The production of a novel poly(vinyl alcohol) hydrogel cryogenic spheres for immediate release using a droplet system

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ABSTRACT

Although freeze-thaw mechanism for producing crosslinked hydrogels is relatively easy and simple, it lacks sufficient integrity and support leading to modifications on its three-dimensional polymeric network arrangement during water removal - drying. Therefore, a strengthening method for obtaining cryogenic spheres of polyvinyl alcohol hydrogels is purposed by dispensing it as liquid droplets into a low temperature solution followed by a modification on its freeze-thaw cycles to confer a good geometry without aggregation. Various materials were incorporated into this hydrogel structure, including poly(acrylic acid) to impart pH sensitivity; hydroxyapatite to improve biocompatibility; and ciprofloxacin as an antimicrobial agent relevant for the treatment of osteomyelitis. The overall network presented a porous structure with a fibrous-like pattern which varied in size and volume, with presence of Ca and P when hydroxyapatite is incorporated. These materials varied the crystalline melting point of the poly(vinyl alcohol) and a linear pH sensitivity function was obtained by the addition of the materials. These hydrogels were found to release ciprofloxacin within 60 min and were able to dissolve at the same time intervals. Therefore, the hydrogel synthesised in this work can be used as an immediate release drug delivery mechanism for the in-situ delivery of active pharmaceutical such as ciprofloxacin for a treatment of osteomyelitis.

keywords: Cryogenic spheres, Polyvinyl alcohol, pH-sensitivity, Immediate release

1. Introduction

Hydrogels are a group of three-dimensional polymeric materials [1,2], able to retain large amounts of water or other liquids and to store chemical compounds due to its hydrophilic structure. It has applications in several fields, in particular as drug delivery systems and cell scaffolds [3,4]. This arises from the soft and rubbery consistency of hydrogels, very similar to living tissues, which makes them an ideal material for a variety of biomedical applications; among other useful properties such as functionality, swelling behaviour reversibility and biocompatibility [5–7]. Hydrogels are denominated cryogenic hydrogels or cryogels when they are physically crosslinked at low temperatures by the freeze-thawed mechanism [8,9].

Poly (vinyl alcohol) (PVA) is commonly used as the main polymeric component for hydrogels in freeze-thawed mechanism. PVA is a hydrophilic polymer used for hydrogel-based drug delivery systems due to its biocompatibility and drug loading capability [10,11]. Several drugs for different biological applications have been used as bioactive compounds of drug delivery systems, such as ciprofloxacin – a multifunctional antibiotic [12,13]. However, there are difficulties related to drug dissolution in water, as many do not completely dissolve, which may affect the final efficiency of such drug delivery systems [14]. Poly(acrylic acid) (PAA) is often added in conjunction with PVA hydrogels to improve mechanical properties and to provide different response modes according to the pH; while hydroxyapatite is added to enhance biocompatibility [15], as this material has approximately the same chemical composition of human bones.

Depending on the crosslinking made in these materials, it is possible to achieve various configurations for drug delivery applications. In the case of crosslinking PVA by freeze-thawing, which occurs a physical crosslink by hydrogen bonding, one can modify the parameters of the thawing profile to achieve the desirable structure for the specific application on biomedical field [16–19]. In this work, a hydrogel designed for the immediate release of ciprofloxacin was investigated while also adding a biocompatible material, hydroxyapatite, for the use of support and prevention for osteomyelitis and bone regeneration as suggested by other authors [20,21].

While PVA hydrogel-based drug delivery systems been extensively studied in recent years, so far there is no research investigating hydrogels produced using a dripping technique on freezing solvents followed by the freeze-thawing mechanism. These spheres are usually prepared by dripping sodium alginate over a calcium chloride solution –

technically named as coacervation [22]. However, it is possible to produce cryogenic spheres by dispensing the polymer as liquid droplets into a low temperature solution, where the droplet upon impact forms a sphere [23]. Nevertheless, this polymeric sphere requires crosslinking to maintain its shape and be used as a drug delivery device.

Nonetheless, the technique performed in this work, to our knowledge, has not been researched due to the technical challenges in achieving desirable shapes. The technique described herein exploits the reports of other researchers that freezing cryogenic spheres at different temperatures might lead to different pore diameters and structures [24,25]. Furthermore, if a cryogenic sphere is produced by the dripping mechanism followed by freeze-thawing and freeze-drying, certain complications will appear as aggregation and loosening of its shape. Within this study we aim to develop a method to vary the temperature in freezing cycles without loosening of the sphere's shape and no aggregation. Therefore, in the present study, hydrogels for drug delivery systems were obtained from different formulations of PVA, PAA, ciprofloxacin and hydroxyapatite aqueous solutions and shaped into spheres through dripping the solution into ethyl acetate at low temperature (-80 °C). Hydrogels were strengthened by freeze-thaw cycles. Characterisation of the samples produced aimed to evaluate the thermal, chemical, physical, mechanical and biological properties of the hydrogel and to investigate the influence of each compound on PVA.

2. Materials and Methods

2.1 Cryogel spheres preparation

2.1.1 Materials

Poly(vinyl alcohol) grade Mowiol 18-88, with average M_w of 130,000 g·mol^{-1}, degree of hydrolysis 99% and Poly(acrylic acid) with average M_v of 450.000 g·mol^{-1} and approximately 0.1% crosslinked was provided by Sigma Aldrich Chemistry Co., USA. Ciprofloxacin was provided by Sigma Aldrich Chemistry Co., China, minimum purity of 98.0%. Hydroxyapatite nanopowder (HAp) was provided by Sigma Aldrich Chemistry Co, Germany, with a minimum purity of 90.0% and particle size lower than 200 nm. NIH 3T3 fibroblast cells were supplied by American Type Culture Collection (ATCC® CRL-1658™).

2.1.2 Hydrogels synthesis

Hydrogels were prepared by dissolving PVA in distilled water on a heater with a magnetic stirrer at 85±5 °C and 500±10 rpm to prepare solutions having a concentration of 5±0.05% per weight in volume. As the polymer was completely dissolved, it was cooled down to room temperature. Drug and PAA, when appropriated, were added. Mass of drug added was 5±0.07% (%w/v), and 20±0.2% (%w/w) of PAA. As the PAA dissolved, the temperature was increased to 50±5 °C. Thus, the hydroxyapatite was added into the necessary solutions in the amount of 5±0.5% (%w/w) of PVA. The solution was then stirred for 24 hours at 600±10 rpm.

At first, the hydrogel was prepared with PVA only to compare each freezing method. Other samples were prepared either with the presence or absence of drug and PAA. However, it is important to note that hydroxyapatite is a low solubility compound. Therefore, it is not completely dispersed in water. Table 1 lists the reagents and their respective proportions to prepare the hydrogels studied in this work.

Table 1. Hydrogel solutions prepared. PVA and CIP were produced based on the volume of water used. HAp and PAA based on the amount of PVA used.

Sample	PVA (%w/v)	PAA (%w/w)	Ciprofloxacin (%v/v)	HAp (%w/w)
PVA	5.00	0.00	0.00	0.00
PVA-PAA	5.00	20.00	0.00	0.00
PVA-HAp	5.00	0.00	0.00	5.00
PVA-PAA-HAp	5.00	20.00	0.00	5.00
PVA-CIP	5.00	0.00	5.00	0.00
PVA-HAp-CIP	5.00	0.00	5.00	5.00
PVA-PAA-CIP	5.00	20.00	5.00	0.00
PVA-PAA-HAp-CIP	5.00	20.00	5.00	5.00

2.1.3 Spheres production

Initially, a solution of PVA was dispensed with a 100 µl micropipette into cooled ethyl acetate (AcOEt) (-80±2 °C). Three freeze-thawing cycles from freezing at -80 °C and cooling at room temperature until defrost was performed. However, it was not possible to produce rounded spheres with this method, and therefore the conditions of freeze-thawing cycles were modified. The frozen spheres were quickly transferred to a refrigerator at -3 °C for 20 minutes and once again quickly transferred to a freezer at -80 °C. This step was repeated three times and was labelled as a freeze cycle. After three freeze cycles at different temperatures (i.e. -80 °C and -3 °C) the frozen spheres were thawed at room temperature until defrost started to occur – the time necessary for this was around 20 min – this step was labelled as freeze cycle with thawing. Ethyl acetate was then removed by filtration, and spheres were dried using a freeze-drier Heto PowerDry® LL1500 Freeze Dryer. Finally, samples were left at 60 °C for five days in an GALLENKAMP Hot Box Oven without fan (65±5 °C) to remove any remaining AcOEt residue.

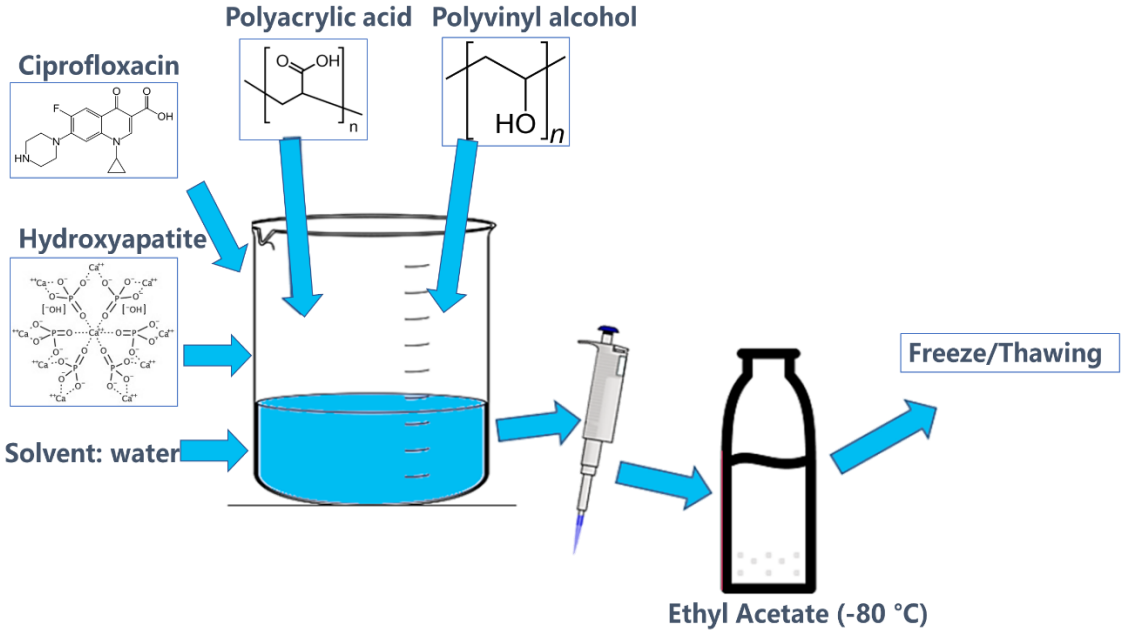

Figure 1. Representation of PVA spheres production (unscaled). Samples were produced according to the different conditions used in this work; hydroxyapatite, ciprofloxacin and polyacrylic acid were added when necessary. After dissolving the correct materials in distilled water, they were dripped with a micropipette into a cooled ethyl acetate (AcOEt) forming a sphere upon contact with the solvent, which was then followed by freeze-thawing technique.

2.1.4 Ciprofloxacin loading by impregnation into the hydrogel structure

Ciprofloxacin was incorporated into the hydrogel by dissolution within the polymeric solution, as described in section 2.1.3. It was also incorporated via the impregnation process. For the impregnation process, a known quantity of ciprofloxacin was dissolved in 5 ml of a 2 N potassium chloride (KCl). Five spheres of each sample condition were added into the solution, with same average weight, and mixed with a magnetic stirrer for 24 h. The following day, the spheres were filtered, washed to remove the excess drug and dried at room temperature.

2.2 Samples characterisation

Characterisation of the spheres produced aimed to evaluate the thermal, chemical and physical properties obtained.

2.2.1 Differential scanning calorimetry

DSC was employed to investigate the influence of HAp, PAA and ciprofloxacin on glass transition, melting temperatures and crystal size distribution of PVA hydrogel spheres in the final dried state. An initial heating cycle to eliminate the samples' thermal history was not performed as the test aimed to evaluate the properties obtained by utilising the freeze-thaw cycles. All samples weighed between 6 and 11 mg, and the specimens were encapsulated into aluminium pans. Tests were carried out in a TA Instruments model Q2000 DSC equipment, with a heating step from 20 °C to 280 °C at 5 °C·min^{-1}.

2.2.2 Scanning electron microscope and EDX analysis

The structure of the loaded PVA spheres cross-section was observed using a Mira scanning electron microscope (TESCAN Performance in Nanospace). To achieve this, the dried cryogenic spheres were immersed in liquid nitrogen and, after some time, were shattered using conventional tools in order to maintain the structural integrity of the materials. Prior to imaging, specimens were sputtered with gold in Baltec SCD 005 for 110 s at 0.1 mbar vacuum yielding a coating of ca. 110 nm. Energy Dispersive X-ray (EDX) analysis was performed with an Oxford instruments detector to confirm the elements presented in the hydrogels.

2.2.3 Swelling and pH sensitivity

The dry weights of the samples (Wd) were determined. Subsequently, samples were placed in distilled water to determine their water uptake (Ws). Tests were carried out in duplicate and data is presented as mean ± SD. The percentage of gel weight was calculated using equation (1).

$$\%\ \mathbf{Swelling} = \frac{\mathbf{Ws - Wd}}{\mathbf{Wd}} \times \mathbf{100} \quad (1)$$

Ws and Wd are the weights of the hydrogels in the swollen state and the dried state, respectively.

To investigate pH responsiveness during swelling, swelling studies were carried out at a pH of 2, 4, 6, 8 and 10 respectively.

2.2.4 Determination of loading efficiency by impregnation

Samples that had incorporated the ciprofloxacin by impregnation after drying were added to the same solution used to dissolve the ciprofloxacin (2N KCl). After 24 hours aliquots of 4 ml were withdrawn and had its absorbance measured by UV-VIS spectrometry in a Perkin Elmer Lambda 2 spectrometer. The experiment was performed in quadruplicate. The calculation of loading efficiency was evaluated using equation (2).

$$\%\ \mathbf{Loading\ efficiency} = \frac{\mathbf{Mass\ of\ drug\ presented\ in\ the\ spheres}}{\mathbf{Theoretical\ mass\ of\ Ciprofloxacin}} \times \mathbf{100} \quad (2)$$

2.2.5 Drug release

Kinetics of drug release of the drug-loaded formulations (Table 1) was evaluated through basket drug release model. Amounts of 10 mg of dried spheres were added into metallic baskets with 450 ml at 37±1 °C with a pH of 7 and a stirring rate of 50 rpm. Aliquots of 4 ml were withdrawn at different time intervals and had its absorbance measured by UV-VIS spectrometry in a Perkin Elmer Lambda 2 spectrometer. The percentage of drug released %RD was evaluated using equation (3).

$$\%\mathbf{RD} = \frac{\mathbf{A}}{\mathbf{A_f}} \times \mathbf{100} \quad (3)$$

where A is the aliquot absorbance, and A_f is the absorbance after it reaches constant values, no variations in the absorbance readings and it was qualified as 100% drug release.

2.2.6 Dissolution tests

For dissolution tests of these spheres, samples containing ciprofloxacin (Table 1) were added in glass vials bottles filled with pH buffer 7 and placed in a laboratory drying oven with glass door (Salvis VC-20 vacuum oven)at 37 °C. Photographs were taken at different time points, i.e. at the beginning of the test, when the sphere is partially dissolved as well as when is fully dissolved. Shperes were photographed to visually indicate the progress of dissolution.

2.2.7 Antimicrobial activity

To examine the antimicrobial activity of the cryogel spheres compositions containing ciprofloxacin (CIP) (Table 1), a Kirby Bauer agar diffusion test was carried out. Two bacteria strains were cultured overnight, *S. aureus* (NCTC 12981) and *S. Typhimurium* (ATCC 14028) and diluted to a working concentration of ~108 colony forming units per mL (CFU/mL). Nutrient agar plate surfaces were inoculated using a swab. For the positive control, the surface of an agar plate was inoculated, and triplicate antimicrobial susceptibility discs of 13mm diameter were placed on the agar surface, a solution of 5% CIP was prepared, and triplicate 50 µL drops were pipetted onto the surface of the inoculated agar plate. For each test sample, after inoculation of the agar, three wells were aseptically punched into the agar with a sterile cork borer, and five microspheres were placed evenly along the inner wall of each well. The microspheres were then rehydrated by the addition of 50 µL of phosphate buffered saline (PBS) to each wells allowing the microspheres to swell. Each condition was repeated on two plates, one for each bacteria strain. The agar plates were then incubated for 24 hours at 37 °C. After incubation, the zone of inhibition around each sample was recorded.

2.2.8 Cytotoxicity

Samples were sterilised prior for cytotoxicity testing with Isopropanol alcohol (IPA) for 30 s, PBS for 30 s and followed by DMEM media for 30 s. After that, the samples were incubated overnight with DMEM. Serial dilution of 10000, 8000, 4000, 2000, 1000, 500, and 100 µg/ml for each sample was prepared on the next day. The cytotoxicity potential of the microspheres was evaluated using an MTT colourimetric assay. 3T3 cells (1×10^4 cells/ml per well) were seeded in a 96-well plate and incubated at 37 °C and in 5% CO_2. Cells were then exposed to different concentrations, for 24 hours or 48 hours.

Cells were then treated with MTT reagent for 3 hours at 37 °C. DMSO was added to each well to dissolve the formazan crystals. The optical density (OD) was recorded at 540 nm in a microplate reader (BioTek Synergy HT, Swindon, UK), and the percentage of residual cell viability was determined.

3. Results and Discussion

3.1.1 Visual inspection

Firstly, samples produced through freezing in AcOEt the spherical shape intended (Figure 2a). As the AcOEt temperature raised (because the recipient containing the liquid was removed from the freezer at -80 °C to allow the PVA solution to be dripped into AcOEt), spheres took longer to freeze.

Despite this, at first, spheres remained separate from each other. However, as the medium temperature increased during the thawing stages, the spheres formed large agglomerates due to secondary interactions, in some cases losing the spherical shape. After the third thawing stage, some spheres could not keep the shape, indicating freeze-thaw cycles into AcOEt had to be improved for producing PVA hydrogel spheres (Figure 2b).

Conversely, if the spheres were produced with freeze cycles only, i.e., freezing the solution at different temperatures followed by freeze-drying, they present a more rounded shape without aggregation on drying. However, they still present some deformities observed at the macroscopic scale (Figure 2c).

Therefore, samples were produced by a combination of freeze cycles and thawing; samples with these modifications presented a more well-defined geometry. Freeze cycles help to maintain the shape of the sphere in the first cycles without losing its geometry which we propose is due to the minimal hydrogen bonding occurring. However, after the third freeze cycle with thawing, spheres could completely keep the shape. This demonstrated that it was a more suitable freeze-thaw method (Figure 2d) and was selected for the formulations containing ciprofloxacin, PAA and HAp. These samples exhibited essentially the same characteristics as the spheres made of PVA only (not shown).

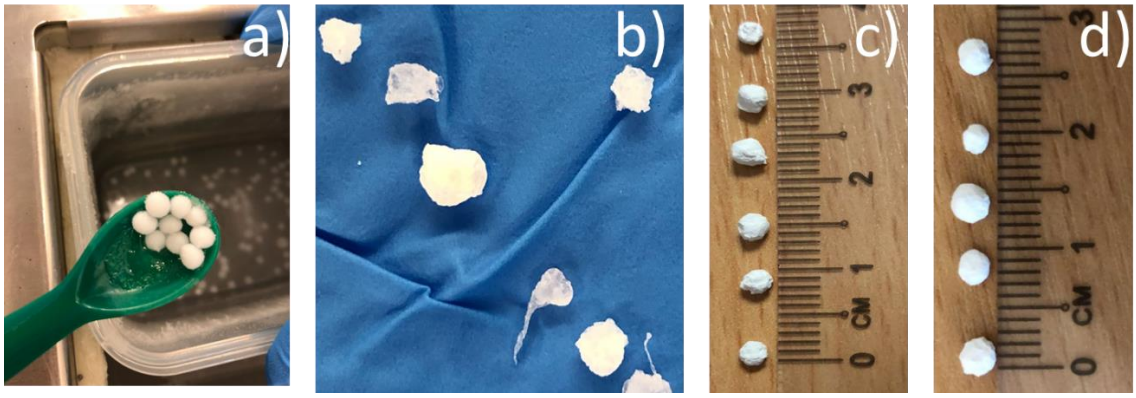

Figure 2. (a) Spheres frozen by the dripping method; (b) Samples after freeze-thawing and following freeze-drying; (c) Samples after freezing cycles and (d) Samples after freezing cycles and thawing following freeze-drying.

3.1.2 Thermal properties

The effect on the polymer, HAp and the drug added to the PVA hydrogel was investigated using a DSC technique. Firstly, the glass transition temperature of PVA and the α relaxation of PAA are, respectively, 84.68 °C and 63 °C. PAA is an amorphous material and has no crystalline melting peak. The PVA and CIP have melting temperatures respectively at 227.88 °C and 276.77 °C. The β-transition of the crystalline PVA domains was identified due to the relaxation of the domains in the crystalline regions. The HAp had no significant thermal transition but both PVA and PAA presented thermal degradation events.

Hydrogels studied in this work (Figure 3a) exhibits first that the incorporation of PAA decreases the melting point and increases the glass transition temperatures compared to PVA. It is known that due to the addition of PAA, the tendency of PVA to crystalize fades due to some strong H-bonding interaction between PVA and PAA in the amorphous phase, which can be seen by the broad melting peak obtained by PVA-PAA samples [26].

The addition of HAp increases the melting point of PVA and PVA-PAA samples, the effect of HAp as an addition to improve the mechanical properties of the hydrogels are known and several researchers have reported this phenomenon [15,27,28]. Conversely, when ciprofloxacin was added to the structure of these materials there is a strong decrease in the crystalline melting temperature of PVA, exhibiting the same characteristics as with samples without the drug. This decrease could be due to the interaction of the PVA and CIP; similar reports for PVA polymer have been shown [29] and for other polymers [30].

In addition, the sample containing HAp still has the highest value of PVA melting point temperature in which sample PVA-HAp-CIP exhibited the highest for samples containing the drug.

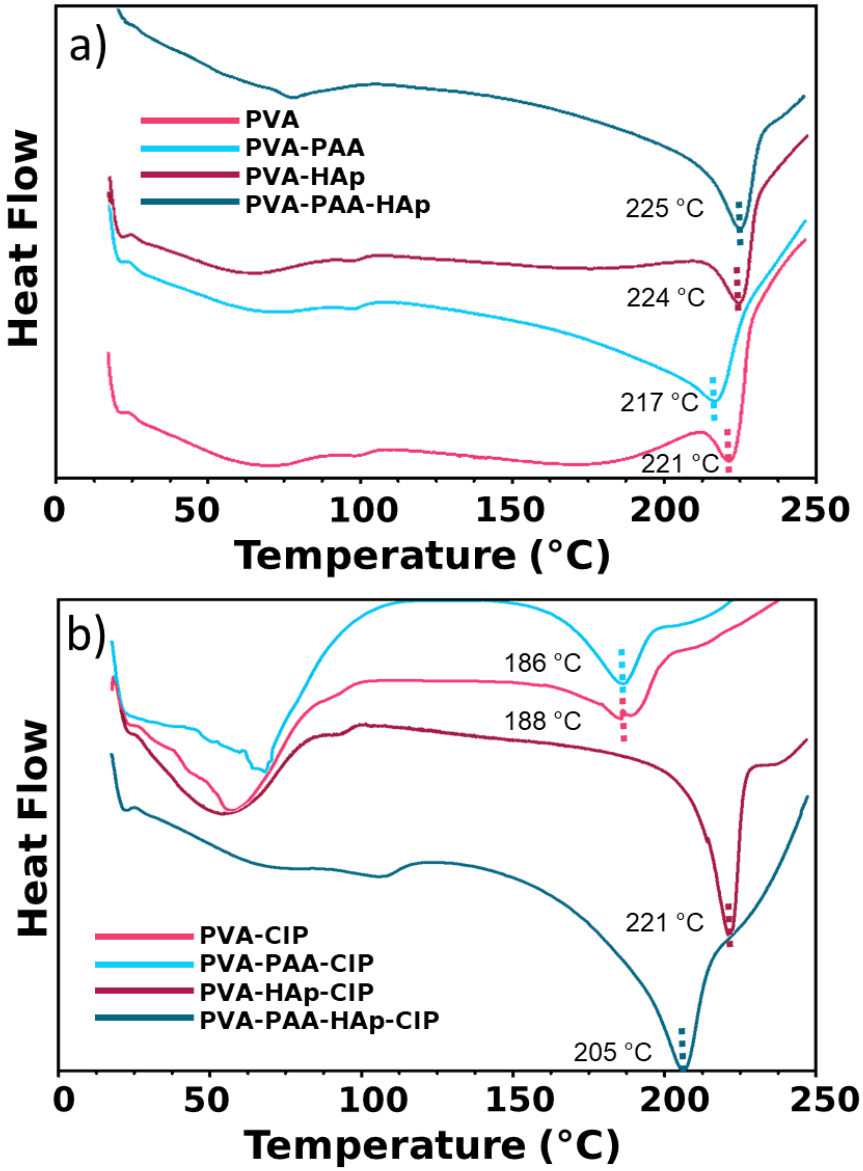

Figure 3. DSC to investigate the effect on Tm and Tg of PVA and PAA when adding HAp (a) and CIP (b) to the hydrogel structure – the scale of heat flow from (b) is 2x higher than (a).

3.1.3 SEM observation

The morphology of the hydrogels was investigated using SEM to understand the effect of freezing using the dripping method; for samples of PVA and PVA-PAA (Figure 4) it is possible to see that the samples maintained the overall spherical shape (Figure 4.a,d) and

upon further inspection of its surface, a porous structure was observed throughout the sample (Figure 4.c,f). This porous structure can be used to create pre-defined geometries for drug delivery vehicles and allow cell infiltration and incorporation of payloads after crosslink [31]. In the cross-section of these spheres, it was possible to observe two different morphologies within the interior of this material - a porous structure, also seen on the surface, and a fibrous pattern-like (Figure 4.b,e). The fibrous structure is beneficial for cellular interaction. The overall structure comparing the addition of PAA was the presence of whiter regions on the cross-section images, which could indicate an increase in diameter of the fibrous pattern and smaller pores on the surface due to the effect of PAA.

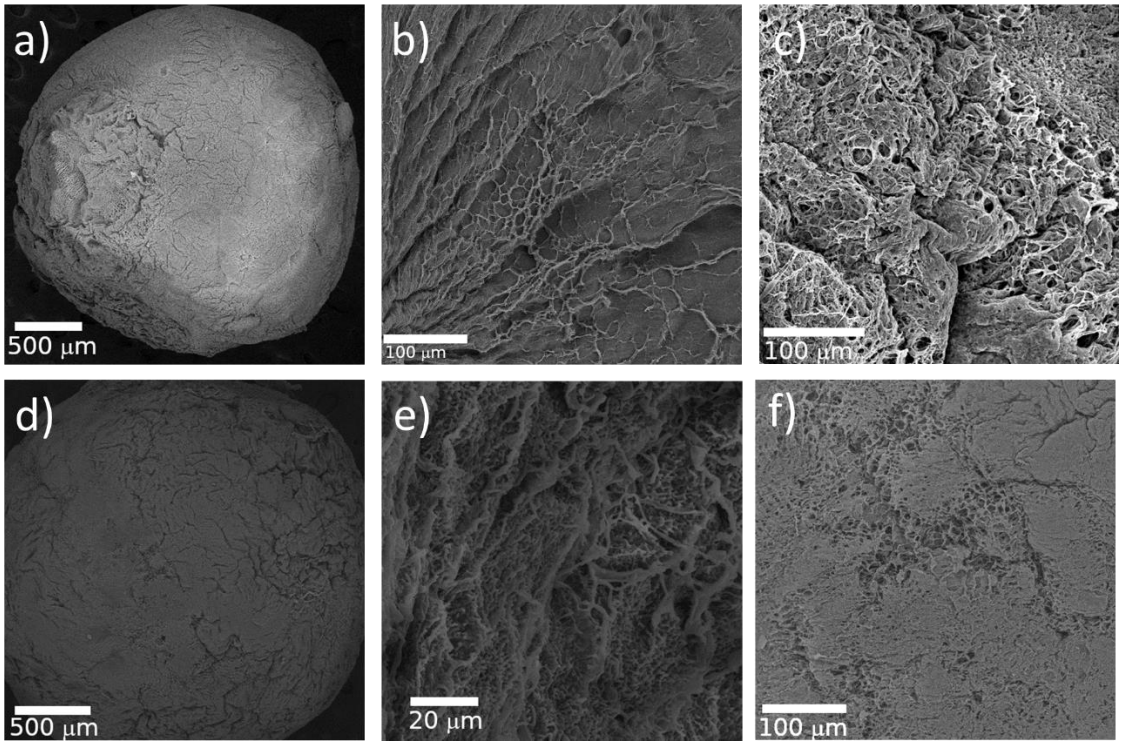

Figure 4. SEM images of hydrogel spheres frozen into AcOEt, the first row are PVA and second row are PVA-PAA samples; (a,d) overall image; (b,e) cross-section and (c,f) zoomed surface.

The addition of HAp to these hydrogels (Figure 5) evidences the formation of smaller sphere sizes and decreased pore sizes. It is also possible to observe the formation of a fibrous like-structure that is slightly different from the hydrogels without HAp; these patterns had different sizes and thickness. This might be due to the interaction of HAp with the polymers. However, even on the surface of this material, it is possible to observe this spiderweb-pattern. We deduced that the technique of freeze-thawing performed here

by dripping mechanism led the HAp particles to interact with the polymer chains forming this structure, which was maintained after drying by the freeze-dryer. It is interesting to note that the morphology results shown here are rather different from the ones shown in literature for freeze casting. This is due to this approach by the freeze-thawing technique before drying the samples where we obtained a porous structure rather than the conventional laminar generally found in these techniques [23,32].

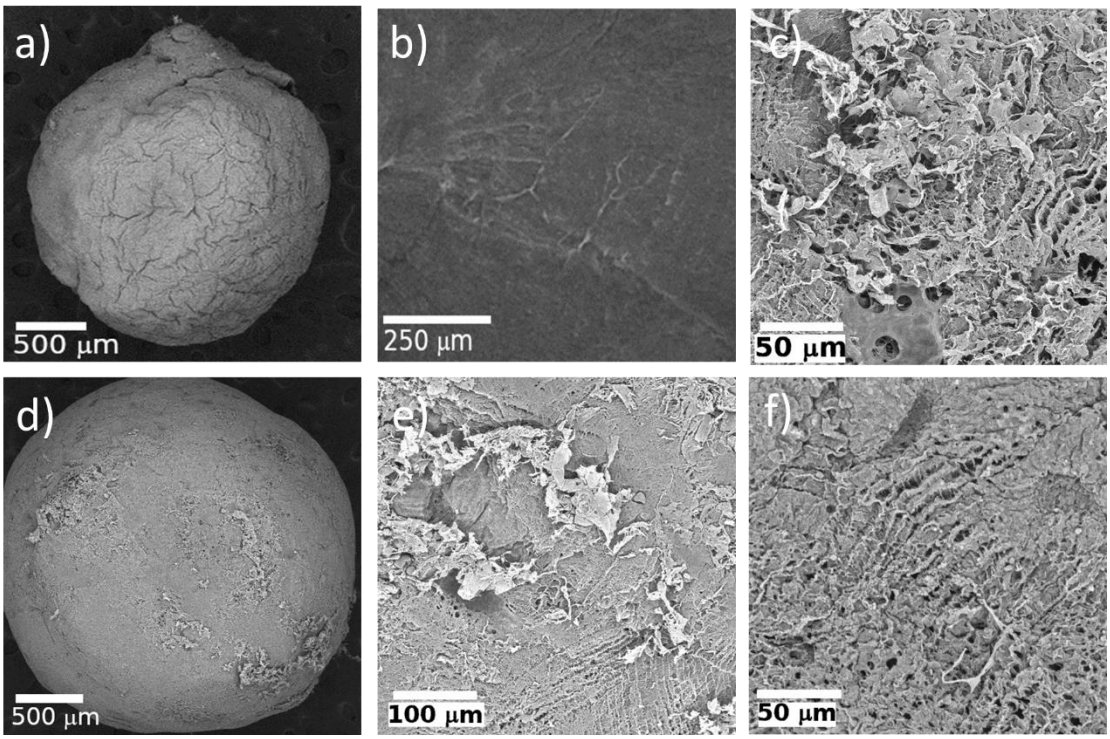

Figure 5. SEM images of hydrogel spheres frozen into AcOEt, first row are PVA-HAp and second row are PVA-PAA-HAp samples; (a,d) overall image; (b,e) cross-section and (c,f) zoomed surface.

Energy dispersive X-ray analysis was applied to samples samples containing HAp on the regions of the fibrous pattern confirmed the presence of hydroxyapatite. Table 2 presents the approximated chemical composition by weight of each sample.

Table 2. Approximated chemical composition obtained by EDX for the hydrogels investigated in this work containing HAp.

Element [%wt.]	Sample	
	PVA-Hap	PVA-PAA-HAp
C	27.02	51.45
O	38.43	44.72
P	11.79	0.33
Ca	22.76	3.50

The calcium characteristic radiation could be identified in all of the four samples but decreasing with increasing the complexity of the formulation by adding PAA.

3.1.4 Swelling Studies

To investigate the ability of the hydrogels for targeted drug delivery, studies on swelling were performed (Figure 6). The results elucidate the effect of the addition of PAA on increasing the swelling value due to the carboxylic acid chains. When HAp was incorporated into the structure, these values were lower for both PVA and with the addition of PAA. These results correlate with the DSC results and its morphology, leading to a more compact and resistant material. Nonetheless, the effect on PAA with the addition of HAp is still noticeable due to the increased values.

It is known that the addition of PAA to PVA can induce a pH-sensitivity in the structure of the materials. Therefore, studies at different pH were conducted. The effect of swelling of PVA is unchanged while varying the pH until the highest number investigated and is comparable to those reported in the literature [33].

Nonetheless, when adding PAA the materials, follow a somewhat linear function while increasing the pH. The effect of controlling the pH sensitivity of these materials is one requirement for biomaterials [14] and the linear function of PVA-PAA have been reported by other authors as well, though not to a wide extent shown in this work [34,35].

The addition of HAp to the PVA also seems to exhibit a pH sensitivity though not following a linear function and swelling less than the PVA alone. When HAp was incorporated in the hydrogel with PAA the linear function is also observed while with lower swelling values. PVA hydrogel is reported to have low pH-sensitivity [36] and could have been benefited from the HAp in the structure by slightly improve its response.

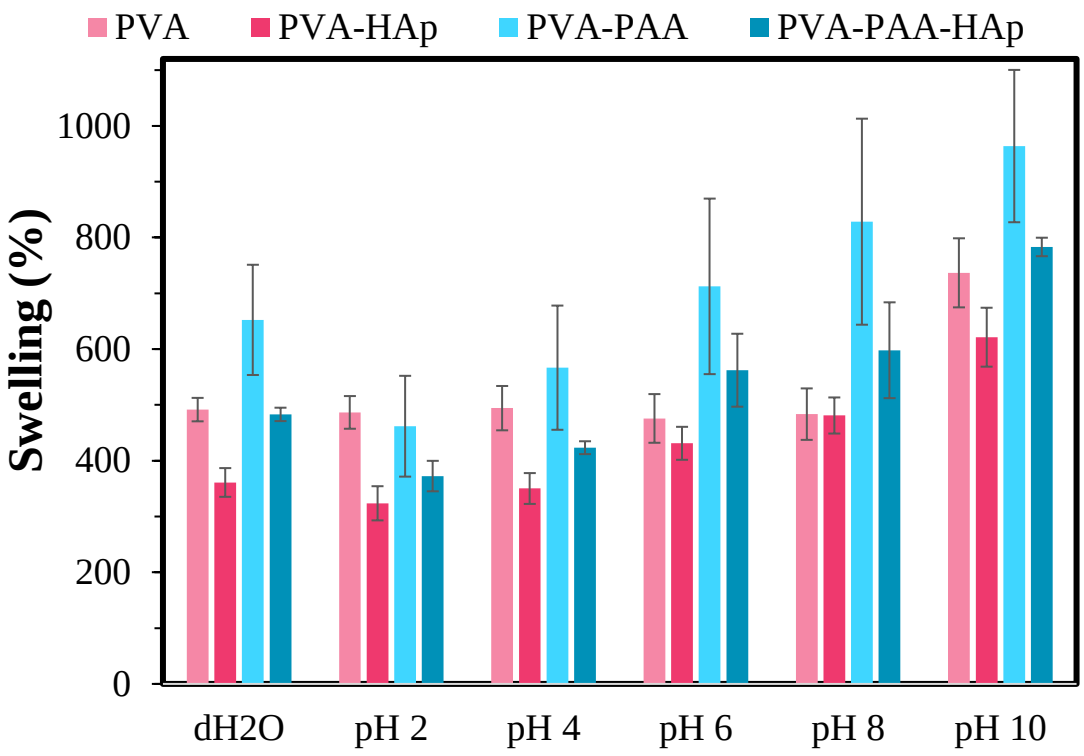

Figure 6. Swelling in distilled water and pH-sensitivity tests of the studied hydrogels; although PVA seems to show sensitivity towards higher pH values, they were not varied towards other pH values.

When the spheres reached swelling equilibrium, they were immediately removed from the buffer solution and photographed (Figure 7). It is possible to observe an increase in volume due to the swelling and pH sensitivity, as expected from hydrogels. It seems to follow the trend shown in swelling graphs (Figure 6).

However, it is harder to perceive differences with the addition of HAp and although all components present hydroxyl groups in their chemical structures, only PVA and PAA contribute significantly to the absorption of water by hydrogen bonds [1].

The ability for pH sensitivity in the point of biomaterials is relevant is due to the different pH values that certain body tissues have, such as the neutral pH in the intestine; also, chronic wounds have pH between 7.1 to 5.4 [37,38].

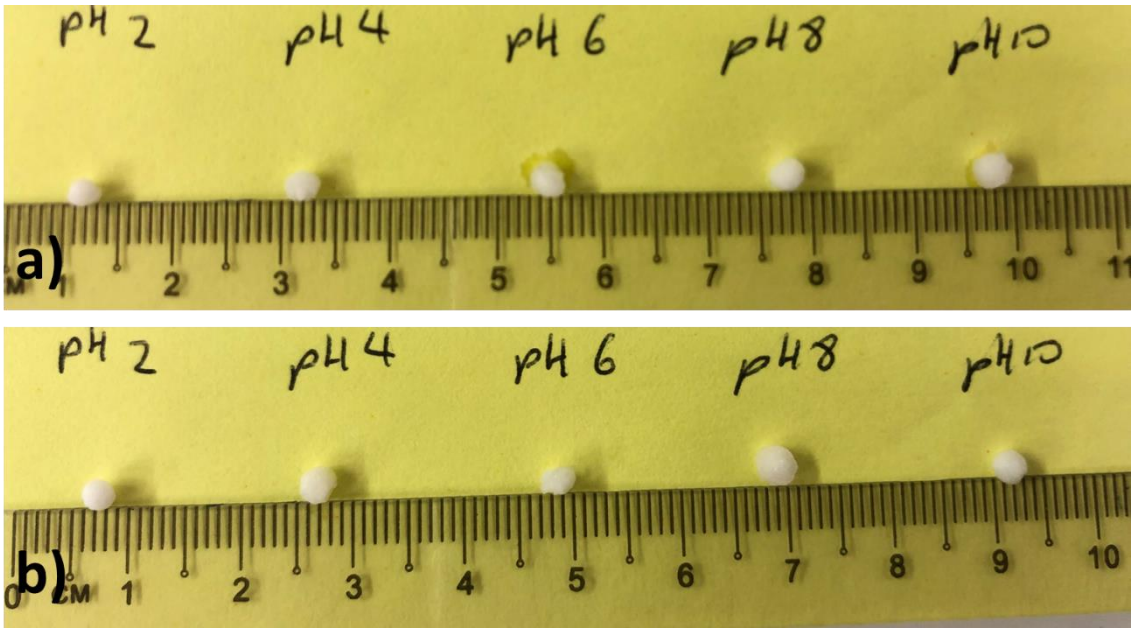

Figure 7. Hydrogel spheres of (a) PVA-PAA and (b) PVA-PAA-HAp after reaching equilibrium swelling at different pH values.

3.1.5 Drug release

The drug release of ciprofloxacin incorporated into the structure of the hydrogel spheres (Figure 8) exhibits that for PVA and PVA-PAA the drug is released nearly at 30 min while when HAp was added to the structure, the drug is fully released only at 120 min for PVA-CIP and 350 min when PAA was added. In contrast, PVA-HAp hydrogels only fully release ciprofloxacin after 6 h – which is in agreement with DSC data. These results suggest that samples without HAp could be used for applications demanding drugs for immediate release.

Another important result was that the PVA and PVA-PAA hydrogels dissolved at the same time that the drug was fully released, i.e., 35 min for PVA and 40 min for PVA-PAA. Hydrogels which incorporated HAp did not dissolve in the timeframe of the test. These materials maintained the integrity in swelling tests at room temperature, however, these spheres slowly dissolved at temperatures higher than 35 °C. There is a desired criteria for immediate release drug delivery system whereas it is important that the carrier should be dissolved within a short period [39,40]. The porous structure observed by the SEM could have probably led to this fast time response delivery. Nonetheless, it is possible to modulate the profile of samples with HAp to dissolve and deliver the drug

under the allowed time frame for immediate release. This can be achieved by controlling the steps on freeze-thawed mechanism, i.e., by controlling the amount of crosslinking and it could be done by either reducing the amount of cycles or the thawing time.

The entrapment efficiency of these hydrogel spheres by impregnation of ciprofloxacin reported values of 0.26 % for PVA-CIP, 0.12 % for PVA-PAA, 0.26 % for PVA-HAp and 0.24 % for PVA-PAA-HAp. These were very low values, and due to the crosslink occurring between the polymeric chain, there was not enough space for the drug to be entrapped after the hydrogel was formed; nonetheless, spheres produced by sodium alginate have reported values of impregnation of 10 % [22]. The increase in this loading was due to the different fabrication method, by coacervation. When the sphere was formed via the dripping mechanism the solution where the sphere will be formed ($CaCl_2$ for sodium alginate) will also contain the drug in a specific concentration and this mechanism may report values on the order of 90 % of entrapment efficiency [41].

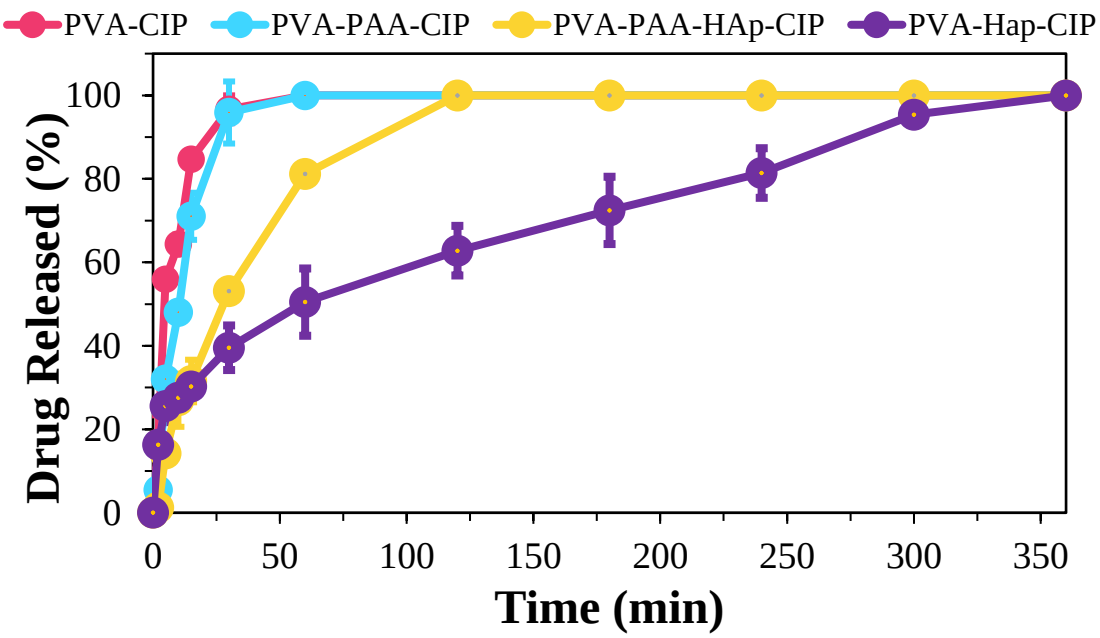

Figure 8. Percentage of released drug in function of time for the studied samples. It is possible to observe the effect of Hap – leading to a slow release of ciprofloxacin.

3.1.6 Dissolution studies

The cryogenic spheres containing ciprofloxacin and without HAp dissolved in the drug release tests. Therefore, these spheres were evaluated at which time it was fully dissolved (Figure 9). For samples without HAp, it is possible to observe that the dissolution follows

the drug release curve of ciprofloxacin from Figure 8. The time for the sphere to be fully dissolved matches the time for the complete release of ciprofloxacin release test. In contrast, samples containing HAp improves the structure of these hydrogels from reinforcement of intermolecular and intramolecular bonds and maintain its integrity within the time of the study. We believe the structure of the samples containing HAp was improved leading to a high crosslink polymeric network which does not led the pH 7 buffer to dissolve this material, as supported by our previous research [15].

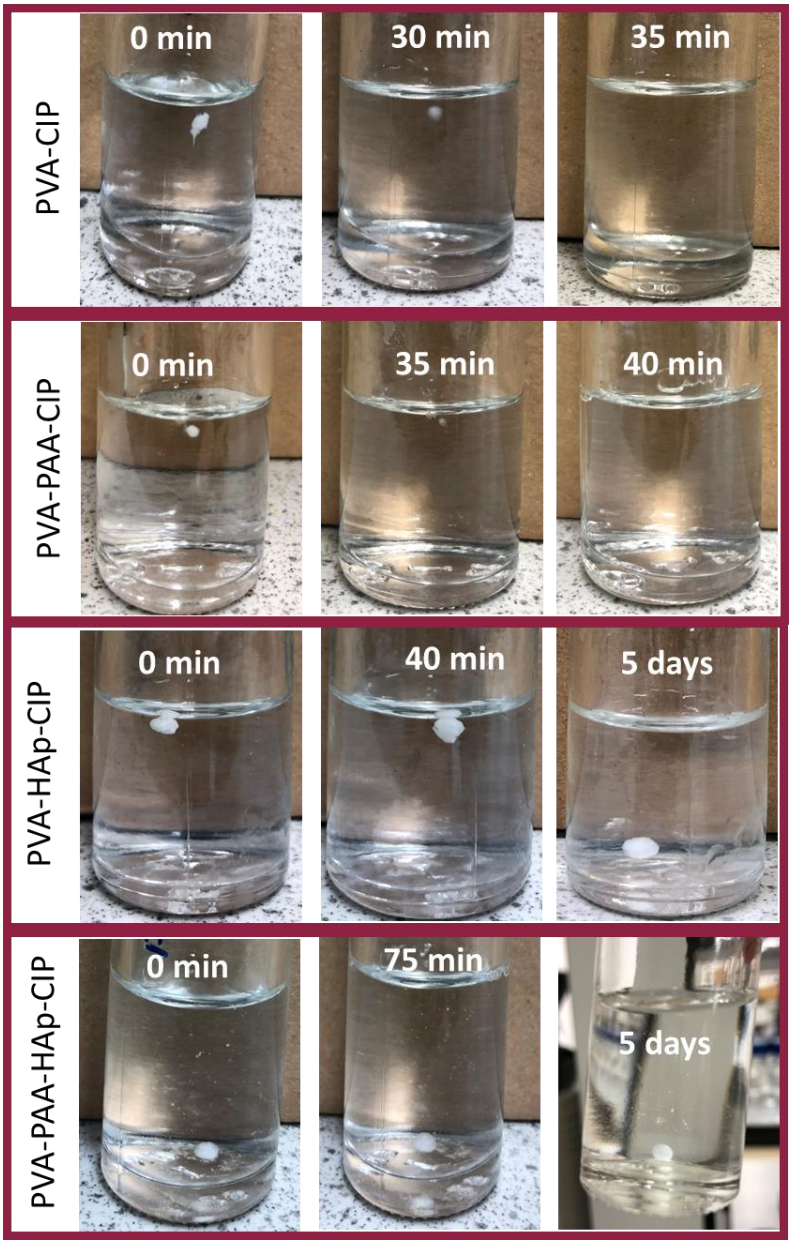

Figure 9. Dissolution of the cryogenic spheres incorporated with ciprofloxacin. For samples without HAp, the last time measurement was performed at the point where there was no visual observation of the sphere.

3.1.7 Antibacterial activity

To test the efficacy of these spheres against known micro-organisms that cause osteomyelitis, *Staphylococcus aureus* and *Salmonella typhimurium* [42–44] (Figure 10). As a standard result, pure ciprofloxacin was effective against these bacteria strains (Fig.10 a-b). Nonetheless, when this drug was incorporated into a hydrogel, they also exhibit a good antimicrobial effect against them. In addition, the effect of dissolution for spheres without HAp, can also be observed (Fig.10.c-f).

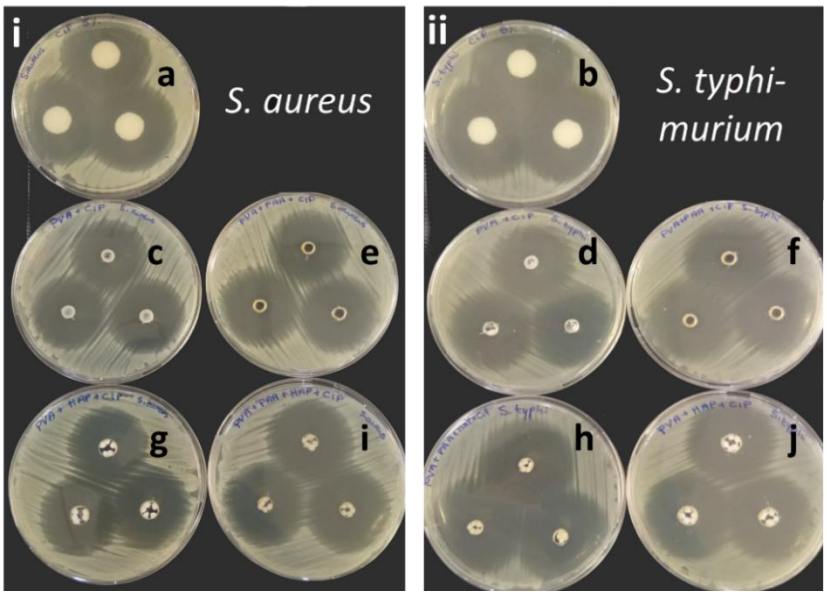

Figure 10. Antibacterial activity of (i) *S. aureus* and (ii) *S. typhimurium* on (a-b) 5% Ciprofloxacin using antibiotic control paper; cryogenic spheres (c-d) PVA-CIP; (e-f) PVA-PAA-CIP; (g-h) PVA-HAp-CIP; (i-j) PVA-PAA-HAp-CIP.

The zone of inhibition diameter can provide an accurate measurement and comparison from groups against specific micro-organisms and according to the standard values provided by NCCLS, pathogens are classified as sensitive (> 21 mm), intermediate resistant (16-20 mm) and resistant (< 15 mm) [45] to the antibiotic used. The effective zone of inhibition of these materials (Table 3) exhibited zone of inhibition higher than 20 mm. Therefore, the bacteria in this work are sensitive to ciprofloxacin and incorporating this antibiotic into the hydrogel also shows sensitivity. In addition, samples containing HAp had the highest values of zone of inhibition. This may be due to the more controlled release of the drug compared to the immediate release that occurs when this ceramic is not within the structure of the hydrogel.

It is worth mentioning that since the method used for the pure drug, disk diffusion method using antimicrobial susceptibility discs of 13mm, it is different than the spheres and a direct comparison was not possible.

Table 3. Average and standard deviation of inhibition zone diameter, n=3 for samples containing ciprofloxacin. 5% CIP represents the pure ciprofloxacin tested antibiotic control paper (13 mm).

Sample	*S. aureus*	*S. typhimurium*
5% CIP	44 ± 0.8	48 ± 0.5
PVA-CIP	34 ± 0.5	36 ± 0.5
PVA-PAA-CIP	35 ± 1.0	37 ± 0.8
PVA-HAp-CIP	36 ± 0.8	38 ± 0.5
PVA-PAA-HAp-CIP	38 ± 0.5	42 ± 0.5

3.1.7 Cytotoxicity

The results for MTT assay of NIH 3T3 cell line following a 24 h and 48 h exposure are shown in Figure 11. After the 24 h exposition, it was possible to observe that all spheres presented to be non-toxic (cell viability > 72%) in all concentrations.

Furthermore, after the 3T3 cell line being exposed for 2 days, it can be observed that there was an increase in cell viability for most of the samples and still exhibiting a non-toxic profile. Although, sphere samples were produced with AcOEt – that is known to be toxic for specific concentrations, the procedure performed in this work shows that any remaining solvent in the hydrogel sphere was not effective to decrease the cellular viability.

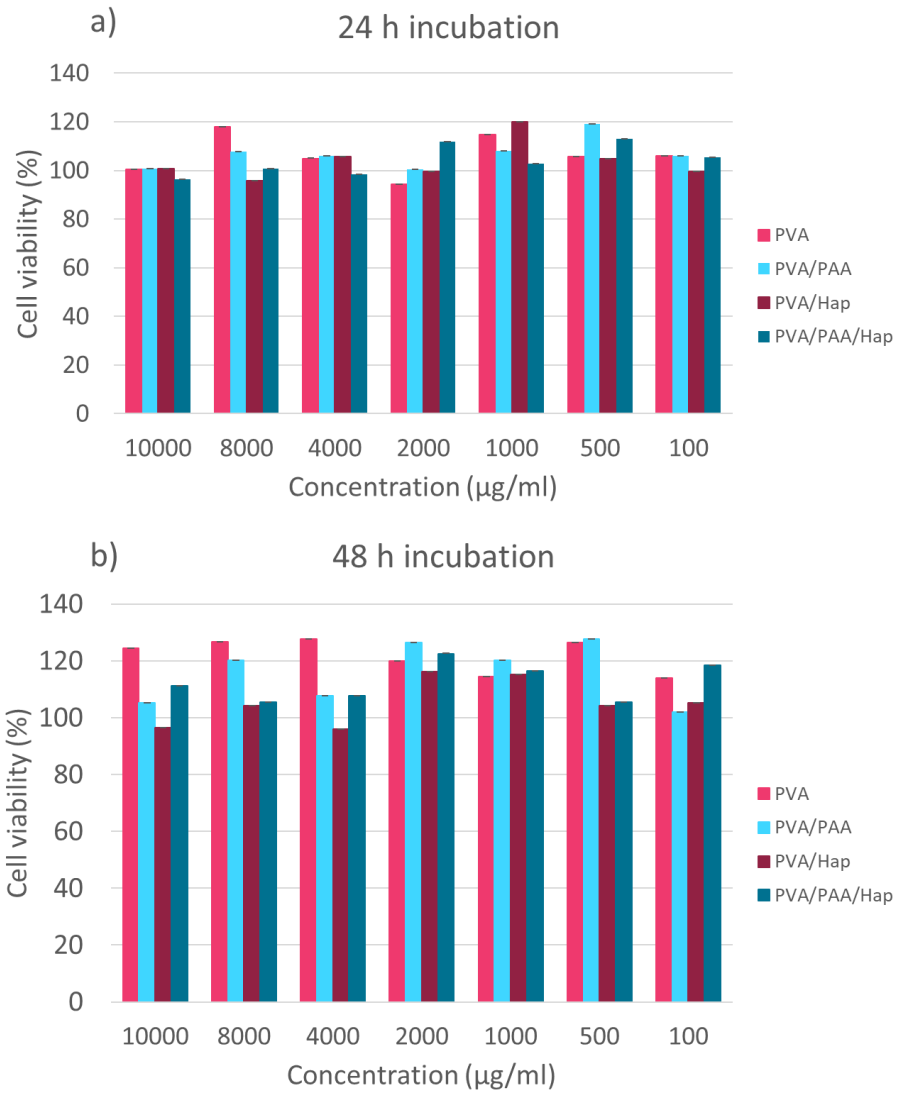

Figure 11. Cell viability tests of the studied hydrogels on NIH 3T3 cells after (a) 24 h and after (b) 5 days.

It is worth mentioning that although we produced these spheres via a droplet method using AcOEt as solvent to form the cryogenic spheres upon impact, it was only chosen due to the easy access to this compound from our lab, nonetheless it is possible to use any other solvent that can freeze below the temperatures studied in this work and that could have lower toxicity values.

Conclusion

In the present study, hydrogels for the immediate release of ciprofloxacin for osteomyelitis treatment was reported. Hydrogels were produced with different formulations of poly(vinyl alcohol), poly(acrylic acid), ciprofloxacin and hydroxyapatite

in aqueous solutions and shaped into spheres by dispensing liquid droplets into ethyl acetate at low temperature (-80 °C) following by formation of hydrogels by freeze and thawing cycles. Samples were able to maintain a spherical shape after the freeze-thawing cycles technique described. The cross-section of these samples revealed different internal structures, depending on the components incorporated into the PVA.

In addition, EDX exhibited quantities of Ca and P into these hydrogels due to the HAp incorporated. These materials modified the melting point and the glass transition temperature of PVA and also led to a pH sensitivity, which improved by the addition of poly acrylic(acid). Hydrogels were able to immediately release ciprofloxacin under 60 min and dissolves under 1 h for samples without HAp. Furthermore, these spheres were effective against two pathogens known to cause osteomyelitis, *S. aureus* and *S. typhimurium*. Finally, these samples presented to be non-toxic from cellular viability tests against NIH 3T3 cell line while also increasing the cellular activity after 2 days. We believe this material can be used as a support for biomaterials intended as load bearing which can be incorporated by the technique proposed herein.

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