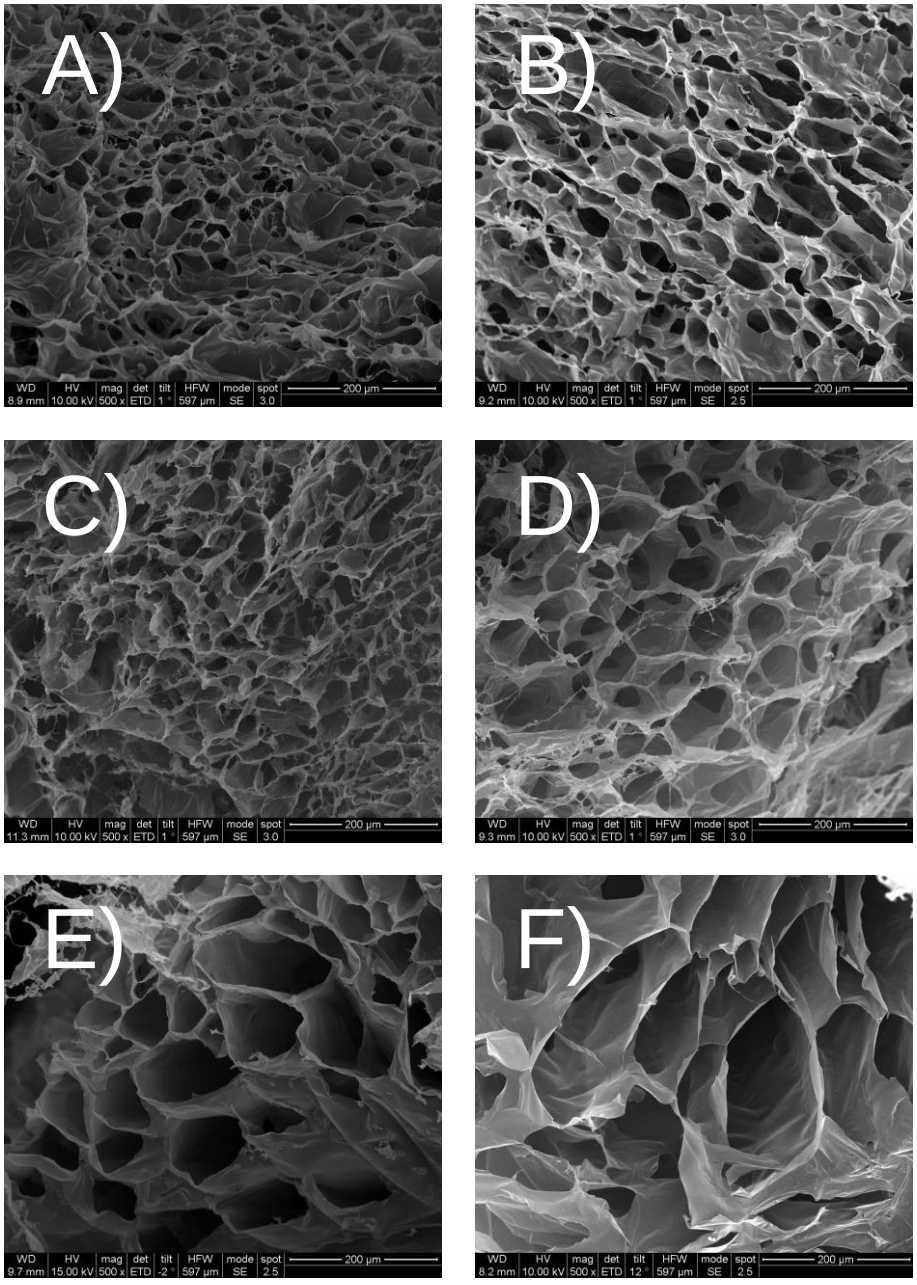

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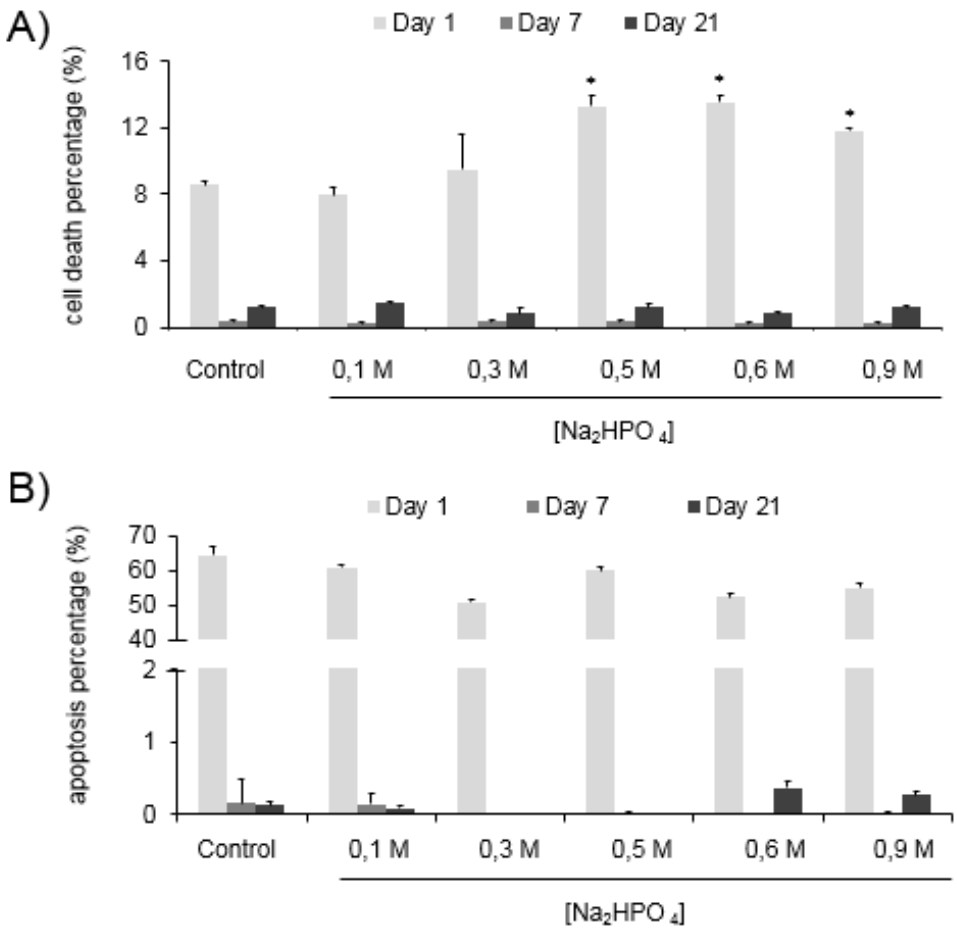

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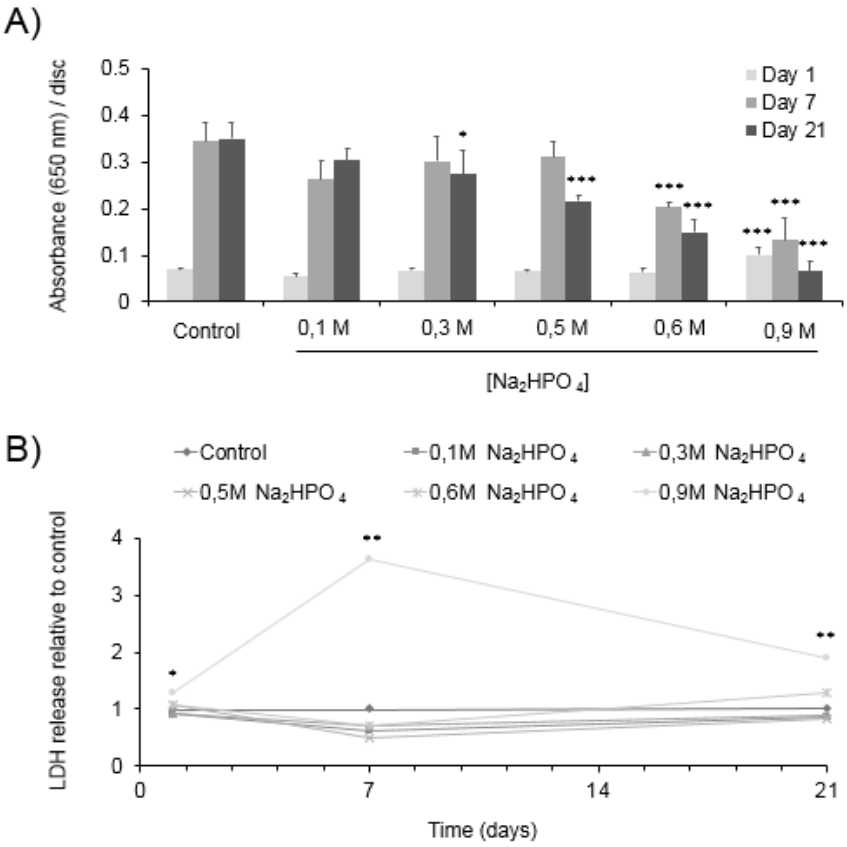

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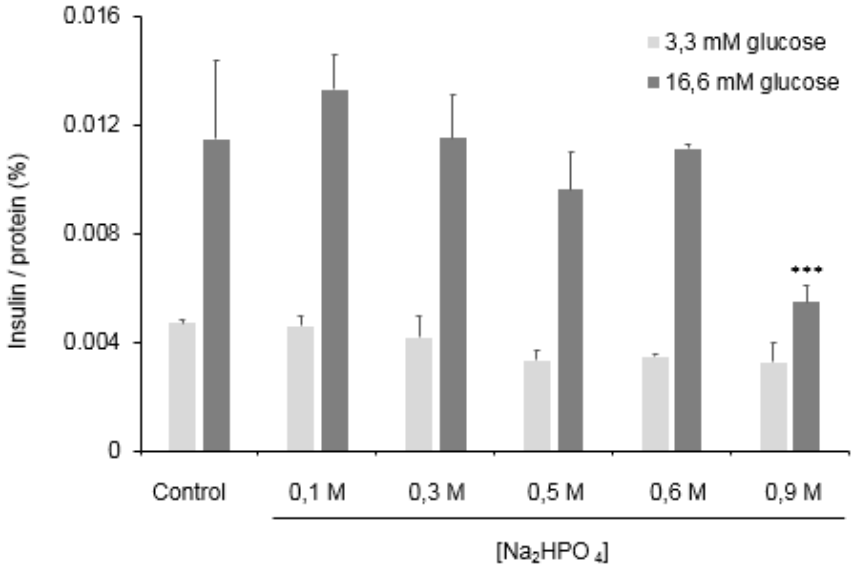

International Journal of Biological Macromolecules, Albert Espona-Noguera, Fig 6.

Table 1. Rheological properties (G' and G'' moduli) and gelation time of alginate hydrogels containing different concentrations of Na_2HPO_4 and control without Na_2HPO_4. Values represent mean±SD. *: $p<0.05$, **: $p<0.01$ compared to control hydrogel.

		[Na_2HPO_4]				
	Control	**0.1M**	**0.3M**	**0.5M**	**0.6M**	**0.9M**
Elastic modulus G'(Pa)	853.2± 110.2	1165.5± 177.8(*)	1445.6 ±55.3 (*)	468.6± 73.9(*)	483.8± 97.2(*)	27.9± 6.4(*)
Viscous modulus G''(Pa)	87.4±9.1	108.1±15.7	120.9±4.5 (*)	31.2±2.8(*)	30.5±4.8(*)	6.8±1.2(*)
Gelation time (min)	4.2±0.2	7.7± 0.4(**)	9.9± 0.2(**)	73.3± 7.6(**)	148.5± 13.1(**)	253.3± 25.2(**)

Table 2. Homogeneity and water content of alginate hydrogels containing different concentrations of Na_2HPO_4 and control without Na_2HPO_4. Values represent mean±SD.

	[Na_2HPO_4]					
	Control	**0.1M**	**0.3M**	**0.5M**	**0.6M**	**0.9M**
Homogeneity	0.3±0.014	0.3±0.007	0.3±0.014	0.3±0.002	0.3±0.014	0.3± 0.010
Water content (%)	97.2±0.7	97.5±0.7	97.2±0.5	97.1±0.6	97.5±0.1	96.1±0.5

Table 3. Compressive properties (Young and Aggregate moduli) and Poisson's coefficient of alginate hydrogels containing different concentrations of Na_2HPO_4 and control without Na_2HPO_4. Values represent mean±SD.***: $p<0.001$ compared to control hydrogel.

	[Na_2HPO_4]					
	Control	**0.1M**	**0.3M**	**0.5M**	**0.6M**	**0.9M**
Young modulus (KPa)	10.1±1.3	9.8±1.0	11.7±2.0	7.6± 0.7(***)	7.9± 0.9(***)	5.4± 1.2(***)
Aggregate modulus (KPa)	1092.7± 110.4	1150.3± 96.8	1183.4± 57.6	741.4± 109.8(***)	724.8± 86.8(***)	785.5± 119.2(***)
Poisson's coefficient	0.4984± 0.0002	0.4986± 0.0001	0.4989± 0.001	0.4983± 0.0002	0.4981± 0.0001	0.4986± 0.0006