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GRADO EN BIOTECNOLOGÍA

Bibliographical review: Alginate-based materials with antiviral properties

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Abbreviation Index

AAD — adipic acid dihydrazide
AIDS — acquired immunodeficiency syndrome
Alg — alginate
ALV — alginate-lipid vesicle
ALTV — alginate-lipid-tremella vesicle
A-OA-GTE — alginate-oleic acid-green tea extract films
AV — alginate vesicle
BMSCs — bone marrow stem cells
BSA — bovine serum albumin
Ca^{2+} — Calcium ion
$CaCl_2$ — Calcium chloride
$CaCO_3$ — Calcium carbonate
$CaSO_4$ — Calcium sulfate
CER cells — chicken embryo-related cells
Chs — chondroitin sulfate
EB/AO — ethidium bromide/acridine orange
FFD — film-forming dispersion
G — α-L-glucuronic acid
Gp120 — envelop glycoprotein 120
GSE — grape seed extract
GSE-1 — general search equation 1
GSE-2 — general search equation 2
GTE — green tea extract
HAD — HIV-associated dementia
HAd5 — human adenovirus type 5
HAV — hepatitis A virus
HBV — hepatitis B virus
HCV — hepatitis C virus
Hela cells — human cervical cancer cells
HIV-1 — human immunodeficiency virus type 1
HSV-1 — herpes simplex virus type 1
IC_{50} — 50% inhibitory concentration
IFN-1 — interferon type 1
IFV — influenza virus
IgG — immunoglobulin G
IgM — immunoglobulin M
IHNV — infectious hematopoietic necrosis virus
IHNV G — infectious hematopoietic necrosis virus glycoprotein
IPN — interpenetrating polymer network
KD — dissociation constant
M — (1-4)-β-D-mannuronic acid
M/G ratio — mannuronate to guluronate ratio
MNV — murine norovirus
MSC — mesenchymal stem cells
MW — molecular weight
Na^{2+} — Sodium ion
NaCl — Sodium chloride
NaOH — Sodium hydroxide
NIPAAm — N-isopropylacrylamide
PAG — poly(aldehyde guluronate)

PAH — poly(acrylamide-co-hydrazide)
PEG — poly(ethylene glycol)
PEG-co-PCL — poly(ethylene glycol)-co-poly(ε-caprolactone)
PG — polyguluronate
PNIPAAm — Poly(N-isopropylacrylamide)
PVX — potato virus X
RGD — cellular recognition peptide arginine-glycine-aspartic acid
Semi-IPN — semi-interpenetrating polymer network
SGF — simulated gastric fluid
SI — selectivity index
sIgA — intestinal secretory immunoglobulin A
SPMG — sulfated polymannuroguluronate
SPR — surface plasmon resonance
SPSE-1 — specific search equation 1
SPSE-2 — specific search equation 2
SPSE-3 — specific search equation 3
SPSE-4 — specific search equation 4
SPSE-5 — specific search equation 5
Tat — transactivator of transcription protein
TC_{50} — 50% toxic concentration
$TCID_{50}$ — 50% tissue culture infectious dose
TI — therapeutic index
TMV — tobacco mosaic virus
UV — ultraviolet radiation
Vero cells — African green monkey cells

Abstract

Alginate is a biomaterial obtained from brown marine algae that presents not only excellent physical, chemical and biocompatibility properties, but also demonstrated antibacterial activity, which makes it an ideal candidate for the development of biomedical applications. However, its use as an antiviral agent has not yet been widely explored in the scientific community, so the objective of this thesis is to gather as much information as possible about the antiviral activity of alginate.

In this thesis, a deep bibliographic review has been carried out on the antiviral activity that alginate can present. For this purpose, different search engines and databases have been consulted, such as Google Scholar and PubMed, using a series of general and specific search equations.

The results of this bibliographic search have shown that alginate possesses antiviral activity against a variety of viruses that can affect different organisms, such as viruses that affect the human species: the human immunodeficiency virus type 1 (HIV-1), the hepatitis B virus (HBV) , sindbis virus, herpes simplex virus type 1 (HSV-1), hepatitis A virus (HAV), influenza virus (IFV), poliovirus type 1 and rabies virus; viruses that affect mice, such as murine norovirus (MNV), and viruses that affect plants, such as tobacco mosaic virus (TMV) and potato virus X. Alginate has also been used in the field of virology as a vaccine delivery system or as a scaffolding material for the development of viral models.

Scientific production in this field has been very uneven, with few articles published in recent years. However, the results presented in this thesis suggest that alginate can indeed be used as an antiviral agent. This, together with the small scientific production regarding this topic, highlights the opportunity involved in the study of this biomaterial as an antiviral agent.

Keywords: *alginate, biomaterials, antiviral activity, biomedicine, bioengineering*

Resumen

El alginato es un biomaterial obtenido a partir de las algas marinas marrones que presenta unas excelentes propiedades físicas, químicas y de biocompatibilidad, además de presentar actividad antibacteriana, lo que lo hace un candidato ideal para el desarrollo de aplicaciones biomédicas. Sin embargo, su uso como agente antiviral aun no ha sido muy explorado en la comunidad científica, por lo que el objetivo de este trabajo es recabar toda la información posible sobre la actividad antiviral del alginato.

En este trabajo se ha realizado una profunda revisión bibliográfica sobre la actividad antiviral que puede presentar el alginato. Para ello se han consultado diferentes buscadores y bases de datos, como Google Scholar y PubMed, haciendo uso de una serie de ecuaciones de búsqueda generales y específicas.

Los resultados de esta búsqueda bibliográfica han demostrado que el alginato tiene actividad antiviral frente a una serie de virus que afectan a diferentes organismos, como virus que afectan a la especie humana: el virus de inmunodeficiencia humana tipo 1(VIH-1), el virus de la hepatitis B (HBV), el virus sindbis, virus herpes simple tipo 1 (HSV-1), el virus de la hepatitis A (HAV), el virus de la influenza (IFV), el poliovirus tipo 1 o el virus de la rabia, virus que afectan a ratones, como el norovirus murino (MNV), y virus que afectan a las plantas, como el virus del mosaico del tabaco (TMV) o el virus X de la patata. El alginato también se ha usado en el campo de la virología como sistema de administración de vacunas o como *scaffold* para el desarrollo de modelos víricos.

La producción científica en este campo ha sido muy desigual, con pocos artículos publicados en los últimos años. Sin embargo, los resultados obtenidos en este trabajo sugieren que el alginato sí que puede ser usado como un biomaterial con actividad antiviral. Esto, junto a la poca producción científica, pone de manifiesto la oportunidad que supone el estudio de este biomaterial como agente antiviral.

Palabras clave: *alginato, biomateriales, actividad antiviral, biomedicina, bioingeniería*

INTRODUCTION

Alginate: general properties

When the first biomaterials were designed, they weren't made to have direct contact with any biological system. Naturally-obtained materials (e.g. wood) have been used throughout the years in therapeutical treatments for dressing wounds and replacing lost limbs. However, as time passed, these natural materials began to be replaced by man-made polymers, like ceramics and alloys. These synthetic materials performed better and had increasingly reproducible properties, as opposed to those obtained from nature (Ratner & Bryant, 2004) e. As a consequence of this development, a new meaning for the word biomaterial was coined: a material made to interact with biological systems in order to asses, treat, increase or restore any part or capacity of the host's body (Williams, 2009). The boundaries restraining the uses of biomaterials are still expanding. New biomaterials focus on replicating the many uses of the extracellular matrices of body tissues, which manage the organism's inner interactions in a determined manner. In addition, materials obtained from nature have gained a lot of attention due to their biocompatibility.

Alginate is a natural anionic polymer that can be extracted from brown algae. Due to its inherent biocompatibility, low toxicity, and reasonable price, it is a biomaterial that has been widely researched in order to use it as a tool in the field of biomedicine. Moreover, the addition of divalent cations such as Na^{2+} (Gombotz & Wee, 1998), causes the formation of alginate hydrogels by cross-linking, whose particular resemblance to the extracellular matrices of body tissues grants many biomedical uses such as wound dressing, small-scale drug and protein delivery, and scaffolding for tissue growth. These hydrogels have many applications and can vary their structure attending to the cross-linker types and the cross-linking methods, which will be further addressed.

Typically, alginate is obtained from brown seaweed comprised in the class *Phaeophyceae*, mainly from the species *L. hyperborea*, *L. digitata, Macrocystis pyrifera* and *Ascophyllum nodosum* (Skjak-Braek & Smidsrod, 1990), treated then with basic solution, usually NaOH. *Ascophyllum nodosum* has a concentration of alginate of 22-30% of its dry weight, while *Laminaria hyperborea*'s varies from 17-33% to 25-30% depending on the part of the algae the alginate is extracted from (Qin, 2008). The extract is filtered and mixed with calcium or sodium chloride to precipitate alginate, which is

treated with watered-down chloride acid to obtain alginic acid. Next, several concentration cycles are succeeded to finally obtain hydrophilic alginate powder.

Another method that yields better alginate properties is bacterial biosynthesis from *Pseudomonas spp* and *Azotobacter vinelandii*. The general process begins with the synthesis of a precursor substrate, followed by polymerization and membrane transfer, the periplasmic transfer and modification, and lastly, the exportation through the external membrane (Remminghorst & Rehm, 2006). Further research undergoing these synthesis processes and our ability to easily change bacteria might allow us to create customized alginate better suited for specific applications.

Basic structure and its characterization

Alginate is a linear polysaccharide composed of subunits of (1-4)-β-D-mannuronic acid (M) and its C-5 epimer α-L-glucuronic acid (G). These monomers can be distributed in several ways that directly affect alginate's physical properties: consecutive M (M-Blocks), consecutive G (G-Blocks), or alternating subunits (MG-Blocks) (Fig 1). Apart from the M/G ratio, other characteristics like the molecular weight and the degree of acetylation also determine alginate's rheological properties (Urtuvia, Maturana, Acevedo, Peña, & Díaz-Barrera, 2017) Different sources produce alginate that vary in G and M contents and the length of each block, creating many possible different structures with different properties. For example, the species *L. digitata* has a M-block content of 49%, while other available alginates range from 15% to 43% (Qin, 2008).

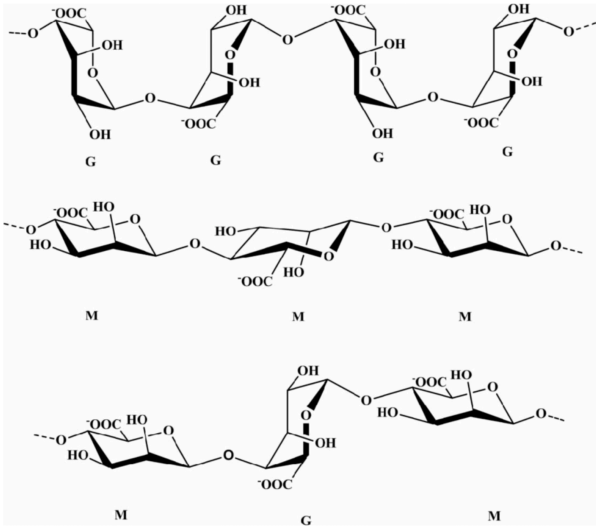


Fig 1. Chemical structures of G-block, M-block and alternating block in alginate (Lee & Mooney, 2012)

However important M-blocks are to determine the physical properties of the different kinds of alginate, only the G-blocks take part in the cross-linking process with divalent cations to form hydrogels. Alginate's physical treats are improved by expanding the length of G-block and molecular weight. These characteristics determine the stability of the gels, their rate of drug release, and the properties of encapsulated cells.

Molecular weight and solubility

Commercially available sodium alginates have a molecular weight (MW) that goes from 32,000 to 400,000 g/mol. According to the Mark-Howink formula ($[\eta] = KM_v^a$) for sodium alginate in NaCl solution, the alginate's viscosity increases as pH decreases, peaking around pH 3–3.5, as carboxylate groups become protonated and form hydrogen bonds (Rinaudo, 1992).

Rising alginate MW can enhance the mechanical characteristics of the resultant gels. However, it also increases its viscosity greatly, an unwanted property for the following processes that can cause, for instance, high shear forces (LeRoux, Guilak, & Setton, 1999). Controlling alginate's MW and its distribution can determine the solution's viscosity pre-gelation and its rigidness afterwards. Using a combination of low and high MW alginate, the elasticity of the gel can be raised significantly, whereas the viscosity barely increases (Kong, Lee, & Mooney, 2002).

Biocompatibility

Even though the biocompatibility of alginate has been thoroughly reviewed *in vivo* as well as *in vitro*, authors still argue about how it is affected by the alginate's composition, although a great deal of them disagree mainly because of the different purity levels of the alginate studied in their reports. For instance, high M content alginates have been reported to be immunogenic and much more efficient in the induction of cytokine production, as opposed to great G content alginates (Otterlei M, Ostgaard K, Skjakbraek G, Smidsrod O, Soonshiong P, 1991). However, other authors reported little to no immunogenic response against alginate hydrogels. This immunogenic reaction might be caused by remaining alginate impurities, like heavy metals or proteins (Zimmermann et al., 1992). This immunogenic reaction might be caused by lingering alginate impurities, like heavy metals or proteins. In addition, highly pure alginate obtained from purification processes didn't cause any reaction when implanted into animals (Orive et al., 2002). In

the same way, hydrogels made with bought alginate didn't produce any immunogenic reaction either (Lee & Lee, 2009).

Hydrogel formation

Alginate is mainly employed in the shape of a hydrogel in biomedicine, typically for dressing wounds, delivering drugs and in tissue engineering. These gels are three-dimensionally cross-linked networks made of hydrophilic polymers with high water content. Hydrogels have a high biocompatibility, as discussed above, due to how deeply its structure resembles that of the macromolecular-based components within the body.

Additionally, they can be applied by minimally invasive methods (Sakiyama-Elbert SE, 2001). In order to create hydrogels, cross-linking hydrophilic polymers, be it physically or chemically, is the main technique used since their physicochemical properties are highly dependent on the cross-linking type and density, molecular weight and chemical composition aside (Varghese & Elisseeff, 2006), (Lee & Yuk, 2007). In the following sections, several ways to cross-link alginate chains in order to create gels are discussed, and how the way they are arranged might affect the hydrogel properties significant to biomedical applications.

Ionic cross-linking

The most utilized method to produce hydrogels using an aqueous alginate solution is to mix said solution with ionic cross-linking agents, in particular divalent cations, namely Ca^{2+} or Na^{2+}. The structure of the guluronate blocks grants a very high degree of coordination of the divalent ions, which are considered to link exclusively to the guluronate blocks of the alginate chains. The guluronate blocks of one polymer then form junctions known as the egg-box model of cross-linking, with the guluronate blocks of adjacent polymer chains being a gel structure the result of the process (Fig. 2) (Grant, Morris, Rees, Smith, & Thom, 1973). Calcium chloride ($CaCl_2$) is one of the most frequently used agents to ionically cross-link alginate. However, it typically leads to rapid and poorly controlled gelation due to its high solubility in aqueous solutions. In order to moderate and control the alginate's gelation, one possibility is to use a buffer with phosphate as a component, since alginate's carboxylate groups in reaction with calcium ions compete with the phosphate groups in the buffer, slowing the process of gelation. Because of their lower solubilities, calcium carbonate ($CaCO_3$) and calcium sulfate

($CaSO_4$) are able to increase the working time for alginate gels, as they can as well slow the gelation rate. For instance, $CaCO_3$, which is not soluble in water at pH 7, can be mixed with an alginate solution. Next, glucono-δ-lactone is added to the $CaCO_3$/alginate blend in order to lower the pH so that the gelation of the alginate solution is started by the dissociated Ca^{2+} in a more steady and progressive manner (Crow & Nelson, 2007).

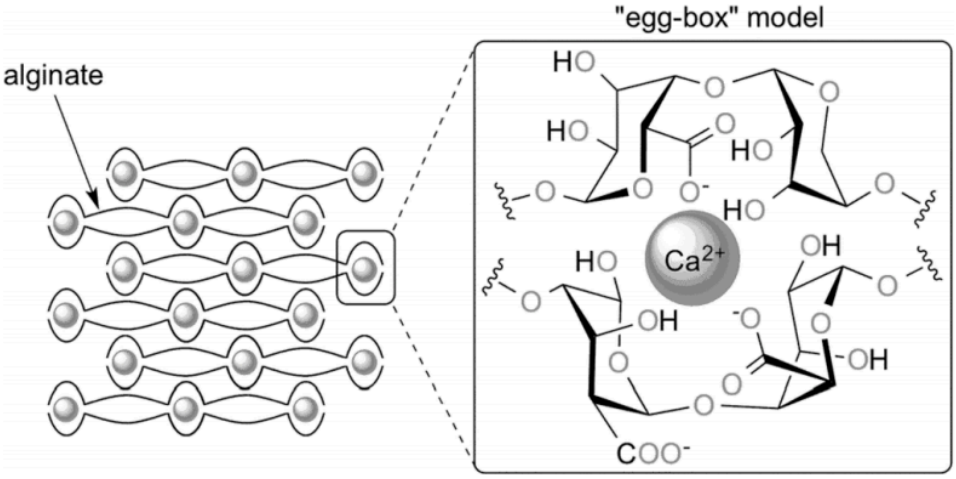


Fig 2. Schematic representation of the egg-box association of the poly-L-guluronate sequences of alginate crosslinked by calcium ions (Kühbeck et al., 2015)

When using divalent cations, the rate of gelation needs to be taken into account in order to control the gel's uniformity and strength. For instance, slower gelation rate makes for more regular structures and an increased mechanical integrity (Kuo & Ma, 2001). In addition, the temperature of the gelation process can alter the gelation rate as well, and the final physical properties of the gels. When the temperature is low, the cross-linking agents (divalent cations) see their reactivity decreased, slowing the cross-linking process altogether. The final gel has a neat and uniform structure, which deeply increases its mechanical characteristics (Augst, Kong, & Mooney, 2006). These characteristics may be affected greatly by the structure of the alginate used in the process. For instance, using alginate with a high amount of G residues increases the gel's stiffness, whereas those with a lesser amount of G residues does not (Drury, Dennis, & Mooney, 2004).

A significant limitation of this kind of cross-linking process is the short stability the gels exhibit when exposed to long-term physiological conditions. Exchange reactions with monovalent cations may cause the dissolution of these gels due to the release of divalent cations into the surrounding media. Furthermore, hemostasis might be caused by the release of calcium ions, making the cross-linking network a matrix where blood

cells (e. g. erythrocytes) can aggregate (Suzuki et al., 1998). This is not necessarily a drawback, but in order to avoid biological reactions such as these, and other problems presented by ionic cross-linking, researchers have turned their sights on covalent cross-linking.

Covalent cross-linking

In order to improve the mechanical characteristics of the gels, researchers have been recently focused on covalent cross-linking. When a mechanical force is applied on an ionically cross-linked alginate gel, the cross-links dissolve and rebuild elsewhere, leading to the dehydration of the matrix and the consequent plastic deformation. Although this dehydration phenomenon is also present in covalent cross-linking, precisely the covalent nature of the bonds allows them to resist dissociation, leading to a significantly more elastic deformation (Zhao, Huebsch, Mooney, & Suo, 2010). Nevertheless, cross-linking agents used in covalent cross-linking may be toxic, and the non-reactive agents may need to be thoroughly removed from the gels.

In order to widen the range of physical properties of the gels, it was first researched the covalent cross-linking of alginate with poly(ethylene glycol)-diamines (PEG) of several molecular weight. The gel's elasticity first increased steadily with an increment in the cross-linking density of PEG in the gel. However, it then decreased as the molecular weight between cross-links descended below the molecular weight of the softer PEG (Eiselt, Lee, & Mooney, 1999). Therefore, it was proved that the physical characteristics of alginate hydrogels are determined by the different types of cross-linking agents used in the reaction, and by regulating the cross-linking densities. The chemical nature of these agents can also alter the swelling of the hydrogel. Using hydrophilic cross-linking agents such as PEG can make up for the dehydration caused by the cross-linking reaction (Lee et al., 2000).

Using cross-linking agents with several functions to male hydrogels allows a broader range and firmer control of the degradation process and physical stiffness than bi-functional cross-linking agents. For instance, the mechanical characteristics and degradation rates of poly(aldehyde guluronate) (PAG) gels made with either poly(acrylamide-co-hydrazide (PAH) as a multi-functional cross-linker or adipic acid dihydrazide (AAD) as a bi-functional cross-linker were compared *in vitro*. PAG/PAH gels displayed greater physical stiffness before degradation and degraded more slowly than PAG/AAD gels. The improved physical stiffness slower degradation rate might be

caused by the many attachment points of PAH in the gel even without changing the concentration of the functional groups (Lee, Bouhadir, & Mooney, 2004).

In situ gelation with covalent cross-linking may be approached with photo cross-linking. This process can be realized in moderate reaction conditions, even with drugs and cells present, with the right chemical starters. Alginate, in combination with methacrylate and cross-linked with an argon ion laser (λ=514 nm) exposure of 30s in the presence of eosin and triethanol amine, makes clean and malleable hydrogels, which were used to seal a corneal perforation in vivo, demonstrating yet another use in sutureless surgery (Smeds & Grinstaff, 2016). Photo cross-linking reactions generally use a light sensitizer or the discharge of an acid, which can be dangerous to the organism. Another approach uses polyallylamine combined partially with α-phenoxycinnamyldiene acetylchloride, which dimerizes at 330 nm of light exposure and doesn't produce any toxic products (Tanaka & Sato, 1972). The physical properties of the hydrogels obtained from this photosensitive polyallylamine and alginate were quite improved by light irradiation, and the gels were significantly permeable to myoglobin and cytochrome c (Lu, Lan, Wang, Chang, & Wang, 2000).

Thermal gelation

Drug delivery systems have prompted the research on thermo-sensitive hydrogels, due to their adaptable swelling characteristics as a result of changing temperatures, leading to personalized regulation of drug release from the gels (Roy, Cambre, & Sumerlin, 2010). Poly(N-isopropylacrylamide) (PNIPAAm) hydrogels have been exploited the most among the thermo-sensitive gels, and these go through a reversible phase transition close to body temperature in hydrophilic media (lower critical solution temperature near 32ºC). The temperature of the transition can be modified by copolymerization with polar monomers like acrylic acid and acrylamide (Rzaev, Dinçer, & Pişkin, 2007). In spite of the potential significance of thermos-sensitive hydrogels in the biomedical field, not many alginate systems have been developed yet, since alginate is not a thermo-sensitive biomaterial itself. Nevertheless, semi-interpenetrating polymer network (semi-IPN) structures were made by in situ copolymerization of of N-isopropylacrylamide (NIPAAm) with poly(ethylene glycol)-co-poly(ε-caprolactone) (PEG-co-PCL) macromer in the presence of sodium alginate by ultraviolet (UV) irradiation (Fig. 3). The gel's swelling behavior escalated alongside the sodium alginate's concentration at a sustained temperature and was diminished as the temperature raised. Using sodium alginate in

semi-IPN structures also enhanced the physical strength and the cumulative discharge of bovine serum albumin (BSA) from the gels, demonstrating potential in drug delivery applications (Zhao et al., 2010).

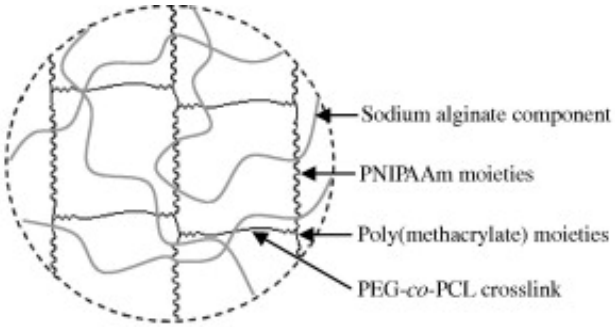


Fig. 3 Schematic representation of thermo-sensitive semi-interpenetrating network hydrogel (Zhao et al., 2010).

Cell cross-linking

Various chemical and physical methods have been developed to make alginate gels, although the cellular ability to add to the gel's formation has been mainly overlooked. If alginate is altered with cell adhesion ligands, the cell's ability to attach several polymer chains can result in a reversible network structure even when there aren't any cross-linking agents. When cells are added to an RGD-altered alginate solution (i.e. altered with cellular recognition arginine-glycine-aspartic acid peptide), they are regularly distributed in the solution, and this structure then makes the cross-linked network structure by specific receptor-ligand interactions without the use of any more cross-linking agents (Fig. 4) (Lee, Kong, Larson, & Mooney, 2003).

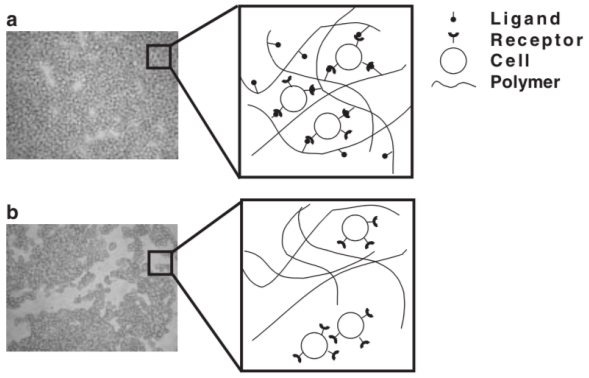


Fig. 4 Cell-cross-linked network structures when cells are mixed with (**a**) RGD-modified alginate and (**b**) non-modified alginate (Lee, Kong, Larson, & Mooney, 2003).

However, when cells are added to unmodified alginate solutions, they pile up forming an irregular structure, due to the heavy interactions that occur between cells in that system. This gelation rate can be reversed by shearing, and thus can be redone many times. When this structure is broken down with shear forces, those structures that have cross-linked can be recovered in a short amount of time. This phenomenon is caused by the non-specific and reversible ligand-receptor interactions in the system. This reaction might prove to be suited for the delivery of cells in tissue engineering, since the gel flows like a liquid at the moment of the injection and then solidifies when distributed in the organism. Moreover, it was demonstrated that cells can add to the physical integrity of RGD-alginate gels ionically cross-linked with calcium ions, through interactions between cells and the ligands paired to the alginate chains (Drury, Boontheeku, & Mooney, 2005).

Biodegradation of alginate compounds

Mammals cannot degrade alginate polymers themselves, since they don't produce the enzyme alginates needed to break the polymer chains. However, ionically cross-linked alginate gels can be dissolved with the discharge of the divalent ions cross-linking the hydrogels to the media around it, because of the exchange reactions with monovalent cations like sodium ions. If the gel did dissolve, the molecular weight of the commercially available alginates is higher than the renal clearance threshold and wouldn't be eliminated from the body (Al-Shamkhani & Duncan, 1995). Another way to turn alginate into a biodegradable polymer involves partial oxidation of the alginate's chains. This kind of alginate can dissolve in a hydrated media and makes for materials that have proved to show potential as carriers for drug and cell delivery. This oxidation process is caused generally by the addition of sodium periodate (Fig. 5), that breaks the bonds between the carbons of the cis-diol group in the uronate residue and turns the chair conformation into an open chain, a reaction that facilitates the alginate's backbone degradation. This minor oxidation, however, doesn't really affect the alginate's ability to produce hydrogels in the presence of divalent cations. The gel's final degradation behavior highly depends on the degree of oxidation, in addition to the pH and the media's temperature (Bouhadir et al., 2001).

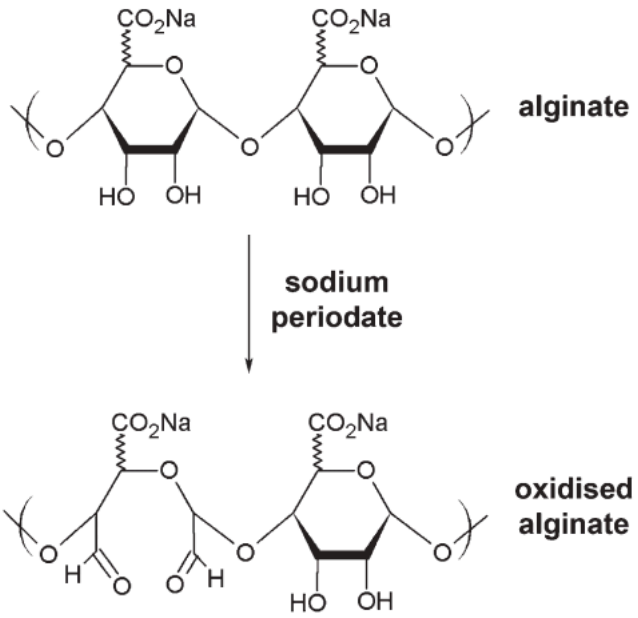


Fig. 5 Partial oxidation of alginate with sodium periodate (Wright, de Bank, Luetchford, Acosta, & Connon, 2014)

Gels can be built with G-blocks isolated from alginate, slight oxidation of the G-blocks allows the formation of degradable gels. For instance, polyguluronate (PG) was extracted from alginate at pH 2.85 and oxidized with sodium peridiotate in order to make PAG. Gels can be prepared with PAG and AAD covalently cross-linked instead of ionically cross-linked (Fig. 6). The reaction between hydrazides and aldehydes happens in a very short time, and the newly-formed hydrazine bonds will be susceptible to hydrolysis, making hydrogels that dissolve in aqueous media. The degradation behavior of these gels became slower as the concentration of AAD increased. The physical characteristics and degradation behavior are properties that play a significant role in the engineering of new tissue, but are often paired, making it hard to modify one without affecting the other. However, PAG gels cross-linked with single-ended AAD molecules demonstrated a prolonged degradation rate, unrelated to the low cross-linking density. The great amount of single-ended AAD molecules enabled re-cross-linking of PAG chains after the hydrolysis of the aforementioned hydrazine bond (Lee, Bouhadir, & Mooney, 2000), indicating that soft gels with that have a prolonged degradation rate can be made.

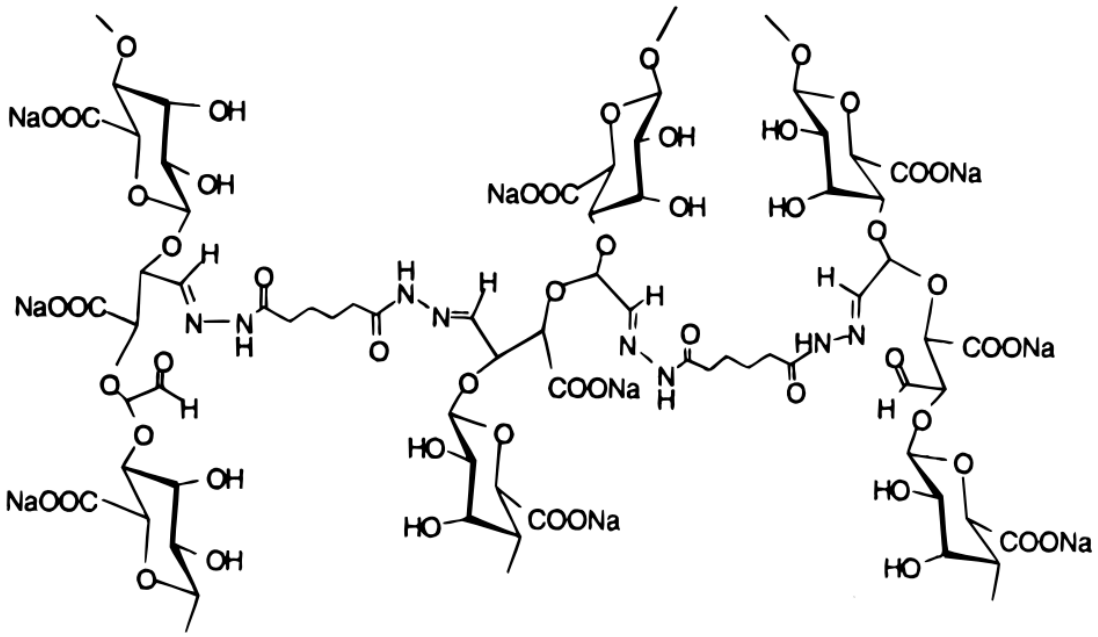


Fig. 6 Chemical structure of poly(aldehyde guluronate) gels cross-linked with adipic acid dihydrazide (Lee, Bouhadir, & Mooney, 2000).

Moreover, the degradation behavior and physical characteristics of alginate gels can be unpaired by regulating the alginate's molecular weight distribution. Gels with low and high molecular weight have been obtained from slightly oxidized alginates via covalent or ionic cross-linking. Alginate's molecular weight was altered with γ-irradiation, leaving the G-block's length virtually unchanged. By raising the low molecular weight alginate's fraction to 0.5 sustained the physical stiffness of the gels, as opposed to the high molecular weight alginate gels, but resulted in s swifter degradation, unrelated to the cross-linking method used (Kong, Kaigler, Kim, & Mooney, 2004). Meanwhile, prepared gels with two kinds of alginate with different G-block length demonstrated a faster ion exchange rate, leading to gel degradation (Kong, Alsberg, Kaigler, Lee, & Mooney, 2004). These several ideas can be effective by themselves or combined, in order to change the mechanical characteristics of several hydrogels in the evolution of drug and cell delivery carriers.

Alginate-based materials with antiviral properties

There remains today a critical need for new antiviral agents, particularly in view of the alarming increase in drug resistance and associated issues. The objective of this thesis is to examine the different ways alginate can be used as an antiviral agent. In order to achieve this goal, a series of different kinds of alginate-based materials will be reviewed

in the following sections of this thesis so as to determine the role of alginate and its uses in today's fight against viruses, and its potential in future applications.

For instance, it has been reported that sulfated alginate with an unique ratio of 1,4-linked β-d-mannuronic acid over α-l-guluronic acid blocks inhibits the binding between the human immunodeficiency virus type 1 (HIV-1) receptor in the human body, CD4+ T lymphocytes, and envelop glycoprotein 120 (gp120), which plays a critical role in the initiation of the entry process of HIV-1 into CD4+ T cells (Meiyu et al., 2003). It was also demonstrated that sulfated alginate also significantly decreased vulnerability of PC12 cells to HIV Tat protein by protecting cells from apoptosis (Hui et al., 2008).

Furthermore, it was also reported that that a marine polysaccharide drug 911 derived from alginate and dominated by heterogeneous fragments (MG) could dramatically inhibit the severe infection of MT4 cells and the chronic infection of H9 cells with HIV-1 (Xianliang, Meiyu, Huashi, & Li, 2000). This inhibition effect was mainly attributed to the inhibition of viral reverse transcriptase, the interference with viral adsorption, and the enhancement of immune function (Xin et al., 2000). Moreover, 911 can also improve the immune function of the body's cells and inhibit the DNA polymerase of hepatitis B virus (HBV), thus indicating that alginate-derived drug 911 can also hinder the replication of HBV (Bao-fa et al., 2003).

When used as encapsulation technique for HuH-7 cells (i.e. human liver cell line), alginate microspheres demonstrated antiviral activity against several viruses when added to the supernatant, namely strain JFH1 of hepatitis C virus (HCV), Sindbis virus, herpes simplex virus type 1 (HSV-1), and Poliovirus type 1. Additionally, the use of calcium alginate hydrogel beads also prevented the release of HCV viral agents when the hepatic cells were previously infected and encapsulated. Using empty calcium alginate hydrogel beads in inhibitory experiments also demonstrated that the antiviral activity was dose- and incubation time-dependent and depended on chemical interactions between the calcium alginate gel and the HCV viral particles (Tran et al., 2014). This interesting approach could have a potential role within the field of regenerative medicine but needs further investigation.

Alginate hydrogel has also been demonstrated to possess antiviral activity against HSV-1 when used as a sulfated compound, showing a dose-dependent antiviral activity and a range of IC_{50} (i.e. 50% inhibitory concentrations) between 0.6 and 10 μg/ml,

lacking cytotoxicity at concentrations up to 200 μg/ml. The antiviral activity was dependent on the sulfate contents of the polysaccharides. The chemical properties of the sulfated alginate and the *in vitro* characteristics reported in this study demonstrate that sulfated alginate is an interesting candidate for further anti-viral research (Bandyopadhyay et al., 2011).

Alginate has also been tested against the rabies virus in chicken-embryo-related (CER) cells, where the initial step in infection of CER cells by rabies virus, i.e. the virus adsorption process, was affected by alginate's antiviral activity, measured on the basis of 50% inhibition of nucleocapsid antigen synthesis, which was observed at concentrations as low as 1.0 μg/ml for alginate, showing 75% inhibition at 100 μg/ml. These results led to the conclusion that the inhibitory effect alginate had on the rabies virus was dose-dependent in nature, while also being far below the cytotoxicity threshold, showing what could be a potential new lead in the development of antirabies virus agents (Pietropaolo et al., 1993).

Additionally, alginate has also demonstrated to show antiviral activity against some plant virus. Alginate inhibited potato virus X (PVX) infectivity by 95% when used *Chenopodium quinoa* as host, and the mechanism of inhibition may be via aggregation of viral particles (Pardee et al., 2004), as observed in other assays seen before where alginate's antiviral activity was studied. In another study, researchers observed that sodium alginate had a high inhibitory activity against tobacco mosaic virus (TMV) infection. When alginate was added to the inoculum solution, the quantity of local lesions formed on Xanthi tobacco leaves was significantly decreased, and the inhibition effect improved as the alginate concentration rose, being greater when the alginate used had a lower composition of mannuronate to guluronate ratio (M/G ratio), which suggests that the strength of inhibition depends on the rigidity of the polymer chain. Researchers concluded that alginate's antiviral activity may caused by blocking the decapsulation process of TMV protein on the cell membrane surface (Sano, 1999).

These findings have been made by using uncombined alginate, which leads to the question of what can be achieved if the natural antiviral activity of alginate is magnified by its combination with other polymers or chemicals.

Alginate-based composites

There hasn't been much investigation regarding the antiviral activity of alginate-based composites throughout the years, as the study of alginate's antiviral activity in combination with other polymers is still a novel idea in need of attention. However, it has been recently reported that the addition of lipids and natural extracts rich in phenolic compounds (i.e. green tea extract (GTE) and grape seed extract (GSE)) into alginate hydrogels produced edible films by emulsion, which have been tested for their antiviral activity against murine norovirus (MNV) and hepatitis A virus (HAV). Interestingly, it was reported that alginate films containing GTE were slightly more efficient on HAV and MNV than the films containing GSE. Moreover, the antiviral activity mainly increased alongside the concentration of phenolic extracts, except in the case of GTE where its efficacy against MNV were similar at both GTE concentrations (Fabra et al., 2018). In a related study, the researchers obtained the same results when the alginate/oleic films containing green tea extract developed for the preservation of strawberries and raspberries were tested against MNV and HAV (Falcó et al., 2019). This indicates that alginate has a potential role in the field of food preservation, which will however require more investigation until it can be successfully applied.

In another study, the cytotoxicity and anti-influenza virus (IFV) activity of calcium and zinc alginate fibers were investigated on African Green Monkey kidney cells (Vero) and human cervical cancer cells (Hela) in order to determine whether they could be used as biomaterials. The antiviral assays carried out on Vero cells with IFV resulted in a maximum of 34.42% protection yielded by the calcium alginate fibers, while the zinc alginate fibers yielded a maximum of 59.42% protection. Overall, the investigators concluded that both alginate fibers had a good cellular biocompatibility, but that the heavier zinc alginate fibers had a better anti-IFV activity than calcium alginate fibers, which could be potentially used in the field of tissue engineering (Gong et al., 2011).

Alginate in the virology field

Alginate is a biopolymer that can be used in many applications. In the virology field, it has been thoroughly employed as a delivery system of many different vaccines that induced both antibacterial and antiviral effects, although not directly. For instance, the encapsulation of recombinant adenovirus into biodegradable alginate microspheres was studied as a delivery system in order to avoid the pre-existing immunity against adenovirus. Mice, both naïve and immunized with human adenovirus type 5 (HAd5),

were inoculated with a HAd5 recombinant containing the bacterial β-galactosidase LacZ gene, either loaded into alginate beads or as a viral suspension. In this assay, it was demonstrated that the immune response against the vector adversely affected transgene expression, and that alginate microencapsulation of recombinant adenovirus effectively circumvented the vector-specific immune response, which could present an effective delivery system of different kinds of antiviral vaccines (Sailaja, HogenEsch, North, Hays, & Mittal, 2002). In a similar study, an oral DNA vaccine against infectious hematopoietic necrosis virus (IHNV) encapsulated in alginate microspheres was demonstrated to induce dose-dependent immune responses and significant protection in rainbow trout (*Oncorrhynchus mykiss*) (Ballesteros et al., 2015), which further proves the utility of alginate used in the delivery of antiviral vaccines.

Alginate has also been used in combination with chitosan as nanoparticles for the encapsulation of hepatitis B antigen as an oral vaccination system in order to assess the virus ability to induce local and systemic immune responses. In this assay, only the mice inoculated with chitosan-coated alginate beads loaded with HBV induced the production of viral antibodies detected in serum (IgG) and in the intestinal washings (sIgA) (Borges et al., 2007).

In a similar way, chitosan-coated alginate-$CaCl_2$ microbeads were used as an oral vaccination delivery system of bacteriophage Felix O1. The therapeutic use of bacteriophages has been thoroughly studied as an alternative to antibiotics in order to treat bacterial infections. Researchers have reported several successful applications including the use of phages to treat *Escherichia coli* in calves and lambs (Williams Smith & Huggins, 1983), and to decrease both *Campylobacter jejuni* (Carrillo et al., 2005) and *Salmonella* (Atterbury et al., 2007) infection of broiler chickens. It has also been reported the effective use of bacteriophage therapy for the control *Salmonella* and *Campylobacter* on the surface of meat (Goode, Allen, & Barrow, 2003). In the bacteriophage Felix O1 study, it was demonstrated that the alginate-chitosan encapsulation method allowed a large fraction of the phage to remain bioactive in an artificial gastrointestinal tract environment, which indicates that these microspheres may enable delivery of therapeutic phage to the gut (Yongsheng et al., 2008).

In another study, it was evaluated a system for oral vaccine delivery of Influenza A virus subtype H5N3, consisting of liposomes coated first with a layer of tremella and then with an outer layer of alginate. In this assay, it was demonstrated that the triple layer was more resistant to an acidic pH and controlled the release profiles at an alkaline pH,

and also improved the mucosal production of intestinal secretory immunoglobulin A (s-IgA), providing protection against H5N3 infection. This system may have potential use as a carrier for oral vaccine delivery (Cheng et al., 2011).

Alginate has also been used as a scaffolding biomaterial in the virology field. For example, a highly porous alginate hydrogel was functionalized with the Tobacco Mosaic Virus (TMV) and a mutant version containing the cellular recognition RGD peptide (arginine-glycine-aspartic acid), which were used because of TMV's well-known genetic/chemical modularity, its multivalence (TMV capsid is composed of 2130 copies of identical subunits), and its well-defined structural characteristics. In this assay, the alginate-based porous composite hydrogels were successfully synthesized and functionalized with TMV, demonstrating their potential as carriers to support 3D stem cell culture and differentiation. It was also demonstrated that TMV surface can be accessed in these hydrogels, as deduced from a significant increasement in cell attachment after functionalization by TMV-RGD. Furthermore, using TMV and its RGD variety can enable bone differentiation of bone marrow stem cells (BMSCs) (Luckanagul et al., 2012).

These results were further reaffirmed in a related study where alginate hydrogel was used as a scaffolding material and functionalized with TMV in order to induce bone regeneration in rodents with cranial imperfections. In this study, the alginate hydrogel was also functionalized with the cellular recognition peptide, arginine−glycine−aspartic acid (RGD), through an incorporation of an RGD mutant of TMV (TMV-RGD). The functionalized alginate hydrogel was demonstrated to enable bone differentiation of mesenchymal stem cells (MSCs) *in vitro*, which enabled the researchers to perform an *in vivo* study on rats with cranial bone imperfections. The results indicated that the TMV-functionalized hydrogel scaffolds didn't cause systemic toxicity, and that the alginate scaffold could support cell localization and could be further optimized for bone regeneration and repair. Paired with more biocompatibility and bone generation studies *in vivo*, this system could have a great potential in the field of tissue engineering. This interesting approach could not only facilitate the localization, delivery, and differentiation of stem cells but also simplify the process of matrix modification (Luckanagul et al., 2016).

OBJECTIVES

The main goal pursued in this work is to carry out a thorough bibliographical review on the topic of alginate-based materials used as antiviral agents. In order to achieve this main objective, the following secondary objectives are proposed:

- Describe the general properties of alginate and the different hydrogels that can be obtained from it.

- Analyze, after a deep bibliographical research, the different ways alginate-based materials have been used up until now, especially in regard to how they have been used against viral activity.

MATERIALS AND METHODS

The methodology used to carry out this final thesis is the classical documentary analysis method, used to detect and analyze the different sources of information in order to obtain the "state of the art" on the subject under study.

In this sense, a thorough bibliographic search was carried out on the biomaterial alginate, its main characteristics, its biomedical applications and its potential antiviral activity.

Databases

This bibliographical review was carried out within the time period that ranges from the 1st of January of 1972 to the 31st of December of 2019. The databases consulted were: Google Scholar and Pubmed.

Google Scholar is a subset of the larger Google search index, consisting of full-text journal articles, technical reports, preprints, theses, books, and other documents, like a select few websites deemed scientific enough. Although this search engine covers a vast range of areas, it specifies on science, particularly medicine, and secondarily in the social science (Gusenbauer, 2019). Google Scholar was first developed in 2004, and contains roughly 389 million documents (including articles, citations and patents) as estimated in January 2018, including documents in more than 20 different languages.

PubMed is a free-access search engine that has been active since 1996 with more than 30 million medical citations from the database MEDLINE, life science journals and online books. Citations may include links to full-text content from PubMed Central and publishers' websites. The National Library of Medicine of the United States maintains the database at the National Institutes of Health as part of the Entrez information retrieval system (Lindberg, 2000).

In order to select the documents that were finally used in this thesis, a series of inclusion and exclusion criteria were defined, which are detailed below.

Inclusion criteria

The following criteria was taken into account in order to select the documents from which the information used in this thesis was obtained:

- Articles published in scientific journals, Web portals, books, conferences or reviews.
- Documents published in Spanish or English.
- Works whose titles are related to the scope of study of this thesis and whose objectives are consistent with the information meant to be presented.
- Bibliographic reviews of related literature in which there has been made a previous selection of information.
- Articles published after 1972.

Exclusion criteria

- Studies in a language other than English.
- Studies whose objectives are not related in any way to any of the objectives of this thesis.
- Articles unrelated to alginate-based materials.
- Works obtained from unsanctioned databases.
- Articles published before 1972.

Search equations

The documents used in this thesis were found by using keywords related to the topic/objective of the study, connected with different Boolean operators such as: “AND”

used to find documents that contain several terms, "OR" used to search for documents that contain any of the terms that are searched, and "NOT" used to avoid an unwanted concept.

In order to present the keywords used, a table was made with the different descriptors and their respective terms and synonyms (Table 1).

Table 1. Used descriptors and synonyms in the searching process

Descriptor	Synonyms and related words
Alginate	Biomaterial, hydrogel, gel, structure, biocompatibility, biodegradation, antiviral, composites, nanocomposites, scaffolds, applications
Antiviral	Antiviral activity, antiviral properties, antiviral capacities, antiviral vaccines
Composites	IPN, semi-IPN, fibers, compounds
Cross-linking	Ionic cross-linking, covalent cross-linking, thermal gelation, cell cross-linking
Delivery system	Carrier, vehicle
Microspheres	Microparticles, nanoparticles, microbeads, beads
Scaffolds	Matrix, net, support system, cell culture
Structure	Composition, formation, distribution, content
Vaccine	DNA vaccine, oral vaccine
Viral	Viral studies, viral assays, viral models

During the bibliographic search, the “subject” field was used mostly, except in some specific cases, where both the “title” and “author” fields were used. The “title” field was also used to further narrow the search when the documents obtained in the first place were not related to the search carried out.

First, a general search was carried out to obtain a more generic view of the information, and then the search was narrowed down using more specific word combinations and search equations (Table 2).

Table 2. General and specific search equations entered into the different databases. Searches carried out between February and June 2020.

Search equation	Abbreviation
Alginate AND Antiviral activity OR antiviral properties OR antiviral capacities	General Search Equation 1 (GSE-1)
Alginate AND Structure OR composition OR formation OR distribution OR content	General Search Equation 2 (SPSE-2)
Alginate AND hydrogel OR gel AND cross-linking OR ionic cross-linking OR covalent cross-linking OR thermal gelation OR cell cross-linking	Specific Search Equation 1 (SPSE-1)
Alginate AND hydrogel OR gel AND biodegradation	Specific Search Equation 2 (SPSE-2)
Alginate AND composite OR IPN OR semi-IPN OR fibers OR compounds AND Antiviral activity OR antiviral properties OR antiviral capacities	Specific Search Equation 3 (SPSE-3)
Alginate AND delivery system OR carrier OR vehicle AND vaccine OR DNA vaccine OR oral vaccine	Specific Search Equation 4 (SPSE-4)
Alginate AND scaffold OR matrix OR net OR support system OR cell culture AND viral studies OR viral models OR viral assays	Specific Search Equation 5 (SPSE-5)

In the first general search equation (GSE-1), the keywords “Alginate” and “antiviral activity” were used as a first search in order to obtain a general view on the information known about alginate’s antiviral activity. The next general search equation (GSE-2) includes the descriptors “alginate” and “structure”, or one of their synonyms or related words, as seen in Table 2, in order to obtain more specific information related to alginate’s structure, molecular weight, solubility and biocompatibility.

Once a general view was acquired, a more narrowed search was carried out with the use of specific search equations (SPSE), with the purpose of focusing the search and obtaining new specific information.

The first specific search equation (SPSE-1) includes the descriptors “alginate”, “hydrogel” and “cross-linking” or one of their synonyms or related words described on Table 1, in order to carry out a more narrowed search on alginate’s different methods of hydrogel cross-linking.

The next specific search equation (SPSE-2) includes the descriptors “alginate”, “hydrogel” and “biodegradation”, or one of their synonyms or related words described on Table 1, in order to focus the search on the biodegradation behavior presented by alginate hydrogels.

The third specific search equation (SPSE-3) includes the descriptors “alginate”, “composite” and “antiviral activity”, or one of their synonyms or related words described on Table 1, in order to finally obtain specific information on the different alginate-based materials and their reported antiviral activity.

The fourth specific search equation (SPSE-4) includes the descriptors “alginate”, “delivery system” and “vaccine”, or one of their synonyms or related words described on Table 1, in order to obtain specific information on the different ways alginate can be used as a delivery system of oral or DNA vaccines in the virology field.

The last specific search equation (SPSE-5) includes the descriptors “alginate”, “scaffold” and “viral studies”, or one of their synonyms or related words described on Table 1, in order to obtain specific information on the different ways alginate can be used as a scaffolding material in the development of viral studies.

Once the searching process had finalized, the studies found and selected were collected in tables, and were classified according to alginate-based material, antiviral activity, tested viruses, cytotoxicity, tested cell lines, tested organism, year and reference.

RESULTS and DISCUSSION

Bibliometric analysis

The results obtained from the bibliometric analysis carried out with the search equations previously described are resumed through tables 3-9.

The results obtained from the search with GSE-1 can be observed in table 3. The search yielded a total of 46,654 studies, but only 10 had relevant information and were selected. From the selected articles, 40% could only be found on Google Scholar, while 60% could be found in both databases. The few selected articles from such a high quantity of found articles indicates that researchers have only just set their sights on alginate as a potential antiviral biomaterial, and the topic need further research. The search yielded a great number of studies, possibly due to the fact that the search was carried out in two different, but very large databases, and the search was too general, and many search results didn't include the descriptor combination used in GSE-1, thus showing irrelevant assays. All of the selected articles were found in both databases

Table 3. Results yielded by the general search equation 1 (GSE-1)

GSE-1	Alginate AND Antiviral activity OR antiviral properties OR antiviral capacities	
Databases	**Studies found**	**Studies selected**
Google Scholar	17,400	10
PubMed	29,654	6
Both	46,654	6
Total	46,654	10

The results yielded by the next general search equation (GSE-2) can be reviewed in Table 4. There was a total of 9,574,099 studies reported in this search, but only 14 studies provided the information necessary, which is indicative of how deeply alginate and its structure and properties have been reviewed. The search yielded a great number of studies, 92.156% obtained from PubMed and 7.844% from Google Search. From the selected articles, 21.428% were only found on Google Scholar, while 78.572% could be found on both databases. Such an imbalance on the quantity of results obtained from the different databases is probably due to the fact that PubMed is a more medically-centered database, and alginate has a great deal of applications in the field of

biomedicine, and also because many search results didn't include the descriptor combination used in GSE-2, thus showing irrelevant assays.

Table 4. Results yielded by the general search equation 2 (GSE-2)

GSE-2	Alginate AND Structure OR composition OR formation OR distribution OR content	
Databases	**Studies found**	**Studies selected**
Google Scholar	751,000	14
PubMed	8,823,099	11
Both	9,574,099	11
Total	9,574,099	14

The results obtained with the first specific search equation (SPSE-1) can be reviewed in Table 5. There was a total of 66,153 studies reported in this search, but only 22 studies provided the information necessary. The search yielded a smaller quantity of articles, 40.96% obtained from Goggle Scholar and 54.04% obtained from PubMed. From the selected articles, 50% were only found on Google Scholar, while 50% could be found on both Google Scholar and PubMed.

Table 5. Results yielded by the specific search equation 1 (SPSE-1)

SPSE-1	Alginate AND hydrogel OR gel AND cross-linking OR ionic cross-linking OR covalent cross-linking OR thermal gelation OR cell cross-linking	
Databases	**Studies found**	**Studies selected**
Google Scholar	27,100	22
PubMed	39,053	11
Both	66,153	11
Total	66,153	22

The results obtained with the next specific search equation (SPSE-2) can be reviewed in Table 6. There was a total of 66,153 studies reported in this search, but only 6 studies provided the information necessary for this thesis. Although this search was focused on a specific topic, the use of only 3 different descriptors in the search yielded a greater quantity of results than in other specific searches. The search yielded a larger quantity of articles, 14.8% obtained from Goggle Scholar and 85.2% obtained from PubMed. From the selected articles, 33% were only found on Google Scholar, while 67% could be found on both Google Scholar and PubMed.

Table 6. Results yielded by the specific search equation 2 (SPSE-2)

SPSE-2	Alginate AND hydrogel OR gel AND biodegradation	
Databases	**Studies found**	**Studies selected**
Google Scholar	42,100	6
PubMed	242,244	4
Both	284,344	4
Total	284,344	6

The results obtained with the third specific search equation (SPSE-3) can be reviewed in Table 7. There was a total of 65,538 studies reported in this search, but only 3 studies provided the information necessary to be included in this thesis. The search yielded a smaller quantity of articles, 24,871% obtained from Goggle Scholar and 75,129% obtained from PubMed. This time, all of the selected articles could only be found on Google Scholar, possibly due to the more specialized and technical nature of the selected articles. Although this search was meant to be specific, more suitable articles were expected to be found, which suggests that there isn't much information on the topic of alginate combinations with antiviral activity.

Table 7. Results yielded by the specific search equation 3 (SPSE-3)

SPSE-3	Alginate AND composite OR IPN OR semi-IPN OR fibers OR compounds AND Antiviral activity OR antiviral properties OR antiviral capacities	
Databases	**Studies found**	**Studies selected**
Google Scholar	16,300	3
PubMed	49,238	0
Both	65,538	0
Total	65,538	3

The results obtained with the fourth specific search equation (SPSE-4) can be reviewed in Table 8. There was a total of 93,523 studies reported in this search, but only 9 studies provided the information necessary to be included in this thesis. The search yielded a greater quantity of articles, 31,22% obtained from Goggle Scholar and 68,78% obtained from PubMed. This time, all selected articles could be found on both Google Scholar and PubMed. This search still yielded a big quantity of articles, from which only 9 could be selected, which suggests many search results didn't include the descriptor combination used in SPSE-4, thus showing irrelevant assays

Table 8. Results yielded by the specific search equation 4 (SPSE-4)

SPSE-4	Alginate AND delivery system OR carrier OR vehicle AND vaccine OR DNA vaccine OR oral vaccine	
Databases	**Studies found**	**Studies selected**
Google Scholar	29,200	9
PubMed	64,323	9
Both	93,523	9
Total	93,523	9

The results obtained with the last specific search equation (SPSE-5) can be reviewed in Table 9. There was a total of 539,954 studies reported in this search, but only 2 studies provided the information necessary to be included in this thesis. The search yielded a greater quantity of articles, 6.36% obtained from Goggle Scholar and 93.64% obtained from PubMed. This time, only one of the two studies selected could be found on both databases, finding the remaining one only on Google Scholar, even though both articles were written by the same author. This search still yielded a big quantity of articles, from which only 2 could be selected, which suggests many search results didn't include the descriptor combination used in SPSE-5, thus showing irrelevant assays.

Table 9. Results yielded by the specific search equation 5 (SPSE-5)

SPSE-5	Alginate AND scaffold OR matrix OR net OR support system OR cell culture AND viral studies OR viral models OR viral assays	
Databases	**Studies found**	**Studies selected**
Google Scholar	34,300	2
PubMed	505,654	1
Both	539,954	1
Total	539,954	2

As a final review of the articles found and selected, in Table 10 is summarized all the data regarding these aspects of this thesis. Of 10,670,265 articles found, only 66 articles were selected in order to develop the initial section of this thesis.

Table 10. Results yielded by all search equations

Search equation	Articles found	Articles selected
GSE-1	46,654	10
GSE-2	9,574,099	14
SPSE-1	66,153	22
SPSE-2	284,344	6
SPSE-3	65,538	3
SPSE-4	93,523	9
SPSE-5	539,954	2
Total	10,670,265	66

Figure 7 and 8 describe the percentage of articles found and selected in the two databases used. In Figure 7 it can be observed that the articles found on PubMed (91%) greatly outnumber the articles found on Google Scholar (9%). This may be due to PubMed showing results that don't include the combination of descriptors used in the search equations, thus showing a great deal of irrelevant results, while Google Scholar usually shows results that include the combination of descriptors. That may be the reason why the articles selected are found usually on Google Scholar rather than on PubMed, although many of these studies can be found on both platforms, as described on Fig. 8.

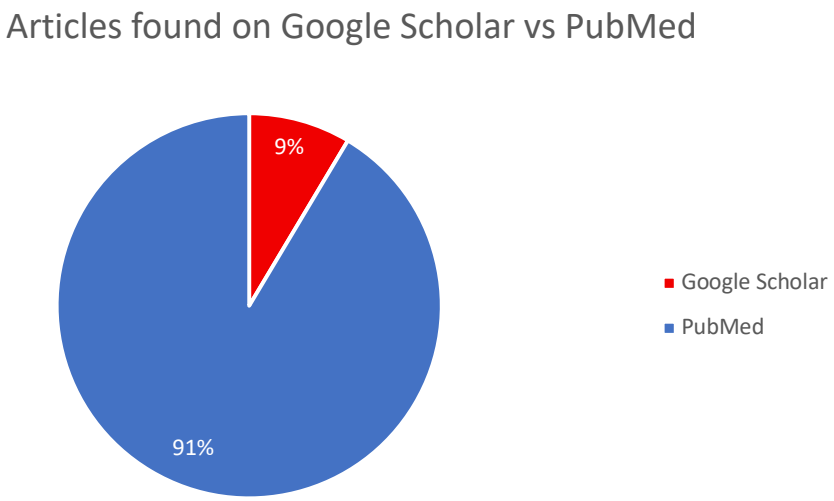


Fig. 7 Articles found on Google Scholar vs PubMed

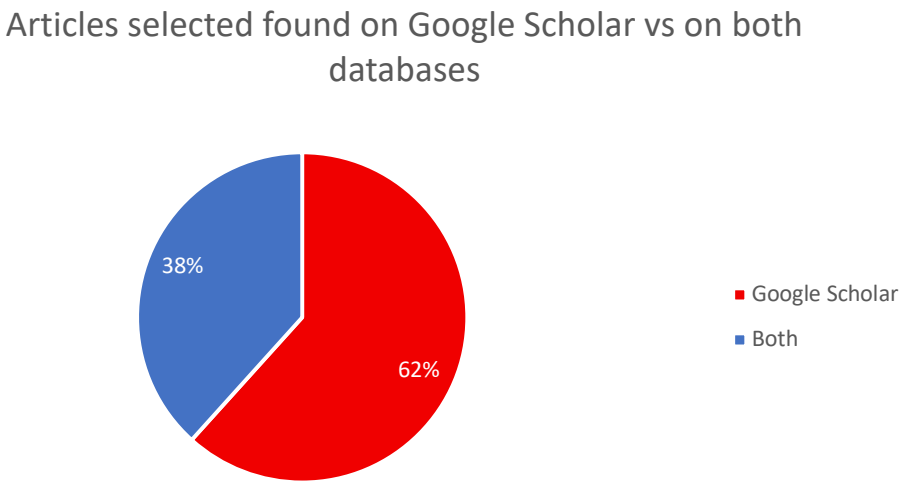


Fig. 8 Articles selected found on Google Scholar vs on both databases

The selected articles range from year 1972 to year 2019, a time period within the described inclusion criteria. Old articles were considered suitable to be included in this thesis in order to thoroughly review all the information regarding alginate and its antiviral properties. Figure 9 describes the yearly scientific productivity within the studied time range.

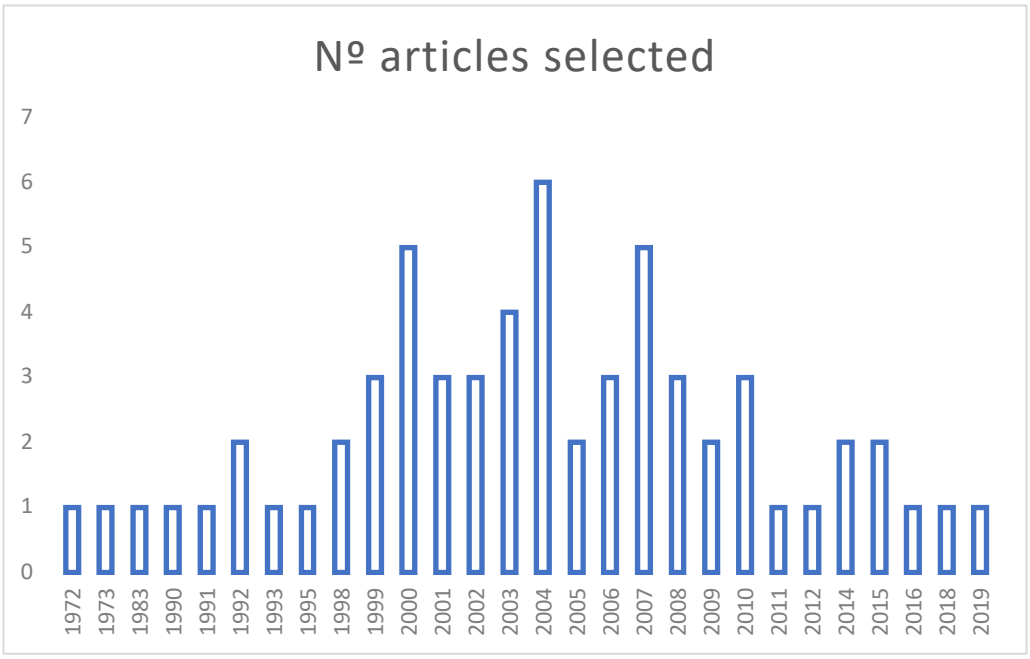


Fig. 9 Selected articles by year

Alginate-based materials with antiviral properties

There has been a lot of information reported regarding alginate, its composition, its antibacterial activity, and, consequently, its many biomedical applications. However, alginate's antiviral activity is an area of study that researchers have yet to fully explore.

The information found and presented in this thesis regarding the antiviral properties of alginate are resumed in Table 11. From the 66 selected articles, only 10 are related to alginate and its antiviral activity and have been classified according to the virus they can repel (Fig. 10). According to Fig. 10, alginate has been proved to have the most antiviral activity against HIV-1 (Meiyu et al., 2003), (Hui et al., 2008), (Xianliang et al., 2000), (Xin et al., 2000), followed by HSV-1 (Tran et al., 2014), (Bandyopadhyay et al., 2011), MNV and HAV (Fabra et al., 2018), (Falcó et al., 2019). Alginate has also demonstrated antiviral activity against HBV (Bao-fa et al., 2003), HCV (Tran et al., 2014), sindbis virus (Tran et al., 2014), poliovirus type 1 (Tran et al., 2014), PVX (Pardee et al., 2004), TMV (Sano, 1999) and influenza virus (Gong et al., 2011).

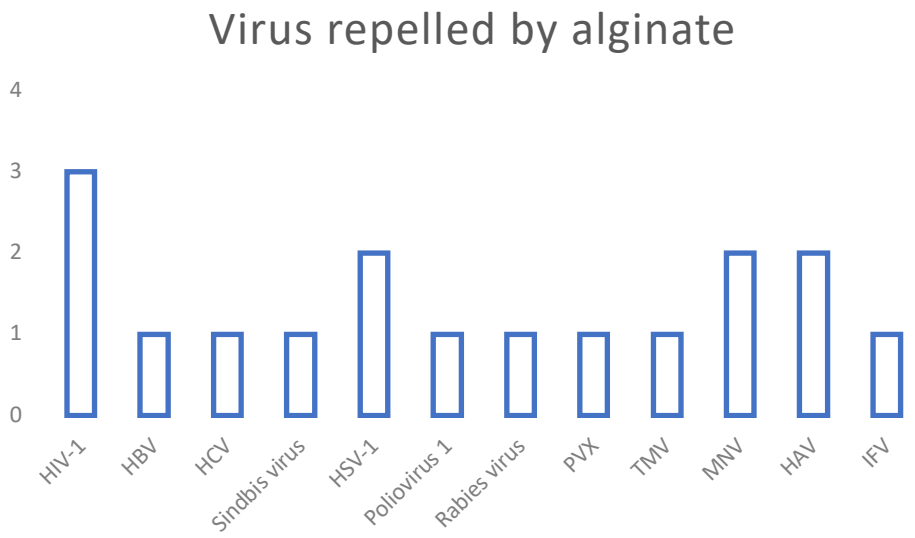


Fig. 10 Virus repelled by alginate

Table 11. Alginate-based materials with antiviral activity

Alginate-based material	Antiviral	Tested viruses	Cytotoxicity	Tested cell lines	Tested on organism	Year	Reference
Sulfated alginate	Yes	HIV-1	No	CD4+ T cells	Human CD4+ T cells	2003	(Meiyu et al., 2003)
Sulfated alginate	Yes	HIV-1	No	P12 cells	Rat pheochromocytoma PC12 cells	2008	(Hui et al., 2008)
Alginate-derived polysaccharide "911"	Yes	HIV-1	No	MT4 cells and H9 cells	H9 (human embryonic stem cell line) and MT4 (human cutaneous T-lymphocyte)	2000	(Xianliang et al., 2000) (Xin et al., 2000)
Alginate-derived polysaccharide "911"	Yes	HBV	No	HepG2. 2.15 cells	Human hepatoblastoma cell line HepG2. 2.15	2003	(Bao-fa et al., 2003)
Alginate microspheres, calcium alginate microspheres	Yes	JFH1 HCV, Sindbis virus, herpes simplex virus type 1 (HSV-1), and Poliovirus type 1	No	HuH-7 cells	Human liver cell line	2014	(Tran et al., 2014)
Sulfated alginate	Yes	HSV-1	No	RC 37 cells	Green monkey cell line (RC 37)	2011	(Bandyopadhyay et al., 2011)
Alginic acid	Yes	CVS (challenge virus strain) fixed rabies virus	No	CER cells	Chicken embryo related cells (CER cells)	1993	(Pietropaolo et al., 1993)
Alginate	Yes	Potato virus X	No	–	*Chenopodium quinoa*	2004	(Pardee et al., 2004)
Sodium alginate	Yes	Tobacco Mosaic Virus (TMV)	No	–	Xanthi NN tobacco leaves	1999	(Sano, 1999)
Sodium alginate with lipids and phenolic extracts (i.e. GTE and GSE)	Yes	Murine norovirus (MNV) and hepatitis A virus (HAV)	No	RAW 264.7 cells (for MNV) and FRhK-4 cells (for HAV)	Murine macrophage cells and primate cell line	2018	(Fabra et al., 2018)

Sodium alginate with oleic acid and GTE	Yes	Murine norovirus (MNV) and hepatitis A virus (HAV)	No	RAW 264.7 cells (for MNV) and FRhK-4 cells (for HAV)	Murine macrophage cells and primate cell line	2019	(Falcó et al., 2019)
Calcium alginate fibers and Zinc alginate fibers	Yes	Influenza virus (IFV)	No	Vero and Hela cells	African Green Monkey kidney cell (Vero) and human cervical cancer cell (Hela)	2011	(Gong et al., 2011)

Alginate in the virology field

Alginate can also be used as a delivery system for vaccines and as scaffolding material designed for viral studies, as can be observed in Table 12. On Table 12 are resumed the results of 7 articles selected regarding alginate's role in the virology field. While alginate is not directly responsible for the antiviral activity observed in these studies, it is interesting to see that alginate can still contribute to the development of more systems that disable viral infection on a number of organisms, which further indicates that alginate ought to be considered as a candidate in the manufacturing of new antiviral biomaterials.

From the 6 selected articles, 66% of them focus on the role of alginate in the development of delivery systems for vaccination purposes, as observed on Figure 11. These studies have different objectives: to avoid the pre-existing immunity against adenovirus (Sailaja et al., 2002), to test alginate's ability to resist degradation in the organism's gastrointestinal tract environment while successfully delivering the vaccine and eliciting an immune response (Ballesteros et al., 2015), (Yongsheng et al., 2008), (Cheng et al., 2011); and to asses HBV ability to induce local and systemic immune responses (Borges et al., 2007).

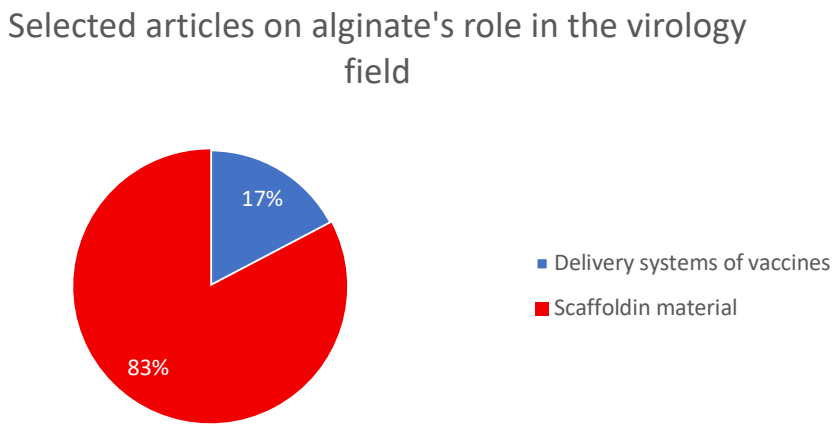


Fig. 11 Selected articles on alginate's role in the virology field

The resting 33% of the selected articles focus on the development of alginate scaffolds functionalized with recombinant variants of TMV. These studies aimed to test alginate's ability as a scaffolding material to support 3D culture and differentiation of BMSCs (Luckanagul et al., 2012), and to induce the regeneration of cranial bones of mice (Luckanagul et al., 2016).

Table 12. Alginate-based materials in the virology field

Antiviral-based material	Used as	Results	Year	Reference
Alginate microspheres	Delivery system of HAd5 into mice	The system effectively circumvented the vector-specific immune response	2002	(Sailaja et al., 2002)
Alginate microspheres	Delivery system of IHNV into rainbow trout	The system induced dose-dependent immune responses and significant protection	2015	(Ballesteros et al., 2015)
Alginate and chitosan microspheres	Delivery system of HBV into mice	The system induced the production of viral antibodies detected in serum (IgG) and in the intestinal washings (sIgA)	2007	(Borges et al., 2007)
Alginate, chitosan and $CaCl_2$ microspheres	Delivery system of bacteriophage Felix O1 into artificial gut	The system allowed a large fraction of the phage to remain bioactive in an artificial gastrointestinal tract environment	2008	(Yongsheng et al., 2008)
Alginate, liposome and tremella microspheres	Delivery system of IFV subtype H5N3	The system was more resistant to an acidic pH and controlled the release profiles at an alkaline pH, and also improved the mucosal production of intestinal secretory immunoglobulin A (s-IgA)	2011	(Cheng et al., 2011)
Alginate and TMV-RGD scaffold	Functionalized scaffolding biomaterial	Successful synthesis and functionalization of scaffolds that supported 3D stem cell culture and differentiation of BMSCs	2012	(Luckanagul et al., 2012)
Alginate and TMV-RGD scaffold	Functionalized scaffolding biomaterial	The scaffold enabled bone differentiation of mesenchymal stem cells (MSCs) *in vitro* and bone regeneration *in vivo*	2016	(Luckanagul et al., 2016)

VIH-1

Meiyu et al., 2003 reported that sulfated alginate (SPMG) employs its anti-HIV-1 effect by hampering the entry process of the viral particles. Therefore, surface plasmon resonance (SPR) and flow cytometric assays were used to identify the potential targets for SPMG regarding the inhibition of the entry process. Results showed that SPMG mainly bound to gp120 through the V3 loop region within the molecule, but also that the SPMG could bind to gp120 through other sites of the protein.

In this assay, the researchers speculated that the interruption of ionic interactions between charged domains of gp120 and the cell membrane were responsible for the anti-HIV-1 of sulfated polysaccharides. In fact, the V3 loop located within gp120 is a highly charged region of the protein and has demonstrated to attract negatively charged molecules (Stanfield et al., 1999; Witvrouw, Desmyter, & de Clercq, 1994). The SPR assay showed that one SPMG molecule bound to three to four 28-amino acid peptides within the V3 loop with high affinity (KD = 1.38 nM), a discovery also demonstrated by the digital docking of the octasaccharide backbone of SPMG and the the V3 loop region (Fig. 12).

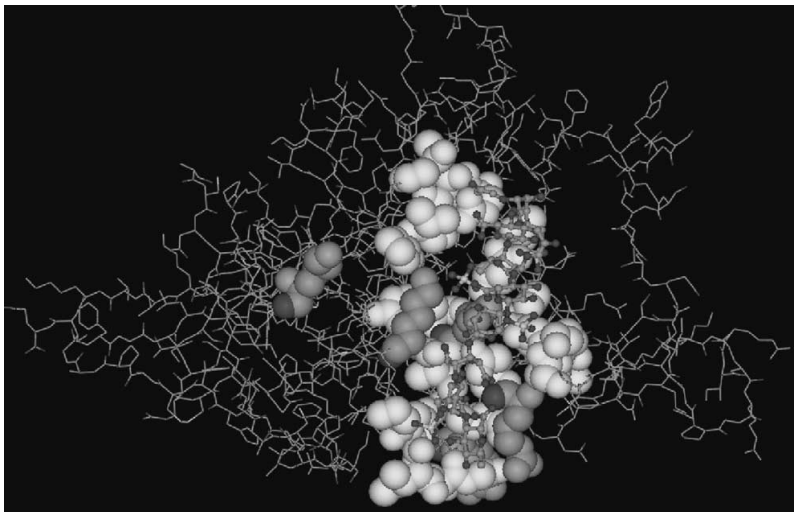


Fig. 12 Computer docking modeling of the V3 loop of gp120 with the octasaccharide unit of SPMG backbone. The binding was mainly due to the electrostatic force (Meiyu et al., 2003).

Hui et al., 2008 also reported that SPMG could be used as treatment for VIH-1 by protecting the cells form apoptosis mediated by the transactivator of transcription protein (Tat) (Eugenin et al., 2007; Gavriil, Cooney, & Weeks, 2000), which has been

demonstrated to cause HIV-associated dementia (HAD). In this assay, the researchers tested the ability of SPMG to protect PC12 cells from Tat-induced apoptosis.

Results showed that the pre-incubation of PC12 cells with SPMG elevated the cell viability after treatment with Tat protein (Fig. 13A), and the number of apoptotic cells was significantly decreased after Tat and SPMG treatment (Fig. 13B).

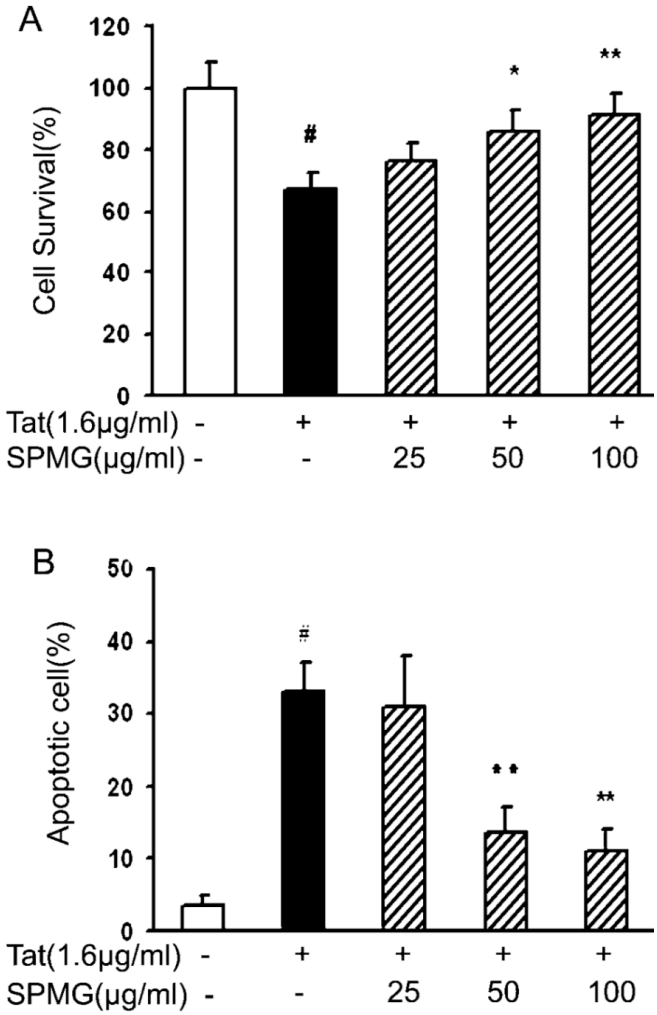


Fig. 13 SPMG inhibits Tat-induced apoptosis in PC12 cells. **A**: PC12 cells were preincubated with the indicated concentrations of SPMG, then treated with Tat or PBS (control). **B**: PC12 cells were preincubated with the indicated concentrations of SPMG, then treated with 1.6 μg/ml Tat. Apoptosis was measured using EB/AO staining assay (Hui et al., 2008).

An increment of cytoplasmic calcium (Ca^{2+}) induced by Tat has been proved to cause apoptosis of human cortical cells in several studies (Brailoiu et al., 2006; Pérez, Probert, Wang, & Sharmeen, 2001; Wallace, 2006). This study also demonstrated that SPMG inhibited Tat-induce apoptosis of PC12 cells through a decrease of the cytoplasmic concentration of calcium (Ca^{2+}), as can be observed in Figure 14.

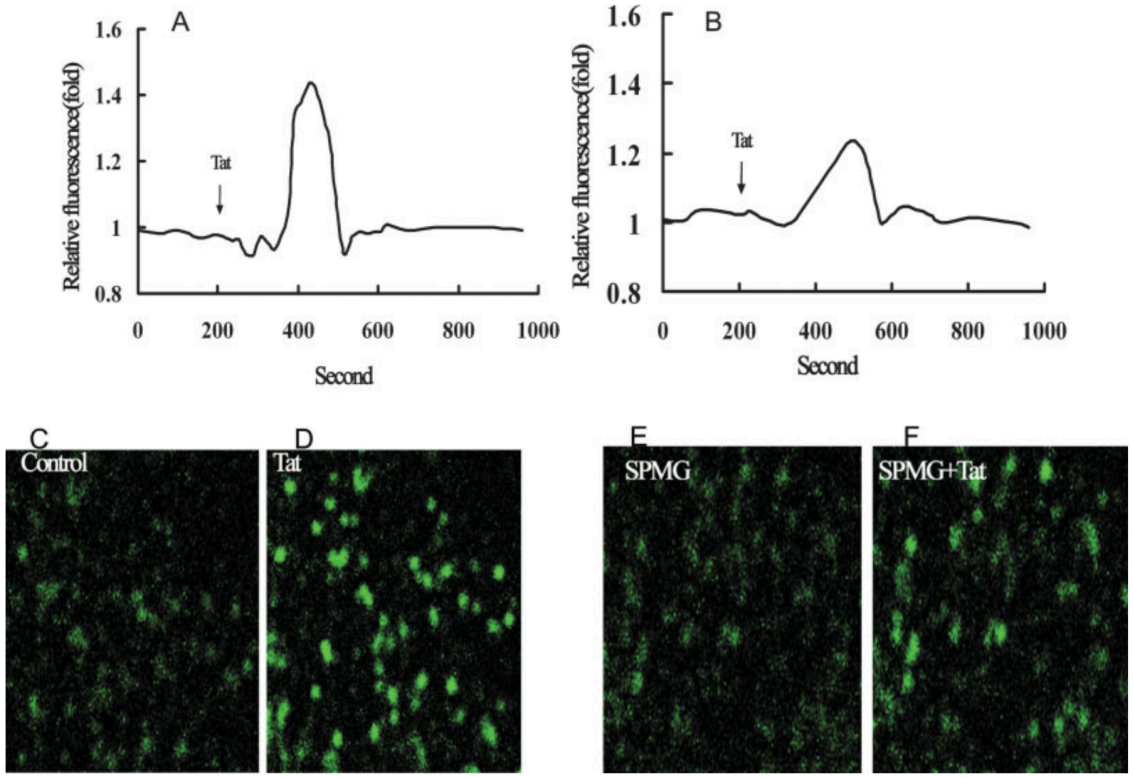


Fig. 14 SPMG decreases Tat-induced calcium overload. **A, B**: Variation of the cytoplasmic calcium concentration by confocal microscopy. PC12 cells were untreated (A) or preincubated with SPMG (B). Then, the cells preincubated with fluo-3-AM were observed with a confocal microscope and then stimulated by Tat at the time indicated by the arrows. **C, E**: Basal levels of Ca^{2+} for control and SPMG before stimulation by Tat, respectively. **D, F**: Maximum levels of calcium stimulated by Tat, respectively. (Hui et al., 2008)

An anti-AIDS effect was also observed by Xianliang et al., 2000 and Xin et al., 2000. In their studies, these researchers observed that the alginate-derived drug 911 inhibited acute and chronic infection of HIV-1 in MT4 and H9 cells in a dose-dependent manner by hampering the activity of HIV reverse transcriptase *in vitro*, with a reported half inhibitory concentration (IC_{50}) of 36.51 $mg \cdot L^{-1}$ for MT4 cells and a half effect concentration (EC_{50}) at 4.44 $mg \cdot L^{-1}$ and 0.32 $mg \cdot L^{-1}$ respectively.

HSV-1

Bandyopadhyay et al., 2011 tested the antiviral activity of alginate-containing fractions obtained from *Sphacelaria indica* against herpes simplex virus type 1 (HSV-1). The alginate used in this study was made of 41% guluronic acid (G) and 59% of mannuronic acid (M), and two sulfated versions were also tested. These researchers reported that alginate inhibited HSV-1 in a dose-dependent manner with a 50% inhibitory

concentration (IC_{50}) that ranged from 0.6 to 10 μg/ml, and the antiviral activity relied on the sulfate contents of the alginates, as can be observed in Table 13.

Table 13. Cytotoxicity, anti-HSV-1 activity, and selectivity index of sodium alginate (B) isolated from *Sphacelaria indica* and its chemically sulfated (BS1 and BS2) derivatives (Bandyopadhyay et al., 2011).

Polysaccharides	TC_{50}(μg/ml)[a]	IC_{50}(μg/ml)[b]	SI (TC_{50}/IC_{50})[c]
B	≥1000	10	≥100
BS1	≥1000	0.65	≥1538
BS2	≥1000	0.6	≥1667

[a] 50% toxic concentration (TC_{50}): compound concentration required to reduce cell viability by 50%, as determined from dose-response curves.

[b] 50% inhibitory concentration (IC_{50}): compound concentration required to reduce virus plaques by 50%.

[c] Selectivity index (SI): ratio between TC_{50} and IC_{50}.

The mechanism of antiviral action was also investigated in this assay. Cells were pretreated with sodium alginate and its sulfated derivatives (B, BS1 and BS2) before infection and viruses were incubated with acyclovir both before and after infection, in order to determine the inhibitory effect of alginate during different stages of the viral replication cycle (Fig. 15). Figure 15-I shows that no significant effect could be observed when the cells were treated with alginates before infection, whereas Figure 15-II shows that pretreating HSV-1 with the different alginate types before infection provoked a dramatic decrease in plaque formation for B, BS1 and BS2. However, Figure 15-III shows that while none of the alginate molecules demonstrated a significant antiviral effect, Acyclovir inhibited the viral replication by 98.6%, which was to be expected, since it inhibits viral DNA as it is synthesized

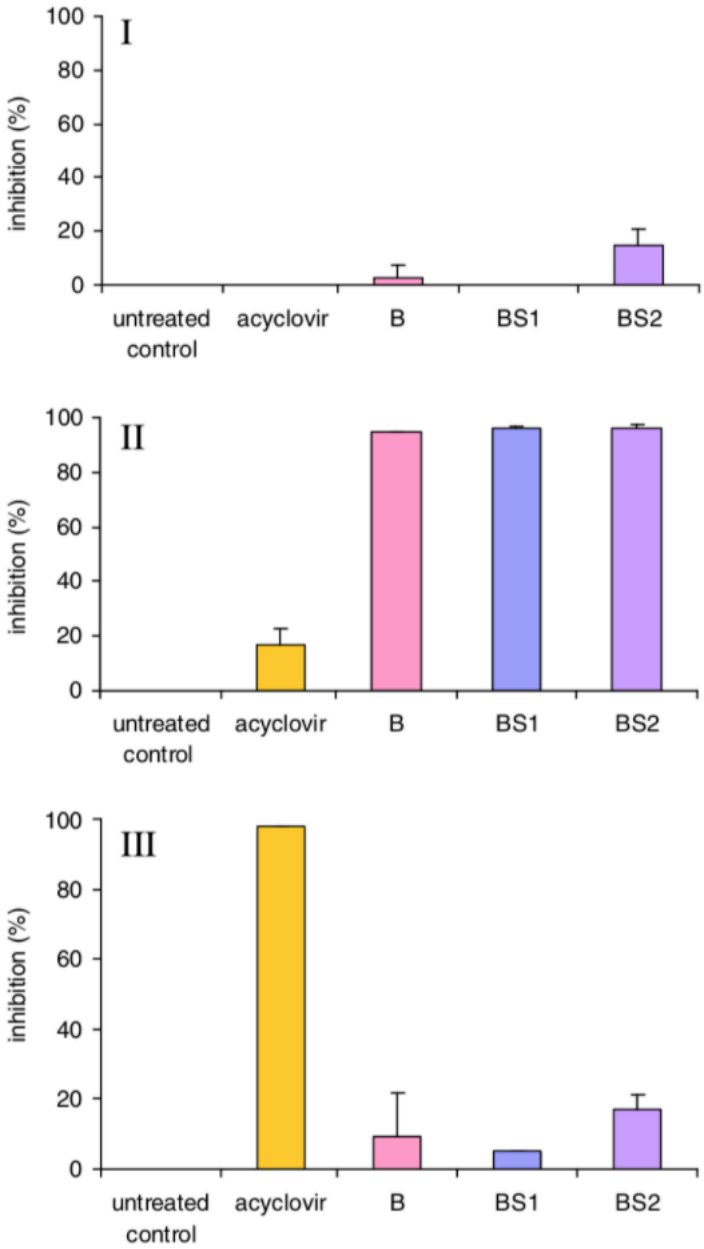


Fig. 15 Inhibitory effect of alginate (B) and its sulfated derivatives (BS1 and BS2), and acyclovir against HSV-1 through different stages of the viral replication cycle. (I) Cells were treated before infection, (II) HSV was preincubated with the aforementioned molecules before infection of cells and (III) HSV-infected cells were incubated for 3 days during viral replication in the presence of the molecules. (Bandyopadhyay et al., 2011)

These results indicate that the antiviral activity against HSV-1 of the tested alginate molecules was employed directly by interfering with virion particles or covering viral structures needed for the adsorption or entry into host cells as had been reported before for plant-derived extracts and isolated compounds (Astani, Reichling, & Schnitzler, 2008; Schnitzler, Schneider, Stintzing, Carle, & Reichling, 2008).

Tran et al., 2014 also reported that alginate presented antiviral activity against HSV-1 on HuH-7 cells. In this study, the researchers were mainly trying to determine if calcium-alginate microspheres presented antiviral activity against HCV. When they were positive alginate did inhibit HCV infection, they also studied alginate's effect on other virus, namely HSV-1, Poliovirus type 1 and Sindbis virus, so as to ascertain whether this alginate property was specific to HCV particles.

HSV-1 was incubated for 4 h with 600 mL of microspheres loaded with HuH-7 cells. Next, the supernatant was extracted and exchanged for new medium. After 2 days of incubation, the extracted supernatants were used to infect two-dimensional cultures of HuH-7 cells. After a day, HSV-1 infectivity was measured through $TDIC_{50}$ (50% tissue culture infectious dose) titration. As depicted in Figure 16, a dramatic reduction in the infectious titer was observed between supernatants extracted from both 2D cultures and encapsulated culture systems, more than 2-fold for HSV-1.

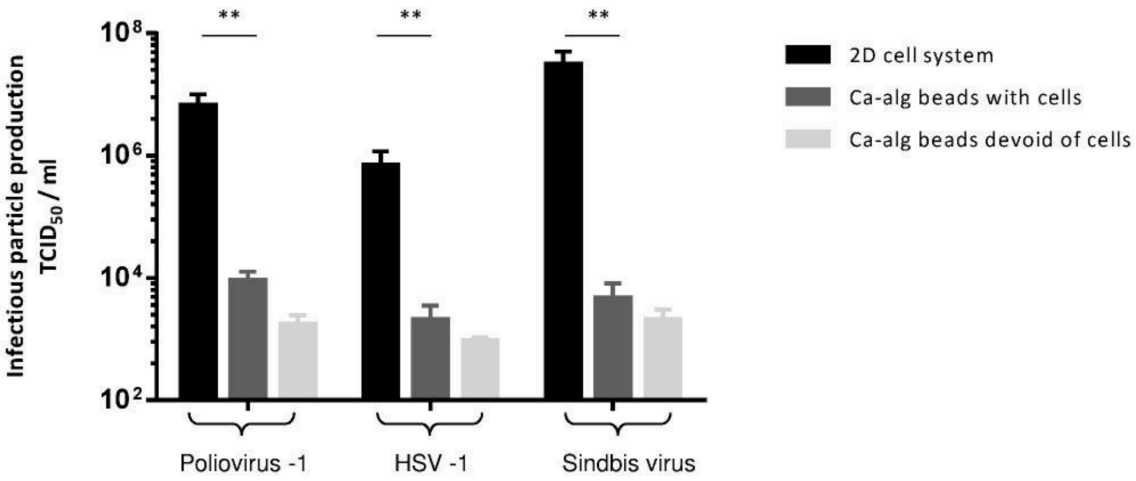


Fig. 16 Protective property of calcium-alginate microspheres against Poliovirus type 1, HSV-1 and Sindbis virus. Empty calcium-alginate microspheres were used as control. (Tran et al., 2014)

These calcium-alginate microspheres were obtained from a sodium alginate solution and then cross-linked with $CaCl_2$, and although the beads did show antiviral activity, alginate molecules in suspension have reported low antiviral effect as opposed to other polysaccharides obtained from marine organisms (Ghosh et al., 2009). Alginate's chemical content of G and M residues, which do not have any sulfate groups, may be the reason alginate solutions present such a lacking antiviral activity (Bandyopadhyay et al., 2011). For instance, sodium alginate's effect on HSV-1 was lower than the antiviral activity presented by other sulfated polysaccharides, as

demonstrated by its 50% inhibitory concentration values (IC_{50}), which ranged from 10 to 15 mg/mL, ten times greater than the IC_{50} of fucoidans, sulfated polysaccharides (Bandyopadhyay et al., 2011; Sinha, Astani, Ghosh, Schnitzler, & Ray, 2010).

MNV and HAV

Fabra et al., 2018 has reported that alginate hydrogels combined with lipids and two natural extracts with a high content of phenolic compounds (i.e. green tea extract (GTE) and a grape seed extract (GSE)) demonstrated antiviral activity against murine norovirus (MNV) and hepatitis A virus (HAV).

Previously, it had been reported that GTE and GSE presented antiviral activity against human norovirus surrogates and HAV (Falcó et al., 2018; Randazzo, Falcó, Aznar, & Sánchez, 2017; Su & D'Souza, 2011, 2013), indicating that these natural extracts could be used in active food packaging in order to inhibit enteric viruses. In this assay, alginate hydrogels combined with 0.75 g extract (GTE or GSE) per gram of alginate were demonstrated to reduce MNV titers by 1.92 and 1.67 log $TCID_{50}$/mL, respectively, while a concentration of 0.50 g extract/g alginate reduced MNV titers by 2.00 and 0.96 log TCID50/mL for GTE and GSE films respectively (Fig. 17A). HAV titers were also reduced by 1.92 and 1.50 log $TCID_{50}$/mL when treated with GTE and GSE with 0.75g extract/g alginate, respectively, whereas lower concentrations of GTE and GSE reduced it by 1.25 and 1.38 log, respectively (Fig. 17B).

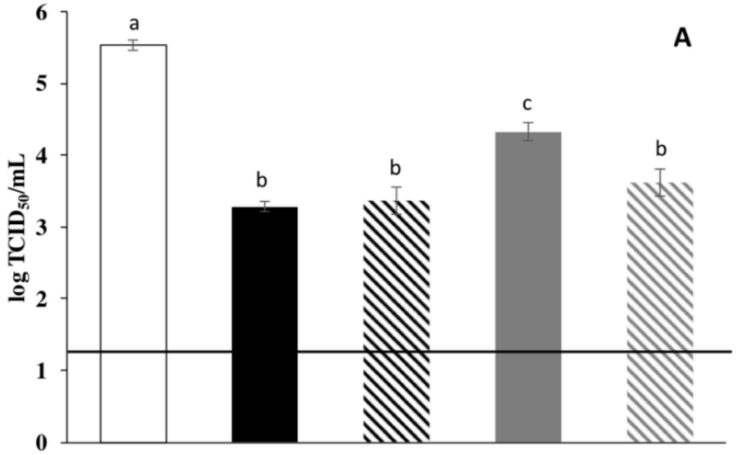
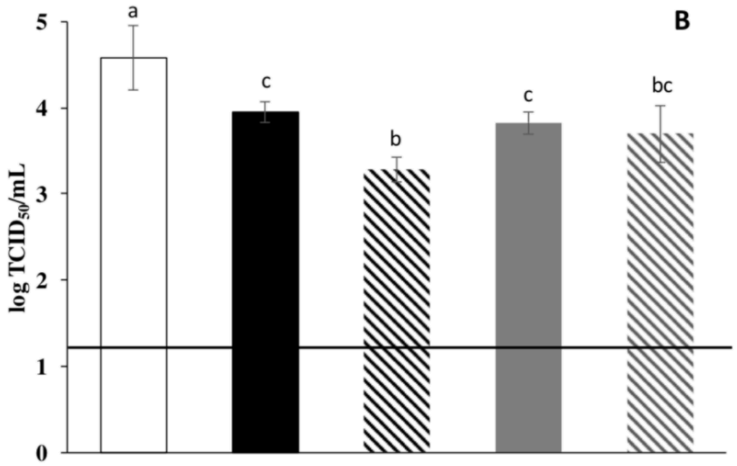


Fig. 17 Represented $TCID_{50}$ per mL of the different concentrations of GTE and GSE-containing alginate films against a control without GTE and GSE. **A.** The white column represents the control alginate film without extract infected with MNV, with *ca.* 5 logs $TCID_{50}$/mL. From left to right are then depicted the log $TCID_{50}$/mL values of 0.5GTE, 0.75GTE, 0.5GSE and 0.75GSE, alginate films infected with MNV.



B. The white column represents the control alginate film without extract infected with HAV, with *ca.* 5 logs $TCID_{50}$/mL. From left to right are then depicted the log $TCID_{50}$/mL values of 0.75GTE, 0.5GTE, 0.75GSE and 0.5GSE, alginate films infected with HAV. (Fabra et al., 2018)

Although alginate biofilms combined with GTE or GSE demonstrated antiviral activity against MNV and HAV, they presented lower antiviral activity than the pure natural extracts (Falcó et al., 2018; Su & D'Souza, 2013), indicating that the extracts could interfere with the full release of the alginate film's active compounds. This assay reported that alginate hydrogels combined with GTE were marginally more successful on the inhibition of MNV and HAV than those combined with GSE. Moreover, the viral inhibition mainly grew with the concentration of phenolic extracts within the film, with the exception of GTE, whose effectivity against both viruses was alike at both concentrations.

Falcó et al., 2019 further studied the antiviral activity GTE presented in the previous study against MNV and HAV by developing alginate edible films containing oleic acid and GTE (A-OA-GTE) for the coating of strawberries and raspberries. In this study, the effect of the pH of the film-forming dispersion (FFD) on the antiviral activity was analysed at different temperatures (10ºC and 25ºC).

On Table 14 and 15 is depicted the activity of edible alginate films against MNV and HAV, respectively, at different temperatures and exposure periods. Generally, A-OA-GTE films prepared at pH 5.5 demonstrated the most antiviral activity at 37ºC. When left overnight at 37ºC, greater reductions were observed for MNV, a 3.42 log $TCID_{50}$/mL and 5.76 log $TCID_{50}$/mL for films prepared at pH 7.0 and 5.5, respectively. Significant reductions on MNV infectivity were also reported for the films prepared at both pH values at 25ºC, whereas no activity could be observed at 10ºC, because viral particles generally thrive at lower temperatures instead of higher temperatures.

Table 14. The antiviral activity of edible alginate films on the infectivity of MNV (Falcó et al., 2019)

FFD/coating	Temperature							
	37°C		25°C		10°C			
	Overnight		Overnight		Overnight		4 days	
	log $TCID_{50}$/mL	Reduction	log $TCID_{50}$/mL	Reduction	log $TCID_{50}$/mL	Reduction	log $TCID_{50}$/mL	Reduction
A-OA	6.91 (0.31)[a]		6.24 (0.26)[a]		7.45 (0.25)[a]		7.32 (0.54)[a]	
A-OA-GTE *7.0*	3.49 (0.14)[b]	3.42	4.49 (0.47)[b]	1.75	6.99 (0.26)[a]	0.46	6.62 (0.14)[a]	0.71
A-OA-GTE *5.5*	1.15 (0.00)[c]	5.76	4.43 (0.19)[b]	1.71	7.53 (0.40)[a]	-0.08	6.53 (0.52)[a]	0.79

Mean value (standard deviation). Within each column for each temperature and time, different letters denote significant differences between treatments. A-OA alginate film containing oleic acid; A-OA-GTE 7.0: alginate film containing oleic acid and GTE at pH 7.0; A-OA-GTE 5.5: alginate film containing oleic acid and GTE at pH 5.5

Table 15. The antiviral activity of edible alginate films on the infectivity of HAV (Falcó et al., 2019)

FFD/coating	Temperature							
	37°C		25°C		10°C			
	Overnight		Overnight		Overnight		4 days	
	log $TCID_{50}$/mL	Reduction	log $TCID_{50}$/mL	Reduction	log $TCID_{50}$/mL	Reduction	log $TCID_{50}$/mL	Reduction
A-OA	4.70 (0.45)[a]		4.53 (0.31)[ab]		6.07 (0.45)[a]		5.74 (0.72)[a]	
A-OA-GTE *7.0*	4.82 (0.25)[a]	-0.13	4.95 (0.22)[a]	-0.42	6.07 (0.66)[a]	0.00	4.87 (0.36)[b]	0.87
A-OA-GTE *5.5*	3.33 (0.07)[b]	1.67	3.32 (0.13)[b]	1.21	5.74 (0.14)[a]	0.33	4.78 (0.59)[b]	0.96

Mean value (standard deviation). Within each column for each temperature and time, different letters denote significant differences between treatments. A-OA alginate film containing oleic acid; A-OA-GTE 7.0: alginate film containing oleic acid and GTE at pH 7.0; A-OA-GTE 5.5: alginate film containing oleic acid and GTE at pH 5.5

In the case of HAV, relevant variations were observed for alginate-GTE films prepared at pH 5.5 after overnight incubation at 25 and 37ºC (Table 15). However, alginate-GTE films prepared at pH 7 didn't have a significant effect on HAV after overnight incubation at either 10, 25 or 37ºC, which doesn't match with what was reported by Falcó et al., 2018 on pure GTE. These researchers observed that pure GTE was highly successful in the inhibition of HAV and MNV at neutral pH but showed no activity at pH 5.5 due to the variation in GTE composition. Therefore, the variations in the antiviral activity between pure extract and alginate-GTE hydrogels may be ascribed to the processes followed in the characterization of the antiviral activity.

In the assay described before, the conditions under which pure GTE was incubated remained unchanged, whereas in the current study, alginate-GTE gels were incubated at 7.0 after having been prepared at 5.5 or 7.0, suggesting that the gel prepared at pH 5.5 was degraded as a consequence of the acidic environment. However, the GTE of the film prepared at pH 7 had already dissolved during FFD and the antiviral activity of residual compounds diminished with time, as reported by Falcó et al., 2018 for pure GTE, which was attributed to a decrease of the contents of phenolic compounds.

Other viruses

Sano, 1999 reported that sodium alginate had a high inhibitory effect on TMV. The antiviral activity increased with the alginate concentration and was greater in alginates where the composition of mannurate (M) was lower than the composition of guluronate (G) (Fig. 18), indicating that the degree of inhibition depends on the rigidity of the polymer chain of alginate, which was consistent with the behavior observed for chondroitin sulfate (Chs) (Sano, 1995, 1997).

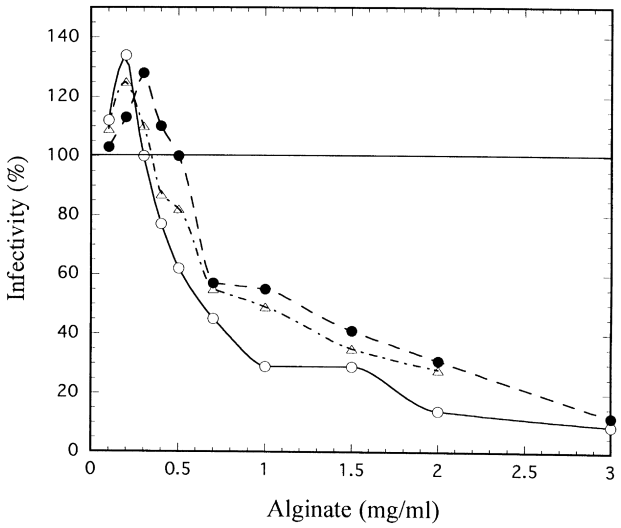


Fig. 18 Infectivity of Alg 500G (M/G = 0.41) (solid line), Alg 500 (M/G = 0.8) (chain line) and Alg 500M (M/G = 1.05) (dotted line). (Sano, 1999)

It was also observed by electron microscopy, that adding alginate to the TMV suspension caused TMV particles to form great raft-like aggregates, which may be the reason behind alginate's effect on infectivity.

Pietropaolo et al., 1993 reported that alginate inhibited rabies virus infection in CER cells. This assay demonstrated that alginate interfered with the initial stage of the rabies infection in CER cells (*i.e.* the virus adsorption process), the antiviral activity being

measured on the basis of 50% inhibition of nucleocapsid synthesis. Alginate demonstrated a dose-dependent antiviral effect at concentrations that ranged from 1 to 100 μg/mL.

Although the results obtained in this assay aren't enough to draw conclusions about the effect the structure of these polymers has on their antiviral activity, the researchers speculated that negatively charged polysaccharides such as alginate could increase the negatively charged glycosylated G protein of the viral envelope and the anionic receptor sites of eukaryotic cells.

Pardee et al., 2004 reported that alginate extracted from *Fucus gardneri* inhibited PVX infectivity by 95%, and electron microscopy indicates that the mode of inhibition may be related to viral aggregation (Fig 19). In this study, many extracts from marine algae were tested against PVX, but only *F. gardineri* completely inhibited local lesion on *Chenopodium quinoa* at a concentration of 10 μg/μL and still demonstrate antiviral effect at 1 μg/μL (94% ± 3%).

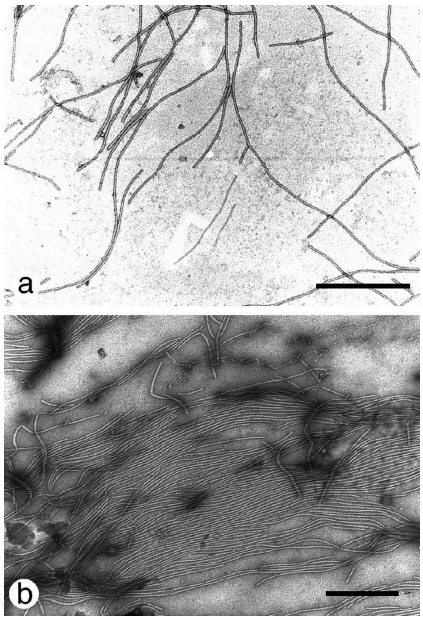


Fig. 19 Transmission electron microscopy images of Potato virus X particles after incubation in test solutions. (**a**) Negative control. Scale bar = 400 nm. (**b**) Virion "rafts" after alginate treatment. Scale bar = 400 nm. Magnification × 31 500. (Pardee et al., 2004)

These results were consistent with those reported by Sano, 1999, who indicated that this aggregation could be considered the responsible for the antiviral mode of action, by decreasing the functional content of viral particles in solution or by interfering with viral uncoating during infection.

Bao-fa et al., 2003 reported that the previously seen alginate-derived '911' drug also demonstrated antiviral activity against HBV on Hep G2. 2.15 cells. In this assay, different concentrations of 911 were added to the Hep G2. 2.15 culture, and the supernatant was later collected and tested for HBV antigens, which turned out positive. TC_{50}, IC_{50} and TI values were measured and can be observed on Table 16.

Table 16. TC_{50}, IC_{50} and TI values for alginate-derived 911 drug. (Bao-fa et al., 2003)

	'911' drug
TC_{50} [a]	44.8 mg/ml
IC_{50} [b]	17.3 mg/ml
TI [c]	3.37

[a] 50% toxic concentration (TC_{50}): compound concentration required to reduce cell viability by 50%, as determined from dose-response curves.

[b] 50% inhibitory concentration (IC_{50}): compound concentration required to reduce virus plaques by 50%.

[c] Therapeutic index (TI): ratio between TC_{50} and IC_{50}.

These researchers determined that the alginate-derived '911' drug first reported to present antiviral activity against HIV-1 (Xianliang et al., 2000), (Xin et al., 2000), also demonstrated antiviral activity against HBV infection on Hep G2. 2.15 cells.

Gong et al., 2011 reported that calcium or zinc alginate fibers demonstrated antiviral activity against IFV on African Green Monkey Vero cells and cervical cancer human cells (Hela), as a previous test to determine if these alginate fibers could be used as biomaterials.

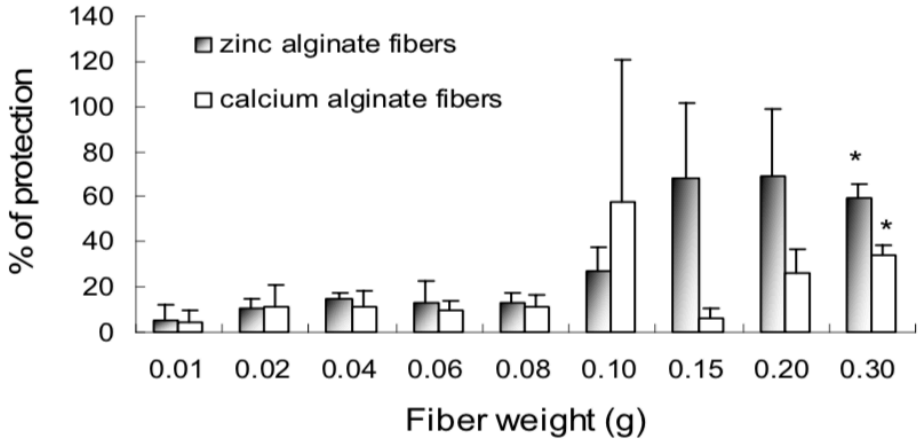


Fig. 20 Antiviral activity against IFV of calcium or zinc alginate fibers on Vero cells. (Gong et al., 2011)

In this assay, the fibers were divided and tested against IFV by their weight and assayed against IFV of $TCID_{50} = 10^{-3.67}$. The highest degree of protection of calcium alginate fibers was 34.42%, while the highest degree of protection of zinc alginate fibers was 59.42% on Vero cells with IFV (Fig. 20). Furthermore, the Vero cells incubated with IFV and 0.3 g zinc fibers presented greater viability than 0.3 g calcium fibers (Fig. 21b and 21c). However, cells only incubated with IFV degraded fast and seemingly detached (Fig. 21a).

Fig. 21 Shape of Vero cells cultured after 48 h exposure to (**a**) IFV control group, (**b**) 0.30g zinc alginate fibers with IFV, (**c**) 0.30g calcium alginate fibers with IFV (original magnification ×100). (Gong et al., 2011)

Lastly, a previously commented study Tran et al., 2014 also reported that calcium-alginate microspheres demonstrated antiviral activity against HCV, Sindbis virus and Poliovirus type 1. In this study, the researchers were mainly trying to determine if calcium-alginate microspheres presented antiviral activity against HCV, but they also studied alginate's effect on other virus in order to ascertain whether this alginate property was specific to HCV particles.

The researchers discovered that encapsulating HuH-7 cells before HCV infection and encapsulating previously infected cells didn't produce any infectious HCV particles, which was caused by the inability of HCV to enter the encapsulated cells. They also found that the antiviral activity of calcium-alginate microspheres depended on the ratio between microspheres and viral particles, and also depended on the time of incubation between them.

These results indicate that the negative charge density of calcium-alginate gels may interact with components within the viral envelope, inhibiting the viral particles in the gel environment or interfering with particular interactions between the virus and the membrane receptors.

Calcium-alginate microspheres loaded with HuH-7 cells also demonstrated antiviral activity against Sindbis virus and Poliovirus type 1, as observed previously on Figure 16. The antiviral activity was measured by $TCID_{50}$, and Sinbis virus and poliovirus both demonstrated a significant decrease of 3-fold in their infectious titer.

Delivery systems

Alginate has also been used as a delivery system of oral or DNA vaccines, and although it doesn't directly present antiviral activity, it is interesting to see that alginate has already been utilized in the virology field.

Sailaja et al., 2002 developed a delivery system of HAd5 loaded in biodegradable alginate microspheres in order to determine if the pre-existing immunity against adenovirus could be avoided. In this assay, both naïve and immunized mice were inoculated with recombinant HAd5 containing the bacterial β-galactosidase (LacZ) gene that could be loaded into alginate microspheres or not.

In immunized mice, the expression of LacZ by the non-encapsulated virus was significantly inhibited as opposed to its expression in naïve rodents, and its degree relied on the level of specific immune response. However, in immunized mice, transgene expression after inoculation with encapsulated HAd5 was partially decreased as opposed to levels from naïve mice (10% in 1x HAd5-immunized, and 20–25% in 2x HAd5-immunized).

In immunized mice there was at least 10–25% reduction in LacZ expression on inoculation with encapsulated virus, which may be due to several reasons: a limited approachability of the virus to the microspheres, a degradation of some beads that released the recombinant virus that was neutralized, or because the immune response may be targeting the cells expressing LacZ.

Ballesteros et al., 2015 reported that an orally-administered DNA vaccine against IHNV encapsulated into alginate microspheres produced protective immune responses in rainbow trout (*Oncorhynchus mykiss*). In this assay, IHNV glycoprotein (G) transcripts were detected by quantitative real-time PCR in different tissues of vaccinated fish (Fig. 22), which indicated that the alginate encapsulation successfully protected the vaccine against the acidic gut pH and delivered it to the intestine, where it was distributed through the organs of vaccinated fish.

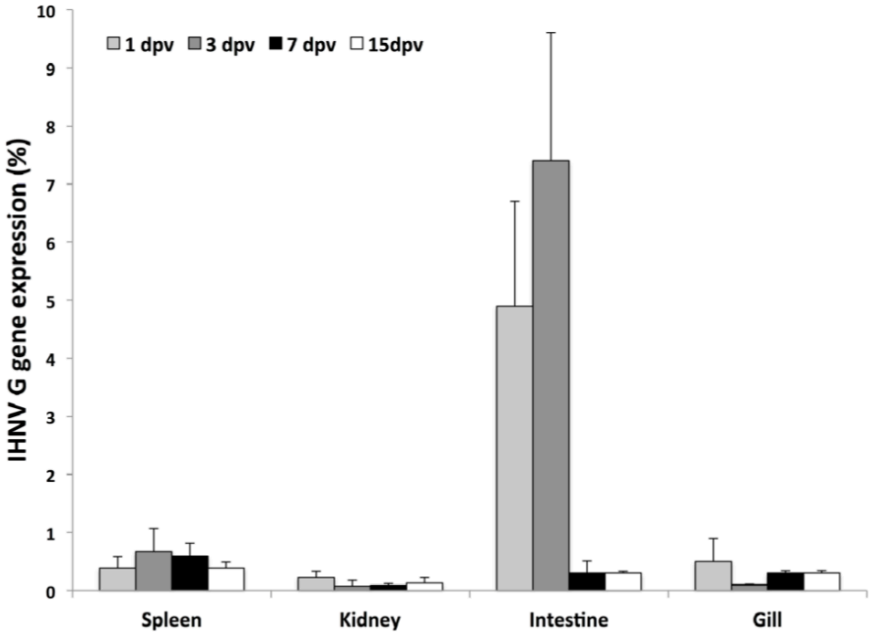


Fig. 22 IHNV G gene expression after oral immunization against IHNV encapsulated in alginate microspheres (10 µg dose) analyzed in gills, kidney, spleen and intestinal tissues collected from four vaccinated rainbow trout 1, 3, 7 and 15 days after immunization. (Ballesteros et al., 2015)

The same DNA vaccine had previously demonstrated a great antiviral effect against IHNV when administered by intramuscular injection (Alonso, Johnson, Simon, & Leong, 2003). However, this administration route is highly intensive and solely taken for specific species.

After vaccination with a 10 µg, 25 µg and 100 µg dose, several markers belonging to the innate and adaptive immune responses, such as IFN-1 and IgM, respectively, were detected in trout tissues in a dose-dependent manner, which was further supported by the results obtained when vaccined trouts where infected with IHNV.

Cheng et al., 2011 tested a delivery system for oral vaccine delivery, consisting of liposomes layered with tremella and alginate for the encapsulation of activated IFV subtype H5N3. *In vitro* studies demonstrated that the triple-layered vaccination system was more resistant to an acidic pH and controlled the release behavior at a basic pH (Fig. 23).

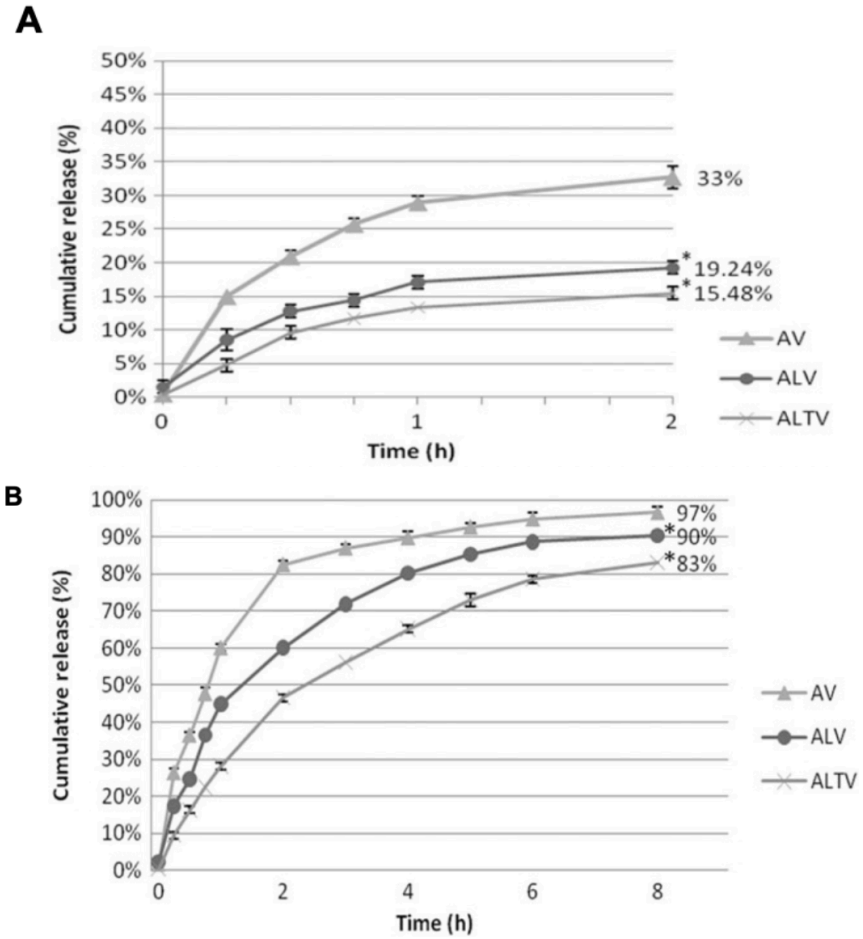


Fig. 23 A Stability of triple-layered vaccine in simulated gastric acid fluid (0.1N HCl, pH 1.2). **B** Release of H5N3 from vaccine in a simulated intestinal fluid (PBS, pH 7.5).
AV: alginate vesicle; ALV: alginate-lipid encapsulated virus; ALTV: alginate-lipid-tremella encapsulated virus (Cheng et al., 2011)

In mice, even though IgG levels weren't expected to rise and hemagglutination inhibition demonstrated that there weren't enough immunoglobulins to provide successful protection, the triple-layered vaccine did induce the production of sIgA to provide protection against virus infection, as it was delivered by the 'M' cells to the posterior cervical lymph node resulting in a mostly mucosal immune response (Mittal et al., 2000; Rebelatto, Guimond, Bowersock, & HogenEsch, 2001).

Yongsheng et al., 2008 developed a chitosan-alginate-$CaCl_2$ oral delivery system of bacteriophage Felix O1 and valuated its resistance to simulated gastric fluid (SGF). While the non-encapsulated phage demonstrated extreme sensitivity to low pH values and was degraded very fast, the viable count of microencapsulated phage decreased over time under the same conditions but was still maintained (Fig. 24), which is consistent with the results obtained in a similar study (Koo, DePaola, & Marshall, 2000).

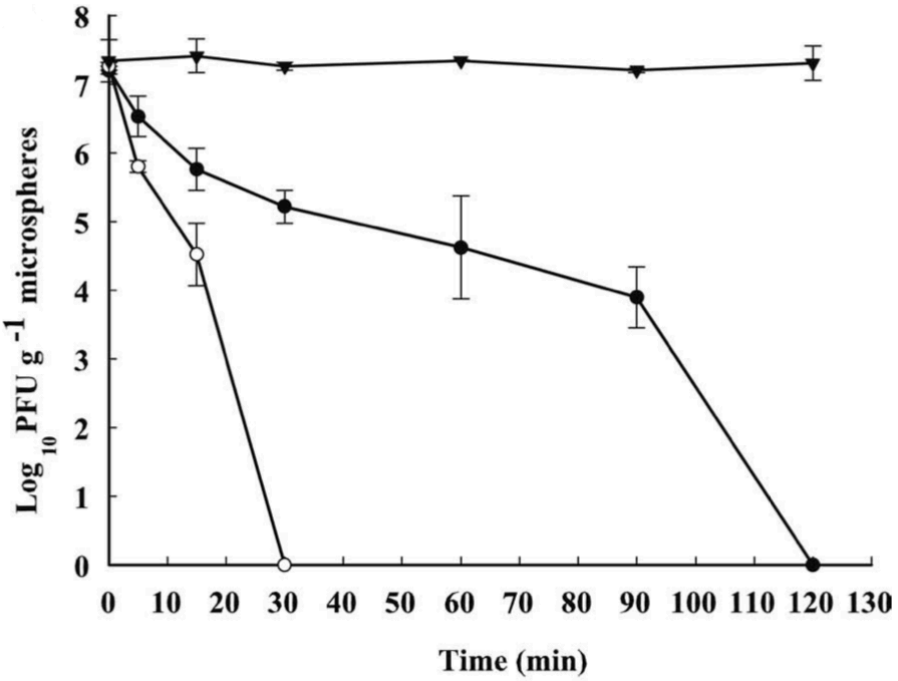


Fig. 24 Survival of microencapsulated phage Felix O1 after exposure to SGF with 3.2 mg/ml pepsin at pH 2.0 (○) and pH 2.4 (●) and SM buffer of pH 7.5 (▼) at 37°C for 0, 5, 15, 30, 60, 90, and 120 min (Yongsheng et al., 2008).

Alginate hydrogels have been demonstrated to resist degradation in acidic conditions, while dissolving in higher pH (George & Abraham, 2006), which enables the protection of phage from harsh gastric conditions and its release at desired locations in a viable form. In this assay, it was reported that encapsulated phages were much more resistant to SGF than free phages, even though there was a slight decrease in viability, which agreed with the results obtained in a related study (Ki Yong Lee & Heo, 2000).

Similarly, Borges et al., 2007 developed a delivery system an HBV vaccine encapsulated into alginate-coated chitosan nanospheres and tested it in order to characterize its ability to provoke local and systemic immune responses after oral vaccination in mice.

After several vaccinations, mice groups I (HVB antigen + nanospheres) and VI (HVB antigen + stimulatory motif CpG + nanospheres) demonstrated improved immune responses, with significantly greater values of the CD69 expression in CD4+ and CD8+ T-lymphocytes and lower values of the same protein in B lymphocytes, as observed in Figure 25. Additionally, only mice of these groups demonstrated production of antibodies against HBV antigen detected in serum (IgG) and in the intestinal washings (sIgA).

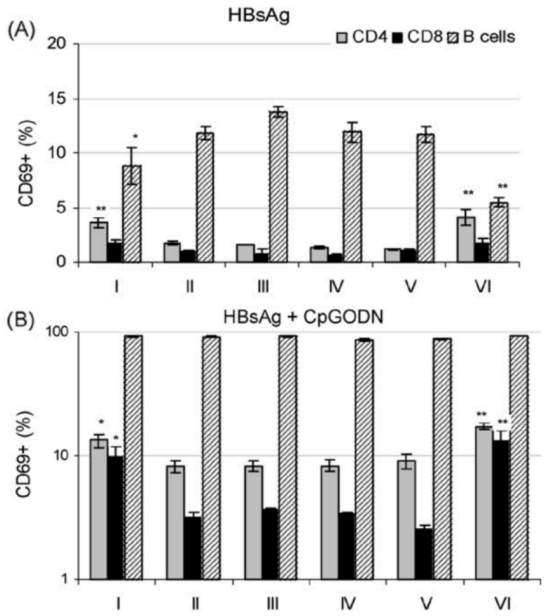


Fig. 25 Expression of the CD69 marker on B and T lymphocytes from different mouse groups in response to different *in vitro* stimulation. **A** Mice stimulated with the HBV antigen + nanospheres. **B** Mice stimulated with the HBV antigen + stimulator motif CpG + nanospheres. (Borges et al., 2007)

However, it was also reported that within these groups were non-responder mice, which has also been reported in other studies, both in mice and humans. (M. J. McCluskie, Weeratna, & Davis, 2000; Michael J. McCluskie, Weeratna, Krieg, & Davis, 2000; Rendi-Wagner et al., 2006), and is solved by administering higher antigen doses with successive vaccine administration.

Alginate-based scaffolds

Alginate has also been used in the virology field as a scaffolding material. Luckanagul et al., 2012 developed a highly porous alginate scaffold and functionalized it with TMV and its mutant containing the cellular recognition arginine-glycine-aspartic acid (RGD) peptide in order to assess their ability to support 3D stem cell cultures.

In this assay, cell attachment and viability of BMSCs were measured in three different hydrogels: alginate, alginate-TMV and alginate-RGD-TMV, as depicted on Figure 26. The hydrogel containing the RGD peptide demonstrated the highest cell attachment after 8h (96%) and also the highest cell viability after 2 weeks.

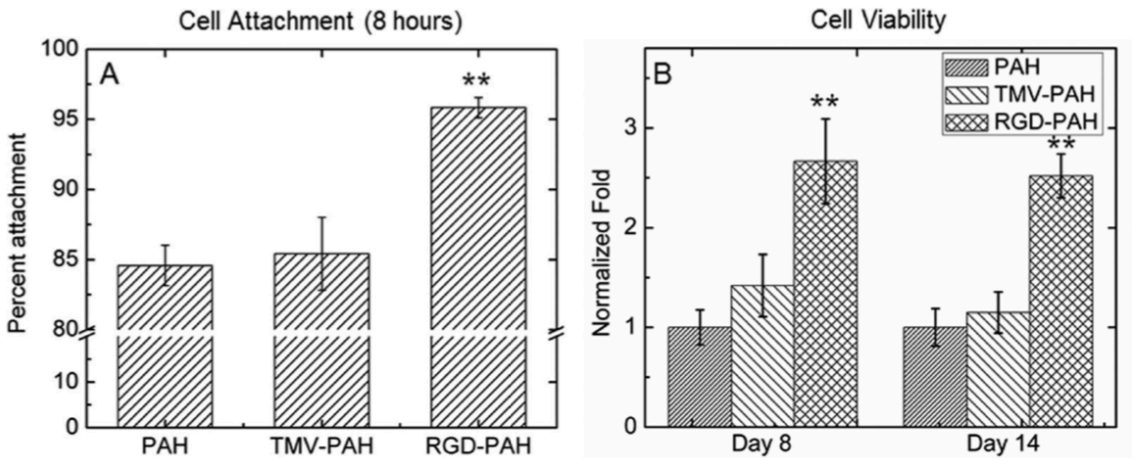


Fig. 26 Cell attachment and viability in different types of porous alginate hydrogels. **A** Attachment degree of BMSCs in each type of hydrogel. **B** CellTiter Blue metabolic activity assay of BMSCs culture in each type of hydrogel at different time points. (Luckanagul et al., 2012)

In a related assay by the same researcher, Luckanagul et al., 2016 successfully used this functionalized scaffold to regenerate bone tissue in rats with cranial imperfections, which indicates that alginate-based scaffolds functionalized with TMV may be an interesting candidate for the development of new applications in the field of tissue engineering.

CONCLUSIONS

From the results obtained on the bibliographical review carried out in this thesis, a series of final conclusions have been drawn:

- Alginate has demonstrated antiviral activity against several viruses: the most reported viruses are HIV-1 (25%), HSV-1 (16,67%), MNV (16,67%) and HAV (16,67%). To a lesser extent, alginate has also demonstrated activity against HBV, HCV, Sindbis virus, Poliovirus type 1, rabies virus, PVX, TMV and Influenza A virus type 1.

- Alginate has also been reported to be used in the virology field as a delivery system for oral or DNA vaccines, and as a scaffolding material used in order to support the 3D culture of stem cells, especially mesenchymal cells (MSCs) and bone marrow stem cells (BMSCs).

- The analysis carried out on the scientific production regarding the evolution of the use of alginate as an antiviral biomaterial shows that the number of articles published has been erratic and inconsistent throughout the years, indicating that, although there isn't much information on the topic as of yet and more investigation is needed, alginate can indeed be considered as a promising candidate for the development of antiviral biomaterials.

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