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Effect of $CaCl_2$ Crosslinker Concentration On The Characteristics, Release and Stability of Ciprofloxacin HCl-Alginate-Carrageenan Microspheres

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Abstract

***Background**: Ciprofloxacin HCl is a broad-spectrum fluoroquinolone antibiotic that has the lowest MIC against Mycobacterium tuberculosis but has limitations in oral use, so inhalation microspheres are made. **Objective**: This study aimed to investigate the effect of $CaCl_2$ crosslinker concentration on the characteristics, release and stability of ciprofloxacin-alginate-carrageenan microspheres. **Methods**: Microspheres were prepared by ionotropic gelation using aerosolization with calcium chloride 0.5M (F1), 1.0M (F2), 1.5M (F3), 2.0M (F4) as crosslinker and then dried using freeze dryer. **Results**: Ciprofloxacin-alginate-carrageenan microspheres formed of yellowish-white powder, smooth morphology and excellent flow properties with the particle size of less than 5µm, drug loading and entrapment efficiency were between 2.05% - 2.42% and 75.34% - 98.09%, yield was between 84.69% - 97.57%, moisture content of less than 10%. Ciprofloxacin-alginate-carrageenan microspheres with 1.5M crosslinker (F3) was the optimal formula. For 12 hours, ciprofloxacin released was 49.89% - 63.78% at pH 7.4, and the kinetics of drug release showed that of Korsmeyer-peppas with a mechanism based on fickian diffusion. The microspheres were discovered to be stable for up to 28 days of storage. **Conclusion**: The increased concentration of the CaCl2 crosslinker from 0.5M to 2.0M decreased the particle size and drug release but increased the yield, drug loading and entrapment efficiency.*

***Keywords**: calcium chloride, characteristics, ciprofloxacin microspheres, release, stability*

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INTRODUCTION

Ciprofloxacin HCl is a broad-spectrum fluoroquinolone antibiotic that inhibits the bacterial DNA replication enzymes DNA-gyrase and topoisomerase IV (Thai *et al*., 2022). Ciprofloxacin has the lowest minimum inhibitory concentration (MIC) against Mycobacterium tuberculosis, which ranges from 0.125 to 2.0 µg/mL (Heifetst & Lindholm-levy, 1987). Ciprofloxacin for oral use has a short half-life of 3-5 hours (Katzung *et al*., 2012). Around 70% of the Ciprofloxacin HCl is absorbed after oral administration. Ciprofloxacin is then metabolized in the liver by 15% and then excreted by 40-50% of the oral dose (Bayer Health Care Pharmaceuticals Inc, 2021). Thus, the delivery system needs to change the route or modify the release to increase the efficacy of ciprofloxacin. Inhalation delivery systems to target the lungs have advantages such as the ability to target alveolar macrophages containing TB bacilli, high drug concentrations in lung tissue maintained, avoidance first-pass metabolism, faster onset of action and smaller doses compared to intravenous and oral administration and ensure limited systemic side effects (Misra *et al*., 2011). Inhalation delivery systems to target the lungs can be achieved by microencapsulation methods that can achieve targets and control drug release (Santa-maria *et al*., 2012). The dosage form with microspheres is needed for delivery of the lungs so that the drug design is encapsulated with microparticle technology (Lengyel *et al*., 2019).

Microspheres have a small diameter of 1 µm to 1000 µm with spherical particles consisting of drugs, proteins or synthetic polymers (Kadam & Suvarna, 2015). For the inhalation route, the particle size ranges from 1 µm to 5 µm for lung deposition (Gaber *et al*., 2021). One of the things that affect the effectiveness of microspheres as a drug delivery system is the selection of polymers and crosslinkers used as matrices and crosslinking of microspheres (Lee *et al*., 2012). The type of polymer used can be derived from nature or synthetic, but natural polymers have the advantages of being cheap, readily to use, biocompatible, biodegradable and able to do a multitude of chemical modifications (Rajeswari *et al*., 2017). A combination of alginate and carrageenan polymer was selected in this research because it is a natural polymer that is biodegradable, economical, widely used for encapsulation (Hariyadi *et al*., 2019) not accumulating in organs and not toxic in its administration (Hariyadi *et al*., 2019). Alginate is a naturally occurring polysaccharide polymer that is generally found in the cell walls of brown algae (Phaeophyceae) species (Lee & Mooney, 2012). Alginate provides the highest mucoadhesive ability compared to other polymers (Adrian *et al*., 2019). However, alginate has poor mechanical properties and a porous structure, which causes the microspheres to burst release, so it needs to be combined with carrageenan, which is capable of providing sustained release (Abdelghany *et al*., 2017). Carrageenans is a natural polysaccharide polymer found in the family Rhodophyceae of marine red algae, and kappa carrageenan is one of the carrageenan types which has many advantages (Tecante & Núñez, 2012).

Crosslinkers in biodegradable polymers are very important in controlling the swelling and degradation rate of the microspheres. Ca^{2+} ions are most preferred for developing microparticles compared to Rb^{+}, Cs^{+}, K^{+}, Na^{+}, Li^{+}, Ba^{2+}, Sr^{2+}, and Mg^{2+}, because Ca^{2+} is the safest for the body (Tecante & Núñez, 2012). The use of $CaCl_2$ crosslinker in alginate and carrageenan polymers is used because sodium alginate has a high carboxyl group content and a high affinity for bivalent cations such as Ca^{2+} ions, whereas kappa carrageenan and Ca^{2+} form crosslinks due to electrostatic attraction forces. Concurrently, neighbouring Ca^{2+} and OSO3 generate a network of crosslinks between macromolecular networks (Yu *et al*., 2019). The combination of alginate and carrageenan polymer with a ratio of 1:1 with $CaCl_2$ crosslinkers has an increased swelling degree at neutral pH. It shows the most optimal encapsulation efficiency with a release degree of 92% so this ratio was chosen to provide a maximum therapeutic effect (Kolesnyk & Burban, 2015). The combination of alginate and carrageenan polymer as a microsphere matrix with a ratio (1:1) at a concentration of 0.9% and a 0.5M $CaCl_2$ crosslinker obtained optimum microsphere characteristics but still had low drug loading and encapsulation efficiency and an increase in polymer concentration above a concentration of 0.9% was thought to be cannot be stirred and forms a matrix of microspheres, so it is suggested to increase drug loading and encapsulation efficiency of these microspheres (Wijaksana, 2022). Microspheres produced with increased crosslinking concentrations resulted in small particle size, high entrapment efficiency, and drug loading (Hariyadi *et al*., 2014).

Several methods can prepare microspheres utilising a combination of alginate and carrageenan polymer with calcium chloride crosslinker. This study used the ionotropic gelation method with aerosolization technique because this method produces microspheres chemically based on the ability of polyelectrolytes to

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cross over the ion counter to form a gel (Hariyadi *et al*., 2019). The advantages of this method that it is easy, fast, relatively inexpensive, does not use organic solvents, and uses low temperature, thereby reducing drug damage (Pasquale *et al*., 2021), and aerosolization techniques can produce small and uniform particle sizes (Hariyadi *et al*., 2018). In the process of making microspheres, drying is required, paying attention to the stability of the active pharmaceutical ingredients. Drying is carried out using the freeze-drying technique and needs to be added with lyoprotectant (Laura *et al*., 2010). The lyoprotectant used in this study was maltodextrin (Hariyadi *et al*., 2016). This study will produce ciprofloxacin HCl-alginate-carrageenan microspheres with various concentrations of CaCl2 crosslinker, which are made using ionotropic gelation technique by aerosolization method to investigate the effect of $CaCl_2$ crosslinker concentration on the characteristics, release and stability of ciprofloxacin-alginate-carrageenan microspheres. Physical characteristics include organoleptic, morphology, particle sizes, drug loading, entrapment efficiency, yield, moisture content and flow properties.

MATERIALS AND METHODS

Materials

Ciprofloxacin HCl (Zhejiang Ltd., China), Sodium Alginate (Sigma Aldrich, USA), Kappa-Carrageenan (KCN, Pasuruan, Indonesia), CaCl2.2H2O (PT.Sumber Utama Kimia Murni, Indonesia), Maltodextrin (Bratachem Chemistry, Indonesia), Aquadestillata (PT.Sumber Utama Kimia Murni, Indonesia), Phosphate Buffered Saline Tablet (Oxoid, England). NaH_2PO_4 (CV. CIMS, Indonesia), KH_2PO_4 (CV. CIMS, Indonesia), NaCl (CV. CIMS, Indonesia), KCl (CV. CIMS, Indonesia). All composition and reagent use pharmaceutical grade.

Tools

UV-Vis spectrophotometer (Shimadzu UV 1800 spectrophotometer, Japan), FT-IR spectrophotometer (Perkin Elmer Instrument, USA), Differential Scanning Calorimeter/DSC (Mettler Toledo FT 900 Thermal System, USA), Analytical balance (Chyo Balance corporation Kyoto, Japan), Spray aerosol, Freeze dryer (Eyela FD-81, Japan), optical microscope (PS AIR FI 31-032, China), stirring plate (Dragon Lab MSPro, China), centrifuge (Rotofix 32, Germany), Scanning Electronic Microscope (JEOL, Japan), Climatic chamber, Moisture analyzer (Mettler Toledo HB43-S, USA) and Thermoshakers (Gerhardt, Germany).

Method

Preparation of ciprofloxacin-alginate-carrageenan microspheres

Microspheres were made by applying the ionotropic gelation method with aerosolization techniques, with a 0.2% drug concentration. The drug was dissolved into a combination of alginate carrageenan polymer solution with a ratio of 1:1 (w/w) at a concentration of 0.9% of each polymer. The ciprofloxacin-alginate-carrageenan solution was sprayed into various $CaCl_2$ cross-agent solutions with different concentrations, namely 0.5M, 1.0M, 1.5M, 2.0M at 40 psi and stirred continuously for 2 hours at a speed of 1000 rpm. The microsphere was collected by centrifugation at 2500 rpm for 6 minutes, followed by two washes with distilled water. The microspheres was resuspended in 5% maltodextrin solution and then dried with a freeze dryer at -80ºC until the humidity met the requirements (Hariyadi *et al.*, 2022). Furthermore, ciprofloxacin-alginate-carrageenan microspheres that form were evaluated. The formula of microspheres can be seen in Table 1.

FTIR spectroscopic analysis

FTIR spectroscopy is a crucial analytical method that identifies several distinctive functional groups in molecules of any substance. FTIR spectra of all raw materials and microsphere formulations were obtained using an FT-IR (Perkin Elmer Instrument) spectrophotometer. The FTIR band spectra were examined to investigate the structural integrity in the different formulations after scanning at wavenumbers 4000 - 500 cm^{-1}.

DSC analysis

The thermal properties of all raw materials were determined using a DSC TGA (Mettler Toledo FP 900). Samples of about 5mg of all formulas were scanned (from 30°C to 250°C) at a heating rate of 10°C/min. The characteristic of the prominent DSC peak, which represents the endothermic peak shown by temperature, was determined.

Table 1. Formula of ciprofloxacin-alginate-carrageenan microspheres

Materials	Functions	F1	F2	F3	F4
Ciprofloxacin HCl	API	0.2%	0.2%	0.2%	0.2%
Sodium alginate	Polymer	0.9%	0.9%	0.9%	0.9%
Kappa carrageenn	Polymer	0.9%	0.9%	0.9%	0.9%
Calcium chloride	Crosslinker	0.5M	1.0M	1.5M	2.0M

*drying with freeze dry added with 5% maltodextrin

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Physical characterization of microspheres

Organoleptics

All formulas of ciprofloxacin-alginate-carrageenan microsphere were observed for their organoleptic appearance, including shape and colour.

Morphology

Morphology was evaluated using Scanning Electron Microscopy (SEM). The gold-coated samples were mounted for 120 seconds and were then examined with an SEM microscope (JEOL) at a working distance of 10 mm and 15mm, with 15 kV beam energy.

Particle size

Microspheres of 300 particles were determined using an optical microscope (PS AIR FI 31-032) and Image Raster Software. The average diameter was calculated using the equation:

$$Diameter\ average = \frac{\sum nd}{\sum n}$$

n = a number of microspheres observed

d = size of the microspheres (Dhakar *et al*., 2010).

Drug loading and entrapment efficiency

50 mg of microsphere was added to Citrate Buffer, which has a pH of 4.4 and was stirred for 7 hours at 1000rpm. The solution was filtered, and then a UV-Vis spectrophotometer was used to measure the absorbance of the solution at a wavelength of 275 nm (taken from in-house validation). The amount of the drug was determined using a validated in-house standard calibrated plot. The drug loading and entrapment efficiency was calculated using the equation (Balagani *et al*., 2011):

$$Drug\ loading = \frac{Weight\ of\ drug}{Weight\ of\ microspheres}\ x\ 100\%$$

$$EE = \frac{Drug\ content\ in\ microspheres}{Theoritical\ drug\ content\ in\ formula} x100\%$$

Yield

The yield was calculated according to the total recoverable final weight of microparticles to evaluate efficiency in producing microspheres. The yield was calculated using the equation (Balagani *et al*., 2011):

$$Yield = \frac{Weight\ of\ dry\ microspheres}{Total\ weight\ materials}\ x\ 100\%$$

Moisture content

Moisture content was measured using the Moisture Analyzer (Mettler Toledo HB43-S). The microspheres are weighed with a minimum weight of 500 mg then distributed evenly in the pan and the sample is burned and the moisture content is measured in the range of 100-140°C (Hariyadi *et al*., 2019).

Flow properties

A graduated cylinder of 10 mL was used to measure bulk and tapped densities. The sample was mechanically tapped 500 times after being put into a cylinder. The tapped volume was recorded, and the bulk density and tapped density were calculated (Lane 2016).

Bulk density

The ratio of powder weight bulk density compared to the initial volume of the untapped powder, as below equation (Lane 2016):

$$\rho\ bulk = \frac{Powder\ weight\ (g)}{Initial\ volume\ of\ powder\ (ml)}$$

Tapped density

Tapped density was determined by the ratio of powder weight compared to final volume of the tapped powder as below equation (Lane 2016):

$$\rho\ tapp = \frac{Powder\ weight\ (g)}{Volume\ of\ powder\ after\ tapping\ (ml)}$$

$$Carr's\ index = \frac{\rho\ tapped - \rho\ bulk}{\rho\ bulk}\ x\ 100\%$$

$$Hausner\ ratio = \frac{Tapped\ density}{Bulk\ density}$$

Parameters of Carr's index and Hausner's ratio are shown in Table 2.

Table 2. Carr's index and Hausner ratio of powder flow

Flow character	Carr's index (%)	Hausner ratio
Excellent	<10	1.00-1.11
Good	11-15	1.12-1.18
Fair	16-20	1.19-1.25
Passable	21-25	1.26-1.34
Poor	26-31	1.35-1.45
Very poor	32-37	1.46-1.59
Very, very poor	>38	>1.60

In vitro release study

Ciprofloxacin release from the microspheres was tested in phosphate-buffered saline (PBS) at a pH level of 7.4 (Karimi *et al*., 2016). The released test was carried out using a Thermoshaker (Gerhardt) at 37ºC with 100 rpm. 500 mg of microspheres were added to 100 mL PBS solution (pH 7.4) on a Thermoshaker at 37 ± 0.5°C and stirred at 100 rpm. A snippet of samples (5.0 mL) was taken at the minutes of 0, 15, 30, 60, 90, 120, 180, 240, 360, 480, 600 and 720 minutes. In each snippet of samples, the same volume of release media was replaced, and the snippet of samples was filtered using millipore filter paper 0.45 µm. The sample absorbance was observed with a UV-Vis spectrophotometer (Shimadzu UV-1800) at the wavelength of 268 nm (Hariyadi *et al*., 2019).

Drug release kinetics

The drug release kinetics were investigated to describe the drug release pattern. The mechanism of

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drug release from microspheres was examined using various kinetic equations, such as zero-order kinetic, first-order kinetic, Korsmeyer-peppas and Higuchi (Bruschi, 2015). The release kinetics of a drug can be determined from the price of R^2 from the linear regression equation obtained from each formula. If R^2 approached one, it could be assumed that the kinetic followed the release of the regression equation from the corresponding kinetics model.

Stability test

Ciprofloxacin-alginate-carrageenan microspheres were tested for accelerated stability. The microspheres were placed into a vial and stored for 28 days at room 25 ± 2˚C and 40 ± 2˚C, RH 75 ± 5%. The stability of the prepared formulations was tested by organoleptic property, moisture content and drug loading (Hariyadi & Hendradi, 2020).

Data analysis

All data are presented in mean ± SD. For physical characteristics and drug release, the statistical data was analyzed using one-way analysis of variance with a confidence degree of 95% ($\alpha = 0.05$). For stability test, the statistical data was analyzed using two-way analysis of variance with a confidence degree of 95% ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Characterization of microspheres

This study investigated the effect of $CaCl_2$ crosslinker concentration on the formation and physical characterization of ciprofloxacin microspheres using a combination of alginate and carrageenan polymers produced by ionotropic gelation methods using aerosolization techniques. Result of characterization by FTIR spectra and analysis of F1 to F4 were presented in Table 3. The functional groups of ciprofloxacin HCl, sodium alginate, kappa carrageenan, all microsphere formulas, and the possibility of wavenumber presence or shifting were investigated.. Referring to the ciprofloxacin, the wavenumber of the characteristic peak of quinolone N-H bending, OH stretching, C=O stretching, C-H stretching and C-F stretching was present in all formulas of microspheres indicated the stability of ciprofloxacin in the microspheres. For formulations F1 to F4, the shift of the wavenumber of quinolone N-H band of ciprofloxacin HCl was from 1612.62 cm^{-1} to between 1602.23 cm^{-1} and 1661.17 cm^{-1}; the OH group from 3516.74 cm^{-1} to between 3529.87 cm^{-1} and 3558.08 cm^{-1}; the CH group from 2914.76 cm^{-1} to between 2915.03 cm^{-1} and 2937.59 cm^{-1}; the C=O group from 1699.31 cm^{-1} to between 1724.68 cm^{-1} to 1731.16 cm^{-1}; the C-F group from 1043.55 cm^{-1} to between 1005.25 cm^{-1} and 1013.94 cm^{-1}. The wave shift in sodium alginate occurred in the C-C group from 1080.87 cm^{-1} to between 1075.11 cm^{-1} and 1113.43 cm^{-1}; the guluronic group from 884.91 cm^{-1} to between 925.44 cm^{-1} and 930.61 cm^{-1}; the mannuronic group from 810.48 cm^{-1} to between 844.94 cm^{-1} and 849.63 cm^{-1}. The wave shift in kappa-carrageenan occurred in the S=O from 1223.52 cm^{-1} to between 1224.16 cm^{-1} and 1247.41 cm^{-1}; the galactose sulfat from 841.94 cm^{-1} to between 844.96 cm^{-1} and 849.63 cm^{-1} and the loss of the C-O-C group. These indicated that there were chemical interactions between the combination of alginate-carrageenan polymer and CaCl2 crosslinker to form microspheres. Chemical interactions between groups that occurs predict that the alginate polymer has carboxyl groups on two opposite G blockchains. In contrast, the kappa carrageenan polymer has a sulphate group in its structure (Yu *et al*. 2019). The combination of alginate-carrageenan polymer with the $CaCl_2$ crosslinker causes the interaction of the carboxyl guluronic group from the alginate polymer and the sulphate group from carrageenan polymer to bind to Ca^{2+} ions to form a network or matrix to entrapped the drug (Wathoniyyah 2016).

Table 3. FTIR of all microspheres formula

Functional group	Wave Number (cm^{-1})						
	Ciprofloxacin HCL	Sodium Alginate	Kappa carrageenan	F1	F2	F3	F4
OH stretch	3516.74	3310.90	3352.32	3532.21	3556.43	3558.08	3529.87
CH stretch	2914.76	2895.76	2892.99	2923.13	2918.83	2915.03	2937.59
C=O stretch	1699.31	1592.77		1729.30	1724.68	1731.16	1728.55
Quinolone N-H bending	1612.62			1604.16	1661.17	1602.23	1602.90
OH bending	1263.03			1245.69	1247.41	1224.16	1239.42
C-F stretch	1043.55			1013.94	1011.97	1005.25	1011.50
C-C stretch		1080.87		1076.84	1075.92	1113.43	1075.11
Guluronic finger		884.91		926.71	925.44	926.29	930.61
Mannuronic finger		810.48		847.20	849.63	848.99	844.96
S=O			1223.52	1245.69	1247.41	1224.16	1239.42
Galactose sulphate			841.94	847.20	849.63	848.99	844.96

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DSC result of all materials showed that the endothermic peak of ciprofloxacin HCL at 153.3ºC, sodium alginate endothermic peak at 127.8ºC, kappa-carrageenan showed a prominent characteristic endothermic peak at 124.8ºC, calcium chloride dihydrate endothermic peak at 175.6 ºC and maltodextrin endothermic peak at 139.4ºC. All of these DSC peaks indicate the raw material is up to standard.

Organoleptics of microspheres

The organoleptic results of ciprofloxacin-alginate-carrageenan microspheres made by ionic gelation method with aerosolization technique and then dried by freeze drying showed that all microspheres formulas F1 to F4 were in the form of yellowish-white powder and did not agglomerate.

Morphology of microspheres

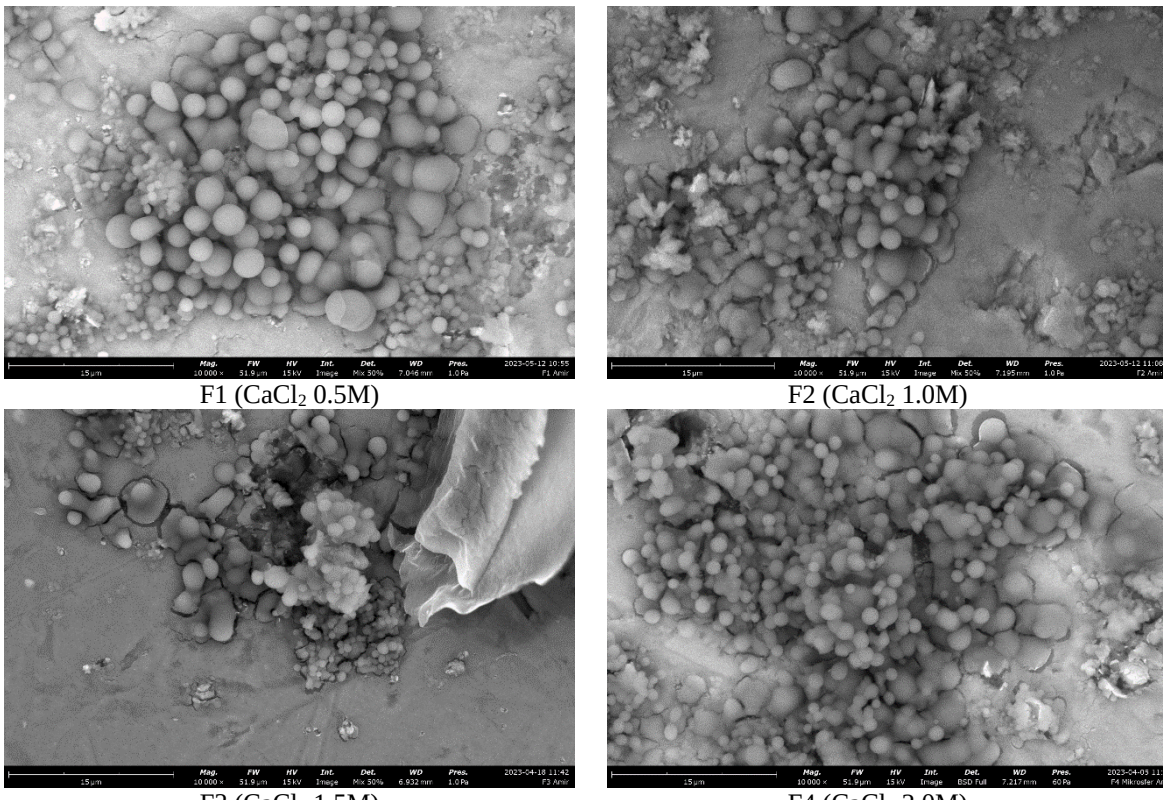
The morphology of the ciprofloxacin-alginate-carrageenan microspheres using SEM can be seen in Figure 1 and Figure 2. All the microsphere formulas F1 to F4 are spherical with smooth surfaces. The microspheres have a smooth texture because the viscosity of the polymer is sufficient to form cross-links with the crosslinker (Hariyadi *et al*., 2019).

Physical characteristics of microspheres

Physical characteristics of ciprofloxacin-alginate-carrageenan microspheres can be seen in Table 4.

Particle size

The result showed that ciprofloxacin-alginate-carrageenan microspheres resulted in particle size between 2.17 ± 0.03 µm and 2.50 ± 0.03 µm (Table 4). The results of the particle size of all formulas are less than 5 µm suitable for the inhalation route so that microspheres can be deposited in the alveoli (Gaber *et al*., 2021). If the particle size of the microsphere is and more significant than 5 µm, the particles will only reach the oropharynx, whereas if it is less than 1 µm, the particles will be expelled with the expired air (Ashish *et al*., 2012). Based on statistical analysis ($p<0.05$), the increase of $CaCl_2$ crosslinker concentration from 0.5M to 2.0M reduced the particle size of the microspheres (Hariyadi *et al*., 2018). According to some reports, gelation occurs immediately when a drop of polymer solution comes into contact with calcium ions. When Ca^{2+} ions penetrate to the inside of the polymer solution droplets, water is forced out of the droplets, which causes the microspheres to contract and resulting in smaller particle sizes being formed until saturation occurs so that the particle size does not shrink again (Manjanna *et al*., 2010; Ra *et al*., 2014). and a PDI of 0.0033, which was <0.30. This indicates a narrow size distribution, and the particle size of the microspheres is homogeneously dispersed (Li *et al*., 2014).



F1 ($CaCl_2$ 0.5M) F2 ($CaCl_2$ 1.0M)

F3 ($CaCl_2$ 1.5M) F4 ($CaCl_2$ 2.0M)

Figure 1. Morphological examination of ciprofloxacin-alginate-carrageenan microspheres formula using SEM at 10000x magnification

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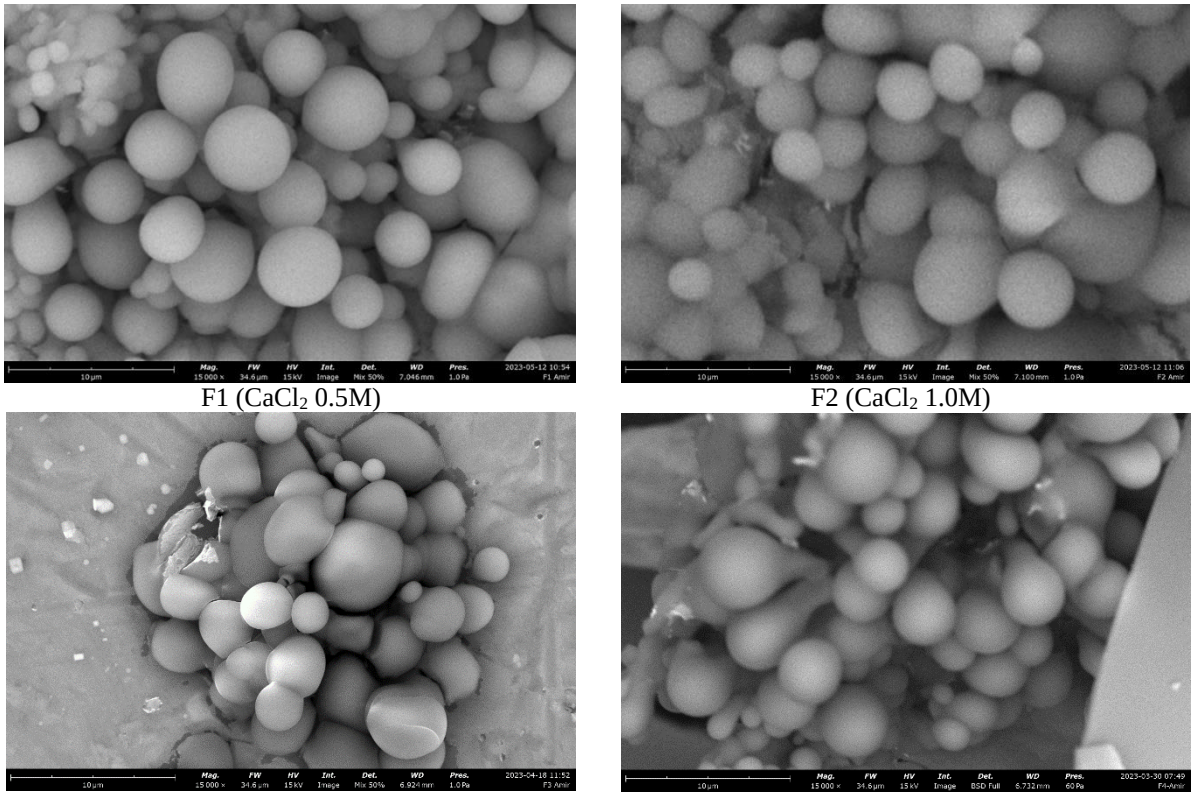

F1 ($CaCl_2$ 0.5M) F2 ($CaCl_2$ 1.0M)

F3 ($CaCl_2$ 1.5M) F4 ($CaCl_2$ 2.0M)

Figure 2. Morphological examination of ciprofloxacin-alginate-carrageenan microspheres formula using SEM at 15000x magnification

Table 4. Physical characteristics of ciprofloxacin-alginate-carrageenan microspheres

Formula	Particle size (µm)	Drug loading (%)	Efficiency entrapment (%)	Yield (%)	Moisture content (%)	Carr's index (%)	Hausner ratio
F1	2.50 ± 0.03	2.05 ± 0.02	75.34 ± 1.46	84.69 ± 0.88	2.59 ± 0.36	5.27 ± 1.11	1.05 ± 0.01
F2	2.29 ± 0.03	2.32 ± 0.01	86.61 ± 2.52	87.07 ± 1.85	2.75 ± 0.75	6.76 ± 0.66	1.07 ± 0.01
F3	2.21 ± 0.02	2.42 ± 0.02	98.09 ± 0.67	93.71 ± 1.09	2.84 ± 0.69	10.32 ± 1.87	1.10 ± 0.02
F4	2.17 ± 0.03	2.37 ± 0.07	96.81 ± 2.19	97.57 ± 0.50	3.16 ± 0.65	14.52 ± 1.53	1.15 ± 0.02

Drug loading and entrapment efficiency

For drug loading and entrapment efficiency, the results are consistent by showing the same pattern of increasing the crosslinker concentration of $CaCl_2$ from 0.5M to 1.5M increased ciprofloxacin-alginate-carrageenan microsphere drug loading and entrapment efficiency (Table 4). Based on statistical analysis, a significant difference was found between the effect of crosslinker concentration on increasing drug loading and entrapment efficiency ($p<0.05$). Drug loading increased from 2.05% ± 0.02 to 2.42% ± 0.02 and a similar result of entrapment efficiency was also found to increase from 75.34% ± 1.46 to 98.09% ± 0.67. The highest drug loading and entrapment efficiency was found in the microsphere formulation with a $CaCl_2$ crosslinker concentration of 1.5M and decreased at a concentration of 2.0M, but the decrease was not significant ($p>0.05$). The increase in drug loading and encapsulation efficiency of ciprofloxacin-alginate-carrageenan microspheres occurs because the higher crosslinker concentration causes more availability of Ca^{2+} ions, which crosslinked with carboxyl guluronic groups from alginate and sulphate groups from carrageenan and forms a network or matrix so that drug loading and entrapment efficiency becomes higher until there is a saturation of the crosslinker and polymer bonds so that drug loading and encapsulation efficiency do not increase anymore (Hariyadi *et al*., 2014).

Yield

The yield of ciprofloxacin-alginate-carrageenan microspheres is seen in Table 4. Yield value showed the smallest value that was for F1 ($CaCl_2$ 0.5M) at 84.69% ± 0.88, and the highest was F4 ($CaCl_2$ 2.0M) at 97.57% ± 0.50. These results indicated that the ionotropic gelation method with aerosolization techniques was a potential method for producing ciprofloxacin-alginate-

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carrageenan microspheres (Hariyadi *et al.*, 2022). Based on statistical analysis ($p<0.05$), this means that there is a significant difference. The increase of $CaCl_2$ crosslinker concentration from 0.5M to 2.0M increased the yield of the microspheres. The high yield value at greater crosslinker concentrations may be due to all the available polymer gelating with the crosslinker (Gedam *et al*., 2018).

Moisture content

The examination of moisture content was done to determine moisture content in the microspheres after the drying process. The moisture content of ciprofloxacin-alginate-carrageenan microspheres showed at about 2.59% ± 0.36 to 3.16% ± 0.65 (Table 4). The moisture content is less than 5%, so it is preferred for better aerosol performance (Saha *et al*., 2022). Microsphere particles with high moisture content can cause degradation of the microspheres and reduce their stability (Varela *et al*., 2022; Shan *et al*., 2016). The statistical analysis found no significant difference between the increase in $CaCl_2$ crosslinker concentration and the resultant moisture content ($p>0.05$).

Flow properties

Carr's index and Hausner ratio measurements were used to determine the flow properties of ciprofloxacin-alginate-carrageenan microspheres. Carr's index ranged from 5.27% ± 1.11 to 14.52% ± 1.53, and the hausner ratio from 1.05 ± 0.01 to 1.15 ± 0.02 (Table 4). According to the values of Carr's index and the Hausner ratio, F1, F2, and F3 have excellent flow properties, while F4 has good flow properties so that it is microspheres are suitable for inhalation routes targeting the lungs. Based on statistical analysis ($p<0.05$), this means that there is a significant difference. The increase of $CaCl_2$ crosslinker concentration from 0.5M to 2.0M increased Carr's index and Hausner's ratio of ciprofloxacin-alginate-carrageenan microspheres. The increase in the Carr's index and Hausner ratio occurs because the higher concentration of $CaCl_2$ crosslinker in the microspheres will affect the compaction of the microspheres, which results in cohesive-adhesive forces of the particles so that agglomeration tends to occur and causes a significant decrease in volume which is observed with an increase in the density of microsphere particles (Lane, 2016).

In vitro release study

Ciprofloxacin HCl was released from microspheres in all formulas for 12 hours in phosphate buffer media at pH 7.4 ± 0.05 can be seen in Figure 3. For 12 hours, the cumulative percentage of ciprofloxacin HCl released from microspheres F1 (63.78 % ± 1.67), F2 (56.86 % ± 2.39), F3 (50.29 % ± 1.74) and F4 (49.89 % ± 0.85). Increasing the concentration of the $CaCl_2$ crosslinker from 0.5M to 2.0M causes a decrease in the release of ciprofloxacin-alginate-carrageenan microspheres. A significant reduction in release ($p<0.05$) occurred; however, at an increase in the concentration of $CaCl_2$ from 1.5M to 2.0M, there was a decrease in the release rate, although there was no significant difference including ($p>0.05$). The observed reduction in drug release with increased calcium chloride is related to the formation of tight junctions between uronic acid residues from alginate and sulphate from carrageenan with calcium ions (Wathoniyyah 2016). The greater the concentration of the $CaCl_2$ crosslinker in the microspheres, the greater the strength of the ionic bonds and cross-links so that the network or bonds that are formed are more stable, stiff and compact and cause the degree of swelling and sensitivity of the matrix to decrease. It causes a decrease in drug release (Berger *et al*., 2004; Ra *et al*., 2014). According to some reports, drug release is also correlated with microsphere characteristics such as particle size and entrapment efficiency (Freiberg & Zhu, 2004; Lin *et al*., 2018). The smaller particle size of the microspheres indicates that the microspheres are rigid and have low matrix porosity so that drug release is slower compared to larger microsphere particle size (Lin *et al*., 2018), and drugs encapsulated in a matrix provide the opportunity for a slower release effect so that low of entrapment efficiency caused a faster of drug release (Freiberg & Zhu, 2004; Jerome *et al*., 2020).

Drug release kinetics

Release kinetics model showed that kinetics follows the korsmeyer-peppas kinetic model with the highest R^2 value and close to 1 for each formula F1 = 0.9942, F2 = 0.9962, F3 = 0.9902, F4 = 0.9915 (Table 5). The release exponent (n) of all formulations showed that it was $n<0.43$, and then the drug release was based on fickian diffusion, which describes the drug controlled by diffusion mechanism (Bruschi, 2015). In diffusion-controlled systems, the polymeric chains, either by inherent semipermeability or by swelling form pores into which the drug can diffuse and be released into the media (Jerome *et al*., 2020).

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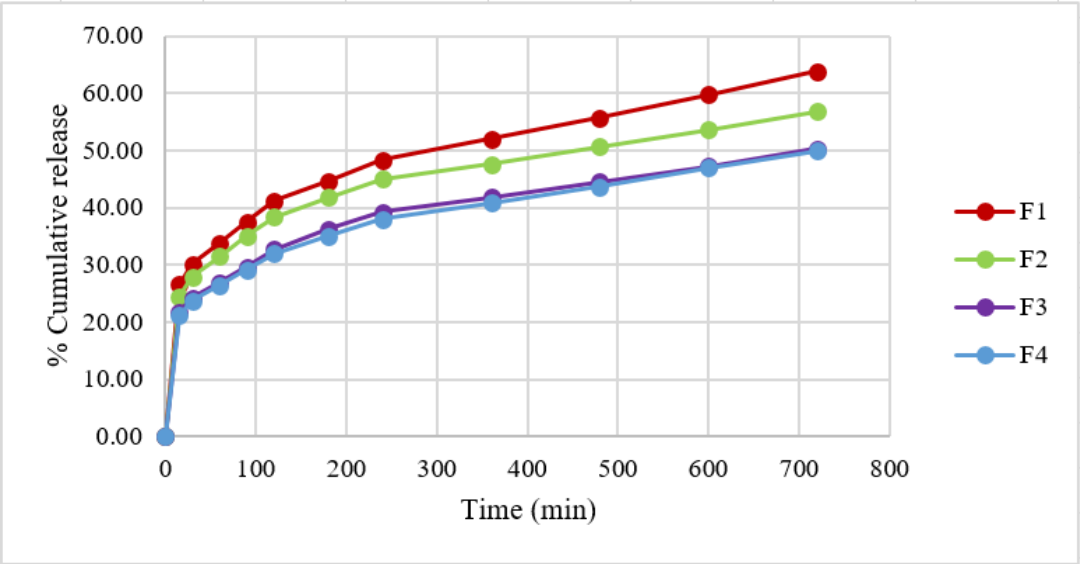

Figure 3. Release profile of ciprofloxacin HCl-alginate-carrageenan microspheres from all formulas

Table 5. Release kinetics with release exponent (n) of ciprofloxacin-alginate-carrageenan microspheres

Formula	Zero order	First order	Higuchi	Korsmeyer-Peppas	
F1	R^2 = 0.7084	R^2 = 0.8469	R^2 = 0.8923	R^2 = 0.9942	n = 0.2272
F2	R^2 = 0.6714	R^2 = 0.7930	R^2 = 0.8699	R^2 = 0.9962	n = 0.2184
F3	R^2 = 0.6941	R^2 = 0.7950	R^2 = 0.8835	R^2 = 0.9902	n = 0.2215
F4	R^2 = 0.7103	R^2 = 0.8096	R^2 = 0.8928	R^2 = 0.9915	n = 0.2254

Table 6. Moisture content and drug loading in stability test at 0 days and 28 days

Sample	Day	Moisture content (%)		Drug loading (%)	
		25ºC	40ºC	25ºC	40ºC
F1	0	2.59 ± 0.36	2.59 ± 0.36	2.05±0.02	2.05±0.02
	28	2.95 ± 0.36	3.00 ± 0.21	2.03±0.07	2.02±0.04
F2	0	2.75 ± 0.75	2.75 ± 0.75	2.32±0.01	2.32±0.01
	28	2.83 ± 0.65	2.84 ± 0.58	2.29±0.15	2.28±0.06
F3	0	2.84 ± 0.69	2.84 ± 0.69	2.42±0.02	2.42±0.02
	28	3.05 ± 0.70	2.99 ± 0.63	2.39±0.14	2.38±0.05
F4	0	3.16 ± 0.65	3.16 ± 0.65	2.38±0.07	2.38±0.07
	28	3.42 ± 0.59	3.44 ± 0.66	2.34±0.08	2.33±0.16

Stability test

Results of stability testing after 28 days of storage at 25ºC and 40ºC can be seen in Table 6. Organoleptic observation of all formulas of ciprofloxacin-alginate-carrageenan microspheres did not show any changes; the colour was still yellowish-white and did not agglomerate. In terms of moisture content and drug loading at 25ºC and 40ºC compared from 0-day to 28 days of storage, it was found that there was no significant difference between the effects of temperature and time on increasing moisture content and decreasing drug loading ($p>0.05$), which indicated that the ciprofloxacin-alginate-carrageenan microspheres remained stable (Kalalo *et al*., 2022). To determine the possibility of microsphere instability, more time and high temperature are required to see a substantial difference in the physical stability of ciprofloxacin-alginate-carrageenan microspheres.

CONCLUSION

Ciprofloxacin-alginate-carrageenan microspheres were successfully formed using the ionotropic gelation with aerosolization technique. Yellowish-white microspheres with small particle size and smooth morphology were obtained from the system. Increasing the concentration of the $CaCl_2$ crosslinker from 0.5M to 2.0M decreased the particle size, drug release and increased the drug loading, entrapment efficiency and yield. The microspheres containing 1.5M $CaCl_2$ crosslinker had the maximum drug loading and entrapment efficiency with excellent flow properties and were stable for 28 days.

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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