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Chitosan as an Antiviral

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Abstract

There is no broad-based antiviral medication available today; there are specific antivirals, for example, the antiretroviral for HIV. However, these specific antivirals are not available in each country and can be problematic for specific patients. Chitosan is proposed as a possible broad-based antiviral, which has already demonstrated antibacterial properties, antiviral properties in plants, is used for wound healing and as a hydrogel among other medical applications. The methods used are transfection of NIH-3T3 cells with GFP-adenovirus with 0.1%, 0.5%, and 1% chitosan added to virus prior to transfection. Fluorescence microscopy and flow cytometry data has validated that the use of 0.1% chitosan added to virus before transfection reduced the percentage of fluorescence of gfp from 72% to 1.9%, suggesting a 70% percentage difference of transfection. Based on these findings, it is proposed that chitosan could be a broad-based antiviral medication. Future directions include the adding chitosan after transfection, use of other viruses, and employing studies in animal models to test in-vivo.

Background

Many viruses of today's world go uncured from a lack of a broad-based antiviral medication that would be comparable to antibiotics for bacterial infections. Treatments are available to patients to help with the symptoms of a viral infection or to help subdue the virus from spreading or remaining active in their body. Currently antiretroviral treatments that target cell entry, transcription of the virus, and viral progression are helping to reduce the mortality rates of viruses such as HIV.¹ This treatment has been made available to 15.8 million of the 36.9 million people living with HIV worldwide.² Although this amount of people receiving treatment is almost 50%, many third world countries do not benefit from the antiretroviral treatment. Also, if a patient does receive the treatment, there can be complications such as resistance and drug toxicity.³ This treatment also requires regular monitoring of the CD4 levels in the blood to adjust medication as needed which is not always feasible for the patient.⁴

Other viruses that are prevalent and outbreaks have occurred recently is Ebola and the Zika virus. Ebola virus has no cure or antiretroviral with the most concentrated outbreaks in West Africa.⁵ The Ebola outbreak has declined in West Africa since 2014, but there is an estimation of 11,316 deaths and 28,639 cases.⁶ The Zika virus also has no treatment or cure with an outbreak that started in 2015 originating in Africa, Southeast Asia and Pacific Islands and now has spread to the Americas and many countries.⁷ The Zika virus has been linked to microcephaly in infants born to mothers that have contracted the Zika virus which is a lifelong disease that can cause many health conditions.⁸

There are many clinical research efforts being made to discover an antiviral treatment or cure for the above viruses and viral infections in general. This study employs the use of chitosan as a possible antiviral medication. Chitosan is a natural polysaccharide biomaterial found on the shells of crustaceans, for chemical formula see figure one.⁹ This material is biocompatible and used in many medical applications. The most common medical applications of chitosan include wound healing, chitosan as a hydrogel, drug delivery using chitosan, and as a diluent.¹⁰ Among chitosan's uses, it also has been shown to display antiviral and antibiotic characteristics.¹⁰

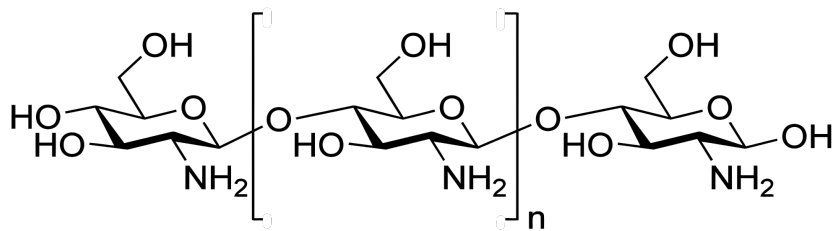


Figure 1. *Chemical structure of chitosan.*

Recently, studies have demonstrated chitosan to not only be effective in delivering antibiotics when loaded with antibiotics for wound healing applications, but has presented to have antibiotic effects alone.¹¹ The molecular weight of chitosan has proven to be a key role in the antibiotic effects.¹² It appears that lower molecular weights of chitosan are more successful for antibiotic activity than higher molecular weights.¹² In addition to antibiotic activity, chitosan has shown antiviral properties in plants.¹³ In a study performed by Kulikov, the molecular weight of

chitosan also was crucial for antiviral effects, also with lower concentration working better than high.¹³

Chitosan is proposed in this study as a material targeted for antiviral treatment with adenovirus in NIH-3T3 mice cells. Adenovirus is a common virus that accounts for common cold like symptoms usually in child respiratory infections.¹⁴

Techniques to measure the transfection rates of the cells with and without chitosan will be by fluorescence microscopy and flow cytometry. The adenovirus used in this study is engineered with green fluorescence protein (gfp), so when the cell is transfected and replicates, the green fluorescence protein will become visible under its excitation wavelengths using a FITC filter.¹⁵ Figure 2 displays the excitation and emission wavelengths for GFP, and the FITC wavelength ranges in figure 3.

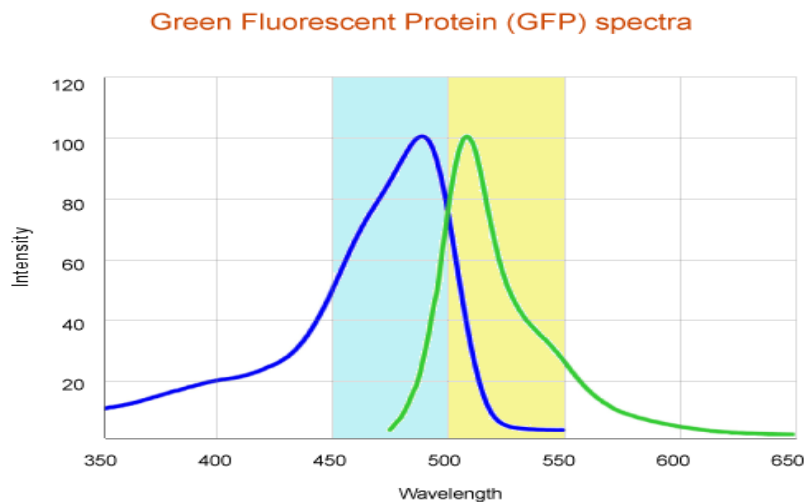


Figure 2¹⁶. *Excitation and emission wavelengths of GFP. Excitation peaks at 490nm, and emission peaks approximately 510 nm.*

FITC (Ex-Max 494 nm/Em-Max 520 nm)

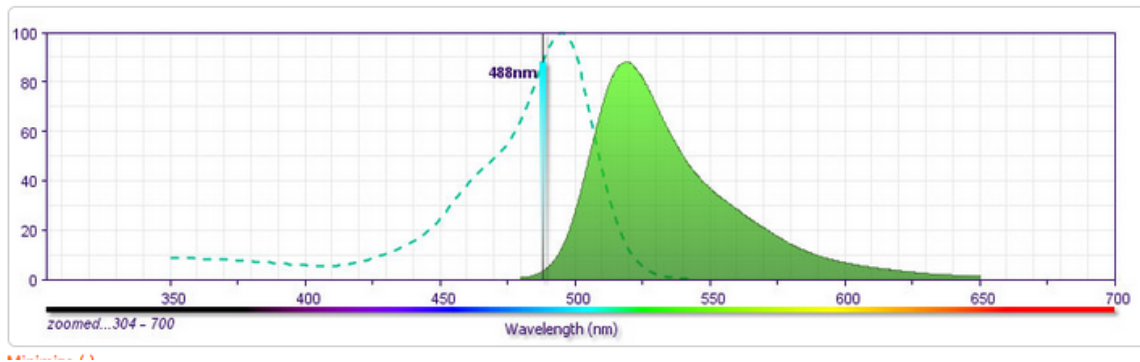


Figure 3¹⁷. *Excitation and emission wavelengths of FITC, excitation peaking around 480nm, emission peaking around 530 nm.*

Once the fluorescent molecule is excited at its excitation wavelength, it jumps up to the excitation energy level. After that the molecule energy decays down to its emission energy level when the image is captured.¹⁸ A few disadvantages to fluorescent imaging is there is no intrinsic qualitative analysis and the risk of photo bleaching the fluorophore molecule is prevalent which prevents prolonged exposure to light and prolonged imaging. Also, photo bleaching can occur before imaging which can result in false negatives.¹⁸

To counterbalance the disadvantages of fluorescent imaging, flow cytometry provides the quantitative results desired. Flow cytometry is a laser based sorting tool for dyed or inherently fluorescent molecules. Since our sample is already tagged with GFP prior to use, no dying was necessary. The machine forces each cell out of the nozzle one by one. As the cells go through one at a time the specified laser, FITC in this case, excites the GFP inside the cell. If the cell fluoresces then it is sorted in one compartment, if it does not, it is sorted differently, displayed in figure 4.¹⁹ The

flow cytometer also counts the number of cells present in each sample and determines their size and shape.

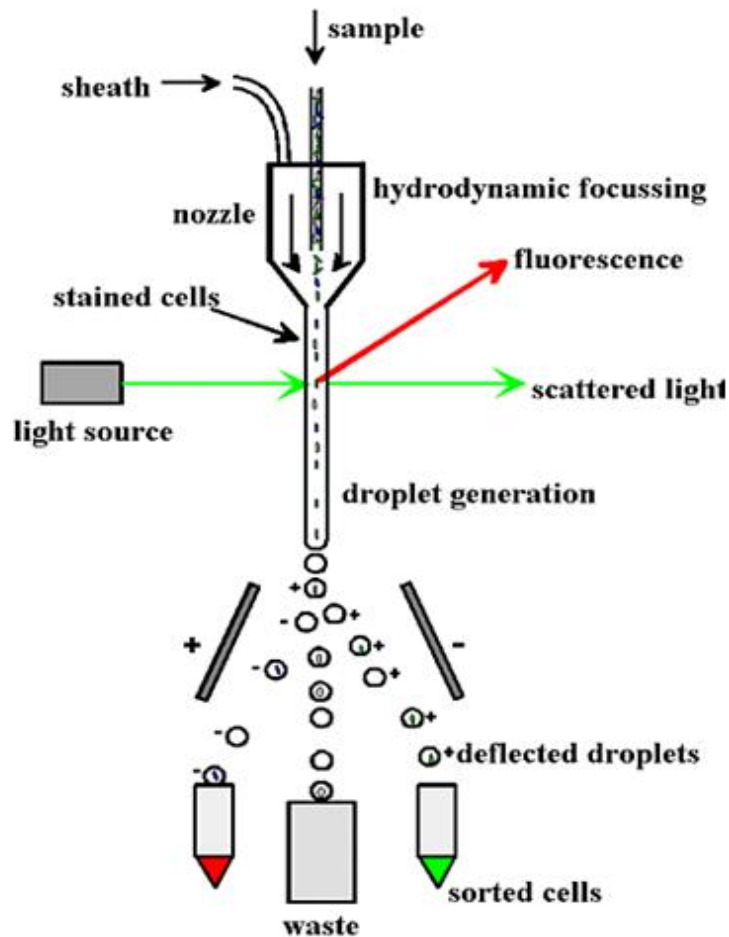


Figure 4¹⁹. *Schematic of flow cytometry using fluorescent exiting laser, FITC.*

Methods

The experiments used to transfect NIH-3T3 cells with adenovirus were to incubate a soluble chitosan (protosan) with the adenovirus prior to exposing the cells to virus. The experimental process consisted of seeding the cells in a 24-well plate, transfecting the cells with virus+chitosan, then analyzing through fluorescence microscopy and flow cytometry.

Cell Passaging

NIH-3T3 cells were housed in a cell culture flask at 37 degrees Celsius for incubation times. DMEM high glucose media with 0.5% L-Glutamate, 0.5% penicillin/streptomycin antibiotic and 10% Fetal Bovine Serum was used for cell culture media. Every 4-5 days, depending on cell population the cells were passaged. The old media was aspirated out, and 3 ml of diluted trypsin was added and incubated for 5 minutes. The cells were collected and centrifuged after adding fresh 7ml of media for 5 minutes at 1400 rpm. The media/trypsin solution was aspirated out, and new media was used for suspension of the cells. The cells were counted using a hemocytometer, and roughly 10% of the population was added to 2 new cell culture flasks with 13ml of fresh media. All cell passaging was done under cell culture hood, and sterile techniques using ethanol sterilization was used to decrease contamination.

Cell Counting

After the cells were centrifuged and suspended in 5 ml of new media after being collected from the cell culture flask, 100 ul of cells were collected and mixed with 100 ul of trypan blue, for cell staining. 10ul of this solution was injected into the hemocytometer device wells, pictured in figure 5.

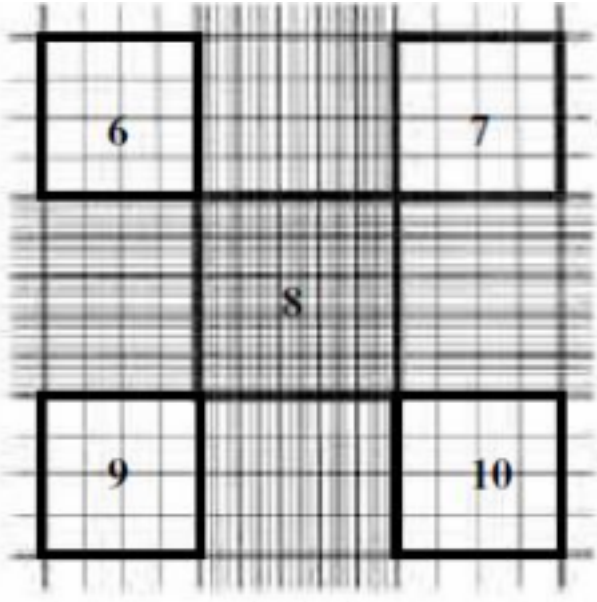


Figure 5. Hemocytometer device wells. Wells 6,7,9, and 10 are used for cell counting.

The cells were counted in wells six, seven, nine, and ten. The numbers of cells in each well were added and averaged. Using equation one, the number of cells per milliliter was calculated.

$$\frac{\text{Cells}}{\text{milliliter}} = \left(\frac{(\text{cells counted in the four wells} * \text{trypan blue dilution factor})}{4} \right) * 10,000$$

(Equation 1)

Cell Seeding

The cells were passaged as above and counted using a hemocytometer. For experiments, 200,000 cells were added to each well in the 24-well plate. When seeding for new culture, approximately 500,000 cells were added to a new flask.

Adenovirus Alloquating

Upon receiving new virus, it was immediately alloquated to avoid repeated freeze-thaw cycles to the virus, which diminishes viral ability. Green fluorescent protein adenovirus, gfp-ad ,cat# 1060, 200ul of 10^6 PFU/ml was used from vectorbiolabs. The virus was alloquated into 200 MOI micro centrifuge tubes with DMEM as the storage buffer media and stored at -80 degrees Celsius.

Transfection without chitosan

Initial transfection tests and upon receiving new virus, the transfection of the 3T3 cells were done. The cells were seeded at 200,00 per well in a 24-well plate with a gap in between the first and third column with no cells seeded to keep the virus from transferring from positive over to negative control wells. The cells are incubated for 24 hours to adhere to the surface of the wells. Following incubation, the virus was thawed in an ice bath and the cell media was aspirated from the wells. The negative control was replenished with new media, the gap remained in between the negative control and transfected cell well columns. The third column was transfected at 20 MOI, the fourth at 50 MOI, fifth at 75 MOI and last at 100 MOI. The cells were put back into the 37-degree incubation chamber and left for 12-24 hours. All transfection was done under the cell culture hood with the lights off to reduce bleaching.

Transfection with Chitosan

Transfection with chitosan differed only at the transfection phase. All experimental steps are held constant with seeding into the 24-well plate and incubation. The virus was thawed in the ice bath per usual, and during this time the chitosan was measured out. Three samples of chitosan were measured out for 0.1%, 0.5%, and 1% chitosan. For 0.1% in 1 ml of virus/media solution, 1mg was weighed, 5mg for 0.5% and 10mg for 1%. The chitosan virus solution was incubated for 15 minutes on the shaker at 37 degrees Celsius or until chitosan was in solution. Once the chitosan/virus solution was ready, the cells were transfected and incubated for 12-24 hours at 37 degrees Celsius. All transfection was done with cell culture hood lights off.

Fluorescence Microscopy

Prep

The cells were washed three times with PBS following the transfection incubation. 200 ul of PBS was kept in the well for imaging. All washing done with cell culture hood lights off.

Imaging

After turning on the microscope and letting it warm up for fifteen minutes, the wells were placed on the microscope-imaging platform. The eyepiece was used in conjunction with the computer software imaging modality. Once a satisfactory region was selected in normal light, the image was captured in 10x and 20x. Then the light source was switched to FIT-C for fluorescent imaging. The gain and exposure time was increased or decreased for a satisfactory image of

fluorescence. There was effort to keep exposure time small so the fluorescence did not bleach out due to prolonged light exposure. This was done with each experimental group multiple times for best image. All imaging was done in the dark, and the cells were disposed of after imaging.

Flow Cytometry

Prep

If flow cytometry was employed after fluorescence imaging, the cells were taken back to the cell culture hood and washed with PBS two more times. The PBS was aspirated off, and 100ul of trypsin was added to each well and incubated at 37 degrees Celsius for 3 or 4 minutes. The cells were collected off of the well bottom with new media added and centrifuged for 5 minutes at 1500 rpm. All media and trypsin was aspirated off and the cells were resuspended in 200ul of PBS for each sample run. Depending on volume of cells collected, usually 3 samples to run for each experimental group. During the time of cell sample collection the flow cytometer was prepared. First it was turned on and warmed up for about fifteen minutes. The flow startup was then initiated. Following the flow startup, the FIT-C mode was calibrated using appropriate calibration beads. Once the calibration test was complete, the flow cytometer was ready for use.

Data Collection

The negative control was ran first, to fix the x and y axis of the flow cytometer plot and to fix the appropriate cell population region. Once conditions were set satisfactory each other sample was ran. Once each sample was ran, the

population two region was adjusted to each run, which separated the fluorescent region from normal non-fluorescent region. Once all samples were ran, the flow cytometer machine was shut down by first completing the flow shut-down process. All of the samples were all disposed of afterward.

Results

Transfection without chitosan is pictured below in figures six and seven. This shows the early transfection experiments using 50, and 100 MOI. The transfection difference from 50 to 100 MOI does make a slight difference in fluorescence intensity shown qualitatively in figures 6 and 7, but not significant difference. This was true for this batch of virus, but subsequent batches required 100 MOI for complete transfection and fluorescence intensity.

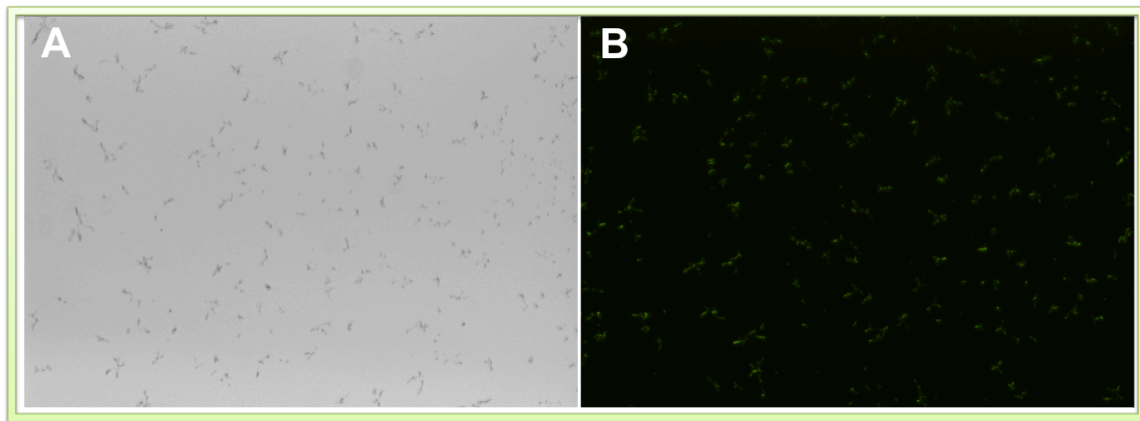


Figure 6. *3T3 cells transfected with 50 MOI adenovirus figureA; regular light image of cells at 10x magnification, B; FITC image of A. Cells were prepared for this image similar to flow cytometry preparation. Cells were not adhered to the well, they were on a glass slide.*

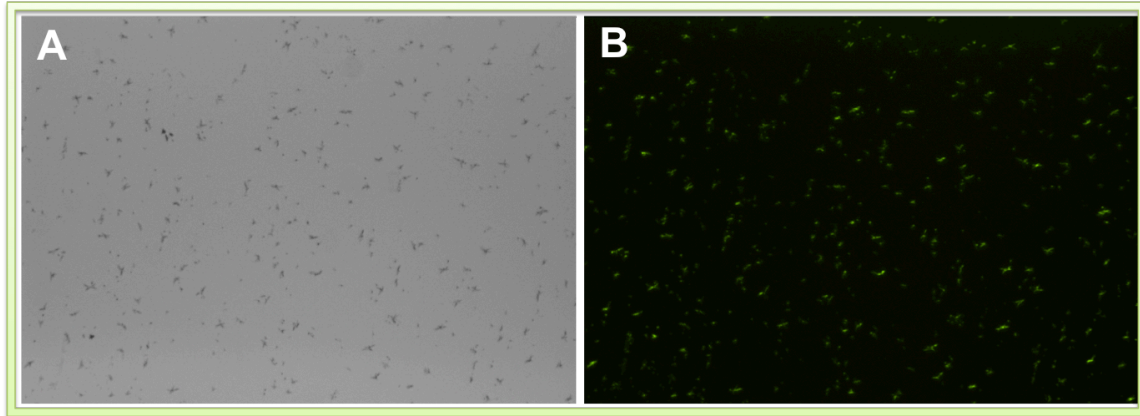


Figure 7. *3T3 cells transfected with 100 MOI adenovirus figureA; regular light image of cells at 10x magnification, B; FITC image of A. Cells were prepared same as figure 6 cells.*

Shown in figures below is the experiment of transfection with chitosan. Figure 8 is the positive control for transfection at 100 MOI, and figure 9 is transfection with chitosan of 0.1% and 0.5%.

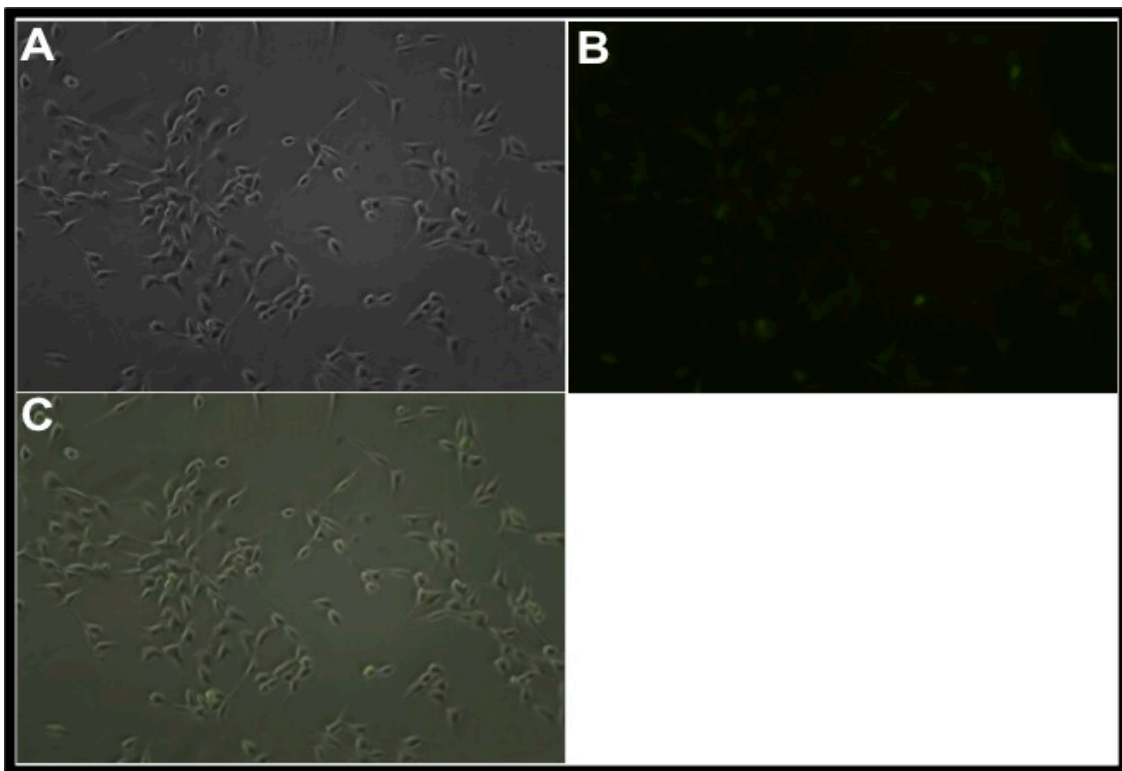


Figure 8. Positive control (100MOI) of experiment with transfecting with chitosan and magnification 10x. Figure A is 3T3 transfected cells, B; FITC image showing fluorescence, C; superimposed image of figures A and B

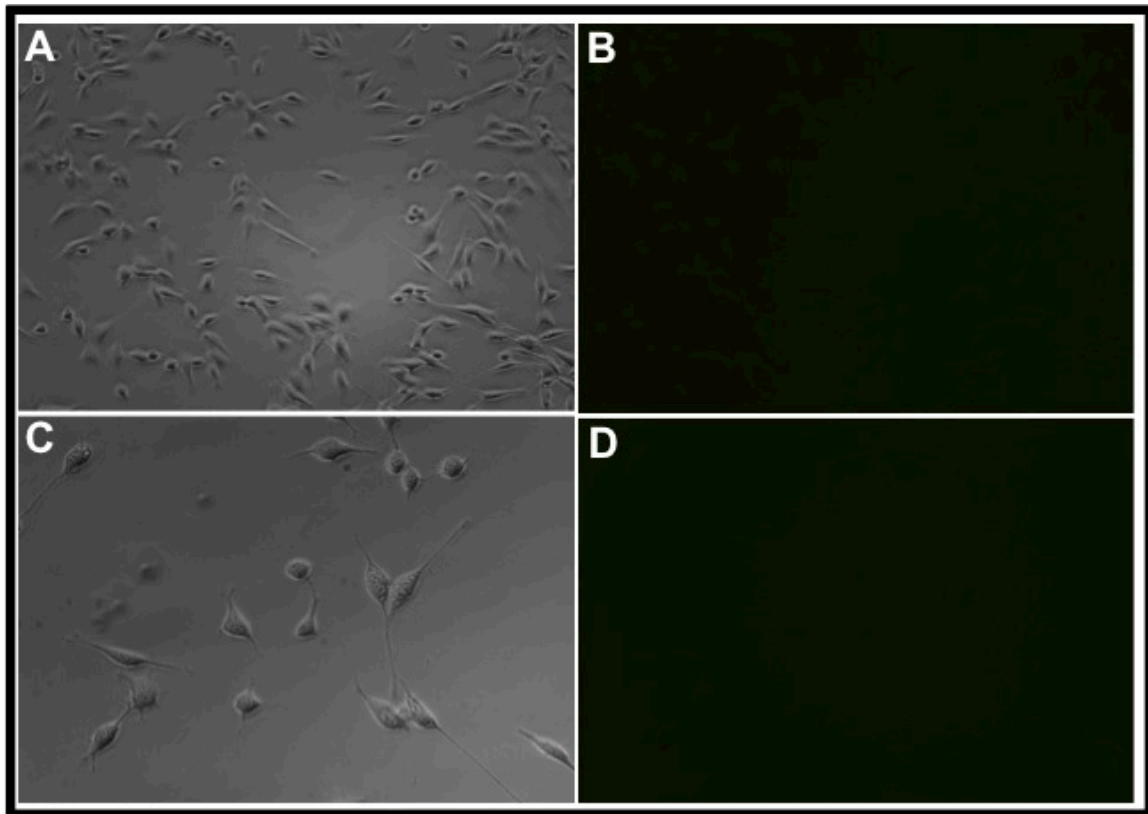


Figure 9. Images of 0.1% chitosan added (A and B) and 0.5% chitosan added, C and D. Figure A is the regular exposure light of 0.1% chitosan transfection at magnification 10x, B is the FITC exposed image of field A. Figure C is the regular light exposed field of 0.5% chitosan at magnification 20x, and D is the FITC exposed field of C.

The data from fluorescent imaging shows promise of chitosan's ability of antiviral with adenovirus. The positive control has visible fluorescence in the FITC

image, where the images taken with chitosan added appear to have no fluorescence in the FITC image. Since the fluorescent intensity was low for the positive control, flow cytometry was employed for standalone quantitative results.

Figures 10-14 are the flow cytometry data of negative and positive control, 0.1%, 0.5%, and 1% chitosan.

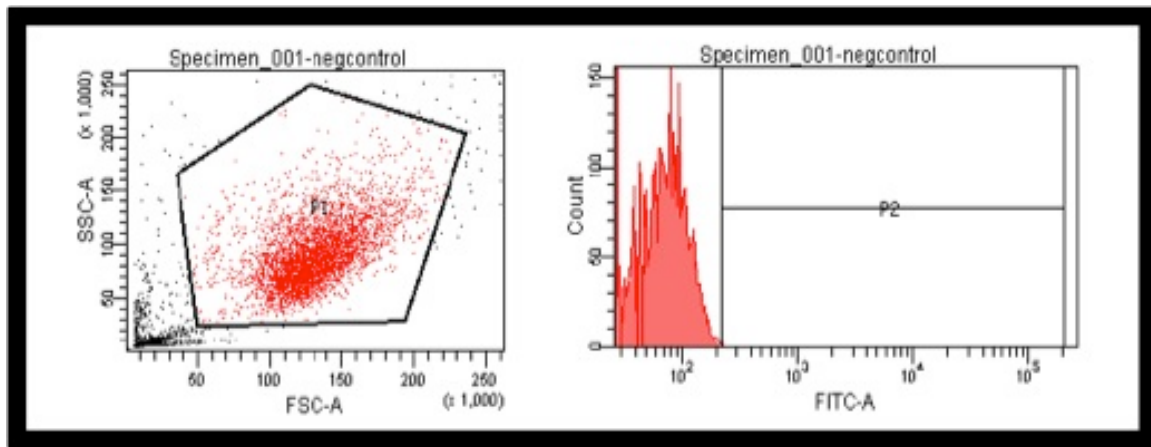


Figure 10. *Negative control of transfection experiment. Population 1 shows most healthy cell growth, with no population in P2, the designated fluorescence population.*

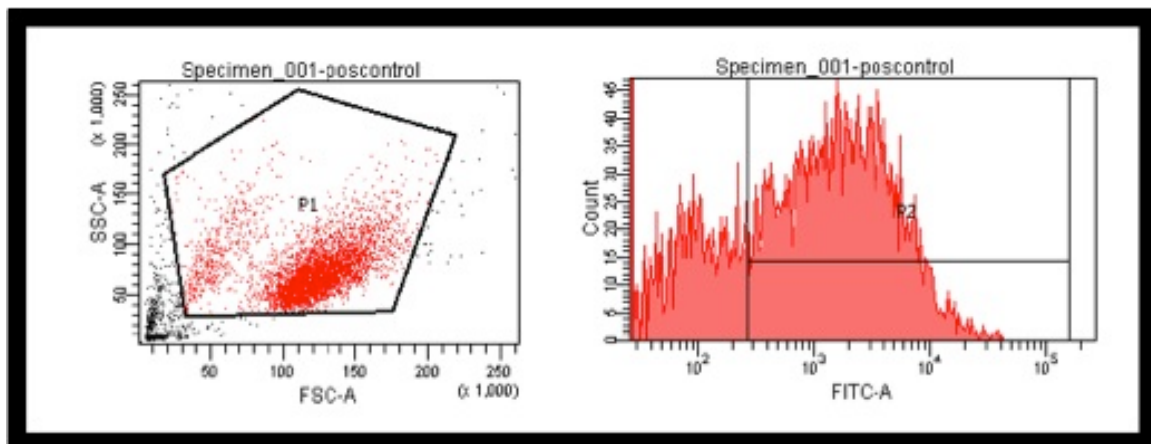


Figure 11. *Positive control of transfection experiment. Significant peak shift to fluorescence region of P2.*

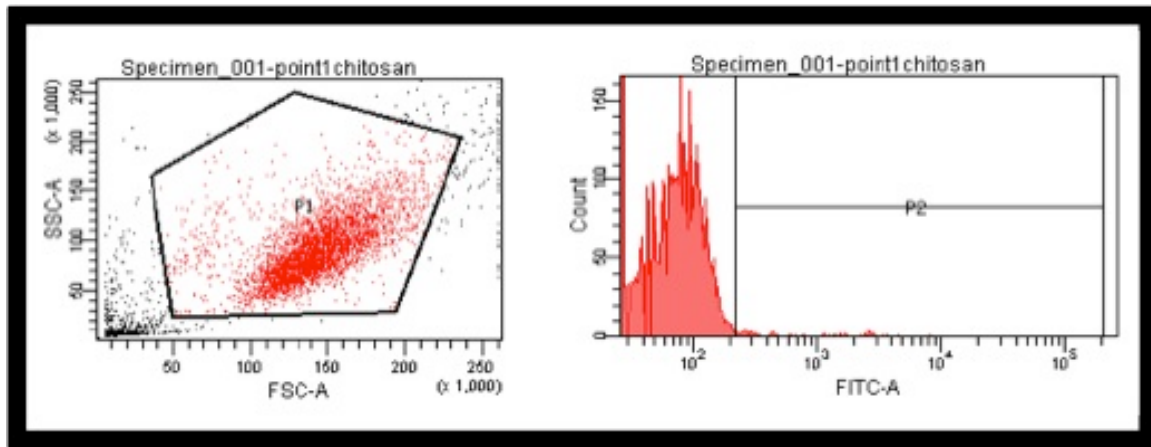


Figure 12. *0.1% chitosan added to adenovirus. Shows little amounts of fluorescence in population 2.*

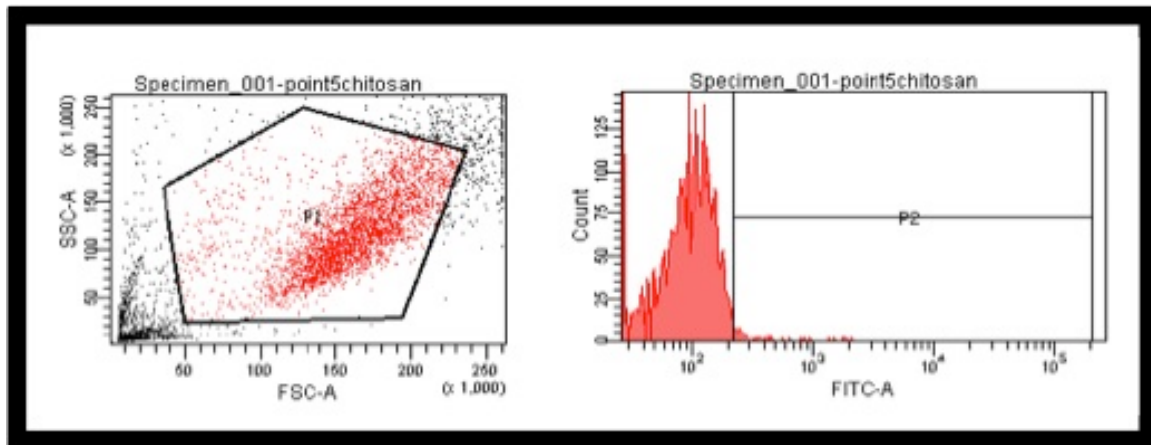


Figure 13. *0.5% chitosan added. Little fluorescence in population 2, although cell status is not similar to negative control, or 0.1%. Abnormal size.*

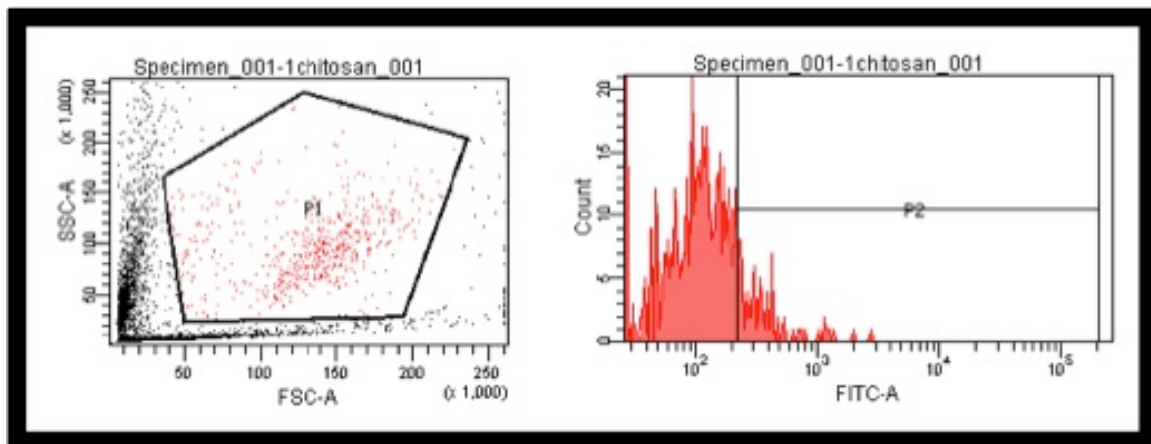


Figure 14. *1% chitosan added. Increased fluorescence and low cell condition and sample size, as seen on right image.*

Table 1.

Fluorescent (Transfection) Percentages		
Sample	Population 1	Population 2
Negative Control	79.3%	0.1%
Postive Control	84.6%	72.3%
0.1% Chitosan	81.1%	1.9%
0.5% Chitosan	67.7%	2%
1% Chitosan	11.4%	16.6%

It is clear from the flow cytometry results that the 0.1% chitosan-virus reduced fluorescence almost to the degree of the negative control, at 1.9% compared to 0.1% fluorescence in population 2. The 0.5% chitosan was roughly the same as the 0.1% in fluorescence at 1.9% and 2%, but the cells were in worse condition. The 0.1% chitosan doesn't seem to influence the cells much in terms of growth, size, and death as the 0.5%. 1% chitosan added to viral transfection resulted in low viability of cells and increased fluorescence up to 16%. Overall, adding 0.1% chitosan to virus before transfection reduced fluorescence, hence transfection, from 72.3% at positive control to 1.9%.

Conclusion

Chitosan has displayed antiviral promise with gfp-adenovirus on NIH-3T3 cells. Qualitative data from fluorescence microscopy displays significant decrease in fluorescence from positive control to when the chitosan is added simultaneously with virus prior to incubation. This find is validated with flow cytometry data showing a 70% difference of fluorescence of positive control to added chitosan, see table 1.

Future directions will need to include replication of this experiment with flow cytometry, to provide more validation of the results. In addition, factors to the experiment can be altered, such as adding the chitosan after viral transfection to observe the antiviral potential after transfection. Also, adding less chitosan ranging from 0.01%-0.1% to find a threshold.

Once experiments are validated and chitosan amount is clarified for maximum antiviral activity, this study can be moved into animal models for further testing in-vivo, and the use of more extreme virus such as HIV and Ebola to test the efficiency of chitosan as an antiviral with a wide range of virus. From this study, it is possible that chitosan may be a broad-range antiviral treatment but further studies are needed to validate.

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