Ceramics International xxx (xxxx) xxx

Contents lists available at ScienceDirect

Ceramics International

journal homepage: www.elsevier.com/locate/ceramint

Emulsion-based multi-responsive microspheres for the delivery of lipophilic Ozoile

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ARTICLE INFO

Keywords:
Hydrogel microspheres
Biomedical applications
Extrusion
Chemical properties
Thermal properties

ABSTRACT

Natural and biodegradable polymers with pH-responsive properties, such as alginate and chitosan, are widely exploited for drug delivery applications, most notably in the form of biocompatible microspheres. In addition, thermo-responsive poly(N-isopropyl-acrylamide) (PNIPAM) is often added to biomedical delivery systems due to its lower critical solution temperature close to human physiological conditions. In this work, multi-responsive microspheres (MRMs) consisting of a hydrogel matrix made of alginate, chitosan, and PNIPAM were successfully prepared by extrusion dripping and proposed as delivery system for Ozoile (Stable Ozonides) derived from biological olive oil. Ozoile acts as a biological inducer, regulating the main metabolic pathways and stimulating the endogenous defence system. The MRMs containing Ozoile were prepared from alginate/NIPAM emulsions integrating the lipophilic bioactive principle. The MRMs were further optimised with a chitosan coating to improve their stability and control the degradation behaviour of the hydrogel matrix, with subsequent release of Ozoile. Results in terms of chemical composition from Fourier transform infrared (FTIR) spectroscopy, thermo-responsive properties from differential scanning calorimetry (DSC) analysis, swelling and degradation behaviour were used to establish the potential use of the MRMs for the therapeutic release of Ozoile at specific inflammatory sites.

1. Introduction

Hydrogels are defined as three-dimensional (3D) networks of cross-linked hydrophilic polymers capable of absorbing and retaining large amounts of water [1–3]. Hydrogels are widely used as drug delivery systems in many branches of medicine [4,5] for their notable properties, such as 3D structure, high biocompatibility, similarity to living tissues and tuneable physical characteristics [6]. Alginate hydrogel microspheres are used as a controlled drug delivery system, releasing the drug in controlled rate and overcoming the problems of conventional drug delivery systems, thus enhancing the therapeutic efficacy of a given drug [7,8]. Stimuli-based drug delivery systems offer enormous and promising advantages in the prevention, diagnosis and treatment of many diseases [9]. These drug delivery systems have the capability of responding to both internal (pH, temperature, and redox conditions) and external (magnetic field, ultrasound field, and light) stimuli [10–12].

Among thermo-responsive polymers, poly(N-isopropyl-acrylamide) (PNIPAM) is widely used in biomedical research due to its biocompatibility and lower critical solution temperature (LCST) close to physiological conditions [13]. PNIPAM is composed of both hydrophilic amide groups (–CONH–) and hydrophobic isopropyl side chains (–CH$(CH_3)_2$) [14,15], and in solution exhibits a LCST value of 32 °C [16,17]. PNIPAM solutions assume a sol state at room temperature and can be transformed to a gel state as they approach 37 °C [18]. PNIPAM has been used for a wide range of applications in biomedical areas as cell therapy [19,20], drug delivery transporters [21], and scaffolds for tissue engineering. In particular, the temperature sensitivity of PNIPAM and the pH sensitivity of alginate [22] would be extremely useful for the release of stable ozonides (such as Ozoile) at specific sites.

Ozoile is a bioactive formulation obtained by ozonation of biological olive oil, which is known to induce the generation of moderate oxidative stress in environments characterised by a proton increase, such as ischemic, hypoxic, or damaged tissues [23]. In particular, the stable ozonides release molecular oxygen with the production of radical species, causing a moderate oxidative stress that stimulates the expression of several antioxidant enzymes by the activation of the nuclear

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https://doi.org/10.1016/j.ceramint.2022.12.095
Received 30 August 2022; Received in revised form 7 December 2022; Accepted 12 December 2022
Available online 13 December 2022

factor-erythroid 2-related factor 2 (Nrf2). The moderate pro-oxidant environment leads to the stimulation of molecular mechanisms that can suppress the inflammatory response [24]. For instance, the oxidative stress may induce the expression of the hypoxia-inducible factor-1α (HIF-1α), which is a transcription factor mainly associated with the adaptive responses of cells to hypoxia. HIF-1α induces the expression of several genes, among these there are growth factors such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor-α (TGF-α), and TGF-β, which are involved in the complex multi-step process of tissue regeneration [24].

In this work, temperature and pH-responsive hydrogel microspheres were fabricated starting from PNIPAM and alginate polymers. Using an emulsion-based approach, Ozoile was embedded into the multi-responsive microspheres (MRMs) with the aim to reach targeted inflammatory sites for therapeutic purposes. The fabrication of the Ozoile-containing microspheres was developed and optimised, and the high-intensity ultrasound (HIU) method used for obtaining stable emulsions. MRMs/Ozoile coated with chitosan were also prepared, with the aim to improve the response to pH. In fact, the presence of primary amines on the glucosamine residues makes chitosan a pH-responsive polycation [25]. The properties of the microspheres are investigated using different techniques. In particular, the chemical composition of the MRMs is examined using Fourier transform infrared (FTIR) spectroscopy, whereas differential scanning calorimetry (DSC) is used to analyse the thermo-responsive properties, determining the critical transition temperature and bound water fraction. In order to evaluate the multi-responsive behaviour of the Ozoile-containing microspheres, degradation tests at different pH values (3, 4, 5, 7.4) and at different temperatures (20 °C, 37 °C) are presented. The role of chitosan coating is also analysed to assess its influence on the controlled release of Ozoile. Results in terms of thermo-pH-responsive behaviour, swelling and degradation are useful to establish the potential use of the emulsion-based MRMs for the therapeutic release of Ozoile in specific inflammatory sites.

2. Experimental methodology

2.1. Materials

Alginic acid sodium salt, calcium chloride ($CaCl_2$) dihydrate, polyethylene glycol sorbitan monooleate (Tween 80), xanthan gum, N-isopropylacrylamide (NIPAM), N,N′-methylenebisacrylamide (MBA) and chitosan (medium molecular weight, >75% deacetylated) were purchased from Sigma-Aldrich (Milan, Italy). Ammonium Peroxodisulfate (APS), N,N,N′,N'-Tetramethyl ethylenediamine (TEMED) were obtained from PanReach AppliChem (Milan, Italy). Ozoile (Stable Ozonides) is a formulation obtained from a patented mixing process in which a defined oxygen-ozone mixture is bound to olefinic bonds of fatty acids of a biological olive oil forming stable ozonides. Deionized water (resistivity 18.2 MΩ·cm) was produced by a Direct-Q3 UV water purification system (Millipore, Molsheim, France) and used in all preparations.

2.2. Synthesis of hydrogel MRMs

Sodium alginate and NIPAM were mixed in deionized water at a ratio of 1:8 (w/w). The solution was mixed for 30 min in a cold-water bath using a magnetic stirrer (C-MAG HS7, IKA, Staufen, Germany). Ozoile (20 wt%), the non-ionic surfactant Tween 80 (5 w/v%), the emulsifier xanthan gum (0.25 w/v%) and catalyst for the polymerization of acrylamide and bis-acrylamide TEMED (0.15 v/v %) were added and a stable emulsion was obtained using the high-intensity ultrasound (HIU) method [26]. The process was performed using the Handheld Ultrasonic Homogenizer (UP200Ht, Hielscher Ultrasonics GmbH, Teltow, Germany) for 5 min at 50% power. For preparation of the microspheres, the emulsion was dripped, using a 0.26 mm gauge syringe connected to a syringe pump, into a gelling bath containing a cold solution with $CaCl_2$ (20% w/v) and APS (2% w/v) previously purged with nitrogen for 15 min to completely remove any residual oxygen. The collecting distance was optimised and fixed at 10 cm to obtain spherical microspheres. A gentle mixing was applied to $CaCl_2$/APS solution to prevent sticking of the microspheres. The hardening time of the MRMs was set at 18 h. The microspheres were washed three times with deionized water to extract unreacted monomer, crosslinker, initiator, and free calcium ions. The chitosan-coated microspheres were prepared by vortex mixing in 0.5 wt % chitosan solution using a stirring rate of 700 rpm for 30 min.

2.3. Characterization methods

A digital camera with a resolution of 12 megapixel was used to evaluate the morphology, shape, and size of the MRMs. The average size of MRMs was measured using the ImageJ software (version 1.53).

To determine swelling ratio and water content, the hydrogel microspheres were first completely hydrated in deionized water and then dried at 50 °C for 18 h. The swelling ratio and water content were calculated using the following equations [27]:

$$\text{Swelling Ratio} = \frac{\text{Ws}}{\text{Wd}} \tag{1}$$

$$\%\ \text{water content} = \frac{(\text{Ws} - \text{Wd})}{\text{Ws}} \times 100 \tag{2}$$

where W_s is the weight of the hydrogel in the equilibrium swelling state, and W_d the weight of the fully dried hydrogel. The test was repeated on 5 samples for each microsphere type and the results averaged.

Fourier transform infrared (FTIR) spectroscopy spectra of MRMs were recorded using a Nicolet Summit Spectrometer (Thermo Fisher Scientific, USA) with a zinc selenide ATR accessory. IR spectra were acquired in the region of 4000–800 cm^{-1}, with a resolution of 4 cm^{-1}, and each spectrum was acquired by averaging 32 scans.

The volume phase transition temperature (VPT) and bound water fraction (X_{BW}) of the MRMs were investigated using differential scanning calorimetry. A Pyris DSC 8500 instrument (PerkinElmer, Waltham, MA, USA) calibrated with high purity indium and tin was used. DSC samples were sealed in aluminium pans with lids and measurements were performed under a constant flow of nitrogen (20 mL/min). An identical empty cell was taken as reference.

For the determination of the VPT, swollen hydrogel samples (3 samples, ~25 mg) were measured in the temperature range from 25 °C to 50 °C at a heating rate of 10 °C/min. For the X_{BW} determination, swollen microspheres (3 samples, ~25 mg) were analysed in the temperature range from −30 °C to 30 °C at a heating rate of 10 °C/min. X_{BW} was calculated according to the following equation (Eq. (3)) [28]:

$$X_{BW} = X_{TW} - \frac{Q_{endo}}{Q_f} \tag{3}$$

where Q_{endo} is the heat of fusion of freezable water which is obtained from the DSC thermogram (J/g) by calculating the peak area with PerkinElmer Thermal Analysis software, Q_f indicates the heat of fusion of pure water (333 J/g) [29] and X_{TW} is the total water fraction and was calculated according to the following equation (Eq. (4)):

$$X_{TW} = 1 - \frac{M_{DRY}}{M_{TOTAL}} \tag{4}$$

with M_{DRY} indicates the dry mass and M_{TOTAL} the total wet mass of the microspheres.

Degradation tests were performed in deionized water and in PBS, monitoring the microspheres behaviour every hour for the first 4 h and after 24 h. For the chitosan-coated MRMs/Ozoile, tests were carried out in PBS solution at different pH (3, 4, 5, 7.4) and different temperatures (20 °C, 37 °C). In addition, the role of the chitosan was determined by

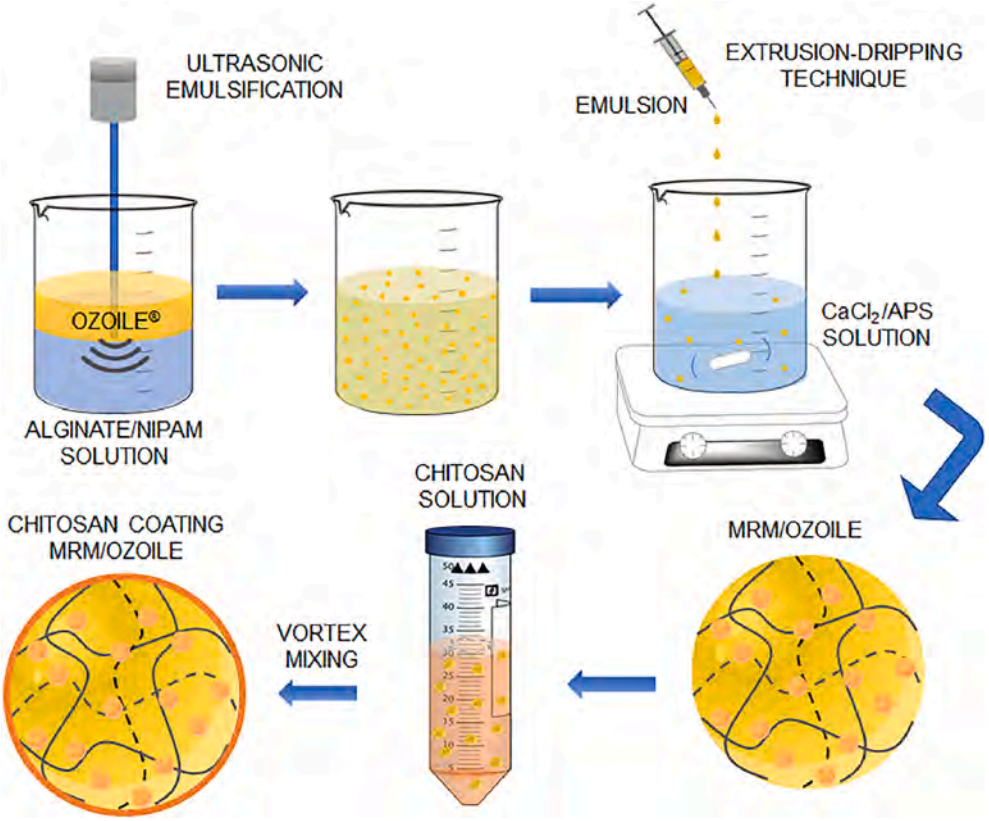

Fig. 1. Schematic representation of the fabrication process of multi-responsive microspheres (MRMs) containing Ozoile and with chitosan coating applied by vortex mixing.

monitoring the MRMs/Ozoile with and without coating at 37 °C in PBS (pH = 7.4) and in deionized water.

3. Results and discussion

3.1. Preparation of MRMs containing Ozoile

The process of fabrication of the Ozoile-containing microspheres was developed and optimised. In particular, the homogeneity of the emulsion prepared with alginate, NIPAM and Ozoile was ensured using the high-intensity ultrasound (HIU) method. The acoustic cavitation induced by ultrasound increases the breakdown of oil droplets, facilitating the formation of stable oil/water (O/W) emulsions with small droplet sizes [26]. Emulsions were prepared through two steps: in the first step, large droplets of the dispersed phase are formed in the continuous phase, and in the second step, continuous physical shearing and acoustic cavitation generated in the medium break up the large droplets [30].

The extrusion technique was used for fabricating the MRMs, and size and shape were optimised using a collecting distance of 10 cm and a

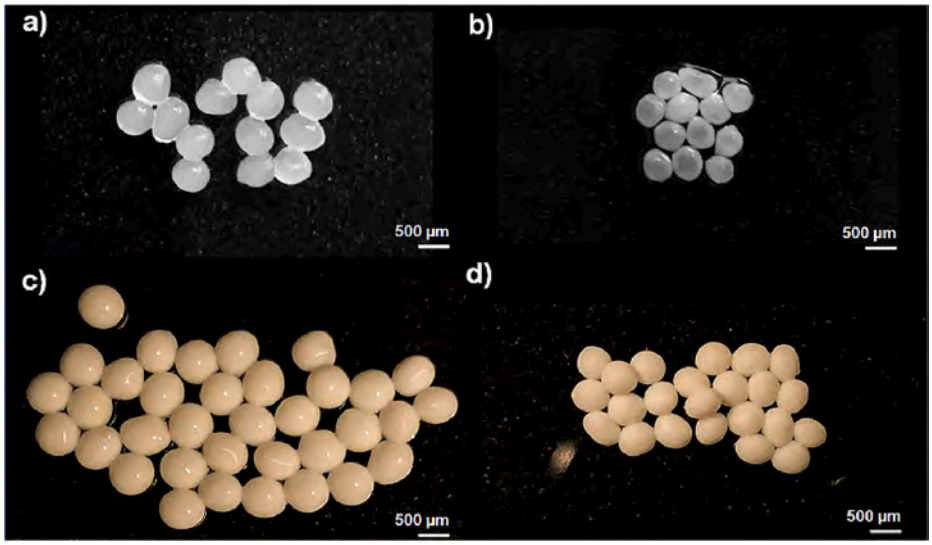

Fig. 2. Optical microscopy images of hydrated a) MRMs, b) MRMs coated with chitosan, c) MRMs/Ozoile and d) MRMs/Ozoile coated with chitosan.

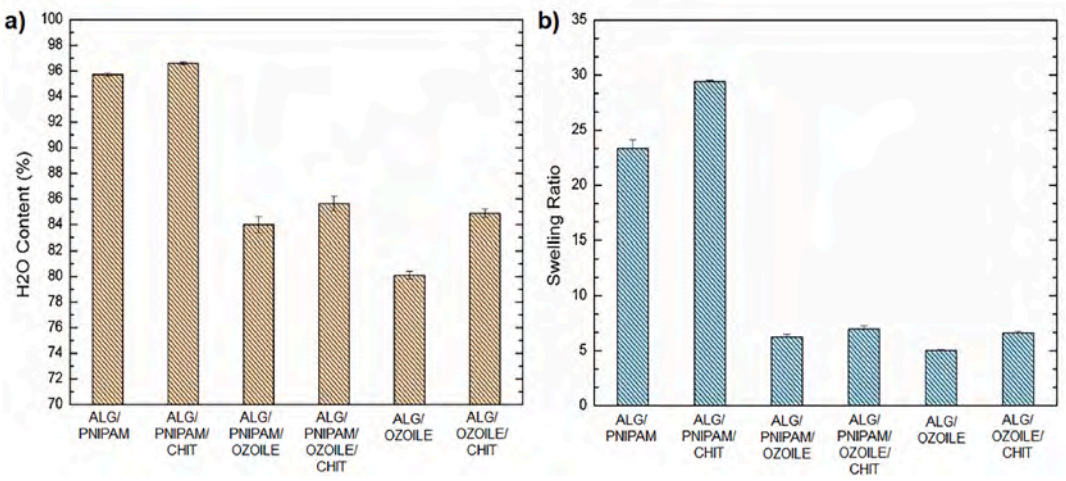

Fig. 3. Swelling ratio and water content of different types of MRMs.

0.26 mm gauge syringe. Determining an appropriate collecting distance is strongly dependent on the viscosity of the emulsion [31]. In this study, the collecting distance was determined by trial and error adjusting the distance between the collection tip and the gelling bath. In general, too small collecting distances lead to microspheres characterised by tails, whilst at large distances microspheres with irregular shape were observed [32].

The gelling bath ($CaCl_2$/APS) should be stirred to prevent aggregation of the microspheres and to improve the homogeneity of the crosslinking reaction [33,34]. Nevertheless, the choice of too high agitation speeds can induce the deformation of microspheres, leading to irregular shape due to the formation of strong centrifugal forces in the gelling bath [33]. Moreover, the speed of stirring should be selected taking into account the impact of droplets that could cause development of nonspherical or coalescent microspheres [35]. In this work, a stirring rate of 300 rpm was found to be optimal for the fabrication of MRMs with spherical shape.

The microspheres were continuously stirred at 20 °C for 18 h to make a complete crosslinking of alginate with Ca^{2+} and polymerize PNIPAM. In the hardening process, the Na^+ ions in alginate structure are replaced by the Ca^{2+} ions [36–38], and the APS starts the free-radical polymerization of PNIPAM forming the microspheres. During the process, the NH_3^+ groups of chitosan bind to the $–COO^-$ of alginate by ionic bonding [39]. The fabrication process of MRMs was schematized in Fig. 1. The chitosan modification was applied to the microspheres by transferring them into a chitosan solution and vortex mixing the suspension at 700 rpm. The stirring rate value was selected after evaluating its effect on the MRMs size and morphology as described in Section 3.2.

3.2. Morphological and swelling properties

Fig. 2 shows images of the hydrated MRMs. These microspheres are spherical in shape and with compact structure. Empty MRMs without chitosan coating (Fig. 2a) have a diameter of 625.7 ± 8.7 μm, whereas those coated with chitosan (Fig. 2b) are 408.9 ± 7.4 μm in size. The Ozoile-containing MRMs without chitosan (Fig. 2c) and those with chitosan modification (Fig. 2d) are 631.6 ± 6.9 μm and 443.4 ± 8.5 μm, respectively. These results show that the diameter of the MRMs is lower in the presence of chitosan (vortex stirring rate of 700 rpm). The effect of the stirring rate was further investigated. Stirring rates from 500 to 700 rpm were experimented, and the resulting size of MRMs/Ozoile measured from the digital images. Results are summarised in Table 3, and they show that the microsphere diameter decreases at increasing stirring rates. The value of 700 rpm was selected for the preparation of small-size MRMs. Indeed, few works in the literature report that the coating applied through stirring may induce the shrinkage of microspheres [40]. In our case, this might be due to the diffusion of chitosan molecules inside the alginate/PNIPAM matrix, which causes a partial collapse of the polymer network as a consequence of electrostatic neutralisation. The chitosan diffusion is enhanced at higher mixing rates, resulting in a size decrease of the chitosan-modified microspheres.

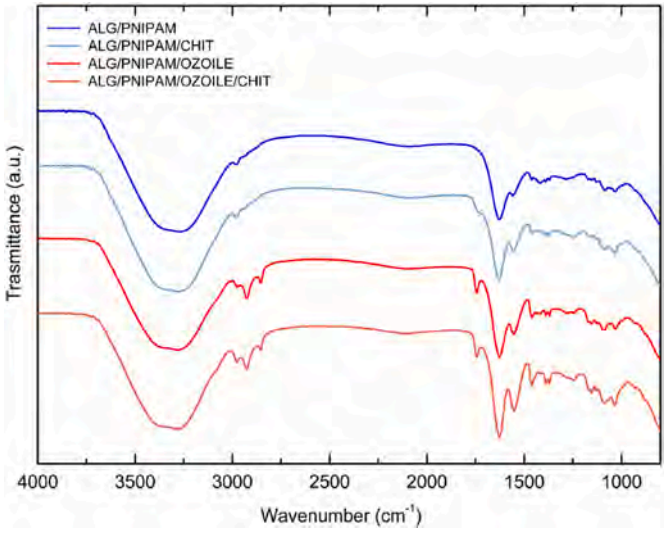

Fig. 4. FTIR spectra of different types of MRMs. Data are offset for clarity.

Fig. 3 shows the swelling ratio (Fig. 3a) and the water content (%) (Fig. 3b) of hydrogel microspheres at room temperature. All microspheres show a water content over 80%, in particular unloaded alginate/PNIPAM MRMs show higher water content (>90%) than those with Ozoile (water content 80–85%). In fact, the hydrophobic nature of Ozoile makes the hydrogel matrix more hydrophobic, lowering the swelling degree of the microspheres. Therefore, the MRMs coated with chitosan exhibit higher water content than those without. This effect had already been observed in similar alginate-based systems [41] and can be ascribed to an increase in the crosslinking density of the polymeric chains due to the chitosan coating [42,43].

3.3. FTIR analysis

Fig. 4 shows the FTIR spectra of different types of MRMs. All spectra exhibit a broad absorption band at approximately 3400 cm^{-1} and 3200 cm^{-1} corresponding to the stretching of O–H, and a band at 2980 cm^{-1}

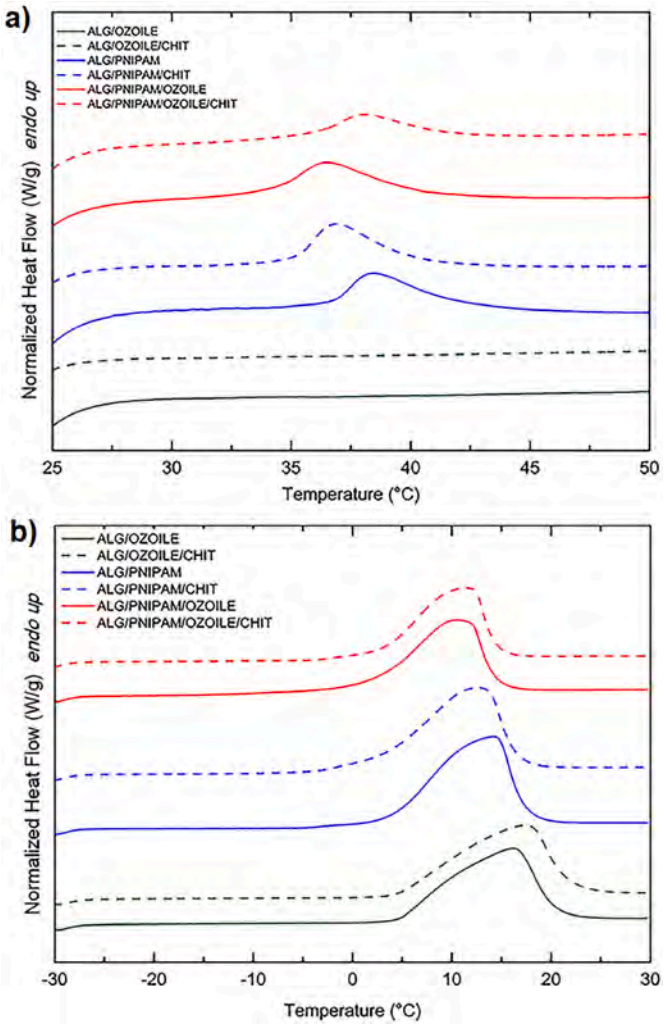

Fig. 5. DSC thermograms for a) VPT evaluation and b) X_{BW} evaluation of different types of MRMs with heating rates of 10 °C/min. Data are offset for clarity.

that can be attributed to the alkyl –CH bonds of alginate [44]. Asymmetric and symmetric stretching vibrations of methylene (-CH_2) and methyl (–CH_3) groups were observed in MRMs/Ozoile with and without chitosan coating at the characteristic bands between 2920 and 2850 cm^{-1}, respectively [45]. Stretching vibration of carbonyl (C=O) from the ester linkage of triacylglycerol was observed in MRMs/Ozoile with and without chitosan coating at approximately 1744 cm^{-1} [46]. The presence of PNIPAM into MRMs was confirmed by the characteristic bands between 1650 and 1500 cm^{-1} that can be attributed at amide I band (C=O stretching) and amide II band (N–H in-plane bending vibration), respectively [47]. The peaks at 1386 cm^{-1} and 1368 cm^{-1} arose from the coupling split of the symmetrical bending vibrations originating from the two methyl groups –$CH(CH_3)_2$ of PNIPAM [48]. In addition, CO–C vibration at the bands around 1033 cm^{-1} was observed for all types of MRMs, due to the presence of guluronic units in sodium alginate [49].

3.4. Thermal response of MRMs by calorimetry

Differential scanning calorimetry (DSC) was used to analyse the volume phase transition (VPT) temperature and the bound water fraction (X_{BW}) of microspheres. Fig. 5a shows the endothermic phenomena in the physiological range 35–40 °C only for the MRMs containing NIPAM due to the volume phase transition (VPT). The VPT is driven by hydrophobic collapse, in particular when the temperature increases, an imbalance between hydrophilic and hydrophobic polymer–water interactions occurs, leading to the collapse of the polymer network and to the fast expulsion of Ozoile [50]. Therefore, reaching VPT temperature can trigger the release of molecules of interest, such as the Ozoile incorporated into the polymer network. Most of the authors reported for the PNIPAM-based hydrogels a VPT at a temperature of ~32 °C [51,52], whilst here we observed a VPT at a temperature in the range of 35–40 °C. This phenomenon can be attributed to the presence of alginate in the polymer matrix. In fact, although there are no chemical bonds or other strong interactions between the alginate and PNIPAM, the alginate is cross-linked and tightly coupled to the PNIPAM network to form the microspheres after the addition of Ca^{2+}. In this case, the main driving force of the phase transition remains the interaction between the isopropyl and amide groups in the PNIPAM backbone, but due to the presence of alginate, the movement of the PNIPAM chains requires more energy to drive the hydrophilic-hydrophobic transition, resulting in higher VPT temperature [53].

Table 1
Volumetric phase transition (VPT) and enthalpy (ΔH_{VPT}) values of microspheres obtained from DSC analysis.

Sample	VPT (°C)	ΔH_{VPT} (J/g)
ALG/PNIPAM/OZOILE	36.1 ± 0.3	0.78 ± 0.03
ALG/PNIPAM/OZOILE/CHIT	37.9 ± 0.2	0.42 ± 0.03
ALG/PNIPAM	38.5 ± 0.2	0.38 ± 0.02
ALG/PNIPAM/CHIT	36.3 ± 0.2	0.19 ± 0.09

Table 2
Bound water fraction (X_{BW}) values and enthalpies of fusion (ΔH_f) of microspheres obtained from DSC analysis.

Sample	X_{BW} (%)	ΔH_f (J/g)
ALG/PNIPAM	13.9 ± 0.6	272.2 ± 1.3
ALG/PNIPAM/CHIT	15.7 ± 0.3	269.4 ± 1.1
ALG/PNIPAM/OZOILE	23.4 ± 0.5	201.7 ± 0.4
ALG/PNIPAM/OZOILE/CHIT	27.8 ± 0.9	192.4 ± 1.4
ALG/OZOILE	7.8 ± 0.6	241.4 ± 1.9
ALG/OZOILE/CHIT	9.1 ± 0.5	252.2 ± 1.7

Table 3
Mean diameter of MRMs/Ozoile coated with chitosan by vortex mixing using different stirring rates.

Stirring rate (rpm)	Mean diameter (μm)
500	785.3 ± 10.6
550	651.1 ± 12.4
600	580.3 ± 9.8
650	510.4 ± 14.1
700	443.4 ± 8.5

The VPT values and the corresponding enthalpy (ΔH_{VPT}) values were calculated and listed in Table 1. In the presence of Ozoile, VPT is anticipated from 38.5 °C to 36.1 °C, and this can be related to the hydrophobic interaction between Ozoile and the monomer units of microspheres [54]. MRMs based on alginate and PNIPAM exhibit lower enthalpy (0.38 J/g) associated with VPT than MRMs/Ozoile (0.78 J/g). This result is due to the predominant effect of alginate, which in the absence of Ozoile induces better and stronger hydration of the polymer chains implying fewer hydrogen bonds broken during the phase transition [55]. Moreover, for the MRMs/Ozoile, VPT peak is delayed when chitosan coating is applied, suggesting a stabilising effect of coating.

Fig. 5b shows the DSC thermogram of fully swollen MRMs. The endothermic peak appears in the range between 5 and 20 °C and it can be attributed to the presence of free water in the hydrogels. The enthalpy of fusion (ΔH_f) and the bound water fraction (X_{BW}) values were calculated and listed in Table 2. MRMs based on alginate and PNIPAM exhibit

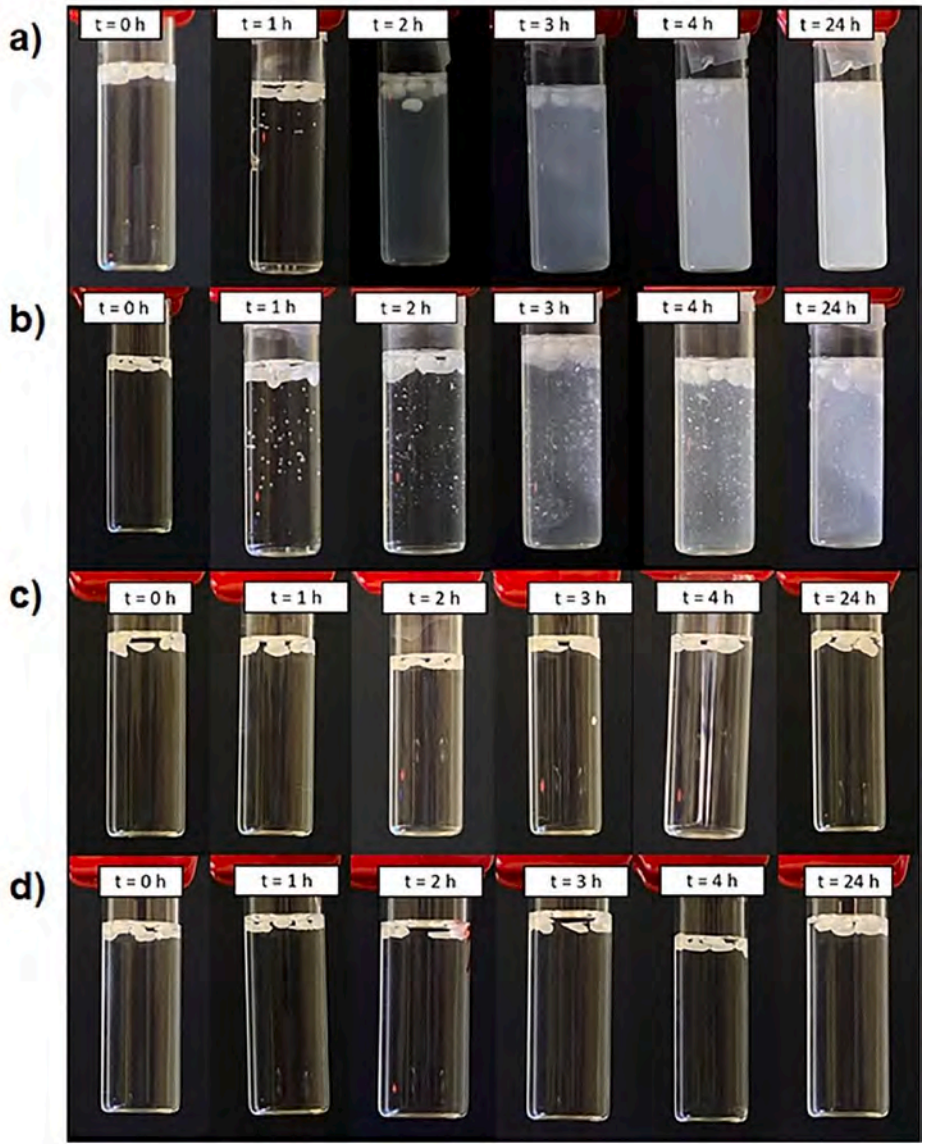

Fig. 6. Degradation tests performed at 37 °C in PBS (pH = 7.4) (a, b) and in deionized water (c, d) on MRMs/Ozoile without coating (a, c) and on MRMs/Ozoile with chitosan coating (b, d). Degradation evaluated every hour for the first 4 h and after 24 h.

lower X_{BW} (13.9%) associated with higher VPT than those observed for MRMs/Ozoile (23.4%). In general, VPT transitions move toward higher temperatures as the amount of bound water decreases and the free and interfacial water content increases [56,57], as mentioned above.

3.5. Degradation tests

The degradation behaviour of the MRMs/Ozoile was analysed at different temperatures (20 °C and 37 °C) and at different pH values (3, 4, 5, 7.4), in deionized water and in phosphate-buffered saline (PBS) solution. The effect of the chitosan addition on the Ozoile-containing microspheres was also evaluated, by comparing the stability of the MRMs in PBS (pH = 7.4) and in deionized water (Fig. 6). In PBS solution, the Na^+ ions initiate an ion exchange process with the Ca^{2+} cations that are mainly bound to the $–COO^-$ groups of the maluronic alginate blocks. In the initial phase of this process, the release of calcium cations is driven by diffusion. As a consequence, the negatively charged carboxylic groups ($–COO^-$) in the polymer matrix become predominant, and the associated electrostatic repulsion between them induces relaxation of the polymer chains and swelling. This result is shown in Fig. 6 and is in agreement with previously reported work [58]. Fig. 6a shows the MRMs degradation process evaluated every hour for 4 h and after 24 h. In the case of MRMs coated with chitosan (Fig. 6b), the degradation process appears delayed in time and this effect can be ascribed to the interaction between $–COO^-$ of alginate and NH_3^+ of chitosan. Otherwise in deionized water, ion exchange does not take place and degradation of MRMs does not occur, as shown in Fig. 6c and d. Fig. 7 shows degradation tests on Ozoile-containing MRMs with chitosan coating performed at different temperatures (20 °C, 37 °C) and at different pH values (3, 4, 5, 7.4). In this case, the degradation of the microspheres is strongly dependent on pH and temperature. In fact, degradation does not occur for pH values of 3, 4, 5 at the two different temperatures (20 °C, 37 °C), whereas it is present at pH = 7.4. Further, we observe that at pH 7.4 the degradation is faster at the higher temperature (37 °C) than at 20 °C. These combined results show that the MRMs are both temperature and pH-responsive and that the Ozoile release is feasible upon degradation of the hydrogel matrix.

4. Conclusions

Multi-responsive microspheres (MRMs) made of alginate/chitosan/PNIPAM hydrogels were developed and applied as carriers of Ozoile,

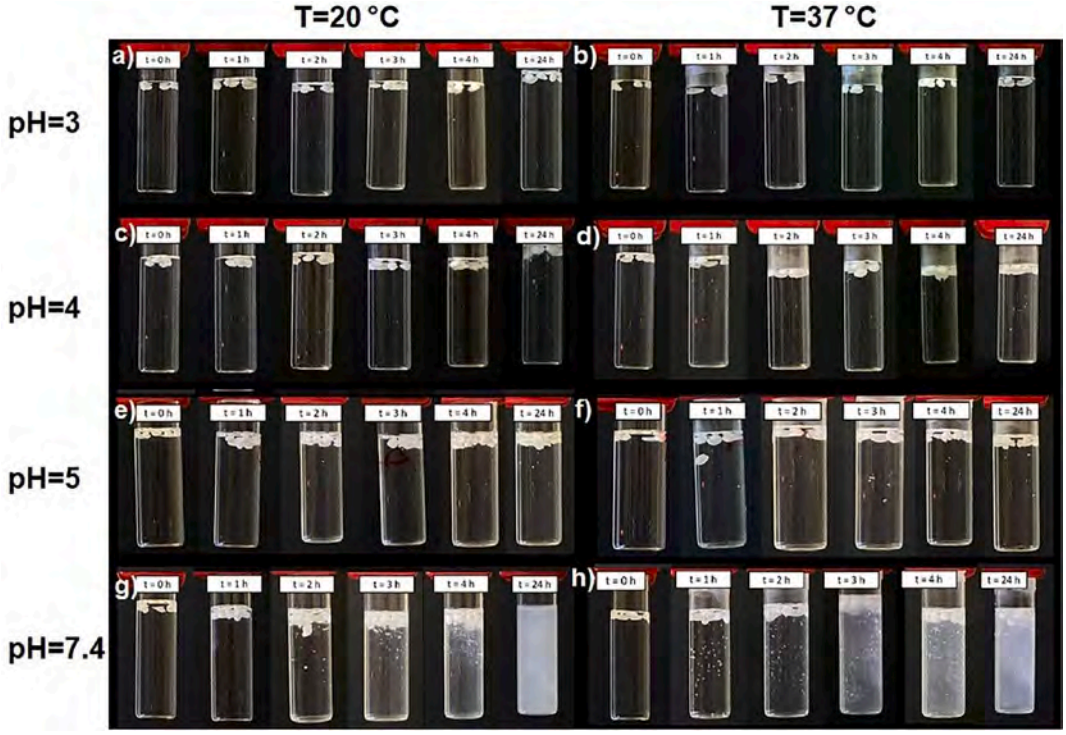

Fig. 7. Degradation tests of Ozoile-containing multi-responsive microspheres with chitosan coating at 20 °C (a, c, e, g) and 37 °C (b, d, f, h) and at different pH values. Degradation evaluated every hour for the first 4 h and after 24 h.

Stable Ozonides derived from biological olive oil in a patented process. The process of fabrication was developed and optimised, exploiting the high-intensity ultrasound (HIU) method for obtaining stable emulsions and the extrusion dripping technique for crosslinking the polymer network.

The properties of the microspheres were investigated using different techniques. In particular, the chemical composition of the MRMs was examined using Fourier transform infrared (FTIR) spectroscopy, which confirmed the presence of Ozoile in the MRMs. Optical analysis revealed the spherical shape with compact structure of the MRMs, whereas the analysis performed using ImageJ software highlighted that the diameter of the MRMs is below 650 μm and lower in presence of chitosan coating (450 μm). Analysis of swelling ratio revealed the high-water content (>80%) of the MRMs, indicating their suitability for mimicking specific aspects of tissue microenvironments.

Differential scanning calorimetry (DSC) was used to analyse the thermo-responsive properties of MRMs, determining the critical transition temperature. The endothermic phenomenon appears in the physiological range 35–40 °C, leading to the collapse of the polymer network and to the fast expulsion of Ozoile. Moreover, the degradation in deionized water and phosphate buffered saline (PBS) solution at different pH and temperatures were evaluated. MRMs/Ozoile show a degradation only in PBS and the tests revealed the influence of the chitosan coating on the process, which acts delaying the release of Ozoile. Therefore, the results in terms of thermo-pH-responsive behaviour, swelling and degradation revealed the potential use of the MRMs for the therapeutic release of Ozoile in specific inflammatory sites.

Author contributions

Conceptualization, M.G.S.; methodology, G.C., S.V., E.T., M.G.S.; validation, S.V., E.T., M.G.S.; formal analysis, G.C., E.T.; investigation, G.C.; writing—original draft preparation, G.C.; writing—review and editing, S.V., E.T., M.G.S.; supervision, M.G.S. All authors have read and agreed to the published version of the manuscript.

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.

Acknowledgement

This work was financially supported by Sapienza University of Rome (Advanced Responsive Polymer Interfaces for Biological Applications, grant n. PH118164368214F8).

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