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Synthesis and characterization of poly(*N*-vinylcaprolactam)-based spray-dried microparticles exhibiting temperature and pH-sensitive properties for controlled release of ketoprofen

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Abstract: Poly(*N*-vinylcaprolactam) (PNVCL) and poly(*N*-vinylcaprolactam-*co*-acrylic acid) (poly(NVCL-*co*-AA)) were synthesized by solution free radical polymerization and displayed thermo-responsive behavior, with lower critical solution temperatures (LCSTs) of 35 °C and 39 °C, respectively. The incorporation of AA unities made the poly(NVCL-*co*-AA) sensitive to both pH- and temperature. They were exploited in this work in preparing microparticles loaded with ketoprofen via spray-drying to modulate the drug release rate by changing pH or temperature. The interaction between polymer and drug was studied using x-ray diffractometry, Raman spectrometry and scanning electron microscopy (SEM). The biocompatibility of pure polymers, free ketoprofen as well as the spray-dried particles was demonstrated in vitro by low cytotoxicity and a lack of nitric oxide production in macrophages at concentrations as high as 100 µg mL⁻¹. The release profile of ketoprofen was evaluated by in vitro assays at different temperatures and pH values. Drug diffusion out of PNVCL's hydrated polymer network is increased at temperatures below the LCST. However, when poly(NVCL-*co*-AA) was used as the matrix, the release of Ketoprofen was primarily controlled by the pH of the medium. These results indicated that PNVCL and the novel poly(NVCL-*co*-AA) could be promising candidates for pH and temperature responsive drug delivery systems.

Keywords: *N*-vinylcaprolactam; acrylic acid; ketoprofen; microparticles; spray-drying; triggered drug release, stimuli-responsive polymers.

Introduction

Polymeric microparticles have been developed as drug delivery systems using different natural and synthetic polymers¹. Chitosan² and alginate³ are some examples of natural polymers that have been used, whereas synthetic polymers comprise poly(lactide-co-glycolide) (PLGA)⁴ and (3-hydroxybutyrate-co-3 hydroxyvalerate) (PHBV)⁵, among others. However, during the last years, stimuli-responsive polymers have attracted great attention due to their applications in controlled and self-regulated drug delivery.

Also termed 'environmental-sensitive' or 'smart', these polymers experience rapid changes in their microstructure in response to environmental changes. The changes are reversible; therefore, the polymer is capable of returning to its initial state as soon as the trigger is removed. These intelligent, stimuli-sensitive materials respond to, for example, physical (temperature, magnetic field), chemical (pH, ionic strength), or biochemical (enzyme) stimulus⁶⁻⁸.

Among others, thermosensitive polymers are responsive to temperature changes. These materials require a signal, i.e. critical temperature, which triggers their phase transition, when water-soluble and hydrophilic polymers become hydrophobic and collapse⁹⁻¹⁵. Polymers, which become soluble upon heating, have a so-called upper critical solution temperature (UCST). Those polymers, which become insoluble in solutions upon heating, possess a lower critical solution temperature (LCST)¹⁶. The drug release from these polymers can then be regulated in response to the changes of temperature.

The polymer of special interest in this work is the poly(*N*-vinylcaprolactam), PNVCL. PNVCL is a non-ionic, water-soluble, thermosensitive polymer that belongs to the group of poly *N* vinylamide polymers, which exhibits an LCST in water close to physiological temperature (35-38 °C), depending on polymer concentration, molecular weight and copolymer composition¹⁷. Thus, PNVCL is considered to be suitable for biomedical applications, as the collapsing temperature is close to the physiological temperature^{5,18-22}. In addition, PNVCL is expected to be a biocompatible polymer. The amide group in the lactam ring is directly connected to the carbon-backbone chain. PNVCL does not break easily hydrolytically, but if the PNVCL is hydrolyzed, a polymeric carboxylic acid builds up and small toxic amide compounds will not form, like is the case with PNIPAAm), one of the most studied thermosensitive polymers^{23,24}.

Our research is focused on thermosensitive PNVCL-based polymers for triggered drug release. In addition to thermo-responsive behavior, growing interest exists in obtaining materials whose aqueous solution and swelling properties can abruptly and reversibly change in response to simultaneous changes in temperature and pH²⁵. Therefore, in order to improve the functionality of PNVCL systems such as temperature- and pH-sensitivity, a hydrophilic monomer, acrylic acid (AA), was incorporated into the PNVCL backbone and this work was conducted in two main parts: synthesis of poly(*N*-vinylcaprolactam) (PNVCL) and poly(*N*-vinylcaprolactam-*co*-acrylic acid) (poly(NVCL-*co*-AA)) by free radical solution polymerization including the in vitro cellular toxicity of both PNVCL based polymers and then the preparation of spray-dried polymeric microparticles loaded with a model drug to investigate their thermo and pH responsive behavior as drug delivery systems for oral administration. Ketoprofen, a nonsteroidal anti-inflammatory agent (NSAIA) with analgesic and antipyretic properties²⁶ was selected as the drug model. Ketoprofen is an appropriate model drug for formulation for controlled delivery due to its short plasma elimination half-life (2-3 h) that requires multiple dosing to achieve and maintain therapeutic concentration²⁷. Also high levels of ketoprofen in the stomach can cause gastric ulceration partly by directly irritating the gastric mucosa and partly by inhibiting the synthesis of cytoprotective prostaglandins²⁸, so that gastro-protective delivery has been gaining a great importance to target ketoprofen only to the desired GIT regions, to reduce the frequent dosing and the gastrointestinal disturbance. Therefore, there is a need to design such a delivery system, with a more effective drug targeting. Microparticulate carriers like polymeric microparticles with different attributes such as the ability to form gels in aqueous medium²⁹, to dissolve in neutral/basic pH³⁰ or responsive to pH³¹ have been investigated to reduce the adverse effects of ketoprofen in the stomach. Our work presents an alternative way exhibiting temperature and pH sensitive properties to manage this challenge.

Materials and methods

Materials

The monomers *N*-vinylcaprolactam (NVCL) (98%, Aldrich) and acrylic acid (AA) (supplied by Rhodia - Brazil) were distilled under vacuum to remove the inhibitor. 2,2'-azobisisobutyronitrile (AIBN) (supplied by BASF, Brazil) was purified by recrystallization in ethanol and isopropanol (99%, Synth) and was further distilled.

n-hexane (99%, Synth) and trioxane (99%, Aldrich) were used without further purification. Chloroform-*d* (99.8%, Aldrich) was used for nuclear magnetic resonance (¹H NMR) analyses.

Cell culture reagents: Dulbecco Modified Minimum Essential Medium (DMEM) and heat inactivated foetal calf serum were obtained from Cultilab, Brazil; Lipopolysaccharide (LPS), Triton x-100 and the Griess reagents were purchased from Sigma Aldrich and the lactate dehydrogenase kit was supplied from Cultilab, Brazil.

Ketoprofen, C₁₆H₁₄O₃ (molecular weight of 254 g/mol, *pKa* 4.6, log P 3.12) was kindly supplied by Sanofi (Brazil) and used as drug model.

Methods

Synthesis of poly(N-vinylcaprolactam) (PNVCL) and poly(N-vinylcaprolactam-co-acrylic acid) (poly(NVCL-co-AA))

PNVCL and poly(NVCL-co-AA) were synthesized by free radical polymerization in isopropanol at 70 °C, according to the procedure reported by Medeiros et al. (2010)²⁰. The NVCL polymerization was performed in a 50 mL glass double-wall reactor under nitrogen atmosphere. First, 3 g of NVCL and 0.39 g of internal standard (trioxane) were transferred to the reactor. Next, 75% isopropanol was added, and the mixture was flushed with argon for 20 min at a moderate gas flow rate. Separately, a solution containing AIBN (0.05 g) and 25% solvent was prepared and purged with argon for 20 min. When the temperature reached 70 °C, polymerization was initiated by the addition of the AIBN solution to the NVCL solution. The polymerization proceeded for 3 hours. Samples were withdrawn from the reaction medium at the beginning and at the end of the polymerization reaction to analyze monomer conversion by ¹H NMR. Following polymerization, the polymeric product was purified by precipitation in *n*-hexane followed by drying at 37 °C for 24 hours under vacuum.

The synthesis of poly(NVCL-co-AA) followed the same procedure, but in this case, 3 g of the monomer mixture (80 mol % of NVCL and 20 mol % of AA) was transferred to the reactor to be polymerized.

Characterization of poly(N-vinylcaprolactam) (PNVCL) and poly(N-vinylcaprolactam-co-acrylic acid) (poly(NVCL-co-AA))

Conversion by Nuclear magnetic resonance spectroscopy (^1H NMR)

Monomer conversion was determined by ^1H NMR. Samples were withdrawn from the polymerization medium at the beginning and end of the reaction, introduced into vials containing hydroquinone, and placed on ice to stop polymerization. ^1H NMR analyses were performed without evaporation of polymerization solvent by mixing 0.2 mL of each sample with CDCl_3 (1:3 v/v). Monomer conversion was determined by comparing the vinyl protons of NVCL and AA with the protons of trioxane as a reference.

LCST measurements

The transmittance of each polymer in aqueous solution was measured using UV-visible spectrophotometry (Genesys 10 Series Spectrophotometer, USA) at 570 nm for PNVCL and 540 nm for poly(NVCL-co-AA). The concentration of each polymer solution was 5 mg mL^{-1} . The light intensity through the solution was measured as a function of temperature. Transmittance measurements were performed from 20°C to 50°C with increasing temperature increments of 1°C every 5 min. The transition temperature (LCST) is defined as the temperature at which the transmittance is 50% of the initial value obtained at room temperature³²

Molar masses and molar mass distributions (MMD)

Gel Permeation Chromatography (GPC) analyses were performed using a Waters GPC device (Waters USA) equipped with a 1515 Waters HPLC pump, a 2414 Waters differential refractometer, and a 717 Plus Waters auto-sampler. To obtain poly(NVCL-co-AA) molar mass, *N,N*-dimethylformamide (DMF) was used as eluent and the calibration was performed using eight polystyrene standards with different molar masses. A set of three Phenogel columns (Phenomenex) with porosities of 10^3 , 10^4 and 10^6 \AA was used in the analyses. However, for PNVCL, the absolute molar mass was determined using the 302 Viscotek dual detectors, which includes a viscometer, a low angle/LALS (7°) and a high angle/RALS (90°) light scattering detector at a wavelength of 670 nm. A solution of tetrabutylammonium bromide in tetrahydrofuran (2.5 g L^{-1}) was used as eluent at a flow rate of 1.0 mL min^{-1} . Polystyrene standards (\overline{M}_w

= 99448 g mol⁻¹, viscosity index = 0.477 and \overline{M}_w = 273773 g mol⁻¹, viscosity index = 0.894) were used to calibrate the detectors.

Determination of copolymer composition by potentiometric titration

Two hundred milligrams of poly(NVCL-co-AA) was dissolved in 10 mL of a 0.1 N sodium hydroxide solution to neutralize the carboxylic groups. The excess NaOH was then titrated (Titralab, France) with a 0.1 N hydrochloric acid solution. The amount of acidic comonomer units was calculated from the amount of NaOH required to neutralize the carboxylic acid groups.

Spray-dried PNVCL and poly(NVCL-co-AA) microparticles

Preparation

PNVCL and poly(NVCL-co-AA) microparticles were prepared by spray-drying. To entrap ketoprofen in the polymers, PNVCL or poly(NVCL-co AA) was first dissolved in water or in an phosphate buffer solution at pH 9.0 resulting in a polymeric solution concentration of 50 mg.mL⁻¹. An appropriate amount of hydrophobic ketoprofen (solubility in water equivalent to 197nmoles.mL⁻¹)³³ was dispersed in this polymeric aqueous solution corresponding to a weight polymer/drug ratio of 1:1. The dispersion was stirred at 500 rpm for 30 min. The aqueous dispersion of polymer and drug was then spray-dried with a 0.7 mm two-fluid pressurized atomizer at a feed rate of 6 mL min⁻¹ in a Büchi-B190 spray dryer (Büchi, Switzerland). The atomizing air flow rate was 500-600 NL h⁻¹. The inlet temperature was controlled at 122-125 °C and the outlet temperature (98 100 °C) was determined by the inlet temperature and relative factors such as air and liquid flow rates.

Characterization

Size, residual water content and morphology

The powder samples were dispersed in mineral oil and their average particles size were analyzed using a Horiba LA-950 laser diffraction analyzer (Horiba Instruments, Inc., Kyoto, Japan) equipped with a Mini Flow circulation system. The residual water content in dried samples was determined by gravimetric tests.

Morphological evaluation was conducted by using a LEO1450VP scanning electron microscope (SEM) (Zeiss, Germany) with a tungsten filament operating at 10

kV. For these analyses, the spray-dried samples were dispersed on a brass support, fixed with a double face 3M tape and coated with a thin gold layer. All samples were sputter-coated with gold before microscopic observations.

X-ray diffractometry (XRD)

XRD patterns of pure ketoprofen, PNVCL, poly(NVCL-co-AA) and spray-dried microparticles containing ketoprofen were recorded using a XRD-6000 X-Ray Diffractometer (Shimadzu, Japan) (Cu α source with 1.5406 wavelength) in thin film mode at 40 kV and 30 mA. Intensity measurements were performed as a function of diffraction angle, which varied from 10° and 80°.

Raman spectroscopy

Raman microscopy analyses were performed with an Alpha 300AR Confocal Raman - AFM microscope (Witec, Germany) equipped with a laser at a wavelength of 532nm. The measurements were recorded using a 50x objective, which allowed 360nm of lateral resolution and 920nm of axial resolution. The spectral range was selected from 200 to 3600cm⁻¹. All spectra were acquired at a total laser light exposure time of 10 x 0.5s per collected spectrum.

Ketoprofen entrapment

The ketoprofen loading in the PNVCL or poly(NVCL-co-AA) spray-dried microparticles was determined according to the following methodology: 50 mg of each sample (MS1 and MS2) was dissolved in aqueous solution at pH 9.0 under magnetic stirring during 24 hours. The ketoprofen content in this aqueous solution (C_{KM}) was then measured by UV spectroscopy at 260 nm using a calibration curve based on solutions of ketoprofen in water at different concentrations.

Biological assays

Assessment of cytotoxicity of PNVCL-based polymers: LDH Assay

The cytotoxicity of empty PNVCL and poly(NVCL-co-AA) polymers and ketoprofen-loaded microparticles was evaluated in vitro according to their capacity to induce cellular release of lactate dehydrogenase (LDH) by damaged cells.

For the assay, mouse peritoneal macrophages were plated at 5×10^5 cells/well in 96-well culture plates and incubated at 37 °C. After 24 hours, the adherent cells were

treated with various concentrations of the ketoprofen or polymers at 1, 10, 100 or 1000 $\mu\text{g mL}^{-1}$ in 200 μl of DMEM culture medium containing 5% HIFCS. The specific release of LDH into the culture medium was measured by a colorimetric assay³⁴ using a commercial kit (Labtest, Brazil), according to the manufacturer's instructions. Maximum and spontaneous release values were determined in cells cultured with 2% Triton X-100 and cells without treatment, respectively. Cytotoxicity was expressed as the % specific release of LDH [(test release - spontaneous release) / (maximum release - spontaneous release)] x 100.

Anti-inflammatory potential of PNVCL and poly(NVCL-co-AA) ketoprofen microparticles: nitric oxide production by macrophages

Activation of nitric oxide (NO) production by macrophages was used as an indication of the anti-inflammatory potential of PNVCL and poly(NVCL-co-AA) ketoprofen microparticles.

For the assays, bone marrow-derived macrophages were plated at 1×10^5 cells/well in 96-well culture plates and incubated at 37 °C. After 24 hours, the cells were treated with various concentrations of ketoprofen or ketoprofen-loaded microparticles at 1, 10, 100 or 1000 $\mu\text{g mL}^{-1}$ in 200 μl of DMEM culture medium containing 5% HIFCS. After 18h, 1 $\mu\text{g.mL}^{-1}$ of lipopolysaccharide (LPS) was added or not to the cells. After 96h, the microplates were centrifuged at 1000 rpm for 5 min, the supernatants were collected, and the nitrite concentration was calorimetrically evaluated by the Griess reaction at 540 nm (exc)/570 nm (em) using a nitrite standard curve³⁵. The polymers were evaluated as controls.

Dissolution studies

The dissolution studies were performed with a pharma test dissolution apparatus (299/6 ATTS; Nova Ética, São Paulo, Brazil), using the USP Method-2 (Paddle Method). Drug release was examined by varying the temperature (25 °C or 37 °C) and the pH (1.2 or 7.4) of the release media to simulate gastrointestinal fluids. The dissolution media was composed of 500 mL of phosphate standard buffers (pH 1.2 or 7.4), simulating in vivo conditions. A speed of rotation of 100 rpm was maintained in each dissolution test. Two hundred milligrams of microparticles were packed into a lock-cap gelatin capsule and added to the dissolution medium. Aliquots were removed at suitable intervals from the dissolution vessel, immediately filtered and assayed

spectrometrically at 260 nm. The polymer used did not interfere in the assay. The drug concentration in the dissolution solution was calculated from a standard curve. The mean of three determinations was used to calculate the drug release from each of the formulations. The in vitro dissolution of pure spray-dried ketoprofen crystals was also determined.

Methods used to compare drug release profiles from PNVCL and PNVCL-AA spray dried microparticles

Drug release profiles were analyzed using independent (Dissolution Efficiency-DE) and model-dependent (curve-fitting) approaches. The dissolution efficiency (DE) was calculated by the free open source software KinetDS[®] ³⁶, according to Eq. 4 (Table 1). The differences for DE were statistically examined by one-way ANOVA followed by Tukey's test, in order to find the source of difference. In this method, $\langle DE \rangle$ (n=2) was the dependent variable, and $\langle \text{Formulation} \rangle$ was the factor. The calculations were performed using the software *Statistica* (Data Analysis Software system, StatSoft, Inc) version 12.0. KinetDS[®] was also used to fit the release curves to the Korsmeyer-Peppas mathematical model³⁷ (Eq. 1, Table 1). The accuracy and prediction ability of the mathematical model were analyzed by calculation of coefficient of determination (R^2), AIC (Akaike's information criterion) and RMSE (root mean square error), described in Table 1 (Eq. 2 and 3).

Results and discussions

Synthesis and characterization of PNVCL and poly(NVCL-co-AA)

¹H NMR spectra (not shown) were used to analyze the NVCL and AA conversion, and it was observed in both homopolymerization and copolymerization that NVCL was totally consumed after 3 hours into the reaction. ¹H NMR revealed the absence of a peak corresponding to the vinylic proton of the AA molecule after 15 min of copolymerization.

The molar masses (\bar{M}_n) obtained for PNVCL and poly(NVCL-co-AA) were 15,220 and 6,640 g mol⁻¹, respectively. This significant difference on molar masses was not expected since the total concentration of monomers was the same in both polymerizations and the ¹H NMR analyses indicated that they were totally consumed in the reactions. However, this result may be attributed to intramolecular interactions

between NVCL and AA unities into the copolymer chains. These strong polymer-polymer interactions will compete with the polymer-solvent interactions resulting in changes on polymer conformation (hydrodynamic volume) and consequently in changes of the polymer solubility in DMF, which was used as solvent for GPC analyses. The content of AA in the poly(NVCL-*co*-AA) copolymers, determined by titration, was equivalent to 17 mol %, which is near the percentage used in the feed (20 mol %).

Poly(*N*-vinylcaprolactam) (PNVCL) and poly(acrylic acid) (PAA) are both water-soluble at neutral pH and room temperature. However, the copolymers are not water soluble, which is attributed to complex formation by hydrogen bonding between the amide groups from PNVCL segments and the non-ionized carboxylic groups from PAA segments. For this reason, it is necessary to ionize the carboxyl groups from AA molecules to break these hydrogen bonds and to dissolve the copolymer. Therefore, to determine the lower critical solution temperature (LCST) of poly(NVCL-*co*-AA), the copolymer was first dissolved in water at pH 9. Fig. 1 shows a comparison between the transmittance as a function of temperature and the LCST obtained for PNVCL and poly(NVCL-*co*-AA). After complete dissolution of the polymers, the pH was adjusted to 4 and 10, respectively, in order to evaluate the influence of this parameter on the polymers LCST.

Fig. 1. Transmittance as a function of temperature and pH for PNVCL and poly(NVCL-*co*-AA).

It is well known that the LCST of PNVCL shifts to higher values when molar mass decreases and when a hydrophilic comonomer is added. As previously mentioned, the presence of carboxylic groups from the pH-responsive segments modifies the hydrophilicity/hydrophobicity balance, leading to LCST changes. Fig. 1 reveals that LCST increased from 35 °C to 39 °C when AA was added to the polymerization.

The hydrophobic effect and hydrogen bonding are the main factors responsible for the LCST behavior of the thermo-responsive-based random copolymers. Salgado-Rodriguez et al.³⁸ synthesized a series of random *N*-isopropylacrylamide (NIPAAm) and methacrylic acid (MAA) copolymers of similar composition that could be split into two groups. One group had a controlled number of units with a free acid group, while the other group was methoxy-protected. These experiments were performed to clarify the effect of possible hydrogen bonding on the LCST behavior. These authors observed that

the carboxylic groups of poly(NIPAAm-*co*-MAA) can form hydrogen bonds with the amide groups of PNIPAAm, and these bonds lead to additional polymer-polymer interactions. These additional interactions make phase separation by heating a less endothermic process than that in pure PNIPAAm because fewer sites are available for water to bind to the NIPAAm units. Thus, the hydrogen-bonding interactions play an important role in these systems, and as a result, the pH of the medium also influences the phase transition, as it can be observed in Fig. 1. At pH 4, when the majority of the carboxylic groups from AA are not ionized (pK_a of AA = 4.17), the LCST was equivalent to 39 °C, while at pH 10, the phase transition was observed only at 42 °C. This increase is due to the higher hydrophilicity of the copolymer at pH 10 and resulted from the ionized state of the carboxylic groups from AA. The copolymerization of monomers that produces thermo-responsive polymers with monomers containing ionizable groups is an interesting approach because it makes possible to adjust the thermo-sensitivity for temperature values close to the human body temperature and to consequently discover available systems for biomedical applications. Concerning the homopolymer PNVCL, no difference was observed in LCST at pHs 4 and 10.

*Spray-dried PNVCL and poly(NVCL-*co*-AA) microparticles loaded with ketoprofen*

Drug loading and residual water, particle size and morphology

In this study, ketoprofen was entrapped in thermo-responsive PNVCL and pH- and thermo-responsive poly(NVCL-*co*-AA) polymers by spray-drying. The physicochemical characteristics of the dry particles are given in Table 2.

The drug loading in the microparticles (C_{kM}) determined by UV analyses are lower than those of the initial formulations C_{ki} (46% for MS1 and 40% for MS2). The differences between C_{kM} and C_{ki} can be attributed to the residual water retained within the spray-dried microparticles, ranging from 4.3 to 9.7%.

Table 2 also shows that particles size was affected by the type of polymer: larger particles were obtained when poly(NVCL-*co*-AA) was used.

Solid state characterization

X-ray Diffractometry

X-ray diffraction was used to analyze the nature of the polymeric microparticles loaded with ketoprofen. The XRD patterns of raw ketoprofen, blank PNVCL and ketoprofen-loaded PNVCL microparticles (sample MS1) are compared in Fig. 2. The characteristic peaks of raw ketoprofen appeared in the 2θ range of $10 - 80^\circ$ revealing its high crystalline structure (Fig. 2-A). However, these peaks disappeared in the ketoprofen-loaded PNVCL microparticles (MS1) (Fig. 2-C). XRD peak depends on the crystalline content, but in the present study, for all the drug loadings, the characteristic peaks of ketoprofen, if present, overlapped with noise of the blank polymer itself (Fig. 2-B). This result suggests that ketoprofen and polymers can interact at the molecular level. Moreover, the drug dissolution followed by solidification process by spray-drying resulted in the amorphization of the most part of ketoprofen. The reflections at 44, 65 and 78 degrees (2-theta) observed in all XRD patterns are probably related to the sample holder.

Fig. 2. Powder XRD patterns of (A) pure ketoprofen (B) blank spray-dried PNVCL microparticles and (C) ketoprofen-loaded PNVCL microparticles (MS1).

In turns, spray-dried microparticles consisting of poly(NVCL-co-AA) loaded with ketoprofen (Sample MS2) in Fig. 3-C provide little or no evidence of ketoprofen crystallinity similarly to the PNVCL microparticles.

Fig. 3. Powder XRD patterns of (A) pure ketoprofen, (B) blank spray-dried poly(NVCL-co-AA) microparticles and (C) ketoprofen-loaded poly(NVCL-co-AA) microparticles (MS2).

Raman analysis

The Raman spectra of pure ketoprofen and selected ketoprofen-loaded poly(NVCL-co-AA) microparticles are compared in Fig. 4. Raman spectrum of pure ketoprofen showed typical -C-H-, -C=C- and -C-O-H- stretching bands at 3061, 1659, 1605 and 1003 cm^{-1} , respectively, which were not evidenced in pure poly(NVCL-co-AA) spectrum. These bands were also observed in the Raman spectra of spray-dried

poly(NVCL-co-AA) particles containing ketoprofen. The peaks present at 1659 and 1605 cm^{-1} , related to C = C band of aromatics appeared in the same region of the bands related to the C = O amide groups of NVCL and AA, but a significant difference between the intensity and width of these bands can be noted. The intensity of the peak at 1452 cm^{-1} related to C-N- (from NVCL) stretching reduced considerably in the spectra for spray-dried particles containing ketoprofen. These findings also suggest the molecular interaction between drug molecule and poly(NVCL-co-AA) matrix.

Fig. 4. Raman spectra of blank poly(NVCL-co-AA) (A), spray-dried poly(NVCL-co-AA) particles containing ketoprofen (B) and pure ketoprofen (C).

SEM images

Scanning electron microscopy (SEM) was also used to investigate the particle microstructures. First, the unprocessed crystals possess an irregular shape and a broad particle size distribution as shown in Fig. 5.

Fig. 5. Scanning electron micrograph of unprocessed ketoprofen crystals.

Samples of the spray-dried powders containing the polymers and ketoprofen were also analyzed by SEM (Fig. 6). Differences in powder topography compared to the unprocessed crystals shown in Fig. 5 can be clearly visualized by observing the images in Fig. 6. The smoother surface of the spray-dried particles, mainly observed in Fig. 6-B (PNVCL) and Fig. 6 D (poly(PNVCL-co-AA)), suggests that the drug and the polymers are well mixed with only few crystals of drug alone observed in MS1 sample (Fig. 6-A), however did not see in the other samples. These findings are consistent with the XRD results which showed no or little drug crystalline structure in the drug-loaded microparticles. In addition, compared to the PNVCL microparticles, the poly(NVCL-co-AA) microparticles have a less spherical shape and a more wrinkled surface.

Fig. 6. SEM images of: ketoprofen-loaded PNVCL microparticles (MS1) (A and B) and ketoprofen-loaded poly(NVCL-co-AA) microparticles (MS2) (C and D).

Biological assays

Assessment of cytotoxicity of PNVCL-based polymers: Lactate Dehydrogenase (LDH) Assay

The capacity of the polymers PNVCL and poly(NVCL-*co*-AA) and ketoprofen microparticles to induce cytotoxicity was evaluated in vitro by examining LDH leakage from lysed macrophages (Fig. 7).

The results show that at concentrations ranging from 0.1-100 $\mu\text{g.mL}^{-1}$, both PNVCL and poly(NVCL-*co*-AA) did not promote significant LDH release during the 48 hour-cell culture (Fig. 7-A).

The poly(NVCL-*co*-AA)-ketoprofen microparticles, but not PNVCL-ketoprofen, promoted significant LDH release at concentrations above 100 $\mu\text{g.mL}^{-1}$ (Fig. 7-B), during the 48 hour-cell culture. The higher cytotoxicity of poly(NVCL-*co*-AA)-ketoprofen microparticles as compared to PNVCL-ketoprofen microparticles may be due to the presence of AA (17 mol %) in its composition. However, these results indicate that PNVCL and poly(NVCL-*co*-AA)-ketoprofen microparticles are not cytotoxic to macrophages in amounts compatible with pharmaceutical use.

Macrophages were used for in vitro cytotoxicity studies because they are the most abundant mononuclear phagocytes in the gut lamina propria³⁹ and are capable of microparticles uptake.⁴⁰

Fig. 7. Cytotoxicity of PNVCL and poly(NVCL-*co*-AA) polymers and microparticles. Mouse peritoneal macrophages (5×10^5) were incubated for 48 hours in the presence of the indicated concentrations of polymers (A) and ketoprofen microparticles (B) in 200 μl of culture medium at pH 7.4. The amount of LDH in the cell supernatants was expressed in relation to 100% LDH induced by 2% Triton X-100. Means \pm SD (n=3). * Significance level $p > 0.05$ in relation to untreated controls.

Anti-inflammatory effect of ketoprofen microparticles: Nitric oxide Assay

Nitric oxide plays an important dual effect in inflammation⁴¹. Ketoprofen and other nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, naproxen, flurbiprofen, ketorolac and diclofenac have been reported to reduce de NO production by macrophages⁴². Here, ketoprofen was used as a highly lipophilic drug prototype for loading into the polymeric microparticles aiming at oral and subcutaneous drug delivery. The capacity of polymers and formulations to induce nitric oxide release and

cytotoxicity was compared in vitro in unstimulated and LPS-stimulated bone marrow-derived macrophages.

We found that encapsulation in PNVCL and to a lesser degree in poly(NVCL-*co*-AA)-microparticles rendered ketoprofen an activating agent of NO production in resting cells (Fig. 8-A). We found that ketoprofen suppressed the NO production by LPS-stimulated macrophages, in accordance with previously reported in LPS-stimulated J774 macrophage cell line⁴¹, and this effect was not affected by encapsulation in microparticles, irrespective of the polymer (Fig. 8-B). These findings indicate that despite the intrinsic NO-stimulating activity, poly-(NVCL-*co*-AA) does not interfere with the in vitro anti-inflammatory effect of ketoprofen.

Fig. 8. Anti-inflammatory activity of PNVCL and poly(NVCL-*co*-AA) polymers and microparticles. Bone marrow-derived macrophages (1×10^5) were incubated for 18 hours in the presence of the indicated concentrations of polymers and ketoprofen microparticles in 200 μ l of culture medium. The cells were incubated without (A) or with 1 μ g.mL⁻¹ of lipopolysaccharide (LPS) (B). After 96h, the cell supernatants were collected and nitric oxide (NO) production was evaluated by Griess reaction in spectrophotometer. Means \pm SD (n=3).

In vitro drug release

Effect of temperature and pH on drug release kinetics

The in vitro ketoprofen release from PNVCL and poly(NVCL-*co*-AA)-microparticles prepared was investigated as a function of temperature (25 °C and 37 °C respectively) and pH (pH 1.2 and 7.4 phosphate buffer solution). The experimental release data are shown in Figs 9 and 11. The quantitative interpretation of the values obtained in the dissolution assays is easier using dissolution efficiency (DE) to describe the release profiles. Dissolution efficiencies during 240 min (DE₂₄₀) are given in Table 3.

Fig. 9 and Table 3 reveal that increasing temperature from 25°C to 37 °C resulted in decrease of the ketoprofen release rate from both PNVCL and poly(NVCL-*co*-AA) microparticles. For example, at pH 7.4 and below the LCST (25 °C), the release of ketoprofen from PNVCL microparticles (F5) was faster (DE₂₄₀ \cong 63%) compared to that released at 37 °C in the same time period (F7, DE₂₄₀ \cong 20%). In general, higher

release rates were obtained from PNVCL microparticles as compared to release rates from poly(NVCL-*co*-AA) microparticles.

Besides temperature, another parameter governing the drug diffusion from the microparticles to the release medium was the pH. It can be seen that ketoprofen release from poly(NVCL-*co*-AA) microparticles increased when the pH was increased from 1.2 (Fig. 9A, F2 in Table 3) to 7.4 (Fig. 9B, F6 in Table 3). F2 and F6 were statistically different as $p < 0.05$, ($DE_{240} = 11.5\%$ and 42.6% , respectively), confirming the positive effect of increasing pH on the ketoprofen release from the particles prepared with poly(NVCL-*co*-AA) at 25°C . However, PNVCL microparticles also showed a pH-responsive behavior at 25°C , with $DE_{240} = 18.5\%$ for F1 significantly different ($p < 0.05$) of F5 ($DE_{240} = 62.9\%$). A same expressive behavior was found at 37°C for PNVCL (DE_{240} F3 = 5.1% against DE_{240} F7 = 19.9%) and for poly (NVCL-*co*-AA) (DE_{240} F4 = 2.0% and DE_{240} F8 = 10.8%).

The pH-responsive behavior of PNVCL was first observed by Vihola et al.³³ who studied the release of ketoprofen from PNVCL hydrogels physically cross-linked with salicylic acid (SA). These authors attributed the pH-dependent behavior of PNVCL-based hydrogels to the acidic nature of ketoprofen and the influence of the pH on its water solubility⁴³. The pH-dependent characteristics of pure ketoprofen were also demonstrated in the present work, as shown in Fig. 10 and Table 3 (DE_{240}).

Fig. 9. In vitro release of ketoprofen from PNVCL and poly(NVCL-*co*-AA) microparticles in acidic medium, pH 1.2 (A) and in phosphate buffer, pH 7.4 (B).

Fig. 10. In vitro dissolution rate of unprocessed ketoprofen crystals in phosphate buffer (pH 7.4) and acidic medium (pH 1.2) at 25°C and 37°C .

Curve fitting and drug release mechanism

To determine the mechanism of drug release, the first 60% cumulative drug release from the PNVCL-based microparticles were fitted in Korsmeyer–Peppas model. Nonlinear regressions were applied for cumulative dissolved drug. The coefficient of determination (R^2), root-mean-square error (RMSE) and Akaike's information criterion (AIC) values were determined using KinetDS 3.0 rev. 2010 software, Krakow, Poland. The data obtained from Korsmeyer–Peppas model (Eq. 1, Table 1) for all dissolution experiments performed in this work, at different conditions of temperature and pH are

listed in Table 4. It can be observed that the drug release mechanism is well described mathematically by the Korsmeyer-Peppas equation, considering the values of R^2 , AIC and RMSE. Korsmeyer-Peppas model is a semi-empirical model, relating exponentially the drug release to the elapsed time. It is used to analyze the release of pharmaceutical polymeric dosage forms, when the release mechanism is not well known or when more than one type of release phenomenon could be involved⁴⁴. There are three primary mechanisms by which the release of a drug molecule can be controlled⁴⁴: erosion, diffusion, and swelling followed by diffusion.

The release rate of ketoprofen (k) decreased as the temperature increased from 25°C to 37°C, and for this reason, drug diffusion was diminished when temperature increased. More specifically, at 25 °C, hydrogen bonding between the polymer chains and water leads to the hydration of the microparticles, resulting in polymer chain relaxation, penetration of water into the matrix, and consequently higher release rates. At 37 °C (temperature higher than LCST for pure PNVCL and near LCST for poly(NVCL-*co*-AA)), water uptake is inhibited due to the collapsed state of the polymer chains resulting from polymer-polymer interactions. The loss of hydrogen bonds between the polymer chains and water limits diffusion of the small drug molecules and decreases the drug release rate.

The low values of the release exponent ($n < 0.48$) at pH 1.2, 25°C and 37°C, indicated a Fickian diffusion mechanism from all formulations, i.e. polymer relaxation followed by diffusion of ketoprofen. The shift of the mechanism from diffusion controlled ($n < 0.5$) to an anomalous or non-fickian transport ($n > 0.54$) occurred by changing the pH of the medium from acid to basic conditions, when the tested carrier polymers switched from an agglomerated state to a swollen state.

The pH-dependent swelling of the polymeric particles evidenced by pH changes can explain the pH-dependent release rate of the entrapped drug. In vitro tests revealed, in fact, that polymeric aggregates are formed and remained stable (Fig. 11-A and 11-C, in the bottom of the tube) when PNVCL or poly(NVCL-*co*-AA) microparticles are dispersed in acidic medium (pH 1.2). Contrarily, Fig. 11-B and 11-D also show that the same particles are found in a gelled (swollen) state when dispersed in pH 7.4. The agglomerated state clearly visualized in Fig. 11-C is probably due to the complexation between PNVCL and PAA segments; therefore, the diffusion of ketoprofen through the polymeric barrier was significantly diminished. At pH 7.4, ionization of carboxyl groups from PAA segments led to the dissociation of the polymeric complex and

swelling of the microparticles due to electrostatic repulsion. Consequently, diffusion of ketoprofen molecules was favored, increasing the drug release. In vivo, by analogy, it could be expected that the PNVCL-based microparticles could pass through the stomach (pH near 1.2) without significant drug release. Furthermore, they could reach the small intestine and the colon (pH near 7.4), increasing drug delivery in this region due to their pH-dependent swelling. Under this perspective, a proper (gastro-protective) release in GIT can be expected from the PNVCL-based microparticles proposed here.

It is important to note that drugs have been found to plasticize or antiplasticize polymers⁴⁵, thus enhancing or decreasing the rate of polymer hydration. In the present case, embedded ketoprofen, if interacting with the polymers as indicated by Raman and XRD analysis, may have changed some key PNVCL-based microparticles features and may have played its part in the release performance found in this work

Fig. 11. Different states of ketoprofen-loaded polymeric microparticles after dispersion in pH 1.2 (at left: agglomerated state) and 7.4 (at right: swollen state): (A, B) PNVCL; (C,D) poly(NVCL-co-AA).

Conclusions

In this work, a series of temperature- and pH-responsive poly(*N*-vinylcaprolactam)-based polymers synthesized by free radical solution polymerization have been used to prepare loaded-drug microparticles by spray drying from aqueous formulations. The polymers PNVCL-co-AA and PNVCL exhibited temperature and pH-induced phase transitions and they were not toxic to phagocytic cells. Therefore, they could be considered potentially safe for biological use as drug delivery systems. Their LCST reached the body's physiological temperature (35-39 °C) and their role as drug delivery carriers could be investigated using ketoprofen as the drug model. It was possible to demonstrate that both PNVCL and poly(NVCL-co-AA) can be used as a novel carrier to encapsulate ketoprofen as a model drug, resulting in extended release of the drug, modulated by pH and temperature. The results reported here encourage pursuing the study by enlarging the number of drugs to be associated to the copolymer to study the nature of their interaction or by modifying the AA content in the copolymer to adjust its LCST closer to the body's physiological temperature.

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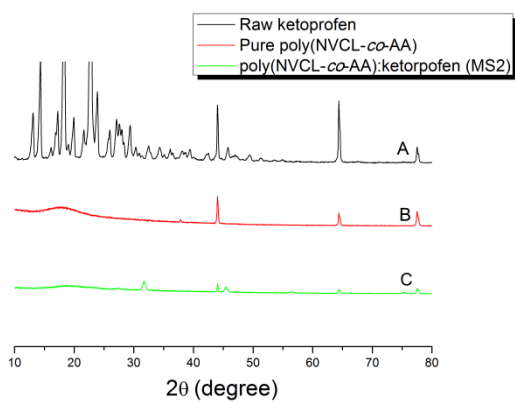
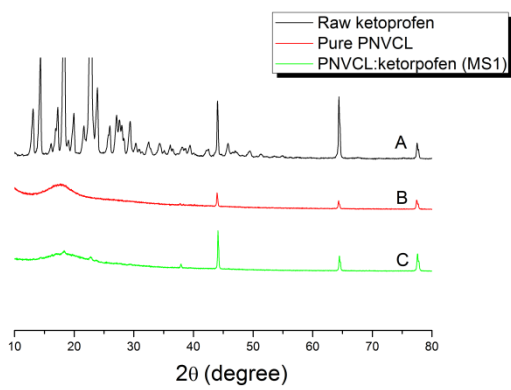
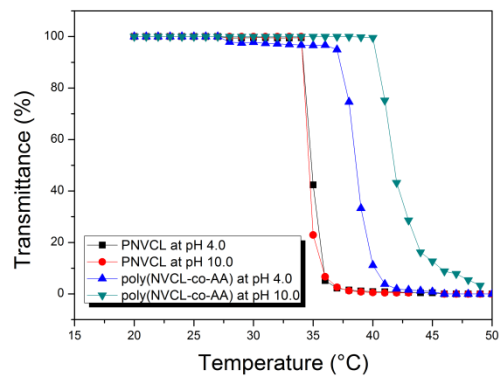
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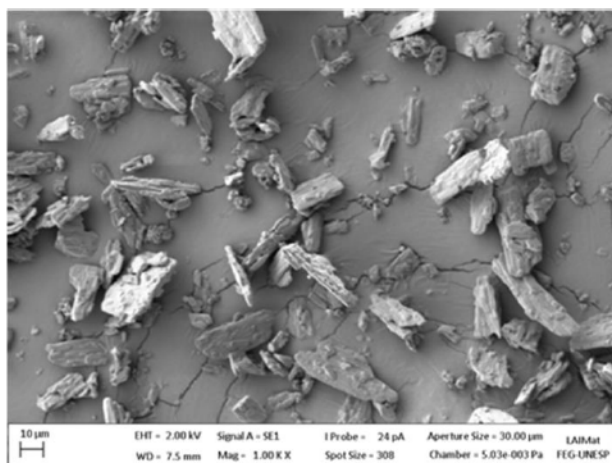
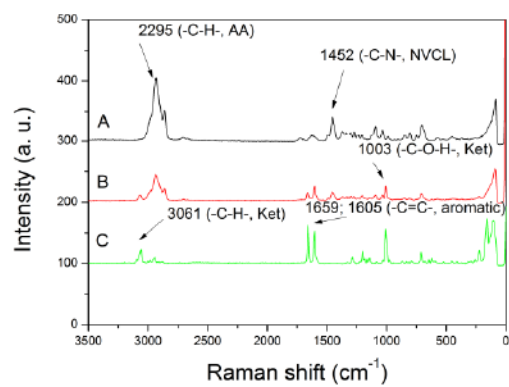
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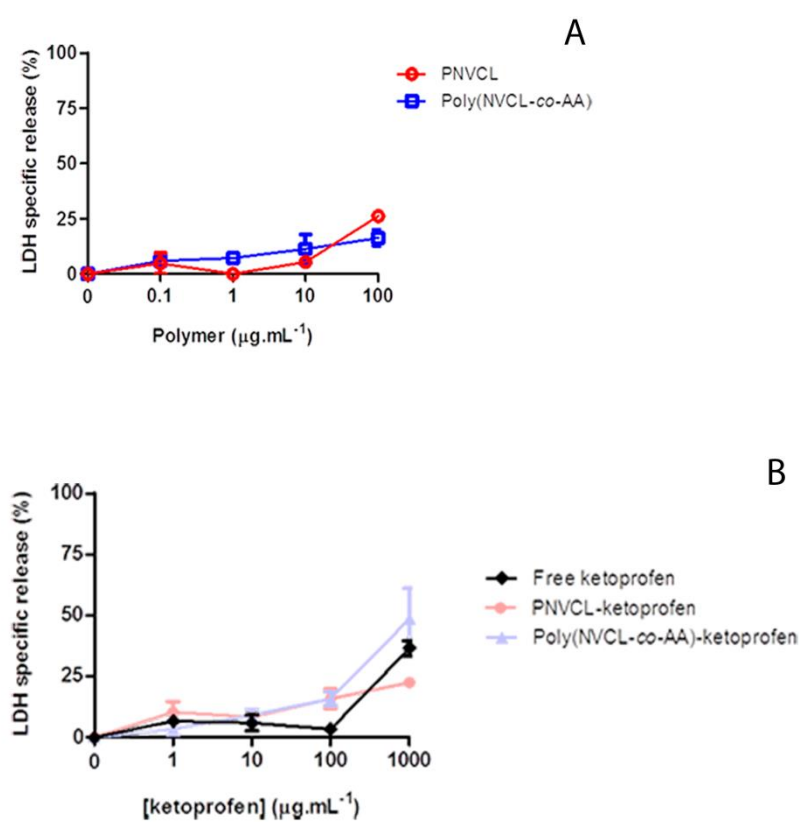
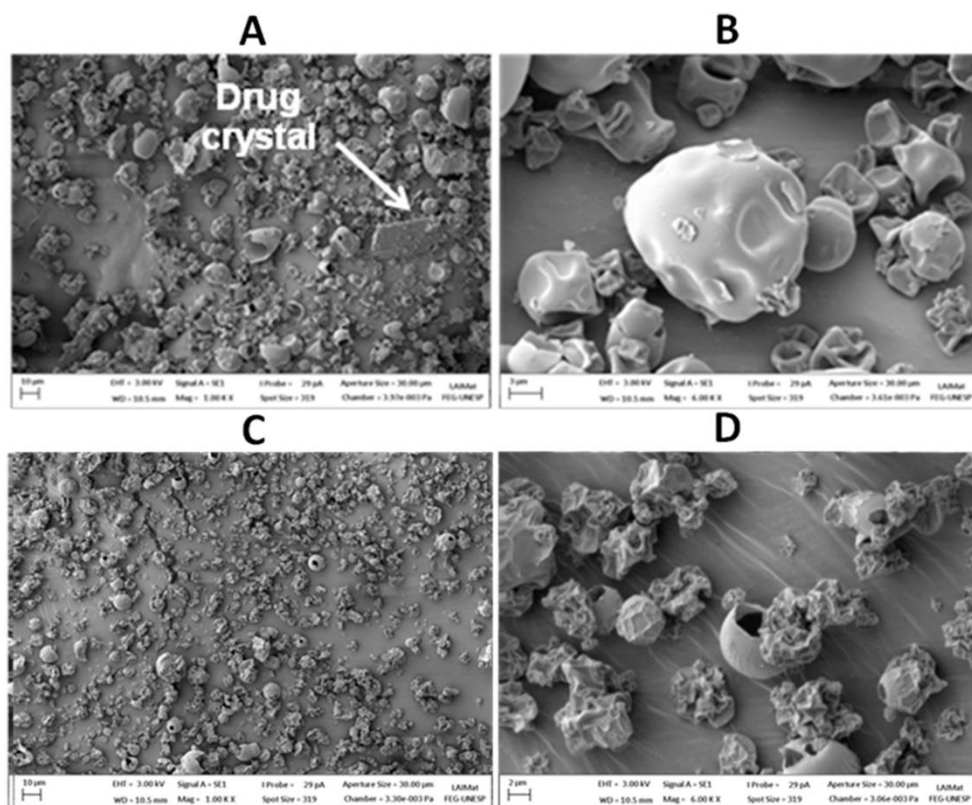
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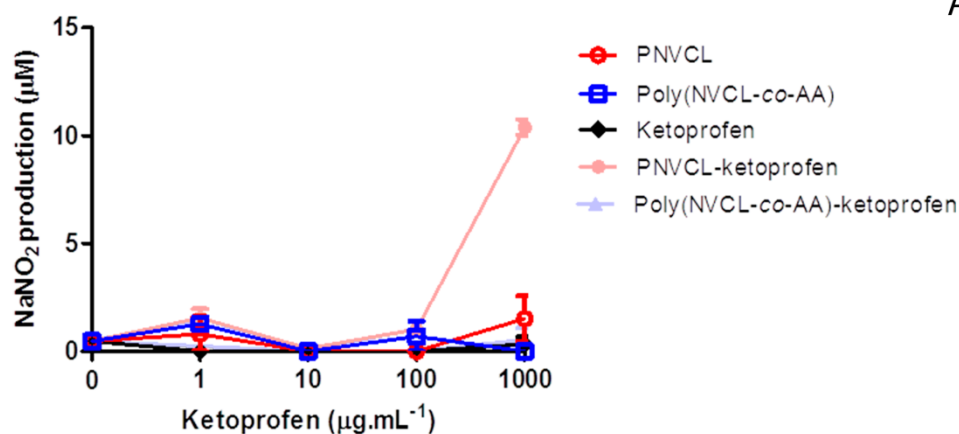
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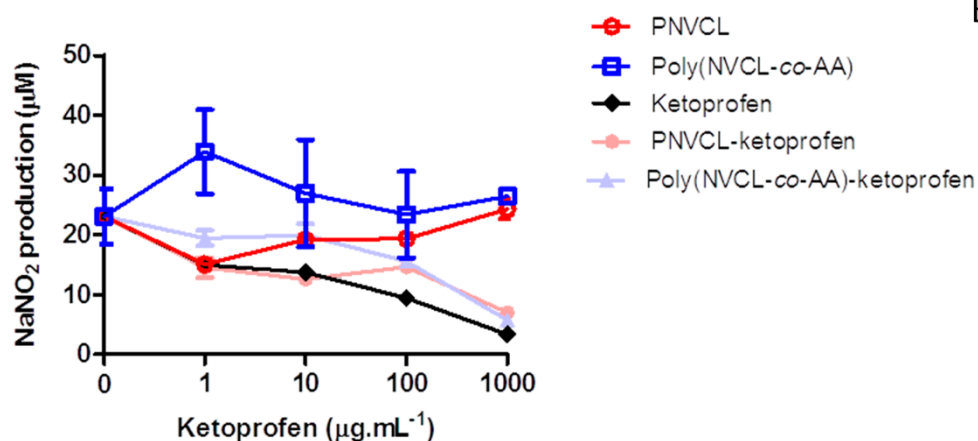




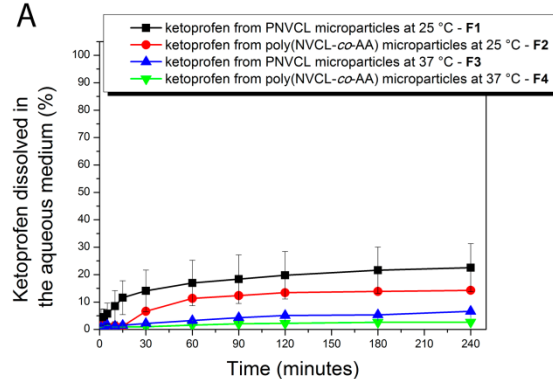
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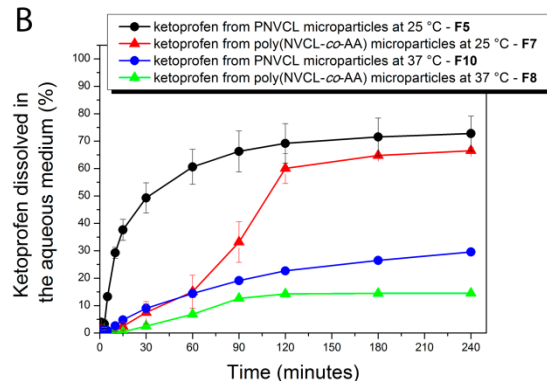
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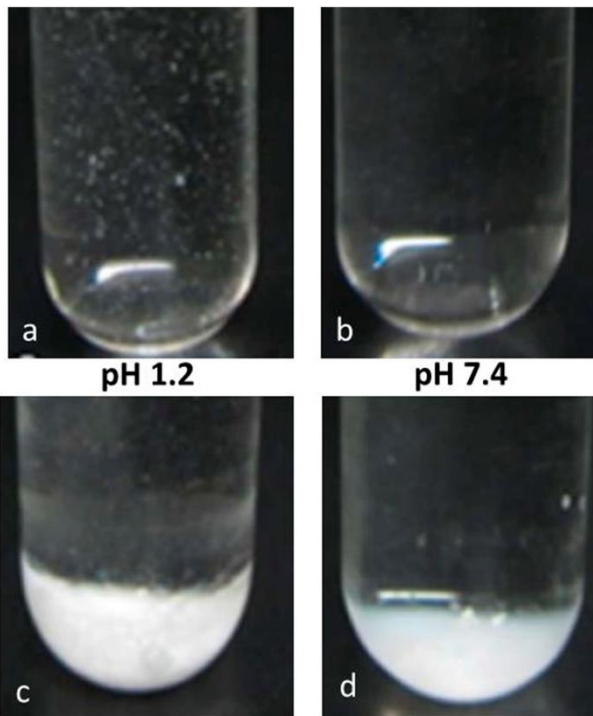
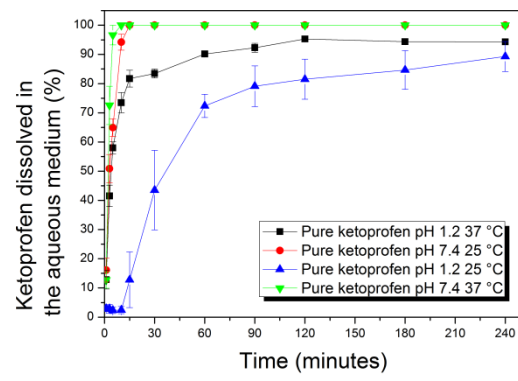


Table 1

Dependent and independent kinetic models applied to analyze the drug release data.

Approach	Method	Equation
Model-Dependent	Korsmeyer-Peppas	$Mt = K_{KP} \cdot t^n$ (Eq. 1)
	<i>Best model criteria</i>	$RMSE = \sqrt{\frac{\sum_{i=1}^n (y_{iobs} - y_{ipred})^2}{n}}$ (Eq. 2)
		$AIC = 2k + n \cdot [\ln(\sum_{i=1}^n (y_{iobs} - y_{ipred})^2)]$ (Eq. 3)
Model-Independent	Dissolution efficiency (DE)	$DE = \left(\frac{\int_0^t Mtdt}{Mt_{max} \cdot t} \right) \times 100$ (Eq. 4)

Mt: amount of drug released in time t

Mo: initial amount of drug in the dosage form

K_{KP} is the release constant incorporating structural and geometric characteristics of the drug-dosage form; n is the diffusional exponent indicating the drug-release mechanism

Mt_{max} = maximum amount of drug released (=100%)

Table 2

Physical characteristics of the spray-dried polymeric microparticles

Sample	Polymer	Initial polyme : drug mass proportion	Ketoprofen entrapped in the spray-dried microparticles, C_{kM} (%)	Residual water content (w/v %)	Particles size (μm)
MS1	PNVCL	1:1	46	4.3	63.8±3.1
MS2	Poly(NVCL-co-AA)	1:1	40	9.7	71.4±2.6

Table 3

Comparison of ketoprofen-loaded microparticle release profiles using the independent model values of dissolution efficiency DE (*one-way ANOVA* followed by **Tukey's test**).

Formulation	Temp. (°C)	Type of polymer	pH	Dissolution efficiency, DE ₂₄₀ mean (n=2)*
F1	25	PNVCL	1.2	18.47 ± 8.15
F3	37	PNVCL	1.2	5.12 ± 0.09
F5	25	PNVCL	7.4	62.86 ± 6.32
F7	37	PNVCL	7.4	19.89 ± 0.27
F2	25	Poly (NVCL-co-AA)	1.2	11.51 ± 5.87
F4	37	Poly (NVCL-co-AA)	1.2	2.04 ± 0.30
F6	25	Poly (NVCL-co-AA)	7.4	42.64 ± 3.08
F8	37	Poly (NVCL-co-AA)	7.4	10.83 ± 1.71
Unprocessed ketoprofen crystals	37	-	7.4	71.62 ± 0.76
	25	-	7.4	62.58 ± 18.70
	37	-	1.2	58.78 ± 7.11
	25	-	1.2	47.57 ± 22.60

* Values represent the mean of two independent determinations. The analysis of variance (ANOVA) revealed statistical differences ($p < 0.05$) between the formulations. To evaluate these differences, a post hoc Tukey test was performed on the results of ANOVA. . Same letters at the same column indicate that there is no statistical difference ($p < 0.05$).

Table 4

Results of curve fitting and kinetic analysis of ketoprofen release data from the spray-dried PNVCL-based microparticles using the **Korshmeier-Peppas** model.

Curve fitting parameters	25°C				37°C			
	PNVCL	PNVCL	Poly(N VCL-co-AA)	Poly(NV CL-co-AA)	PNVCL	PNVCL	Poly(N VCL-co-AA)	Poly(NVCL-co-AA)
	pH 1.2	pH 7.4	pH 1.2	pH 7.4	pH 1.2	pH 7.4	pH 1.2	pH 7.4
R ²	0.9791	0.9971	0.9822	0.9862	0.9853	0.9957	0.9737	0.9814
RMSE	1.07	5.31	1.83	6.99	0.27	1.37	0.204	1.93
AIC	35.84	47.35	41.35	80.50	2.46	41.40	-4.33	49.63
K(min ⁻¹)	4.51	7.13	1.21	0.73	0.50	1.11	0.49	0.54
n	0.30	0.54	0.48	0.81	0.47	0.61	0.31	0.63

Table 5

Kinetic analysis of ketoprofen release data from the spray-dried PNVCL-based microparticles using the **Korshmeier-Peppas** model.

In vitro study conditions		Ketoprofen PNVCL		Ketoprofen : Poly (NVCL-co-AA)	
pH	Temp (°C)	K(min ⁻¹)	n	K(min ⁻¹)	n
1.2	25	4.51	0.30	1.21	0.48
1.2	37	0.50	0.47	0.49	0.31
7.4	25	7.13	0.54	0.73	0.81
7.4	37	1.11	0.61	0.54	0.63