Universidade de Lisboa

Faculdade de Farmácia

3D printing-based technology using mesenchymal stem cells for personalized psoriasis therapy

Rita de Oliveira Barros

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Trabalho de Campo de Mestrado Integrado em Ciências Farmacêuticas apresentado à Universidade de Lisboa através da Faculdade de Farmácia

Orientador: Professora Auxiliar, Joana Marques Marto

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Resumo

A psoríase é uma doença inflamatória crónica que afeta cerca de 2-3% da população mundial. Esta doença é caracterizada pela hiperproliferação aberrante dos queratinócitos e infiltração de células inflamatórias na derme que resultam no aparecimento de lesões cutâneas ruborizadas, pruriginosas e em placa. A sua patogénese tem sido intensamente estudada ao longo dos anos, no entanto o mecanismo exato da doença é bastante complexo e permanece elusivo. Esta complexidade inclui fatores genéticos, epigenéticos e ambientais e uma forte interação entre o sistema imunitário inato e adaptativo. O desequilíbrio de vias inflamatórias leva a uma forte ativação das células dendríticas e produção de péptidos antimicrobianos, citocinas e quimiocinas que exacerbam a resposta inflamatória.

Atualmente, o tratamento tópico desta patologia passa pela aplicação de cremes emolientes, queratolíticos e anti-inflamatórios para tratar as zonas afetadas e diminuir a inflamação da pele. Nos casos mais severos da doença, muitas vezes é necessário recorrer a terapêutica sistémica, que pode desencadear efeitos adversos mais graves. Assim sendo, as terapêuticas tópicas são preferíveis, contudo, atualmente apenas aliviam os sintomas e são insuficientes no tratamento da patologia. Por este motivo, e considerando que a indústria farmacêutica está em crescente desenvolvimento, é incentivado o desenvolvimento de abordagens terapêuticas inovadoras com tecnologias mais avançadas. O secretoma das células estaminais mesenquimais (MSC) poderá ser uma boa opção para contornar este problema.

As células estaminais mesenquimais (MSC) pertencem a um subgrupo de células estaminais e podem ser isoladas a partir de vários tecidos como a medula óssea, o cordão umbilical, os dentes, a pele ou mesmo o tecido adiposo. Estas células possuem propriedades regenerativas e imunomoduladoras, através da secreção de fatores bioativos que atuam de forma parácrina, induzindo a remodelação tecidular. Tendo em conta a fragilidade destas células, vários estudos referem vantagens na utilização do secretoma celular em substituição das mesmas. O secretoma constitui os biofatores produzidos pela maquinaria celular como proteínas, hormonas, lípidos e ácidos nucleicos que exercem funções importantes não só no seu desenvolvimento, mas também no de outras células. As suas mais-valias incluem uma fácil utilização e armazenamento, uma menor imunogenicidade e acessibilidade económica. Considerando estas suas propriedades, o secretoma das MSC é um potencial candidato para uma terapêutica de inovação tópica.

Atualmente, o desenvolvimento de biotintas para aplicação tópica recorrendo à impressão 3D tem sido revolucionária no sentido em que permite a produção de formas farmacêuticas melhoradas e personalizadas. A impressão 3D é uma técnica utilizada em diferentes áreas incluindo produção de órgãos, próteses, implantes, modelos anatómicos e formas farmacêuticas. Esta tecnologia baseia-se na criação de um ficheiro modelo 3D através de um software digital de design, em inglês denominado por *computer aided design* (CAD) software. Depois de desenvolvido, este ficheiro modelo é convertido num formato reconhecido pela impressora a utilizar e procede-se a impressão 3D da biotinta com o design pré-definido. Dentro das várias tecnologias de impressão 3D, a extrusão é a mais adequada para a bioimpressão de células porque permite a impressão de formulações semissólidas à temperatura ambiente, mantendo a viabilidade celular; permite também a utilização ampla de vários polímeros e é facilmente personalizável, ajustando parâmetros da formulação e do processo.

A escolha do polímero para a formulação da biotinta é de extrema importância porque os seus parâmetros físico-químicos irão influenciar significativamente a qualidade do produto final. Um polímero ideal deve ser biocompatível, biodegradável, pouco tóxico e possuir boas propriedades mecânicas para manter a integridade do impresso 3D. Há dois principais tipos de polímeros: os polímeros naturais e os polímeros sintéticos, sendo que os primeiros geralmente possuem melhor biocompatibilidade, mas piores propriedades mecânicas relativamente aos últimos, que possuem pior biocompatibilidade, mas melhores propriedades mecânicas. O alginato e o quitosano são dois polímeros naturais frequentemente utilizados por investigadores na impressão 3D de células porque, além de possuírem as características previamente descritas, também são relativamente económicos. Após seleção dos polímeros, é necessária a produção de uma formulação otimizada que seja imprimível, mantenha a sua integridade após impressão e que constitua um meio adequado para a incorporação do secretoma celular. No sentido de evitar gastos excessivos de tempo e dinheiro no desenvolvimento de várias formulações e apenas posteriormente averiguar a sua qualidade numa abordagem *quality by testing*, a indústria farmacêutica tem apostado numa abordagem muito mais eficiente denominada *quality by design* (QbD).

A QbD é uma abordagem sistemática, proativa e baseada na gestão de risco que incentiva a definição dos padrões de qualidade antes do processo de manufatura propriamente dito e a adoção de procedimentos consistentes para atingir o objetivo de qualidade previamente definido.

O principal objetivo deste trabalho é a otimização de uma forma farmacêutica tridimensional (3D) para futura libertação tópica de secretoma de MSC, utilizando uma estratégia de QbD.

Formulações iniciais de alginato e quitosano foram desenvolvidas para avaliar as suas propriedades físico-químicas, estudo esse que resultou na exclusão das formulações de quitosano por apresentarem valores de pH incompatíveis com a viabilidade celular. Subsequentemente, um estudo de otimização foi desenvolvido, onde se avaliou o impacto da concentração de alginato, da concentração do agente de gelificação e do efeito pré-*crosslink* no tempo de gelificação, que foi definido como a característica de qualidade a controlar. Os resultados demonstraram que apenas o agente de gelificação apresentou um impacto significativo no tempo de gelificação. Esta abordagem de QbD é importante para perceber em que concentrações e condições se deve trabalhar para atingir a qualidade do produto pretendida.

A formulação otimizada foi utilizada como prova de conceito para corroborar a sua sustentabilidade para incorporar o secretoma celular das MSC. A produção de formas farmacêuticas que requerem a manipulação celular, deve ser realizada em condições asséticas. Quando isso não é possível, opta-se pela esterilização do material e meio de cultura e adição de conservantes à formulação. O impacto do método de esterilização, bem como da incorporação de meio celular na formulação foi avaliado recorrendo a uma análise reológica. Verificou-se uma menor interferência nos valores iniciais de viscosidade com o método de esterilização UV-C e assistiu-se a um aumento de viscosidade com a adição de meio celular. Também foi estudada a influência da incorporação na viscosidade da biotinta e a biocompatibilidade de cinco conservantes diferentes, tendo sido selecionado o Nipagin™/Nipazol® como o mais adequado. O processo de impressão e a qualidade do produto final também foram avaliadas e concluiu-se que a formulação otimizada está efetivamente apta para ser utilizada na incorporação de secretoma celular de MSC. Trabalhos futuros nesta área podem incluir o estudo e otimização de formulações com misturas de polímeros naturais e sintéticos, a fim de se obter um melhor comprometimento entre biocompatibilidade e propriedades mecânicas e também estudar a libertação de secretoma celular de MSC através da forma farmacêutica 3D.

Palavras-chave: Células estaminais mesenquimais (MSC); impressão 3D; Alginato; Quality by Design; Aplicação tópica

Abstract

Psoriasis is a chronic inflammatory disease characterized by hyperproliferation of keratinocytes and infiltration of inflammatory cells manifested by characteristic skin lesions typically red, itchy, and scaly. Enhanced understanding of the underlying pathomechanisms and the interplay between the innate and adaptive immune system resulted in the development of many therapeutical options. Nevertheless, psoriasis remains without cure and this lack of curative therapeutics in the market demands a search for novel therapeutical approaches.

Mesenchymal stem cell (MSC) derived secretome presents regenerative and immunomodulatory properties and therefore may be a suitable option if embedded in the adequate bioink.

In the pharmaceutical industry, the development of hydrogel bioinks for topical application using semisolid extrusion-based 3D printing has been revolutionary since it allows the production of improved pharmaceutical forms with tuneable properties.

Alginate and chitosan are natural polymers commonly used by researchers in this area due to their biocompatibility, biodegradability, non-toxicity, and relatively low cost.

The main goal of this project was the optimization of a 3D system for future application for topical delivery of secretome using a Quality by Design (QbD) approach. Initial formulations were developed to understand physicochemical properties of both polymers and chitosan formulations were excluded for not presenting biocompatible pH values. Then an optimization study was performed where the impact of different parameters on gelation time was evaluated. The results showed that only the crosslinking agent had a significant impact on gelation time.

A proof of concept was performed to guarantee the suitability of the optimized hydrogel in encapsulating cells secretome. The rheological influence of two sterilization methods, incorporation of preservatives and cell culture medium were studied, as well as the biocompatibility of five different preservatives. The printability process and the quality of the final product in terms of structural properties were also evaluated. The results obtained confirmed the potential use of this bioink for secretome delivery.

Keywords: Psoriasis; Mesenchymal Stem Cells (MSC); 3D printing; Alginate; Quality by Design (QbD); Topical delivery

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Index

Resumo 3

Abstract 6

Agradecimentos 7

List of abbreviations 13

Introduction 14

1.Psoriasis 14

1.1 Introduction 14

1.2 Pathogenesis 14

1.3 Treatment 16

2. Mesenchymal Stem Cells (MSC) 16

2.1 MSC-derived secretome delivery systems 17

3. Three-dimensional printing (3DP) 18

3.1 3D Extrusion-based 3D printing 19

3.2 Bioink properties 20

3.3. Polymers 22

3.3.1 Natural polymers 22

3.3.1.1 Alginate 22

3.3.1.2 Chitosan 23

3.3.1.3 Gelatin 24

3.3.1.4 Collagen 24

3.3.1.5 Fibrin 25

3.3.1.6 Hyaluronic acid 25

3.3.2 Synthetic materials 26

4. Quality strategies 26

4.1 Quality by testing (QbT) 26

4.2 Quality by design (QbD) 27
Aim and hypothesis 28
Materials and Methods 29
1. Materials 29
2. Methods 29
2.1 Process mapping 29
2.2 Selection of Polymers 30
2.3 Alginate and Chitosan Hydrogel Inks 30
2.4 Physicochemical Characterization of Alginate and Chitosan Hydrogel Inks 30
2.5 3D Printing Process 31
2.6 Validation and/or exclusion methods 31
2.7 Formula optimization and establishment of Design Space 32
2.8 Hydrogel 3D Patches Characterization: Gelation time 33
2.9 Proof of concept: Secretome incorporation in alginate 3D hydrogel patches 33
2.9.1 Alginate sterilization 33
2.9.2 Alginate-based hydrogels 34
2.9.3 Viscosity measurements 34
2.9.4 Cytotoxicity assay 34
2.9.5 3D printing 34
Results and discussion 35
1.Process mapping 35
2. Selection of Polymers 36
3.Formulation development 37
4.Validation and/or Exclusion method 38
5.Quality by design approach 39
5.1 Establishment of quality target product profile (QTPP) 39
5.2 Adjustment of CPPs 40
5.3 Formula Optimization and Establishment of Design Space 41

6. Proof of concept: Topical delivery of cells' secretome 43
6.1 Influence of the sterilization process in alginate viscosity 43
6.2 Influence of preservatives in alginate viscosity 44
6.3 Influence of cell culture medium in alginate viscosity 45
6.4 Citotoxicity assay of the preservatives 46
6.5 3D printing 47
Conclusion 48
References 50

Table of contents – Figures

Figure 1- Pathogenesis of psoriasis 15
Figure 2-3D printing techniques a) Laser-assisted printing; b) Inkjet-based printing; c) Extrusion-based printing 19
Figure 3-Extrusion mechanisms a) pneumatic force; b) piston force; c) screw force....20
Figure 4-Quality by design: Product and process understanding.....28
Figure 5-Decision-making scheme: validation and/or exclusion method. 32
Figure 6-Ishikawa diagram illustrating factors that may have impact on the development of a 3D hydrogel patch. 36
Figure 7-Viscosity for F1 to F13 versus shear rate. Alg-aginate; CS-chitosan; LMW-Low molecular weight; MMW-Medium molecular weight; HMW-High molecular weight..38
Figure 8-Response contour plot of the fitted model for gelation time (a) and design space (b). 42
Figure 9-Influence of sterilization process in alginate hydrogel viscosity.....44
Figure 10-Influence of preservatives in alginate hydrogel viscosity. 45
Figure 11-Influence of cell culture medium on alginate viscosity. CF-control formulation (water as solvent); TF-test formulation (cell culture medium as solvent). 46
Figure 12-Cytotoxicity of alginate solutions containing different preservatives. Data expressed as mean ± SEM. *** $p < 0.01$ ** $p < 0.01$ and * $p < 0.1$. CF-control formulation (water as solvent); TF-test formulation (cell culture medium as solvent). 47
Figure 13-Evaluation of the printability and printing accuracy (PA) of the bioinks. 48

Table of Contents – Tables

Table 1-Quantitative and qualitative composition of the initial formulations..... 38
Table 2-Summary of regression analysis results for measured response (Full Factorial Design composed by 3 levels), for formula optimization. 41
Table 3-ANOVA parameter summary of fitted model's characterization..... 41

List of abbreviations

- APC-antigen presenting cell
- TLR- toll-like receptor
- pDC-plasmacytoid dendritic cell
- IFN-interferon
- mDC-myeloid dendritic cell
- TNF-α-tumour necrosis factor alpha
- IL-interleukin
- MSC-mesenchymal stem cell
- CAM-cell adhesion molecules
- TDDS – Transdermal drug delivery system
- 3DP-three-dimensional printing
- CAD-computer aided design
- CMA-critical material attributes
- CPP-critical process parameters
- CQA-critical quality attributes
- QTPP-quality target product profile
- DS-design space
- QbT- quality by testing
- QbD-quality by design
- RSM-response surface methodology
- RA-risk analysis
- FFD-full factorial design
- CE-cetrimide
- NN-Nipagin^{TM}/Nipazol®
- BKC-benzalkonium chloride
- CHX-chlorhexidine
- SHMG- sodium hydroxymethylglycinate
- PA-printing accuracy

Introduction

1.Psoriasis

1.1 Introduction

Psoriasis is a chronic inflammatory skin disease affecting 2-3% of the worldwide population. It is characterized by hyperproliferation and abnormal differentiation of keratinocytes and infiltration of inflammatory cells into the dermis, which result in characteristic plaque skin lesions typically red, itchy, and scaly. Psoriasis is a complex disease because it can emerge in different forms, affect different body areas and therefore can be categorized in five main types: plaque, guttate, inverse, pustular, and erythrodermic (1,2). Despite being a skin disease, it may also affect joints, leading to arthritis; the cardiovascular system, increasing the risk of cardiovascular events and, moreover, promote mental disorders due to the psychological stress and poor quality of life (3).

Many studies have reported various factors contributing to the pathogenesis of psoriasis, including genetic factors, the immune system, and environmental conditions, thus describing it as a multifactorial disease (1).

1.2 Pathogenesis

The immunopathological mechanisms of psoriasis are still not very well described exactly because it is a multifactorial disease. Nevertheless, it is reported that innate immune responses start with antigen presenting cell (APC) activation by a skin antigen. When under stress or injury, keratinocytes secrete antimicrobial peptides, such as LL-37, which are inherently overexpressed in psoriatic skins. These peptides connected to DNA stimulate toll-like receptor (TLR) 9 in plasmacytoid dendritic cells (pDC). This activation is marked by the production of type I interferon (IFN) which has two forms, IFN-α and IFN-β, and is important in the development of the characteristic psoriatic plaques. Myeloid dendritic cells (mDC) are maturated and activated through type I IFN signalling and, subsequently, they migrate into draining lymph nodes. There they secrete several cytokines in which are included tumour necrosis factor (TNF-α), interleukin (IL)-23, and IL-12. The former exerts pleiotropic effects on a wide array of cells from different lineages (4) and the latter two modulate the differentiation and proliferation of Th17 and Th1 cell subsets, respectively, which in turn, will activate the adaptive immune response (5).

Subsequently, the activated T-cells produce other psoriatic cytokines, such as IL-17, IL-22, interferon (IFN)-ɣ, and tumour necrosis factor-α (TNF-α) which lead to uncontrolled keratinocyte proliferation. The crosstalk between keratinocytes, adaptive immune cells, and innate immune cells, mediated by cytokines and chemokines, sustain the persistence of psoriasis in a chronic phase (1,2,4).

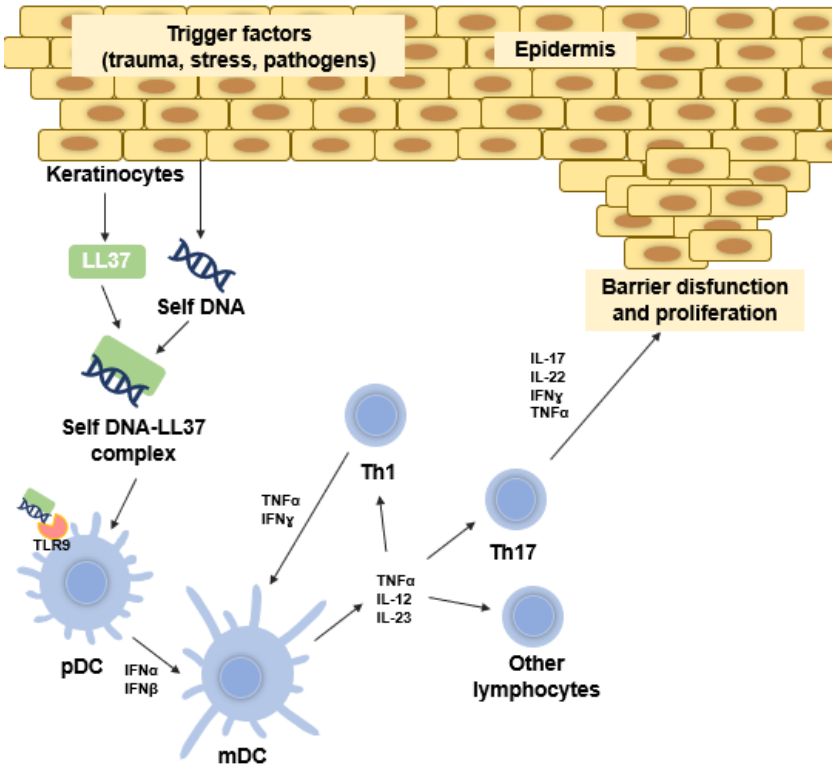


Figure 1- Pathogenesis of psoriasis. Retrieved from reference (5)

Psoriasis can also be caused by genetic mechanisms concerning about 40 loci associated with psoriasis susceptibility, skin barrier functions, and innate and adaptive immunity.(6) Epigenetic factors also play a considerable role in psoriasis pathogenesis: including dysregulated DNA methylation levels, abnormal histone modification and microRNAs expressions (2).

In addition to genetics and altered immune function, several other risk factors may predispose patients to psoriasis. These include environmental triggers (infection and stress); medications (lithium, beta-blockers), other immune-mediated diseases (Crohn's disease, multiple sclerosis) and psychogenic stressors (emotional or mental stress) (6).

1.3 Treatment

Earlier treatments have included application of emollients or keratolytic agents to hydrate or shed off the skin, whereas later treatments are more focused on treating the underlying T-cell proliferation. Topical treatments like coal tar, vitamin D, retinoids, topical calcineurin inhibitors for treating mild psoriasis, systemic treatments including methotrexate, cyclosporine, acitretin, hydroxyurea, as well as light therapy for severe psoriasis have become more prominent. Development of biologics such as TNF-α inhibitors (infliximab, adalimumab), IL-17 inhibitors (secukinumab, ixekizumab and brodalumab) and anti-interleukin (IL)-12/23p40 antibody (ustekinumab) have revolutionized the treatment for this pathology, helped understanding the pathogenesis of the disease and provided excellent results in efficacy. However, and as with all biologic therapies, their safety has raised concern among clinicians (7,8).

Due to the non-curative profile of current topical therapies and the safety issues concerning the biologics, there is an urgent need to develop novel medicines and personalized therapies. The secretome of Mesenchymal Stem Cells (MSC) embedded in the ideal biomaterial may be the answer.

2. Mesenchymal Stem Cells (MSC)

MSC belong to a subgroup of stem cells that can be isolated from various tissues including bone marrow, umbilical cord, teeth, skin, or even adipose tissue (9). These cells are a promising therapeutic approach in psoriasis because they offer tissue regenerative and immunomodulatory properties by inhibiting the differentiation into the Th1 and Th17 subsets of helper T cells and by promoting the generation of regulatory T cells (10).

MSC present three main assets: firstly, they are proficient in migrating to the sites of injury due to chemoattraction mediated via chemokine receptors present in plasma membrane. The mechanism through which MSC interact with the endothelium at the damaged sites remains elusive, but it is related to cellular adhesion molecules (CAMs), specially integrins and selectins (9).

Secondly, they present *in vivo* differentiation skills. Many authors reported that these cells may induce tissue regeneration without local engraftment and differentiation prior to administration. For example, MSC have been proved to promote angiogenesis by easily

migrating to damaged sites and undergoing differentiation *in vivo* into endothelial cells, a very useful property in treating cardiovascular diseases (11–14).

Lastly, MSC secrete bioactive factors. The majority of MSC loses their viability when transplanted into the host tissue, and still, their regenerative and immunomodulatory effects are very impressive. This may indicate that their beneficial effects are not a consequence of the MSC themselves, but of their secreted factors that act via paracrine activation of neighboring cells (12).These factors, also known as MSC-derived secretome, include all excreted molecules to the extracellular space: growth factors, cytokines, lipids, nucleic acids, and proteins (TGF-β, NO, IDO, TSG6, prostaglandin E2, IL-1 receptor antagonist and IL-10, among others). The secretome has been preferred over MSC because it offers several advantages (15) like avoiding unwanted differentiation of engrafted MSCs, which could lead to immunogenicity; better control in dosage and potency; better storage; lower price; potential for being commercialized and therefore available for acute disease treatment (12,16).

Secretome composition differs between tissue sources and between different donors depending on the health condition and age. Therefore, different types of secretome may be indicated depending on the desired clinic uses. Other parameters should be taken into consideration such as the type of media, temperature, pH, cell density, oxygenation, and mechanical, electromagnetic, or biochemical stimuli. For instance, in case of hypoxia, MSCs increase the production of angiogenic and anti-apoptotic factors, such as VEGF, IL-6, CCL2, and STC-1. As such, it is possible to personalize secretome-derived products according to the patients' needs (12)

2.1 MSC-derived secretome delivery systems

Secretome delivery systems should guarantee their adequate release, ergonomic administration, and good stability. Topical drug delivery systems, such as, topical patches are a suitable option and have been gaining popularity in the pharmaceutical industry because of their controlled release, painless administration and few side effects (17). Despite their designation, these systems are aimed not only for drug delivery, but also for bioactive factors or cell delivery.

Hydrogels have been selected as the best material for topical drug delivery patches in biological applications owing to their biocompatibility and biophysical similarity to natural tissues and good porosity, which allows permeability to biochemical molecules (18,19).

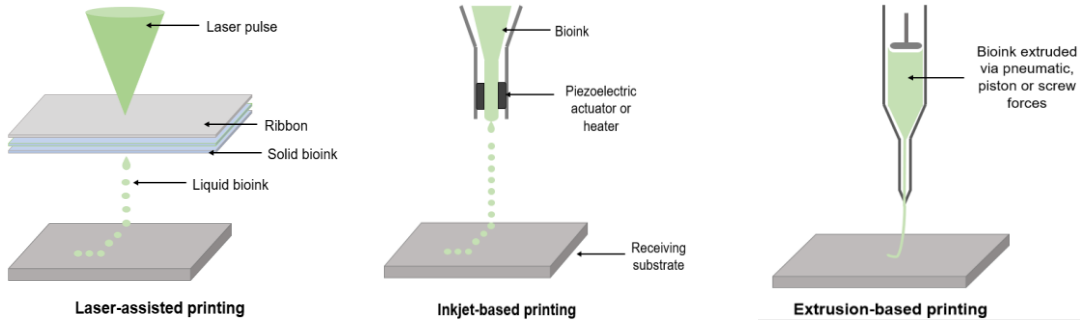
Hydrogels are three-dimensional networks composed by hydrophilic polymers mixed in aqueous media that can hold large amounts of water in their structure and therefore can act as biomaterials. In a psoriasis therapeutic context, MSC-derived secretome could be encapsulated in a hydrogel for future production of a patch. Three-dimensional printing appears in this context as a promising and modern technique for the fabrication of these patches (12).

3. Three-dimensional printing (3DP)

3DP is recognized as an additive manufacturing process that is rapidly expanding and creating new opportunities in health care. It comprises a wide range of areas such as tissue and organ fabrication; production of prosthetics, implants, and anatomical models; and research in drug delivery systems. In pharmaceutical industry, 3DP has revolutionized personalized therapy, especially for topical application, as it provides improved pharmaceutical dosage forms for different groups of people through design flexibility and accurate drug or bioactive loading (20–22).

Summarily, this technique is based on the construction of a three-dimensional system from the digital creation of a 3D model file with a computer-aided design (CAD). This file is then converted into a printer recognizable format (eg. STL) and imported to the software where the number and height of printed layers are generated. These steps are transversal to all 3DP strategies. What varies between them are the processes through which the 3D construct is assembled i.e, the printing techniques (20).

Since the introduction by Charles Hull in 1980 of stereolithography, a technique for converting CAD into real objects by projecting light patterns into a pool of photopolymers, there has been a remarkable increase of the reported publications on this area and different types of printing technologies have been developed. Currently, the three main 3DP technologies are laser-assisted (figure 2a), inkjet-based (figure 2b), and extrusion-based printing (Figure 2c). Among these, extrusion-based printing will be the main focus of this project, since it constitutes the most researched and appropriate technique for bioprinting (23,24).



a) **b)** **c)**

Figure 2-3D printing techniques a) Laser-assisted printing; b) Inkjet-based printing; c) Extrusion-based printing. Retrieved from reference (22)

3.1 3D Extrusion-based 3D printing

Extrusion-based 3DP method is becoming increasingly popular as it is relatively cheap and ergonomic (25). This method is characterized by the continuous extrusion of a bead of material from a nozzle and its deposition layer by layer. During this process, the dispensing head is moved along X and Y axes controlled by the robotic system to deposit the bioink onto a stage, and moved up or down along the Z axis (21).

Bioprinting technology requires four main components: polymer solution, viable cells or their secretome, gelation agent and 3D printers. The polymer solution together with the cells/secretome constitute the bioink. The bioink is generally inserted in disposable plastic syringes and extruded out of the nozzle either pneumatically or mechanically (piston- or screw-driven) (Figure 3). The piston approach offers advantages over the pneumatic system in terms of flow control owing to delays associated with the compressed gas volume. Screw-based deposition provides more spatial control and is capable of dispensing bioinks exhibiting higher viscosities. However, the larger pressure drops generated by this extrusion method can be harmful for the suspended cells due to possible disruption of the cell membranes which results in cell death. Due to its simple structure and the possibility to adjust the air pressure, pneumatic deposition systems can be used for a broad range of bioink types and viscosities (26).

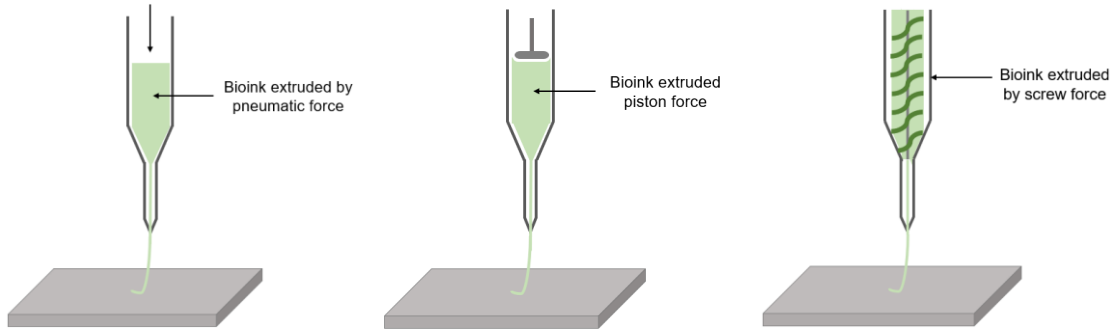


Figure 3-Extrusion mechanisms a) pneumatic force; b) piston force; c) screw force. Retrieved from reference (21)

Advantages of extrusion-based 3DP include the ability to print viscous bioinks with higher cell densities. However, this technique has some inherent limitations— potential nozzle clogging, shear stress applied to the bioink at a small orifice leading to cell death, and a limited selection of materials because of viscosity and rapid encapsulation of cells (23,27–29).

Along these lines, materials used in the bioink must be carefully selected according to their properties. On one hand, the printing process must occur at room temperature, since the cells and their secretome cannot tolerate high temperatures. On the other hand, the bioink must be semi-solid at printing temperature so that it is viscous enough to maintain the three-dimensionality and support subsequent layers and fluid enough to allow the ink flow through the nozzle and provide a viable environment for the cells/secretome (26,28,30).

3.2 Bioink properties

Bioinks require a set of physicochemical properties such as biocompatibility, printability, and viscosity among others, that will ensure the quality and functionality of the 3D patch (24,30).

Biocompatibility refers to the capacity of a biomaterial to perform an adequate host response, without causing negative effects. A biocompatible material should support cell proliferation, migration, and differentiation, facilitate signaling pathways, protect cells from the immune system, and be biodegradable without originating harmful degradation byproducts. Bioinks must be developed considering the sensitivity of cells and their secretome. Therefore, printing processes must be performed with low pressure and temperature environments, within a range that guarantees cell viability (21,22,28,31).

Another essential property of bioinks is printability, which refers to the ability to assemble and maintain a structure with high shape fidelity to the desired CAD. Printability depends on several factors, among which are viscosity, surface tension, and crosslinking mechanisms (28,31).

Viscosity describes the internal resistance of a fluid to flow under shear stress. The viscosity of a polymer solution is determined by its concentration, molecular weight, and temperature. Higher polymer concentration and molecular weight are associated with higher viscosity. Bioinks must be fluid enough to properly flow through the nozzle and maintain cell viability but viscous enough to maintain the integrity of the 3D construct and avoid the collapse of the 3D layers and/or merging of adjacent strands.(30–32)

Shear thinning is another property of bioinks that promotes printability and biocompatibility. It illustrates the natural decrease in viscosity with the increase of shear rate. This feature is more notorious in formulations of higher concentrations. When inside a printer nozzle, the shear stress increases and thus viscosity decreases, which improves cell viability. Nevertheless, when the tension is suddenly released, there is an increase in viscosity, resulting in a high printing fidelity (31).

The interaction between biomaterials and substrate is essential because the attachment of the 3D construct to the printing substrate guarantees its stability and avoids deformation and movement during the layer-by-layer deposition process (31).

Crosslinking mechanisms also play an important role in printability. Crosslinking is a stabilization process in which, by action of a crosslinking agent to a hydrogel, different polymer chains are connected by covalent or ionic bonds, leading to solidification of the structure. A crosslinking process should be rapid enough to maintain shape fidelity but slow enough to guarantee all polymer chains are correctly crosslinked. Currently, ionic, photo, and thermal crosslinking are the most frequently used crosslinking mechanisms in bioprinting (30,31,33).

In some cases, hydrogels are submitted to pre-crosslinking process because it can considerably improve their viscosity properties and thus improve their printability. The amount of crosslink should be appropriately selected to avoid alterations in the flow properties of bioinks due to phase change (30). To ensure bioinks possess all the mentioned properties, it is important to study their composition and thus select a polymer that will meet the desired specifications.

3.3. Polymers

Polymers are large molecules made up of chemically bonded smaller repeating units, called monomers. They can be divided into natural and synthetic groups according to their origin (34).

3.3.1 Natural polymers

Natural polymers originate from various living organisms and exhibit highly biocompatible characteristics. The majority of these polymers are water soluble and present common biological and physiological properties such as being flexible, easy to handle, and suitable for cell administration, protecting the cells during the printing process. Examples of these are alginate, chitosan, gelatin, collagen, fibrin, hyaluronic acid—which are formulated in the form of hydrogels (27).

3.3.1.1 Alginate

Alginate, also called alginic acid, constitutes a biodegradable, biocompatible, non-immunogenic, non-toxic, and low-cost anionic polysaccharide obtained from seaweed. It is frequently employed in bioprinting techniques due to the ability to gelate at physiological conditions, its viscosity properties, fast gelation rate and low toxicity. Alginate polymer has two monomers as a repeating unit, namely (1–4)-β-D-mannuronic acid and α-L-guluronic acid. α-L-guluronic acid promotes gel assembly, whereas the (1–4)-β-D-mannuronic acid and a combination of (L–4)-β-D-mannuronic acid and α-L-guluronic acid help in increasing the flexibility of the material (28,35). Water and other molecules can be trapped by capillary forces in an alginate matrix, but because this polymer has a wide pore size distribution (5-200mm) the diffusion of large molecules in and out of the gel is facilitated. Consequently, alginate is also commonly used for drug/growth factor delivery, where the degradation rate of the alginate can be tuned by altering the molecular weight of the alginate, which can in turn vary the release rate of the drug/growth factor encapsulated (29,36,37). Extrusion-based bioprinting of alginate has been one of the most popular techniques in the bioprinting community. Gelation of alginate is usually induced by divalent cations, such as calcium (Ca^{2+}), barium (Ba^{2+}) and strontium (Sr^{2+}) ions.(38) (35) In extrusion-based printing, calcium chloride ($CaCl_2 \cdot 2H_2O$) is the most frequent choice of gelation agent because it induces rapid post-printing cross-linking due to its high solubility in aqueous solutions. The divalent calcium ions form a bridge, due to the attraction of negatively charged carboxylic acid groups between two neighboring alginate chains (29).

Alginate can be extruded in one of two ways, precursor and pre-crosslinked by mixing it with low concentrations of the cross-linker, with the latter increasing its printability. The concentration of alginate determines the viscosity of the solution, porosity and crosslinking time (29).

3.3.1.2 Chitosan

Chitosan is the second most abundant polymer on earth after cellulose. It is a linear amino polysaccharide derived from deacetylation of chitin, composed of glucosamine and N-acetyl glucosamine units by linking with β (1–4) glycosidic bonds. Chitosan has a wide range of applications in tissue engineering (e.g cartilage regeneration), formation of sponge scaffolds, and wound healing owing to its biocompatibility, biodegradability, non-toxicity, and low cost (39). Besides providing cell support and differentiation, chitosan also plays a bactericidal role by interacting with negatively charged substances on the surface of bacteria and increasing cell wall permeability (35). The molecular weight and degree of acetylation are the two major properties of chitosan which affects its use as a matrix molecule for drug delivery. These properties affect its aqueous solubility and hydrophobicity thereby altering its drug encapsulation efficiencies (40).

Gelation of chitosan is usually processed by cross-linking agents such as glutaraldehyde, glyoxal, and ethylene glycol diglycidyl ether, however these may generate undesirable effects. To overcome these disadvantages, the ionic gelation with sodium tripolyphosphate (TPP), which has been approved as a “generally recognized as safe” ingredient by Food and Drug Administration, has been preferred by researchers. Protonated chitosan and the anionic phosphate groups of TPP can form biocompatible constructs through electrostatic interactions (41).

Chitosan is insoluble in aqueous environments above neutral pH and, in acidic environment it is completely soluble due to protonation of free amino acids (40). This is, however, not compatible with cell viability as cells only tolerate neutral gaps of pH. Studies on chitosan neutral solution were first proposed by Chenite et al.(2000) (42). They found that the addition of glycerophosphate salt promotes chitosan dissolution below room temperature, even with pH values within a physiologically acceptable neutral range from 6.8 to 7.2.

Moreover, chitosan presents low mechanical properties and, therefore, only highly viscous hydrogels are able to maintain the shape integrity (43).

3.3.1.3 Gelatin

Gelatin is a water-soluble protein extracted from bones, skin, and connective tissues of animals. It is derived from collagen through hydrolytic degradation, which involves the breakage of triple helix of collagen to obtain a single stranded gelatin molecule. Gelatin has been effectively used in development of bioink for bioprinting of materials owing to its unique properties including high biocompatibility and biodegradability, less immunogenicity, promotion of cell adhesion, differentiation, migration, and proliferation, considerable cross-linking potential, and better thermal stability in physiological environment (30). Gelatin is a thermoresponsive protein with a sol-gel temperature of around 30 °C depending on the gelatin concentration applied. Below 30ºC, gelatin strands start self-associating to form helical structures leading to a gel-like form, which reverts back to a random coil conformation as temperature increases. When applied to regenerative medicine**,** various functional groups corresponding with constituting amino acids can be easily modified with (meth)acrylate groups to prevent liquefying of gelatin at physiological temperature. This results in the formation of gelatin methacrylate (26) which is a versatile matrix, closely resembling the properties of natural extracellular matrix (ECM) and provides characteristics features such as cell attachment sites, proteolytic degradability, contains matrix metalloproteinase responsive peptide motif, and can be used to engineer tissue analogs (35).

3.3.1.4 Collagen

Collagen is the most abundant protein present in the ECM of mammalian cells and is highly conserved cross-species resulting in low immunogenicity. This protein presents similar physicochemical properties to human tissues, and high biocompatibility, and therefore has been widely used in biomedical applications. Collagen forms a hydrogel at physiological conditions by triple helix formation (29,30).

Collagen is a suitable material for cell encapsulation purposes because of the presence of cell-interactive RGD (Arginine-Glycine-Aspartic acid) sequences in their backbone, which stimulate cell adhesion (26).

Its crosslinking mechanisms are diverse and can go from temperature or pH change to vitamin Riboflavin. Collagen crosslinking promotes increased and improved mechanical and viscosity properties when comparing to non-crosslinked collagen. However, the crosslinking or gelation of collagen requires a minimum of 30 mins for gelation at 37 °C. Therefore,

employment of collagen directly in 3DP is difficult and thus, it is generally combined with different other gelation material (28).

3.3.1.5 Fibrin

Fibrin is a fibrous protein that is naturally present in native ECM and is enzymatically formed by polymerization of thrombin and fibrinogen. After polymerization, fibrin combines with platelets resulting in blood clotting at an injury site, a phenomenon known as coagulation. For this reason and due to its capacity of supporting cell growth and proliferation, it has been extensively used in wound healing and fabrication of skin grafts. However, the use of fibrin for *in vivo* applications can also result in highly immunogenic reactions or transmission of infectious diseases when heterologous proteins are used (24,28,29,44).

Fibrin gels present nonlinear elasticity, and thus their rheologic properties have been studied. These gels usually present high flexibility because the fibrin network allows a high degree of deformation without breakage. Despite presenting excellent biocompatibility, the fragile nature of fibrin and its high degradation rate create a limitation in maintaining the structure integrity of scaffolds. In addition, thrombin and fibrinogen present non-shear thinning behavior and thus, fibrin is not commonly used in extrusion 3DP (29,45). Also, it is difficult to manipulate fibrin after gelation because of its weak mechanical properties and therefore pre-crosslinking printing methods are not possible when using this polymer. Nevertheless, extensive efforts have been devoted to overcome this issue by combining fibrin with various polymers (28).

3.3.1.6 Hyaluronic acid

Hyaluronic acid (HA) is a non-sulfated linear glycosaminoglycan that is naturally present in ECM and in most connective tissues. It is composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine linked by alternating β-1,4 and β-1,3 glycosidic linkages. HA is an interesting polymer for bioprinting applications because it possesses appropriate elasticity, non-immunogenicity, biocompatibility, and degradability (29,35).

The modification of hydroxyl and carboxylic acid functional groups of hyaluronic acid enable the introduction of photocrosslinkable moieties which can be photopolymerized in the presence of cells (26).

Its main limitations are related to a slow gelation rate, poor mechanical properties and high hydrophilicity which makes scaffolds less stable. This dilemma has been solved through the adoption of strategies such as incorporation of hydrophobic moieties (28,29,35).

3.3.2 Synthetic materials

Even though natural polymers provide great biocompatibility by mimicking the ECM microenvironment for cell proliferation, their tunable properties are low. For this reason, natural polymers can be combined with synthetic polymers such as PEG, PCL and Pluronic. These polymers can be modified depending on the aims of bioprinting processes. For example, they can be tuned by introducing crosslinkable functional groups or domains that improve mechanical properties of scaffolds, according to the specifications of the target native tissue (29,35).

Usually, synthetic polymers are not used individually in bioprinting because they present poor biocompatibility, are usually bioinert materials without cell adhesion properties, and moreover, may generate toxic degradation byproducts. Hence, to make them more suitable for cells, they must be modified with the necessary binding peptides and enzymatically degradable groups. An example of this is the addition of methacrylate groups to gelatin to prevent its liquefaction at physiological temperatures (30).

Summarily, selecting the polymer is a key step in the production of bioinks because it will significantly affect their physicochemical properties and quality. When referring to quality, industries generally follow two main strategies to achieve it: quality by testing (QbT) which is usually assessed after the product has been manufactured and quality by design (QbD) which addresses issues before the production of the product, leading to a more efficient manufacturing process (46).

4. Quality strategies

4.1 Quality by testing (QbT)

Quality by testing technique used by the pharmaceutical industry to guarantee the quality of a drug product is a strict process with inflexible specifications. These specifications are not essentially based on critical quality attributes of the materials and critical process parameters but based upon intensive observation of produced batches. Quality by testing is targeted toward repeated reproducibility with narrow room for flexibility. Raw materials are

only employed if they meet the manufacturer's, FDA and USP standards. Out of specification drug products must be discarded and any change in operating parameters during manufacturing in a batch record must be notified to the FDA. This approach is not very comprehensive comparing to QbD because there is not a global understanding of the product and process and, moreover, it may generate waste owing to post-production quality assessment. If quality issues are detected using QbT, it is generally too late to fix the batch (47).

4.2 Quality by design (QbD)

Currently, the main goal of pharmaceutical industry is to develop high quality products, with low manufacturing costs associated, in a short interval of time. The application of QbD appears in this context as a tool to improve and accelerate formulation and manufacturing process, thereby enhancing the production of high-quality 3D constructs (3D patches). Pharmaceutical QbD is a systematic, holistic, risk-based and proactive approach to pharmaceutical development that encourages producers to establish their quality goals before the manufacturing process and develop features and consistent processes to achieve these goals (48).

According to W.A. Shewhart, quality is characterized by dependent variables and can be only attained through the optimization of the process inputs, i.e., the independent variables (49). In QbD, inputs are defined as the Critical Material Attributes (CMAs) and the Critical Process Parameters (CPPs) which refer to the properties of raw materials and process variables, respectively, that by appropriate selection and interaction, will deliver a high-quality product with its Critical Quality Attributes (CQAs). These refer to physical, chemical or biological properties that must always be within certain specifications since they affect patient security and efficacy and that will be necessary to achieve the predefined Quality Target Product Profile (QTPP). QTPP must be the first parameter to be determined, since it represents a prospective summary of the characteristics of the patch that will expectedly be achieved to ensure its desired quality, efficacy, and safety. (48,50,51) The multidimensional combination and interaction of CMAs and CPPs mentioned above is defined as Design Space (DS) (50).

Design of Experiments (DoE) has also been extensively used in pharmaceutical industry. It is a process optimization technique, which plans, experiments and analyzes the information from an output response when intentional changes are performed to the input variables. The technique allows using a minimum number of experiments, in which several experimental parameters are varied systematically and simultaneously to obtain sufficient information. Based

on the obtained data, a mathematical model of the studied process is created (52). Different methodologies may be used when creating a DoE such as the Taguchi method or Response Surface Methodology (RSM), among others (53).

Another key component of QbD approach is the Risk Assessment (RA), which consists of a systematic process of organizing information to support a risk decision. In RA, the criticality of parameters affecting the process is identified and ranked with the aid of adequate techniques such as Risk Estimation Matrix; Failure Mode and Effects Analysis; Failure Mode, Effects and Criticality Analysis; Fault Tree Analysis; Cause and Effect diagrams, amongst other tools described in the ICH Q9 guideline (51). The Cause and Effect diagram, also known as Ishikawa diagram, is a graphical tool capable for highlighting all possible variables which could pose a potential risk for the CQAs of a drug product. Resorting to these previous tools, it is possible to plan the production process, avoid wasting resources, mitigate the associated risks and attain high quality products (50).

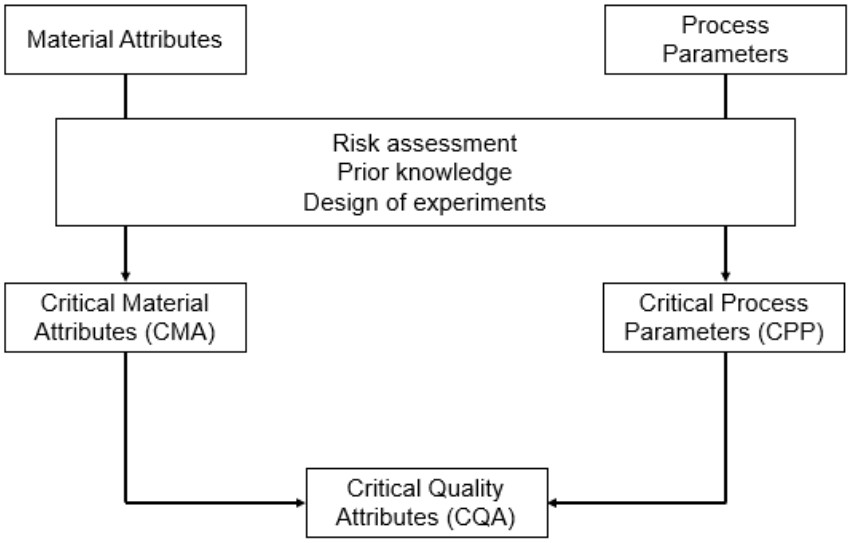


Figure 4-Quality by design: Product and process understanding. Retrieved from reference (54)

Aim and hypothesis

The present study aimed at developing a hydrogel patch for future application for topical delivery of MSC-derived secretome, as active principle for the treatment of psoriasis, resorting to an extrusion-based printing process. The hydrogel formulation and process parameters were optimized using a QbD approach. The different factors that can affect gelation time and construct integrity during the printing process were evaluated, including the percentage of

alginate, and $CaCl_2 \cdot 2H_2O$ and the effect of pre-crosslink. A proposal for a design space was also included.

To prove the usefulness of this hydrogel in integrating MSC-derived secretome, a proof of concept was designed and tested. The effect of the addition of secretome to the optimized formulation was studied as well as the rheological influence of two sterilization methods and the biocompatibility of different preservatives. The printability process and the quality of the final product in terms of structural properties were also evaluated.

Materials and Methods

1. Materials

Alginic acid sodium salt powder (medium-viscosity: ≥2000 cP), calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), chitosan low molecular weight (50,000-190,000 Da), chitosan medium molecular weight (190,000-310,000 Da), chitosan high molecular weight (310000-375000 Da), glycerophosphate and sodium tripolyphosphate (TPP) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Acetic acid (Acetic acid) and lactic acid (Lactic acid) were obtained from AppliChem (Spain). Benzalkonium chloride, α-MEM, DMEM high glucose were purchased by Sigma-Aldrich. FBS and PenStrep was obtained by Gibco®. Cetrimide was purchased by FeF Chemicals; Nipagin™ and Nipazol® were obtained by Fagron, Chlorhexidine was obtained by Acofarma and Hydroximethylglicinate 0.1% was purchased by Ashland. Purified water was obtained by reverse osmosis and electrodeionization (Millipore®, Elix 3), followed by filtration (filter pore 0.22 µm) and sterilization. The MSCs were isolated from human umbilical cord tissue, which was adapted to ATMP standards according to EMA guidelines (animal component-free, endotoxin-free, mycoplasma-free) and GMP-compliant.

2. Methods

2.1 Process mapping

An Ishikawa diagram was used to visually and graphically identify the causes of potential problems that may occur during the 3D hydrogel patch printing process using a 3D Focus printer (ByFlow Eindhoven, The Netherlands) by a semi-solid extrusion printing method. This approach enabled the establishment of the 3D printing technique workflow and the identification of the parameters that have the greatest chance of leading to product failure and that may influence the quality of the final product.

2.2 Selection of Polymers

The first step of the development of the bioink itself for the hydrogel patch was the selection of the polymer. For this project, natural polymers were preferred owing to their biocompatibility and non-immunogenicity. There is a wide range of natural polymers such as alginate, chitosan, hyaluronic acid, gelatin, among others, and therefore other selection criteria were included to narrow the final options: 1) low cost of the materials, and 2) the frequency of use of these materials in bioprinting. After applying these criteria, the selected polymers for this study were alginate of medium viscosity and chitosan of low, medium and high molecular because they were the most convenient for the desired viscosity target. An initial study was performed where several formulations were developed to understand the polymers physicochemical properties for future optimization.

2.3 Alginate and Chitosan Hydrogel Inks

Alginate hydrogels were prepared by dissolving alginic acid sodium salt in purified water in concentrations ranging from 1.5% to 7% (w/v) (55–57). Chitosan hydrogels were prepared by dissolving chitosan powder of low, medium or high molecular weight in concentrations ranging from 1% to 3% (w/v) and a mixture of lactic acid and acetic acid (1:1) 1% (w/v) in purified water (58–61). All formulations were magnetic stirred overnight (12 h) to obtain homogeneous hydrogels. The cross-linking solutions for alginate hydrogel inks were calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) in purified water, with concentrations ranging from 0.7% to 3.0% (w/v) (62,63). The cross-linking solutions for chitosan hydrogel inks were tripolyphosphate (TPP) in purified water with concentrations ranging from 1% to 3% (w/v) (64,65).

2.4 Physicochemical Characterization of Alginate and Chitosan Hydrogel Inks

Measurements of pH were determined and controlled using a digital pH-Meter with a glass electrode (SevenEasy™ by Mettler Toledo). Viscosity measurements were performed using a controlled stress Kinexus Lab +Rheometer (Malvern Instruments, Malvern, UK) employing a cone-and-plate geometry (truncated angle 4◦ and radius 40 nm). The measurements were performed between 1 and 1000 Pa on a logarithmic increment, ranging from 0.1 to 100 s−1.

In order to determine the gelation time, a single frequency strain-controlled time event sequence was designed. The measurements were carried out with a plate–plate geometry and a

gap of 0.5 mm (patch height simulation). Hydrogel inks were mixed with the crosslink solution at a ratio of 1:10 and vortexed for 30 s before sample loading to simulate a non-saturation gelation process. The onset of gelation time was recorded as the time when a minimum of 7 successive data points showed the sol/gel transition point. All measurements were performed at 25 ◦C.

2.5 3D Printing Process

The 3D Focus printer (ByFlow, Eindhoven, The Netherlands) was used to evaluate the printability of the generated inks. All hydrogels were printed using a 0.8-mm needle. The structure corresponds to a pad with 20 mm × 20 mm × 0.5 mm. Printed patches were generated with Ultimaker Cura slicing software, version 4.4 (Utrecht, The Netherlands). The geometry was sliced in two layers (0.25 mm each, including the first one) and printed at first with two contours and the middle filled at 100% with directions of ±45◦. The feed rate was defined as constant for all the contours and experiments (10 mm/s). The printing parameters were adjusted considering the rheological properties of the different alginate or chitosan aqueous solutions. The polymeric aqueous dispersions were printed and the $CaCl_2 \cdot 2H_2O$ or TPP solution, respectively, were added immediately after (ionic cross-linking method). In some experiments the alginate solutions were pre-crosslinked by mixing the polymeric solutions with $CaCl_2 \cdot 2H_2O$ for 30 minutes prior to printing. Glass was selected as the support material to improve adhesion during patch printing.

2.6 Validation and/or exclusion methods

After selecting the polymers, several initial formulations were developed and their physicochemical properties were studied, to understand their effect in the desired product quality. A Validation and/or Exclusion method was performed to select the combination of factors that allowed the best conciliation between physical and chemical properties and, consequently, the best printability and stability.

This method was performed based on the following sequential tests: 1- Determination of pH and 2 –Determination of viscosity. According to the decision-making scheme presented in Figure 5, it is essential to observe that, if the formulations do not pass one of the tests, they are automatically excluded, but can be reintroduced in the study by returning to formulations development using a different strategy. For example, in case of low viscosity, a strategy of

increasing the polymer concentration could be performed. In case of low pH, a strategy of introducing an alkaline excipient could be implemented.

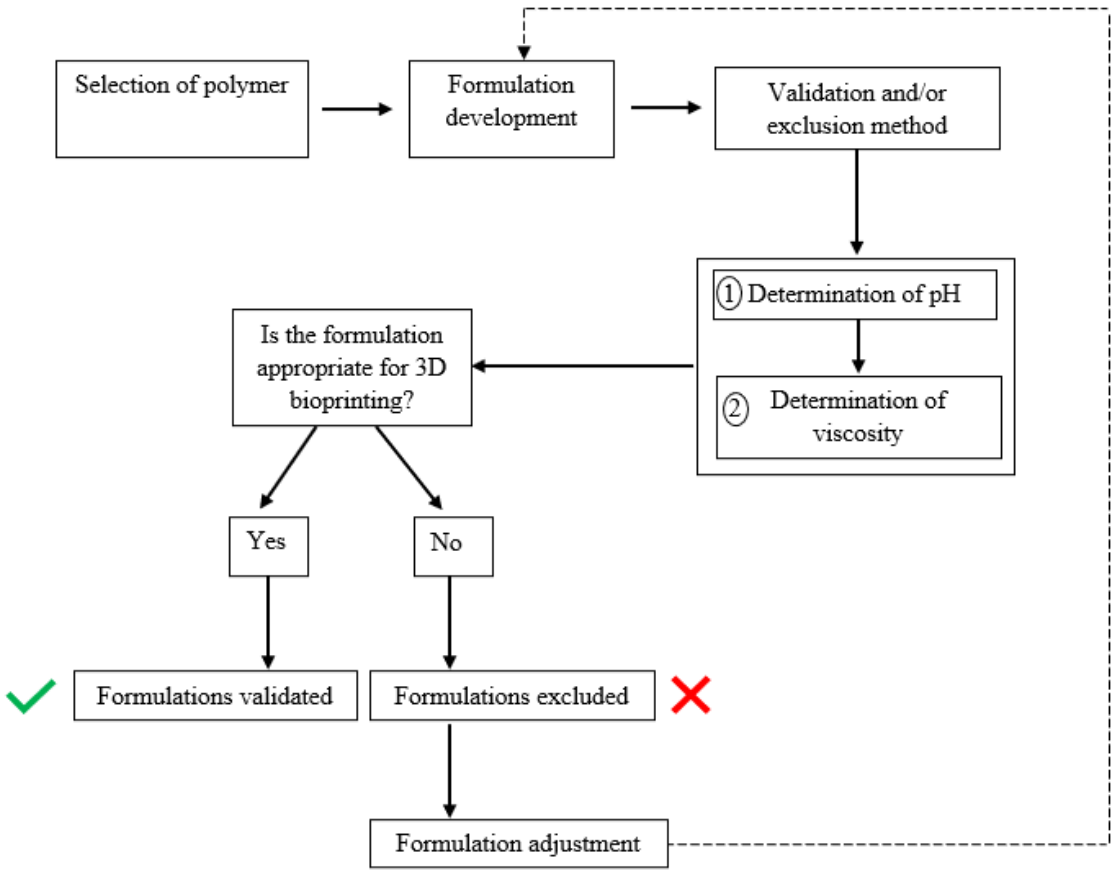


Figure 5-Decision-making scheme: validation and/or exclusion method.

After the validation and exclusion studies, alginate was selected as the fittest polymer and an optimization study was performed.

2.7 Formula optimization and establishment of Design Space

The formula of the Alginate 3D patches was optimized using a Full Factorial Design (FFD), composed by 3 levels (-1, 0, +1). The data were statistically analyzed using the MODDE® software (Umetrics, Sweden) and differences were considered significant for $p < 0.05$. According to preliminary studies and risk assessment analysis, the percentages of alginate (X_1) and $CaCl_2.2H_2O$ (X_2) were defined as the factors to be analyzed, while gelation time (Y_1) was defined as the responses. This design required 11 experimental runs, including three replicated center points for the estimation of the prediction variance over the entire design space. The following mathematical model was fitted to the data:

$$Y_1 = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 \quad (1)$$

where Y_1 denotes the response associated with each factor level combination; β_0 depicts the arithmetic average; β_1 and β_2 represent the first order coefficients of the respective independent variables; and, β_{12} typify the interaction coefficients.

2.8 Hydrogel 3D Patches Characterization: Gelation time

Gelation time was recorded during the design assay, and the macroscopic properties of alginate hydrogels were visually analyzed. The time at which the gel did not flow/spread was recorded as the gelation time (minutes).

2.9 Proof of concept: Secretome incorporation in alginate 3D hydrogel patches

In order to demonstrate the practical potential of the optimized formulation in supporting cell viability, a proof of concept was designed. The main goal of this clinical study was to test if the optimized hydrogel ink was, in fact, suitable in terms of biocompatibility and printability. As living cells are very fragile and more complicated to work with, they were replaced by their culture medium (the same matrix of their secretome), which has several advantages such as its ease of use and storage, its higher stability, less immunogenicity and lower cost. Cell culture medium manipulation requires either working in aseptic conditions or addition of preservatives to the formulations. Thus, rheologic studies allowed to infer about the influence of different sterilization methods and presence of preservatives in the quality of bioink. The influence of the addition of cell culture medium, as well as the biocompatibility of five different preservatives were also examined. Finally, the printability process and the quality of the final product regarding structural properties were assessed. This study was carried out in parallel with the optimization study and therefore the formulation used to assess the influence of the sterilization process, preservatives and cell culture medium in alginate viscosity and the cytotoxicity of the preservatives is different from the optimized one.

2.9.1 Alginate sterilization

Two methods of alginate powder sterilization were tested: (i) moist heat sterilization (121 °C, 20 min); (ii) sterilization by short-wavelength ultraviolet (UV-C) irradiation (15 W; 16 h).

2.9.2 Alginate-based hydrogels

Alginate solutions were prepared by dissolving medium viscosity alginic acid sodium salt powder (Sigma-Aldrich) in sterile water (control formulations - CF) or cell culture medium (α MEM with 10% FBS; Sigma-Aldrich and Gibco®) (test formulations - TF) to obtain an alginate concentration of 1.5% (w/v). The cross-linking solution was prepared by dissolving 3% (w/v) of calcium chloride ($CaCl_2.2H_2O$) in sterile water. Preservatives were incorporated at the recommended concentrations of efficacy: cetrimide (CE) 0.1% (FeF chemicals); Nipagin™ 0.18%/ Nipazol® (NN) 0.02%; benzalkonium chloride (BKC) 0.1%; chlorhexidine (CHX) 0.3%; and, sodium hydroxymethylglycinate (SHMG) 0.1%.

2.9.3 Viscosity measurements

The measurement of the viscosity was performed according to the procedure previously described in section 2.4.

2.9.4 Cytotoxicity assay

A cytotoxicity assay (MTT) was performed to test the biocompatibility of the test formulations with five different preservatives. HaCaT cells (human skin keratinocytes) cultured in DMEM high glucose (Sigma-Aldrich) supplemented with 10% FBS and 1% PenStrep (Gibco®) were seeded at 3.0 x 105 cells/cm^2. Each condition was assayed in six replicates in three independent experiments and results were expressed as mean ± SEM. Absorbance was measured at 570 nm using a SPECTROstar Omega spectrophotometer (BMG Labtech).

2.9.5 3D printing

The 3D Focus printer (ByFlow Netherlands) was used to evaluate the printability of the generated hydrogels. All hydrogels were printed using a 0.8 mm needle. The structure and dimension of the printed patches correspond to a 10 mm x 10 mm square (z = 0.3 mm). The optimum printing parameters were optimized considering the rheological properties of a 6% alginate hydrogel. The aqueous/cell culture medium alginate solutions were printed and the $CaCl_2.2H_2O$ solution was added immediately after printing (ionic cross-linking method). The printing accuracy (PA) was evaluated by measuring the spreading behavior in percentage, using ImageJ® software.

Results and discussion

1.Process mapping

3D printing is a complex process, composed of several steps that need to be previously established and organized. The workflow of the semi-solid extrusion printing started with the formulation development, where different parameters were selected such as the polymer, the gelation agent, the pre-crosslink, the mixing rate in the hydrogel production, among others. Afterwards, the 3D construct model was digitally designed with a computer-aided design software and was conversed to a STL file with specific instructions for the printer by Slic3r, a slicing software. After setting up the printing design and number and height of the layers, the hydrogel formulation was placed into a syringe in the 3D printer for subsequent extrusion-based printing. During this process, there were several parameters that, if not properly tuned, could have led to poor product quality. As such, an Ishikawa diagram was used in the present work to establish which variables could influence the hydrogel patch printing process using a 3D Focus printer (ByFlow Eindhoven, The Netherlands) by a semi-solid extrusion printing method. Also known as fishbone diagram, the Ishikawa diagram is considered one of the seven basic tools of quality management, and is a graphic problem-solving tool applied in a wide range of areas. To begin, a brainstorm session was performed where potential risk parameters were listed on paper. Subsequently, the list was reviewed to extract relevant causes in the context of the main presenting problem, the bioink, which is represented in the 'fish head'. The potential causes of the problem, derived from the brainstorming session and research, were then organized in the 'fish bones' of the Ishikawa diagram (Figure 6). A total of nine potential causes were listed: people, environment, materials, drug, pre-manufacturing, design and slicing, printing, characterization, and packaging. Each 'fish bone' was subdivided into smaller 'bones' to show the relationship of all potential causes to the presenting problem. Even though all these could influence the quality of the final product, the most relevant parameters to consider were the ones related to the formulations' development (materials). According to the literature, the selection of polymers for the formulation is of paramount importance in the quality of the 3D printed constructs, and therefore, the present work focused on the optimization of the formulation (66).

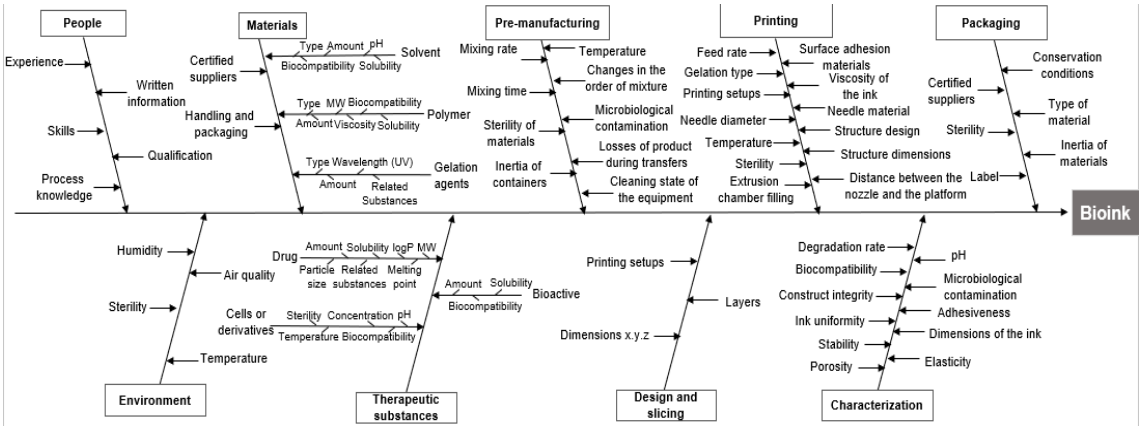


Figure 6-Ishikawa diagram illustrating factors that may have impact on the development of a 3D hydrogel patch.

2. Selection of Polymers

As previously stated, the selection of polymers is of great importance in the formulation development for 3D printing process. There are two main types of polymers for bioprinting: natural and synthetic polymers. Both are widely used in this area, nonetheless they have very different properties. Natural polymers offer great biocompatibility, biodegradability, non-immunogenicity but relatively poor mechanical properties as opposed to the synthetic ones who offer great mechanical properties but poor biocompatibility (28). Since the aim of this project was to administrate cell secretome in the hydrogel, natural polymers were selected over synthetic ones to ensure cell viability. Alginate and Chitosan were the final selected options because of their good biocompatible properties, their high frequency of use in 3DP and low-cost (37,67). Apart from being the most commonly used polymer in 3DP for regenerative medicine, alginate also gelates quickly and has a wide pore size distribution which helps in the diffusion of bioactive factors. Alginate of medium viscosity was chosen because it was known by previous projects that it granted the desired viscosity target (16).

The crosslinking solution selected for alginate was constituted by $CaCl_2 \cdot 2H_2O$ because it is the most frequently used by researchers in alginate formulations due to its fast but controlled gelation rate. This happens because divalent calcium ions form a bridge, due to the attraction of negatively charged carboxylic acid groups between two neighboring alginate chains (35).

Chitosan of low, medium and high molecular weight (MW) were selected to analyze the differences in physicochemical properties when using different MW. Since chitosan is not

soluble in pure water, there was a need to add acid excipients. The solubility of chitosan is attributed to the presence of amine groups in its structure, which are protonated in acid media, resulting in positive charges distributed along their chains. The higher the value of charged groups present, the greater its solubility. The selected acid excipients were acetic acid and lactic acid because the former is the most used solubilization agent for chitosan hydrogels and the latter is thought to improve their flexibility and decrease their stiffness. The study of different combinations of these acids, resulted in the proportion of acetic acid-lactic acid (1:1) (68). Sodium tripolyphosphate (TPP) was the selected crosslinking agent for chitosan formulations because unlike other substances that may generate undesirable effects, TPP is the safest and therefore has been preferred by researchers (41).

3.Formulation development

In order to explore which was the best formulation for the preparation of alginate and chitosan hydrogels, a preliminary study was made. The concentration of the two polymers in the formulations needed to be tested. So, the first alginate hydrogels developed had concentrations ranging from 1.5% to 7.0% and chitosan hydrogels had concentrations ranging from 1.0% to 3.0% (table 1).

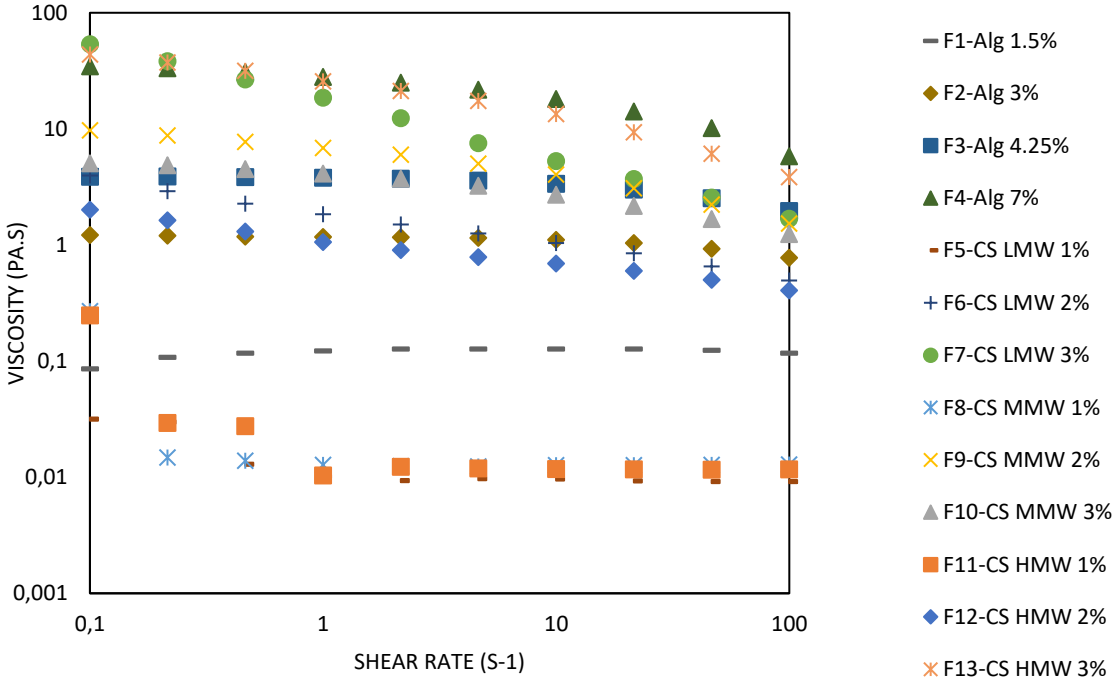
After the development, the hydrogels obtained were characterized by measuring their pH and viscosity. Alginate pH measurements are not shown because they were slightly basic and very stable from formulation to formulation. On the other hand, chitosan hydrogels presented rather acid pH values, which constitutes a limitation that will be discussed in the next section. In viscosity measurements (figure 7), it is evidenced that increasing the polymer concentration but maintaining the molecular weight results in an increase of viscosity. However, maintaining the concentration and varying the molecular weight, does not have a linear correlation in viscosity values. According to Chattopadhyay DP et al (69), higher molecular weight chitosan solutions suffer higher variances in viscosity with time. Another reason for this non-linearity may be the fact that chitosan is not only characterized by the molecular weight but also by degree of deacetylation. Degree of deacetylation determines the number of free amino groups present in the chitosan macromolecule, which in turn determines the functionality, polarity, and water solubility of the polymer. (69) Some authors claim that differences in the deacetylation degree of chitosan lead to different viscosity and flow properties of the solutions. Shear thinning properties could also explain this viscosity value, because under increasing shear stress higher viscosity formulations tend to be the most affected (70).

Table 1-Quantitative and qualitative composition of the initial formulations.

	Concentration (%, w/w)												
Ingredients	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**F11**	**F12**	**F13**
MMW Alginate	1.5	3.0	4.25	7.0									
LMW chitosan					1.0	2.0	3.0						
MMW chitosan								1.0	2.0	3.0			
HMW chitosan											1.0	2.0	3.0
Acetic acid:lactic acid (1:1)					1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Purified water	q.s. 100	q.s. 100	q.s. 100	q.s. 100	q.s. 100	q.s. 100	q.s. 100	q.s. 100	q.s. 100	q.s. 100	q.s. 100	q.s. 100	q.s. 100

LMW-Low molecular weight; MMW-Medium molecular weight; HMW-High molecular weight

Figure 7-Viscosity for F1 to F13 versus shear rate. Alg-aginate; CS-chitosan; LMW-Low molecular weight; MMW-Medium molecular weight; HMW-High molecular weight



4.Validation and/or Exclusion method

As shown in table 1, 13 formulations were developed to assess the physicochemical behavior of both polymers in different concentrations and select the best combination of factors for biocompatibility and printability. On step 1 of the decision-making scheme, pH values were measured, and all chitosan formulations failed this test. The addition of acetic and lactic acids to chitosan to promote its solubility, led to an abrupt decrease in the pH of the chitosan formulations, which constituted a problem for cell viability, as cells only support neutral gaps of pH (6.8-7.2). Due to this problem, all 9 chitosan formulations were automatically excluded on step 1. The solution to overcome this limitation, was to return to formulations development, eliminate the use of these acids and introduce another excipient in the formulation that would

improve chitosan solubility without significantly varying the pH. Glycerophosphate was a good candidate because it acts as a catalyst to cause a sol-to-gel transition in chitosan solutions at physiological pH and temperature (71). After including glycerophosphate, formulations became more neutral, but not enough to support the cells, which led to the final and definite decision of excluding all chitosan formulations. On the other hand, alginate-based formulations presented similar and basic pH values (data not shown), which could be easily adjusted by adding HCl solution, when applicable, without significantly affecting the viability and viscosity of the gel. In the end, only 4 formulations passed to step 2.

In step 2, viscosity measurements were performed. Formulations with high viscosities were not viable for the cells and resulted in nozzle clogging which in turn led to low printability. On the other hand, formulations with low viscosities resulted in weak mechanical properties and limitations in maintaining a 3D structure. Hence, formulations with alginate concentrations below 1.5% and above 5% were excluded, however the 7% alginate formulation (F4) was still included for the formula optimization study, to avoid losing data and obtain a more comprehensive study.

5.Quality by design approach

5.1 Establishment of quality target product profile (QTPP)

In this study, a QbD approach was performed to identify the influence of different factors on the formulation and physicochemical properties of alginate 3D patches for topical application.

When using QbD to optimize formulation and process development, the first step is to predefine the QTPP, i.e, the product characteristics expected to be achieved to ensure its quality. This study focused on the gelation time, a critical formulation attribute that will significantly influence the quality of the hydrogel patch for topical application. Gelation time was set at 10-30 min because previous studies revealed that a rapid gelation time can result in nonuniform crosslinking, which may lead to undesirable effects on the rheological and mechanical properties of the hydrogel patch (72).

In this study, despite focusing on the formulation properties, it is important to consider that quality is not only influenced by the critical properties of the raw materials (CMAs), but also by the process parameters (CPPs). For instance, mixing time and rate influence the gelation time due to the dispersion of the polymer in the aqueous solution. Several examples exist in

literature describing the importance of mixing on material performance. Cohen et al. performed a study in which they tested the power of increasing the number of mixing cycles in hydrogel homogeneity and they came to the conclusion that it reduced material inconsistency and improved geometric fidelity and stability of hydrogels (73). On the other hand, Piskounova et al. showed that insufficient mixing resulted in softer and not fully crosslinked hydrogels, while excessive mixing led to weaker hydrogels most likely due to defects in the 3D network (74).

Dimension and number of printed layers also have an impact on the gelation time. The gelation rate is a ratio between the thickness of the layer and the gelation time. According to Long et al, a thin gel is much easier to process to achieve a uniform gelation throughout the gel (75). It takes more time for the crosslinking solution to reach inner layers. Thus, the number of layers needs to be set in a compromise between providing good construct integrity and a uniform crosslinking process.

Other process parameters such as air pressure related to the extrusion chamber filling, feed rate and the distance between the needle and the platform, are also very important factors because they will influence the printing accuracy and quality. Thus, they may be adjusted either by reduction of the extrusion rate or by acceleration of the moving speed (76).

Concerning the needle diameter, a smaller needle size results in higher printing resolution, however it is prone to clogging. Moreover, the needle diameter can influence the ink viscosity during printing, which in turn can have an impact on gelation time (77).

5.2 Adjustment of CPPs

After understanding how the process parameters influence the QTPPs, preliminary studies were carried out to adjust them. Thus, mixing time was adjusted to 12 h to assure the homogeneity of the formulations, and mixing rate was adjusted considering the viscous properties of formulations. The final structure corresponds to a 3D patch with 20 mm × 20 mm × 0.5 mm. The geometry was sliced in two layers of 0.25 mm each and printed at first with two contours and the middle filled at 100% with directions of ±45° to guarantee the construct integrity and bulk structure. Furthermore, and according to preliminary studies, a 0.8 mm needle was selected to minimize the impact on the ink viscosity during printing. A delay between the printing of subsequent layers was also performed. Low printing delays may lead to merging of adjacent layers and consequently, low dimensional accuracy. On the other hand, samples printed with a sufficient delay between layers present higher mechanical strength and higher fidelity to the CAD (78).

5.3 Formula Optimization and Establishment of Design Space

As previously stated, the printability of the bioinks will highly influence the quality of the hydrogel patch. The printability is highly dependent not only on polymer but also on crosslinking agents' type and concentration. In this study, the gelation time was set up as the QTPP. Through RSM, it was possible to understand the interactions between alginate and $CaCl_2$ concentration and their influence on gelation time (the output response). In retrospective, this mathematical model extracted information about the input variables concentrations necessary to achieve the desired gelation time. This optimization study was performed using the final formulations and different concentrations of $CaCl_2 \cdot 2H_2O$. The impact of alginate (X1) and $CaCl_2 \cdot 2H_2O$ (X2), and their interactions on the predefined gelation time was studied. At different factor levels combinations, a total of 11 runs (including 3 center replicates) were performed. To evaluate the effect of X1 and X2 variables on alginate hydrogels patches, the gelation time were characterized. The data obtained by the experimental designs were analyzed with the MODDE® software, and first-order polynomial models were obtained. The adequacy and significance of each model are summarized in Table 2. The summary of regression analysis results for measured responses for formula optimization is presented in Table 3.

Table 2-Summary of regression analysis results for measured response (Full Factorial Design composed by 3 levels), for formula optimization.

Response	**Regression**	**β_0**	**β_1**	**β_2**	**β_{12}**
Gelation Time	Coeff	12.085	0.368	-5.146	-1.094
	± SE	0.467	0.527	0.527	0.527
	p	1.3349e-05	0.5233	0.0006	0.1069

β_0 – arithmetic average; β_1-first order coefficient of the percentage of alginate; β_2-first order coefficient of the percentage of $CaCl_2 \cdot 2H_2O$; β_{12}-interaction coefficient (alginate-$CaCl_2.2H_2O$); Coeff-coefficient; ± SE-standard error; *p*-probability

Table 3-ANOVA parameter summary of fitted model's characterization

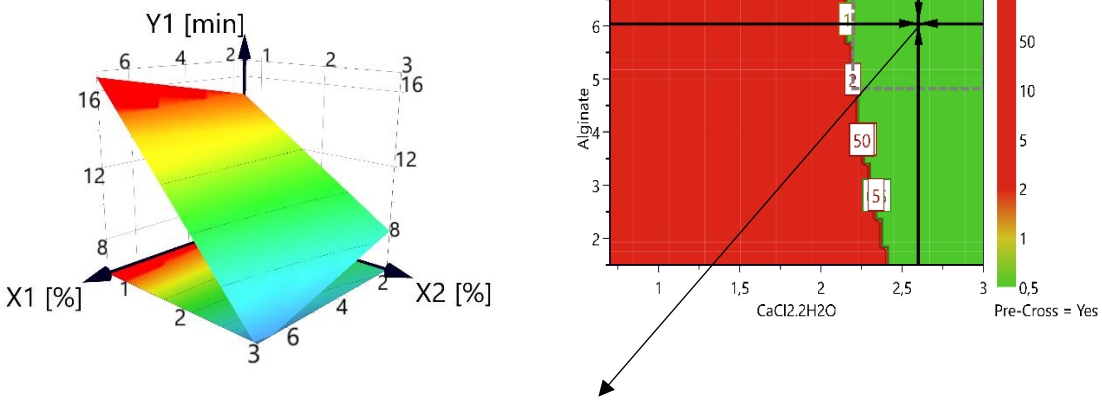
CQAs	**Regression**				**Lack of fit**	
	F	*p*	R^2	Reproducibility	F	*p*
Gelation Time	17.516	0.008	0.963	0.992	21.907	0.044

F-Distribution value; *p*-probability; R^2-square regression coefficient

To statistically analyze the adequacy of the fitted models, an analysis of variance (ANOVA) was also performed. As shown in Table 3, the regression data shows that the fitted model for gelation time responses is statistically significant ($p < 0.05$), highlighting the importance of the variables on the considered CQA. However, a significant model does not necessarily mean a correct explanation of the variation in results. The maximum square regression coefficient (R^2) evaluates the model fit. Furthermore, the reproducibility is an additional parameter of analysis that infers over the variation of the replicates compared to overall variability, where the closer the value is to 1, the better is the fit. The reproducibility value obtained (>0.8) further supports the sensitivity and the adequacy of the fitted models presented.

Moreover, the information derived from the models was expanded graphically by using isoresponsive curves. Figure 8 shows the response contour plot of the fitted model for gelation time and design space. All plots were adjusted against X1 and X2 as they were defined as the most significant variables in this study, apart from gelation time.

(a) **(b)**



Factor	Setpoint	Contribution
X_1 (Alginate)	6%	14.9
X_2 ($CaCl_2.2H_2O$)	2%	85.1

Figure 8-Response contour plot of the fitted model for gelation time (a) and design space (b).

As evidenced in figure 8, gelation time decreased with the increase of $CaCl_2.2H_2O$ concentration and increased with increasing polymer concentration. However, only $CaCl_2.2H_2O$ concentration was considered significant ($p > 0.05$). In addition, previous studies have shown that a rapid gelation time leads to nonuniform crosslinking, which in turn can have a negative impact on the final properties of the product (36). Hence, this type of parameter must be

carefully evaluated, and for an adequate adjustment, it is necessary to tune the ratio $CaCl_2 \cdot 2H_2O$–alginate in function of the QTPP defined. The formula critical parameters that were shown to affect gelation time were used to build the design space shown in figure 8b. A broad and wider design space leads to a more robust and flexible formula. Every single point corresponds to a combination of a percentage of $CaCl_2 \cdot 2H_2O$ (X_2), with alginate (X_1) defined as constant. This overlay plot provides a range within the values of a critical formula parameter that will not affect the final responses. From these results, it was possible to define the optimized formula, of which composition was the reflection of the target inputs. To guarantee a gelation time around 10–30 min, the alginate patch should be composed by 6% of alginate (factor contribution = 14.9) and 2% of $CaCl_2 \cdot 2H_2O$ as the crosslink solution (factor contribution = 85.1).

6. Proof of concept: Topical delivery of cells' secretome

6.1 Influence of the sterilization process in alginate viscosity

The assay was conducted with control formulations (i.e. aqueous alginate solutions) in comparison with two sterilization processes: moist heat sterilization method and UV-C sterilization method. None of the sterilization processes showed a significant impact on the viscosity properties of the alginate formulation (figure 9). Nonetheless, the sterilization by UV-C light seems to be a more suitable alternative given the similarity of results compared to the control (alginate at 1.5%). This is concordant with research by Stoppel et al. in which the UV-light treatment appeared to preserve the mechanical properties of alginate hydrogels for wound healing applications. However, it was demonstrated to be ineffective in bacterial inactivation in 1 mm-thick samples. Since the patches produced in this project present a 0.5 mm thickness, that is not a concern. Moreover, their results indicated that autoclave stem heating compromised the mechanical behavior and led to significant water loss, which is not accordant with the obtained results (79).

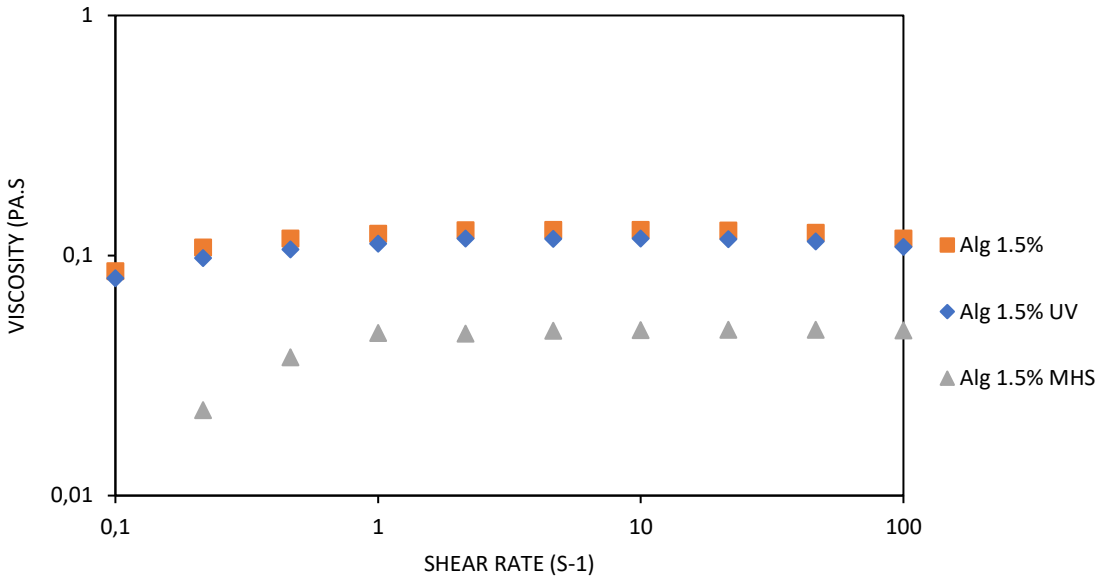


Figure 9-Influence of sterilization process in alginate hydrogel viscosity.

Recently, a study performed by Yu et al.(80) also showed that the UV treatment could be an effective method to sterilize alginate powder with low impact on the mechanical properties and that cell behavior in alginate-based hydrogel could differ depending on the duration of the UV treatment. In this same study, an autoclave treatment was also employed, but it strongly decreased the viscosity values, and the shear thinning behavior was lost. The results obtained in the present study do not show a shear thinning behavior in any of the formulations, including the control, which may indicate that sterilization methods did not have a significant impact on the shear thinning properties of the hydrogels (80). Nevertheless, Yu et al. concludes that alginate is difficult to sterilize and that all used sterilization methods may lead to chain scission and hemolysis of alginate due to the high pressure and temperature which the process of it is called de-polymerization. Thus, it is important to stablish well-defined and optimized sterilization conditions to avoid degradation of the materials, discoloration, embrittlement, odor generation or toxic effects (81). Due to the previously referred limitations of sterilization processes and because working in aseptic conditions was very challenging, preservatives were added to the formulations instead, to maintain their microbiologic quality.

6.2 Influence of preservatives in alginate viscosity

Five preservatives were added to the formulation and their influence in viscosity was determined. The results showed that the addition of preservatives to the alginate formulation leads to a slight increase in viscosity (figure 10). Chlorhexidine formulation presented a more

significant shear thinning process and revealed the highest increase in viscosity comparing to the control. The other preservatives presented very similar behaviors. To select among these the best preservative for alginate hydrogel patches, it was performed a cytotoxicity assay.

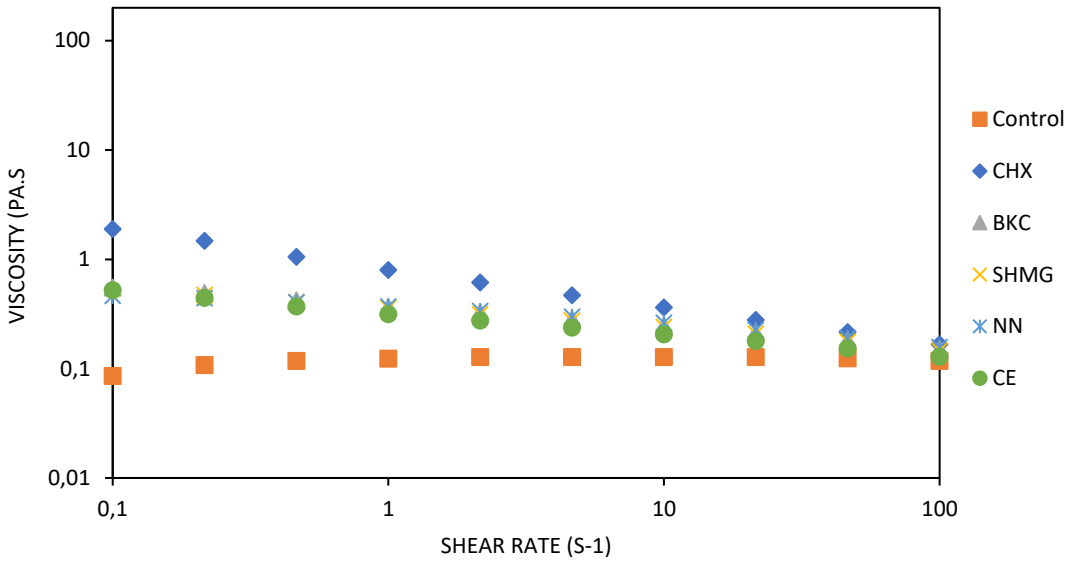


Figure 10-Influence of preservatives in alginate hydrogel viscosity.

6.3 Influence of cell culture medium in alginate viscosity

As shown in Figure 11, the incorporation of cell culture medium (TF- test formulation) has influence on the rheological properties of the alginate formulations (CF-control formulation), increasing the viscosity. This founding needs further testing to determine whether it has a significant impact on printability or not. Hence, printing accuracy was determined after the 3DP process.

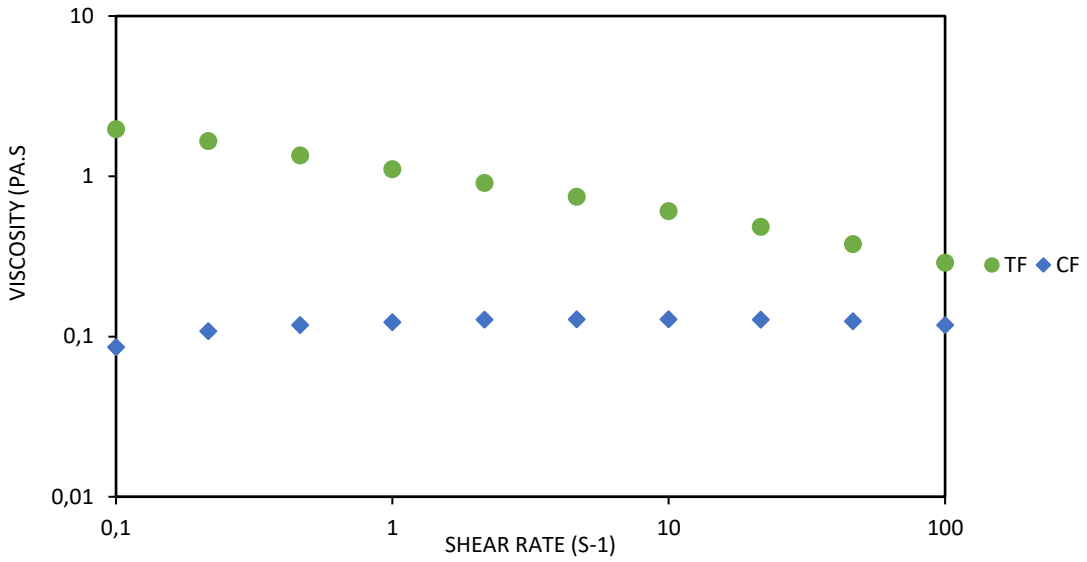


Figure 11-Influence of cell culture medium on alginate viscosity. CF-control formulation (water as solvent); TF-test formulation (cell culture medium as solvent).

6.4 Citotoxicity assay of the preservatives

The results show that all preservatives present some degree of cytotoxicity and thus, none is perfectly fit to maintain cell viability. Nonetheless, they suggest that the preservative system Nipagin^{TM}/Nipazol® is the most biocompatible with HaCaT cells, keeping cell viability above 50%, reflecting a reduction of 20% cell viability when compared to the corresponding control (TF). Thus, this preservative system was selected for the following studies. These results are in accordance with a study performed by Smith CN et al. (82), in which parabens were shown to have lower cytotoxicity on BALB/C mouse fibroblast cells after 1 hour of exposure than the other preservatives tested. In contrast, research carried out by Carvalho et al. (83) evaluated the potential of some preservatives for the induction of apoptosis, necrosis, and genotoxicity against human fibroblasts following the 24hours of exposure and they determined that methyl and propylparaben have increased genotoxic potential. These results are conflicting possibly because of the use of different cell lines, reagents, methodologies, exposure times, and concentrations of preservatives.

On the other hand, CE and BKC presented high toxicity for cell viability. According to a study done by Ayaki M. et al(84), concerning cytotoxicity of ophthalmic solutions with and without preservatives, BKC revealed high cytotoxicity with cell survival decreasing to 20% at the concentration estimated in commercial ophthalmic solutions, which is in accordance with

the significant decrease in cell viability obtained in this study. Another study performed by C. Dirain (85), showed that BKC reduced human fibroblast survival in a dose-and time- dependent manner.

CHX also showed significant decrease in cell viability. This is concordant with a study performed by Liu JX et al (86) where its cytotoxicity was evaluated in fibroblasts, myoblasts, and osteoblasts, with different concentrations and exposition times. Their results showed that an exposition of CHX even at minimal concentrations led to a decreased cell survival across all cell types, demonstrating the profound cytotoxic ability of CHX.

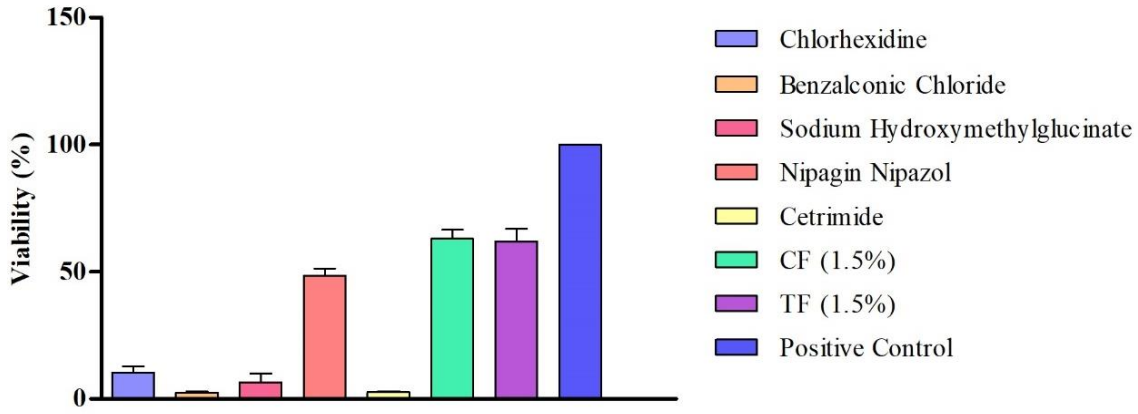


Figure 12-Cytotoxicity of alginate solutions containing different preservatives. Data expressed as mean ± SEM. *** $p < 0.01$ ** $p < 0.01$ and * $p < 0.1$. CF-control formulation (water as solvent); TF-test formulation (cell culture medium as solvent).

6.5 3D printing

The patches showed in Figure 13 were printed with the optimal concentrations of alginate (6%, w/v) and $CaCl_2 \cdot 2H_2O$ (2%, w/v), where the control formulation patch is shown on the left and the formulation with cell culture medium on the right. The former is colored in blue due to the addition of brilliant blue dye, which helps in the visualization of the patch limits without impacting its viscosity (data not shown), since its solvent (purified water) is transparent. In the latter, the cell culture medium has a turbid appearance, therefore there was no need to add a dye. No relevant modifications in patch geometry were observed between the two patches.

Through the area measurements analyses it is possible to conclude that the incorporation of cell culture medium did not influence the printability and the construct integrity of alginate patches. The viscosity differences have no influence on such properties. However, in both patches there is a shrinkage of the structure after gelation of 24.8% and 25.8% for CF and TF,

respectively. During the ionic cross-linking gelation process, patches suffer a contraction of the structure in volume, which is directly related to the crosslink concentration and the viscoelastic properties of the hydrogels. According a study performed by Kuo et al., a high concentration of calcium ions resulted in shrinkage, while a low concentration resulted in swelling of the gel (87). In both patches there was a slight shrinkage in the structure, which reveals that the addition of $CaCl_2 \cdot 2H_2O$ has an impact on the retraction of the structure regardless of its composition. Overall, both formulations present good printability and, considering all performed tests, it can be concluded that the optimized hydrogel patch constituted by alginate 6% and $CaCl_2 \cdot 2H_2O$ 2% is able to sustain cell secretome.

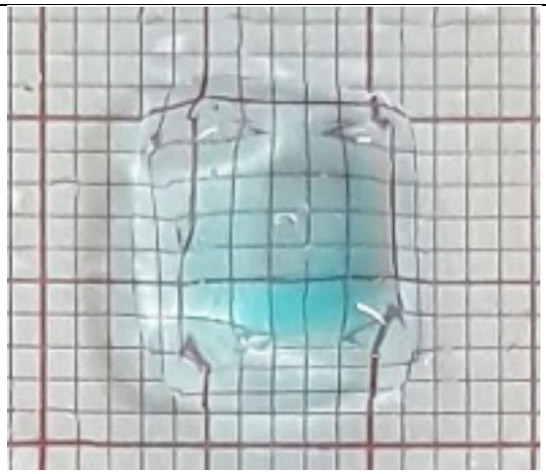
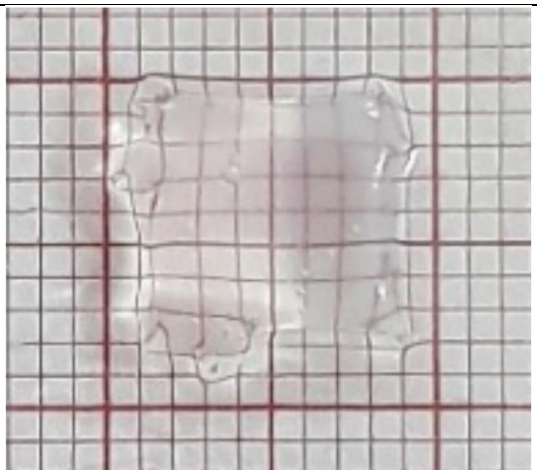
CF (6%)	**TF (6%)**
	
PA=75.2%	PA=74.2%

Figure 13-Evaluation of the printability and printing accuracy (PA) of the bioinks.

Conclusion

Psoriasis is a chronic inflammatory disease that exists in 5 different forms and affects 2% of the population worldwide. Over the years, several therapeutics have been developed but none of them guarantees a long-term effect and are mostly centered in symptoms relieve. Thus, it is important to study this disease and develop modern therapeutics. MSC incorporated in an appropriate vehicle were a good candidate because they secrete bioactive factors that reduce inflammation and stimulate tissue regeneration.

The work presented in this dissertation, aimed at developing an optimized 3D system for future application for topical delivery of cell's secretome (as active principle) using a semisolid extrusion 3D printing technique and a quality by design approach. To achieve this goal, a process mapping was created to organize the steps of the process and extract relevant

parameters for the quality of the final product. In this step, the formulation development was considered of utmost importance because its optimization was the highlight of this study. Alginate and chitosan were selected as the fittest polymers and initial hydrogel formulations were developed to understand their physicochemical properties. Chitosan hydrogels were then excluded because they presented acid pH values, incompatible with cells viability.

A Quality by Design approach was employed to optimize the formulation, using the previously developed formulations. Gelation time was selected as the QTPP and the role of the different factors that can affect it was evaluated, including percentage of alginate, percentage of $CaCl_2.2H_2O$ and the effect of a pre-crosslink. Alginate-based 3D patch were successfully printed by an extrusion-based bioprinter. The gelation time was concluded to be dependent on the concentration of the crosslink solution $CaCl_2.2H_2O$, while the other factors were not considered significant in the tested concentrations. The printability and the printing accuracy were significantly affected when a pre-crosslink stage was adopted, mainly with the alginate % increase.

Subsequently, a proof of concept was developed to corroborate that the optimized formulation was, in fact, fit for cells encapsulation. This was only tested with cell culture medium, not with living cells. However, it was possible to conclude that the optimized hydrogel patch was effectively able to encapsulate living cells or their secretome. Since manipulating cell culture medium requires microbiologic control, the effect of different sterilization methods and preservatives on hydrogel viscosity was analyzed. It was shown that UV-C sterilization was the most appropriate method to be adopted, as it presented no influence on the rheological properties of alginate-based formulations. Because working in aseptic conditions was very demanding, preservatives were preferred. As such, Nipagin™/Nipazol® system was selected as preservative because it presented higher biocompatibility. The incorporation of cell culture medium with 10% FBS did not influence the printability and the construct integrity of the alginate patches confirming the potential use of this bioink for secretome delivery.

In conclusion, the development of cost-effective 3D hydrogel patch resorting to semisolid extrusion 3D printing technology might represent an opportunity to increase and personalize different therapeutic approaches. The versatility of the developed alginate 3D patches makes them promising systems to be used for tailored-made drug delivery. Thus, the results obtained in this study provide an excellent baseline for future drug release modulation strategies.

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