Liposomal delivery of Remdesivir for localized and targeted treatment of COVID-19

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Liposomal delivery of Remdesivir for localized and targeted treatment of COVID-19

By

Anupama Melam

A thesis presented to the McKelvey School of Engineering of Washington University in St. Louis in partial fulfillment of the requirements for the degree of Master of Science

January, 2021

St. Louis, Missouri
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Abstract

Liposomal delivery of Remdesivir for localized and targeted treatment of COVID-19

By

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Master of Science in Biomedical Engineering

Washington University in St. Louis, 2021

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COVID-19 is a serious, and in many cases lethal, disease that is caused by infection of the upper respiratory tract by the novel betacoronavirus, SARS-Cov-2 virus. This disease has a very high mortality rate and has affected the world in a global pandemic. SARS-Cov-2 binds to the ACE2 receptor via the receptor-binding domain (RBD) in the S protein. After this, the virus fuses with the cell membrane by the formation of a six-helix bundle. Thus, the S protein plays a major role in ensuring that the virus attaches to the ACE2 receptor and enables the viral fusion, entry and transmission. The ACE2 receptor is essentially the main point of entry for the virus. Some research efforts have been focused on drugs that can be used for viral-entry inhibition by targeting human ACE2 receptors. Remdesivir, an antiviral, is a nucleoside RNA polymerase inhibitor which causes premature termination of viral transcription. Although it is not FDA-approved, it is the current standard of care as some studies have indicated that Remdesivir use leads to reduced mortality. However, the possible side effects observed are increased levels of liver transaminase, hypersensitivity, anaphylactic reaction and potential renal failure. The goal of this study is to improve the therapeutic effects and reduce the side effects by the localized delivery of Remdesivir using liposomes that target ACE2 receptors in the body.
Chapter 1: Introduction

Coronaviruses have been around the world for a while and in most cases cause mild to moderate cold symptoms in patients that are infected. However, some viruses of this family have caused widespread disease. The 2003 outbreak of Severe Acute Respiratory Syndrome\(^\text{36}\) (SARS) and the Middle East Respiratory Syndrome\(^\text{30}\) (MERS) outbreak, first identified in Saudi Arabia in 2012, are two viral outbreaks that were caused by coronaviruses. In 2019, patients in Wuhan, China were infected by another new type of Coronavirus, SARS-Cov-2, which has plagued the world with Coronavirus Disease 19 (COVID-19) and lead to the Global pandemic of 2019-2020. This chapter gives a brief introduction to coronaviruses and the SARS-Cov-2 virus.

**Coronaviruses**

Coronaviruses are a family of RNA viruses called Coronaviridae\(^\text{8}\) that cause mild to moderate infection in the upper-respiratory tract of the body leading to diseases like the common cold. However, three new viruses have impacted the world greatly over the past 20 years by causing widespread breakouts of diseases such as SARS, MERS, and COVID-19.

The Coronaviridae family has four genera of which two (alphacoronavirus and betacoronavirus) are known to infect human beings\(^\text{31}\). The viruses within this family have a ring of spikes on the outside which resemble a crown. This has earned them the name coronaviruses (“corona” means crown).

Presently, the mode of transmission for these viruses is through animals – SARS was transmitted from civet cats, MERS from camels and COVID-19 from bats. Once in humans, these viruses are transmitted from human to human via coughing, sneezing, touching hands and close personal contact\(^\text{31}\).
SARS-Cov-2 virus

COVID-19 is a serious, and in many cases lethal, disease that is caused by infection of the upper respiratory tract by the novel betacoronavirus, SARS-Cov-2 virus. This disease has led to almost 270,000 deaths in the United States alone\textsuperscript{14} and has affected the daily lives of everybody in the world with the pandemic.

SARS-Cov-2 like other coronaviruses is transmitted between people through particles that are aerosolized when an infected person coughs or sneezes and is transmitted through touch and close contact within distances $<$ 2m\textsuperscript{31}. It has been proposed that the virus may also be transmitted from contaminated surfaces since laboratory studies have indicated that the virus can live on plastics, cardboard and stainless steel for days\textsuperscript{39}.

Figure 1: Structure of the SARS-Cov-2 virus\textsuperscript{18}

This figure shows the 4 main structural proteins of the virus – the N, S, M and E proteins. The spikes on the outside of the virus have earned it the name Coronavirus, corona meaning crown.

This figure is adapted from a publication by Florindo et al\textsuperscript{18}. 
SARS-Cov-2 virus is an “enveloped, non-segmented, positive sense RNA virus” that has a diameter of 65–125 nm, containing single strands of RNA and has spikes on the outside that resemble a crown. The SARS-Cov-2 virus has 4 main structural proteins namely, spike (S) glycoprotein, envelope (E) glycoprotein, membrane (M) glycoprotein, and nucleocapsid (N). Like SARS-Cov-1, the virus that caused the SARS outbreak, SARS-Cov-2 binds to Angiotensin Converting Enzyme 2 (ACE2) receptors in the body. The virus first binds to the ACE2 receptor via the receptor-binding domain (RBD) in the S protein. After this the virus fuses with the cell membrane by the formation of a six-helix bundle. Thus, the S protein plays a major role in ensuring that the virus attaches to the ACE2 receptor and enables the viral fusion, entry and transmission. Many research efforts, especially in the development of monoclonal antibody-based treatments, are focused on studying the structure of this S protein in order to develop treatment that target this S protein and its subunits.
Chapter 2: COVID-19 disease

The outbreak of COVID-19 began in Wuhan, China in late 2019 and by February 2020 China had reported 75,000 cases and 2,000 deaths\(^2\). Since then, the disease spread rapidly resulting in nearly 63.5 million cases and 1.5 million deaths worldwide\(^1\) as of December 1, 2020. These statistics are expected to be an underestimate of the actual numbers of cases and deaths due to the limited access to testing leading to unreported cases and delayed testing of infected patients. Presently, the only actions people have been taking to prevent the spread of the virus are isolation and quarantine.

COVID-19 has impacted people irrespective of age. There are, however, some populations that are at higher risk than others. People that are immunocompromised, have other risk factors such as diabetes and hypertension, and those that are of advanced age are considered to be most “at risk”. Recent studies suggest that people that have blood type A are at a higher risk compared to other non-A groups while with people with blood type O have the lowest risk\(^4\).

The disease has also impacted people’s daily lives outside of the physiological impact to their physical health. People’s mental health has taken a toll with many health care workers and families suffering from Post-Traumatic Stress Disorder (PTSD) due to the quarantines and isolation. The lockdown amidst the global pandemic has impacted people’s livelihood – small businesses are shutting down; many people are suffering from lay-offs and furloughs and the economy saw a recession in early 2020.

This section describes what the disease looks like in patients – the symptoms and the pathophysiology of the infection and how the disease progresses through the patient’s body.
Symptoms

In most patients, signs and symptoms appeared 2-14 days after exposure to the virus. The virus has an incubation period of about 14 days after which symptom onset begins. Some of the most common symptoms are:

- Fever
- Cough
- Tiredness
- Loss of the sense of smell usually accompanied by the loss of sense of taste

The report published by the Chinese Center for Disease Control (Chinese CDC) in the Journal of the American Medicine Association (JAMA) in February 2020 divided the clinical manifestations of the disease by the severity in symptomatic affected patients:

- Mild disease: no to mild pneumonia (81% of cases)
- Severe disease: dyspnea, respiratory frequency ≥30/min, blood oxygen saturation ≤ 93%, partial pressure of arterial oxygen to fraction of inspired oxygen ratio (i.e., P/F ratio) < 300, and/or lung infiltrates > 50% within 24-48 hours (14% of cases)
- Critical disease: respiratory failure, septic shock, multiple organ failure (5% of cases)

These statistics were reported based on data collected about the 72,314 (confirmed, suspected, diagnosed and asymptomatic) cases reported by the Chinese CDC in the paper. Other symptoms that have been identified include weakness, respiratory distress malaise and
muscle pain\(^2\). The length of infection varies from person to person – some people recover within weeks of symptom onset while others take much longer (sometimes even months) to recover.

Although many people, especially in the United States, are required to perform regular health checks to identify a fever or notice any other symptoms, the issue is complicated by the fact that many people that have COVID-19 are asymptomatic. People that have been exposed to the virus may be asymptomatic and can also infect other people. Therefore, the lack of symptoms gives people a false sense of security.

The only way COVID-19 is clinically diagnosed is through a combination of clinical symptoms, testing for presence of viral RNA via RT-PCR through nasal swabs, chest x-ray or CT scans and quantifying antibodies produced by the body via a blood sample\(^2\). There are 2 main types of tests that are being administered in patients – the PCR tests in which a nasal swab is taken from a person to detect for viral RNA and the blood serology test which detects the antibodies that the patient’s body produced in response to the viral infection. While there are severe limitations to testing such as - false positives and lack of enough tests for people around the world, it is currently the best method to check for infection. When used in conjunction with other methods like the chest x-ray the tests have been very helpful for identifying infected patients and to start contact tracing so other people that may have been exposed to the virus may be informed.
Pathophysiology

Since the outbreak of COVID-19 many researchers have been trying to identify the pathophysiology of the disease and learn about the virus transmission in the hope to find ways to stop the spread. Early in the outbreak, positive cases were observed mostly among older populations – specifically in older men with comorbidities. As the disease has spread and more people have been affected more has been learned about the disease and its pathophysiology.

Infection Route

SARS-Cov-2, the virus that causes COVID-19, enters the body through aerosolized droplets often released into the air by other infected patients when they cough, speak, sneeze, touch surfaces, through physical contact etc. Once the virus has entered the body it goes through 5 main steps –

- Attachment – virus binds to the receptor
- Penetration – virus enters the cells (either through membrane fusion or through endocytosis)
- Biosynthesis – viral proteins are made in the host using the viral RNA
- Maturation – new viral particles are made using the viral proteins
- Release – newly made viral particles are then released back and the whole process repeats

The Spike (S) protein plays a major role in the virus attaching and fusing with the host cells. The S protein has two main subunit – the \( S_1 \) subunit, which is responsible for the virus binding with the host cells’ receptors (attachment) and the \( S_2 \) subunit which is responsible for the
fusion of the virus with the host cells’ membranes (penetration). The S1 subunit of SARS-Cov-2 binds to the Angiotensin Converting Enzyme 2 (ACE2) receptor. The ACE2 receptor is highly expressed in pulmonary epithelial cells. After binding with the ACE2 receptor, the S protein goes through a 2-step protease cleavage – the first cleavage to prime the S1/S2 cleavage site by the Transmembrane serine protease 2 (TMPRSS2) and the second activates a position adjacent to the fusion in the S2 subunit.

Once the fusion is complete, the virus enters the pulmonary alveolar epithelial cells where the viral RNA is released. The virus then begins replication based on the viral RNA template. Then transcription is completed via RNA polymerases and the newly formed replicate can be used to produce new proteins in the cell cytoplasm through translation. This process is called biosynthesis.

The virus’ N protein then binds the newly made RNA material and the M protein enables their integration into the host cell’s endoplasmic reticulum (ER). The N proteins are then enclosed in the ER and transported to the lumen from where the Golgi transports it to the cell membrane. During this time the newly made viral particles made from the viral proteins are undergoing maturation.

After the viral particles reach the cell membrane, they then are taken outside the cell via exocytosis and released so that they can invade new epithelial cells and continue proliferation. Once the viral particles have been released, they can also be transmitted outside the patient’s body through aerosolized droplets to infect the next person.
Figure 2: Infection route for SARS-Cov-2\textsuperscript{45}

Disease Progression

According to the Treatment guidelines published by the NIH and updated with the latest findings, COVID-19 disease progression is thought to have two main stages – the initial stage post infection where the disease primarily involves replication of the SARS-Cov-2 viral particles and the later stage where the disease is driven by inflammation in response to the virus particles which leading to tissue damage\textsuperscript{1}.

The SARS-Cov-2 viral particles are spread primarily through respiratory droplets or by surface contamination. Namely, face-to-face exposure with an individual with COVID-19 puts one at higher risk of being infected, especially if the infected individual coughs, sneezes, or talks in a way that spreads saliva droplets. The average time from exposure of the virus to the first symptoms averages around 5 days, with 97.5% of people who do develop symptoms develop them within 11.5 days from exposure. The most common symptoms include fever, shortness of breath, and dry cough. Patients developing symptoms may also develop weakness, fatigue, nausea, vomiting, diarrhea, changes to taste and smell.

However, severe complications can occur affecting primarily the heart and lungs in addition to the liver, kidney and coagulation system. COVID-19 can also lead to myocarditis,
cardiomyopathy, ventricular arrhythmias and hemodynamic instability. About 17-35% of patients suffering from COVID-19 are treated in ICUs most often due to Acute Respiratory Distress Syndrome (ARDS), pneumonia or hypoxemic respiratory failure. Of these patients in the ICU, anywhere from depending on the severity of the disease, the extent of disease progression and the combination of risk factors.
Chapter 3: Treatments for COVID-19

The magical cure for COVID-19 has been a priority for research efforts across the globe since the outbreak in Wuhan, China. However, there are no drugs approved by the Food and Drug Administration (FDA) for the treatment. The SARS-Cov-2 viral genome undergoes high error rates and recombination (homologous and non-homologous). This is because of the virus’ short replication time and high viral yields. This section summarizes some of advancements that have been made towards the treatment of COVID-19 including prophylactic measures (to prevent infection) and post exposure treatments.

Vaccines (Pre-exposure)

Many scientists and pharmaceutical companies are working on finding a vaccine for the disease so that healthy people can be protected from getting infected when exposed to the vaccine. This way people would not have to suffer any of the potentially severe symptoms and the world can go back to functioning per usual back to what is now considered “pre-COVID-19”. There are 3 main types of vaccines that are currently in varying stages clinical trials. These types are Adenovirus-based vaccines, Vesicular Stomatitis Virus-based vaccines, mRNA vaccines.

![Figure 3: Summary of the different types of vaccines](image)

[Image of a tree diagram showing the different types of vaccines: Vaccines, Virus based, mRNA, Adenovirus, VSVs]
**Adenovirus-based vaccines**

Adenovirus-based vaccines work by inserting a segment of the SARS-CoV-2 viral genome into a rare virus, that does not cause any significant harm, so it can produce SARS-CoV-2 protein. This rare virus loaded with the SARS-CoV-2 genome is then injected into the body to trigger an immunogenic response in the body. The biggest challenge with this process is that the transport virus (adenovirus) must be rare to humans, i.e., most people have not been exposed to it, in order to prevent the body from recognizing it as a threat and destroying it before the virus can infect other cells with the SARS-CoV-2 proteins. An alternative to using rare human adenoviruses is to use monkey adenoviruses but these have to be capable of infecting humans (which is unlikely to be naturally occurring or they would have infected humans by now).

Johnson & Johnson (J&J) and Janssen Pharmaceuticals are currently working on a potential vaccine of this type called JNJ-78436735 (also called Ad26.COV2-S). They are in their second round phase III clinical trials. Their vaccine uses the replication-defective adenovirus type 26\textsuperscript{23}. AstraZeneca in collaboration with the University of Oxford has also developed a potential vaccine called AZD1222 (also known as ChAdOx1 nCoV-19). They are using a simian adenovirus. They are doing their phase 3 of clinical trials\textsuperscript{10}.

**Vesicular Stomatitis Virus-based Vaccines**

Vesicular stomatitis virus (VSV) can be used as a delivery vehicle to transport SARS-CoV-2 viral genes that trigger antigen-production in human cells. VSVs are used as vaccine vectors because they induce a strong T cell immune response in the body after just a single dose. The cytopathic nature of VSV makes it a strong candidate for use in the production of vaccines. VSV infections in humans are rare (it primarily affect cattle and livestock) and once infected the
patient experiences mild influenza-like symptoms. Additionally, the genetic flexibility of VSV enables the production of rVSVs that express high levels of foreign viral proteins – in this case SARS-Cov-2 proteins\textsuperscript{20}.

Merck is developing a potential oral antiviral vaccine called MK-4482. This is a vaccine based on the rVSV vector. This vector has been successful in combatting Ebola. Currently they are in Phase II/III of clinical trials for this vaccine\textsuperscript{29}.

**mRNA Vaccines**

mRNA vaccine is a new type of vaccine that works, not by inserting live or weakened viruses like the previous two types, but by inducing cell production of the protein or a piece of the protein that triggers an immune response\textsuperscript{12}. mRNA in itself is a relatively safe vector since it only carried information for a short while and does not affect the body’s genome. mRNA vaccines produce a balanced immune in the body and the combination of the desired immunogenic response induced in the body and the minimal side effects makes mRNA vaccines are very attractive option\textsuperscript{6}. The only major limitation with the production of mRNA vaccines is that since viral vectors are not used the mRNA needs to be enclosed in a complex lipid delivery system to prevent it from degrading either outside the cell or in the cytoplasm\textsuperscript{33}.

Currently, the two vaccines with the most potential and the highest efficacy (94.5\%) are mRNA vaccines. Moderna has developed mRNA-1273, a potential vaccine made by encapsulating the mRNA is a lipid nanoparticle. This mRNA encodes for a “full-length, prefusion stabilized spike (S) protein of SARS-CoV-2” triggering the production of antigens that target this S protein. Presently, this vaccine has completed Phase III of testing\textsuperscript{5}. Moderna is awaiting FDA approval for use in the United States and Europe.
Another major vaccine candidate is BNT162b2 developed by BioNTech, Fosun Pharma and Pfizer. Like the vaccine candidate developed by Moderna, this is also a lipid nanoparticle-encapsulated mRNA vaccine. This vaccine has also completed Phase III trials and has filed for an Emergency Use Authorization (EUA) with the FDA. This vaccine has been approved and is currently being deployed particularly to high-risk populations like people over the age of 65 and frontline healthcare workers. It has already been authorized for use in the UK on December 2, 202034.
Drugs (Post exposure)

Drug treatment of patients with COVID-19 depends on the stage and the severity of the disease and on potential risk factors and co-morbidities that the patient has. Earlier in the disease progression antivirals and antibody-based treatments are very effective. However later stages in the disease progression are often marked by hyperinflammatory responses and coagulopathies. In these stages anti-inflammatory drugs and anticoagulants are more effective than antivirals4.

While there are no FDA approved drugs to treat COVID-19, a large variety of drugs have been tried both in research labs and in hospitals. Since COVID-19 is caused by a coronavirus and the spread was so rapid, many drugs that worked in the past have been repurposed for the treatment of COVID-19. The Infectious Diseases Society of America (IDSA) published their guidelines on the use of various treatments for COVID-19. These guidelines are regularly updated with latest findings from ongoing studies. This section summarizes some of the most popular treatment types, outlined by IDSA, that have been tested and the benefits and challenges with each type.

Repurposed treatments

Early on the search for the treatment of COVID-19, Chloroquine (CQ) and its less toxic counterpart Hydroxychloroquine (HCQ) were tested to check their efficacy against SARS-Cov-2. Both CQ and HCQ are 4-aminoquinoline drugs that were previously used in the treatment of Malaria. HCQ has an extra hydroxyl group (hence the name) which leads fewer severely adverse effects with use overtime. These drugs have been tested in vitro and these studies have shown that these drugs have some activity against SARS-Cov-224. A study that aimed to optimize the dose of HCQ and CQ by testing pharmacological activity in vitro found that HCQ is more potent.
that CQ in vitro\textsuperscript{43}. One non-randomized study reported a reduction in mortality in patients suffering from COVID-19 when treated with HCQ\textsuperscript{44}.

However, while HCQ was considered a potential drug candidate for the treatment of COVID-19 it is no longer under consideration due to side effects such as – longer time until discharge, risk of QT prolongation, arrythmias and hemolysys among others. Furthermore, there were concerns about the validity of the studies because there was no measure taken to control for critical cofounders of disease severity, to factor steroid uses given their additional benefits (steroid use thus is a confound that should have been removed to understand the effect of HCQ alone) etc.

**Antibody treatments**

The second stage of COVID-19 involves a hyperinflammatory response often characterized by a “cytokine storm”\textsuperscript{7}. Descriptions of infected patients and their immunopathology indicated that those with higher levels of IL-6 and hyperinflammatory response had more severe disease symptoms\textsuperscript{3}. Tocilizumab, a monoclonal antibody, is being considered a potential drug candidate due to its anti-IL-6-receptor blocking property thereby reducing hyperinflammatory response.

While there was some evidence of lower clinical deterioration in one study, the results were of low certainty due to bias and imprecision. To make things worse, Tocilizumab use for treatment of COVID-19 in patients led to case reports of bowel perforations. Since the drug did not provide any significant benefit when compared to outcomes of untreated patients and there was significant risk to its use, there is a conditional recommendation against the use of Tocilizumab.
Corticosteroid treatments

In order to reduce the hyperinflammatory response observed in the later stages of COVID-19 investigations began to identify potential immunomodulatory approaches, involving the use of steroids, to treat COVID-19. The hyper-inflammatory response is potentially what causes Acute Respiratory Distress Syndrome (ARDS) in some patients. Patients with ARDS required prolonged use of ventilators. Corticosteroids are known to alleviate non-viral ARDS. Hence, they were considered as a potential treatment for the later stages of COVID-19.

Dexamethasone is most effective in critically ill patients – odds of mortality were 34% less in patients treated with glucocorticoids vs those that were not. Also, patients that received Dexamethasone were more likely to be discharged from the hospital. In patients with severe illness, odds of mortality were 17% in patients treated with glucocorticoids vs those that were not. In patients that were not suffering from severe or critical illness, Dexamethasone did not produce any significant benefit or harm. It is therefore advised to be used only in patients with severe or critical illness. If Dexamethasone is unavailable, other glucocorticoids are to be used.

Antivirals

Remdesivir (aka Veklury, GS-5734) is one the most important anti-viral drugs that is being considered for COVID-19 treatment. Remdesivir, while currently not fully authorized for a treatment, it is currently approved by the FDA under Emergency Use Authorization (EUA) and is the current standard of care, especially for patients early on in the disease progression.

Remdesivir is a nucleoside RNA polymerase inhibitor which causes premature termination of viral transcription. In vitro studies have shown that Remdesivir is effective in preventing replication of SARS-Cov-1 and MERS-Cov viruses in Human Airway Epithelial
(HAE) cells\textsuperscript{19}. An in vivo study in Rhesus Macaques infected with SARS-Cov-2 showed that the animals had improved pulmonary lesions, reduced viral load titers in bronchoalveolar lavage 12 hours post treatment and reduced viral load in lungs after 7 days\textsuperscript{41}.

When used in patients some studies have shown decrease in mortality and improved clinical outcomes after being treated with Remdesivir\textsuperscript{1}. However, some patients reported side effects such as increased levels of liver transaminase, hypersensitivity, anaphylactic reaction and potential renal failure\textsuperscript{38}.
Chapter 4: Hypothesis and Research Objective

Remdesivir is the current standard of care for patients suffering from COVID-19 per the FDA. However, the less-than-ideal efficacy and the risk of possible side effects from increased liver transaminase levels to potential renal failure has rendered the drug subpar.

The goal of this study is to improve the therapeutic effects and reduce the side effects by the localized delivery of Remdesivir using liposomes that target ACE2 receptors in the body.

Figure 4: Schematic representation of the liposome and its route
Liposomes are small spherical particles that typically have one or more hydrophobic layers surrounding a hydrophilic core. This hydrophobic shell is typically made up of a phospholipid bilayer. Liposomes have become very important and widely used vehicles for drug transport. This popularity is due to multiple factors including how well drugs can be encapsulated inside these nanoparticles, the ease of preparation and the standardization of size, and most importantly the dual regions. The dual regions are the key benefit since drugs can be loaded in either (or both) of the regions based on the hydrophilicity of the drug. Further, antibodies and proteins can be added to the surface of the liposome particles adding more functionality to the particles.

**Figure 5: Schematic representation of a liposome**

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*References:*

35 [Source]
**Preparation of liposomes**

Liposomes were prepared using the thin layer evaporation method\textsuperscript{16}. The hydrophobic layer is made up of DPPC, Cholesterol and PEG-succinyl. Since Remdesivir dissolves in hydrophobic solutions (soluble in DMSO, insoluble in water and ethanol), it was added along with the lipids. The lipids and Remdesivir were dissolved in 1 ml Chloroform. 10ul of Ethanol was added to ensure the lipid film was unilamellar. This solution was evaporated through a rotary evaporator (Heidolph, Schwabach, Germany) to form a thin lipid film. The lipid film was hydrated with PBS (to produce liposomes) and then extruded to ensure liposomes were uniform and of a specific size. The extruded set by Avanti Polar Lipids was used for this and the liposomes were extruded though 200nm and 100nm filters. The size, polydispersity index (PDI) and the zeta-potential of the liposomes were determined using the Zetasizer Nano ZS which measures these parameters using Dynamic Light Scattering (DLS).

![Diagram of liposome preparation](image-url)

**Figure 6: Steps involved in the preparation of liposomes\textsuperscript{17}**

To make targeted liposomes with anti-ACE2 receptors on the surface of the liposomes, carbodiimide chemistry. Appropriate volumes of EDC and NHS with original concentrations of 200uM (in Milli-Q® water) were added and the liposomes were incubated at room temperature
for 15 minutes with gentle stirring. The solution of liposomes with EDC and NHS was then split into two groups – one group of liposomes with anti-ACE2 receptors and one group of non-targeted liposomes. To the first group anti-ACE2 receptors were added. Both groups of solutions were placed in dark bottles in 4°C in a light-protected environment and incubated overnight with gentle stirring. Unbound anti-ACE2 receptors were filtered out using ultracentrifuge to remove the supernatant solution.
Characterization of liposomes

Dynamic Light Scattering (DLS) analyzers are machines that measure the size of really small particles that are in solution. These analyzers detect the particle sizes based on the fluctuations in the scattered light created by the Brownian motion of the particles. This is why it is critical to have the particles in solution so that they exhibit Brownian motion. DLS analyzers are able to characterize liposomes based on a number of parameters. For this experiment the most important were size, PDI and zeta potential.

Table 1: Characteristics of the liposomes identified by the DLS analyzer

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The DLS analysis showed that the mean size of the liposomes was 117nm with a standard deviation of 1.15 which makes sense since the liposomes were extruded through 100nm membrane filter. According to a review by Danaei et al while there is no ideal size for the treatment of systemic disease determined yet, in order to maximize protein interaction, transepithelial transport and to delay lung clearance the particle size must be less than 150nm\textsuperscript{15}. With the particle size at 117nm, the liposomes are ideal for delivery of Remdesivir specifically to the lungs.

The Polydispersity Index (PDI) of the liposomes were determined to be 0.09 (with a standard deviation of 0.03). PDI describes the particle size dispersion. So, a smaller PDI means all the particles are the same size while a larger PDI means that there is a lot of variability. For drug delivery through nanoparticles and liposomes, a PDI $\leq$ 0.3 is considered acceptable\textsuperscript{15}. Based on these guidelines a PDI of 0.09 is really good and indicates that the liposomes are uniform in size around 117nm.

The average zeta potential of the liposomes was -42.03mV. The zeta potential is a measure of the surface charge on the particles and indicates how stable the nanosuspensions are. Zeta potentials that are greater than $+30$mV and less than $-30$mV indicate good physical particle stability\textsuperscript{4}. This is because the magnitude of the surface charge on the particles is sufficiently large to repel neighboring particles minimizing agglomeration. Thus, the zeta potential of the liposomes prepared (which is $< -30$mV) indicates that the liposome particles are very stable in solution and do not aggregate.
Testing liposome receptor binding

In order to test if the anti-ACE2 receptors on the surface of the liposomes were indeed successful at binding to the ACE2 receptors on the epithelial cells liposomes with no drug were coated with anti-ACE2 receptors and recombinant ACE2 protein. These liposomes were prepared in the exact same way as before – however no Remdesivir was added with the lipids and DiD was added to the lipids (before dissolving in Chloroform) to make fluorescent liposomes. When conjugating with EDC and NHS, the solution of liposomes with EDC and NHS were split into three dark brown bottles – one group with anti-ACE2 receptors, and the other group with no targeting.

A 96-well plate was prepared with 2 different solutions, 1 solution in each row – BSA (1mg/ml) and recombinant ACE2 protein (5.7ug/ml). To coat the wells with each solution, 35ul was added to each well, just enough to cover the bottom of the well, the plate was sealed and placed in 4°C for 48 hours. The plate was then rinsed with PBS and the liposomes were added (down the column). The plate was covered with aluminum foil and placed at room temperature for 1 hour. The fluorescence was measured using a SpectraMax i3 Spectrophotometer.

![Figure 8: 96-well plate arrangement of the solutions and the liposomes](image-url)
This experiment was repeated 3 times for most accurate results since ensuring that the binding did indeed take place was the most important step. In the first trial, the initial concentration of liposomes of 28.57ug/ul was used. 50ul of liposomes were pipetted into each well. After allowing the liposomes to bind to the solutions, the fluorescence was measured first with liposomes in the wells, then after they were aspirated and after each rinse with PBS. The fluorescent dye used to color the liposomes was DiD which has an excitation wavelength of 648nm and an emission wavelength of 675nm. The fluorescence was then compared across rinses and plotted on a graph. However, in this trial, some of the data from the early PBS washes was lost and the only data recovered was from the fourth wash with PBS. The results were not accurate, and a second trial of the experiment was carried out.

In Trial 2 some of the mistakes from Trial 1 were fixed. The first change was the use of a black 96-well plate. This was used in order to reduce internal reflections when using the fluorescence plate reader. The black plate was also covered by an aluminum when the solutions were coating the bottom for 48hours. The second change was that the liposomes were diluted approximately 1:3.5. The new concentration of liposomes used was 8.729ug/ul (compared to the original concentration of 28.57ug/ul). Another big change made was that after the 48hours of incubating the solutions on the wells, the 96 well plate was rinsed with BSA to minimize non-specific binding. The fluorescence data was collected only after PBS was added between washes, not after aspirating it. The results from the fluorescence plate reader were then plotted as shown in Figure 1.
Figure 9. Plot of relative fluorescence for Trial 2 of the affinity binding experiment

This figure indicates that the targeted liposomes with the anti-ACE2 antibody (Red) binds almost 2x more to the recombinant ACE2 protein solution than the non-targeted liposomes (Blue). The lower non-specific binding to BSA indicates that the liposomes with anti-ACE2 antibodies bind specifically to the recombinant ACE2 protein.
The results from Trial 2 looked very promising. BSA was used in order to compare the non-specific binding of the liposomes with the specific binding of liposomes. Recombinant ACE2 protein was used as the binding solution so it can mimic the ACE2 receptors that are present in the human body. As expected, the anti-ACE2 targeted liposomes had almost 2x more specific binding than the non-targeted liposomes indicating that the liposomes with the anti-ACE2 antibody was able to successfully bind specifically. In order to fine-tune the results, one more trial was conducted to show repeatability and to improve accuracy.

In the Trial 2, the data indicated that the fluorescence for each well was too high. So, for Trial 3, the liposomes were diluted 1:10 for a final concentration of 0.8729ug/ul. The plates were placed in the cold room for 24 hours and similar to Trial 2, the fluorescence was only read after the PBS was added and not after it was aspirated. This data was again collected and plotted in Figure 2.
Figure 10. Plot of relative fluorescence for Trial 3 of the affinity binding experiment

This figure also shows that the targeted liposomes with the anti-ACE2 antibody (Red) binds >2x more to the recombinant ACE2 protein solution than the non-targeted liposomes (Blue). The lower non-specific binding to BSA indicates that the liposomes with anti-ACE2 antibodies bind specifically to the recombinant ACE2 protein. The fine tuning of the experiment made the difference between specific and non-specific binding clearer.
The results from Trial 3 proved that the liposomes with anti-ACE2 antibody were indeed capable of targeting and binding specifically to the recombinant ACE2 protein. Since the recombinant ACE2 protein was used as a binding solution, the significantly higher fluorescence, indicating higher binding, means that the experiment works successfully and liposomes with the drug are capable of targeted drug delivery at the ACE2 receptors.

**Lentiviral transfection of A549 cells**

First the optimal dose of antibiotic (Hygromycin) was determined. This is the concentration of Hygromycin that kills all of the A549 cells (lung carcinoma cells from human alveolar basal epithelium). The lentivirus, hACE2 Hygro was rapidly thawed from -80°C and was diluted to prepare a final 1:5 concentration in DMEM media. In a 6-well plate, a control with no lentivirus and the 1:5 dilution of the lentivirus were plated first. The healthy A549 cells (50,000 per well) were then plated on top for reverse transduction. The cells were incubated with the virus for 72 hours. Media was changed and incubated again for another 72 hours. Images of the cells were taken at this point (Figure 11 b, c). Since the cells were over 70% confluent, they were trypsinized and moved to a T-25 flask. Hygromycin (600ug/ml) was added to the media in both the flasks. The plate was observed every day and regular fluid changes were performed, and cell growth was monitored. The cells in the first flask, without any lentivirus are expected to all die and the cells that have been successfully transfected, should survive since the virus provides antibiotic resistance. After this is completed, the ACE2 expression on the transfected cells will be measured using Flow cytometry.
Figure 11: 6-well plate arrangement for lentiviral transfection.

Fig 11a shows the 6-well plate arrangement where well A1 has A549 cells with no lentivirus and well B3 has the 1:5 dilution of lentivirus with the A549 cells. Fig 11b is an image captured of the A549 cells without lentivirus post transfection, before being selected using Hygromycin. Fig 11b is the image captured of the A549 cells that have been transfected with the lentivirus before adding Hygromycin. At this point the cells in the well were over 70% confluent and were moved to a T-25 flask.
COVID-19 has become a top priority among researchers across the globe with the race for the vaccine and drugs being the forefront of research efforts. In vitro studies show that Remdesivir is effective as a treatment for COVID-19. However, the low efficacy in patients and the risk of side effects that could be fatal, along with the limited data available on it has prevented it from being approved by the FDA. Enveloping the drug in the hydrophobic layer of the liposomes was done successfully. These liposomes had anti-ACE2 antibodies on the outside which could bind specifically to the ACE2 receptors. This design would allow the drug to be encapsulated in the liposome that specifically targets the same ACE2 receptors as the SARS-Cov-2 virus. Thus, the drug delivery will be localized improving the efficacy of the treatment of COVID-19. Since the highest dose of this antiviral will be where the virus is located, it would reduce the side effects in the kidneys and liver and reduce mortality making this an appealing treatment option for patients suffering from COVID-19.

While this is the first step in potentially improving patient outcomes by addressing existing concerns with the use of Remdesivir this targeted delivery system needs to be tested in vitro and in animal models. The first step would be to measure the uptake and binding of the drug in the treatment of human epithelial cells (A549) that have been transfected with the lentivirus in order to express ACE2 receptors. The targeted liposomes are expected to bind to the ACE2 receptors that the cells are expressing.

In order to test the efficacy of these liposomes in vivo in small animals, the ideal animals are hamsters according to a study published by Imai et al. According to another publication by Sia et al, indicated that “the features associated with SARS-CoV-2 infection in golden hamsters resemble those found in humans with mild SARS-CoV-2 infections”. Thus, testing the efficacy
of Remdesivir encapsulated in targeted liposomes in COVID-19 infected hamsters would be a good way to understand the therapeutic effect of the drug in vivo before testing in patients.

While the vaccine has an important role in the prevention of COVID-19 infection, this treatment will help alleviate the symptoms of thousands of patients worldwide that are suffering from the disease.
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