Prisma Sains: Jurnal Pengkajian Ilmu dan Pembelajaran Matematika dan IPA IKIP Mataram
https://e-journal.undikma.ac.id/index.php/prismasains/index
e-mail: prismasains.pkpsm@gmail.com

October 2024. Vol. 12, No. 4
p-ISSN: 2338-4530
e-ISSN: 2540-7899
pp. 706-713

Harvesting and Evaluating Uptake Machropage Induced by Ciprofloxacin HCl-Alginate-Carrageenan Pulmosphere

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Received: January 2024; Revised: August 2024; Published: October 2024

Abstract

Ciprofloxacin HCl-Alginate-Carrageenan Pulmosphere has emerged as a therapy for lung infections. Ciprofloxacin HCl-Alginate-Carrageenan Pulmosphere consists of Ciprofloxacin HCl, Sodium Alginate, Kappa Carrageenan, and CaCl2 made using the ionotropic gelation method with the aim of delivering the drug via inhalation. Ciprofloxacin HCl-Alginate-Carrageenan Pulmosphere exhibits good physical characteristics that can be further studied in vitro using alveolar macrophage cells. Alveolar macrophages demonstrate a high level of effectiveness in removing pathogenic microorganisms from the lungs. More than 80% of alveolar macrophages can be easily obtained through lung lavage for study. This research aims to determine the uptake of Ciprofloxacin HCl-Alginate-Carrageenan Pulmosphere by alveolar macrophages. The extraction of alveolar macrophage cells was carried out through the utilization of the BAL (Bronchoalveolar Lavage) method. Pulmosphere was labeled with a fluorescent dye, Rhodamine-B, for visualization. Uptake evaluation was performed using the Nikon Eclipse TS2R Inverted Microscope. The findings suggest that alveolar macrophages have the ability to uptake Ciprofloxacin HCl-Alginate-Carrageenan Pulmosphere. This research significantly enhances current knowledge by revealing previously unidentified variables. The results not only question established theories but also provide practical insights for the field of pharmaceutics. As a result, this study deepens our comprehension of certain aspects and sets the stage for future research in related domains.

Keywords: Pulmosphere, Ciprofloxacin HCl, Alveolar macrophage uptake, Bronchoalveolar Lavage, fluorescence

How to Cite: Aqsha, A., Rahmadi, M., & Hariyadi, D. (2024). Harvesting and Evaluating Uptake Machropage Induced by Ciprofloxacin HCl-Alginate-Carrageenan Pulmosphere. *Prisma Sains : Jurnal Pengkajian Ilmu dan Pembelajaran Matematika dan IPA IKIP Mataram*, *12*(4), 706-713. doi:https://doi.org/10.33394/j-ps.v12i4.10674

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INTRODUCTION

Respiratory disorders encompass a variety of illnesses that impact the respiratory system, which includes the airways and lungs, resulting in deviations from normal physiological functioning. The emergence of these conditions can be attributed to diverse factors, including a weakened immune system, smoking, microbial infections, environmental factors such as pollution, or a genetic predisposition. Microbial fragmentation in the alveoli occurs with the help of immune cells and chemical factors such as alveolar macrophages, natural killer cells, surfactants and complement (Alipour & Suntres, 2014; Mehta et al., 2019).

Alveolar macrophages are phagocytic cells that originate from monocytes and are plentiful within the lungs. They serve as a defense against microorganisms entering the lungs and contribute to resistance against certain groups of bacteria, making alveolar macrophages a common treatment target. Various pathogens that can cause infections in this area and attack the lungs interact with alveolar macrophages. Upon inhalation of pathogenic organisms, alveolar macrophages initiate the synthesis and release of various inflammatory mediators,

including cytokines, chemokines, arachidonic acid metabolites, lysozyme, and antimicrobial proteins. This process enhances the inflammatory response and facilitates phagocytosis. Additionally, alveolar macrophages play a role in recruiting activated neutrophils to the alveolar region (Allard et al., 2018; El-Sherbiny et al., 2015).

Diverse mechanisms of phagocytosis have been identified, and the primary initial step involves the recognition of foreign particles through opsonization, a process in which the particles are coated with soluble proteins. After opsonization, the opsonized particles, bound to specific receptors such as complement or immunoglobulin receptors on the phagocyte membrane through adsorbed surface opsonin proteins, initiate receptor activation. This activation, in turn, induces cytoskeletal rearrangement or the formation of pseudopodia on the phagocyte surface, ultimately leading to the engulfment of the particles (Dunn et al., 2011; Patel et al., 2015).

Ciprofloxacin HCl inhalation has demonstrated good tolerability and microbiological activity. The benefits of inhaling include elevated antibiotic levels at the infection site, heightened bacterial elimination, and a decrease in systemic toxicity with prolonged administration. The uptake of particulate carrier systems by alveolar macrophages can be modulated by deceiving their phagocytic function and foreign body recognition ability. Pulmosphere formulation was chosen because it can be targeted to alveolar macrophages and has a small size (1-5µm) so it is suitable for drug delivery to the lungs. Pulmospheres were made using a combination of sodium alginate and kappa carrageenan polymers, and CaCl2 crosslinker using the ionotropic gelation method. Macrophage uptake was observed to assess the pulmonary absorption of Ciprofloxacin HCl, labeled with fluoresceinamine, by macrophages. The method used to determine the internalization of compounds into living cells is fluorescence labeling. The labeling requirement is that the compound must produce fluorescence due to its ability to absorb light or electromagnetic radiation. In this study, rhodamine-B was used as a fluorochrome which can provide fluorescence equivalent to ciprofloxacin HCl. Macrophage uptake was observed using a Nikon Eclipse TS2R Inverted Microscope (Ragazzi et al., 2014). The aim of this research is to determine which Ciprofloxacin-alginate-carrageenan pulmosphere will be uptaken by macrophages.

METHOD

Materials

Ciprofloxacin HCl pharmaceutical grade (Sigma Aldrich); Natrium Alginate (Sigma Aldrich); Kappa Carragenan (Sigma Aldrich); $CaCl.2H_2O$ pharmaceutical grade (Solvay chemicals International); Rhodamine B; Maltodextrin pharmaceutical grade (BrataChem); Aquademineralisata (BrataChem); Natrium citrate pharmaceutical grade (Weifang Ensign Industry Co.I.td), Rat (8-13 weeks), PBS pH 7,4 (Thermo Fisher Scientific), Trypan blue (Sigma-Aldrich), RPMI 1640 (PAN Biotech).

Preparation of Fluorescent Ciprofloxacin HCl-Alginate-Carrageenan Pulmosphere

Microspheres containing ciprofloxacin HCl were prepared using the ionotropic gelation method with a drug concentration of 0.5%. Ciprofloxacin HCl was dissolved in a mixture of alginate and kappa carrageenan polymers, after which a cross-linking agent, $CaCl_2$, was added at a precise concentration. Rhodamine B, previously dissolved in water, was added to the drug and polymer mixture. Each formula was cross-linked for 120 minutes, and stirring was conducted at a speed of 1000 rpm. The microspheres were washed using centrifugation techniques and dried using a freeze dryer for 7 days, with the addition of 5% maltodextrin lyoprotectant for stabilization. This method was chosen because Rhodamine B together with antibiotics can reach intracellular targets. However, unfortunately this method has a drawback, namely the large molecular weight of Rhodamine B so that the penetration of the compound into the cytosol, especially in gram-negative bacteria, is less than optimal.

Collecting Alveolar Macrophages using Lung Lavage

The alveolar macrophages were obtained from male Wistar rats (8-12 weeks ; 180-200 grams). Conical tubes of 15 ml capacity were prepared and filled with 3 ml complete media (RPMI 1640, 1x glutaMAX, 1x Pyrivate, 1x Penicillin, 10% FBS, and LPS). The BAL buffer (PBS, 2mM EDTA, 0.5% FBS) was heated to 37°C in a water bath and maintained at this temperature until the procedure was completed. Wistar rat were anesthetized with intraperitoneal injections of ketamine (50 mg/kg BW) and xylazine (2 mg/kg BW) in a supine position. The skin on the abdominal area was cleaned with 70% alcohol and then opened with a dissection set. A small incision was performed in the upper trachea, situated just below the larynx. The tracheal portion facing downward should remain intact, avoiding cutting the entire trachea. Utilize the incision to introduce a slightly blunt 18-G cannula, directing it 5 mm deeper towards the lungs. Exercise caution to avoid damaging lung tissue. Connect a 5 ml syringe, containing 2 ml of warm BAL buffer, to the inserted cannula. Administer 2 ml of the buffer while stabilizing the cannula position with the other hand. Pull the plunger to gather BAL fluid in the syringe, approximately 800-900 µL. Maintain moderate pressure to prevent alveolar rupture and loss of BAL fluid. Following injection and collection, observe the lungs inflating and deflating. Filter the obtained BAL fluid through a 70 µm cell strainer into a 15 ml conical tube containing 3 ml of complete media. Repeat steps 4-8 two to three more times using warm and fresh BAL buffer, accumulating in the same conical tube (Busch et al., 2019; Chavez-Santoscoy et al., 2012).

Processing of Harvest Results Alveolar Macrophage Lung Lavage

Collect the cells and centrifuge at 300 xg for 5 minutes at 4°C. Discard the supernatant, ensuring the cell pellet appears white. If there's a red/pink color, it indicates inadvertent blood collection; in such cases, add 1 ml of cell lysis buffer and incubate for 2 minutes at room temperature to lyse any remaining red blood cells. Halt the lysis process by filling the tube with complete media and collect cells through centrifugation as previously instructed. Discard the supernatant, and now the cell pellet should exhibit a white color. Suspend the cellular pellet in 500 µl of BAL buffer and extract a sample for enumeration using a hemocytometer. After staining with Trypan Blue to eliminate dead cells, tally only the live cells identified as Trypan Blue negative. Determine the total cell count per BAL. Dilute the cells to 5 x 10^5 live cells per rat using RPMI media in a 6-well plate. Incubate for 48 hours in a 5% CO_2 incubator to optimize cell culture.

Addition Ciprofloxacin HCl-Alginate-Carrageenan Pulmosphere

Prepare a stock solution with a concentration of 1000 ppm. Dilute the stock solution to a concentration of 150 ppm. Vortex the Ciprofloxacin HCl-Alginate-Carrageenan Pulmosphere suspension before adding it to the well plate. Tilt the well plate at approximately a 45° and pipet approximately 1 ml of media. Pipet 1 ml of Pulmosphere suspension. Avoid pipetting up and down to mix. Incubate for 1 to 2 hours to allow cell interaction with Pulmosphere.

Evaluate the Macrophage Uptake of Ciprofloxacin HCl-Alginate-Carrageenan Pulmosphere Using an Inverted Nikon Eclipse TS2R Microscope

After incubation, discard 500 µl of media, wash the cells once with 500 µl of PBS, then block with 1 ml of PBS. Observe under a fluorescence microscope with a TxRed filter (Wijagkanalan et al., 2008).

RESULTS AND DISCUSSION

In this study, Wistar rats were used as test animals which were anesthetized using Ketamine 50 mg/kg and Xylazine 2 mg/kg BB intraperitoneally in the supine position (Underwood & Anthony, 2020). The medium used in the macrophage cell culture process is RPMI 1640. This medium is used because it can keep cultured macrophages alive. RPMI medium contains several ingredients for culture such as $CaCl_2$, KCl, NaCl, NaHCO, glucose, glutathione, phenol red, amino acids (tyrosine, valine, and vitamins (Biotin,pantothenate)

(Santoso et al., 2013). The incorporation of BAL buffer is indispensable as alveolar macrophages tend to stick to the alveolar wall, using PBS alone proves inefficient in cell removal and leads to a significantly reduced yield (Nayak et al., 2018). The uptake process that occurs in macrophages is included in the immune system process that occurs in the body between antigens and antibodies (Wijagkanalan et al., 2008).

Collecting alveolar macrophages from lung lavage fluid and administering various pulmonary formulations of ciprofloxacin can offer valuable insights into the chemical properties of different particles in activating macrophages, subsequently influencing antigen presentation. Moreover, these investigations serve as valuable tools for evaluating the ability of particulate adjuvant formulations to activate alveolar macrophages, serving as a preliminary step before embarking on more extensive and intricate studies (Torres et al., 2011).

Macrophage medium preparation was carried out 48 hours before the uptake process was carried out so that culturization could be optimal. The process of mixing the ciprofloxacin HCl pulmosphere and macrophages was carried out after the cell culture process in a 5% CO_2 incubator. The cultured macrophage cell suspension will then be mixed with ciprofloxacin pulmosphere at a concentration of 150 ppm. The suspension was then left in a 5% CO_2 incubator for 1 hour so that absorption could occur optimally. The absorption process requires a fairly fast time because macrophages have an active response in the body to antigens because the stimulation of antibody formation in the body against antigens occurs quickly. The mixture of macrophages and pulmospheres was cleaned using PBS so that when the research was carried out using a microscope, no impurities or debris were visible. Observations were carried out on 3 samples, namely macrophage cells, macrophage cells and rhodamine-B and macrophage cells with ciprofloxacin HCl pulmosphere (Ragazzi et al., 2014).

Figure 1 illustrates Pulmosphere suspensions prepared both before and after sonication, emphasizing the necessity of sonication to achieve proper particle dispersion. Pulmosphere morphology examination using a Nikon Eclipse TS2R Inverted Microscope. The results of the examination can be seen in **Figure 2.**

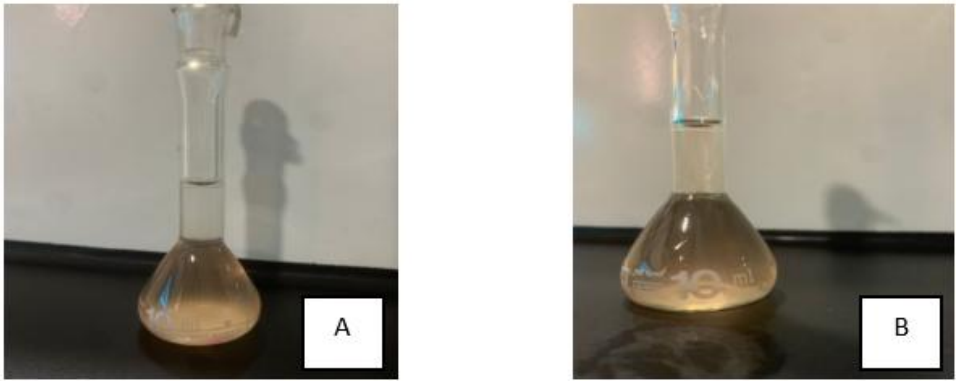

Figure 1. Pulmosphere Ciprofloxacin HCl (A) Before Sonication and (B) After sonication

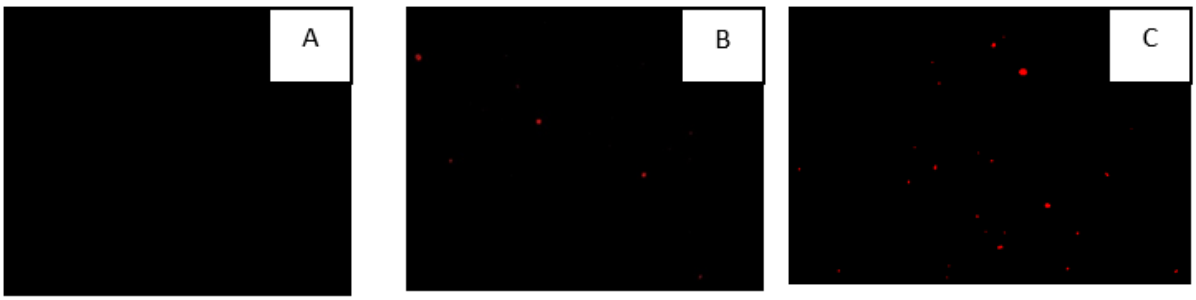

Figure 2. Results of Pulmospheric Macrophage Uptake of Ciprofloxacin HCl Labeled with Fluorosceinamine Using an Inverted Nikon Eclipse TS2R Microscope at 4x Magnification and a TxRed Filter. (A) Control Cells; (B) Rhodamine B control; (C) Ciprofloxacin HCl-Alginate-Carrageenan Pulmosphere

Ciprofloxacin HCl pulmosphere absorption results show that the pulmosphere formula shows a red color caused by the Ciprofloxacin HCl pulmosphere which produces red fluorescence due to the presence of Rhodamine B as the fluoresceinamine used. The intensity of the pulmosphere formula increased compared to the control as indicated by the red color in the cells. This means that if observed using an inverted microscope, ciprofloxacin absorption in the lungs occurs by alveolar macrophages (Jiang et al., 2017; Song et al., 2015). The absorption of Pulmosphere may be attributed to the particle size and morphology, which are advantageous for AM absorption (Marcianes et al., 2019; Parikh et al., 2014; Soni et al., 2024). Next, overlay the non-fluorescent cell observations with the fluorescent cells.

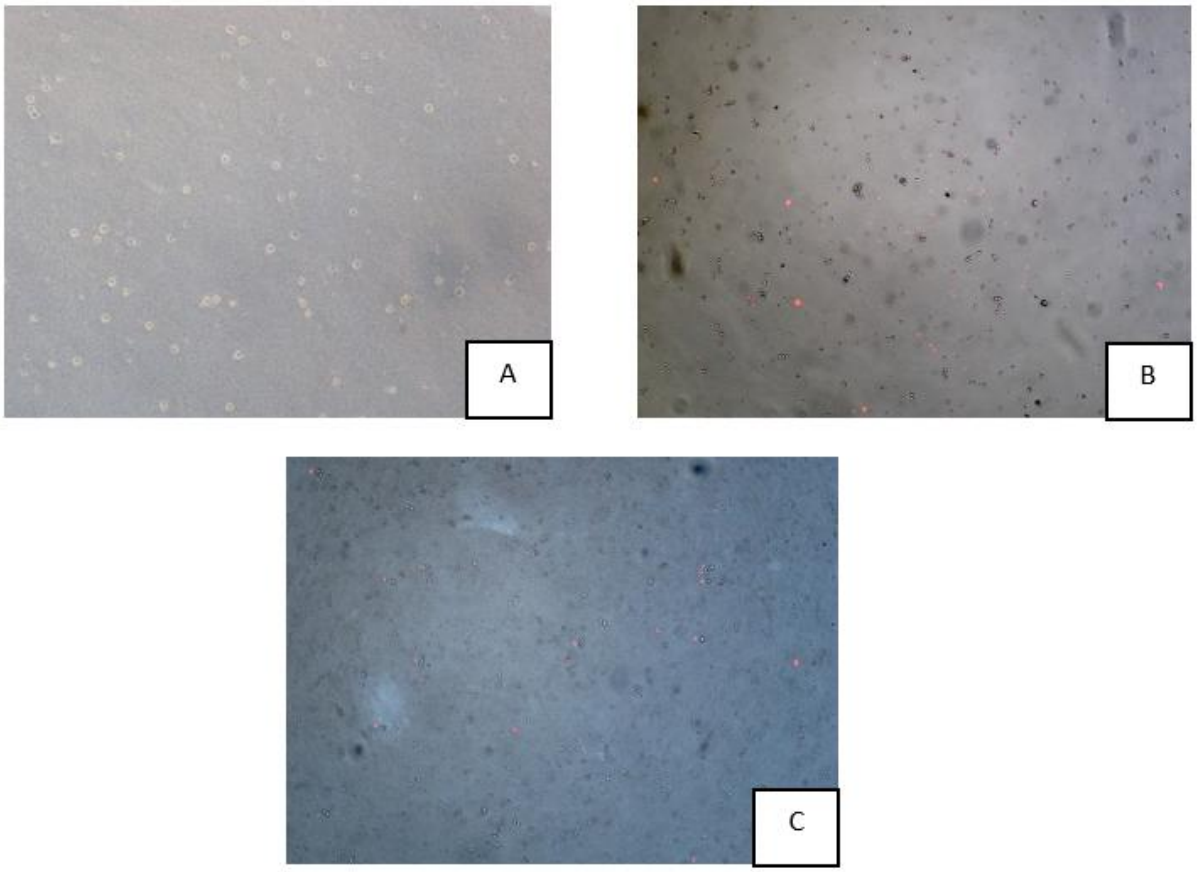

Figure 3. Overlay Results of Pulmosphere Macrophage Uptake of Ciprofloxacin HCl Labeled with Fluorosceinamine Using a Nikon Eclipse TS2R Inverted Microscope at 4x Magnification and a TxRed Filter. (A) Control Cells; (B) Rhodamine B control; (C) Ciprofloxacin HCl-Alginate-Carrageenan Pulmosphere

Inverted microscopy showed that the morphology of alveolar macrophages did not change with the addition of ciprofloxacin HCl to the pulmosphere (Park et al., 2013). Stack macrophage cells with pulmospheres to count cells that take up macrophages. The results of cell calculations obtained a pulmosphere formula with a total of 152 cells that could be absorbed by alveolar macrophages. This value is higher than the control cells which can be absorbed by alveolar macrophages totaling 72 cells. In summary, the method outlined here provides a streamlined approach for isolating rat AM in a cell culture. While the protocol can be fine-tuned to align with specific experimental requirements, it serves as an initial guide for investigating the functional significance of AM in the context of respiratory diseases and medicine (Nayak et al., 2018).

Future research should focus on specific areas, such as the polymer-drug ratio concentration used, as this study has identified several promising avenues to explore. Investigating the variable combination of alginate-carrageenan polymer with drug

concentration may shed further light on the Ciprofloxacin HCl pulmosphere and improve our understanding of the drug delivery field.

CONCLUSION

Ciprofloxacin HCl-Alginate-Carrageenan Pulmosphere has the capability to be absorbed by alveolar macrophages. However, future research could benefit from using fluorochromes conjugated with the active ingredient or those with similar properties. Additionally, it is essential to conduct macrophage uptake assays using more advanced tools and methods to accurately determine the intensity differences among various formulations. This research has the potential to contribute significantly to the further development of Ciprofloxacin HCl Pulmosphere using alginate-carrageenan polymer combinations.

RECOMMENDATION

While the recommendations offered in this study are valuable, there remains substantial opportunity for further investigation in specific areas. Future research could greatly benefit from exploring drug delivery systems, which may lead to significant advancements in the pharmaceutical field. Examining aspects such as the comparison of drug concentrations in alginate-carrageenan polymer combinations, selecting fluorochromes conjugated to or resembling the active ingredient, and performing macrophage uptake assays with methods that can differentiate intensity in various formulations could provide new insights and propel progress in drug delivery systems. By focusing on these areas, subsequent research has the potential to expand on current findings and make more profound contributions to related applications.

ACKNOWLEDGMENTS

The authors would like to thank grant from DRTPM DIKTI, Faculty of Pharmacy Universitas Airlangga for all facilities and supports. Also support of PNF for assisting researchers.

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