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Formation of Antiviral Calcium Alginate Layers Analyzed by Combining Theory, Computational Chemistry and Experiments

Ali Youssef[1], Michail Georgakis[2], Daniel Yaxley[3] and Eva Künnemann[4]*

[1,4]*MVS Pharma GmbH, Germany*
[2] *Sinodos Chemistry, Greece*
[3] *BluTest Laboratories, United Kingdom*

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CORRESPONDING AUTHOR
eva.kuennemann@mvs-pharma.com

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A B S T R A C T

Alginate, a sugar polymer derived from algae, crosslinks with calcium ions to form a stable gel or film. Several studies already analyzed the antiviral properties of calcium alginate, whereby only some studies showed viral inhibition. This research investigates the biochemistry and conditions of calcium alginate networks to form gels and membranes by a combination of literature analysis, computational simulations, and spraying experiments. Cell culture assays were applied to test the potential of calcium alginate to inhibit viral entry into cells. These investigations demonstrate that protective effects on cultured cells depend on the specific alginate substance, the concentrations and the manner of deposition. The results confirmed conditions so that the calcium alginate forms effectively gel-like networks and thin membranes. Additionally, the experiments proved that over 50% of the infections of cells with viral particles can be inhibited easily by calcium alginate overlaying cells.

1. Introduction

During the corona pandemic Rainer Proksch, CEO of MVS Pharma, had the idea to develop a protection by oral-nasal spray. In a review in the ACS Applied Bio Materials Journal, it was concluded that use of the antiviral properties of alginate-based biomaterials is a promising strategy for SARS-CoV-2 prevention [1]. Therefore, this substance and its properties are analyzed in this study.

Alginates are natural long chains of a mixture of two polyuronic acids and thereby form polysaccharides [2]. They contain 50 – 3500 of the saccharide subunits. The molecular weight distribution and the length of the alginate chains can be studied by analytical ultracentrifuge and light scattering [3] [4]. Atomic force microscopy and ^{1}H NMR spectroscopy are the main methods used to study the composition and structure of alginates [5] [6].

The name alginate comes from the brown algae from which it is obtained. Alginate also occurs in the extracellular polymeric substance of bacteria of the species *Pseudomonas aeruginosa*, whereby the bacterial alginate is partially acetylated. Commercially available alginate is mostly isolated from algae because it is easier to obtain than from bacteria. A method to extract alginic acid in pure form is acid precipitation with subsequent treatment with alkaline solution [6]. Dry seaweed is crushed, water and acid are added for washing and swelling. After clarification, extraction is performed by filtration. The alginic acid is precipitated under acidic conditions and ion exchange leads to sodium alginate or other salts of alginic acid.

Humans have extracted and used alginates since a long time for many technical and medical applications, like surface coating in the paper industry, stabilizers in food and filling material for wound swabs [4]. Due to the gelling behavior these substances are also used in molecular cuisine. In this context, there is a workshop in the Swiss Science Center Technorama in which visitors can take part [7]. The molecular mechanisms are clearly illustrated and explained.

Advantages of alginates as natural polymers are their biocompatibility and biodegradability. They can be processed into various forms by using their ability to polymerize. There are numerous brown seaweeds suitable for alginate production [6]. Different species like *Laminaria hyperborea*, *Laminaria digitata*, *Macrocystis pyrifera*, *Ascophyllum nodosum*, *Ecklonia maxima*, and *Sargassum* spp are used for commercial production.

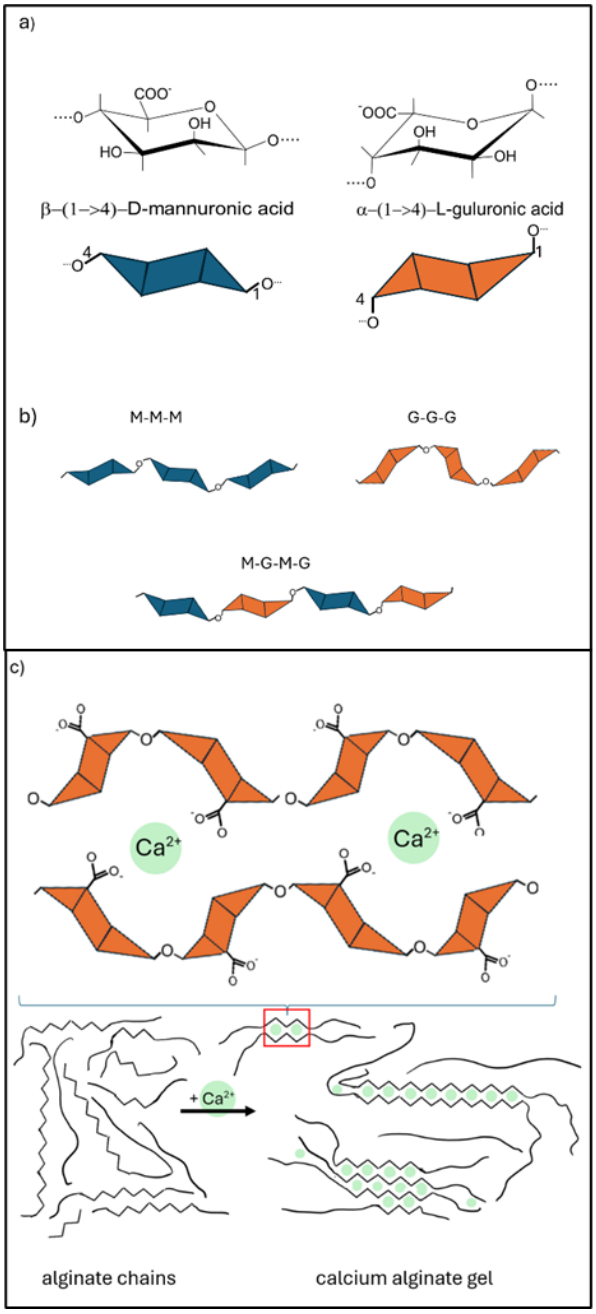

Figure 1. a) Chemical structure of mannuronic acid and guluronic acid, the subunits forming of alginate, b) Depending on which subunits bind to each other, different geometries are formed, M-M and M-G binding forms rather straight chains whereas G-G binding results in zigzag chains. c) Two G-G-G-G chains complex two calcium ions according to the egg-box-model. Additionally, below a model of the formation of calcium alginate is depicted, compare references [6] [8].

Sodium alginate consists of the sodium salt of alginic acid. This substance is soluble, forming viscous solutions when diluted in water whereby the singly loaded sodium ions only can attach to one chain. Mannuronic acid units (M) and guluronic acid units (G) are the two types of uronic acid, the subunits. These form three different kinds of polymer segments in blocks (Figure 1a) [6]. There are three possibilities for blocks containing either several M or a row of G units, and as a third pattern stretches where M and G alternate occur (Figure 1b). M is (1→4)-linked β-D-mannuronate and G is (1→4)-linked α-L-guluronate. Therefore, in the M subunits the links to the oxygen bridges at C-atoms 1 and 4 are equatorial whereas both bonds linking to the oxygen bridges from the G subunits are axial. This leads to different geometries of the subunits to each other. In M-blocks the sugar molecules form a straight line while in G-blocks a zig-zag pattern occurs. Blocks containing alternating M and G subunits form lines similar to M-blocks.

The free carboxyl groups within the sugar units are in different positions for M and G subunits. These are the positions that bind metal ions. They can react with several kinds of cations and are easily ion-exchanged, as is the case for sodium. Calcium ions bearing two positive loads fit well into the holes formed in zigzag-line of G-blocks [8]. The term egg-box model was used for this as in this structure the calcium ion is binding two negative loads of two alginate chains together and geometrically fits into the hole built by a top and a bottom zigzag-G-block that protects like a box used for eggs. When several chains come together, calcium links the alginate to a network and determines the microstructure of alginate (Figure 1c).

Sodium alginate is a white to pale powder that is slowly soluble in water to form a viscous colloidal solution. Sodium alginate substances with different viscosities are available commercially. The viscosity of sodium alginate depends mainly on the length of the polymer chains. By addition of calcium salts alginate solutions increase the viscosity and form a gel. The possibilities of various arrangements concerning relative abundance and distribution of M and G subunits as seen above and the fact that the chains can have variable lengths are the reason that the properties and functionalities of different alginate substances vary. Subsequently, properties like thickening and gelling capability with calcium and other ions and gel strength are affected and the physicochemical properties of alginate show significant alterations [9]. A more rigid molecular structure is observed in alginates enriched with G units, while substances with an abundance of M units form more flexible structures [10] [11]. It was observed that high G content produces strong brittle gels with good heat stability, however it is prone to destruction upon freezing and thawing. On the other hand, high M content produces weaker more-elastic gels with good freeze-thaw behavior. There are exceptions for very low molecular weight polymers.

In a review Serrano-Aroca and colleagues report 32 studies with 18 different viruses where alginate-based material showed antiviral properties [1]. In 25 studies antiviral activity was observed, whereby this was not clearly dependent on the virus, nor on the type of alginate-based material [1]. It was suggested that viral inhibition by calcium alginate depends on the

mechanical properties of the biopolymer chain. For some viruses it was shown by electron microscopy that aggregation occurred, and it was indicated that the antiviral action could be related to decreased functional content of viral particles in solution or that it interfered with viral uncoating during infection. If a network of calcium alginate covers large parts of the cell surface viral particles cannot pass through and might stick to the polymer [16]. Thereby the number of viral particles entering and infecting the cells is reduced.

Seeing that alginate does not always inhibit viruses, we speculate that for antiviral activity alginate must polymerize and form a molecular network, a gel-like film over the cells so that the viral particles cannot pass this network and infect the cells and might even stick to the polymer. It was shown before that the physicochemical properties of alginate as a biofilm are related to the antiviral activity [12]. In several articles in the literature antiviral properties of alginates have been described [13] [14] [1] [15]. For alginates extracted from *Laminaria hyperborean*, *Laminaria digitata*, Laminaria japonica, Ascophyllum *nodosum* and *Macrocystis pyrifera* antimicrobial activities were published. Antiviral tests have shown that alginates inhibit besides SARS-CoV2 also HSV-1, HSV-2 and tobacco mosaic virus [16] [15]. A further study shows antiviral properties for alginate-based materials against different types of viruses, including enveloped viruses like SARS-CoV-2 [1]. Additionally, biocompatible films of alginate showed antiviral activity [16], but not all experiments were totally clear and in most studies the mechanism of inhibition was not unequivocal.

With the goal to achieve consistent antiviral inhibition by calcium alginate, this study investigates the process of film and membrane formation through crosslinking reactions between sodium alginate and calcium chloride ($CaCl_2$) using both computational simulations and practical experiments. The aim is to assess the potential of the substances to form a networked alginate gel. The final target of this study is to find conditions to build a very thin antiviral layer by spraying onto the mucous membranes in mouth and nose. This film is designed to trap small particles like viruses, preventing their passage through the structure.

This research reports on simulations of calcium alginate network formation. Additionally, the spraying behavior of alginate solutions is investigated to assess how alginate can be sprayed to form calcium alginate, that is a network where small particles like viruses cannot pass. Furthermore, cell culture experiments were done for antiviral testing using different approaches to find optimal conditions for antiviral properties.

2. Materials and Methods

2.1. Materials

Sodium alginate was purchased from Sigma-Aldrich (Product Number 180947 and A1112), Harihatamaja Industries (Sodium Alginate BP) and Amit Hydrocolloids (M/SAG/24/A/131). Calcium chloride and all other chemicals were of analytical grade.

2.2. Chemical simulations

A series of sodium alginate systems has been created in HyperChem and Molecular Dynamics and Single Point calculations were run under MM+ force fields. Chain placement (in real time), inter chain distances (for various conformations), QSAR properties (surface area of the polymers before and after crosslinking, volume of the polymers before and after crosslinking, electronic charge and potential 3D distributions, etc.) were calculated via the computational chemistry simulations., compare also patent document WO2023052310A1 [17]. The characteristic entered is that the chains are crosslinked at the end of the COONa groups, when the sodium ion is removed and substituted with a calcium cation which connects the opposing COO^- -groups.

2.3. Experimental gel formation by spraying

For spraying experiments a stock solution was made by adding 2 g of sodium alginate to 75 ml of water. Samples were stirred until everything was dissolved. Water was added to get finally 100 ml of 2% w/v sodium alginate solution. This was diluted further 1:1 to get 1% w/v and 0.5% w/v sodium alginate. Calcium chloride solutions were prepared in various concentrations ranging from 1 mM – 1 M. Spraying tests were conducted on conventional Petri dishes, diameter 60 mm, as well as alternative substrates, including human skin, to explore practical applications.

Gel and membrane formation was visually monitored and quantitatively assessed using mechanical pull-off tests to confirm the integrity and strength of the membranes. To visualize the diffusion between sodium alginate and $CaCl_2$, yellow and blue pigments were added to the sodium alginate and $CaCl_2$ solutions, respectively, resulting in green membrane formation. The pigment concentration was 0.04% (0.1 ml pigment per 25 ml solution).

2.4. Antiviral cell culture assays

Antiviral testing was first done at BluTest Laboratories (www.blutest.com) according to standard procedures with all samples done in triplicates. For pretreatment to PBS containing feline coronavirus 2% alginate had been added 1:1. CRFK cells were seeded at a density of 8.0×10^4 cells/ml in EMEM + 10% FBS into as a triplicate for each sample. Calcium was added to the medium to obtain a concentration >1.5 mM. Media was removed from each well and replaced with 200µl of media (negative control, uninfected), 200µl of

untreated virus suspension (positive control, treated) or 200µl of the pretreated virus suspension was added. Cells were incubated at 37°C, the optimal incubation temperature for virus replication with gentle shaking. After 1 hour incubation, each well had its contents removed and was washed three times in 0.5ml PBS to remove any virus which had not adsorbed to the host cells. 1.0 ml whole medium was added, preincubated to the optimal temperature for virus replication to each well. The cell culture media was removed after 24 hours and plated out. For the cell viability assay 100µl of AlamarBlue was added. The plate was incubated further for 2 hours and values were read on a plate reader (BioTek Synergy HT) for fluorescence.

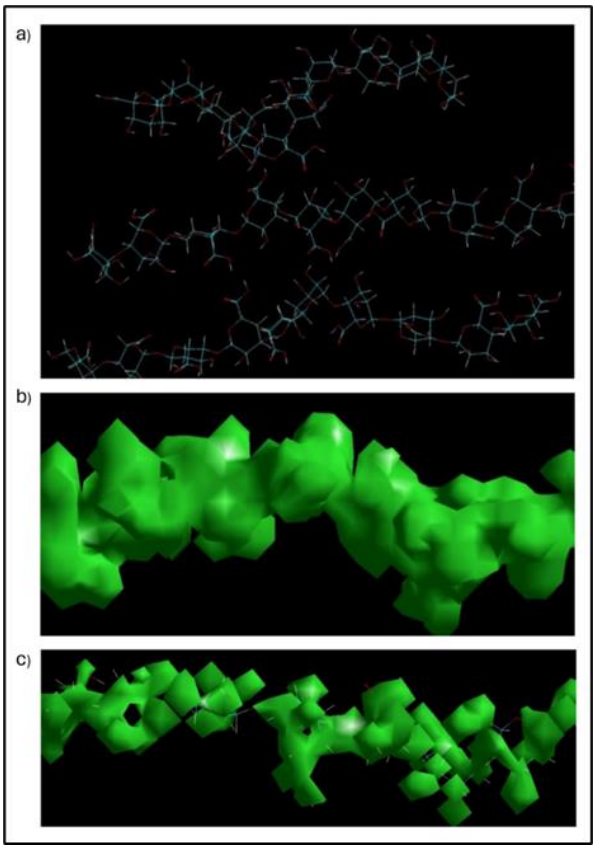

Figure 2. a) chain, MMMGGG, loosely crosslinked sodium alginate, b) electronic density of an MMMGGG sodium alginate chain at the end of loose crosslinking, c) electronic charge distribution of the same chain before crosslinking. This visualization of the molecular model shows that electronic density and the charge distribution of the crosslinked alginate chains includes all three chains in one band.

For further antiviral testing performed at the University of Ulm a modified protocol was used. 1% Alginate was prepared in HEPES 10 mM/0.9% Saline and pH adjusted to 7 using NaOH. The medium contains 1.8 mM calcium. For infection assay, VeroE6 cells were seeded one day prior in 24 well format (B, 100k cells) or 96 well format (C, 6k cells) one day prior. The next day, medium was mostly removed, but 100 µl (24 well) or 50 µl (96 well) left on. An equal volume of test substances was then applied by spraying or pipetting. Virus (VSVΔG(Fluc/eGFP) pseudotyped with SARS-CoV-2-Spike EG5.1 as previously described, [18]) or medium only (for metabolic activity testing) was then added and infection rate determined the next day by measuring Fluc activity. All data in triplicates, means with SEM shown.

3. Result and Discussion

The present approach combines various techniques. Literature data in comparison with in silico modeling and with empirical validation yields data to advance the understanding of the structure of thin calcium alginate layers. Sodium alginate chains are crosslinked at the end of the COONa groups, when the Na is removed and substituted with a calcium cation which connects the opposing COO^- groups and calcium alginate is formed. If all of the available COO^- groups are connected, the result is a tight, non-flexible rigid membrane with a complete loss of water on the sodium alginate side. However, also partial substitution of the sodium ions in the COONa groups can be achieved, when about half of the COO^- groups are connected via calcium ions. Then a loose crosslinking occurs and instead of a tight membrane a flexible gel layer is generated with lower water loss on the sodium alginate side. With the goal to develop an oral-nasal spray, we are interested to achieve this gel-like layer that can be well tolerated in the mouth and nose.

Computational models using HyperChem (HyperCube Inc.) simulated the crosslinking process aiming to determine the optimal conditions to advance the understanding of the structure of thin calcium alginate layers as described above. The used computational models include molecular volume, and mass of the alginate chains and number of calcium crosslinks per chain. In Figure 2 an example shows the electronic charge distribution of chains consisting of six sugar subunits in the order MMMGGG that are loosely crosslinked. Only half of the COO^- groups interact with calcium, however, the electronic density and the electronic charge distribution show the crosslinked molecules as one band, where the single carbohydrate strands interact. A flexible network is formed.

These simulations were validated against existing visualizations and are in accordance with the following data from the literature. Using atomic force microscopy and dilute solution viscometry, it was shown before that polymerization follows three critical steps: The first is monocomplexation of alginate with Ca^{2+} which directly leads to the second step that is dimerization. Finally, in the third step, multimerization occurs [5]. Overall, the formation of alginate gels is a very complex process [6]. The three steps are influenced by the type and length of the alginate chains and the various possibilities of alternating patterns of M and G units and their ratio. Therefore, to assure similar outcomes for different tests with alginates, experimental quality controls are necessary to check and monitor the process.

Sodium alginate can be diluted in water; however, the solution is viscous whereby the viscosity is dependent on the concentration and the exact substance that is used. In our first spraying experiments samples of 2% (w/v) sodium alginate were used where the carbohydrate chains showed solubility and a viscosity that can be sprayed. A shot was sprayed into petri dishes containing calcium chloride (1mM, 3mM, 10mM and 1M calcium chloride). Gel formation occurred at 3mM, 10mM and 1M calcium chloride, however, mostly a bulk aggregate was formed. The intension was to obtain a thin layer.

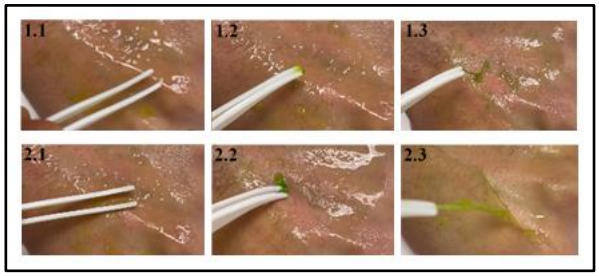

Figure 3. Film formation by spraying sodium alginate and calcium chloride on skin (vertical: Sodium alginate sample 1 Harihatamaja Industries, Sample 2 Amit Hydrocolloids; horizontal: (.1, .2, .3) 10, 50, 100 mM $CaCl_2$. In all samples calcium alginate networks were formed. At 10 mM $CaCl_2$ a gel is formed that is very loose, at 50 mM $CaCl_2$ sample 2 is a fragile membrane, at 100 mM both samples form a membrane layer, whereby the membrane of sample 2 was so solid that it detached from the skin as a whole layer.

As in Petri dishes surface tension formed droplets of aequous solutions, skin was used as a model to form a thin layer in subsequent experiments (Figure 3). It is shown that a thin film or membrane is formed if there is only a very small amount of calcium solution. Two different calcium alginate samples were used, one from Harihatamaja Industries (sample 1) and one from Amit Hydrocolloids (sample 2), to analyze what can be used for the production of an oral-nasal spray. Each of these samples was sprayed with $CaCl_2$ at 10, 50 and 100 mM. Upon spraying the alginate solution in well-nebulized form onto liquid layers containing calcium chloride, very thin films of calcium alginate in the range of micrometers occur. To analyze the strength of the networks mechanical pull-off tests were carried out to confirm the integrity and strength of the membranes (Figure 3). At a low concentration of 10 mM $CaCl_2$ a gel is formed that is very loose, at 50 mM $CaCl_2$ sample 2 is a fragile membrane, at 100 mM both samples form a membrane layer, whereby the membrane of sample 2 was so solid that it pulled of the skin.

The results of the spray tests reveal a clear correlation between the strength of film formation and the solution concentration. These findings align with the data from chemical simulations, which show that rigid membranes form when all COO^- groups coordinate with calcium ions. In contrast, loose, gel-like networks are generated when only a subset of the COO^- groups -approximately half - are bound to the calcium ions.

Additionally, we analyzed crosslinking kinetics to see how long the film forming reaction takes. These crosslinking reactions are very fast, but so are also diffusion kinetics for this system and competitive action is taking place. When sodium alginate is sprayed into a large volume of medium containing calcium ions and is not well nebulized, it will lead to mass formation deep in the liquid rather than the formation of a jellylike film. This has been observed in the first experiments above. Therefore, the sample must be applied in the appropriate manner by fine spraying and the concentrations of alginate and $CaCl_2$ must be controlled.

Our goal is to produce a thin gel layer as a very flexible film of alginate within mouth and nose. Saliva and nasal secretion contain calcium ions in the range of 1-3 mM. The first spraying tests show that the formation of a calcium alginate is at the limit at these concentrations. Therefore, an approach to add calcium ions by additional spraying is feasible. We found that sodium alginate can be sprayed onto liquid containing calcium ions or the two solutions are sprayed together, data not shown.

For a preventive spray a solid membrane as it occurs at 100 mM calcium chloride would not be pleasant in the mouth. Additionally, solid parts of calcium alginate could be dangerous in the airways. 100 mM calcium chloride is the maximum concentration that is tolerated in the mouth. Here, we see, that a concentration well below would be used. These data help to control concentration and application of an oral-nasal spray. Calcium chloride concentrations in the range 10 - 50 mM with 1-2% sodium alginate can yield a thin gel layer in mouth and nose. The aim is that it is well-accepted by users.

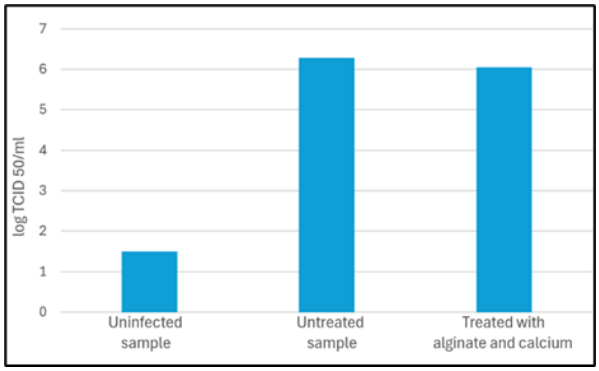

Figure 4. When added in bulk to the cell culture medium calcium alginate showed no inhibition of infection with feline corona virus in comparison to uninfected control and control where virus was added without further treatment.

Of large importance is also the antiviral activity of the calcium alginate layer. Therefore, cell culture assays were performed. In experiments performed at BluTest Laboratories with feline coronavirus alginate was added to the medium of the cells that contained >1.5 mM calcium ions. The formation of a film was not controlled and observed. The result was that no viral inhibition was observed (see Figure 4). Cell culture assay data show

that treatment with neither 2% nor lower concentrations of sodium alginate inhibited the replication of feline coronavirus, as increases in virus titre were consistent between the treated and the untreated cultures.

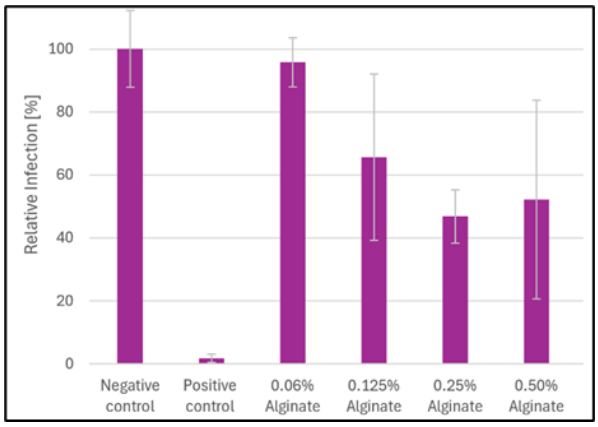

Figure 5. Calcium alginate showed at final concentration 0.1-0.5% inhibition of infection with pseudotyped VSV when added as a layer on top of cells. At 0.06% alginate no. so significant inhibition was observed. (Negative Control: Buffer, Positive Control: Viruseptin.)

Further viral assays were performed with a harmless virus-like particle containing similar size and similar spike proteins like corona virus. The medium contained 1.8 mM calcium chloride. Sodium alginate was pipetted so that a layer was formed above the cells. No significant blockage of viral entry was observed at concentrations below 0.1% calcium alginate. However, viral infection was clearly prevented at alginate concentrations 0.1 - 0.5% (see Figure 5). Up to around 50% inhibition was observed. Spraying the alginate solution was found to yield the same inhibition but with quite high variability.

It was suggested viral inhibition by calcium alginate depends on the mechanical properties of the biopolymer chain [1]. For some viruses it was shown by electron microscopy that aggregation occurred, and it was indicated that the antiviral action could be related to decreased functional content of viral particles in solution or that it interfered with viral uncoating during infection. If a network of calcium alginate covers large parts of the cell surface viral particles cannot pass through and might stick to the polymer [16]. Thereby the number of viral particles entering and infecting the cells are reduced.

The manner of application and the concentration of calcium ions or the use of different viral particles might be responsible for the different results. It has been reported before that only part of viral with alginate-based material showed antiviral properties [1]. This was not clearly dependent on the virus, nor on the type of alginate-based material. We suggest from our data that the right combination of substances and the use of the appropriate conditions to form a calcium alginate network is important to achieve viral inhibition.

Originally, this research started to find a prevention against SARS-CoV-2. Still this virus causes many infections but epidemics with other respiratory viruses are also important like influenza that infects each year millions of people and causes the flu and in worse cases death. Influenza virus has many similarities to corona viruses. As the prevention by calcium alginate is generally unspecific it might be possible to achieve prophylaxis against the entrance of different airborne viruses into human cells.

Pollen from flowering plants is responsible for many allergic reactions. They are natural particles similar in size to viruses. A thin layer of calcium alginate deposited by oral-nasal spray might also prevent their entry into the human body and be of help for lots of allergic persons.

A further aspect that must be considered in the development of an antiviral oral-nasal spray is the biocompatibility of the material. Alginates have been known for a long time and used in food. However, as the carbohydrate chains can vary in length and sequence of subunits, it has been described that molecular weight has an impact. Although the biocompatibility of alginate gels and films has been demonstrated, the purity of the substance remains important [16] [19]. Therefore, assessment of biocompatibility of the substance for future use in the human body is recommended.

Analysis of surface area of the mouth and nose and the expected amount of saliva could be used to calculate the required amount of sodium alginate and calcium to form a gel and cover the mucous membranes. The specific sodium alginate substances should always be tested with appropriate cell culture assays for biocompatibility and antiviral properties before application.

4. Conclusion

The final goal of the current project is to have a spraying system and exact formulations of sodium alginate and calcium chloride solutions that can form a very thin protective antiviral film when sprayed into the user's mouth.

Here, computational chemistry simulations visualized calcium alginate network systems at molecular level using exact surfaces and volumes before and after crosslinking. This helps to calculate the requirements of the ratio of calcium cations to alginate mass for different levels of crosslinking. The results of these in silico analysis showed analog results as experiments spraying sodium alginate into liquid containing calcium chlorid. Depending on the ratio of calcium to COO^--groups in alginate solid membranes or loosely formed networks build gel layers are formed.

Conditions were established so that the calcium alginate forms effectively gel-like networks and thin membranes. As previous results did not show consistent antiviral properties of calcium alginate, we analyzed with a variety of techniques sodium alginate substances and the formation of calcium alginate networks. In cell culture conditions were found where experiments proved that

over 50% of the infections of cells with viral particles can be inhibited easily by calcium alginate overlaying cells. These antiviral tests show that a thin gel layer of calcium alginate can inhibit viral entry into cells.

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