Ibuprofen loaded Chitosan Films: *in Vitro* Assessment of Drug Release Profile and Cell Viability on Primary Neurons Culture

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Abstract: *The success of a drug delivery system relies heavily on its interaction with cells from the target tissue. The range of applications for ibuprofen-loaded chitosan (ICH) films is widening, mainly due to the biodegradability of chitosan (CH) films and ibuprofen's safety and versatility, with a particular interest in exploring it as a neural drug delivery system. In this study, CH and 12% (w/w) ICH films were prepared through the solvent cast and characterized regarding their physicochemical composition, surface and bulk morphology, drug release profile, and cell viability of primary neurons from the rat spinal cord. Fourier transform infrared spectroscopy (FTIR) analyses demonstrated that both groups had a similar composition. According to scanning electron microscopy (SEM) images, ibuprofen particles were entrapped on the surface and inside the polymeric matrix. In vitro drug release profile indicated that release starts as diffusion within the first hours, is best fitted by the Higuchi model, and continues for at least 30 days, in agreement with the Korsmeyer-Peppas model. Therefore, ibuprofen is first released through the diffusion process of the particles found on the surface and later through a combination of diffusion and erosion of the chitosan matrix. Regarding in vitro cell viability of primary neurons, CH and ICH extracts are non-toxic, as both groups displayed cell viability over 50%. ICH films are mildly reactive in neuronal cells but do not cause severe cell death i.e., it allowed non-cytotoxic neuronal and glial differentiation. These findings enhanced our understanding of ICH films as a safe neural drug release system to be explored.*

Keywords: *Biomaterial, Chitosan, Ibuprofen, Drug Delivery System, Primary Neurons.*

1. INTRODUCTION

The development of drug delivery systems (DDS) helps to provide alternative administration routes for well-described drugs. This expands their applications and offers new treatment options for several conditions [1]. The success of a DDS relies heavily on its interaction with cells from the target tissue. Therefore, *in vitro* studies need to take the target system into account in their design [2].

Ibuprofen (IBU) is a long researched, non-steroidal anti-inflammatory drug with analgesic properties, usually orally administered [3]. However, standard oral administration comes with downsides, such as loss of drug availability [4], and gastrointestinal ulcers after prolonged use [5]. The delivery of IBU through DDSs not only avoids these side effects but allows for new applications for the drug. Recently, IBU has been associated with neuroprotective effects in Alzheimer's [6] and Parkinson's [7] disease and also has been shown to contribute to the control of neuroinflammation post-spinal cord injury (SCI) by inhibiting the RhoA pathway, a cell pathway cascade involved in axonal growth inhibition and cell apoptosis following SCI [8–10]. Such innovative purposes for IBU benefit from its delivery through a DDS, as systemic administrations would not guarantee the necessary dosages for IBU to be effective in the nervous system [11].

Chitosan (CH) is a polysaccharide that has been widely explored as a DDS because of its biodegradability, low toxicity, and capacity to entrap biomolecules before cross-linking [12]. CH films have been applied as a system for the release of anti-inflammatory drugs [13],

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antibiotics [14], and gene delivery [15]. When compared to other biopolymers, CH stands out as it is the only cationic biopolymer, and the availability of amino groups facilitates the interaction with hydrophobic anionic drugs, such as IBU [16].

Both IBU and CH had their use on neural cells individually evaluated [17–19], but to our knowledge, there is no report of both combined as a DDS for such application. Considering the importance of DDS-cell interaction, this study evaluated the drug release profile and cell viability of IBU-loaded chitosan (ICH) films in primary neurons from rats' spinal cords. We hypothesize that ICH films would be an effective and non-cytotoxic drug delivery system for neural cells.

2. MATERIALS AND METHODS

2.1. Materials

Analytical grade acetic acid was purchased from Synth (Diadema, Brazil). Highly purified chitosan powder (Medium Molecular Weight) with 75–85% degree of deacetylation, IBU sodium salt powder (98% purity), Tripolyphosphate (TPP) (technical grade– 85% purity), fetal bovine serum, antibiotic solution (penicillin-streptomycin), MTT [3- (4, 5- dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium], propidium iodide (PI); 4', 6- Diamidine- 2';-phenylindole dihydrochloride (DAPI) were purchased from Sigma Aldrich (St Louis, USA).

2.2. Fabrication of CH and ICH films

CH powder was dissolved in an aqueous acetic acid solution (1% v/v) to form a 2.5% (w/v) solution. For ICH films, IBU was added at a 40% (w/w) ratio to chitosan [20, 21]. The solutions were stirred until homogenization. 45 mL of each solution was spread on glass Petri dishes (95 mm in diameter) and stored at 40ºC for solvent evaporation. Samples were immersed in an aqueous TPP solution (1% m/v, pH 9.0) at 4ºC for 30 minutes for cross-linking [22]. Finally, the films were rinsed with distilled water, dried at 37ºC for 48 hours, and stored in a vacuum desiccator (10 kPa).

2.3. Morphological and Physicochemical Characterization

The film surfaces were analyzed by scanning electron microscopy (SEM) (Quanta 250 SEM-FEI- Oregon, USA). The samples were coated with Au (Leica EM ACE200, Wetzlar, Germany) [5]. To confirm the encapsulation of the drug, CH and ICH samples were cryogenically fractured and cross-section images were acquired by SEM. To characterize the composition of CH and ICH films, attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) was employed using a frontier spectrophotometer (PerkinElmer, Waltham, USA), with 60 scans, 0.4 cm^{-1} resolution, within 6000– 450 cm^{-1} spectra region.

2.4. Drug Loading Content and In Vitro Drug Release Profile

The amount of drug incorporated in ICH films was quantified by soaking and stirring in PBS a previously weighted sample until complete drug removal. The UV-VIS absorbance was measured using a Lambda 25 spectrophotometer (PerkinElmer, Waltham, USA) at the wavelength of 221 nm, and compared against a calibration curve [21, 23]. All subsequent measures were based on this initial value.

For the *in vitro* drug release profile, previously weighted ICH films were immersed in 20 mL of PBS (pH 7.4), at 37ºC and slightly stirred. The release medium was collected at predefined times and replaced with fresh media to maintain the original volume [24]. The cumulative release of IBU was evaluated.

The curve of IBU released from ICH films was fitted into four mathematical models: zero and first-order kinetics, Higuchi, Hixson–Crowell, and Korsmeyer–Peppas. The coefficient of each mathematical model (R^2) was used to evaluate the mechanism of drug release [22, 24].

2.5. Primary Neuronal Cell Culture from Rat Spinal Cord

Primary neuronal cell cultures from Wistar rats, male and female, with 0-3 days postnatal, were used for cytotoxicity analyses. The spinal cord tissue was mechanically dissociated with a scalpel blade and enzymatically dissociated using a 0.15% trypsin solution at 37°C for 10 min. Trypsin action was inhibited by the addition of a Neurobasal medium containing 10% fetal bovine serum. Cells were centrifuged for 4 min at 1510 rpm. The supernatant cells were seeded into a 24-well plate at a density of 2.5 x10^4 cells/well in cell

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growth medium (neurobasal media supplemented with B27, 10% fetal bovine serum, and 1% antibiotic) and incubated for 14 days [25].

2.6. MTT assay

Cell viability of CH and ICH films was evaluated using the standard MTT assay by an extract method [26]. Samples were incubated in a cell-free growth medium for 24 h.

 Negative control (sterile, pure cell growth medium) was adopted to validate the results. Cells were kept in the extract for 24 h, and the MTT solution replaced the medium for 2 h of incubation. Then, the medium was removed, and 300 µL of DMSO was added to each well to dissolve purple formazan crystals. The absorbance of the solution was measured at 570 nm. Aliquots of negative control were assumed as 100% of metabolic activities. Cell Viability was calculated using Equation (1):

$$Cell\ Viability\ (\%) = {OD_s}/{OD_c} * 100 \qquad (1)$$

where OD_s and OD_c are the optical density of the sample and the negative control, respectively.

2.7. Propidium Iodide (PI) Assay

PI labeling was used to quantify the percentage of dead and alive cells. After 24 h of incubation with the extract, 250 µL of PI was added to each well, and the cell culture was incubated for 2 h. Cells were washed twice with PBS and fixed with 300 µL of paraformaldehyde 4%. Cell culture was counterstained with DAPI, washed with PBS, and observed in fluorescent microscopy (Leica, Wetzlar, Germany). Viable neurons were blue-stained and counted using ImageJ software (Bethesda, USA) cell counter plugin. The same statistical analysis procedure previously described was applied.

3. RESULTS

Drug dispersion and its interaction with the polymeric matrix have an important effect on release kinetics. Figure 1 shows the surface and bulk morphology of CH and ICH samples. CH films (Figures 1a and 1c) had their surface and interior with a smooth aspect (stretches were caused by cryofracture), but the addition of IBU resulted in ICH films with rougher surfaces and well-dispersed particles (Figures 1b and 1d). Figure 2 presents the ATR-FTIR spectra of samples. The chemical structure of each component is shown next to the spectrum for illustration purposes, with their main reactive site highlighted.
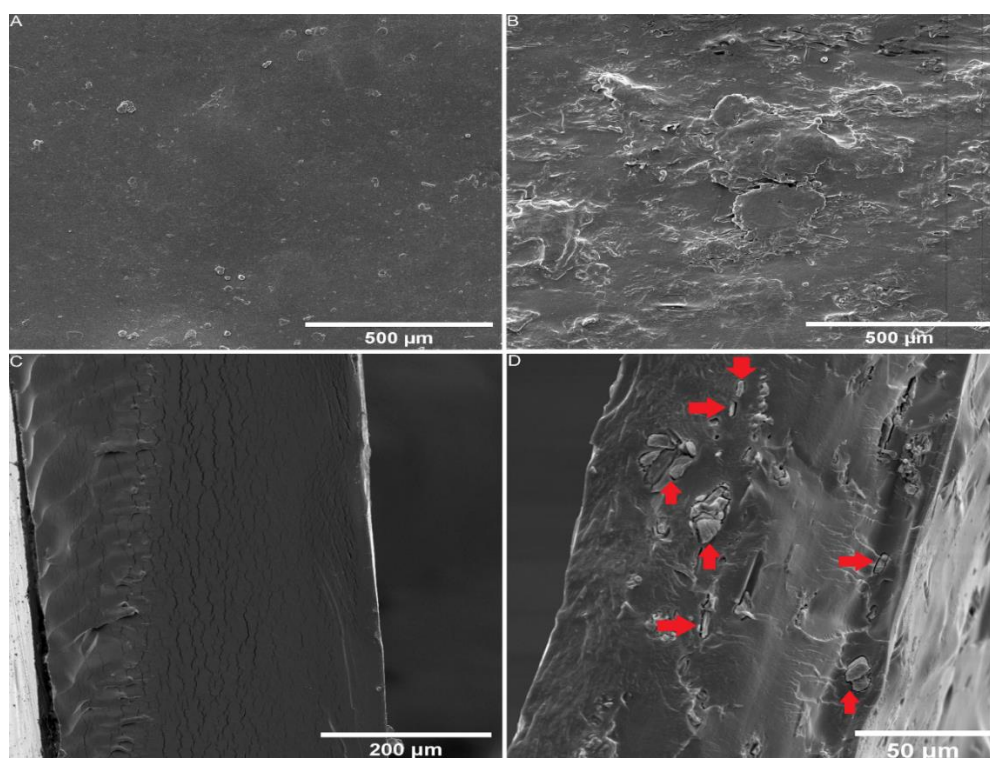
Fig. 1. SEM micrographs of: (a) CH, (b) ICH film, (c) cross-section of CH, (d) cross-section of ICH. Red arrows indicate the IBU particles entrapped inside the chitosan matrix.

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Fig. 2. Average of Fourier transform infrared spectroscopy (FTIR) spectra: (a) IBU, (b) CH and (c) ICH. Chemical structures were adapted from the material's specification sheet provided by the manufacturer.

Comparisons between spectra (a) and (c) indicate that IBU asymmetrical carbonyl group (C=O) shifted from a sharp peak at 1545 cm^{-1} to a less intense peak at 1535 cm^{-1}. Likewise, IBU aromatic ring (C=C) vibration that was identified at 1413 cm^{-1} on the spectrum 2(a) shifts to 1410 cm^{-1} on the spectrum 2(c) and is less apparent [30, 37].

The spectra presented in Figure 2(c) show that IBU addition did not cause severe differences in the chitosan matrix. Therefore, CH physicochemical properties, which have already been confirmed as a safe material for drug delivery systems [17–19, 27], are likely to remain unchanged. Moreover, the comparison between spectra 2(b) and 2(c) shows that chitosan's peak at 1625 cm^{-1} is also less intense, further indicating the ionic complexation between the free amino and carboxyl groups [23, 28].

Finally, TPP cross-linking in both CH and ICH films was identified by the presence of the P=O peak at 1150 cm^{-1}, related to the presence of phosphoric ions [29].

ICH films were found to have a maximum concentration of 12% (w/w) in the dispenser, as the TPP crosslinking and water rinsing steps caused the removal of non-adhered IBU particles [30]. The percentages of drugs released from the IBU-loaded chitosan films are displayed in Figure 3, which shows that a burst effect was initially observed, and a subsequent slow sustained release in the following days. 45% of its original content was released within the first 24 h of incubation, as presented in the close-up, and cumulative release reached 83% within a month.

Microscopic analysis was conducted to support this data, therefore micrographs of ICH films after 24 h and 15 days of immersion in PBS were present in Figure 4. After 24 h (Figures 4a and 4b), several IBU particles were still on the surface and inside the CH matrix. However, after 15 days (Figures 4c and 4d), no particles were observed, as 70% of the drug-loaded content had already been released (Figure 3). The increase observed from the 15th to the 30th day was likely due to the CH matrix's surface leaching, which exposed and released IBU particles that were initially encrusted within the film.

Fig. 3. Ibuprofen's cumulative release from the ICH. Data shows the absorbance and concentration for each sample ± standard error. In detail: Cumulative release of ibuprofen from ICH in the first 24 hours.

Fig. 4. SEM images of ICH after drug release. Representative images of (a) Surface and (b) cross-section after 24 hours of immersion in PBS. (c) surface, and (d) cross-section after 15 days of immersion in PBS.

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The drug release profile (DRP) was determined by fitting the curves on distinct models and comparing the determination coefficients (R^2). Table 1 shows the R^2 values for the curves associated with 24 h and 30 days of *in vitro* release.

Table 1. Determination coefficients (R^2) for different drug release systems

Model	R^2	
	24 h	30 days
Zero-Order	0.81	0.55
First-Order	0.86	0.82
Higuchi	0.95	0.78
Korsmeyer-Peppas	0.70	0.98
Hixon	0.84	0.51

Based on R^2 values, DRP starts by diffusion of the superficial particles, following the Higuchi model, and is later combined with the erosion-oriented release, as described by the Korsmeyer-Peppas model [5, 16, 20].

The effect of IBU-loaded chitosan films on neurons from the rat spinal cord was evaluated through assessments of cell viability and the types of neuronal cells found in the culture. Regarding MTT assay, the percentage of viable cells cultured on the extracts obtained from CH and ICH films can be observed in Figure 5a. Although ICH films showed a significant decrease in cell viability it is still considered non-cytotoxic because both the ibuprofen loaded in ICH and therefore, the maximum concentration in the dispenser (12% m/m) were below inhibitory concentration (IC50) [31–33]. Dead/alive assay revealed a similar behavior, as presented in Figure 5b. Figures 5c-e provide PI images counterstained with DAPI, where red nuclei indicate apoptotic cells. These findings indicate that CH and ICH extracts are non-toxic, as both groups displayed cell viability over 50% [26, 34].

Statistical data analyses were conducted using an unpaired T-test with Welch's correction (does not assume that both groups have the same standard deviation) to establish differences between CH and ICH samples at a significance level of 5% ($p < 0.05$). For the MTT analysis, ICH was only statistically different from the control (p-value of 0.0005). It did not present a significant difference from CH (p-value of 0.4431), nor was CH statistically different from control (p-value of 0.8138).

Fig. 5. Cell viability of spinal cord cells cultured on extracts of sterile culture media (control), CH and ICH. A: MTT analysis. B: Propidium iodide (PI) assay. Data represent the mean for each group and ± standard error (below 1%). Representative images of PI labeling counterstained with DAPI: C – control (sterile culture media), D – CH sample, and E – ICH sample. The asterisk indicates that values obtained from ICH films were statistically different from control. Scale bar = 50 μm.

For the dead/alive assay, there wasn't a significant difference between ICH and CH (p-value of 0.3780), nor between ICH and control (p-value of 0.9932) or CH and control (p-value of 0.4143).

4. DISCUSSION

This study demonstrates that ibuprofen-loaded chitosan is a suitable material for drug delivery in neuron-based systems. In the specific context of neural applications, IBU has been associated with neuroprotective effects [7], axonal regrowth [8], and control of neuroinflammation [10, 35]. Chitosan was selected as a matrix material due to its previous success as a drug delivery system [5, 36], and biocompatibility with neural cells [9, 19]. IBU dispersed throughout the chitosan film and its chemical carboxylic groups interacted electrostatically with the free chitosan amino groups. The loss of peak intensity in FTIR analysis is associated with an electrostatic interaction. In acidic media, chitosan's amino groups were protonated and, therefore, positively charged and available to interact with IBU sodium salt's negatively charged sodium-carboxylate ion, which transforms into an ammonium-carboxylate ion pair, indicating the electrostatic nature of the IBU-chitosan interaction [28]. The conjugation between chitosan's free amino groups and IBU carboxylate groups leaves fewer characteristic groups free to absorb the IR spectrum, leading to lower absorbance values [24, 28, 37]. In addition, the presence of phosphoric ions confirmed the crosslinking process effectively.

A burst effect was observed in the initial hours of IBU *in vitro* release from films. The release was sustained for 30 days. The driving force behind the burst effect observed in the first 24h of IBU release was the drug concentration gradient. The release was mostly slow and steady, mainly because IBU was well distributed in the polymeric matrix (Figure 1). In a previous study, Puttipipatkhachorn indicated that sustained drug release from chitosan films was especially credited to drug-polymer interaction [20]. By adding IBU directly into the chitosan solution, the drug and polymer could interact without interference from additional solvents that could also leave residues in the final material [16,20,38,39]. The sustained release is also explained by the high drug loading content [30]. After all the surface particles were removed, there

were still the ones entrapped inside the matrix, which started to diffuse through it (Figure 4), causing another increase in the release rate from 15 to 30 days [23, 30, 38]. Although the mass percentage found in the dispenser (12% w/w) was not the full drug amount added to the ICH (40% w/w), it was similar to values found by Pang *et al.* [24, 30].

The drug release profile is crucial for anti-inflammatory drug release because most inflammatory peaks occur in the first 24 h after injury. Therefore, drugs that aim to lessen the damaging effects of the inflammatory response must act within this period. Furthermore, IBU prolonged administration at the lesion site has been associated with controlled inflammation, tissue protection, and axonal myelination [40]. In this way, ICH films demonstrated an adequate DRP.

Although ICH films caused a mild reaction in neuronal cells, they allowed non-cytotoxic neuronal and glial differentiation. ICH films were considered non-toxic but did display a mild reaction [26, 32, 33]. As chitosan did not contribute to cell death (Figure 5a and 5b), one possible explanation for this reduced viability could be the IBU dosage used in this study. IBU concentrations up to 200 μM are reported to be safe in cellular and animal models, with progressive cytotoxic behavior in increasing concentrations [32, 41]. However, it is important to point out that available studies were performed on other cell types, and this is the first study of primary neurons cultured on ICH extracts.

As IBU has low solubility in acidic media (pKa 4.4) [42], a high amount of drug-loaded was necessary to guarantee a sustained drug release profile throughout the 30 days immersion [29]. However, according to dead/alive assessment (Figure 5b), ICH extracts did not induce a severe apoptotic effect. These two results together are an indication that the 12% (w/w) IBU concentration of ICH films might have affected the mitochondrial metabolism of the spinal cord neurons, however not to the point of causing acute cell death. As primary neuron cells do not represent the complete *in vivo* conditions, and the *in vitro* viability evaluation was conducted in a static method, it can thus be suggested that it is more damaging to cell cultures than it would be to a natural neural tissue in a clinical application [41].

Fig. 6. Graphical Abstract

5. CONCLUSIONS

This study confirmed that IBU release from chitosan matrixes occurs mainly by diffusion, but erosion-oriented release appears later on prolonged release. It was also the first time that primary neurons from the rat spinal cord were cultured on extracts of IBU-loaded chitosan films. Cell viability tests showed that a 12% (w/w) concentration of IBU-loaded chitosan films caused mild reactivity in primary neurons, but not to the point of provoking severe apoptotic effect. This concentration is higher than most previously indicated in the literature and might guide further studies in balancing drug concentration to balance sustained IBU release and improved cell compatibility.

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