Magnetized Fibers for Cancer Therapy

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Dissertação orientada pela Professora Doutora Maria Henriques Lourenço Ribeiro

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Summary:

Colon cancer is the third most common cancer worldwide and the fourth most common cause of death. It affects men and women almost equally. Due to its invasive nature and evidenced poor patient adherence to chemotherapy post-resection, there is a need for alternative adjunct colon tumor therapies, especially ones which allow controlling tumor growth/recurrence.

The main goal of this thesis work was to develop a nanofibrous system for treating phase III colon cancer patients. Research was directed for creating a containment system which could enclosure metastatic cells within the tumor mass region, slow down tissue's ECM remodelling and eliminate any altered cells in the system's vicinity. The system envisaged, PVA electrospun fibers, loaded with lysozyme, cross-linked with fluorophenylboronic acid and functionalised with magnetic nanobeads, to be applied pre- and post-surgery on the tumor and ablation site respectively, since it will promote both tumor shrinkage, metastatic cells containment and cancer cells death.

The system was successfully built and tested, and the following conclusions were reached:

a) Lysozyme activity was dependent of its concentration following a quadratic trend;
b) Using electrospinning, fibers containing Lysozyme encapsulated were produced;
c) Lysozyme was successfully encapsulated in lenses and fibers, and could be released gradually to the buffer;
d) Lysozyme release from the systems tested was independent of the pH, although minor variations in the initial release can be explained by PVA degradation;
e) Lysozyme encapsulated both in the lens and fibers was able not only to retain its activity, but this was also enhanced and was independent on the type of modification induced to the system;
f) Fibers containing lysozyme encapsulated, cross-linked with fluorophenylboronic acid and/or with IONPs adsorbed, were able to reduce viability of Caco-2 tumor cells seeded on them.

Keywords: Electrospinning, Colon Cancer, PVA, FluorophenylBoronic Acid, Lysozyme
Resumo:

O cancro do cólon é o terceiro tipo de cancro mais comum em todo o mundo, e a quarta causa de morte mais comum. Afeta de igual maneira ambos os sexos. Devido à sua natureza invasiva e à existência de casos de não aderência à quimioterapia pós-rescisão, torna-se necessário desenvolver novas terapias adjuvantes para o cancro do cólon, especialmente se conseguirem permitir o controlo do crescimento/recorrência do tumor.

A interacção entre as células e a matriz extracelular pode despoletar comportamentos celulares importantes, como a proliferação e a diferenciação, e respostas tecidulares como a morfogênese, homeostase e regeneração. No entanto, foi também demonstrado que a arquitetura e remodelação da matriz extracelular tem um papel importante na promoção da tumorigénese, influência na migração celular, e capacidade de invasão tecidual e metastização.

A lisozima humana é uma proteína sintetizada e secretada pelos granulócitos e monócitos do organismo humano para grande parte dos fluidos corporais. Ensaios clínicos com a proteína simples demonstraram que esta potencial actividade anti-tumoral, evidenciada pela associada diminuição da massa tumoral e pelo aumento da esperança média de vida em 20-30% em doentes tratados.

Está descrito que a actividade anti-tumoral da lisozima está dependente da activação do sistema imunitário, quer directamente por receptores específicos, quer indirectamente pela degradação de peptidoglicanos bacterianos capazes de posteriormente activar as células imunitárias. Também estão descritos potenciais efeitos tumorigénicos directos da lisozima, possivelmente dissociados da sua actividade catalítica.

Ao nível da matriz extracelular, a lisozima é ainda capaz de se ligar especificamente à elastina presente em tecidos danificados e proteger-la da degradação por elastases, diminuindo por isso o potencial de remodelação da matrix. Dado que a remodelação da matriz extracelular é uma condição imperativa para ocorrer a metastatização de um tumor, a inibição local deste processo pode retardar, em teoria, o surgimento de metástases e forçar as células tumorais a um crescimento mais controlado.

O principal objectivo desde trabalho foi desenvolver um sistema nanofibroso para o tratamento de doentes com cancro do cólon em fase III. A pesquisa foi direccionada para a criação de um sistema de contenção, que confinaria as possíveis células metastáticas na zona do tumor; abrandaria a remodelação da matriz extracelular e eliminaria as células tumorais que se encontrassem na vicinidade desse sistema. As nanofibras idealizadas, compostas por PVA e obtidas por electrospinning, com lisozima encapsulada, reticuladas com ácido fenilfluoroborónico e esferas magnéticas à superfície, podem ser aplicadas antes ou depois da cirurgia ao tumor e ao local de ablação respectivamente dado que permitirão a redução do tamanho do tumor, retenção de células metastásicas e morte das células cancerosas.
O sistema de interesse conseguiu ser produzido com sucesso. No final do trabalho determinou-se que a actividade da lisozima, determinada pela velocidade de degradação de uma suspensão de extrato de *Micrococcus Lysodeikticus* a 3mg/mL, estava dependente da concentração da primeira, e estas correlacionam-se por uma função quadrática. Em simultâneo foi adaptado o método de Bradford para a determinação de concentrações proteicas em microplaca, permitindo fazer uso de pequenas quantidades de amostra.

A lisozima foi encapsulada com sucesso em lentes de PVA, as quais foram utilizadas como modelo de estudo de modificações ao PVA (comparação do efeito da reticulação com ácido fenilborónico e do ácido fluorofenilborónico e da presença de nanopartículas magnéticas na libertação da lisozima das lentes de PVA) a aplicar nas fibras, e como modelos de previsão de libertação da lisozima a valores de pH diferentes.

O racional por detrás da libertação da lisozima a valores de pH diferentes prende-se com o facto de que o pH do microambiente tumoral, devido também à elevada actividade metabólica das células, é mais baixo do que o fisiológico. Dado que tanto a taxa de degradação do PVA como a actividade da lisozima são influenciados pelo pH, escolheu-se fazer ensaios de libertação a valores de pH ligeiramente mais ácidos e cujos valores estão de acordo com a literatura (tâmpões fosfato a 6.8, 7.0 7.2 e 7.4, com consequente avaliação da massa de lisozima libertada e da respectiva actividade). É interessante referir que todos os perfis de libertação da lisozima para as fibras a vários valores de pH e/ou com modificações diferentes não são diferentes estatisticamente, apesar de se observarem ligeiras diferenças na libertação inicial da enzima. Pensa-se que as nanopartículas magnéticas poderão auxiliar na libertação da proteína através de interacções electrostáticas, promovendo a movimentação destas para a superfície do sistema através da sua carga negativa.

Por forma a comparar as concentrações obtidas pelo método de Bradford com as dos ensaios de actividade, foram calculadas variações relativas ao primeiro. Curiosamente observou-se um aumento geral da mesma, o que significa que a lisozima encapsulada não só não perde actividade significativamente, mesmo após o processo de reticulação, como a sua actividade catalítica está potenciada. Tal significa que o processo de encapsulação e secagem não têm efeito na conformação da proteína.

Utilizando a técnica de electrospinning foi possível encapsular lisozima em fibras de PVA. Para tal fez-se uso de um coletor rotativo de 3.4cm de diâmetro e de condições específicas de produção (17.4kV, 8cm de distância da agulha ao colector, 21ºC, colector a 150rpm).

As fibras foram posteriormente analisadas por microscopia óptica e microscopia electrónica que evidenciaram, com graus diferentes de precisão, a presença de fibras e com características diferentes. De facto, a imobilização de lisozima nas fibras não produziu efeitos visíveis nas fibras, enquanto que a reticulação com ácido fluorofenilborónico diminuiu a porosidade e aumentou a resistência das fibras, e a adsorção de nanopartículas magnéticas provocou o aparecimento de agregados de nanopartículas por toda a fibra. Foi ainda testado o efeito do tamanho do coletor na
morfologia das fibras, e chegou-se à conclusão que quanto maior o seu diâmetro, mais direitas são as fibras obtidas e mais poroso é o tecido produzido.

Foi também possível obter libertação controlada da lisozima tanto a partir das fibras, independentemente da modificação introduzida ao sistema. Também de forma similar ao que aconteceu nas lentes, os perfis de libertação da lisozima a diferentes valores de pH eram estatisticamente semelhantes e as variações das concentrações calculadas pelo método de Bradford versus as concentrações obtidas pela curva de calibração da actividade da lisozima apontaram também para uma potenciação da actividade da lisozima encapsulada.

As fibras contendo lisozima encapsulada, reticuladas com ácido fluorofenilborónico e com nanopartículas magnéticas adsorvidas (IONPs) foram capazes de diminuir a viabilidade de células Caco-2 (linha tumoral de epitélio do cólon) cultivadas em cima das primeiras. Através do ensaio de MTT, foi possível determinar que a viabilidade das células Caco-2 estava bastante diminuída. Estes resultados foram posteriormente confirmados pela coloração de Faloidina-DAPI, que evidenciou a quase ausência de células vivas ou viáveis a crescer sobre as fibras. De facto, a presença de pontos azuis brilhantes irregulares fragmentados aponta para que as células tenham morrido por apoptose.

Este trabalho deixa portas abertas a outros futuros trabalhos associados, já que nem todas as questões científicas puderam ser respondidas. Por exemplo, a interferência do SDS na determinação da lisozima adsorvida nas lentes/fibras poderia ser contornada através da quantificação da lisozima por HPLC. Para além disso, já que o sistema será sujeito a temperaturas locais relativamente elevadas derivadas da hipertermia induzida às nanopartículas magnéticas (40-60°C), deve futuramente ser estudada a influência da temperatura na libertação da lisozima do nosso sistema. Finalmente, a cinética do crescimento celular e o tipo de morte celular deverão ser ainda avaliados para as células semeadas nas fibras.

**Palavras-Chave:** Electrospinning, Cancro do Cólon, PVA, Ácido Fluorofenilborónico, Lisozima.
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“I can not go back to yesterday because I was a different person then” (Lewis Carol in Alice in Wonderland)

It has been nearly 4 years since I finished writing my last Master Degree thesis to finish the course of Pharmaceutical Sciences. In that thesis I thanked everybody that supported me along the process, including those who I would only meet in the future and that would bring success with them. Four years later I can confirm those acknowledgements were worthy.

Starting with my job at Centro de Apoio Social do Pisão, followed by a number of unsuccessful applications to scholarships in various institutes, namely to Fundação para a Ciência e Tecnologia, and by countless sleepless nights dreaming upon a better future, I completed the biggest achievement so far in my life: to conclude this Master Course! To these institutions, and persons from them, who indirectly helped me to grow as a person and as an aspiring researcher, I thank them!

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BSA – Bovine Serum Albumine
CAM – Cell Adhesion Molecules
CS - Chitosan
DMEM - Dulbecco's modified Eagle's medium
DSC - Differential Scanning Calorimetry
ECM - ExtraCellular Matrix
EDTA - Ethylenediaminetetraacetic Acid
EFSA - European Food Safety Authority
EGF - Epidermal Growth Factor
FGF - Fibroblast Growth Factor
FTIR - Fourier Transform Infrared Spectroscopy
IONP - Iron Oxide NanoParticle
LD50 - Lethal Dose for 50% of the studied animals
LDH - Lactate DeHydrogenase
MAPK - Mitogen Activated Protein Kinase
min - Minutes
MTT - (3-(4,5-dimethylthiazol-2-yl)-2,5-DphenylTetrazolium Bromide)
NOAEL - No Observed Adverse Effect Level
Osc/min – Oscilations per minute
PVA - Poly(vinyl alcohol)
SEM - Scanning Electronic Microscopy
TACS - Tumor Associated Collagen Signatures
TGFβ - Transforming Growth Factor β
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Introduction
I - Introduction

I.1. Extracellular matrix (ECM)

It was verified by many studies that the ECM plays a major role in modulating *in vivo* cellular proliferation, differentiation and metabolism (Dvir T 2010). One key aspect in tissue engineering is to recreate this structure, so that cells can adopt the morphological and physiological features required for the construction of the biomimetic system envisaged. Therefore, understanding the anatomy and biology of the ECM *in vivo* is vital.

The ECM is a natural meshwork of diverse insoluble fibbers and fibrils, assembled in diverse structures depending on the tissue and its composition (Hynes R 2014), which include (Shekaran A 2011) (1) Structural Proteins, such as collagens, elastin and laminin; (2) Glycoproteins, such as fibronectin and vitronectin; (3) Glycosaminoglycans, such as chondroitin sulphate. Among this cell-knitted web several soluble factors, such as growth factors, cytokines and chemokines; and cell-cell receptors, such as cadherins, CAMs and ephrins, can also be found.

ECM's composition, immobilization and spatial arrangement varies for each type of tissue or organ, depending on its function. For example, while the bone is richer in collagen, hydroxylapatite and non-collagenous proteins such as osteocalcin, fibronectin and vitronectin, the cartilage is mainly composed of collagen II and chondroitin sulfate proteoglycan (Shekaran A 2011).

Engineering functional tissue requires that cells organize in with morphological and physiological features resembling those *in vivo* in a desired scaffold. When growing, cells tend to differentiate and migrate according to the topography, mechanical properties (matrix stiffness, elasticity and viscosity) and concentration gradients of immobilized soluble factors of the ECM. This was showed by Ott and co-workers (2008) who observed that cardiomyocytes and epitheliocytes were able to migrate and self-organize in their natural location when seeded on a cardiac ECM structure previously decellularized and under physiological conditions.

Although apparently static and lifeless, the ECM is in fact a dynamic and complex cell communication platform that regulates both cell survival, growth, migration and differentiation through activation of different cell signalling pathways, mediated by integrin receptors (Shekaran A 2011).

Integrins are a family of transmembrane receptors which bind to several types of ECM molecules, allowing cells to anchor to them. Upon binding to the extracellular domain, ECM makes intregrins to cluster and associate with both cytoskeletal and intracellular signalling proteins, allowing the initiation of various downstream signalling cascades such as protein kinase C, Ras and MAPK pathways. On the other way, cellular intracellular signalling can also affect the conformation of integrin molecules, modulating the affinity to ECM molecules (Shekaran A 2011).
Integrins bind to precise short peptide sequences within the ECM's proteins' backbones. These domains can be found mainly in ECM's structural proteins, such as in collagen I, II and III (GFOGER peptide sequence), fibronectin and laminin (Shekaran A 2011). Because the interactions between integrins and these sequences can trigger crucial cell behaviours, such as proliferation and differentiation, and tissue-level responses such as morphogenesis, homeostasis and regeneration (Shekaran A 2011). However, it has also been shown that ECM architecture and remodelling plays a pivotal role in promoting tumorigenesis, influencing cell migration, invasion and metastasis (Malik R 2015).

**I.2. Cancer (overview)**

Cancer is a multietiologic disease associated with the over-proliferation of altered cells within the human body, either forming a mass (tumor) or not, and consequent invasion of other tissues, leading to the formation of secondary tumors (metastization) (National Cancer Institute 2015). There are six distinctive and complementary characteristic of a tumor that enable its growth and dissemination: (1) Sustaining proliferative growth, (2) Evading growth suppressors, (3) Resisting cell death, (4) Enabling replicative immortality, (5) Inducing angiogenesis, and (6) Activating invasion and metastasis. Additionally, due to recent research work, two extra emerging hallmarks may also be considered: (7) Deregulating cellular energetics and (8) Avoiding immune destruction. In order to facilitate the acquirement of these hallmarks, both genomic instability, leading to mutations and genetic alterations, and tumor-promoting inflammation, which promotes cell survival and play a critical role (Hanahan D 2011).

Many studies have shown that during fibrosis and epithelial tumor progression the ECM's collagen fibbers are rearranged from a loose, random and isotropic conformation to an organized/aligned, anisotropic and stiffer one, named "tumor-associated collagen signatures" (TACS). This leads not only to the creation of the cancer's pathological microenvironment (desmoplasia) but also to natural trails which cancer cells can use to migrate and metastasize (Malik R 2015). In fact, properties of invasive cancers also include decreased cell-cell adhesion, cytoskeletal remodelling, increased motility, increased ECM's proteases, and synthesis of new ECM components (Krishnamachary B 2003).

**I.2.A. ECM remodelling in cancer**

The ECM turn-over is performed mainly by activated stromal cells, i.e. fibroblasts, which react to the mechanical pressure, induced directly by the growing tumor, and to growth factors secreted by the cancer cells, such as Transforming Growth Factor β (TGF-β). This signals lead to increased integrin-ECM interactions, which through activation of the Rho-Rock pathway promotes cytoskeletal reorganization and cell motility (Malik R
TGF-β signaling also promotes excessive collagen deposition and fibrosis (Rahimi R 2007).

An example of this occurs in pancreatic cancer, the fourth leading cause of cancer related death in developed countries, with an overall survival rate of about 5-6%. Since the clinical symptoms are vague, patients are often diagnosed with already established regional invasion or distant metastasis. In fact, the best treatments available can only prolong life by 8-16 weeks. Because pancreatic cancer is characterized by a highly fibrotic phenotype, causing local hypoxia, the cancer cells undergo "Epithelial-Mesenchimal transition", turning them more resistant to chemotherapy (Xu Z 2014, McCarroll J 2014, Dangi-Garimella S 2012).

This remodelling process is also seen in colon adenocarcinome, the major cause of cancer mortality worldwide (Schetter AJ 2008). Stromal cells present in a colon tumor (myofibroblasts) have been known for interacting with tumor cells and consequently induce remodeling of ECM, through production of lytic enzymes and production of abnormal ECM components. Ultimately, these cells promote invasion and metastasis in colon cancer (Martin M 1996). In fact, a recent study on phase II/III colon cancer patients has shown that the ratio of stromal cells present in the tumor are an important prognostic factor to disease progression (Huijbers A 2013).

I.3. Lysozyme - anti-tumoral agent

Lysozyme is a highly cationic mucolytic enzyme present in nearly all living beings and it is synthesized and secreted by granulocytes and monocytes during degranulation and phagocytosis (Ralph P 1976). As a result lysozyme can be found in body fluids and tissues, especially in those who suffered injury (Park P 1996). This enzyme is capable of hydrolysing the peptoglycan of bacterial cell walls, acting as a defence mechanism against bacteria (Park P 1996).

In the past, lysozyme was studied as a possible intravenous administered anti-cancer agent. Sava G and colleagues (1989) reviewed this subject, and overall found that lysozyme is able to reduce the mass of a tumor and extend survival time in several different animal tumor. And although the degree of anti-tumor effect was dependent on the tumor type, remission was observed even in every kind of cancer, including lymphoproliferative and metastasizing types (Sava G 1989).

According to Sava G and colleagues, in vitro assays demonstrated that lysozyme’s anti-tumoral mechanism of action is more associated with its ability to activate the immune system, either through the activation of specific receptors in macrophages and lymphocytes (direct pathway) or through the production of bacterial-wall antigens, which can even be immunostimulatory when lysozyme is administered by the oral route.

In animal models for cancer, and even those whose tumor growth was not decelerated, survival time was also found improved by 20-30%. This was hypothesized to be related
to additional actions such as (1) Increased specific and unspecific host responses against pathogenic infections, (2) Control of allergenic and anaphylactogenic reactions associated with tumor masses, (3) Control of inflammatory diseases, (4) Control of haemorrhagic episodes associated with malignant tumors, and (5) control of pain (Sava G 1989).

In more recent studies, lysozyme is shown to possess direct anti-tumoral effects. Using a self-assembled nanostructured hen egg white lysozyme Mahanta S and colleagues were able to induce 95% cell death in 24h on MCF-7 breast cancer cells, mainly by inducing oxidative stress. The spherical nanosystem, consisting on partly folded monomeric lysozyme crosslinked with glutaraldehyde and functionalised with folic acid, were found to be stable at pH 7.4 and resistant to proteinase-K degradation. However, due to its preparation process, lysozyme was found to have lost most of its biological activity, meaning cell death was not dependent on its enzymatic activity (Mahanta S 2015). Though the authors did not studied the cell death process in a more detailed manner, the cytotoxic effect of the nanosystem is not completely elucidated; in fact, protein aggregates, such as in Huntington's disease, are able to generate oxidative stress in cells and ultimately leading to cell death (Kaplan A 2012). Until these results are confirmed and/or further studied, focus should be given to monomeric lysozyme if anti-tumoral effect is to be considered.

I.3.A. Lysozyme - interaction with the ECM

Elastin, due to its extensive cross-linking and hydrophobic properties, is considered to be one of the most stable proteins known under physiological conditions. They are sensitive to elastases and metallo- and cystein-proteases, being the last ones present in the interstitium and secreted by inflammatory-cells. Lysozyme associates with elastin in a tissue-injury-degree dependent way, possibly caused by the increased deposition of abnormal elastic fibbers (Park P 1996). The reason why this happens is still unknown, but the collocalization of lysozyme on elastic fibbers may be a reliable marker for tissue damage.

The reason why elastin is unique is because its' synthesis occurs mainly during the fetal and post-natal period. In fact, the fibbers present in the aorta artery have an half-life of approximately 40 years, and no appreciable synthesis was ever observed in adulthood (Martyn CN 1997, Larroque-Cardoso P 2014). Lysozyme was shown to specifically bind reversibly to insoluble elastin. However, this enzyme actually protected elastin from degradation by elastase and other serine-, cystein and metalloproteases. The authors refer that high concentrations of lysozyme are required for full inhibition of elastin degradation to be observed; this is explained by the fact that lysozyme, a positively charged protein, interacts with the wide-distributed anionic domains in elastin, blocking access of elastase, another cationic protein, to its substrate (Park P 1996). However, this also raised the problem of toxic doses and its effects which might compromise its application in clinical therapy (Park P 1996). However, previous studies have shown that lysozyme is a well-
tolerated protein, with high doses as 2.500mg/Kg/day not being associated with local or systemic effects on mice or humans (Sava G 1989).

Lysozyme binding to elastin is not always beneficial. When tissue damage occurs, lysozyme is considered responsible for incorrect elastin-fibbers deposition, compromising normal elastic fiber functions (Parker P 1996). Considering the possible applications of lysozyme in cancer therapy, this side-effect might be used to inhibit tumor growth and metastization through ECM stabilization to degradation. In fact, in a review by Cantor J and Shteyngart B (2015) it is hypothesized that lysozylmes's binding to elastin might disrupt its interaction with hialuronic acid, reducing water content and increasing stiffen of ECM in lung and ultimately promoting chronic obstructive pulmonary disease progression (Cantor J 2015), which supports its possible application in cancer therapy.

I.4. Delivering therapeutic agents to cancer

Artificial ECMs are usually the start-point for designing these delivery systems. This should be done so as to provide both mechanical and environmental support (serve as an adhesive substrate, provide structure, store and present growth factors) while the complete tissue is not structurally stable. Ultimately they should also guide cell attachment and immobilization (Kim BS 2011).

The specific design of an artificial ECM is mainly dependent on the material and biological requirements of the used cells, in order to mimic faithfully their natural environment in the organs. Therefore, artificial ECMs, even when cancer applications are considered, should (1) be biocompatible and non-immunogenic, (2) have the required bioactivity, (3) have an appropriate degradation rate, surface properties, processability, porosity and mechanical properties, and (4) allow access to nutrients and growth factors (Kim BS 2011).

I.5. Artificial ECMs production method - electrospinning

Nano and micro materials have unique characteristics, such as higher area/mass ratio and higher reactivity among others, which are very different properties of the same material in a higher scale (Zhang L 2008). These advantageous properties of nanomaterials have been exploited in controlled release of drugs, site specific targeting of biomolecules for different diseases treatments, as cancer and thrombosis (Emerich DF 2007).

Presently the production of PVA for biomedical applications is being made by the inexpensive method electrospinning. This technique allows obtaining very thin and continuous polymer fibbers in the range of nanometres, with large surface areas and porosity, ease of functionalization and superior mechanical properties. It can both be
applied to natural and synthetic materials, although process parameters must be adjusted to different polymers and systems used (Rogina A 2014).

In electrospinning the monomers of the polymer are dissolved in an appropriate solvent. The solution is then pumped through a tube system, which ends on a thin nozzle wide enough to allow a single droplet to form. To that droplet an electric field of about 10-30kV is applied (Rogina A 2014).

The basic electrospinning device (Figure 1) has two electrodes, positioned in the nozzle and in the collector. When the applied voltage (repulsive electrostatic force) is strong enough to overcome the superficial tension of the solution (cohesive force), the droplet starts deforming in a cone-like shape (Taylor's cone). From the cone, a single jet of the solution begin to form and grows uniaxially in a continuous manner.

As the jet migrates, due to rapid whipping, the solvent evaporates and the monomer is forced to polymerize, forming solid fibbers (Greiner A 2007) which migrate to the collector (distance of 10 - 20 cm) (Rogina A 2014).

The optimization of the electrospinning process material is dependent of many parameters (Koski A 2004, Song WK 2005 ). Briefly, these can be divided in 3 groups (Rogina A 2014):

1. Solution parameters (viscosity, concentration, molecular weight, surface tension, conductivity, dipole moment, dielectric strength);
2. Processing parameters (feed flow rate, electrical field strength, needle-to-collector distance, needle nozzle shape, type of collector)
3. Ambient parameters (Temperature, humidity, air flow)

Each one of these parameters must be optimized for a specific polymer/co-polymer, in order to optimize the obtained fibbers. This is well perceived in various studies, namely
by Jia YT and colleagues (Jia YT 2007) who optimized the production of a PVA/Chitosan co-polymer, and Islam MS and colleagues who worked with the co-polymer PVA/Alginate (Islam MS 2010).

The methods used were (1) Scanning Electronic Microscopy (SEM), which allowed to analyse fibbers' diameter and porosity; (2) Fourier Transform Infrared (FTIR), (3) X-ray diffraction and (4) Differential Scanning Calorimetry (DSC), all of which allowed to measure the strength of the chemical interaction between the two polymers used (Jia YT 2007); and (5) Stress-strain measurements through determination of the tensile strength and elongation at break, which allowed to measure the fibber's mechanical properties (Islam MS 2010).

Some interesting features observed by Jia YT and colleagues (2007) were the existence of a minimal concentration of monomers, below which beads were formed instead of fibbers, a maximal concentration of monomers, above which fibbers could no longer form, and a direct correlation between the concentration of monomers and fibbers' diameter and uniformity.

I.6. Materials for nanofibbers design

As far as their structural components are concerned, artificial ECMs can be classified in two groups (Kim BS 2011):

- Natural polymer-based ECMs: consist of structural and functional proteins, proteoglycans, glycoproteins and glycosaminoglycans found in natural tissues, and therefore have excellent biological properties, due to the presence of natural cell-binding motifs, mechanical properties similar to those of natural ones, biodegradability and biocompatibility. However, antigenicity, instability and complexity of purification are still drawbacks to their safe application.

- Synthetic polymer-based ECMs: Fabricated from biocompatible and biodegradable polymers, they can be made up from one or several different monomers. Usually have modifiable mechanical properties, excellent processability, low cost and controllable degradation time. However, they lack natural cell-recognition signals.
Some examples of natural and synthetic polymer-based materials for tissue engineering are summarized in table 1. Most of them have been already studied for drug delivery (Moroni L 2008).

The most appropriate materials for tissue engineering are naturally-derived ECM macromolecules. However, extracting and purifying these polymers in a large scale is still challenging; furthermore their immunogenic properties and inability to be modified, characterized and controlled easily, limits their applications (Shekaran A 2011). Therefore synthetic non-foiled materials, which can also be functionalised and tailored in composition for each tissue-specific application (Shekaran A 2011), offer a better alternative for tissue engineering applications.
I.6.A. Poly (vinyl alcohol)

Several polymers and co-polymers have been studied for scaffold fabrication in tissue engineering (Rogina A 2014). One of them is poly(vinyl alcohol) (PVA), a synthetic water-soluble polymer largely used by the pharmaceutical industry for eye lenses, implants and artificial organs development and production (Hyon SH 1994, Juang JH 1996).

It is an electrospinnable hydrophilic polymer, with good mechanical properties and described as being a highly biocompatible. In fact, it has an acceptable toxicological profile, with LD50 above 15-20g/Kg, NOAEL of 5g/Kg, bad gastrointestinal absorption, lack of accumulation in the body, no subchronic or chronic toxicity events reported, and no mutagenic or carcinogenic effects observed on in vitro assays. It is regarded as a safe food additive as it has been by EFSA (DeMerlis C 2003).

In order to improve PVA’s biophysical properties several authors have blended PVA with other biocompatible biopolymers, including chitosan (Jia YT 2007, Alhosseini SN 2012), alginate (Islam MS 2007), collagen, hydroxypapate (Song W 2012), silk-fibroin (Lee SY 2012), and adhesion-peptides (Schmendlen RH 2002).

In general, coating/blending of PVA leaded to an increase in the polymer’s bioactivity. An in vivo trial for a PVA matrix used as a dermal substitute has also been reported. Electrospun PVA-Chitosan matrices seeded with fibroblast have demonstrated not only to be able to maintain cell viability and respective metabolic capacity intact, but also to improve wound healing without scar formation (Sundaramurthi D 2012).

Another study made by Lee SY and colleagues (Lee SY 2012) compared the bioactivity of PVA matrices coated with diverse biocompatible polysaccharides (chitosan and hialuronic acid) and proteins (collagen and silk fibroin). Overall, both types of coating agents were able to improve PVA's mechanical properties; on the other hand biological responses varied according to the coating agent's hydrophylicity. In the case of hialuronic acid, the most hydrophilic, cell adhesion and proliferation of keratinocytes and fibroblasts to the matrix were similar to non-coated PVA fibbers; as for silk fibroin, attachment and proliferation were increased when compared even with collagen (Lee SY 2012).

In fact, although different cells have different requirements, it was showed in previous studies that they tend to prefer more rigid and less hydrophilic matrixes to adhere, which may explain great improvements observed for cell growth in PVA-(Silk-Fibroin) and PVA-Collagen matrixes (Agarwal S 2008) and might help explain why cell adhesion improved with the addition of multiwalled carbon-nanotubes (1%wt) to a PVA-Chitosan matrix (Liao H 2011).

In many studies, through the use of cell-binding motifs from ECM molecules, it was possible to enhance cell adhesion to synthetic materials. There are many of these studied (Shekaran A 2011), but the most relevant is the RGD sequence, which has proved to be a universal and unrestricted sequence.
I.6.B. Chitosan (CS)

CS is a natural polysaccharide, composed of polymerised glucosamine and N-acetylglucosamine obtained from the partial deacetylation of quitin, a compound present on crustaceous shells. Biologically this polymer has several properties that enable its use on scaffold fabrication, which include biocompatibility (LD$_{50}$ of 16g/Kg) and biodegradability (Rudzinski W 2010).

CS has been widely studied for the design of nanosystems for central nervous system delivery. However, its applications on drug delivery are also an important field of study in cancer therapy and wound healing. According to several authors, chitosan can either be electrospun alone (Lin TC 2012) or with other polymers such as PVA (Charernsriwilaiwat N 2012).

In their work, Charernsriwilaiwat N and colleagues describe the production of an electrospun CS-EDTA/PVA nanofiber, successfully loaded with 30% (w/w) lysozyme, for wound dressing. The obtained fibers were smooth and with no beads, and lysozyme was able to release well from the nanofibers, although in a burst manner (90% loading mass released in 4 hours). This was explained to be the result of two phenomena: (1) Lysozyme diffusion and (2) Fibber erosion nevertheless, the system showed biocompatibility and even allowed faster healing of wounds in the treated mice (Charernsriwilaiwat N 2012).

I.7. Lysozyme - incorporation for cancer therapy

The incorporation of diffusible/soluble signals in tissue engineered scaffolds is another strategy for promoting cell survival, proliferation and differentiation. Some of these signals include growth factors such as Epidermal Growth Factors (EGF), Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor (FGF), as well as cytokines and chemokines (Shekaran A 2011). They can be used in combination with adhesive peptides to direct cell functions in tissue engineering and/or drug delivery (Shekaran A 2011).

In order to deliver lysozyme to the targeted tumor, encapsulation in electrospinning-made nanofibbers can be performed. Using an adaptation of the electrospinning method, in which coaxial- (Jiang H 2006) and emulsion-electrospinning (Yang Y 2008) are examples, several authors such as Yang Y and colleagues were able to synthesise core-sheath fibbers capable of releasing functional monomeric Lysozyme in a controlled pattern. Also, by using the emulsion-electrospinning, the authors even managed to improve the releasing pattern of lysozyme, reducing the amount released in the initial burst and prolonging the release for 8-12 days (Yang Y 2008).
I.8. Magnetization of electrospun nanofibbers

Magnetization is another concept of functionalization in which magnetic beads are adsorbed and/or trapped in the intertwined fibbers of the scaffold. Once immobilized, and due to their paramagnetic properties (ability to magnetize when the material is exposed to a magnetic field and have no remnant or residual magnetization when the magnetic field is removed), magnetic beads can be used in various biomedical applications, such as (1) Drug delivery (Selective delivery to the inner ear, brain and stented blood vessels, these last with the possibility to perform cell delivery simultaneously), (2) Tissue engineering and regenerative medicine (mimetize mechanical stimulation, allow even distribution of seeded cells, control fibber alignment for scaffolds) (Sensening G 2012).

The use of magnetized scaffolds, through the use of magnetic beads, both as a therapeutic system and as a drug delivery system has recently been reviewed in many articles for cancer therapy. Iron oxide nanoparticles (IONPs), in which magnetite (Fe₃O₄) is an example, have the ability to heat the surrounding environment up to 45°C when an alternating magnetic field is applied to them (hipertermia); when cancer cells are exposed to these high temperatures, inactivation and cell death occur; normal cells are affected into a lesser extent (Lin TC 2012). However, even when intravenously administered IONPs are magnetically targeted to the tumor site, the local and precise delivery are still major challenges (Huang C 2012).

Encapsulating IONPs into electrospinning-made nanofibbers is one possible strategy to improve delivery to the tumor site, allowing to directly place the nanoparticles into the vicinity of the tumor. This enables the system to slowly release IONPs to the tumor and to trap possible metastatic cells. As a result, cancerous cells will be enclosed by the nanofibbers and killed by hipertermic IONPs. In their work, Huang C and colleagues used this principle to induce cell death in cultures SKOV-3 ovarian cancer cells; the results were promising, as the target temperature of 45°C was kept constant over the course of the experiment (1 hour) in which 100% cell death was achieved (Huang C 2012). Nevertheless, in vivo studies should be envisaged in order to understand better how these systems will react in the presence of the natural ECM and how will it affect surrounding tissues.
Scientific question and overall research strategy
II - Scientific Question and Overall Research Strategy

Being colon adenocarcinoma a typical invasive tumor in latter stages of development, partial resection of the intestine is in most of the cases the curative measure to take. Prior or/and after surgery, so to reduce tumor size or to eliminate completely cancerous cells, patients may receive chemotherapy and/radiotherapy (Watanabe T 2011). However, a recent survey (Shayeb D 2012) on oncologist and phase III colon adenocarcinoma patients who had their tumors resected revealed that this post-surgery procedure was not being performed. In fact, of a total of 613 patients, 9% refused chemotherapy, mainly because of concerns about toxic effects, and 18% were advised by their oncologist not to take the treatment, mainly due to the presence of co-morbidities and/or complications of surgery.

To summarise there is a need for alternative adjunct colon tumor therapies, which can be administered prior or after tumor resection, that do not have pronounced toxic effects on patients and that allow controlling tumor growth/recurrence.

The main goal of this thesis work was to develop a nanofibrous system for treating phase III colon cancer patients. Because phase III colon adenocarcinoma is an invasive tumor, research was directed for creating a containment system which can (1) enclosure metastatic cells within the tumor mass region, (2) slow down tissue's ECM remodelling and (3) eliminate any altered cells in the system's vicinity. The nanofibers envisaged can be applied pre- and post-surgery on the tumor and ablation site respectively, since they will promote both tumor shrinkage, metastatic cells containment and cancer cells death.

The system built is composed by 3 important components.

The first component are the PVA electrospun nanofibers. Because they are obtained by electrospinning technique, they possess have high specific area, meaning cellular adhesion can be promoted. The fibbers were produced through simple electrospinning. Although no specific apparatus are needed besides a normal electrospinning one, process parameters such as polymer concentration, tip-to-collector distance and applied tension were necessary to adjust in order to evaluate the quality of the obtained fibbers. Morphology, evaluated through simple optic microscopy and Scanning Electron Microscopy (SEM), were used to observe the fibers structure. In the end, cross-linking with boronic acid was performed to improve the fibbers' stability.

The second component is hen egg white lysozyme, which was incorporated within electrospun fiber through previous mixing with the polymer solution. Because PVA naturally degrades in water, controlled release of lysozyme is expected to occur. To evaluate the success of the encapsulation process, quantification of lysozyme released in vitro, and quantification of its activity will be determined.

The third component are the magnetic beads, composed of magnetite (Fe₃O₄), which were adsorbed to the fibers through simple mixing. In order to evaluate their successful adsorption to the fibers, simple optic microscopy and SEM will be used. Finally, their
effect on the release of lysozyme from the fibers will be also evaluated through its quantification and the determination of its activity.

Finally, to test the efficacy of our system Caco-2 cells were placed in contact with the envisaged system, and their viability and cell adhesion, determined through the MTT assay and DAPI-Phaloidin staining respectively, was evaluated. used as a model for colon cancer. It was expected that cell death rate increased as the complexity of the system increased.
Materials and methods
III - Materials and methods

In order to achieve the goals proposed for this thesis, intermediate milestones were followed by this order:

1. Lysozyme analytical methods development;
2. PVA Lens production and lysozyme release kinetics;
3. PVA nanofibber production and lysozyme release kinetics;
4. In vitro evaluation of cell death in Caco-2 cells;

To attain these milestones different analytical and experimental methods were developed:

- Lysozyme mass – Bradford Method;
- Lysozyme activity – Degradation rate of *Micrococcus Lysodeikticus* extract;
- PVA lenses production – Solvent evaporation of PVA solution into a mold;
- PVA nanofibers production – Electrospinning;
- PVA lenses/fibers quality control – Optic and electronic microscopy;
- Lysozyme release assay – Drip lenses/fibers in appropriate solution, take aliquots at predetermined time-points and measure lysozyme mass and activity;
- Evaluate cell death – MTT assay and DAPI-phalloidin staining.

### III.1. Lysozyme analytical methods development

#### III.1.1. Bradford assay

The first step for analysing lysozyme, the active substance of our envisaged system, was to develop and/or adapt analytic methods described in the literature that could be used to analyse it. Using first BSA (NZY-Tech) as a model, followed next by lysozyme (Sigma-Aldrich), a Bradford calibration curve was established using the Bradford method for protein determination. Briefly, different lysozyme standard solutions (2.0mg/mL, 1.5mg/mL, 0.75mg/mL, 0.50mg/mL, 0.25mg/mL, 0.125mg/mL) were prepared in phosphate buffer (pH = 7.4); Simple phosphate buffer was used as a standard.

Bradford assay was performed following the instruction of BioRad for Bradford reagent. Briefly, 20uL of protein solution was added to 1mL of Bradford reagent (Biorad®) previously diluted in bidestillated water (1:4) and its absorbance at 595nm was measured (Macromethod). The method was later adapted using a modified protocol described by Nunes MAP 2010 for Bradford in microplates. Briefly, 50uL of protein solution was transferred in triplicate to a 96 well-microplate, to which was added a mixture of 50uL of Bradford reagent and 200uL of phosphate buffer (pH = 7.4) (Micromethod); After a 5min incubation period at room temperature, absorbance was measured in triplicate at 595nm. Results were plotted against concentration using Microsoft Excel® and the calibration curve was determinated.
III.1.2. Lysozyme activity assay

Lysozyme’s physiological function was evaluated through its ability to degrade *Mycococcus Lysodeikticus* ATCC Nº4698 (Sigma-Aldrich). Briefly, different lysozyme standard solutions (2.0mg/mL, 1.0mg/mL, 0.75mg/mL, 0.50mg/mL, 0.25mg/mL, 0.125mg/mL, 0.0625mg/mL, 0.03125mg/mL, 0.015625mg/mL, 0.0078125mg/mL) were prepared in phosphate buffer (pH = 7.4). 50uL of each solution was transferred in triplicate to a 96 well-microplate. To evaluate the effect of *Micrococcus Lysodeikticus* concentration on the assay, 200uL of different concentrations (3mg/mL, 2mg/mL, 1mg/mL, 0.5mg/mL, 0.25mg/mL) were added to the samples. Lysozyme activity was assessed at room temperature through the measurement of the variation of its absorbance at 450nm for 7 minutes since the *Micrococcus Lysodeikticus* solution was added. The obtained slopes for each sample were plotted against lysozyme concentration and a calibration curve was obtained.

III.2. PVA lenses production and lysozyme release kinetics

III.2.1. Lens production

Polyvinyl alcohol (99% hydrolyzed, average MW 4,441) (Sigma) was used as the polymer for the lens. Similarly to what has been described by Nunes MAP 2014, PVA was firstly dissolved (10% m/v) in phosphate buffer (pH = 7.4) under vigorous stirring for 45 minutes and left to rest for one hour at 4ºC. Lysozyme entrapment was made at room temperature through direct addition of solid lysozyme to the PVA solution (300mg of lysozyme for each 1g of PVA) under continuous stirring for 30 minutes.

Lens production was made through solution pumping using a syringe pump (NewERA model NE-300, USA) volume dispenser. A fixed amount of PVA solution was dripped for several wells in a 96 well-microplate and were left to dry at 60ºC for 60 minutes and then 24h at room temperature. After this, lenses were stored at 4ºC. Average lens volume was calculated according to the total number of lens produced and the total volume of solution dispensed.

III.2.2. Lens cross-link and IONPs adsorption

Following the protocol of Nunes MAP 2014, lenses were cross-linked either with boronic acids. Briefly, phenylboronic acid 0.50% or 4-fluorophenylboronic acid 0.50% were dissolved in bidestilled water under vigorous stirring. PVA lens cross-link was made by dripping the lens directly in the solution, under constant agitation (40osc/min) for 10 minutes. After lenses were dried at 50ºC overnight and stored at 4ºC.

Iron(II, III) oxide magnetic nanoparticles (nanopowder - Sigma) were dispersed in bidestilled water (2mg/mL) and used readily for nanoparticle adsorption on the lens.
Briefly, cross-linked PVA lenses were dripped in an iron oxide nanoparticle solution under constant agitation (40osc/min). After lenses were dried at 50ºC overnight and stored at 4ºC.

III.2.3. Lens release assay

For this assay, six different samples were tested:

1. PVA lenses
2. PVA lenses + encapsulated lysozyme;
3. PVA lenses + encapsulated lysozyme + cross-link with phenylboronic acid;
4. PVA lenses + encapsulated lysozyme + cross-link with phenylboronic acid + with magnetic nanoparticles adsorbed;
5. PVA lenses + encapsulated lysozyme + cross-link with fluorophenylboronic acid;
6. PVA lenses + encapsulated lysozyme + cross-link with fluoroboronic acid + with magnetic nanoparticles adsorbed.

Adapting the protocol from Yang Y and colleagues 2007, each lens was submerged in 2mL of phosphate buffer and kept at room temperature in an oscillatory platform at 40osc/min during the first 60min and at 5-10osc/min until the end of the assay. At each pre-determined timepoint, 1mL of the sample solution was collected and replaced by the same amount of phosphate buffer (pH = 6.8, 7.0, 7.2 or 7.4).

Lysozyme concentration and activity were evaluated using Bradford assay (absorbance at 595nm) and measurement of *Micrococcus Lysodeikticus* lysis (absorbance variance at 450nm for 7 minutes) protocols for microplate described earlier. Lenses with no lysozyme encapsulated were used as controls. Triplicates for each timepoint were analysed per assay.

III.3. PVA nanofibber production and lysozyme release kinetics;

III.3.1. Fiber Production

PVA fibers were produced using a home-made electrospinning device. The electrospinning set-up was contained in a hermetically sealed box, which provided a safe and stable environment, and where it was possible to operate under different conditions.

The gel polymer solution was fed with 1mL, fitted with 25G plastic micro-tip needles (Terumo), that were connected to the positive terminal of a high-voltage supply (73030DC, Genvolt, Shropshire, UK), able to generate DC voltages up to 30 kV. The solution was delivered to the needle by a syringe pump (New Era, NE-1000, UK).
Electrospinning conditions were the following for both PVA and PVA-LYZ fibers: 17.4V for voltage, 8cm for tip-collector distance, 140μL/minute for flow-rate, and 7 minutes for total production time. Rotating disks collectors (3.4 cm diameter) were used for collecting the fibers. During electrospinning the collector was rotated at a constant speed of 150 rpm by a DC motor to collect the developing nanofibers. The obtained fibers were then observed under optic microscopy and SEM for quality checking.

### III.3.2. Fiber Cross-link and IONPs adsorption

Electrospun PVA-LYZ nanofibers were immersed for 10 minutes in an aqueous solution of 0.5%(w/v) fluorobenzeneboronic acid for 10 minutes, after which were collected, dried at 40-50ºC for 50 minutes in a pre-heated oven and stored at 4ºC.

After cross-linking, PVA fibers with lysozyme encapsulated and cross-linked with fluorophenylboronic acid (PVA-LYZ-FB) were immersed in an aqueous suspension of 2mg/mL Iron(II,III) oxide nanoparticles (Sigma-Aldrich) and left agitating (40osc/min) for 1 hour. The obtained PVA-LYZ-FB fibers with IONPs adsorbed (PVA-LYZ-FB-MN) were then washed with bidistillated water trice, dried at 40-50ºC for 50 minutes and storage at 4ºC.

### III.3.3. Fiber Release assay

Four different samples were used in this assay:

1. PVA fibers;
2. PVA fibers + encapsulated lysozyme;
3. PVA fibers + encapsulated lysozyme + cross-link with fluoroboronic acid;
4. PVA fibers + encapsulated lysozyme + cross-link with fluoroboronic acid + with magnetic nanoparticles adsorbed.

Each different fiber was submerged in 2mL of phosphate buffer and kept at room temperature in an oscillatory platform at 40osc/min during the first 60min and at 5-10osc/min until the end of the assay. At each pre-determined timepoint, 1mL of the sample solution was collected and replaced by the same amount of phosphate buffer (pH = 6.8, 7.0, 7.2 or 7.4). Fibers with no Lysozyme encapsulated were used as controls. Triplicates for each timepoint were analysed per assay.

Lysozyme concentration and activity were determined using Bradford assay (absorbance at 595nm) and Measurement of Micrococcus Lysodeikticus lysis (absorbance variance at 450nm for 7 minutes) protocols for microplate described earlier. Lenses with no lysozyme encapsulated were used as controls. Triplicates for each timepoint were analysed per assay.
III.4. In vitro evaluation of cell death in Caco-2 cells

III.4.1. Caco2 cell culture

Cell culture medium RPMI-1640 already supplemented with L-glutamine, trypsin-EDTA solution, stabilized antibiotic-antimycotic solution (100x) and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma, and fetal bovine serum (FBS) from VWR.

Human colorectal adenocarcinoma (CaCo-2) cell lines were purchased from the American Type Culture Collection (ATCC). Following the protocol described by Frade R and colleagues 2013, the cells were cultured in RPMI-1640 supplemented with 10% FBS and antibiotic-antimycotic solution in 75 cm² tissue culture flasks, and kept at 37°C and 5% CO₂ until confluency.

III.4.2. Caco2 – Fiber culture and Cell Assays

PVA fibers PVA-LYZ-FB and PVA-LYZ-FB-MN were sterilized by exposing them to UV radiation for 40 min. After this, the fibers were transferred to a 96 well microplate (Nunc®) and kept dried until cell seeding.

After reaching confluency, Caco2 cells were trypsinized, diluted 1:5 in RPMI-1640 medium and transferred directly over the fibers. Cells were left to grow at 37°C and 5% CO₂, and their expansion was accompanied by optical microscopy for 10 days.

Cell viability was measured using the MTT assay, and was carried out in RPMI-1640 cell medium with 0.5% FBS. Stock solutions were prepared in ethanol or dimethylsulfoxide (DMSO) and then diluted with the cell culture media to obtain the desirable concentrations. The fibers were treated with RPMI-1640 0.5% FBS supplemented with MTT 0.1 g/L and returned to the incubator for 3–4 h. Media was removed and the purple formazan crystals dissolved in DMSO. The absorbance of the plates was read at 570–595 nm in a plate reader and the ratio of the absorbance of treated cells and the absorbance of control cells was determined (viability). To observe cell morphology and attachment to the nanofibers, DAPI-Phalloidin staining was used.

III.5. Statistical analysis

All in vitro results were analyzed with Student’s t-test, using microsoft excel. For the release assays, the reference used were the values obtained for pH = 7.4. A p-value of less than 0.05 was considered to demonstrate statistical significance.
Results and Discussion
IV – Results and Discussion

IV.1. Lysozyme analytical methods development

IV.1.1. Lysozyme bradford assay

Using Bradford assay, the first step on this work was to develop a calibration curve for lysozyme, to easily calculate its concentration in any sample measured. Other proteins such as BSA or γ-globulin could have been used, although accuracy in the measurements would be compromised due to the fact that these proteins all have different contents in cationic aminoacids (arginines and lysines) (Ku HK and colleagues 2013), to which Coomassie Brilliant Blue binds (Tal M and colleagues 1985). For BSA, linearity between absorbance at 595nm and concentration (mg/mL) was achieved with concentrations between 0.125mg/mL and 1.000mg/mL, while with γ-globulin the same was achieved for concentrations between 0.125mg/mL and 1.500mg/mL. For lysozyme, a wider set of concentrations was chosen (2.000mg/mL and 0.00391mg/mL) to find the best set of concentrations where linearity was observed.

Using the conventional Bradford method for the determination of protein content in a solution (c.f. materials and methods) calibration curves were set and an example can be seen in chart 1

![Chart 1: Calibration curve for lysozyme standards using the macromethod.](image)

Because this method consumed a lot of Bradford reagent and was time consuming to read multiple samples, it was decided to adapt the method to a 96-well microplate. Because absorbance measurements were too low to be accurately measured, due to the reagent being too concentrated, a previous dilution on 1:5 in phosphate buffer (50mM, pH = 7.4) was performed. This buffer was used instead of destillated water because it can maintain its pH constant during the time required to prepare the samples and measure the absorbance, it was easy to prepare and ready to use, with little or non pH adjustment necessary prior to use, and it did not interfere with Comassie Brilliant Blue’s conversion to it’s blue form.
Using the adapted micromethod for lysozyme determination, 50uL of each lysozyme standard were added to 250uL of Bradford reagent diluted in phosphate buffer 1:5 (pH = 7.4). After a 5 minutes’ incubation, absorbance was measured at 595nm and plotted against the respective concentration (Chart 2).

Linearity between absorbance and lysozyme concentration was observed for concentration values between 0.00mg/mL and 0.10mg/mL, which was acceptable since the values for lysozyme release from the fibers were not expected to be above the maximal value. In fact, Yang Y and colleagues 2008 refer that this method could be used to quantify lysozyme up to 25mg/mL. Although this was not confirmed in this assay, available data showed that for concentrations above 0.1mg/mL a new calibration curve must be determined.

Since lysozyme is the single protein to be encapsulated in our system, the problem of low specificity for the Bradford assay did not compromised the quality of the obtained results. Therefore, the calibration curve present in Chart 2 was used to calculate lysozyme’s concentration in all the samples collected.

According to the literature, several chemical can interfere with the absorbance readings in the Bradford method (Walker JM 2002 (pp18)). Because one of those chemicals is SDS, which was envisaged to be used to measure adsorbed lysozyme, three measurements were made in the presence of 2%, 0.1% and 0.02% of SDS. In fact, linearity for low concentrations of lysozyme (0.00 – 0.50mg/mL) was only achieved when SDS concentration was lower (data not showed), making impractical the use of SDS 2% to determine the quantity of lysozyme adsorbed on our system.
IV.1.2. Lysozyme activity assay

Determining lysozyme’s activity allowed not only to determine whether the enzyme’s catalytic function was preserved during the assay, but also to calculate its concentration.

As it happened for the Bradford assay, the method was adapted for 96 well-microplate. Adapting the methodology from Shugar D 1952 and Lee Y 2002, 50uL of different Lysozyme standards (2mg/mL to 0.00193mg/mL) were added to a 96 well-microplate, followed by 200uL of 3mg/mL *Micrococcus Lysodeikticus* suspension. Although the reactions started as soon as the *Micrococcus* suspension was added, the values corresponding to the initial minute were discharged so to give time for the reaction to start. Absorbance was read every 17 seconds at 450nm, which was the spectrophotometer’s minimum reading time, for 7 minutes (25 reading cycles). This methodology allowed to evaluate optical density decrease over time as the *Micrococcus Lysodeikticus*’ peptidoglycans are degraded by lysozyme, and the rate at which this happens was correlated with lysozyme’s concentration. The result can be seen in Figure 2, where the different absorbances at 450nm have been plotted against time, in Chart 3, where the different slopes have been plotted against concentration, and in Chart 4, where the slopes were converted to U/mg of enzyme.

![Figure 2](image.png)

*Figure 2:* Charts showing the variance in the absorbance at 450nm for lysozyme activity determination (7 minutes) using *Micrococcus Lysodeikticus* for different lysozyme concentrations (A) 2mg/mL, (B) 1 mg/mL, (C) 0.5 mg/mL, (D) 0.25 mg/mL, (E) 0.125 mg/mL, (F) 0.0625 mg/mL, (G) 0.03125 mg/mL, (H) 0.015625 mg/mL, (I) 0.0078125 mg/mL, (J) 0.00 mg/mL.
Contrary to what was expected, lysozyme activity tended to decrease for higher concentrations. The mechanism by which this happens has not been sufficiently explored to offer a plausible explanation, but it seems that this concentration-dependent decreasing in activity might indicate a possible self-inhibition mechanism. However, more assays are necessary to justify this trend.
As was described by Shugar D 1952, lysozyme activity profile is also dependent of the quality of *Micrococcus Lysodeikticus*’ peptidoglycans. In fact, variation in the obtained slopes for different lysozyme activity assays were observed every time (data not showed). This justified the preparation of a calibration curve prior to every activity determination assay.

Slope variation was also observed when different concentrations of *Micrococcus Lysodeikticus* (3mg/mL, 2mg/mL, 1mg/mL, 0.5mg/mL, 0.25mg/mL and 0.00mg/mL) were used to determine lysozyme activity at 0.0625mg/mL. **Figure 3** shows the obtained results.

![Figure 3: Charts showing the variance in the absorbances at 450nm for Lysozyme activity determination (7 minutes) using different concentrations of Micrococcus Lysodeikticus (A) 3mg/mL, (B) 2mg/mL, (C) 1mg/mL, (D) 0.5mg/mL, (E) 0.25mg/mL, (F) 0.00mg/mL.](image)

Initially, it was thought that a concentration of 0.5mg/mL of *Micrococcus Lysodeikticus* would be enough to perform the assay, since linearity was observed when this concentration was used. However, when a new set of calibration curves for lysozyme were made with this concentration, the obtained $R^2$ ranged from 0.19 to 0.85 (data not showed), which were unfit to build an accurate calibration curve. Therefore, *Micrococcus Lysodeikticus* concentration of 3mg/mL was used for all the assays performed since.

**IV.2. PVA lenses production and lysozyme release kinetics**

**IV.2.1. PVA lens production and total encapsulated protein quantification**

In order to simulate the effects of our system’s constructing blocks on the release profile of lysozyme, a simpler kind of construct was needed. Therefore, following the
protocol described by Nunes MAP 2014, PVA lens were produced using PVA 10% dissolved in phosphate buffer (pH 7.4).

The production technique was simple. A defined volume of PVA solution was dropped on several wells of a flat-bottom 96 well-microplate and were left to dry in an oven for 60 minutes at 60°C and then for 24 hours at room temperature. The obtained lens were analysed on the optic microscope, but besides the homogeneous film of PVA noting relevant was observed (data not showed).

Similarly to what has been done with naringinase by Nunes MAP in 2014, lysozyme was incorporated in the PVA solution (0.3g of lysozyme for each gram of PVA). The obtained solution was used to prepare lenses using the same procedure as in the PVA ones. The obtained lenses were labelled as “PVA-LYZ”, and were observed under optical microscopy after being stained with Bradford reagent to confirm that ILysozyme has been encapsulated (see Figure 4).

![Figure 4: PVA-LYZ lens shards observed under optical microscopy (magnification of 400X). Arrows show location of dyed inclusions believed to be of Lysozyme.](image)

After production, the lenses were tested for total lysozyme load using (1) extraction methods for ILysozyme, followed by determination of concentration using Bradford assay, (2) direct Bradford method on the lens and (3) lysozyme release profile in a phosphate buffer solution (pH 7.4). The goal was to test whether the calculated amount of immobilized lysozyme (0.95mg) corresponded to the real value.

In the first method, 3 solvents were used to dissolve PVA lens: chlorophorm, DMSO and distillated water. Neither of them was able to dissolve the lens after 24h of incubation under constant agitation, so the incubation period was prolonged for 1 week. After this period, only DMSO was able to breakdown PVA lens but not completely dissolving it. Distillated water at 90°C was able to dissolve the lens. However, at such high temperatures protein denaturation occurred and different aminoacidic residues are exposed to react with Bradford reagent, altering the concentration value obtained. Because none
of the solvents was able to completely dissolve PVA-LYZ lens at room temperature and without altering the protein, this method was not used anymore.

Direct Bradford assay on the PVA-LYZ lens was also tested but the obtained concentrations were lower than the expected value. Lysozyme release profile was the method that gave best results, despite PVA dissolution was not achieved, and was used in the following assays.

IV.2.2. PVA lenses – lysozyme release kinetics

IV.2.2.A PVA lenses + lysozyme

In order not only to assess total protein content encapsulated but as well to evaluate Lysozyme’s release profile from the lens, a release assay was designed. Because the designed system was designed to be inserted inside ablation areas, care was taken with the pH of the phosphate buffer used, which was 7.4. The timepoints (minutes 0, 1, 3, 5, 10, 15, 30, and 60, and hours 24, 48 and 96) were chosen to evaluate not only the overall release profile but also to assess the extent of the initial burst release, as was described by Charernsriwilaiwat N and colleagues 2012. Lysozyme’s activity was also evaluated at strategic timepoints (minutes 0, 5, 60, and hours 24, 48 and 96) to evaluate whether lysozyme’s function was not affected by the encapsulation process.

The literature states that pH in a tumor’s microenvironment is more acidic than normal tissues. In fact, Lee E and colleagues 2008 state in their review that the overall pH of a tumor is about 7.0; although not all tumors have the same pH value ([5.7 – 7.8]), they reached to the conclusion that about 80% of them, which include colon carcinoma, have pH values inferior to 7.0. Other authors observations were also similar, including Fan L 2008, which observed the pH to be inferior to 7.2, and Danhier F 2010, which observed the pH values to vary between 6.0 and 7.0. Aside from being a consequence of the elevated metabolism, low pH was also described by Silva AS 2009 to promote tumor’s metastization. Bearing this in mind, it was decided to evaluate also lysozyme’s release profile and activity at different pHs. Therefore, lysozyme’s release kinetics was also evaluated at pH 6.8, 7.0 and 7.2. The obtained release profiles can be analysed in Charts 5 and 6.
Comparatively to pH 7.4 release profile, the releases' for pH 7.2 ($p = 0.60$) and 7.0 ($p = 0.77$) were very similar, meaning that no statistical difference was observed between them. However, the $p$-value obtained for pH 6.8 was inferior to 0.05 ($p = 0.0185$). This
statistical difference between these two profiles can be explained due to the degradation of PVA being accelerated in acidic pH, as was described by Chen Y and colleagues 2001.

A burst effect was observed for lenses immersed in phosphate buffer pH = 6.8 for the first 60 minutes, contrary to what was observed for the other pH. Although it might seem interesting of the lens to unload such a high amount of lysozyme in acidic pH, a more constant and sustained release, such as was observed for the other pHs, is more desired.

Charernsriwilaiwat N 2012 and colleagues also obtained a similar profile for lysozyme release for PVA-CS nanofibers, although the observed burst effect was visible for the first 90% of Lysozyme’s release. From the analysis made to the chart (colocar o numero), the burst effect was observed to last between 60min and 24h and releases only 25% to 70% of total lysozyme. Although the release patterns obtained by the authors can’t be directly compared with the ones obtained in this work because of different polymers used, the basic behaviour of lysozyme’s release could be also predicted and evaluated in the same way.

IV.2.2.B PVA lenses + lysozyme + cross-linkers

In order to modulate lysozyme release from the lens, boronic acid derivates were used to cross-link PVA. As was observed by Nunes MAP 2016, boronic acid derivates can increase the stability of PVA, including to temperature, and are more biocompatible than boronic acid or other cross-linker used for PVA (Dianhydrides, Glutaraldehyde, Hexamethylene and diisocyanate). More important is that hydrogels obtained from PVA cross-linked with boronic acid derivates are more thermal-resistant than simple PVA, making them capable of being sterilized by heat (Nunes MAP 2016). These unique properties make them excellent building block for drug encapsulation systems or as scaffolds for tissue engineering.

In this work, boronic acid derivates phenylboronic acid (PhB) and fluorophenylboronic acids (FB) were used to increase PVA stability in aqueous solution and control lysozyme’s release from the lens. They were selected because in the work of Nunes MAP 2016 both boronic acid derivates showed to maintain a porous structure on the PVA lens, favouring mass transfer between the encapsulating system and the buffer. However, a worse profile for phenylboronic acid was expected due to increase membrane thickness, which could overcome their pores-maintaining properties on mass transference.

After lens were cross-linked with phenylboronic acid and fluorophenylboronic acid, their release profile was assessed. The results can be seen in Charts 7, 8, 9 and 10.
There is statistical difference in the first 60 minutes of the release profiles for pH 6.8 ($p = 0.0273$) when comparing with pH 7.4, possible due to decreased initial lysozyme release which just began 30 minutes after the assay was started. This can be explained by the fact that, as was described by Nunes MAP 2016, phenylboronic acid can originate a thickest layer of cross-linked PVA, and this in return retards lysozyme’s release from the

**Chart 7:** Release profile of lysozyme from PVA lens, cross-linked with PhB, for 60 minutes. Cumulative relative mass corresponds to the amount of lysozyme released when compared to its theoretical load.

**Chart 8:** Release profile of lysozyme from PVA lens, cross-linked with PhB, for 96 hours. Cumulative relative mass corresponds to the amount of lysozyme released when compared to its theoretical load.
system. As for pH 7.0 ($p = 0.98$) and 7.2 ($p = 35$) there was no statistical significant difference from pH 7.4.

There is no statistical significant difference between the release pattern for 96 hours of pH 7.4 and pH 7.2 ($p = 0.78$), pH 7.0 ($p = 0.89$) and pH 6.8 ($p = 0.63$), meaning that treatment of PVA with phenylboronic acid homogenized lysozyme release and ablated the initial burst effect in the first 60 minutes. A smaller cumulative amount of lysozyme was released after 96 hours (29% - 40%) but no plateau value was reached, meaning that no predictions could be made to the maximal release capacity of lysozyme from the lenses cross-linked.

![Chart 9](image1.png)

**Chart 9**: Release profile of lysozyme from PVA lens, cross-linked with FB, for 60 minutes. Cumulative relative mass corresponds to the amount of lysozyme released when compared to its theoretical load.

![Chart 10](image2.png)

**Chart 10**: Release profile of lysozyme from PVA lens, cross-linked with FB, for 96 hours. Cumulative relative mass corresponds to the amount of lysozyme released when compared to its theoretical load.
For the first 60 minutes of release of lysozyme from PVA-LYZ-FB lenses, and when comparing with pH 7.4, there is statistical difference for pH 6.8 ($p < 0.05$) and 7.2 ($p < 0.05$), whether for pH 7.0 ($p = 0.39$) there is not any difference. For pH 7.2, where no release was observed for 60 minutes, the explanation revolves around the fact that (1) little or none of lysozyme was adsorbed on the lens surface, ablating the burst effect release observed for PVA-LYZ lens, and (2) due to cross-linking PVA takes longer time to adsorb water and swell, making more difficult for encapsulated lysozyme to find a diffusion path out of the lens. As for pH 6.8 the most likely explanation is that a larger amount of lysozyme remained adsorbed and/or trapped on the surface of the lens; according, the acidic pH could have enhanced lysozyme’s release.

For the total time of the assay (96 hours), there was no statistical significant difference between pH 7.4 and pH 6.8 ($p = 0.15$), 7.0 ($p = 0.85$) and 7.2 ($p = 0.76$), meaning that the initial differences observed in the release profiles (first 60 minutes) had no effect on the total yield. However, when comparing with to simple PVA-LYZ lens, the profile was more linear and no significant burst effect was observed, although the total amount of lysozyme released was lower.

Although statistical differences were found at some timepoints between the same type, overall the lens tended to have a more similar release profile independent of the pH that matched with the expectation that cross-linking would retard lysozyme’s release, and would create a controlled release system capable of delivering small but constant doses over time. Despite the assays were ended 96 hours after they had begun, it would be relevant for the cross-linked lens release profile to have more timepoints and determine when a plateau phase would be achieved.

**IV.2.2.C PVA lenses + lysozyme + cross-linkers + magnetic nanoparticles**

The effect of Fe$^{2+}$/Fe$^{3+}$ magnetic nanoparticles on the release of lysozyme was also investigated. This is relevant due to the fact that, accordingly to Liu F 2015, these nanoparticles are negatively charged (pI of 4.85) and can therefore interact/attract the positively charged lysozyme (pI of 11.2 according to Shareghi B 2015), promoting its release from the lenses.

The concentration of IONPs used for adsorbing on the lens was adapted from the literature, mainly from the work of Hainfield JF and colleagues 2013. In their research, it was found that the maximum concentration of PEG-coated iron nanoparticles to reach the tumor site was 1.9mg Fe/mL. This together with magnetic stimulation 24h after the dose was injected (38kA/m, 980kHz, 2 minutes), was able to ablate 78%-90% of all implanted...
squamous carcinoma in the mice legs. The temperatures reached varied from 65ºC inside the tumor and 44ºC in the surroundings, making this treatment very specific for solid tumors. Therefore, this concentration measured in situ was used as the reference so as to adsorb an appropriate amount of iron nanoparticles to our fibers (approximately 3.00mg/mL).

The results for the release of lysozyme from PVA-LYZ-PhB-MN lens, for 60 minutes and 96 hours, can be seen in charts 11 and 12.

**Chart 11:** Release profile of lysozyme from PVA lenses, cross-linked with PhB and with magnetic nanoparticles adsorbed, for 60 minutes. Cumulative relative mass corresponds to the amount of lysozyme released when compared to its theoretical load.

**Chart 12:** Release profile of Lysozyme from PVA lens, cross-linked with PhB and with magnetic nanoparticles adsorbed, for 96 hours. Cumulative relative mass corresponds to the amount of Lysozyme released when compared to its theoretical load.
In the case of PVA lens with lysozyme encapsulated, cross-linked with phenylboronic acid and with magnetic nanoparticles adsorbed, the release profiles of the first 60 minutes for pH 7.2 and 6.8 are statistically different from pH 7.4 ($p < 0.05$ and $p < 0.05$ respectively), contrary to what happens to pH 7.0 ($p = 0.07$). For the others assays (96 hours), only pH 7.2 remains statistically different from pH 7.4 ($p < 0.05$), whereas pH 6.8 and 7.0 are not ($p = 0.14$ and $p = 0.40$ respectively).

There is a tendency for the release rate to stabilize when a plateau is reached, which was not observed in the total length of the assay. However, it can be observed from the fold increase analysis in chart 13 that by minute 60 and hours 24, 48 and 96 the overall quantity of lysozyme release was higher in PVA-LYS-PhB-MN lenses than in the PVA-LYZ-PhB ones, meaning that probably the adsorbed magnetic nanoparticles enhanced lysozyme release from the lenses. However, there is only statistically significant difference between the release profiles of PVA-LYZ-PhB and PVA-LYZ-PhB-MN for pH 7.4 ($p < 0.05$). For pH 6.8 ($p = 0.22$), 7.0 ($p = 0.25$) and 7.2 ($p = 0.56$) the respective p-values do not support this enhancement theory.

![Chart 13: Fold increase in total lysozyme release from the PVA-LYZ-PhB-MN lenses at minute 60 and hours 24, 48 and 96. Values superior to 1 indicate higher quantity released, whether values inferior to 1 indicate lower quantity released.](image)

As for the lens cross-linked with fluorophenylboronic acid, whose results can be analysed in charts 14 and 15, the results are very similar to those of PVA-LYZ-PhB-MN. Briefly, there is a significant statistical difference between pH 7.4 and 7.0 for the first 60 minutes ($p < 0.05$) but not with 7.2 ($p = 0.99$) and 6.8 ($p = 0.62$). When the total 96 hours are analysed, no statistical difference was found between pH 7.4 and pH 7.2 ($p = 0.93$), 7.0 ($p = 0.16$) and 6.8 ($p = 0.90$).
Despite the absence of statistical significance, the analysis to the fold increase of lysozyme released from PVA-LYZ-FB-MN lenses, (chart 16) reveals that more lysozyme was released. This means that there is a tendency for lysozyme’s release be enhanced by the presence of magnetic nanoparticles.
In order to study the effect of pH and the lenses modifications on lysozyme activity, samples from predetermined timepoints (0min, 5min, 60min, 24h, 48h, 96h) had their lysozyme’s activity determined using *Micrococcus Lysodeikticus*. The respective concentrations were calculated according to the calibration curve for lysozyme using the obtained slopes for each sample. Finally, the obtained concentrations were compared with the obtained from the Bradford method and the variance of each one was analysed (Bradford method concentrations were used as reference). The results can be seen in Tables 2, 3, 4 and 5.

**IV.2.2.D PVA lenses + modifications – lysozyme activity**

![](chart.png)

*Chart 16: Fold increase in total Lysozyme release from the PVA-LYZ-FB-MN lens at minute 60 and hours 24, 48 and 96. Values superior to 1 indicate higher quantity released, whether values inferior to 1 indicate lower quantity released.*
Table 2: Lysozyme concentration, released from the PVA lens at pH = 6.8, and determined through Bradford method and lysozyme activity calibration curve. Variation > 0% indicates increase in activity, whether ≤ 0% indicates no variation or decrease in activity. P-Value was calculated with PVA-LYZ as the reference.

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Table 5: Lysozyme concentration, released from the PVA lens at pH = 7.4, and determined through Bradford method and Lysozyme activity calibration curve. Variation > 0% indicates increase in activity, whether ≤ 0% indicates no variation or decrease in activity. P-Value was calculated with PVA-LYZ as the reference.

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</table>
Using different lysozyme’s standard solutions to perform a new lysozyme activity assay, it was possible to construct a new calibration curve for lysozyme’s activity, which was used to calculate lysozyme’s concentration. The concentration obtained by this method corresponds to the equivalent amount of active lysozyme in solution. Comparing this value with the ones obtained from the lysozyme’s activity calibration curve, it was possible to determine to which extent the activity of the enzyme was influenced by the different modifications to the lenses.

The variation present in tables 2, 3, 4 and 5 were the measure used for evaluating the influence of the modifications on lysozyme. They were calculated through the difference between the concentration of the lysozyme activity and the Bradford method, divided by the concentration calculated by the Bradford method. The analysis made to different the different p-values obtained for the timepoints (0min, 5min, 60min, 24h, 48h, 96h) demonstrated that there is a fine tendency for lysozyme’s activity to increase, independently from the pH of the phosphate buffer used. In fact, when compared, the p-values for the variations in the same pH were not statistically different from each other, meaning that this increase happened naturally independently of the pH.

Overall, lysozyme’s activity was not affected by the pH of the phosphate buffer used. When PVA-LYZ lens’ variations for pH 6.8, 7.0, 7.2 and 7.4 were tested for each other with a t-test, all p-values obtained were superior to 0.05 (see table 6), making these similar statistically. Therefore, although a decrease in lysozyme’s activity was observed along the duration of the assay, the overall decrease profile was not affected by the modifications induced on the lens, meaning that lysozyme’s physiological function is intact.

<table>
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<th>PVA-LYZ 7.2</th>
<th>PVA-LYZ 7.4</th>
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<td>PVA-LYZ 7.4</td>
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</table>

Table 6: Double entry table for the t-test values for each correlation for PVA-LYZ’s different buffers. When tested against the same, p-value obtained was 1. No p-values smaller than 0.05 were obtained.
IV.3. PVA Fiber production and lysozyme release kinetics

IV.3.1. PVA fibers production and total encapsulated protein quantification

Electrospun nanofibers were chosen as the encapsulation system for our device to use on colon cancer. Because of their higher superficial area and proneness to be modified, the release of lysozyme from the fibers can be optimized so as to obtain the minimal effective dose of 0.1mg/day to kill the cancer cells.

Before use, the fibers were observed under optic microscopy to quickly assure that fibers were produced with electrospinning. However, confirmation of fiber production could only be made by SEM analysis. Optic microscopy and SEM images can be compared in table 7; fiber analysis is also summarized in Figures 5, 6, 7 and 8:

The sample of PVA fibers used for SEM analysis didn’t have the envisaged characteristics for the fibers, since they fused together. This phenomenon has already been described in the literature (Raghavan BK 2011) and is dependent on all factor that affect solvent evaporation, namely relative humidity, temperature and voltage. The electrospinning device some problems on the deliver of an optimal voltage to the fibers, which made the fibers have more moist than expected and therefore enabling them to fuse together.

Figure 5: PVA fibers observed under electronic microscopy. Magnifications used were 1000X (A), 5000X (B) and 15000X (C).
In this case non-aligned PVA nanofibers were obtained. Process optimization was not as straightforward, and although tip-to-collector had been already optimized, flow-rate and voltage still had to be fine tuned in order to obtain fibers with few agglomerates. However, the main problem was the fibers were being wet by non-electrospun PVA solution. This was minimized through decreasing the flow-rate from 4mL/h to 1mL/h, and decreasing voltage from 26kV to 17.4kV. Solution volume and drying time had no effect on preventing this effect. Nevertheless the technique was improved with the increase in collector size allowed the production of fibers with higher porosity and also made them more straight (Figure 6A, 6B and 6C).

Figure 6: PVA-LYZ fibers observed under electronic microscopy. Magnifications used were 1000X (A, D), 5000X (B, E) and 15000X (C, F). Fibers in pictures A, B and C were collected using a rotating circular collector with 10cm diameter, while those in pictures D, E and F were using a collector with 3.4cm diameter.

Figure 7: PVA-LYZ-FB fibers observed under electronic microscopy. Magnifications used were 1000X (A), 5000X (B) and 15000X (C). Small inclusions can be seen in B and C, which might correspond to cross-linked PVA debris.
As was described by Nunes MAP and colleagues 2014, the cross-link of PVA with fluorophenylboronic acid decreased the porosity of the obtained fibers, making them more resistant to high temperatures. The changes that occurred in their work for the PVA fibers are very similar to the ones obtained in this work, as it can be seen in figure 7A-C. In fact, as was described, a decrease in porosity is observed when these fibers are compared with those from figure 6D.

The inclusions visible in Figures 7 and 8 are believed to be of different natures. While the ones present in figure 8 have a more spherical shape, and are believed to be the IONPs adsorbed on the fibers, in figure 7 these are believed to be PVA debries that cross-linked to the fibers.

Although the main last goal was achieved, the obtained fibers were not as homogeneous as one would have expected. This was due to failure in completely electrospin all the PVA solution due to some equipment problems, which allowed some solution to deposit directly on the fibers. Because the toxicological profile is not altered by this set back, and also due to the impossibility to synthesize more fiber samples, the “imperfect fibers” were used in the subsequent analysis, which included the release assay and the cell proliferation assay.
Table 7: Comparison between optical microscopy and SEM imaging of the obtained electrospun PVA fibers (PVA), with lysozyme encapsulates (PVA-LYZ), cross-linked with fluorophenylboronic acid (PVA-LYZ-FB) and with magnetic nanoparticles adsorbed (PVA-LYZ-FB-MN). Scale according to picture indication.

<table>
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<tr>
<th>Sample</th>
<th>Optical Microscopy</th>
<th>SEM</th>
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<tbody>
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<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td>PVA + LYZ</td>
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<td><img src="image4" alt="Image" /></td>
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<td>PVA + LYZ + FB</td>
<td><img src="image5" alt="Image" /></td>
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<td>PVA + LYZ + FB + MN</td>
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<td><img src="image8" alt="Image" /></td>
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IV.3.2. PVA fibers – lysosyme release kinetics

The results of the release assay for the simple PVA fibers with Lysozyme encapsulated can be analysed in chart 17.

![Chart 17: Release pattern of Lysozyme encapsulated in PVA fibers and incubated at room temperature in phosphate buffers at different pH.](image)

In this case, lysozyme release profile was heterogeneous across the different pH values. It was previously observed in the lenses that no statistical significant different was observed between the different buffers. In the case of the fibers, and using pH 7.4 as reference, pH 7.2, 7.0 and 6.8 did not gave a statistical significant similar profile (p < 0.05 for each of them). Curiously, release extension increased as pH decreased, meaning that possibly PVA degradation might be an important mechanism for lysozyme release when fiber encapsulation was involved. The same tendency was observed for one week of release (data not showed)

The next step was to evaluate the influence of cross-linking with FB and the adsorption of on lysozyme’s release profile at different pH values. The results can be seen in charts 18, 19, 20 and 21.
Chart 18: Release pattern of lysozyme encapsulated in PVA fibers, either simple (pH 7.4), cross-linked with FB (pH 7.4 + FB) and incubated with MN (pH 7.4 + FB + MN), evaluated at room temperature in phosphate buffers at pH 7.4.

Chart 19: Release pattern of lysozyme encapsulated in PVA fibers, either simple (pH 7.2), cross-linked with FB (pH 7.2 + FB) and incubated with MN (pH 7.2 + FB + MN), evaluated at room temperature in phosphate buffers at pH 7.2.

Chart 20: Release pattern of lysozyme encapsulated in PVA fibers, either simple (pH 7.0), cross-linked with FB (pH 7.0 + FB) and incubated with MN (pH 7.0 + FB + MN), evaluated at room temperature in phosphate buffers at pH 7.0.
During manufacturing, the fibers used for pH values of 7.4 and 7.2 coiled and formed a compact aggregate. Due to this change in the systems conformation, release profile of lysozyme might have been greatly altered. Even so, statistical analysis determined that, as was observed in the lenses, lysozyme release profile depended on the modification made to the system. In fact, no statistical significant difference ($p < 0.05$) was observed between the unmodified system and the modified ones for each pH.

Curiously, for pH values of 7.0 and 7.2 the release profile of lysozyme gave higher values than for pH 6.8. This could mean one of two things: (1) magnetic nanoparticles may assist in lysozyme release from the fibers, as was observed for the lenses, or (2) alternatively they are interfering with the Bradford readings of lysozyme, something found during the experiments but only observed for IONPs in suspension. Therefore, the evaluation of these charts must be careful and future more accurate procedures to measure Lysozyme concentration should be envisaged, such as one that uses High Performance Liquid Chromatography (HPLC).

### IV.3.3. PVA fibers – lysozyme activity and release kinetics

Similar to what was done to the lens, the activity of the lysozyme released from the fibers was evaluated using a 3mg/mL *Micrococcus Lysodeikticus* suspension. Using the obtained slopes for each sample, a concentration was calculated and the variance from
the concentration obtained by the Bradford method was evaluated. The results are summarized in table 8.

<table>
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<th>pH</th>
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<th>Concentration based on Lysozyme Activity (ug/mL)</th>
<th>Concentration Bradford Method (ug/mL)</th>
<th>Variance (%)</th>
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<td>60</td>
<td>2.61</td>
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Table 8: Lysozyme concentration, released from the PVA fibers at pH values 6.8, 7.0, 7.2 and 7.4 at the timepoints 0min, 5min and 60min. Concentrations were determined through Bradford method and lysozyme activity calibration curve. Variation > 0% indicates increase in activity, whether ≤ 0% indicates no variation or decrease in activity. P-Value was calculated with PVA-LYZ as the reference.

Overall, variance for lysozyme concentration was superior to 100%. In fact, it was surprising that samples with no lysozyme detected by the Bradford method were actually able to degrade *Micrococcus Lysodeikticus* to an extent of more than 100% when compared to the predicted values. This could mean that the detection limit for Bradford method is too high for the determinations made in this assay, meaning that a new and more sensible detection method (HPLC for example) should be employed.
Also, the fact that some of the determined concentrations for lysozyme activity were inferior to the lower value used for the calibration curve (7.8µg/mL) induced some error in the assumptions made before. Confirmation of the obtained concentrations using a more sensitive method (HPLC) should be envisaged for future works.

Finally, the fact that the obtained concentrations for lysozyme using the Bradford method were inferior to the ones obtained from the activity calibration curve was positive. This confirms the results obtained from the assays using the lenses, which demonstrated a tendency for a positive increase in the variance independently of the pH. This means that the encapsulated lysozyme not only retains its activity after the encapsulation and cross-linking processes, being therefore physiologically active to act on cancer cells, but also had superior activity than the predicted. This could mean that lower lysozyme concentrations can be tested without compromising its tumorigenic activity.

IV.4. PVA fibers – cell assays

After cells were grown for 10 days on the fibers, overall viability was assessed, by the MTT assay, only for the fibers. The results can be seen in chart 22:

The obtained values for the fibers tested were not exactly the expected ones. Cells were expected either to adhere to the fibers and enter in senescence, meaning that the expected viability would be lower than the adherent cells but not much, or to be killed due to the tumorigenic activity of lysozyme. From the analysis of these results, it seemed obvious that cells were more prone to follow the second route.
Confirmation for the MTT assay had to be done. Using the well-known protocol for staining Caco2 cells, phalloidin-DAPI combination was chosen and employed for staining both the cells and the fibers. The results can be analysed in figure 9.

![Figure 9](image)

**Figure 9:** (A-C) PVA-LYZ-FB and (D-F) PVA-LYZ-FB-MN fibers stained with DAPI-Phalloidin and observed under optic/fluorescence microscopy. (A, D) DAPI staining under fluorescence microscopy. (B, E) Phalloidin staining under fluorescence microscopy. (C, F) Fiber structure observed under optic microscopy. (G) Adherent Caco-2 cell’s nuclei stained with DAPI and observed under fluorescence microscopy.

Figure 5-G corresponds to the staining of Caco-2 cells with DAPI. Although phalloidin was also used to observe the cells cytoplasm, no signal from the cells was obtained. This probably means that there was a problem with the reagent’s quality, since this staining protocol is commonly used for these cells (Figueiredo PMS 2006, Zhou X 2014). However, the nuclei were still visible, which allowed to confirm the presence of the cells.

For PVA-LYZ-FB and PVA-LYZ-FB-MN fibers different magnifications were used due to the fibers’ topography that didn’t allow to focus on a single plane. Therefore, for the first one a smaller magnification was used so to focus better on the bright blue inclusions, whether for the second one a higher one was more appropriate.

The bright blue dots present in the DAPI pictures for both fibers (**Figure 9-A and 9-D**) might indicate the presence of genetic material. Despite PVA fibers are also stained by DAPI, the fluorescence intensity was lower and not as bright as seen for Caco-2 nuclei
in figure 9-G. Therefore, the bright blue dots in both fibers indicates the presence of cells within the fibers, possibly dead ones.

The question remains whether the cells on the fibers remain viable. MTT results show that viability was greatly reduced on the fibers. This can in fact be confirmed through the analysis of the bright blue dots morphology in figures 9A and 9C, whose irregular shape and fragmentation resembles more of apoptotic nuclei than one typical of viable cells. It is possible that cells adhered in a first step to the fibers, but eventually died due to the tumorigenic properties of our system. A closer follow-up should be planned to accompany this process should be planned so as to measure the cell growth kinetics. At the same time, cell death should also be confirmed using more specific assays, such as caspase-3 activity determination or Annexin-V.
Conclusion and future trends
V. Conclusions and future trends

From the work presented here, the following conclusions can be made:

1. Lysozyme activity was dependent of its concentration following a quadratic trend;
2. Using electrospinning, fibers containing lysozyme encapsulated were produced;
3. Lysozyme was successfully encapsulated in lenses and fibers, and could be released gradually to the buffer;
4. Lysozyme release from the systems tested was independent of the pH, although minor variations in the initial release can be explained by PVA degradation;
5. Lysozyme encapsulated both in the lenses and fibers was able not only to retain its activity, but this was also enhanced and was independent on the type of modification induced to the system;
6. Fibers containing lysozyme encapsulated, cross-linked with fluorophenylboronic acid and/or with IONPs adsorbed, were able to reduce viability of Caco-2 tumor cells seeded on them.

These observations conclude the preliminary studies of our system in the presence of tumoral cells, and point to a promising research project to be developed in the following years. Much work is still missing to fully understand the interaction between the envisaged system and tumor cells.

Some assays are still missing to complement the in vitro studies of this system. For example, being our system exposed to relative higher temperatures due to nanoparticle’s hipertermia (45-60°C) it would be important to study the effect of temperature on the release and remanescent activity of lysozyme from the fibers (30°C, 37°C, 40°C and 45°C and 60°C). It is a known fact that cross-link of PVA with fluorophenylboronic acid enable the first to widstand high temperatures (121°C), but the question remains if this protection was also extended to the encapsulated proteins.

To confirm the obtained concentrations for the release assays, more sensible detection methods for lysozyme should also be used in order to explain in depth the differences observed between Bradford determined and lysozyme activity evaluated concentrations. One hypothesis would be to use HPLC as was described by Charernsriwilaiwat N 2012.

Also, PVA-Lysozyme interaction was not analysed in depth. Therefore, future studies should also aim to evaluate this interaction inside of the fibers (FTIR, DSC), before and after cross-link with fluorophenylboronic acid.

One of the drawbacks of this work was the inability to accurately measure individually fibers’ lysozyme content, due to the inability of PVA to dissolved in the solvents described in the literature (Chloroform), and the quantity of lysozyme adsorbed to the fibers, since SDS, which was used by Yang Y 2007, interfered with Bradford method for determining total protein concentration. Maybe HPLC could be used to evaluate SDS
interference more efficiently, since the method has enough resolution to distinguish between SDS and Lysozyme.

Finally, cell assays should be repeated. A cell growth kinetic profile should be established to evaluate cell proliferation on the fibers (MTT assay or Alamar Blue) along 10 days. Cell staining should be repeated also for each of the timepoints used in the kinetics, using DAPI and fresh phalloidin, so as to see the cells structure and distribution along the fibers. Cell death should also be investigated, using caspase-3 activity determination, TUNNEL assay and Annexin-V assay to confirm that cells dye through apoptosis.
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