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Research Paper

Effect of Sodium Alginate-Carrageenan Concentration in Rifampicin Pulmospheres on Physical Characteristics, Release, and Anti-Tuberculosis Activity

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Abstract
Pulmonary tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*, which mostly attacks the lungs, but can also affect other organs. Tuberculosis is one of the biggest health problems worldwide. Conventional oral rifampicin preparations have several limitations, such as poor bioavailability, low solubility, and drug instability in the gastrointestinal fluid. Only a small portion of the tuberculosis drug can reach the alveoli, the main target of the tuberculosis drug. Drug delivery Systems are one of the solutions to this problem. They are a formulation or system that can mediate the delivery of therapeutic substances in the body to increase therapeutic effects, reduce drug side effects, increase bioavailability, and improve patient compliance. Pulmonary drug delivery requires a small dose and particle size, so microspheres are selected for lung delivery. This research aims to study the effect of sodium alginate concentration and carrageenan (0.75%, 1%, 1.25%) with a ratio of 1:1 on physical characteristics, in vitro release, and anti-tuberculosis activity. Preparation of Rifampicin Sodium Alginate-Carageenan Pulmospheres with Ionotropic Gelation-Aerosolization. Pulmospheres were evaluated for entrapment efficiency, morphology, yield, particle size, drug loading, in vitro release, and *Mycobacterium smegmatis* activity. Increasing concentrations of sodium alginate and carrageenan produce rifampicin pulmospheres with good physical characteristics, increase rifampicin release, and result in inhibitory activity against *Mycobacterium smegmatis*.

Keywords
Pulmospheres, Rifampicin, Sodium Alginate-Carrageenan, Anti-Tuberculosis, *Mycobacterium smegmatis*

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1. INTRODUCTION

Each year, tuberculosis affects more people worldwide. The disease is typically transmitted via airborne particles containing *M. tuberculosis* from a person who has tuberculosis. This bacterium is an acid-resistant gram-positive bacterium with very slow growth (Patricia, 2020). Four first-line medications are used in the six-month course of standard TB treatment: isoniazid (INH), rifampicin (RIF), ethambutol (ETH), and pyrazinamide (PZA) (Khadka et al., 2022). Rifampicin is a first-line drug for anti-tuberculosis therapy. Despite its effectiveness, oral rifampicin in its traditional form suffers from low bioavailability, poor solubility, degradation in the digestive system, and only a small percentage of anti tuberculosis drug that can reach the alveolus as the main target of anti tuberculosis drugs and have hepatotoxicity effects (Rajabnezhad et al., 2016). To achieve bactericidal concentrations at the infected site, substantial dosages of anti-TB medications and extended treatment regimens are required, which necessitates strict adherence and results in drug resistance. Thus, a focused drug delivery approach may be able to overcome obstacles to TB treatment. (Rani et al., 2024).

One strategy to address the drawbacks of rifampicin instability, limited solubility, and bioavailability is by administering drugs directly to the lungs through the inhalation route and with controlled release. By administering drug through inhalation, the drug reaches the respiratory tract directly, increasing its concentration in the lungs and lowering the potential for systemic side effects such as hepatotoxicity (Paranjpe

and Goymann, 2014). These multiparticulate systems, known as pulmospheres, are engineered to provide controlled drug delivery, improving bioavailability, stability, and site-specific targeting. Additionally, pulmospheres can mask offensive flavors and scents and shield medications from environmental factors including heat, moisture, and oxidation (Uyen et al., 2019). When compared to traditional dosage forms, this delivery method offers numerous benefits, including improved patient comfort, enhanced compliance, reduced toxicity, and higher efficacy (Kadam and Suvarna, 2015). Active ingredients from herbal medicines, including rutin, camptothecin, cedar oil, tetrandrine, and quercetin, have been successfully incorporated into microspheres to improve their therapeutic delivery. Information on entrapped microspheres and magnetic microspheres has also become widely available in recent years (Lakshmana et al., 2009).

In a study analyzing ibuprofen within the context of the Biopharmaceutics Classification System (BCS), it was identified as a class II drug, indicating low solubility, consequently, absorption becomes slow, irregular, and incomplete, requiring the development of formulations like microspheres to improve solubility and overall effectiveness. The research demonstrated successful formulation of ibuprofen microparticles, improving its solubility (Ferdiansyah et al., 2017). To increase the bioavailability of domperidone, it can be modified into a microsphere that can hold the drug longer in gastric to improve the bioavailability (Apridamayanti et al., 2020). In research carried out by Patil and Sawant (2009), alginate provides mucoadhesive ability of around 70-85%, and an increase in the polymer ratio will increase the mucoadhesive properties of alginate. Gavini et al. (2005) reported that alginate microspheres exhibit a mucoadhesive percentage of 78%. In addition, the advantage of using alginate as a copolymer in microspheres is that alginate has biocompatibility, biodegradability, and physicochemical properties that can be modified for the formulation of microsphere preparations (Bakhsi et al., 2017).

The stabilization, bioavailability, and targeted administration can all be improved by using polymers as carriers. The primary purpose of these polymers is to improve drug stability and dissolution, while different delivery approaches are being studied. However, when designing polymer-based delivery systems, it is important to consider factors such as biocompatibility, toxicity, and reason for use (Kurniawan et al., 2024). Sodium alginate is a polymer with very low toxicity. It is mainly used in the pharmaceutical industry and can be used to prolong drug release (Hariyadi and Islam, 2020). Alginate offers several advantages when used as a copolymer in pulmospheres, including its compatibility with biological systems, biodegradability, and the ability to modify its physicochemical properties for optimal formulations (Bakhsi et al., 2017). Carrageenan is a natural polymer with multiple functions, such as acting as a bulking agent, thickener, stabilizer, and gelling agent, making it a popular ingredient in the food industry. In the pharmaceutical industry, tablets formulated with carrageenan as an excipient have demonstrated excellent performance in controlled and sustained release formulations, along with favorable biocompatibility and viscoelastic properties (Qureshi et al., 2019). Carrageenan has good mucoadhesive properties because carrageenan is a hydrophilic polymer that can absorb water and has good swelling ability. Carrageenan itself can form hydrogels and interact with the mucous layer of the lungs so that it has good mucoadhesivity and can stick to the lungs (Alavi and Mortazavi, 2018). According to Rastogi et al. (2007), increasing polymer concentration can increase particle size, increase encapsulation efficiency, reduce release rate, and extend the Duration of Action (DOA) of the delivery system used.

The purpose of this study is to investigate how the physical properties, release, and anti-tuberculosis action of rifampicin pulmospheres are affected by changing the concentration of sodium alginate and carrageenan. The formulation of rifampicin made in the form of pulmospheres is expected to increase the effectiveness of rifampicin as an anti-tuberculosis agent delivered through the lungs. The variations in total polymer concentration used in this study were 0.75%, 1%, and 1.5%. This is based on the research of Hariyadi et al (2021), which stated that a kappa carrageenan concentration of 0.75% can produce high yield, drug loading, and entrapment efficiency. According to the results of this study, the pulmosphere yield increased from 52.53% to 63.19% as the concentration of carrageenan polymer was elevated from 0.25% to 0.75%. Drug loading increased from 15.63% to 38.72%, and entrapment efficiency also increased from 25.38% to 51.61%.

2. EXPERIMENTAL SECTION

2.1 Materials

The equipment used in the study included an analytical balance (Chyo Balance Corporation, Kyoto, Japan - 160), a magnetic stirrer (Dragon Lab MS-Pro), a centrifuge (Rotofix-32), a freeze dryer (Biobase BK-F12P), and a scanning electron microscope (FEI Inspect S50), thermoshaker (Gerhart), aerosol spray (pore 35 μm, pressure 40 psi), FTIR spectrophotometer (Pelkin Elmer Instrument), optical microscope (Axioscop 40 -Zeiss), autoclaf (Huxley HL340). The materials used were rifampicin (Kimia Farma), sodium alginate (Sigma-Aldrich Inc,), carrageenan (Danisco-Cultor), $CaCl_2$ (Solvay Chemicals International), lactose (Himedia), distilled water, wellplate 96 (NEST). All materials are of pharmaceutical grade.

2.2 Preparation of Rifampicin Alginate-Carrageenan Pulmospheres

The production of rifampicin alginate-carrageenan pulmospheres is based on the ionotropic gelation-aerosolization metho d with modifications (Hariyadi et al., 2018). The initial stage of producing rifampicin alginate-carrageenan pulmospheres started with the preparation of formula consisting of 0.75%, 1%, and 1.25% sodium alginate and carrageenan solutions (w/v) of each 100 mL of water was agitated using a magnetic stirrer. To prepare the solution, rifampicin was dissolved in 50 mL of phosphate-buffered saline (PBS). After rifampicin dissolves, the rifampicin solution is added to a solution of sodium

Table 1. Pulmospheres Formula

Components	Function	Formula 1	Formula 2	Formula 3
Rifampicin	Active Agent	0.25% b/v	0.25% b/v	0.25% b/v
Alginate	Polymer	0.75% b/v	1% b/v	1.25% b/v
Carrageenan	Polymer	0.75% b/v	1% b/v	1.25% b/v
$CaCl_2$	Cross Linker	0.5 M	0.5 M	0.5 M
Laktosa	Lyoprotectant	5% b/v	5% b/v	5% b/v

alginate and carrageenan while stirring with a magnetic stirrer until it is homogeneous. A 0.5 M $CaCl_2$ solution in 200 mL of water (w/v) was made as a crosslinker solution. $CaCl_2$ is easily crosslinked to sodium alginate because Ca ions are bound to guluronic acid residues, which are components of sodium alginate (Hariyadi et al., 2013). After the rifampicin solution, sodium alginate and carrageenan have been mixed homogeneously, Aerosol spray is used to apply the solution at a steady speed into the prepared $CaCl_2$ solution with an 8 cm distance and 40 psi of pressure, stirring continuously for two hours at 1000 rpm using a magnetic stirrer. After the stirring process has been completed, the rifampicin alginate-carrageenan pulmosphere is formed. Following a 6 minute centrifugation at 2500 rpm, the pulmosphere was removed from the $CaCl_2$ solution and given twice water washes. After being centrifuged, the results of the wet pulmosphere were resuspended in a 5% lactose solution as a lyoprotectant. Furthermore, the rifampicin alginate-carrageenan pulmosphere was dried with a freeze-dryer at -80°C. Table 1 showed formulation of rifampicin-alginate-carrageenan pulmospheres.

2.3 Particle size

Particle measurement was carried out using an optical microscope. The measurement stage is to place a certain amount of dry pulmosphere on the microscope glass object and measure as many as 300 particles using Raster Image software. Furthermore, the grouping of pulmospheric particles was carried out from the smallest particle size to the largest particle size. The particles are further organized into groups according to their size.

2.4 Morphology

The shape and surface of rifampicin were observed through observation using scanning electron microscopy (SEM). In observing the morphology of microspheres, the shape of the particles and the surface of the particles can be seen, such as whether they are porous or not (Fang et al., 2019).

2.5 Yield

The yield or recovery is determined by contrasting the weight of the pulmosphere-forming material with the overall weight of the pulmosphere that was produced. High yield values indicate an efficient manufacturing process, thus providing the potential for development in large-scale manufacturing processes (Jin et al., 2009).

2.6 Moisture Content

The calculation of moisture content was carried out with the Moisture Content Analyzer tool. A pulmosphere of 500 mg was put into a container, tapped, and then closed to start the heating process.

2.7 Drug Loading

The content of rifampicin was ascertained by dissolving 50 mg of alginate-carrageenan pulmosphere in 50 ml of phosphate buffer (pH 7.4) using sonication for 60 minutes. The sample was then screened, and a spectrophotometer was used to measure its absorbance at 473 nm. Three replications of the experiment were conducted (Hazra et al., 2015).

2.8 In Vitro Release

The release profile was tested using a thermoshaker at 37°C with a speed of 100 rpm. A 100 mL phosphate buffer solution with a pH of 7.4 was added to the sample. At each sampling, replacement of the release media with the same media was carried out. Samples underwent filtration using a Millipore membrane with a pore size of 0.45 μm. A UV-Vis spectrophotometer was used to measure the sample's absorbance at a wavelength of 473 nm. Rifampicin levels are determined by putting the sample absorbance value into the previously created standard Rifampicin curve equation (Hariyadi et al., 2013).

2.9 Anti-Tuberculosis Activity

Mycobacterium tuberculosis antibacterial test was performed with Resazurin Microtiter Assay (REMA). The anti-tuberculosis activity test was carried out using a suspension of *Mycobacterium smegmatis* OD 0.06 as much as 60 μL into wellpate 96, then each well was added to the pulmosphere formula of rifampicin alginate-caragenan, and rifampicin as a control, as much as 20 μL, then incubated for 24 hours at 37°C. After incubation, resazurin solution was added to wellplate 96 as a color indicator to determine bacterial growth inhibition. The pulmosphere formulas used are listed in Table 1.

3. RESULTS AND DISCUSSION

The formulation of rifampicin pulmospheres is expected to increase the effectiveness of rifampicin as an anti-tuberculosis agent delivered through the lungs. The production of rifampicin alginate-carrageenan pulmospheres is based on the ionotropic gelation-aerosolization method. The effective use of many conjugate matrices to increase stability has improved drug encapsulation utilizing the ionic gelation process. By forming a

stable gel network, the ionic gelation process effectively protects encapsulated compounds from degradation due to environmental stresses such as fluctuating pH, elevated temperatures, and mechanical agitation (Nahrowi et al., 2024). Table 2 provides detailed information on the physical characteristics of the rifampicin pulmospheres.

3.1 Particle Size

The particle size results obtained from the pulmosphere were in the range of 2.324 ± 0.128 to 2.625 ± 0.200 µm with a polydispersity Index (PDI) of 0.0033. The low PDI value (<0.1) confirmed the monodispersity of the particles. The size achieved falls within the ideal particle size of less than 5µm for delivery to the lungs (Vishwa et al., 2021). The results showed that when the concentration of polymer increased, so did the size of the particles. This is because the viscosity of the polymer increases with increasing concentration, leading to an increase in the size of the pulmonary droplet (Purwanti et al., 2018). The particles will only reach the oropharynx if it is larger than 5 μm; if it is less than 1 μm, the particles will emerge with the expiration of air. The formation of microparticles or micronization can increase drug delivery in the body. The decrease in particle size that occurs causes the surface area between particles to increase, causing its solubility to increase (Ferdiansyah et al., 2017).

Hariyadi et al. (2019) used aerosolization procedures with varying polymer concentrations to create ciprofloxacin microspheres with alginate polymer for inhalation delivery, resulting in microspheres with sizes ranging from 1.23 μm to 1.43 μm. A greater amount of alginate in the formulation contributes to an increase in particle size. Sig = 0.087 (>0.05) was the value obtained from the Way Anova statistical analysis, indicating that an increase in polymer concentration of 0.25 % had no significant effect on the particle size of the pulmospheres.

3.2 Morphology

Organoleptic observation in Figure 1 reveals that the rifampicin pulmonary morphology is a reddish-orange, odorless powder. The SEM micrographs demonstrated that the particles in all formulas were uniformly spherical and had smooth surfaces, which can be seen in Figure 2.

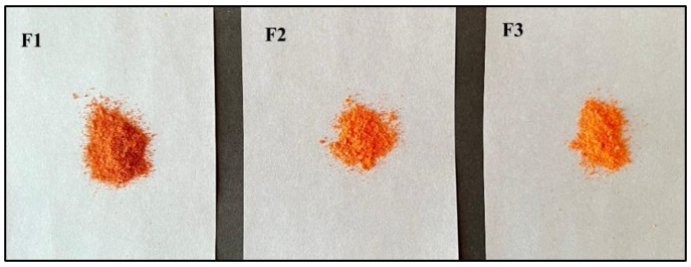

Figure 1. Organoleptic Evaluation of Dry Pulmospheres

The spherical morphology of the pulmosphere and smooth surface can increase acceptability for lung delivery, and does not irritate the pulmonary mucosa. Additionally, the particles must have a spherical form because this spherical shape has good flow properties, so that it allows the drug to reach the respiratory tract. On the inhalation route, the drug will go directly to the target organ (respiratory tract) and minimize the first-pass metabolism experienced by the active agent, so that there is an increase in drug concentration in the respiratory tract.

3.3 Overlay Formula and Rifampicin IR Spectra

The functional groups of kappa carrageenan are identified in the FTIR spectrum at 921.19 cm^{-1} (3,6-anhydrogalactose) and 840.79 cm^{-1} (galactose-4-sulfate). The similarity in functional groups between the sample and the standard spectrum indicates that the kappa carrageenan complies with the established standard. The FTIR spectrum of sodium alginate shows distinct peaks at 886.64 cm^{-1} and 758.17 cm^{-1}, corresponding to guluronate and manuronate fingerprints, respectively. Figure 3 demonstrated the IR spectra corresponding to each formulation.

This spectral result was conducted to prove the interaction that occurs in the pulmospheres formed between the rifampicin, polymer, and crosslinker. Figure 3 provides the spectral wavenumber information for the rifampicin-alginate-carrageenan pulmosphere formulation. The change in wavenu mber suggests a chemical interaction occurring between rifampicin, the polymer, and the crosslinking compound. It also demonstrates that the formation of pulmospheres was achieved as intended.

3.4 Yield

The results showed that the yield varied from 75.18 ± 3.64 to 78.09 ± 2.19. A yield close to 100% suggests that the pulmosphere preparation method is highly efficient and maximizes production (Kumar et al., 2011). The yield data obtained were then analyzed statistically using One Way ANOVA, which has a value of Sig = 0.431 (>0.05), which means that the increase in polymer concentration did not provide a significant difference to the pulmospheres yield value. Durgapal et al. (2017) reported that increasing polymer concentration leads to higher yields, as stronger interactions between the polymer and crosslinker enhance drug entrapment and result in greater pulmosphere formation compared to lower concentrations.

3.5 Moisture content

The moisture content of microparticles intended for pulmonary delivery is maintained below 10% (Rui, 2016). Meanwhile, according to Rey and May (2004), moisture content that can guarantee the stability of powder preparations over a long period ranges from 1-3%. Each pulmosphere formulation showed moisture content values ranging from 2.09% to 3.21%. The moisture content value of all formulas showed that all the pulmospheres have met the requirements for pulmonary delivery preparations. According to the One Way ANOVA statistical analysis, the increase in polymer concentration significantly af-

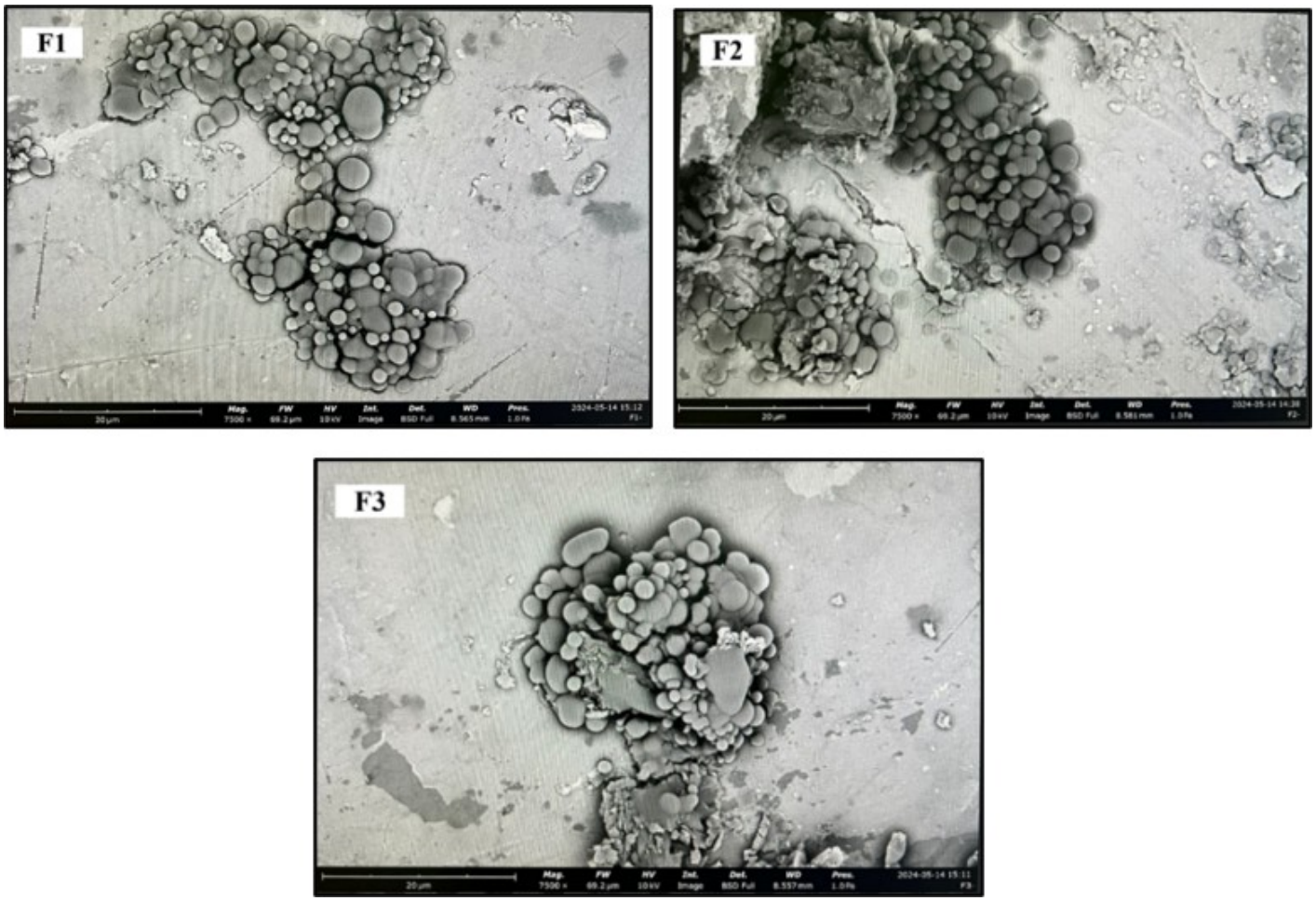

Figure 2. Morphology of Rifampicin Loaded Alginate- Carrageenan Pulmospheres in Magnification 7500 ×

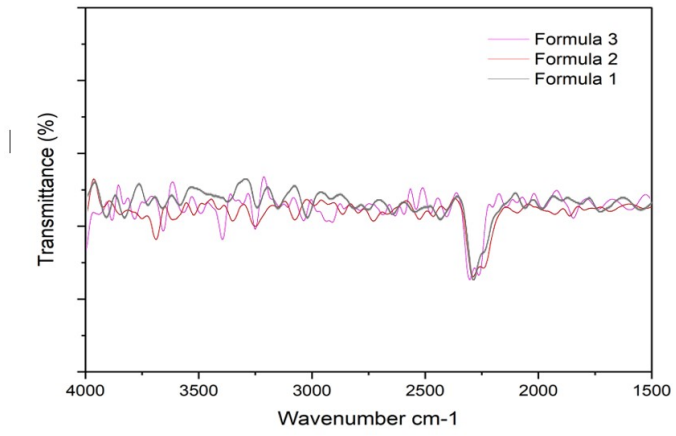

Figure 3. IR Spectra of Formula 1, Formula 2, and Formula 3 of Rifampicin Pulmospheres

fects moisture content, with a value of Sig = 0.003 (<0.05). The Post-Hoc Tukey HSD test was used to continue the investigation which showed that F1 and F2 had a significant difference, where the value of Sig = 0.006 (<0.05). In addition, F1 and F3 also had a significant difference, where the value of Sig = 0.005 (<0.05). High moisture levels in inhalation powders can lead to condensation and particle agglomeration, making moisture control a key aspect in formulation development, which may affect drug loading, entrapment efficiency, and poor powder flowability due to cohesion between particles (Jung et al., 2018).

3.6 Drug Loading

Based on the results of drug loading testing, F1, F2, and F3 obtained values of 1.68% ± 0.13, 1.83% ± 0.02, and 2.05% ± 0.06. Based on these results, it is known that there is a tendency to increase drug loading with an increase in polymers. According to the findings of the One Way Anova statistical study, Sig = 0.006 (<0.05). This figure indicates that the increase in polymer concentration makes a significant difference to the drug loading pulmosphere. According to the results of the Tukey HSD Post-Hoc test, Formula 1 differed significantly from Formula 3, with a significance value of 0.005, which is below the 0.05 threshold. The analysis indicated that neither the comparison between Formula 1 and Formula 2 (siq = 0.162), nor

that between Formula 2 and Formula 3 (siq = 0.056), reached statistical significance. In drug loading testing, The ratio of polymers increases in tandem with an increase in drug loading caused by more rifampicin that can bind to polymers and fill the remaining empty egg-box. These results can be concluded when the polymer ratio rises, drug loading also rises. An increase in alginate concentration during pulmosphere formation enhances the drug entrapment efficiency, leading to higher drug loading. Low drug loading values (less than 50%) were observed, possibly due to inadequate cross-linking between $CaCl_2$ and the alginate–carrageenan matrix, leading to poor drug absorption. In the event of an increase in polymer concentration, the concentration of $CaCl_2$ as a crosslinker must be increased to adequately bind to the polymer and be able to trap more rifampicin (Manjanna et al., 2010).

3.7 Entrapment Efficiency

Based on the results of entrapment efficiency in all formulas, ranging from 70.69% ± 1.17 to 88.18% ± 2.78. Based on these results, it is known that there is a tendency to increase entrapment efficiency with an increase in polymer concentration. This is following other studies that say that increasing the amount of polymer used can increase the efficiency of drug absorption in the microsphere. This effect is attributed to the improved ability of the polymer matrix to encapsulate the drug at higher concentrations (Apridamayanti et al., 2020). According to the findings of the Way Anova statistical study, Sig = 0.000 (<0.05) indicates that the increase in polymer concentration makes a significant difference to the entrapment efficiency in the pulmosphere. The analysis demonstrated that Formula 1 and Formula 2 differed significantly, according to the Post-Hoc Tukey HSD test. (Sig value 0.007 <0.05), between formula 1 and formula 3 (Sig value 0.00 <0.05), and between formula 2 and formula 3 (Sig value 0.001 <0.05). Among all formulations, formula 3 showed the greatest entrapment efficiency. Higher concentrations of carrageenan and sodium alginate will make the solution more viscous, which will increase the size of the droplets and boost the entrapment efficiency (Hariyadi et al., 2014). The tendency for increasing drug loading and entrapment efficiency can be due to the increase in cross-connection points along with the increase in polymer concentration, which can affect the drug loading value and entrapment efficiency (Dashora et al., 2007). The impact of the drug-to-polymer mass ratio on the drug loading and entrapment efficiency of sodium alginate-paclitaxel microspheres for inhalation was discovered by (Alipour et al., 2010).

3.8 In Vitro Release

Based on the results of the release test, there was an increase in drug release from the pulmosphere along with the increase in polymers. The cumulative % of released drugs for each formula was 81.07% ± 3.619, 87.70% ± 1.964, and 92.32% ± 3.761, respectively, for formula 1, formula 2, and formula 3, as shown in Figure 4. The results of the Way ANOVA statistical analysis showed a significant difference between F1 and F3 with a Sig value of 0.027 (<0.05), while F1 with F2 and F2 with F3 did not show a significant difference. This releasing capability is significantly influenced by the use of polymers. There is an immediate release (burst release) at the beginning of the 15th minute. This occurs because there are drug molecules on the surface of the polymer matrix that diffuse out faster than drug molecules in the core. Based on the results of the release test, there was an increase in drug release from the pulmosphere along with the increase in polymers. Compared to the other formulations, Formula 3 shows superior drug release in terms of both speed and extent. This is thought to be due to the crosslinking of polymers with $CaCl_2$ crosslinkers. The amount of the polymer binding site with $CaCl_2$ increases with the concentration of alginate and carrageenan polymers. If the number of Ca^{2+} ions is insufficient to cross-link with all polymer binding sites, this can affect the process of pulmosphere formation and lead to a faster and more efficient release of the drug from the matrix (Mandal et al., 2010), then, increasing the polymer concentration must be followed by an increase in $CaCl_2$ as a cross-linker so as not to cause a decrease in the density of the polymer matrix which causes the drug to be easily released from the system (Rastogi et al., 2007). Based on the results of determining the release kinetics, the selected release kinetics model is a Higuchi release model because it has a relation coefficient value (R^2) that is closest to 1. Diffusion-controlled systems allow the drug to diffuse and be released into the medium through pores formed by the polymeric chains' intrinsic semipermeability or swelling (Amiruddin and Hariyadi, 2023).

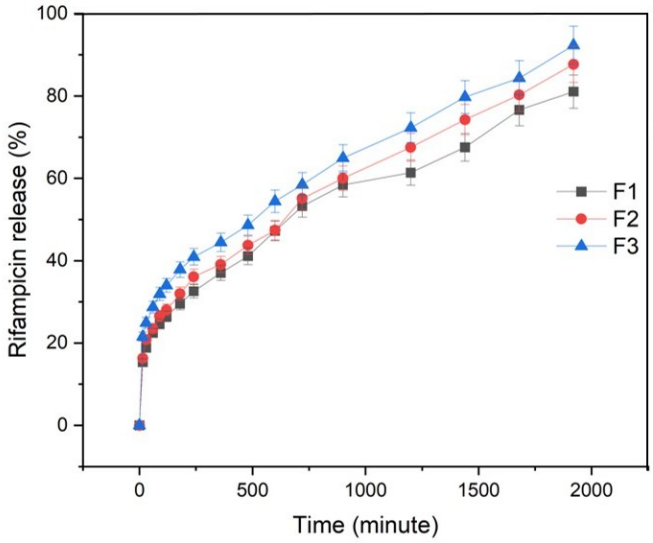

Figure 4. Cumulative release (%) of Rifampicin From the Pulmospheres. One-way ANOVA Statistical Analysis Showing a Significant Difference Between F1 and F3.

The release kinetics and physical characteristics of microspheres are shaped by variables like polymer type and ratio, polymer concentration, the crosslinker used, the concentra-

Table 2. Physical Characteristics Examination

Formula	Particle Size (μm)	PDI	Yield	Drug Loading	Entrapment Efficiency (%)
Formula 1	2.324 ± 0.128	0.033	75.18 ± 1.64	1.68 ± 0.13	70.69 ± 1.17
Formula 2	2.547 ± 0.039	0.033	76.50 ± 1.25	1.83 ± 0.02	77.64 ± 0.26
Formula 3	2.625 ± 0.200	0.033	78.09 ± 1.19	2.05 ± 0.06	88.18 ± 2.78

tion of crosslinker, and the method of manufacture. A higher sodium alginate content increases the solution's viscosity, which contributes to the development of microspheres with greater thickness and surface density, because the pace at which the release medium diffuses into the microspheres slows down, making it take longer for quercetin to be released from them (Kalalo et al., 2022).

3.9 Anti-Tuberculosis Activity

The observation made in testing the anti-tuberculosis activity was the inhibitory power of each formula in inhibiting the growth of *Mycobacterium smegmatis* bacteria. *Mycobacterium smegmatis* is often used as a model to study tuberculosis (TB) because it has many similarities with TB-causing bacteria. *Mycobacterium smegmatis* is a non-pathogenic species, so it is safer to use in research and can grow faster compared to *Mycobacterium tuberculosis*. *Mycobacterium smegmatis* has many genetic similarities to *Mycobacterium tuberculosis*, including similarities in core proteins.

Based on the statistical analysis results using Two-Way ANOVA, the value of Sig = 0.000 (<0.05) was found in the formula and concentration. This indicates that the formula and concentration significantly affect the resistance of *Mycobacterium smegmatis* bacteria. Furthermore, the Post-Hoc Tukey HSD test showed a significant difference between each formula with standard rifampicin, formula 1 with formula 3, and formula 2 with formula 3. Post-Hoc Tukey HSD showed significant differences between each formula with standard rifampicin, formula 1 with formula 3, and formula 2 with formula 3.

This test is also conducted to identify the minimum inhibitory concentration (MIC) within the pulmosphere. It can be seen that the MIC of each formula and standard rifampicin is at a concentration of 0.5 ppm, which is the lowest drug concentration where no bacterial growth can be seen again in Figure 5. Another study explained that MIC rifampicin was in the range of 0.5-1 ppm (Taneja and Tyagi, 2007). IC_{50} (inhibitory concentration) is the concentration of sample solution needed to inhibit 50% of bacterial growth. Standard rifampicin has an IC_{50} value of 2.681 ppm; rifampicin alginate-carrageenan pulmosphere has IC_{50} values of 2.191 ppm, 2.051, and 1.945 in formula 1, formula 2, and formula 3, respectively. In the pulmosphere formulation, IC_{50} values are noticeably lower. This study proves that the pulmosphere delivery system is one way to increase the effectiveness of a drug.

Resazurin is blue when it is oxidized and turns pink when reduced by living cells. Suppose the replacement of the *Mycobacterium smegmatis* test bacteria is carried out. In that case, it must be re-optimized related to the protocol for using REMA Assay, such as the time of culture of the test bacteria, the incubation time of wellplate 96 before and after adding resazurin, and the concentration of bacterial inhibition to be used. The concentration of drugs that inhibit the growth of *Mycobacterium smegmatis* was determined by comparing color changes in drug-containing wells with color changes in control wells. The color change after the addition of resazurin can be seen in Figure 6. The color change to pink indicates that there is still activity from *Mycobacterium smegmatis*. The color change that occurs due to lactate dehydrogenase in living cells converts resazurin into resorufin, which mediates the coupling of enzymatic reactions, and produces a strong fluorescence signal with a pink color. Fluorescence readings were taken using a microplate reader set to an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

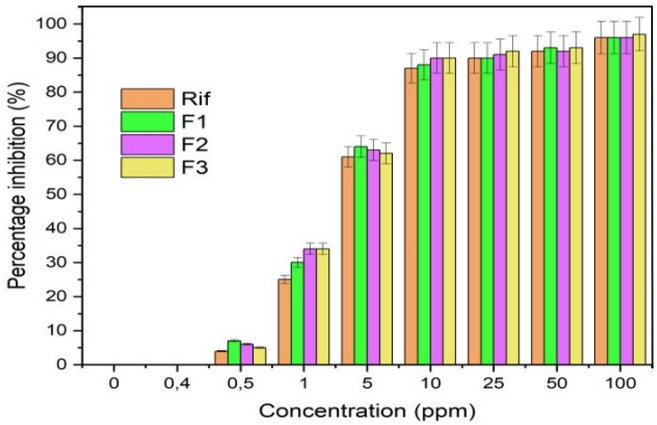

Figure 5. Inhibition Activity of Rifampicin Alginate-carrageenan Pulmospheres Against *Mycobacterium smegmatis*

A significant difference exists between each formula with rifampicin standard, formula 1 with formula 3, and formula 2 with formula 3. Based on the results of the statistical analysis using Two-Way ANOVA showed a Sig value = 0.000 (<0.05) in the formula and concentration. This indicates that the formula and concentration significantly affect the inhibition of *Mycobacterium smegmatis* bacteria. Furthermore, the Post-Hoc Tukey HSD test showed a significant difference between each formula with standard rifampicin, formula 1 with formula 3, and formula 2 with formula 3.

If the test bacteria, *Mycobacterium smegmatis*, are replaced, then the REMA Assay usage protocol must be re-optimized,

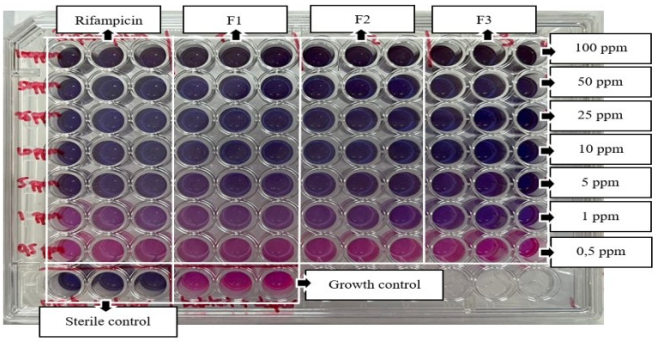

Figure 6. Inhibitory Activity of Rifampicin and Pulmospheres Against *Mycobacterium smegmatis* After the Addition of Resazurin and Incubation for 6 Hours

such as the culture time of the test bacteria, the incubation time of the 96 plates before and after resazurin is added, and the bacterial inhibition concentration to be used.

4. CONCLUSIONS

Inhalation microspheres delivery systems increase the stability and bioavailability of drugs while permitting control release. Size, shape, concentration, and polymer selection are important determinants of the efficacy. Sodium alginate and carrageenan polymers can be a combination of choice as a delivery system. Increasing concentrations of sodium alginate polymers and carrageenan from 0.75% (F1), 0.1% (F2), and 1.25% (F3) produce rifampicin alginate-carrageenan pulmosphere with physical characteristics (particle size, good yield) and provide increased drug loading value, as well as entrapment efficiency. The best formula of rifampicin alginate-carrageenan pulmosphere is F3, where it has the best physical characteristics, with the highest release of rifampicin.

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