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Influence of Polymer Combınatıon Concentratıon on the Characterıstıcs, *In Vıtro* Release, and *In Vıvo* Lung Deposıtıon of Algınate-Carrageenan Mıcrospheres Encapsulatıng Cıprofloxacın HCl

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ABSTRACT

Inhaled Ciprofloxacin HCl microspheres were formulated with a combination of alginate and kappa carrageenan with a total of 1% (Formula 1), 1.5% (Formula 2), and 2% (Formula 3). The aim of this research is to examine the effect of polymer concentration on the characteristics, release, and lung deposition of microspheres. The latter are characterized by qualities including loading, efficiency, yield, size, moisture content, mucoadhesivity, and release. Deposition was studied by means of fluorescence microscopy, the results of which showed it to be spherical, smooth and less than 2 μm in size. Loading was between 21.29% and 38.18%, while entrapment efficiency ranged from 52.86% to 76.29%, and the yield amounted to between 49.83% and 70.72%. The swelling index was less than 10. For moisture content, all formulas recorded less than 6.5%. In terms of mucoadhesivity, F1, F2, and F3 showed 0.0090 kg, 0.0217 kg, and 0.0329 kg respectively. Increasing polymer concentration was found not to affect the size, loading, efficiency, and yield of microspheres. Cumulative release of F1, F2, and F3, with a burst release profile, was 72.64%, 59.25%, and 47.66% respectively after 10 min at PBS pH 7.4. *In vivo* analysis demonstrated that all formulas could be deposited in the lungs of rats, while a reduction in intensity occurred in the fourth hour due to increased levels of polymer. The remaining intensity indicated that microspheres were able to maintain the drugs loaded.

Keywords: Ciprofloxacin HCl, Lung Deposition, Alginate, Carrageenan, Microspheres.

INTRODUCTION

Cystic Fibrosis (CF) is a genetic disorder caused by mutations in the gene which encodes the chloride channel in the CF transmembrane conductance regulator (CTFR). The CTFR is responsible for fluid transport on the surface or in the ducts of several epithelial organs, for example the lungs, digestive system, and pancreas (Saravia and Riley, 2018). Infections occurring in CF patients can be caused by several types of bacteria. However, in approximately 60% of cases an additional infection caused by Pseudomonas aeruginosa bacteria occurs (Jain *et al*., 2012). A combination of excess mucus secretion and infection can exacerbate the poor health of CF patients (Geller, 2009). Ciprofloxacin HCl of the fluoroquinolone class was used to treat infections in such individuals. However, the bioavailability of ciprofloxacin in the respiratory tract accounts for only about 10% of the 0.5-5% of the drug transported by the systemic circulation (Peltola *et al*., 1998). The excess mucus production that occurs in CF patients also further reduces the bioavailability of ciprofloxacin in the respiratory tract (Geller, 2009). Therefore, it is necessary to develop a delivery system that can increase the bioavailability of ciprofloxacin while protecting the drug against the effects of the excessive mucus commonly found in CF patients.

Several delivery systems have been developed for ciprofloxacin, including formulation of the drug in the form of a liposome inhaler (Geller,

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2009). Study findings relating to its use strongly suggest that this type of formulation can increase the bioavailability of ciprofloxacin in the respiratory tract (Geller, 2009). However, liposome delivery systems are produced using oil solvents rendering them more expensive and less environmental friendly (Yeo *et al*., 2001). A Dry Powder Inhalation (DPI) delivery system has also been developed to overcome the identified shortcomings of the existing one. However, reports suggest that the DPI delivery system cannot deliver the full prescribed dose resulting in fluctuating levels of ciprofloxacin present in the respiratory tract. Therefore, the developing of more suitable delivery systems is imperative.

Microspheres are delivery systems capable of shielding the active agents from the environment. Microencapsulation can protect drugs from environmental damage (Jantarathin, 2017), while also providing a physical barrier between core compounds and the environment. The formation of this system requires biocompatible and biodegradable polymer properties (Hariyadi *et al*., 2016). Microsphere delivery systems can also be administered in the form of inhalation preparations. These have mucoadhesive properties that reduce the clearance of materials due to the effect of mucus in CF patients, thereby increasing the bioavailability of the active ingredients (Dastidar *et al*., 2018; Patil *et al*., 2009). Developing a microsphere delivery system demonstrating the properties of mucoadhesiveness can be achieved using a mucoadhesive polymer in combination with those possessing polyanion properties known to be more mucoadhesive (Patil *et al*., 2009). Alginate is a polymer belonging to the polyanionic copolymer group derived from brown algae consisting of a heteropolysaccharide molecule of D-mannuronic acid and L-guluronic acid (Jantarathin, 2017; Atalla *et al*., 2020). The presence of a carboxyl group at the end of the guluronic alginate chain places this polymer within the anionic mucoadhesive polymer group whose members possess mucoadhesive properties of between 70% and 85%, the highest level compared to all other polymers (Patil *et al*., 2009; Al-kafaween *et al*., 2020). Alginates will cross-link with divalent cations, such as Ca^{2+} to form an "egg-box" structure (Voo *et al*., 2011). Ca^{2+} cation will bind to the –COO- group of two opposite (adjacent) G-block chains. However, the –COO- group is free from nonadjacent mannuronic and guluronate blocks that are not bound by Ca^{2+} cation enabling it to produce porous microspheres (Purwanti *et al*., 2018). This can trigger a burst effect on the microsphere system leading to the rapid release of the drug (Abdelghany *et al*., 2017; Setti *et al*., 2018). One means of minimizing the occurrence of this scenario is to combine alginate with other polymers.

The research conducted by Abelghany *et al*., (2017) and Zhang and Zhang, (2012) indicated that a combination of alginate and kappa carrageenan can reduce the release of the drug in such a way as to enable its being controlled. Carrageenan, a high molecular weight sulfate anion polysaccharide composed of galactose and anhydrous galactose linked by glycosidic bonds (Liang *et al*., 2013), possesses bioadhesive properties by forming hydrogels and binding to mucus which can, simultaneously, enhance the adhesiveness of the delivery system employed (Song and Eddington, 2012). There are three forms of Carrageenan, namely: iota-carrageenan (i-carrageenan), kappa-carrageenan (κ-carrageenan), and lambda-carrageenan (λ-carrageenan). In addition to lowering the burst release of alginate, the combination of alginate and carrageenan can increase the swelling index and entrapment efficiency of the formulated microsphere system (Roh and Shin 2006; Mohamadnia *et al*., 2012). With regard to the ratio between polymers, the concentration ratio of 1:1 alginate-carrageenan was selected because it can provide optimal entrapment and drug release efficiency (Kolesnyk *et al*., 2015). The highest polymer concentration of 2% was adopted since any stronger concentration would have reduced entrapment efficiency (Hariyadi *et al*., 2014). A maximum amount of 0.3 M $CaCl_2$ crosslinker was used because it can produce spherical particles. Ca^{2+} cation will bind to adjacent guluronic groups of alginate forming an "egg-box" structure (Kolesnyk *et al*., 2015).

As for the production of microspheres, the ionotropic gelation method was selected since it can form spherical structures within a narrow size range (Manjanna *et al*., 2014). This ionotropic gelation method has the advantages of being rapid, uncomplicated, and cost-effective (Yeo *et al*., 2001), while also avoiding the use of organic solvents. The process can be executed at room temperature, thereby minimizing potential damage to the drug from extremes of heat or cold (Yeo *et al*., 2001). After the wet microspheres are formed, they are subsequently freeze-dried to render them more stable (Hariyadi, *et al*, 2020).

Table 1. The formula for ciprofloxacin microspheres in a polymer combination of Na-alginate and carrageenan with different total polymer concentrations

Compounds	Function	F1	F2	F3
Ciprofloxacin HCl	Active agent		1.2%	
Na-Alginate	Polymer	0.5%	0.75%	1.0%
Carrageenan	Polymer	0.5%	0.75%	1.0%
$CaCl_2$	Crosslinker		0.3 M	
Maltodextrin	Lyoprotectant		5%	

Microspheres are characterized by their polymer concentration and polymer-drug ratio, both of which are the subjects of study (Zafar *et al*., 2013). Differences in the characteristics of these microspheres can affect the release of drugs from the microsphere system. Consequently, determining the optimal polymer concentration is necessary in order to increase the effect of the drug on the microspheres (Zafar *et al*., 2013).

In vivo evaluation of microsphere drug deposition in the lungs is prerequisite to evaluating the success of the system in cases of inhalation. The effectiveness of this form of administration is strongly influenced by the amount of drugs deposited in the lungs. Drug deposition testing can be undertaken by resort to a histological approach, one of which involves the use of a fluorescence microscope (Tell *et al*., 2015). This method can both confirm the presence of microspheres and calculate the number that enter and become localized in lung tissue through the intensity of the luminescence. The deposition and distribution of a drug delivered to the lungs by inhalation are also largely determined by the size of its particles. It is anticipated that this research will identify the optimum characteristics of the Ciprofloxacin-HCl microspheres vis-a-vis inhalation.

MATERIALS AND METHODS

Ciprofloxacin HCl pharmaceutical grade (Sigma Aldrich); Kappa Carragenan (Sigma Aldrich); Natrium Alginate (Sigma Aldrich); $CaCl_2.2H_2O$ pharmaceutical grade (Solvay Chemicals International); Natrium citrate pharmaceutical grade (Weifang Ensign Industry Co.Ltd); Na_2HPO_4 pro analysis (Merck); KH_2PO_4 pro analysis (Merck); NaCl pro analysis (Merck); Maltodextrin pharmaceutical grade (Bratachem); HCl pro analysis (Merck); NaOH pro analysis (Merck); and Aquademineralisata (BrataChem).

Formulation of microspheres with aerosolization technique

Separate solutions of sodium alginate and kappa carrageenan in distilled water were produced. The two polymers having been mixed, the resulting solution was sprayed by means of an aerosol at a pressure of 40 psi into the $CaCl_2$ crosslinker solution (0.3 M) from a distance of 8 cm. The polymer mixture solution and the crosslinker were stirred for 120 min at a speed of 1000 rpm, at which point the ciprofloxacin HCl solution was added by dissolving 0.6g of ciprofloxacin into 50mL of distilled water. Stirring was resumed for the same duration as before and at an identical speed (Table I). Microspheres were added to maltodextrin (5% w/v) before being freeze-dried at -80°C for 29 h.

Evaluation of Microspheres

Moisture Content

1 gram of dry microsphere was placed above the sample pan which was set at a temperature 105°C, while the Moisture Content Analyzer was programmed to start measuring automatically for ten minutes. The moisture content was then recorded.

Particle Size and Morphology

The size was established by use of an optical microscope and software at a magnification of 100x. For the purposes of morphological examination, a Scanning Electron Microscopy (SEM) FEI S50 was used.

Drug Loading Ciprofloxacin HCl in Alginate-kappa Carrageenan microspheres

Drug loading was calculated using the following equation on the basis of the total number of microspheres obtained:

$$Drug\ Loading = \frac{Total\ weight\ of\ ciprofloxacin\ HCl\ in\ the\ microspheres\ (mg)}{Microspheres\ weight\ (mg)}\ x\ 100\%$$

Entrapment Efficiency (EE)

Entrapment Efficiency was calculated from the ciprofloxacin HCl content of the microspheres by means of the equation below:

$$\%\ EE = \frac{Ciprofloxacin\ mass\ in\ the\ microspheres\ (mg)}{Ciprofloxacin\ mass\ of\ the\ formula\ (mg)}\ x\ 100\%$$

Yield

The yield of microspheres was calculated from the total number of dry microspheres using the following equation:

$$\%\ Yield = \frac{Total\ weight\ of\ formmicrospheres\ (mg)}{Polymer\ weight + ciprofloxacin + lyoprotectant\ (mg)}\ x\ 100\%$$

Swelling index

A solution of 100 mL Phosphate Buffer Saline (PBS) pH 7.4 ± 0.05 was prepared as a medium to which 100 mg of previously weighed ciprofloxacin HCl microspheres were added. A swelling process was then carried out at 37°C with the weight changes of the microspheres being recorded at specific intervals. The microsphere sample was dried using filter paper and the post-swelling microsphere weight measured. The swelling index score was calculated using the following equation:

$$Swelling\ percentage = \frac{Dt-D0}{D0}\ x\ 100\%$$

Notes: D0: dry microspheres weight; Dt: post-swelling weight of microspheres

Mucoadhesivity Test

A TA-XT stage Plus Texture Analyzer (Stable Micro Systems, Godalming, UK) with a load cell set on "Adhesive mode" was used. The sample was placed in a cylindrical container attached to the base of the analyzer with double-sided adhesive tape. The lung mucosal that had been equilibrated for 15 minutes at pH 7.4 on a holder was subsequently emersed in a media solution of pH 7.4. The probe (cylinder) was lowered onto the mucosal surface at a specific rate. Contact between the surface of the probe and the sample was subjected to a constant downward force (0.05 N) applied by the probe for 60 seconds. The probe was then re-applied with a turning distance of 20 mm at the same speed. The released force and the adhesion were automatically measured by the texture analyzer software.

***In Vitro* Release Study**

Ciprofloxacin HCl release from the alginate-kappa carrageenan microspheres was carried out in PBS pH 7.4 ± 0.05. A release profile test was conducted using a thermoshaker at 37°C and 100 rpm. The sample was taken from the PBS pH 7.4 at 10, 30, 60, and 120 minutes, a process repeated every two hours for 24 hours, while the volume of media (5mL) was replaced using PBS pH 7.4. The sample was filtered through 0.45 m millipore paper. Calculation of the cumulative amount of ciprofloxacin HCl released from the microspheres within the sampling time range was undertaken using the equation below:

$$\%\ \text{Ciprofloxacin HCl cumulative} = \frac{Ciprofloxacin\ mass\ in\ the\ 100\ mL\ of\ media\ (mg)}{ciprofloxacin\ mass\ (mg)}\ \text{x}\ 100\%$$

The release profile of ciprofloxacin HCl was made in the PBS pH 7.4 media with sampling time as the x-axis and the concentration of ciprofloxacin HCl released as the y-axis. From the profile of drug release previously obtained, a steady-state regression equation was developed. The profile of the release of ciprofloxacin HCl from microspheres was shown by the value of b (slope) of the regression equation.

Drug Deposition in the Lungs

***In Vivo* Animal Study**

Wistar rats were used as experimental animals in accordance with specific inclusion and exclusion criteria. The rats were placed in an insulated cage and underwent a one-week period of adaptation in a room at a constant specified temperature. The minimum number of experimental animals per group was six, giving a combined total research population of 48 across the various treatment groups. The research has passed the Airlangga University Ethical Clearance (Protocol number 038/HRECC.FODM/II/2019) from Airlangga University.

Drug dosage

The inhaled dosage of Ciprofloxacin for experimental animals was 20 mg/Kg BW (Liu, 2013). Several ciprofloxacin HCl alginate-kappa carrageenan microspheres given to the experimental animals were calculated by considering the drug loadings of ciprofloxacin in the microspheres. The number of ciprofloxacin alginate-kappa carrageenan microspheres administered can be calculated using the following formula:

$$Ciprofoxacin\ content = \text{the amount of ciprofloxacin in the formula} \times \%\ drug\ loading$$

$$\textit{Ciprofloxacin microspheres dosage} = \frac{\textit{Average amount of dry microspheres}}{\textit{ciprofloxacin content}} \textit{ x Rat dosage}$$

Administering microspheres to experimental animals

The experimental animals were subjected to six different treatments. Administration of microspheres to their lungs by nose-only exposure involved the use of a modified tool in the same manner as the DPI tool using ciprofloxacin with single kappa carrageenan polymer as previously described (Hariyadi *et al.,* 2021). The apparatus employed was adopted from Kaur *et al*. (2008). The dose administered was equivalent to 30 mg of ciprofloxacin/kg BW of rats.

Drug deposition observation

Ciprofloxacin-alginate-carrageenan microspheres with rhodamine B labeled were inhaled by the subjects (Kaur *et al*., 2008) which were then euthanized and their lungs removed. While still fresh, the lungs were rinsed with normal saline before being sliced longitudinally and horizontally to a thickness of 6 um by means of a cryotome (Tissue-Tek Cyro3, Sakura) at a temperature of -59°C at the Anatomical Pathology Laboratory, Integrated Central Surgical Building, Soetomo Hospital. The cryostat preparations were stored at -15°C to -20°C for subsequent analysis. Finally, observation of drug deposition was carried out using a Fluorescent Microscope (FSX 100, Olympus) fitted with a red filter at a magnification of 42x and an exposure time of 1/1.2 ms.

Data Analysis

For the physical characteristics and in vitro drug release tests, statistical data analysis was performed using the one-way ANOVA method with a confidence degree of 95% ($\alpha = 0.05$). Deposition of the drug was observed by means of a fluorescent microscope with the intensity of each group subsequently being compared. Data analysis was carried out using two methods. Firstly, by comparing qualitatively and quantitatively, by means of ImageJ software, the intensity of fluorescence in the tracheal and lung tissue of rats. Secondly, by analyzing the movement of the microspheres at the end of first and fourth hour through observation of the fluorescence and luminescence in the lung tissue of the rats. The red luminescence intensity data obtained was then subjected to statistical comparison using the Kruskal-Wallis non-parametric analysis.

RESULTS AND DISCUSSION

Particle size and morphology of ciprofloxacin microspheres

Examination of the ciprofloxacin microspheres confirmed particle sizes between 1.24 µm and 1.72 µm (Table II). It was evident that, compared to the other formulas, F3 had the largest particle size which was due to the increase in polymer concentration potentially resulting in a larger number of crosslinking points in the formula (Jin *et al*., 2009). Furthermore, increasing the polymer concentration can render the solution more viscose which, in turn, produces a larger droplet which is related to the final particle size of the microspheres. Prior to observation of the surface morphology of the microsphere particles using SEM, the fact that the microsphere particles were spherical in shape and had a smooth surface (Figure 1) was taken into account.

Moisture content of Ciprofloxacin HCl-Alginate-Kappa Carrageenan microspheres

These confirmed that F1 had the highest moisture content compared to the other microsphere formulas (Table II).

Yield, drug loading, and encapsulation efficiency

The yield, drug loading, and encapsulation efficiency of all formulas (Table II). From the entrapment efficiency of the ciprofloxacin content of microspheres, F3 was found to have the highest ciprofloxacin loadings and entrapment efficiency of all the formulas (Table II). F3 had the highest polymer concentration compared to the others, possibly due to an increase in crosslinking points occurring as a result of an increase in polymer concentration. The yield of microspheres ranged from 49.8% to 70.7%.

The low yield of the F3 formulas could be due to the more viscous polymer requiring a larger amount of energy in order to squeeze the wet microspheres prior to the freeze-drying which results in lighter microspheres and a low yield. Consequently, optimization of the microsphere washing process is necessary. The yield value of each formula should rise following an increase in polymer concentration which would create more potential sites for bonding/crosslinking points. The heavier the weight value obtained, the larger the resulting yield (Patil *et al*., 2010).

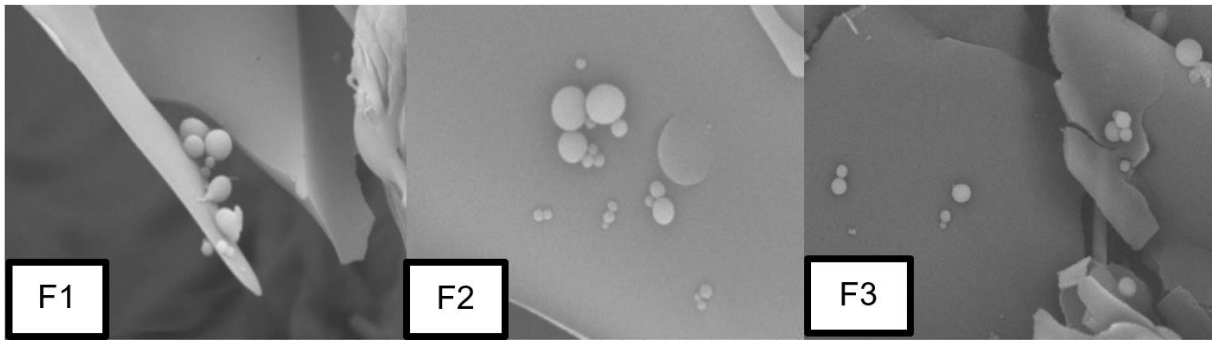


Figure 1. Surface morphology of Ciprofloxacin HCl-Alginate/Carrageenan microspheres of F1, F2, and F3 using SEM (5000x magnification).

Table II**.** The particle size, drug loading, efficiency entrapment, yield, mucoadhesive, moisture content, and swelling index, based on the mass and particle size of ciprofloxacin HCl-alginate-carrageenan microspheres.

Formula			**F1**	**F2**	**F3**
Diameter (µm) ± SD			1.24 ± 0.006	1.35 ± 0.035	1.72 ± 0.137
Drug Loading (%)			21.29 ± 0.90	37.97 ± 3.63	38.18 ± 0.25
Entrapment Efficiency (%)			52.86 ± 4.89	72.78 ± 9.72	76.29 ± 2.15
Yield (%)			70.72 ± 1.77	55.10 ± 1.18	49.83 ± 3.87
Muco adhesive (kg)			0.0090	0.0217	0.0329
Moisture Content (%)			6.40 ± 1.00	4.26 ± 1.26	3.57 ± 0.89
Swelling Index (%)	**Based on mass**	**24 h**	144.00 ± 13.53	125.67 ± 8.62	107.33± 0.30
		30 h	177.67 ± 8.02	140.67 ± 6.11	115.33 ± 0.24
	Based on particle size	**24 h**	169.54 ± 13.86	101.29 ± 3.05	70.67 ± 0.04
		30 h	183.03 ± 11.31	117.64 ± 6.68	80.55 ± 0.01

Table III. Comparison of 1st and 4th hour Luminescence Intensity in tracheal tissue from the left and right lung

Formula	**Average intensity in Trachea (unit)**		**Average intensity in Left Lung (unit)**		**Average intensity in Right Lung (unit)**	
	1st hour	**4th hour**	**1st hour**	**4th hour**	**1st hour**	**4th hour**
F1	42.0070	29.6957	39.7433	32.2717	37.1791	33.6603
	±8.1169	±12.4595	±7.1807	±7.8176	±11.2396	±8.6740
F2	32.60833	28.2218	36.7834	33.7992	32.8559	34.7603
	±7.2431	±10.4518	±6.8599	±4.0498	±8.5113	±6.5422
F3	28. 6393	5.5350	18.3908	19.1184	26.8886	18.5156
	±8.7471	±1.1959	±10.2372	±12.9448	±17.7272	±11.3105

Swelling index

Results of the swelling index of the microsphere formulas according to mass (Table II). Observation of the swelling index indicated that F1 registered the highest compared to other microsphere formulas.

Mucoadhesivity

The observations (Table II) conducted indicated that F3 demonstrated the highest mucoadhesiveness compared to other microsphere formulas.

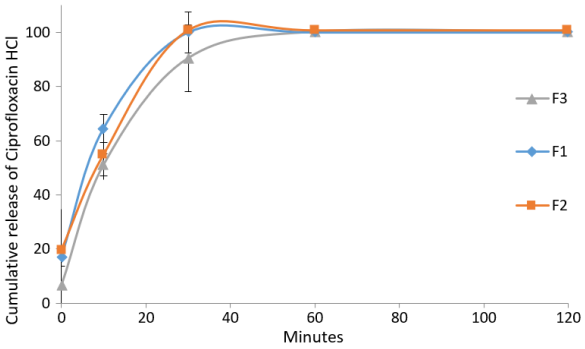
***In Vitro* Release Study**

Profile of ciprofloxacin release from microspheres

From the release evaluation results (Figure 2), it was evident that within ten minutes all the ciprofloxacin HCl present had been released from the microspheres. Alternatively, it could be argued that the microsphere particles had a burst release model.

The cumulative drug release for each formula was as follows: F1= 82.20 ± 13.04; F2= 76.79 ± 9.24; and F3 = 69.58 ± 13.85%. Statistical

analysis showed that there was no significant difference in the cumulative percentage (%) of ciprofloxacin released in each formula. All formulas were known to have smaller correlation coefficient values with the result that no linear correlation existed between the cumulative (%) drug release and the release kinetics model employed. This low correlation coefficient value could be caused by less frequent sampling during the first ten minutes because, in the initial hypothesis, this polymer combination formula was intended to be released continuously for a period of 24h.



Burst release can be caused by microsphere particles undergoing a process of extremely rapid swelling, thereby increasing their volume. This swelling will, in turn, trigger the dissolution and release of the drug from the polymer (Siegel and Rathbone, 2012).

In terms of formulation, the occurrence of this burst release can be due to a number of factors, among others the presence of carboxylic ester bonds and sulfate esters between alginate and kappa carrageenan which are easily hydrolyzed due to the presence of water causing the burst release of drugs. In addition, this burst release can be due to the low concentration of polymer and crosslinker used. The low concentration of polymer can cause a smaller particle size in the formed microspheres causing, in turn, a reduction in the release rate. This is because more drug particles are distributed close to the particle surface with the result that the solvent (water) requires less time to penetrate and dissolve the drug in the system (Dashora *et al*., 2007). The absence of cross-linking concentration can cause the formation of fragile microsphere particles that break easily (Jin *et al*., 2009). Moreover, it is possible that the two polymers employed compete with each other to bond with the Ca^{2+} crosslinker as the result of a negative charge. Carrageenan itself can bind to a Ca^{2+} crosslinker, although the weakness of the bond formed can lead to the formation of fragile microsphere particles that readily release the drug. Therefore, several suggestions for further development of this microsphere formula include replacing the combined polymer (e.g. polymers with different charges); changing the ratio of the polymers combined to ensure they do not compete with each other in bonding with the crosslinker used; increasing the concentration of $CaCl_2$ to obtain a stronger microsphere matrix; and increasing the concentration of the polymer.

Drug deposition of microspheres

Observation of drug deposition was conducted during the first hour after the experimental animals had inhaled 80 mg of ciprofloxacin microsphere with a polymer combination of 1% (F1), 1.5% (F2), and 2% (F3) respectively. The tissues observed had been excised to a thickness of 6 µm from the trachea, left lung lobe, and the caudal lobe of the right lung. Observation of drug deposition sought to determine the ability of the system to reach the lungs of experimental animals. Therefore, the tissue observed was derived from both lung lobes and the trachea. Observations were made at 4.2x magnification and with an exposure time of 1/2.5 seconds.

The following are pictures of normal tissue from the trachea and respiratory tract (bronchioles) of the left lobe of an untreated rat (Figure 3).

For microspheres containing the drugs, the results of drug deposition of each microsphere formula at the end of the first hour can be observed in Figure 3. Observation of the trachea and lung tissues indicates that the higher the polymer concentration, the lower the intensity of fluorescence in these respiratory organs. This is confirmed by the image of lung tissue and tracheal tissue of F3 (2%) which demonstrate lower red fluorescence intensity than those of F2 (1.5%) and F1 (1%).

The calculation of the intensity of the tracheal and lung tissue made it apparent that the higher the polymer concentration, the lower the red luminescence produced. The results of a statistical analysis produced by a non-parametric Kruskal Wallis test confirmed significant differences between the three formulas, most particularly, between F1 and F3.

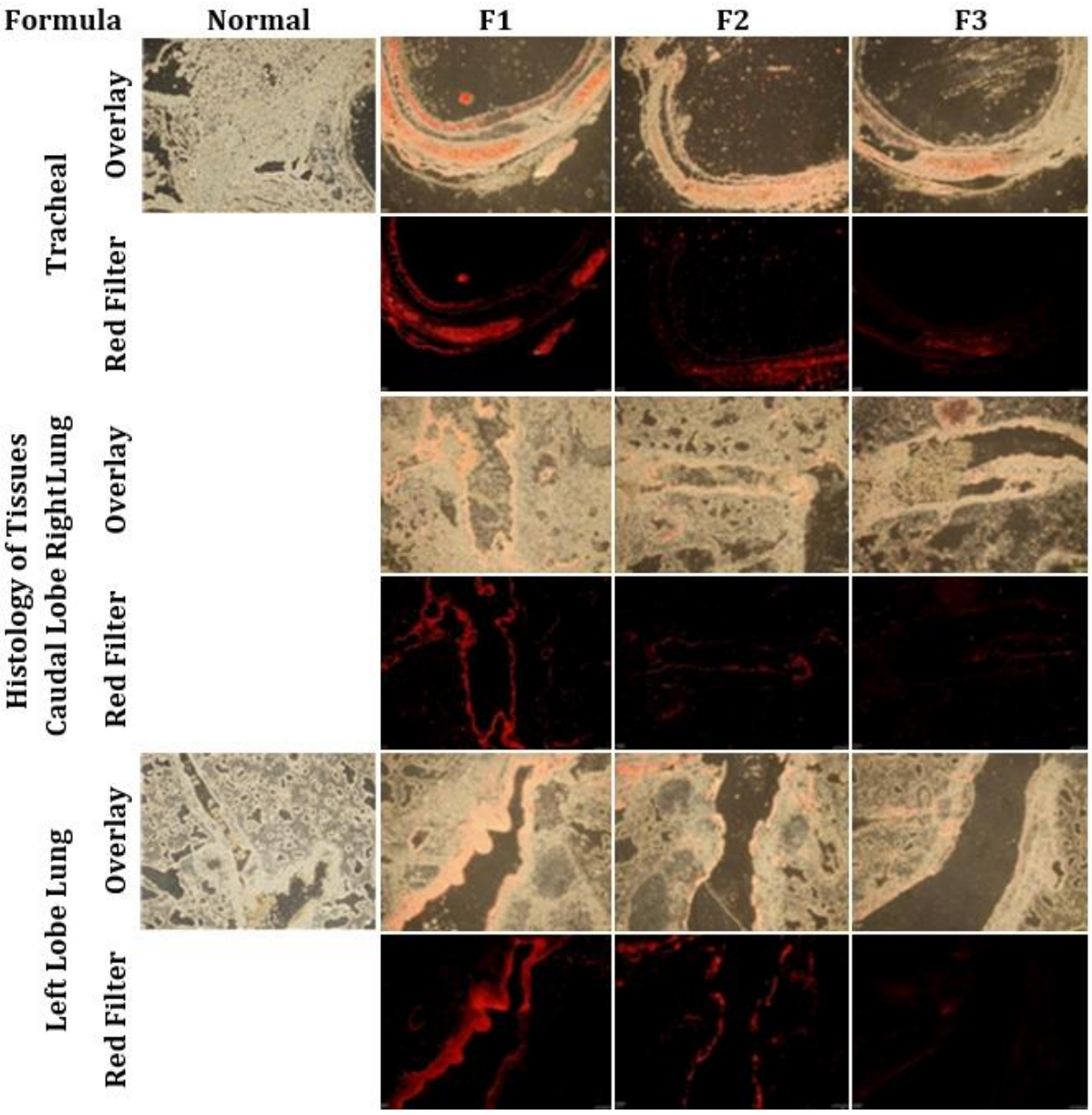


Figure 3. Histology of the tissue without treatment and deposition of Microspheres of F1 (1%), F2 (1.5%), and F3 (2%) in Various Tissues.

Based on these results, it is clear that the higher the polymer concentration, the lower the red luminescence produced in the right lung tissue. There was no significant difference between the three formulas. This was because the increasing concentration of polymer caused the particle size of the microspheres produced to be larger. Consequently, the microspheres experience increasing difficulty in reaching the lungs because they become trapped in the oropharynx and nasopharynx of the upper respiratory tract.

A comparison graph of the intensity of F1, F2, and F3 in the tracheal tissue, caudal lobe right lung, and left lung (Figure 4). The fluorescence intensity of the formula at a concentration of 2% (F3) was very low compared to F1 and F2. This could be due to the very small amount of powder entering the lungs in the case of F3 compared to F1 and F2. Based on the results relating to particle size, it was found that the largest size, that of F3, was significantly different from that of F1. However, it must be borne in mind that the particle distribution

used when describing inhaled particles may be distorted. Therefore, measuring the particle size distribution using *in vitro* an artificial human respiratory tract, by means of a cascade impactor or Twin Stage Impinger is highly recommended.

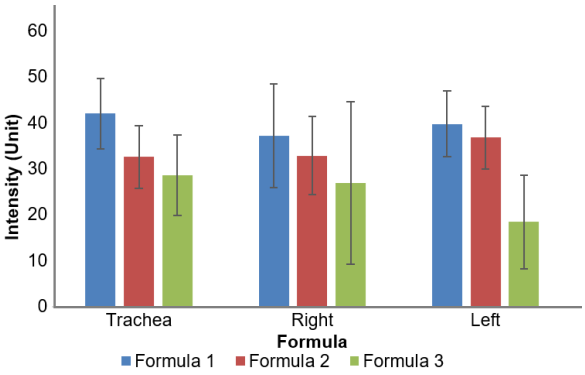


Figure 4. Intensity Comparison Chart Microspheres F1 (1%), F2 (1.5%) and F3 (2%) in Tracheal and Lung Tissue

Again, it can be seen that the fluorescence intensity of F1 and F2 in the left lung tissue faded after the fourth hour compared to the first, while for F3 the intensity at the fourth hour was slightly higher (Table III). Based on the results of the movement of microspheres in the lungs and tracheal tissue at the first and fourth hours, it can be seen that the intensity of the fluorscent luminescence of F1, F2, and F3 faded at the fourth hour compared to first. In general, the observation of the qualitative movement of microspheres in the trachea and lung tissue indicated a decrease in the intensity of the red luminescence at the fourth hour. From the statistical analysis using the non-parametric Mann-Whitney test on tracheal tissue, a significant difference was found between the first- and fourth-hour groups in F1 and F3. With regard to the left lung, F3 showed an increase in the intensity of red luminescence at the fourth hour, but no significant difference between the first- and fourth-hour groups of F2 and F3. The only significant difference occurred in F1.

No significant difference was found in the caudal lobe of tissue from the right lung between the first and fourth hours. At the fourth hour, luminescence could still be detected in the lung bronchioles and trachea, indicating that microspheres remained attached to the tissue due to the mucoadhesive nature of the polymer. This finding is confirmed by the mucoadhesive test results which showed that the three formulas had high mucoadhesive properties which intensified with increasing polymer concentration.

This system can maintain the drug in the lungs for longer than unencapsulated ciprofloxacin HCl because, as highlighted by the research of Liu *et al*. (2015), ciprofloxacin HCl delivered to the lungs is no longer present there after an hour. However, to support these conclusions, it is necessary to determine the levels of ciprofloxacin HCl in the lungs after a significant period of time. In this study, it was not possible to determine these levels because an appropriate assay method was lacking. In this study, the level of aggressiveness of the experimental animals, in addition to their weight and age, greatly influenced the drug deposition. Therefore, it is necessary to modify the advanced inhalation device that allows the tool to adhere continuously to the rat's head so that it can inhale the powder in an uninterrupted manner.

This study also detected that fluorochrome material was not conjugated with the drug ciprofloxacin HCl. In this method, Rhodamine B stains and is entrapped in the microspheres' system of ciprofloxacin, with the result that the ciprofloxacin HCl position cannot be ascertained inside the cell after the microspheres system has been taken up by it (Hariyadi, *et al* 2017). Finally, another type of fluorochrome conjugated with the drug or similar to the drug is recommended.

CONCLUSION

Inhaled Ciprofloxacin HCl microspheres were formulated and the effect of polymer concentration on the characteristics, release, and lung deposition of microspheres was examined. The use of alginate-kappa carrageenan combination polymer, produced by ionotropic gelation method including an aerosolization technique with $CaCl_2$ 0.3M crosslinker concentration and a cross-linking time of four hours, proved successful. This formula resulted in an inhalation delivery system of ciprofloxacin HCl microspheres possessing spherical characteristics; a smooth, flat surface; small particle size; high yield; entrapment efficiency; and high drug loading. Optimal mucoadhesive properties and swelling index were also demonstrated by the microspheres. One study found that increasing sodium alginate-kappa carrageenan concentration had no effect on particle size, drug loading, entrapment efficiency, or ciprofloxacin HCl-alginate-kappa carrageenan microsphere yield. The decrease in polymer concentration affected neither the cumulative release nor the activity duration of

the ciprofloxacin HCl released by the microspheres. Although the activated burst release mechanism produced these microspheres within ten minutes, the ciprofloxacin HCl microspheres were proven capable of delivering ciprofloxacin to the right and left lung tissue of the experimental animals. Ciprofloxacin HCl entered the system to be metabolized or excreted while some of the microspheres were still attached to the lung mucosa due to the mucoadhesive nature of the polymer. Further development of inhaled microspheres of ciprofloxacin HCl with polymer combination and the precise method of quantification of drug levels in the lung is recommended.

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