TREBALL FI DE GRAU

Grau en Enginyeria de Materials

BIOCOMPATIBLE ALGINATE HYDROGELS FOR STORAGE AND DELIVERY OF PLASMA-GENERATED REACTIVE SPECIES

Memòria

Autor: Jordi Pino Contreras
Director: Dr. Cristina Canal
Co-Director: Dr. Francesco Tampieri

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Resum

En els darrers anys a la Medicina de Plasmes s'ha convertit en un camp a estudiar amb molt de potencial donats els resultats que ha mostrat en diversos estudis realitzats a tot el món. El plasma es defineix com un gas ionitzat que es genera en aplicar una descàrrega elèctrica a un gas, heli (He) en aquest cas, i que conté, entre d'altres, nombroses espècies reactives d'oxigen i nitrogen (RONS). Aquestes espècies indueixen un augment d'estrès oxidatiu a les cèl·lules que s'està estudiant com a nou tractament contra el càncer, menys invasiu i altament selectiu, que evita danyar les cèl·lules sanes del pacient.

Aquest projecte final de grau es centra en l'optimització d'hidrogels d'alginat com a vehicles per alliberar aquestes espècies reactives generades per plasma. Això es fa estudiant que paràmetres afecten en el seu alliberament d'espècies a partir d'un tractament amb plasma no tèrmic. L'objectiu consistiria a poder fer servir aquests hidrogels per produir implants que mantinguin una concentració d'aquestes espècies més perllongada que altres vehicles líquids al lloc del tumor. La totalitat dels materials utilitzats en aquest treball és biocompatible evitant així que es produeixin complicacions per degradacions o reaccions dels materials amb els fluids corporals.

Les espècies reactives generades pel plasma no tèrmic són molt variades, i en aquest treball ens centrarem en dues en particular, H_2O_2 i NO_2^- que generem amb la descàrrega de plasma d'Heli amb 0.3% d'aire sintètic permet la formació d'aquestes espècies, entre d'altres. Per analitzar l'alliberament es fan uns assajos d'alliberament o de degradació, podent així comprovar el comportament d'aquest biomaterial i comparar els resultats amb el propi biopolímer.

Durant el treball s'han pogut avaluar diferents mètodes de preparació i reticulació dels hidrogels aconseguint un alliberament esperat d'aquestes espècies en un període de temps de 70 minuts en un medi amb condicions similars a les fisiològiques, a més es va poder analitzar la influència de la microestructura i les propietats reològiques de l'hidrogel al procés d'alliberament.

Resumen

En los últimos años en la Medicina de Plasmas se ha convertido en un campo a estudiar con mucho potencial dados los resultados que ha mostrado en varios estudios realizados en todo el mundo. El plasma se define como un gas ionizado que se genera al aplicar una descarga eléctrica a un gas, helio (He) en este caso, y que contiene, entre otros, numerosas especies reactivas de oxígeno y nitrógeno (RONS). Estas especies inducen un aumento de estrés oxidativo en las células que se está estudiando como nuevo tratamiento contra el cáncer, menos invasivo y altamente selectivo, que evita dañar las células sanas del paciente.

Este Proyecto Final de Grado se centra en la optimización de hidrogeles de alginato como vehículos para la liberación de estas especies reactivas generadas por plasma. Ello se lleva a cabo estudiando que parámetros afectan en su liberación de especies a partir de un tratamiento con plasma no térmico. El objetivo consistiría en poder emplear estos hidrogeles para producir implantes que mantengan una concentración de estas especies más prolongada que otros vehículos líquidos en el lugar del tumor. La totalidad de los materiales utilizados en este trabajo es biocompatible evitando así que se produzcan complicaciones por degradaciones o reacciones de los materiales con los fluidos corporales.

Las especies reactivas generadas por el plasma no térmico son muy variadas, y en este trabajo nos centraremos en dos en particular, H_2O_2 y NO_2^- que generamos con la descarga de plasma de Helio con 0.3% de aire sintético permite la formación de estas especies, entre otras. Para analizar la liberación se realizan unos ensayos de liberación o de degradación, pudiendo comprobar así el comportamiento de este biomaterial y comparando los resultados con el propio biopolímero.

Durante el trabajo se ha podido evaluar distintos métodos de preparación y reticulación de los hidrogeles consiguiendo una liberación esperada de estas especies en un periodo de tiempo de 70 minutos en un medio con condiciones similares a las fisiológicas, además se pudo analizar la influencia de la microestructura y las propiedades reológicas del hidrogel en el proceso de liberación.

Abstract

In recent years, Plasma Medicine has become a field to study with great potential due the results that it has shown in some studies realized around the world. Plasma is defined as an ionized gas that is generated by applying an electrical discharge to a gas, helium (He) in this case, and which contains, among others, numerous reactive oxygen and nitrogen species (RONS). These species induce an increase in oxidative stress in cells that is being studied as a new treatment against cancer, less invasive and highly selective, which avoids damaging the patient's healthy cells.

This Final Degree Project focuses on the optimization of alginate hydrogels as vehicles for the release of these reactive species generated by plasma. This is carried out by studying what parameters affect the release of species using a non-thermal plasma treatment. The aim for this work consists in use these hydrogels to produce implants that maintain a concentration of these species longer than other liquid vehicles at the site of the tumor. All the materials used in this work are biocompatible, thus avoiding complications due to degradation or reactions of the materials with body fluids.

The reactive species generated by non-thermal plasma are very varied, and in this work, we will focus on two in particular, H_2O_2 and NO_2^- that we generate with the Helium plasma discharge with 0.3% synthetic air that allows the formation of these species, among others. To analyze the RONS delivery, some release or degradation tests are carried out, thus being able to verify the behavior of this biomaterial and compare the results with the biopolymer itself.

During the work it has been possible to evaluate different methods of preparation and reticulation of the hydrogels, achieving an expected release of these species in a period of time of 70 minutes in a medium with conditions similar to the physiological ones, in addition it was possible to analyze the influence of the microstructure and the rheological properties of the hydrogel in the release process.

Agraïments

M’ agradaria poder donar les gràcies a la Dra. Cristina Canal per la oportunitat d’aprendre i formar-me en un àmbit de l’enginyeria com el dels biomaterials des del grup de recerca del BBT. També per l’oportunitat de poder entrar en el món de la recerca en el camp de l’enginyeria ja que ha sigut una experiència molt enriquidora a nivell personal i professional.

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Es poden incloure agraïments relatius a ajuts en la realització del treball i en la preparació del document. No és habitual agrair les contribucions com ara un control de rutina, un petit ajut o unes recomanacions de tipus general.

El reconeixement d’altres treballs emprats ha de fer-se en forma de referències. Els agraïments que fan referència a un text citat i a l’ús de taules i il·lustracions poden requerir el reconeixement de drets d’autor.

Índex

1. Introduction

1.1 - Cancer

Cancer is one of the deadliest diseases worldwide. In 2020, 19.3 million new cases were registered and around 10 million deaths [Ferlay,2020]. Studies show that the risk of getting cancer in a lifetime period is around 20%, and the probability of dying from it rounds 10 % [Ferlay,2020]. The estimated costs for cancer treatment in European continent were estimated in 2018 to account for up to 199 billion €, 378 € per capita [Hofmarcher, 2020].

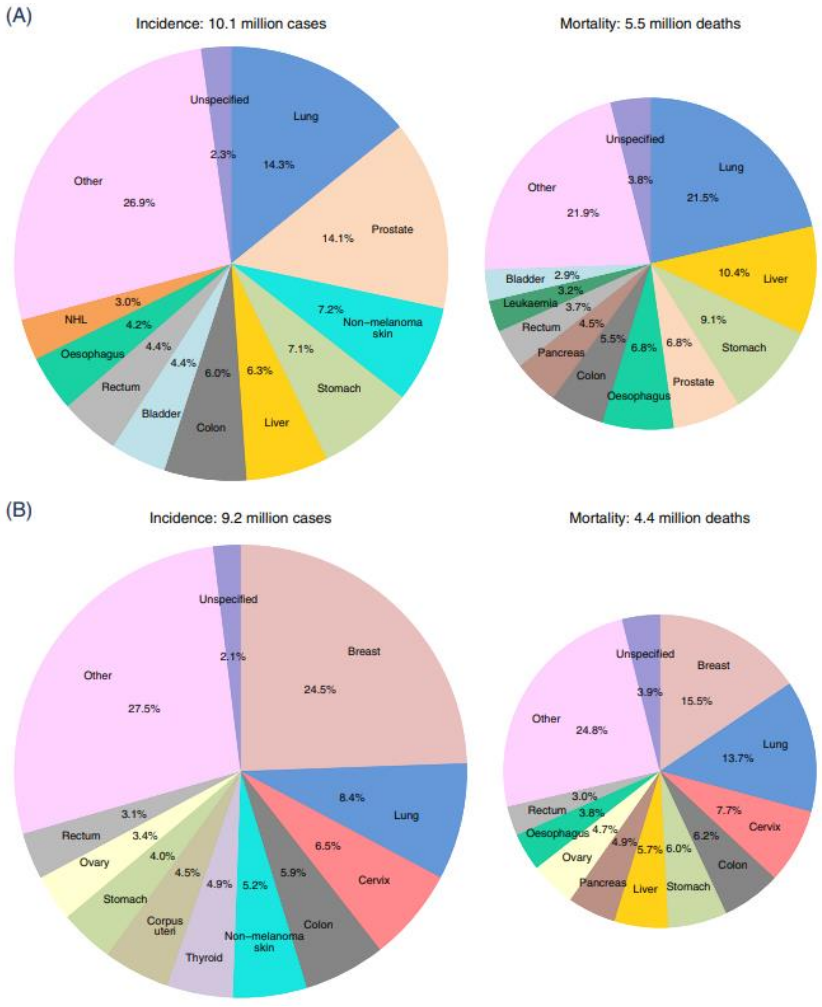


Figure 1.1. Representation of the incidence and mortality of cancer is different for different sexes [Ferlay, 2020].

As shown in the figure above, in males, lung cancer is the most common with 1.44 million cases (14.3%), followed by prostate cancer with 1.42 million cases (14.1%) and non-melanoma skin cancer on third

place with 727 hundred thousand cases (7.2%). When looking at mortality, the number of deaths caused by lung cancer is the highest with 1.8 million deaths (21.5%) followed by liver with 572 hundred thousand deaths (10.4%) and stomach in third place with half million deaths in 2020 (9.1%).

On females the most diagnosed cancer is the breast one, with 2.26 million cases in 2020 (24.5%), in second place lung cancer with 772 hundred thousand cases (8.4%) and in third place cervix with 600 hundred thousand cases (6.5%). Women's most deadly cancer type is breast one with 682 hundred thousand deaths (15.5%) followed by lung cancer with 603 hundred thousand deaths (13.7%) and on the third-place cervix cancer with almost 340 hundred thousand deaths (7.7%) in 2020.

In our group we are interested in osteosarcoma, the most common primary malignancy of bone, in which mesenchymal cells produce osteoid. Every year an average of 2-3 million cases are detected, is even more common in teens who have an incidence between 8 to 11 million cases for year, in the age range of 15 to 19 years of age and its more common un males than females. Osteosarcoma affects bones, any bone can be affected but mainly the long ones like distal femur, proximal tibia, and proximal humerus.

Three main therapies are commonly used to treat cancer, in general, and osteosarcoma, in particular, which can be grouped in 2 main groups: the systemic treatments, which affect not just the tissue affected by the tumor but the whole body and the local treatments, which just affect the tumor region. These therapies are:

1. Surgery: consists in removing the primary tumor by resecting the affected tissue.
2. Chemotherapy: in this kind of treatment the aim is to decrease the tumor size, and by introducing drugs that kill stem cells by intravenous way. The main issue of this treatment is that introduces toxic agents in the body that kill both cancer and non-malignant cells, causing undesirable side effects.
3. Radiotherapy: consists in the destruction of the cancer cells by treating them with high-frequency radiation, the main problem of this treatment is that the radiation not only affect stem cells, but it may also damage healthy cells too. But DNA is damaged in adjacent tumors.

Apart from these three main therapies types many other types are used in base of the patient characteristics and needs [Types of cancer treatment]:

- Biomarker Testing: this group is not a treatment itself, but, thanks to searching on body substances more information of cancer is collected and the doctor may choose a better treatment for the illness.
- Hormone therapy: treatment that uses hormones to slow or stop the growth of cancer cells, main breast, and prostate cancer.
- Hyperthermia: therapy using heat to kill cancer cells with little or no harm.
- Immunotherapy: in this treatment the basis consists in helping the immune system to fight against cancer

- Photodynamic Therapy: the main component on this treatment is light, used for activating a drug that kills not just cancer cells but other malign cells.
- Stem Cell therapy: this method consists in transplanting some stem cells to a patient that has lost many of them due to previous cancer treatments applied.
- Novel therapies, in this group we could include plasma treatments with cold atmospheric plasma

Non-thermal plasmas can generate reactive oxygen and nitrogen species that can interact with cells. Cancer cells have a higher basal level of RONS than healthy cells, so the addition of exogenous RONS (from plasma) causes their death. The same level of exogenous RONS does not affect significantly healthy cells due to a lower basal level of RONS. This explains the selectivity of non-thermal plasma in the treatment of cancer[figure 1.2].

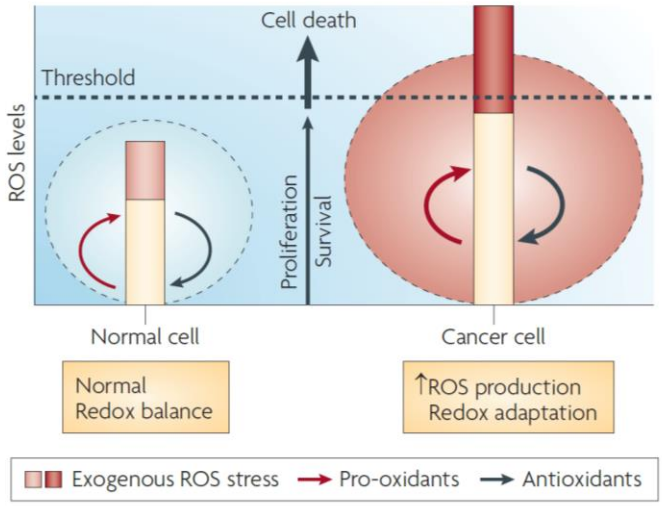


Figure 1.2. Effect of RONS in healthy and cancer cells. [Trachootham, 2009].

1.2 - Plasma

1.2.1 - Definition of plasma

Plasma is commonly known as the “fourth state of matter”, other than solid, liquid and gas (Fig. 1.3). It consists in an ionized gas and can be generated by delivery energy to a gas up to a point where free electrons and positive ions are generated. These free electron and ions are extremely reactive and start a cascade of physic-chemical processes that produces other reactive species and electromagnetic

radiation. So, a plasma is composed by free electron, ions, radicals, excited atoms and/or molecules, neutral atoms and/or molecules and electromagnetic radiation (UV-Vis-IR). which is composed by charged radicals, ions, and neutrals. Overall, a plasma has a zero net charge.

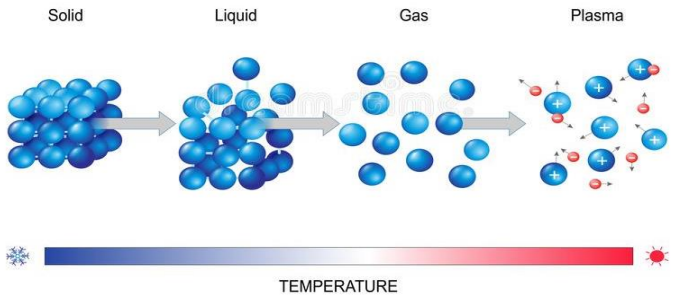


Figure 1.3. The figure shows the bonds between atoms depending on the energy/ temperature that they have in the system and its interaction between them.

Plasmas can be differentiated according to the energy distribution of their components and the pressure in which occurs:

- **thermal plasmas:** in a thermal plasma all the components have approximately the same energy/temperature that is very high if compared to room temperature ($E_{e^-} \cong E_{ions} \cong E_{neutrals} \gg room\ temp$). These plasmas are characterized usually by a high ionization degree and a high electron density. Thermal plasmas occur naturally in the stars of our galaxy
- **non-thermal plasmas (NTPs):** Can be differentiated from the thermal one due to the difference of the energy between electrons and ions **(**$E_{e^-} \gg E_{ions} \cong E_{neutrals} \cong room\ temp$ **),** indeed electrons energy is much higher than neutrals or ions, also the pressure at which the NTPs occur is close to atmospheric pressure Non-thermal plasmas can be generated by applying a high voltage discharge to a gas at atmospheric pressure and room temperature. This make them a very versatile tool for many applications., some examples could be the surface modifications in material science or for coating processes this kind of plasma is also known as Cold Atmospheric Plasma (CAP).

1.2.2 Plasma Applications

Plasma Medicine. Its use in medicine is mainly for treating chronic, infected wounds and skin diseases. For the infected wounds, plasma helps for accelerate the healing process at closing wound, when wound healing is treated with CAP, we can highlight two important effects, one of them is the inactivation of a broad spectrum of microorganisms including the resistant to multidrug, the other one is the stimulation of cell proliferation and angiogenesis resulting in the wound healing effect that we were explaining.

Also applied in cardiology for treating superficial infections or transcutaneous exit ports of the drivelines of ventricular assist devices. Can be used for coagulating blood to prevent hemorrhaging. In the field of dentistry, plasma is used for disinfection of tooth root canals and bones, and, treating teeth or dental implants, mainly to eliminate biofilms.

Other applications. Other applications in which we can found Cold atmospheric plasma(CAP) can be surface coating, in this field the plasma is used to coat a surface with some sputtered ions taken from a target and then they cover the surface giving some needed properties. CAP is also used in hygiene field, used for cleaning surfaces from bacteria, used for air purification, and also, water treatment to make it able for drink.

1.2.3 Reactive Oxygen and Nitrogen Species (RONS)

During a plasma treatment, the high-energy free electrons that are produced, can interact with molecules in the environment to generate a variety of other reactive chemical species (ions, radicals, excited species and neutrals). The nature and amount of these species depends on the composition of the feed gas and on the presence of a target next to the plasma region. If oxygen or nitrogen are present in the feed gas or in the plasma region (e.g. plasma generated in open air), most of the reactive species will contain oxygen and/or nitrogen atoms, in this case the term RONS (Reactive Oxygen and Nitrogen Species) is commonly used. A typical case in plasma medicine applications is to perform experiments in open air atmosphere and in presence of an aqueous-based target. In this case, an example of the possible RONS that are generated, the processes involved and the distribution between gas and liquid phase is depicted in Figure 1.4.

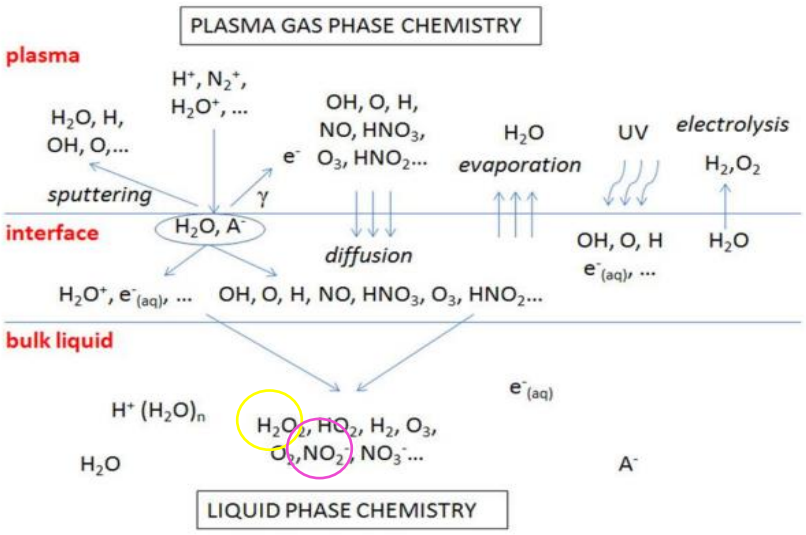


Figure 1.4. Reactive oxygen and nitrogen species generated when a non-thermal plasma is in contact with air and water image extracted from [*Samukawa, 2012].*

The reactive species may be separated depending on its lifetime (Fig. 1.5), if its lifetime is longer than a minute, we can call this species long-lived species and if its lifetime is lower than a second, we are going to call it short-lived species. This short or long lifetime depends on its reactivity with the medium and its stability through time.

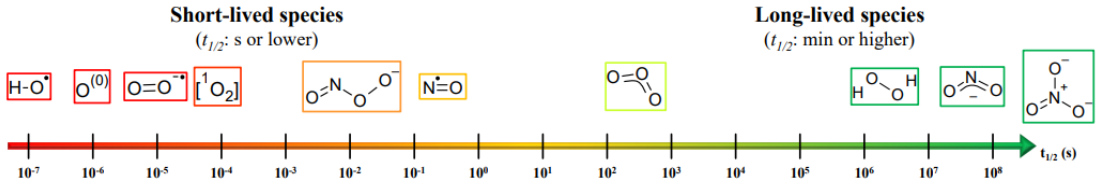


Figure 1.5. Scale of the lifetime for some of the reactive oxygen and nitrogen species.

In particular, in our Project we are going to work with H_2O_2 and NO_2^-, both of them are long-lived species, due to the preparation process of the hydrogel that is going to take longer times than a minute. Thanks to this RONS and its characteristics some treatments based on plasma have been developed, they are briefly explained in the following words.

1.2.4 - Plasma Treatments

Plasma treatments consists in generating RONS with the ionization of certain gas, and in a correct media, that assures the generation of the RONS that we are going to work with, but for treating a patient we know two different ways of working:

- direct treatment: in this kind of treatment, the plasma is applied directly over the intended target, in the case of plasma medicine application, on the tissue or the cells that we want to treat. Thanks to this type of application, all the RONS that are generated (short-lived and long-lived) (Fig. 1.5) can react with the tissue and become involved in the treatment process.
  The main disadvantages of this type of treatment in plasma medicine is that it limited to external tissues (skin, teeth, eyes, …) or requires an open surgery, that can be very expensive and invasive for the patients.
- indirect treatment: in this case the plasma is applied over a liquid and then this plasma-treated liquid (PTL) is put in contact with the target.

With this treatment type only long-lived species are stable enough to reach the target, due to the time that the preparation process takes. The advantages compared to the direct treatment, is that PTLs can be administered by injection, so they can be used to reach internal tissues with minimum invasiveness and allow repeated treatments. The main disadvantage of PTLs is that, if injected in an organism, they would quickly be washed away and diluted by body fluids, making a real local treatment hard to achieve.

To overcome this last issue that emerges from the use of PTLs, in recent years, polymers with the ability to form hydrogels have been extensively studies, since they allow to achieve local treatment by crosslinking in the site of interest and then release plasma-generated RONS.

1.3 - Biopolymers

Biopolymers are worldwide extended and either in the biomaterials and plasma treated patients, they can have different origins and are usually classified in two big groups, synthetic and natural. The first group has many examples like polyvinyl alcohol extracted from the crude and examples of the other group are polysaccharides or proteins.

In this work we are going to focus on alginate, composed by two comonomers such as guluronic acid and mannuronic acid (Fig. 1.6).

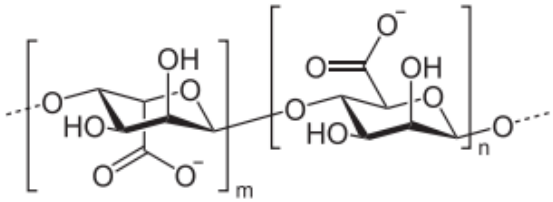


Figure 1.6. Chemical structure of alginate showing the repetitive units.

Alginate is a natural biopolymer that can form hydrogels due to its capacity to crosslink thanks to an ionic bond, a characteristic bond that make carboxylic group react with the Calcium ions (Fig. 1.7). The crosslinking could be explained, thanks to the work done by A. Jejurikar et al. [Jejurikar,2011], as the divalent cations bind to the carboxylic group, assuming the participation of every one of that group the highest molar ratio is 0.5. But the crosslinking happens more commonly in GG di-blocks but it's rare in MG/GM di-blocks. The work concluded too that a relatively high concentration of the ion solution generates a gradient of polymer and the mentioned ion solution, and this crosslinking could be completed within 2h.

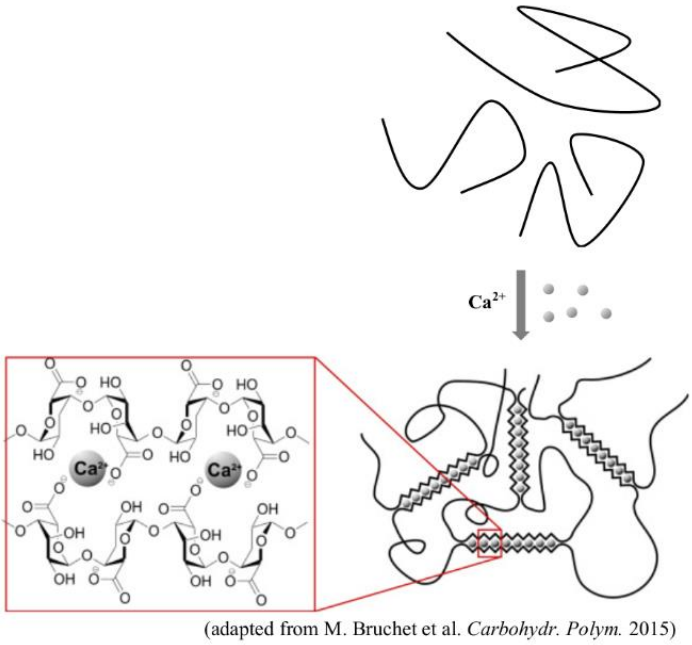


Figure 1.7. Process for alginate ionic crosslinking. Image adapted from [Bruchet, 2015].

Also, the hydrogel has its potential due to the capability to absorb water remain stable, this happens because two processes take part in here, the first one is that the most hydrophilic groups form primary bound water, followed by the formation of secondary bound water through hydrogen bonding to these centers. After that water uptake takes part thank to the swelling pressure that generates an opposite force to the polymer network generating a retraction force until the equilibrium swelling is reached. In the case of the calcium crosslinked hydrogels this equilibrium arrives before any degradation, in mass terms, happens.

Thanks to these materials we can avoid that some RONS injected by liquids in the human body could be washed away by the body fluids, when we add the RONS to the hydrogel we make that body fluids have to degrade them and make the hydrogel release RONS more slowly than if there was not a hydrogel.

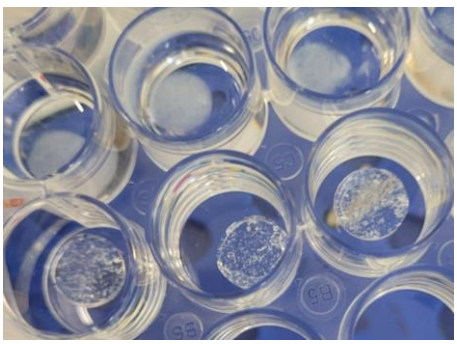


Figure 1.8. Picture of alginate hydrogels.

1.3.1 - Biopolymers and hydrogels treated by non-thermal plasma

The research on the use of biopolymers and hydrogels in plasma medicine is quite recent as well as that focused on the effect of direct plasma treatment on the biopolymer structures. Most of this research was done in the group where this project has been developed.

In 2019 C. Labay et al. analyzed and compared the RONS loading in alginate hydrogels between two plasma jets in order to create a vehicle that could allow local confinement and delivery of RONS in the diseased site.[Labay 2019].

In 2020 C. Labay et al. studied the process for RONS loading in gelatin hydrogels in order to study how these release affects cancer cells in release experiments. These experiments were promising due to the high amount of RONS generated in gelatin compared to water. [Labay 2020].

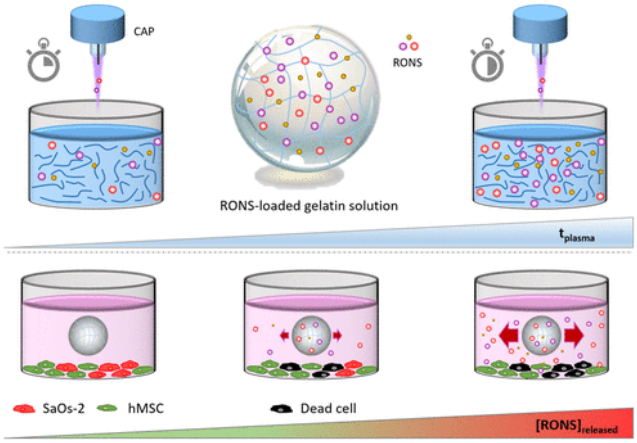


Figure 1.9. process of RONS loading in gelatin solution for a following ions release [C.Labay 2020].

In 2021 E. Bonnefoy and L. Sheid studied the composite hydrogels based on gelatin and agarose mixed with plasma treated biopolymers like dematan, hyaluronic acid, chondroitin, heparin and albumin [Bonnefoy 2021, Sheid 2021]. They showed that these biopolymers are able to enhance the amount of plasma generated reactive species and to store them for many days. They also studied the release ability of these composite hydrogels in physiological conditions.

Finally, X. Solé published a work in 2022 [Solé 2022] studying methylcellulose hydrogels, a very interesting class of hydrogels that can crosslink reversibly with the temperature and shown their promising use and selectivity in experiments with malignant and healthy cells.

2. Aim of the Work

The main objective of this final degree work is to know and study the alginate Hydrogel in all its phases during the plasma treatment, because we aim to know how this Hydrogel absorbs RONS when treated whit plasma and its max capacity, also the capacity of being absorbed if the treatment is done by adding in the hydrogel an RONS solution before the crosslink.

Another of the aims that we have, consist in studying the different parameters that affect in the crosslinking process for knowing which composition and which parameter and how they affect to the final hydrogel, variating the volume of crosslinker or alginate solution and also with the concentration of these solutions, this parameters are studied due the aim of knowing

3. Experimentals

3.1 - Materials

Sodium alginate (10-600 kDa) was purchased by Panreac. Sodium dihydrogen phosphate dihydrate ($NaH_2PO4{\cdot}2H_2O$, 98.0 % - 102 %, MW = 156.01 g/mol) and disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$, >98.0 %, MW = 358.14 g/mol) were purchased by Merk. Calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$, ≥96.0% anhydrous, powder), sodium nitrite ($NaNO_2$, powder, ≥ 98.0%), sulfanilamide ($H_2NC_6H_4SO_2NH_2$, powder, ≥ 98.0 %), N-(1-napthyl) ethylene diamine (NED), titanium(IV) oxysulfate (for detection of hydrogen peroxide), hydrogen peroxide solution (30 $\%_{w/w}$ in water) were purchased by Sigma Aldrich. Ortophosphoric acid (H_3PO_4 ,99.99% purity , MW = 98.00 g/mol) purchased by sigma aldrich. Calcium sulfate ($CaSO_4$ 99% purity, MW = 136.14 g/mol) . Phosphate buffer saline (PBS) purchased by Gibco.

Ultrapure water (milliQ) was obtained by filtration using 0.22-µm pore size MILLEXGP filter unit (Merck Millipore Ltd., Ireland). Helium gas (99.998%, maximum impurities of O_2 and H_2O 5 ppm_v) was provided by Praxair, Spain. Synthetic air (N_2 80 % and O_2 20 %) was purchased by Praxair, Spain.

3.2 - Preparation of the solutions

Phosphate buffer (PB, 5 mM, pH = 7.4) was prepared by dissolving the appropriate amounts of $H_2PO_4^-$ and HPO_4^{2-} in milliQ water. The correct amount of the two salts was obtained by using the following equations:

$$pH = pK_a + \log_{10}\frac{[HPO_4{}^{2-}]}{[H_2PO_4{}^-]}$$

$$[HPO_4{}^{2-}] + [H_2PO_4{}^-] = C_0$$

The final pH, if necessary was adjusted using small volumes of concentrated NaOH or HCl solutions.

Phosphate buffer saline (PBS) was prepared by dissolving one tablet in 0.5 L milliQ water, as indicated by the provider.

Alginate solutions were prepared by dissolving the appropriate amounts of alginate powder in miliQ water or in PB. We prepared solutions ranging from 0.25 % to 2.5 $\%_{w/vol}$. Table 3.1 shows the weights used to prepare 15 mL of alginate solution for each concentration.

Table 3.1. Range of concentration of alginate solutions used in this project and amount solid sodium alginate used to prepare 15 mL aliquots.

Concentration ($\%_{w/vol}$)	Concentration (g/mL)	Mass (g) for 15 mL
0.25	0.0025	0.0375
0.50	0.0050	0.075
0.75	0.0075	0.1125
1.00	0.0100	0.15
1.50	0.0150	0.225
2.00	0.0200	0.3
2.50	0.0250	0.375

Calcium chloride solutions ($CaCl_2$, MW = 147.01 g/mol), were prepared dissolving the appropriate amount of $CaCl_2 \cdot 2H_2O$ powder in milliQ water or PB. Different concentrations were used, the range goes from 10 mM to 1000 mM. The weights of each concentration can be found in Table 3.2 for a solution of 10 mL.

Table 3.2. Range of concentration of Ca^{2+} solutions used in this project and amount of solid $CaCl_2 \cdot 2H_2O$ (147.04 g/mol) used to prepare 10 mL aliquots

Concentration (mM)	Number of moles (mmol)	Mass (g) for 10 mL
10	0.0001	0.0147
100	0.001	0.147
250	0.0025	0.368
500	0.005	0.735
1000	0.01	1.47

The solutions of the retardant (Na_2HPO_4, MW = 178 g/mol), was prepared by dissolving the appropriate amount of $Na_2HPO_4 \cdot 12H_2O$ in milliQ water. Different concentrations were used ranging 100 mM to 200 mM and for preparing 10 mL the weights are collected in Table 3.3.

Table 3.3. Range of concentration of HPO_4^{2-} solutions used in this project and amount of solid (178 g/mol) used to prepare 10 mL aliquots

Concentration (mM)	Number of moles (mmol)	Mass (g) for 10 mL
100	0.001	0.178
125	0.00125	0.223

150	0.0015	0.267
175	0.00175	0.312
200	0.002	0.356

$NaNO_2$ standard solutions were prepared by dissolving the appropriate amount of $NaNO_2$ powder in milliQ water or PB. H_2O_2 standard solutions were prepared by diluting commercial hydrogen peroxide solution (30 $\%_{w/w}$, 9.8 M) with milliQ water or PB.

3.3 - Preparation of the hydrogels

After preparing all the solutions of alginate, Ca^{2+} and retardant (HPO_4^{2-}), as described in the previous section, the following step, to obtain alginate hydrogel, was the crosslinking step. Different protocols were tested by varying several parameters, like the concentration of alginate and Ca^{2+}, the presence and concentration of retardant, the relative ratio of the components, the mixing method, the size and shape of the support used for crosslinking and the crosslinking time.

We used two different mixing methods. In the first one, the alginate solution was added first in a well of a 48-well plate and the crosslinker solution, with or without the addition of the retardant, was added drop-by-drop on top. In some cases, the resulting solution were mixed with a spatula and in others it was just left untouched during the crosslinking period.

In order to achieve a better homogeneity in the hydrogels and to speed up the mixing step, for the second method, we used two 1 mL Luer Lock syringes and a connector. The alginate solution was loaded on one syringe and the crosslinker and retardant solution on the second one. Then the two syringes were connected using a Luer Lock connector (Fig. 3.1) and the two solutions were rapidly mixed by pushing back and forth the syringes several times. All the process was done trying to avoid the incorporation of air bubbles in the solutions. After the mixing, the partially crosslinked hydrogel was extruded from the syringe into a well of a 48-well plate or into a 200 µL 3D-printed mold (Fig. 3.2), in order to obtain disks and left into the fridge at 4 °C to complete the crosslink process.

Figure 3.1. Two Luer Lock syringe connected by the connector.

Figure 3.2. 3D-printed mold used to prepare alginate hydrogels.

3.4 - Qualitative characterization of the hydrogels

3.4.1 - Visual characterization and handling

All the hydrogels produced were inspected visually to see if they were transparent or containing any small bubbles or precipitated salts. Then, they were handled by hand or with spatula and tweezers to determine if they were able to retain the shape of the container and to estimate their stiffness.

3.4.2 - Scanning Electron Microscopy

Some selected hydrogels were analyzed by scanning electron microscopy (SEM) to have information about their morphology and porosity. To prepare the samples for the SEM we mixed, using Luer Lock syringes, 800 µL of alginate solution, 100 µL of Ca^{2+} solution and 100 same volume for retardant. With the total volume (1 mL) three samples can be prepared of 300 µL each.

We repeat this process for each concentration of calcium solution and the retardant, which are 100/125, 150/150 and 250/200 mM. These concentrations are used for the alginate solution 1 $\%_{w/vol}$ and 2 $\%_{w/vol}$. Hydrogels were prepared in the 3D-printed mold.

Once the samples are crosslinked, the hydrogels are dehydrated by lyophilization along one night. After the lyophilization (Fig. 3.3a), due to lack of electrical conductivity in the samples, aluminum strip was placed to connect the hydrogel to the base used for good functioning of the SEM.

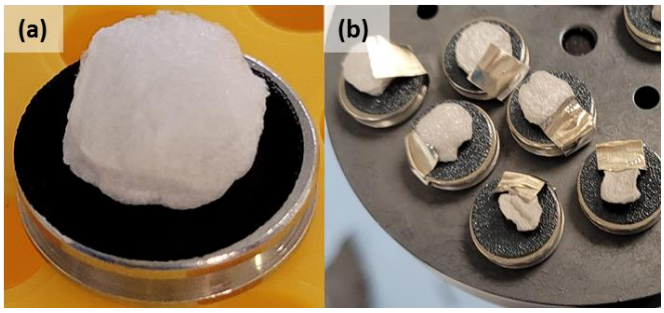


Figure 3.3. a) Alginate sample after the lyophilization process and b) after the carbon coating process.

Once connected, and aiming to get a better resolution of the SEM images, we used a carbon coater EMITECH K950 X to produce a carbon coat that will guaranteed us a better result (Fig. 3.3b). Once all these processes are done, the samples are analyzed in SEM PhenomXL (PHENOMWORLD) (Fig. 3.4).

Figure 3.4. SEM microscope PhenomXL (PHENOMWORLD).

3.5 - Quantitative characterization of the hydrogels

3.5.1 - Gravimetric and volumetric experiments

After crosslinking, the hydrogels were carefully removed from their containers (well plate or 3D mold) with spatula and tweezers and weighted on an analytical balance. Any volume of liquid remaining in the container after the removal of the hydrogel was quantified using micropipettes.

3.5.2 - Rheology experiments

For rheology experiments, hydrogels were prepared as described in section 3.3, but using a 12-well plate as container. The idea is to obtain solid disks with 2 mm height. The difference is that instead of

preparing 3 samples for one Luer lock mixing process, we use all the volume (1 mL) to produce one hydrogel in the 12-well plate that is 2 mm high.

Once the samples are crosslinked, they are extracted and placed over the rheology tester with the rough surface to avoid the sliding of the hydrogel while the experiment (Fig. 3.5).

With the rheometer 2 different type of analysis were done:

1. Time sweep: for this test the objective consist in making an oscillation test in some linear range of the hydrogel in order to obtain the value of the storage modulus (G') of the different concentrations of the alginate, crosslinker and retardant.
   The parameters used for the test are described in the following words:
   - Gap : 1500 µm
   - Axial force for compression 1N
   - 20 mm rough plate, Peltier plate Steel-115417
   - Time: 60 seconds

2. Amplitude Sweep: During this experiment we aimed to evaluate at which oscillation displacement the storage modulus (G') and the loss modulus (G") got the same value, when this happens the hydrogel start the chain rupture and loss his structural properties.
   The parameters used for the test are described in the following words:
   - Gap : 1500 µm
   - Axial force for compression 1N
   - 20 mm rough plate, Peltier plate Steel-115417
   - Range oscillatory displacement: from 0 rad/s to 1·E-4 rad/s

Figure 3.5. Hydrogel sample in the rheometer with the rough surface.

3.6 - Loading hydrogels with reactive species

Two strategies were used to load the alginate hydrogels with RONS. In the first one, we used standard solutions of RONS, hydrogen peroxide and nitrite ions. This was done to obtain hydrogels with an easy controllable, high amount of RONS, in order to ease the release study and to obtain some general results. The second method for loading RONS was the use of a non-thermal plasma jet. In both cases, the RONS were loaded to the alginate solutions, before the crosslinking.

3.6.1 - Reactive species from standard solutions

Stock solutions of hydrogen peroxide and nitrite ions were prepared in phosphate buffer, starting from a commercial 30% H_2O_2 solution and solid $NaNO_2$, respectively. Then, small volumes of these stock solutions were added to alginate solutions in order to obtain the final concentrations of 5 mM for hydrogen peroxide and 2 mM for nitrite ions. These RONS-containing alginate solutions were used to prepare hydrogels according to the previously described protocols.

3.6.2 - Reactive species from plasma treatment

Plasma treatments were performed using an atmospheric pressure plasma jet (APPJ) working with helium as feed gas (Fig. 3.6).

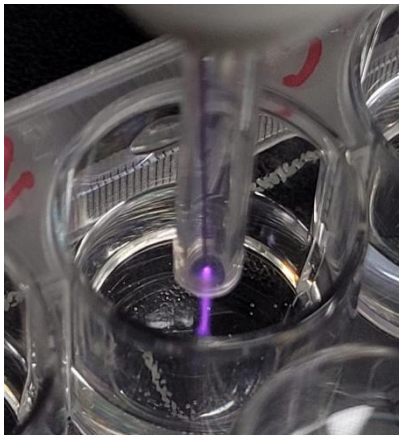


Figure 3.6. Plasma beam generated by the APPj.

The active electrode is a copper wire (0.1 mm diameter) embedded inside a quartz tube (ID = 1.2 mm) and connected to a high voltage power supply. The discharge was operated with a sinusoidal waveform

at 23 kHz with (U) ~ 2 kV and (I) ~ 3 mA. The average power delivered to the discharge was 1 W. He with 0.3% synthetic air flows through the tube and serves as plasma feed gas. Two Bronkhorst EL-FLOW Select flow controllers were used to set the flow rate of the gases. Experiments were performed in open air, at room temperature and with an average relative humidity of 70%. All the treatments, if not otherwise stated, were done using the following conditions: feed gas flow rate 1 L min^{-1}, distance between plasma nozzle and target 5 mm, sample volume 1 mL, sample support 24-well plate. Treatment time was 10 min. The He flow was started 15-20 min before each treatment in order to ensure a good purge of the gas line. The plasma was started at least 5-10 min before the treatment to let it stabilize. Each plasma treatment was repeated four times to ensure reproducibility.

3.7 - Quantification of reactive species

The detection and quantification of hydrogen peroxide was performed using the titanium(IV) oxysulfate method. This method exploits the reaction of a Ti(IV) complex that reacts with H_2O_2 in acidic conditions to generate a yellow complex with absorbance maximum at 410 nm, according to the following reaction:

$$[Ti]^{4+} + H_2O_2 \rightarrow [Ti(OH)_3(H_2O_2)]^+ + 3H^+$$

100 µL of H_2O_2-containing solutions were added to 50 µL of Ti(IV) probe solution in a 96-well plate. Then, the absorbance in the range between 370 nm and 600 nm was read in a microplate reader. An example of the spectra obtained is reported in Figure 3.7.

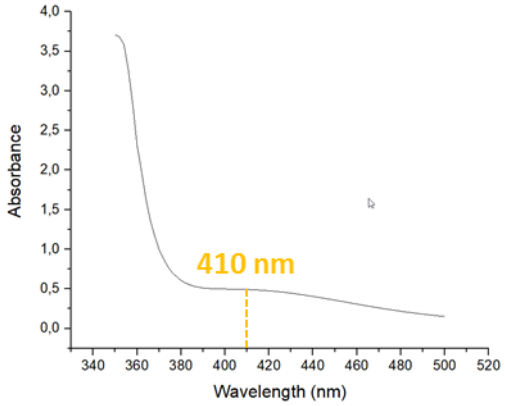
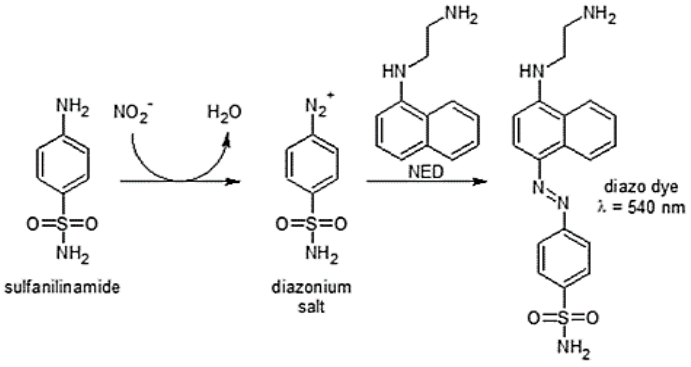


Figure 3.7. UV-vis absorption spectrum of a hydrogen peroxide-containing sample after the addition of the Ti(IV) probe. The maximum of the peak at 410 nm is indicated.

The detection and quantification of nitrite ions was performed using the Griess method. This method exploits the reaction of a sulfanilinamide that reacts with NO_2^- to generate a diazonium salt that, in turn, can react with N-(1-naphthyl)ethylene diamine to generate a diazo purple dye with absorbance maximum at 540 nm, according to the following reaction:



50 μL of NO_2^--containing solutions were added to 50 μL of Griess reagent in a 96-well plate. Then, the absorbance in the range between 400 nm and 650 nm was read in a microplate reader. An example of the spectra obtained is reported in Figure 3.8.

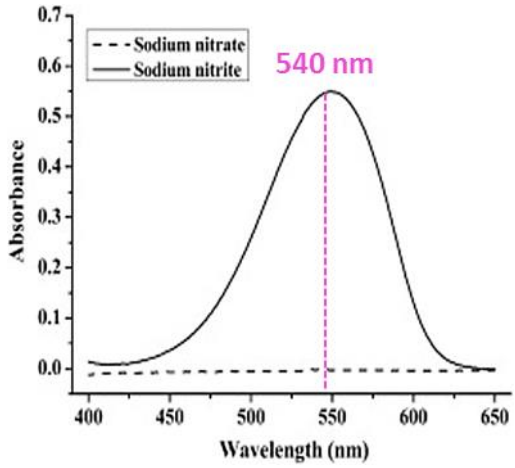


Figure 3.8. UV-vis absorption spectrum of a nitrites-containing sample after the addition of the Griess reagent. The maximum of the peak at 540 nm is indicated.

Calibration lines for hydrogen peroxide and nitrite ions were obtained by using standard solutions of the two RONS in the media of interest. The slope and intercept values of the calibration lines were used to convert absorbance data into concentrations.

3.8 - Release experiments

Once obtained the RONS-loaded hydrogels, as described in the previous sections, the release experiments were performed to determine the percentage of release and the kinetics of the process.

First, the hydrogels were transferred in 2 mL of PBS medium at 37ºC in a 24-well plate. Then, at selected time points (up to 90 min), the release medium was sampled and the RONS analyzed according to the methods described in the previous paragraph. The final data were corrected taking into account for the small variations of release medium volume during the experiments. The data were reported as number of moles released as function of the release time or as release percentage with respect of the total amount of RONS contained inside the hydrogels before starting the release experiments. To obtain an estimate of the kinetic of the release process, the data were interpolated using the exponential function:

$$n(t) = n_{\infty} - Ae^{-\ln 2 \cdot t/\tau_2}$$

where n_{∞} is the amount in moles released at infinite release time, A and τ_2 are respectively the amplitude and the doubling time of the exponential.

3.9 - Data treatment

For the data treatment we worked with an average of 3 independent replicates for each experiment, in order to ensure the reproducibility of the results. As error, associated to the experimental values, we used the standard deviations of the 3 replicates.

To calculate the errors associated to derived quantities, we use the maximum error propagation formula, in which, for a given function, as the following one, depending on *n* parameters:

$$f(x_1, x_2, \dots, x_n)$$

We can obtain the error by knowing all the errors associated to the *n* parameters:

$$\delta f = \left|\frac{\partial f}{\partial x_1}\right| \cdot \delta x_1 + \left|\frac{\partial f}{\partial x_2}\right| \cdot \delta x_2 + \cdots + \left|\frac{\partial f}{\partial x_n}\right| \cdot \delta x_n$$

To analyze the data, excel program was used, and to produce the graphs showed in this work we used Origin Pro 9.0 as well as to obtain all the fitting of the results.

4. Results and Discussion

In this section, the results obtained during this project are presented and discussed aiming at drawing some conclusions on the dependence of the release properties of the hydrogels from the parameters explored during their preparation. For clarity, we first introduce the codes used to classify the samples.

4.1 - Samples coding

In the following sections we will compare different samples, obtained varying parameters such the alginate concentration, the calcium ions concentration, the presence and concentration of crosslinking retardant, the mixing method and the vessel used to prepare the hydrogels, so a code was defined, in order to unequivocally identify each sample/experiment. The codes used have the following format:

Alg_AC%_CC/RC_Ves_MM

Each part explains one characteristic of the hydrogel, at the beginning appears the biopolymer type, in this work just alginate was used so the abbreviation (**Alg**) is used to make it shorter, next to it is shown the concentration, in weight percentage, of the alginate solution (**AC%**) followed by the concentration of the crosslinker (**CC**) and the retardant (**RC**) if used, both in millimolar. Next, is reported the vessel used for the crosslinking (**Ves**) and at the end appears the mixing method (**MM**). A summary of the parameters used and the relative codes is reported in Table 4.1.

Table 4.1. Summary of the codes used for the hydrogel samples in this work.

Parameter	Abbreviation	Range	Notes
Alginate conc.	AC%	0.25% - 2.5%	Concentration in weight/volume
Crosslinker conc.	CC	10 mM - 1000 mM	Ca^{2+} from $CaCl_2 \cdot 2H_2O$
Retardant conc.	RC	0 mM - 500 mM	Na_2HPO_4
Vessel	Ves	48WP 3DM	48-well plate 3D-mold
Mixing method	MM	Top Mix LL	Ca^{2+} solution poured on top Mixed with spatula Mixed with Luer Lock syringes

4.2 - Preliminary experiments

In this section, we report the qualitative and semi-quantitative results obtained from some preliminary experiments aimed at scanning quickly some of the parameters mentioned in the previous section in order to select the most promising conditions for preparing hydrogels in further experiments. The parameters analyzed in this section are weight, size and shape of the hydrogels, the rigidity/manipulability, the transparency/opacity, the presence of precipitates or air bubbles trapped inside the hydrogels.

The first group of hydrogels was prepared in 48-well plates using alginate solutions in phosphate buffer and Ca^{2+} solutions in deionized water. The volumes of the two solutions were 500 µL of alginate solution and 100 µL of Ca^{2+} solution. First the alginate solution was added in the well, then slowly the Ca^{2+} solution was added on top, trying to cover all the surface homogeneously. The well plate was then stored in the fridge at 4 °C overnight. The following day, the hydrogels were carefully removed from the wells using tweezers and spatula and weighted on an analytical balance. In those cases, where some liquid was remaining in the wells after the removal of the hydrogels, its volume was quantified using micropipettes.

The alginate concentration was varied between 0.25 and 2.5 $\%_{w/vol}$ (0.25 %, 0.5 %, 0.75 %, 1.0 %, 1.5 %, 2.0 %, 2.25 %) and the concentration of Ca^{2+} between 50 and 250 mM (50 mM, 100 mM, 150 mM, 200 mM, 250 mM). In Figure 4.1 is reported a picture of all the hydrogels taken after removing them from the well plate.

Figure 4.1. Picture of the hydrogels with different alginate and Ca^{2+} concentrations after removing them from the 48-well plate.

As shown in the Figure 4.1 some quick results can be extracted, the first one is that in general the hydrogels tend shrink with high Ca^{2+} concentrations. According to this, hydrogels with low Ca^{2+} concentration were able to retain better the shape of the well. At the same time is also seen that they are less opaque and became more translucent while Ca^{2+} rises in concentration. The last trend that could be commented is that the manipulability rises with the calcium solution concentration because of the stiffness of the structure. In the experiment we calculated the mass of each hydrogel and the volume of non-crosslinked liquid remaining in the well. These data are reported in Figure 4.2.

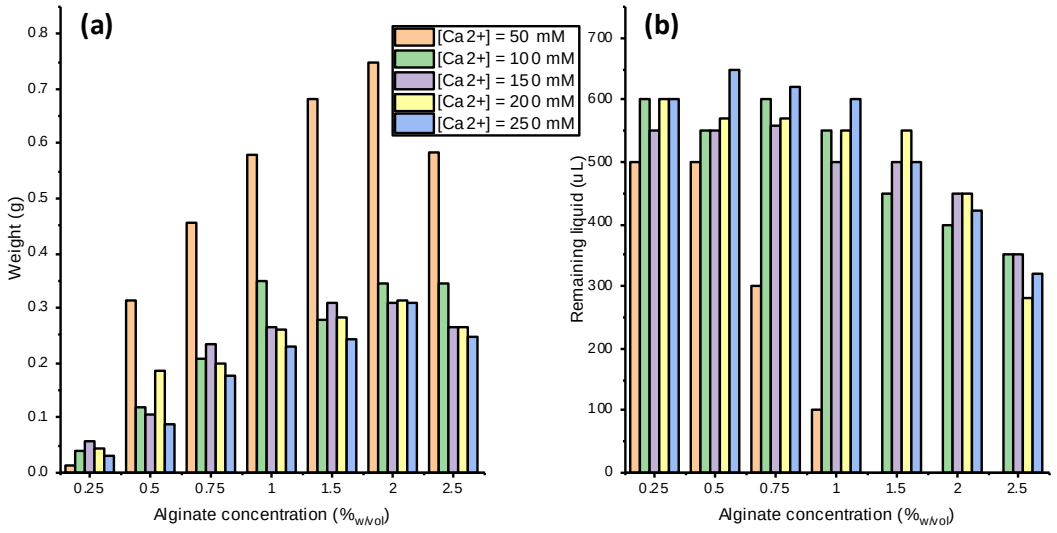


Figure 4.2. Weight (a) and volume of the liquid remaining inside the well (b) for all the alginate hydrogels as function of the alginate concentration and of the Ca^{2+} concentration. For these preliminary experiments only one replicate was performed.

These preliminary results allowed to select the best hydrogels to work with in the following experiments. We selected concentrations of alginate between 0.5 to 2.0 $\%_{w/vol}$, concentrations lower than 0.5 % gave us very small hydrogels with irregular shapes and high non-crosslinked liquid volume. Concentration higher than 2 % are difficult to handle due to high viscosity and therefore were excluded as well. Regarding the concentration of Ca^{2+} ions, we notice differences when the concentration was low (50 mM). In the range from 100 to 250 mM, we did not observe substantial differences, so in the next experiments we selected a larger range, from 10 to 1000 mM. In all the cases presented in this section, the homogeneity of the hydrogels was not optimal. We however tested some of them for the release of long-lived RONS commonly generated by plasma treatment. The results are presented and discussed in the next section.

4.3 - Release experiments with hydrogels prepared in 48-well plate

The aim of the release studies is to find the optimal conditions that allow to have a release of RONS from the hydrogels as high as possible and slow in time, in order to have a continuous therapeutic effect. Also we expect that if we choose a relatively high concentration of alginate solution because of the Na+ ions that will be in the release media, driving the release of Ca^{2+} ions high crosslinking degree, we also expect a slower ions release with a higher concentrations due to the degree of crosslinking that makes the exchange between Na+ and Ca^{2+} ions slower.

Alginate hydrogels were loaded with hydrogen peroxide and nitrite ions, separately, as described in section 3.1. We loaded the hydrogels using standard solutions of these species at first in order to have higher and well controlled concentrations. We moved to hydrogels loaded by plasma treatment only for the optimal conditions that we found (see section 4.6). The RONS-loaded hydrogels were transferred in 2 mL PBS solutions (in a 24-well plate) and the release was monitored at 37 °C for 60-90 min. We first started with 2 % alginate solutions since they allowed to obtain the hydrogels with best shapes and sizes. In Figure 4.3 are reported the H_2O_2 (a) and NO_2^- (b) release data for 2% alginate hydrogels obtained with different Ca^{2+} amounts. For Ca^{2+} concentration lower than 100 mM, the resulting hydrogels were not rigid enough to be manipulated and transferred without breaking them, so the relative release experiments were not done.

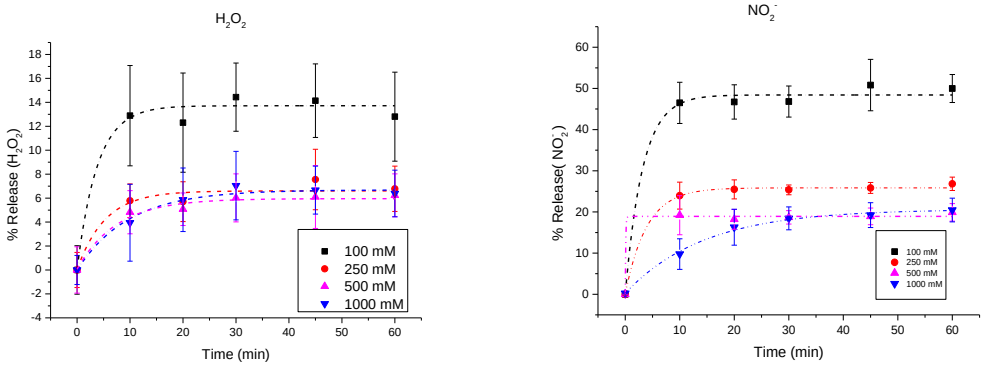


Figure 4.3. Release percentage of hydrogen peroxide and nitrite ions from alginate hydrogels (0.5 $\%_{w/vol}$) in PBS, as function of the treatment time for different concentration of crosslinker (Ca^{2+}). 100 % release would correspond to 0.0015 mol for hydrogen peroxide and to 0.0006 mol for nitrite ions. Each point is represented as average ± standard deviation, n = 3. The dashed lines are the exponential fit of the experimental data. The data obtained from the fittings are collected in Table 4.2.

As evident from the data in Figure 4.2, the release is very fast in all cases. In most cases the concentration of RONS reaches a plateau value already after 10 min. From the exponential fit of the data we could obtain the values of the plateau and the time constants of the process. These parameters are collected in Table 4.2. In some cases, the fitting procedure didn't allow us to obtain reasonable results (e.g. the 500 mM case in Fig. 4.3b) because of the too fast release process and the high error bars. In the table we collected also the results obtained from the hydrogels with alginate 1 $\%_{w/vol}$ and 0.5 $\%_{w/vol}$ that were obtained following the same procedure.

Table 4.2. Parameters obtained from the exponential fit of the experimental data reported in Figure 4.3.

Sample	Hydrogen peroxide (Fig. 4.3a)			Nitrite ions (Fig. 4.3b)		
	n_∞ (%)	n_∞ (mmol)	τ_2 (min)	n_∞ (%)	n_∞ (mmol)	τ_2 (min)
Alg_1%_100/0_ 48WP_Top	50.5 ± 0.8	1.10 ± 0.02	1.2 ± 0.3	100 ± 60	0.9 ± 0.6	0.014 ± 0*
Alg_1%_1000/0_ 48WP_Top	23 ± 2	0.51 ± 0.04	4.5 ± 1.6	34.8 ± 0.5	0.320 ± 0.005	3.9 ± 0.3
Alg_0.5%_100/0_ 48WP_Top	13.7 ± 0.5	0.310 ± 0.011	2.7 ± 1.3	4.4 ± 1.0	0.040 ± 0.009	2.2 ± 0.9
Alg_0.5%_250/0_ 48WP_Top	6.6 ± 0.6	0.150 ± 0.011	3.6 ± 1.6	25.8 ± 0.3	0.230 ± 0.003	2.7 ± 0.7
Alg_0.5%_500/0_ 48WP_Top	6.0 ± 0.3	0.130 ± 0.007	5.2 ± 1.4	19 ± 11	0.170 ± 0.010	0.061 ± 0*
Alg_0.5%_1000/0_ 48WP_Top	6.7 ± 0.3	0.150 ± 0.006	6.7 ± 1.4	20.7 ± 0.5	0.19 ± 0.005	10.0 ± 0.9

The values with a 0 value mean that the error is low enough to be considered negligible.

The results reported in the table show that, in general, the release is higher when the concentration of crosslinker is higher, but at the same time it is also faster. In the two cases with higher Ca^{2+} concentration (1000 mM), the total release is achieved after 30 min from the beginning of the experiment.

Apart from the parameters obtained from the release experiments, we realized that the method of pouring the Ca^{2+} solution on top of the alginate solution in the 48-well plate, didn't allow us to obtain homogeneous hydrogels because the crosslinking was too fast. This was confirmed also by the presence of large volumes on non-crosslinked liquid in the wells after removal of the hydrogels. This lack of homogeneity is the cause of the low reproducibility of some of the experiments reported in this section and could in principle affect the release properties of the hydrogels. In the next section we

describe some modifications introduced in the protocols used to prepare the hydrogels in order to obtain higher homogeneity of the samples and better reproducibility.

4.4 - Preliminary experiments with Luer-lock syringes

In order to achieve a better homogeneity of the hydrogels we followed two strategies: (i) changing the mixing method and (ii) trying to slow down the crosslinking reaction using different salts.

Regarding the mixing of the samples, first we simply tried to mix the samples, inside the 48-well plate, using a spatula, after pouring the Ca^{2+} solution. This method improved a little the resulting hydrogels, but was not satisfactory enough. So we completely changed the procedure, using syringes with Luer Lock connectors (see Figure 3.1). The alginate solution was loaded on the first syringe (800 μL) and the Ca^{2+} solution (0.1 mL) on the second one. Then the solutions were rapidly mixed before pouring them into the crosslinking vessel.

To slow down the crosslinking reaction we tried using insoluble salts of Ca^{2+} that are less soluble than $CaCl_2$ (solubility 740 g/L). The first set of tests was done using calcium sulfate (solubility 2.6 g/L). These hydrogels were done using alginate concentrations from 0.5 to 2 $\%_{w/vol}$ and $CaSO_4$ concentration from 1000 to 3000 mM, the mixing was done with the syringes as explained in the previous paragraph. In Figure 4.4 is reported the picture of one of the hydrogels obtained with this method.

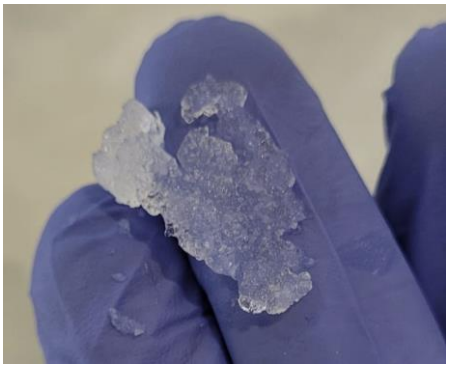


Figure 4.4. Picture showing the texture of an alginate hydrogel obtained using $CaSO_4$ as crosslinker and the Luer Lock syringe to mix before crosslinking.

As can be observed from the picture, the preliminary results obtained using $CaSO_4$ solution were not satisfactory. Precipitates were observed within the samples and the hydrogels that were not able to keep the shape of the container. We then went back to calcium chloride but we mixed it with disodium

hydrogen phosphate. Ca^{2+} ions in presence of hydrogen phosphate generate a very insoluble salt ($CaHPO_4$, solubility 0.1 g/L) so the hydrogen phosphate acts as a retardant in the crosslinking process.

The concentrations chosen where selected according to some previous experience of the group and in order to stablish the release property with the new type of crosslinker and how does it affect to the final hydrogel, also along the whole preparation of its. The value of each concentration

In Figure 4.5 is reported the release curve of nitrite ions from alginate hydrogel 2 $\%_{w/vol}$ obtained using Ca^{2+} 100 mM and HPO_4^{2-} 125 mM; the solutions were mixed using Luer Lock syringes and the crosslink was done in 48-well plate.

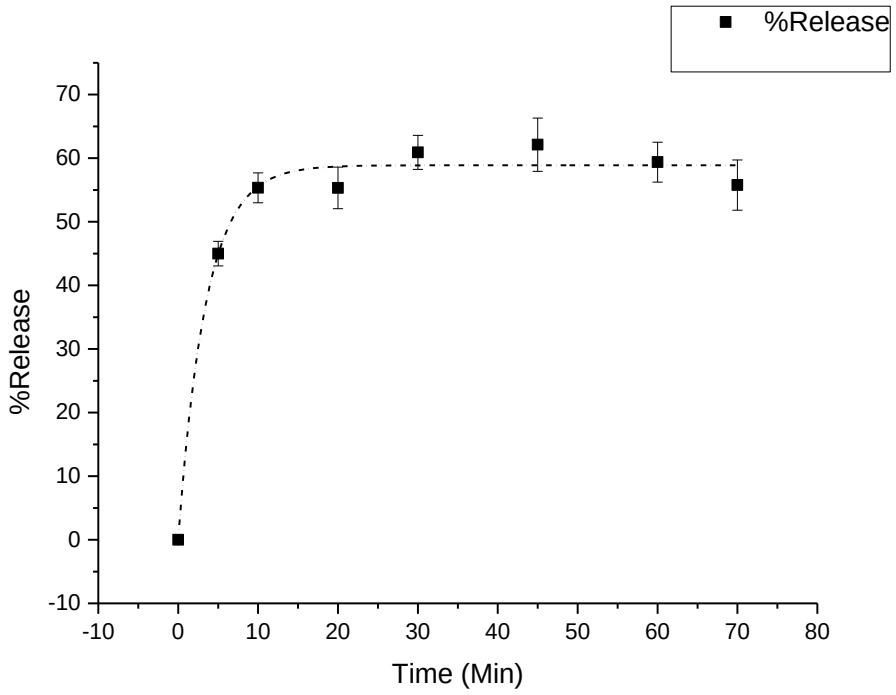


Figure 4.5. Release percentage of nitrite ions from alginate hydrogels (2 $\%_{w/vol}$) in PBS, as function of the treatment time for Ca^{2+}/retardant concentrations 100/125 mM. 100 % release would correspond to 0.5 mmol. Each point is represented as average ± standard deviation, n = 3. The dashed line is the exponential fit of the experimental data.

At last, since the syringe method coupled with the 48-well plate did not allow a good reproducibility in terms of size and shape of the hydrogels, we replaced the well plate with a 3D-printed mold (see Figure 4.6). The mold is composed by a base and a holed plate kept together by four screws in the corners. In this way we were able to produce hydrogels all with the same volume (300 μL) and also to remove them from the mold without risk of breaking them as it was happening with the well plate (Fig. 4.6b).

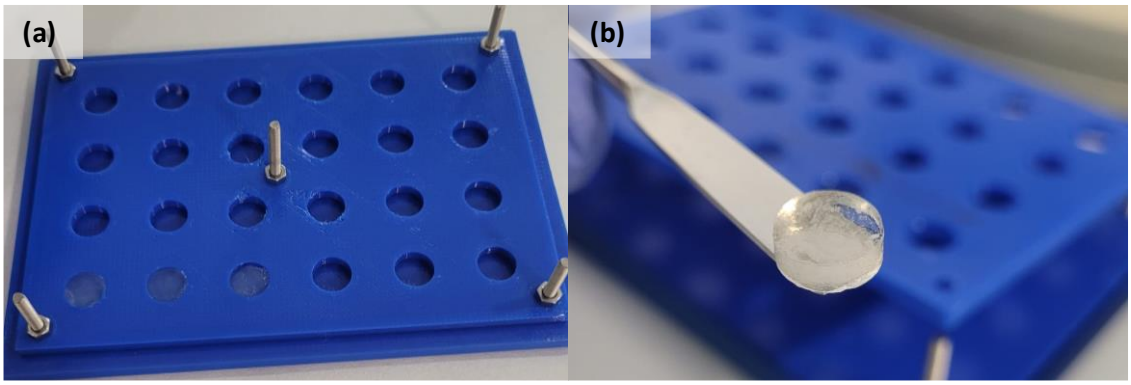


Figure 4.6. 3D-printed mold (a) for a better reproducibility and an easier extraction of the hydrogels (b).

4.5 - Release experiments with hydrogels prepared using Luer-lock syringes and 3D mold

In this section we report the release experiments of hydrogen peroxide and nitrite ions from the hydrogels selected in the previous section. The hydrogels were loaded with RONS using standard solution as described in section 3.3. The release data for hydrogels are reported in Figure 4.7 and the parameters obtained from the fit of the data in Table 4.3.

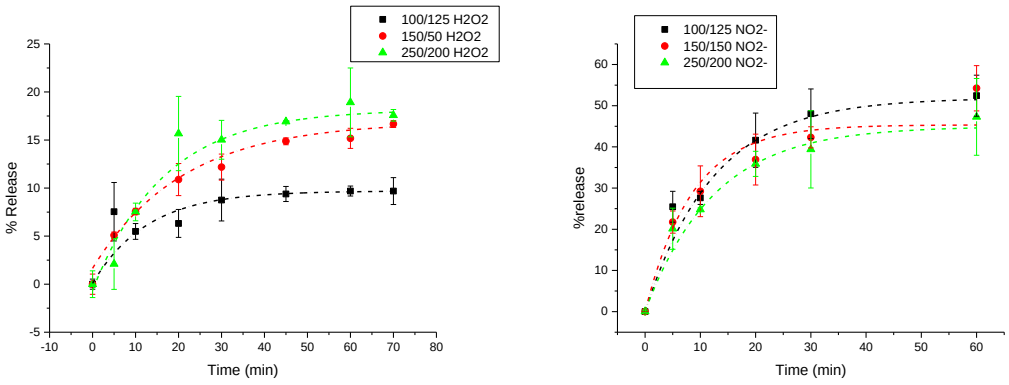


Figure 4.7. Release percentage of hydrogen peroxide (Left) and nitrite ions (Right) from alginate hydrogels (2 $\%_{w/vol}$) in PBS, as function of the treatment time for different concentration of crosslinker (Ca^{2+}) and retardant (HPO_4^{2-}). 100 % release would correspond to 5 mmol for hydrogen peroxide and to 2 mmol for nitrite ions. Each point is represented as average ± standard deviation, n = 3. The dashed lines are the exponential fit of the experimental data. The data obtained from the fittings are collected in Table 4.3.

Table 4.3. Parameters obtained from the exponential fit of the experimental data reported in Figure 4.7.

Sample	Hydrogen peroxide			Nitrite ions		
	n_∞ (%)	n_∞ (mmol)	τ_2 (min)	n_∞ (%)	n_∞ (mmol)	τ_2 (min)
Alg_2%_100/125_	18.2 ± 0.4	0.410 ± 0.009	11.5 ± 1.3	50 ± 3	0.450 ± 0.024	5.1 ± 0.8

48WP_LL						
Alg_2%_150/150_ 48WP_LL	16.9 ± 0.5	0.380 ± 0.012	14 ± 2	56.15 ± 0.01	0.51 ± 0.09	12.31 ± 0*
Alg_2%_250/200_ 48WP_LL	9.7 ± 0.4	0.220 ± 0.008	8.8 ± 1.7	45 ± 4	0.40 ± 0.04	8.6 ± 1.2
Alg_2%_100/125_ 3DM_LL	**	**	**	52 ± 6	0.47 ± 0.06	8.6 ± 1.9
Alg_2%_150/150_ 3DM_LL	**	**	**	45 ± 3	0.41 ± 0.03	5.9 ± 1.4
Alg_2%_250/200_ 3DM_LL	**	**	**	45 ± 4	0.40 ± 0.04	8.6 ± 1.2

The values with a 0 value mean that the error is low enough to be considered negligible.
** amount of species' release too low to be detected with reliability

Analyzing all the results obtained, the main trend we can obtain is that the release of nitrites is much more homogeneous than the hydrogen peroxide, this may happen due to the reactivity that the Hydrogen peroxide has, the time of the release shown doesn't seem to show any trend, this may be due two quick hypothesis, the first one is due to the different distribution of the Ions in the release moment or the different way in which each hydrogel is degraded and the second one is due to the lack of high concentrations that made some release tests fail (**) because the microplate reader couldn't read such low concentrations generating a bigger error or bad data, this is another reason why the lack of hydrogen peroxide in the 3D mold, some experiments were tried but not high enough concentration was found.

The good point comparing the mixing methods is that using the luer lock ensures a slower ions release for Nitrites and also for Hydrogen peroxide, we corelate this due to the better distribution of the bounds in the microstructure and the lower gradient of concentrations along the hydrogel radius. We suppose that with the top mixing the ions were focused on more surface levels and when this surface starts degradation much higher quantity of ions are released, but with the luer lock this seems not to happen, maybe thank to the distribution obtained on mixing.

4.6 - Release experiments with plasma-treated hydrogels

Once the hydrogels loaded with standard solutions of RONS were done, we investigated plasma treatment of the alginate solutions to obtain loaded hydrogels. Our main aim was to obtain the parameters using the same hydrogels described in the previous sections but using plasma treated

alginate. Alginate solutions with different concentrations of crosslinker and retardant were treated by APPJ for 10 min using the parameters described in section 3.3. The amount of hydrogen peroxide and nitrite ions were quantified in alginate solutions after 10 min treatment and then the solution was used to prepare hydrogels. The release data are reported in Figure 4.8 and the parameters obtained from the fit of the data in Table 4.4.

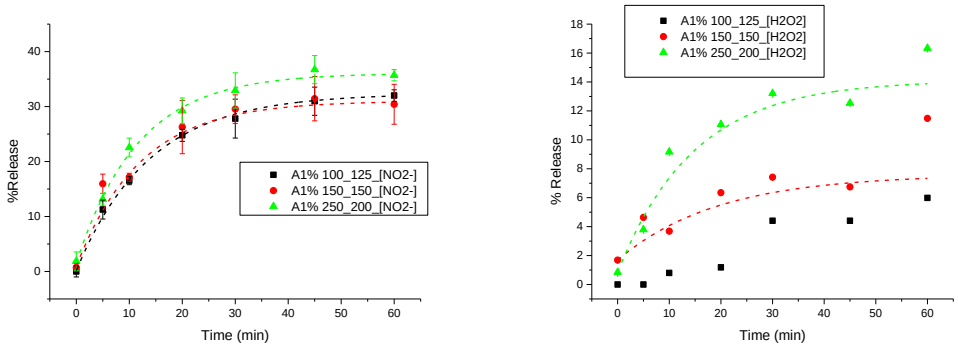


Figure 4.8. Plasma treated release of Alginate 1% mixed with luer lock syringe, the treatment time is about 10 minutes, and the release is done in PBS media.

Table 4.4. Parameters obtained from the exponential fit of the experimental data reported in Figure 4.7.

Sample	Hydrogen peroxide			Nitrite ions		
	n_∞ (%)	n_∞ (mmol)	τ_2 (min)	n_∞ (%)	n_∞ (mmol)	τ_2 (min)
Alg_1%_100/125_3DM_LL	*	*	*	32.3 ± 0.5	0.0360 ± 0.0005	9.6 ± 0.4
Alg_1%_150/150_3DM_LL	7.6 ± 1.1	0.070 ± 0.010	13 ± 6	31 ± 4	0.034± 0.004	8.4 ± 1.9
Alg_1%_250/200_3DM_LL	14.1 ± 1.3	0.130 ± 0.012	10.3 ± 2.8	36.1 ± 0.5	0.040 ± 0.0006	8.1 ± 0.5

*The fit did not converge in proper way so the data could not be calculated

Comparing the results of the plasma treated hydrogels with the standard solutions hydrogels we can observe a much lower amount of RONS released, due to the less concentration at the beginning but, the average release has a slower trend making the plasma hydrogels better for a long period release, at least hydrogen peroxide, in nitrites the values are at least in τ_2 values , the main issue to highlight is that on hydrogen peroxides, the average release is even lower and has given many problems to work with due to the lack of concentration or bad reactivity with the probe.

4.7 – Rheological characterization of the hydrogels

To complete the characterization of the selected hydrogels and to identify any correlation between the release properties and the structure, we performed rheology experiments (this section) and scanning electron microscopy analysis (next section, 4.8).

Different alginate hydrogels were prepared using the Luer Lock syringes mixing method and the 3D mold. The alginate concentration was 1 and 2 $\%_{w/vol}$ and the Ca^{2+}/retardant concentrations were 100/125 mM, 150/150 mM and 250/200 mM; the hydrogels were left 4h in a fridge for crosslinking. The first experiment performed was a time sweep experiment aiming to evaluate the storage modulus of the hydrogels in the linear range. The test was done using a rough surface to have a better grip of the hydrogel and using the parameters described in section 3.3.

The storage modulus values for all the hydrogels analyzed, obtained from the time-sweep experiments are reported in Figure 4.9. The results, as can be seen in the graph just confirm that when the alginate concentration rises, the storage modulus does it as well and show that a high concentration of crosslinker will impart to the hydrogel a more rigid structure.

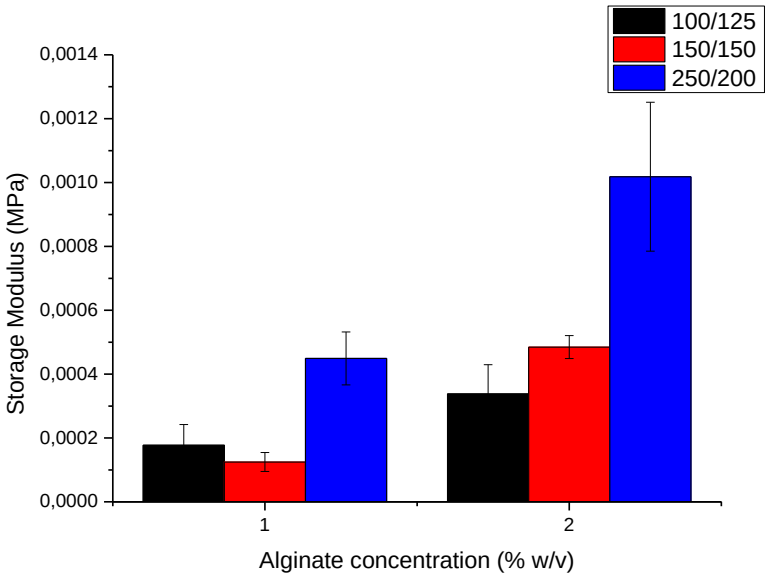


Figure 4.9. Storage modulus of the hydrogels with alginate concentrations 1% and 2 $\%_{w/vol}$ as a function of the concentration of crosslinker and retardant obtained from the time sweep essays.

The second test performed was an amplitude sweep test, the objective of this test is to find the conditions where the hydrogel structure is stable and where it breaks and if it is correlated with the

concentration of alginate or of crosslinker/retardant. In Figure 4.10 is reported an example of the results of an amplitude sweep experiment for one of the samples; the data are reported as storage and loss moduli as a function of the oscillation displacement.

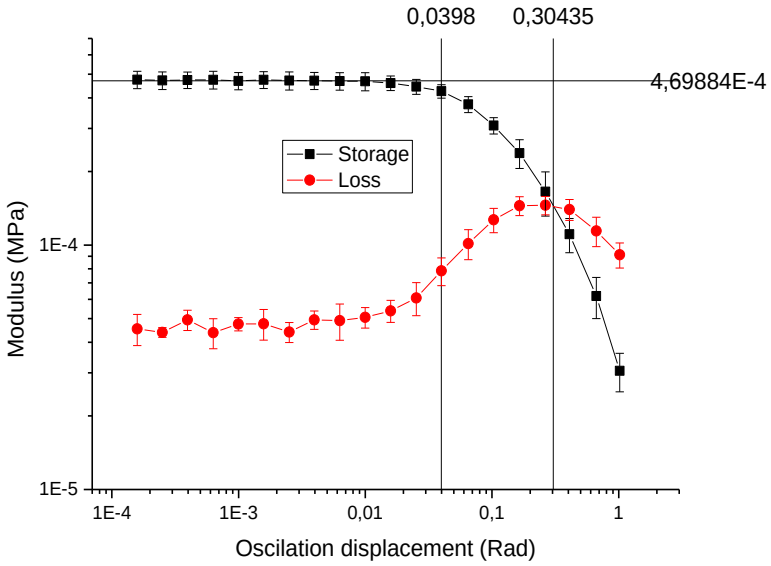


Figure 4.10. the graph shows the amplitude time sweep essay and the values that are obtained from the study, from left to the right the values are: Decay point, crosslinking point and the value at the Hydrogel's linear range.

From this graph we can extract three different information: (i) the value of the storage modulus for low oscillation displacements, that should correspond to the value obtained in the time-sweep experiment; (ii) the angle value at which the storage modulus start deviating from the linear range and (iii) the angle value corresponding to the crossing of the storage and loss moduli. All these parameters are indicated in Figure 4.10 with straight lines and are collected in Table 4.5 for all the samples analyzed.

Table 4.5. Parameters obtained from the amplitude sweep experiments. The procedures to obtain the parameters are explained in Figure 4.x. All the hydrogels described in this section were prepared using the Luer Lock mixing method and the 3D mold.

Sample	Storage modulus in linear range (MPa)	Deviation from linearity (rad)	Crossing point (rad)
Alg_1 %_100/125	$1.3059 \cdot 10^{-4}$	0.1 ± 0.009	0.234 ± 0.0211
Alg _1 % _150/150	$1.192 \cdot 10^{-4}$	0.025 ± 0.0025	0.166 ± 0.0149
Alg _1 % _250/200	$4.542 \cdot 10^{-4}$	0.016 ± 0.0015	0.387 ± 0.0348
Alg_2 %_100/125	$3.19845 \cdot 10^{-4}$	0.08 ± 0.0072	0.414 ± 0.0373
Alg_2 %_150/150	$(4.7 \pm 0.4) \cdot 10^{-4}$	0.040 ± 0.004	0.305 ± 0.0275

Alg_2 %_250/200	$9.9507 \cdot 10^{-4}$	0.018 ± 0.0016	0.156± 0.014

Analyzing the data obtained we can extract some points in the table 4.5, the first conclusion is that the storage modulus grows when the alginate concentration and the crosslinker concentration do it too, that could mean that the hydrogel gets stiffer. Another conclusion can be that the hydrogels with 1% Alginate are stiffer enough for some further applications, and the 2% Alginate hydrogels keep their injectability in a good way so depending on the application both hydrogels could be functionals.

The crossing point, in which the bounds broke does not show any trend in Alginate 1% but it with the alginate 2% seems to arrive on lower oscillation displacements, this could be attributed to the chains flexibility, having more crosslinking points make it less flexible and forcing to break earlier.

4.8 – Morphological characterization of the hydrogels

To complete the characterization of the hydrogels, we decided to analyze some samples with SEM microscopy. First of all, the hydrogels were lyophilized (Fig. 4.11a) and then a conductive coating made of graphite was deposited on them (Fig. 4.11b).

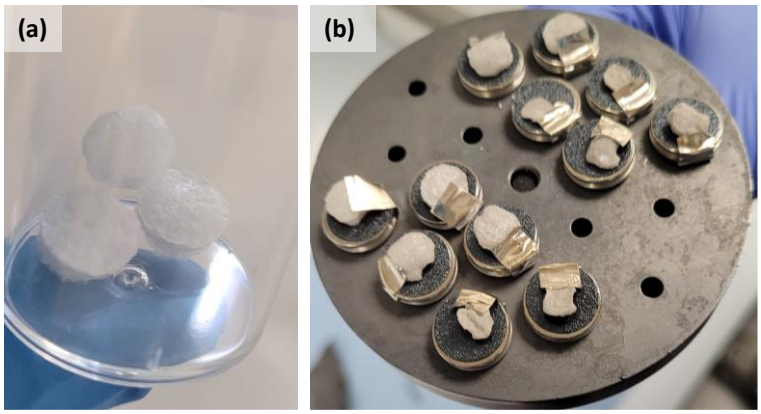


Figure 4.11. Image of the alginate hydrogels after lyophilization (a) and after fixing them in a SEM holder and covering them with conductive carbon layer (b).

Finally, some SEM pictures were taken to investigate the surface structure of each hydrogel on the surface and study the porosity in each concentration. As expected, the samples show high variability, and it is hard to draw conclusions from them. For future experiments it would be recommended to perform cross-section views of the hydrogels.

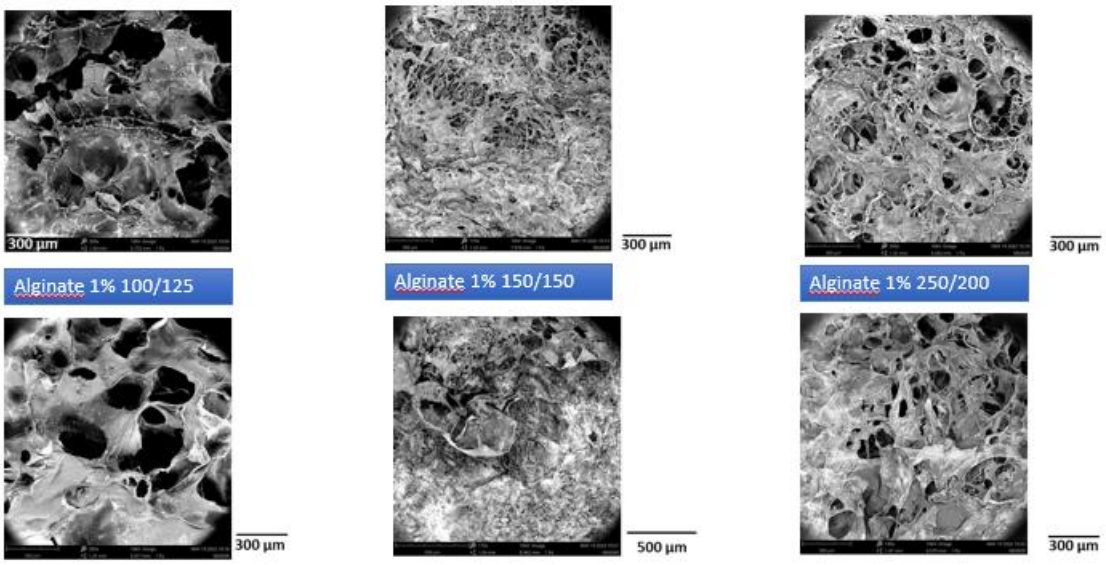


Figure 4.12. From left to right, the SEM images with the concentration of crosslinker/retardant concentration (in mM) for alginate 1% w/w% in which the porous structure can be observed in all cases, with important differences among samples but no clear trends.

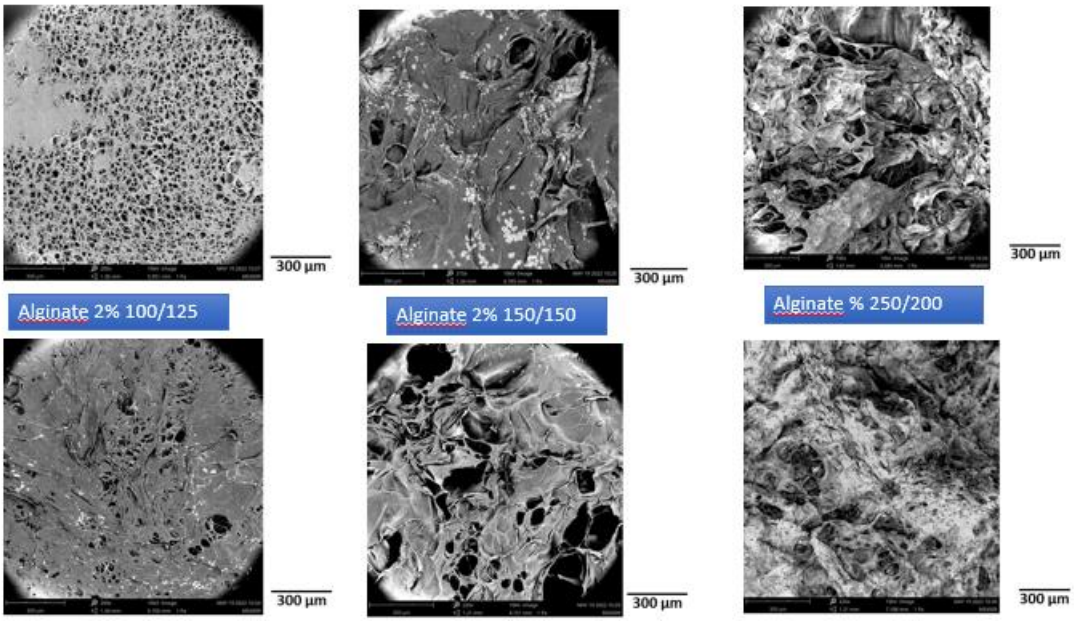


Figure 4.13. From left to right, the SEM images with the concentration of crosslinker/retardant mM for alginate 2% w/w% in which can be observed the reduction of the size that the pores have.

SEM showed that for a low concentration from alginate and crosslinking with retardant, the pores on the microstructure were bigger, showing an inverse proportion behavior, that permitted us to speculate that maybe the higher release of the low concentrations could due to the bigger pores in the microstructure.

5. Conclusions

Making an overview about the results obtained in this work, the alginate hydrogels may be a good option for an implant loaded with RONS to treat cancer. One of the good points of this kind of hydrogel is that with a concentration of 2 $\%_{w/vol}$ or 1 $\%_{w/vol}$ like the used for this work, we can obtain an implant that has a good manipulability and a great injectability because of the fast crosslinking, this may be helpful for instance in a surgery because it can be prepared and injected to the patient at the moment without waiting too long for the crosslinking.

Comparing the methods of mixing, clearly the Luer Lock syringe system works better, due to higher homogeneity obtained in the samples that leads to the longer τ_2 time, this could be associated with a denser crosslinker that, once again is thank to the mixing method.

Comparing now the results between the hydrogels crosslinked in the 3D-printed mold using standard solutions with the hydrogels treated with plasma, the τ_2 values are close, but in terms of moles released standard solutions show better results due to a higher loading. So, the method that would work better could be the 3D mold and the hydrogels that are crosslinked with standard solutions because the τ_2 is the highest, and the released mols are more compared with the two other types as explained before. Finally, and with all the results compared above the concentration of Ca^{2+} solution that worked better would be 250 mM and 200 mM for the Na_2HPO_4 as retardant, the best mixing method is by far the Luer Lock system due to the homogeneity that gives to the hydrogels in shape, weight and amount of RONS, but these RONS are generated by standard solutions.

The time sweep rheology study showed the trend that at higher Ca^{2+} solutions the storage modulus value increased, that can be related with the crosslinking density that makes the hydrogel more rigid. The amplitude sweep showed a growth comparing the alginate 1% with the alginate 2%, the crossing point results are not as good as expected but in the case of alginate 2% that has a clear trend showed that when the concentration of crosslinker rises the crossing point arrives at lower oscillation displacements (rad).

The results in SEM showed a reduction of the pores' size while the concentration of Ca^{2+} solution rises, but the surfaces studied didn't have a clear trend so one of the conclusions are that some cross sections studies in SEM should have been done too in order to understand if the whole structure of the hydrogel has the same pores or it changes in order to obtain a clear result.

To conclude, alginate hydrogels may be a serious good option for future implants for release of reactive species generated by plasma, thanks to its biocompatibility, its relative stiffness, their loading and releasing capacity and its manipulability. Manufactured in proper way and in industrial scale can be cheap and easy. Its release properties are great and with the optimal concentrations, the release could

last long enough to help the patient to clean cancer cells in the implant region accomplishing the aim of the work of finding a vehicle to carry RONS that are going to be released in a tumoral region for killing them healing the patient.

SEM and Rheology essays helped us to complement the information of the release, but in the case of the SEM a cross section essay would be helpful to understand why and how the release of a hydrogel happens in that way. Following the last explanation, rheology helped us to know the viscoelastic properties, and thank to that if the hydrogels are manipulable enough or not in a real environment. In general terms.

Bibliography

Bajpai, S., & Sharma, S. (2004). Investigation of swelling/degradation behaviour of alginate beads crosslinked with Ca2+ and Ba2+ ions. *Reactive and Functional Polymers*, *59*(2), 129–140. https://doi.org/10.1016/j.reactfunctpolym.2004.01.002

Bekeschus, S., Favia, P., Robert, E., & von Woedtke, T. (2018). White paper on plasma for medicine and hygiene: Future in plasma health sciences. *Plasma Processes and Polymers*, *16*(1), 1800033. https://doi.org/10.1002/ppap.201800033

Bonnefoy.E, how gelatin-based composite hydrogels influence the stability and release of reactive species generated in biopolymer solutions by non-thermal plasma treatment, TFM, UPC-EEBE, 2022

Crini, G. (2019). Historical review on chitin and chitosan biopolymers. *Environmental Chemistry Letters*, *17*(4), 1623–1643. https://doi.org/10.1007/s10311-019-00901-0

Cvelbar, U., Walsh, J. L., ČErnák, M., de Vries, H. W., Reuter, S., Belmonte, T., Corbella, C., Miron, C., Hojnik, N., Jurov, A., Puliyalil, H., Gorjanc, M., Portal, S., Laurita, R., Colombo, V., Schäfer, J., Nikiforov, A., Modic, M., Kylian, O., . . . Weltmann, K. (2018). White paper on the future of plasma science and technology in plastics and textiles. *Plasma Processes and Polymers*, *16*(1), 1700228. https://doi.org/10.1002/ppap.201700228

Ferlay, J., Colombet, M., Soerjomataram, I., Parkin, D. M., Piñeros, M., Znaor, A., & Bray, F. (2021). Cancer statistics for the year 2020: An overview. *International Journal of Cancer*. https://doi.org/10.1002/ijc.33588

Guo, T. X., Zhao, Y., Ma, S. C., & Liu, S. T. (2012). Decomposition Characteristics of Hydrogen Peroxide in Sodium Hydroxide Solution. *Advanced Materials Research*, *610-613*, 359–362. https://doi.org/10.4028/www.scientific.net/amr.610-613.359

Hofmarcher, T., Lindgren, P., Wilking, N., & Jönsson, B. (2020). The cost of cancer in Europe 2018. *European Journal of Cancer*, *129*, 41–49. https://doi.org/10.1016/j.ejca.2020.01.011

Jejurikar, A., Lawrie, G., Martin, D., & Grøndahl, L. (2011). A novel strategy for preparing mechanically robust ionically cross-linked alginate hydrogels. *Biomedical Materials*, *6*(2), 025010. https://doi.org/10.1088/1748-6041/6/2/025010

Keidar, M., Shashurin, A., Volotskova, O., Ann Stepp, M., Srinivasan, P., Sandler, A., & Trink, B. (2013). Cold atmospheric plasma in cancer therapy. *Physics of Plasmas*, *20*(5), 057101. https://doi.org/10.1063/1.4801516

Khlyustova, A., Labay, C., Machala, Z., Ginebra, M. P., & Canal, C. (2019). Important parameters in plasma jets for the production of RONS in liquids for plasma medicine: A brief review. *Frontiers*

of Chemical Science and Engineering, *13*(2), 238–252. https://doi.org/10.1007/s11705-019-1801-8

Kuo, C. K., & Ma, P. X. (2001). Ionically crosslinked alginate hydrogels as scaffolds for tissue engineering: Part 1. Structure, gelation rate and mechanical properties. *Biomaterials*, *22*(6), 511–521. https://doi.org/10.1016/s0142-9612(00)00201-5

Labay, C., Hamouda, I., Tampieri, F., Ginebra, M. P., & Canal, C. (2019). Production of reactive species in alginate hydrogels for cold atmospheric plasma-based therapies. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-52673-w

Labay, C., Roldán, M., Tampieri, F., Stancampiano, A., Bocanegra, P. E., Ginebra, M. P., & Canal, C. (2020). Enhanced Generation of Reactive Species by Cold Plasma in Gelatin Solutions for Selective Cancer Cell Death. *ACS Applied Materials & Interfaces*, *12*(42), 47256–47269. https://doi.org/10.1021/acsami.0c12930

Metal Deposition: Plasma-Based Processes. (2016). *Encyclopedia of Plasma Technology*, 722–740. https://doi.org/10.1081/e-eplt-120053919

Moody, C. T., Palvai, S., & Brudno, Y. (2020). Click cross-linking improves retention and targeting of refillable alginate depots. *Acta Biomaterialia*, *112*, 112–121. https://doi.org/10.1016/j.actbio.2020.05.033

Mørch, R. A., Donati, I., Strand, B. L., & Skjåk-Bræk, G. (2006). Effect of Ca^{2+}, Ba^{2+}, and Sr^{2+} on Alginate Microbeads. *Biomacromolecules*, *7*(5), 1471–1480. https://doi.org/10.1021/bm060010d

Park, H., Kang, S. W., Kim, B. S., Mooney, D. J., & Lee, K. Y. (2009). Shear-reversibly Crosslinked Alginate Hydrogels for Tissue Engineering. *Macromolecular Bioscience*, *9*(9), 895–901. https://doi.org/10.1002/mabi.200800376

Rastogi, P., & Kandasubramanian, B. (2019). Review of alginate-based hydrogel bioprinting for application in tissue engineering. *Biofabrication*, *11*(4), 042001. https://doi.org/10.1088/1758-5090/ab331e

Reuter, S., von Woedtke, T., & Weltmann, K. D. (2018). The kINPen—a review on physics and chemistry of the atmospheric pressure plasma jet and its applications. *Journal of Physics D: Applied Physics*, *51*(23), 233001. https://doi.org/10.1088/1361-6463/aab3ad

Rich, J. N., & Bao, S. (2007). Chemotherapy and Cancer Stem Cells. *Cell Stem Cell*, *1*(4), 353–355. https://doi.org/10.1016/j.stem.2007.09.011

Ritter, J., & Bielack, S. (2010). Osteosarcoma. *Annals of Oncology*, *21*, vii320-vii325. https://doi.org/10.1093/annonc/mdq276

Scheid.L, Optimization of agarose-based composite hydrogels for delivery of reactive species generated in biopolymer solutions by non-thermal plasma treatment, TFM, UPC-EEBE, 2022.

Siddiqui, S. A., Favaro, G., & Berkes Maros, M. (2022). Investigation of the Damage Mechanism of CrN and Diamond-Like Carbon Coatings on Precipitation-Hardened and Duplex-Treated X42Cr13/W Tool Steel by 3D Scratch Testing. *Journal of Materials Engineering and Performance*. https://doi.org/10.1007/s11665-022-06812-6

Solé-Martí, X., Vilella, T., Labay, C., Tampieri, F., Ginebra, M. P., & Canal, C. (2022). Thermosensitive hydrogels to deliver reactive species generated by cold atmospheric plasma: a case study with methylcellulose. *Biomaterials Science*. https://doi.org/10.1039/d2bm00308b

Supramaniam, J., Adnan, R., Mohd Kaus, N. H., & Bushra, R. (2018). Magnetic nanocellulose alginate hydrogel beads as potential drug delivery system. *International Journal of Biological Macromolecules*, *118*, 640–648. https://doi.org/10.1016/j.ijbiomac.2018.06.043

van de Velde, K., & Kiekens, P. (2002). Biopolymers: overview of several properties and consequences on their applications. *Polymer Testing*, *21*(4), 433–442. https://doi.org/10.1016/s0142-9418(01)00107-6

Venkatesan, J., Bhatnagar, I., Manivasagan, P., Kang, K. H., & Kim, S. K. (2015). Alginate composites for bone tissue engineering: A review. *International Journal of Biological Macromolecules*, *72*, 269–281. https://doi.org/10.1016/j.ijbiomac.2014.07.008

Voo, W. P., Ooi, C. W., Islam, A., Tey, B. T., & Chan, E. S. (2016). Calcium alginate hydrogel beads with high stiffness and extended dissolution behaviour. *European Polymer Journal*, *75*, 343–353. https://doi.org/10.1016/j.eurpolymj.2015.12.029

Yadav, P. (2015). Biomedical Biopolymers, their Origin and Evolution in Biomedical Sciences: A Systematic Review. *JOURNAL OF CLINICAL AND DIAGNOSTIC RESEARCH*. https://doi.org/10.7860/jcdr/2015/13907.6565

Environmental Impact

This project has been carried out with alginate, a non-polluting and biocompatible natural polymer, which reduce the possibility of contamination. All the solutions were prepared in pure water or water-based solutions. The salts used for the preparation of standard solutions and buffers are all non-toxic and were used in low concentrations.

Furthermore, inert gas which do not present any risk to the environment have been used during plasma treatment. In addition, a chemical hood was always used when plasma treatment was performed in order to remove any ozone generated during the treatment.

The plastic containers have been reused as much as possible to minimize pollution. In cases where this was not possible, they have been recycled in the appropriate container.

The laboratory establishes a functional waste separation system, depending on whether halogenated or non-halogenated agents, aqueous solutions, acids, bases or oils are involved. The only possible environmental risks are the plates and pipette tips that were in contact with the chemical reagent. Therefore, appropriate safety measures were taken at all times during handling and the remaining traces were properly separated

Cost of the project

In this part, the economic analysis will be done, we decided to divide the analysis in three parts, the first one the cost of the used material for doing the experiments such as tips, well plates and solutions. In second place the cost of getting the results, which means all the machines used to obtain the data like the SEM or the rheometer. In third place the personal cost, considering the salary of the workers related to the work.

Table 1. Cost of the material used during the work

Product	Quantity	Price	Costs (€)
12 - well plate	4	188 € / 75 plates	10.03
24 - well plate	8	162€ / 75 plates	17.28
48 - well plate	10	191€/75 plates	25.47
96 – well plate	12	159€/50 plates	38.16
Tips 200 µL	800	59.28€/960 tips	49.4
Tips 1000 µL	300	54.17€/960 tips	16.97
Tips 5000 µL	100	66.17€/196 tips	33.76
Helium	500 L	250€/4000 L	31.25
Synthetic air	5 L	250€/4000 L	0.32
Distilled water	7 L	15€ /25 L	4.2
Alginate	20 g	118 € / 1 Kg	2.36
Sodium dihydrogen phosphate dihydrate	10 g	66.50€/500 g	1.33
Titanium Oxysulfate (IV)	80 mL	56.50€ /500 mL	9.04
Sodium nitrite	10 g	92€/25 g	36.8
Total Price			276.37 €

Table 2. Cost of the machinery used for obtaining the data

Equipment	Time used (h)	Cost/ hour	Cost
Rheometer	6	100	600
SEM	6	100	600
Total Price			1200 €

Table 3. Personal Cost of the work.

Staff	Hours of work (h)	Price (€/h)	Cost (€)
Director	15	60	900
Co-director	350	42	14700
Thecnical team	2	32.97	65.94
Degree Student	450	20	9000
Total Price			25466

Table 4. Total amount of cost for the project

Section	Price
Material	276.37
Machinery	2400
Staff	24666
Total Cost	27342.31 €