UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Escola Tècnica Superior d'Enginyeria Agronòmica i del Medi Natural

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School of Agricultural Engineering and Environment

Pre-clinical evaluation of a bi-functional patch with antibiotic and anti-inflammatory action for chronic skin wounds

End of Degree Project

Bachelor's Degree in Biotechnology

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"Evaluación preclínica de un parche bifuncional con acción antibiótica y antiinflamatoria en heridas crónicas de la piel"

"**Pre-clinical evaluation of a bi-functional patch with antibiotic and anti-inflammatory action for chronic skin wounds**"

FINAL DEGREE PROJECT

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Abstract

Chronic wounds, burns and several other skin disorders like atopic dermatitis or psoriasis are highly associated to chronic inflammation and recurrent infections. In an antibiotic resistance climate where traditional antibacterial treatments are not able to eliminate the emerging strains of multi-drug resistant bacteria, the use of bacteriophages is one of the main focuses. Phages, along with nano-encapsulated curcumin to provide anti-inflammatory and antioxidant activity, would represent a promising alternative to traditional treatments. The presence of a hydrogel matrix, where the active agents would be included, inside a porous scaffold, would provide the hydrating and mechanical properties necessary. Having selected several formulations, the pre-clinical assessment of these devices was performed. Firstly, through MTT cytotoxicity tests in human BJ fibroblasts and HEKn keratinocytes cultures. Secondly, in a reconstructed human epidermis model ($SkinEthic^{TM}$RHE), as a validated alternative to animal experimentation, where skin irritation and tissue morphology, architecture and proliferation was measured. In this sense, none of the curcumin or phage formulations generated a negative cell response. Similarly, most of the hydrogel and scaffold compositions were deemed safe for topical use, except for PVA/HACC. The quaternised chitosan hydrogel exhibited questionable effects, though only in the HEKn monoculture, along with an inferior structural stability and lower curcumin release. Additionally, both studied PGS scaffolds were found to be innocuous but the ability of Strat-T to prevent cell migration positions it as the preferable option. These results suggest that it is possible to produce a patch formulation that is both functional and biocompatible for therapeutic use, although additional studies are required to enhance its performance.

Keywords Skin patch; Scaffold; Hydrogel; Bacteriophages; Controlled release device; Curcumin; Fibroblasts; Keratinocytes; Human reconstructed epidermis model; Cytotoxicity; Skin irritation.

Resumen

Las heridas crónicas, las quemaduras y diversas condiciones cutáneas, como la dermatitis atópica o la psoriasis, están estrechamente asociadas a la inflamación crónica y las infecciones recurrentes. En un contexto de resistencia antibiótica, donde los tratamientos antibacterianos tradicionales ya no logran eliminar las cepas emergentes de bacterias multirresistentes, el uso de bacteriófagos como terapia es uno de los principales focos. Los fagos, junto con curcumina nanoencapsulada para proporcionar actividad antiinflamatoria y antioxidante, representan una alternativa prometedora a los tratamientos convencionalmente usados para estos trastornos. Además, la presencia de un hidrogel que incluyera a los agentes activos, dentro de un andamiaje elastomérico, proporcionaría las propiedades hidratantes y mecánicas necesarias. Con ese objetivo y tras seleccionar varias formulaciones, se llevó a cabo la evaluación preclínica de estos dispositivos. Se realizaron primero ensayos de citotoxicidad con MTT, en cultivos de fibroblastos humanos BJ y queratinocitos HEKn. Por otro lado, se utilizó un modelo de epidermis humana reconstruida ($SkinEthic^{TM}$ RHE), como alternativa validada a la experimentación animal, para evaluar si los parches generaban irritación cutánea, así como si se presentaban cambios en la morfología, arquitectura o proliferación del tejido. Finalmente, ninguna de las formulaciones de curcumina o bacteriofagos generó una respuesta celular negativa. De manera similar, la mayoría de las composiciones de hidrogel y andamiaje se consideraron seguras para su uso cutáneo, excepto PVA/HACC. Este hidrogel de quitosano cuaternizado provocó efectos cuestionables en las células que, aunque únicamente fueron negativos en el monocultivo de HEKn, se sumaron a su menor estabilidad estructural y una liberación menor de curcumina. Además, los dos andamiajes de PGS estudiados resultaron inocuos, pero la capacidad de Strat-T para prevenir la migración celular dentro del parche lo posiciona como la opción preferible. Estos resultados sugieren que es posible desarrollar una formulación que sea funcional y biocompatible para su uso terapéutico, aunque se necesitarán estudios adicionales para optimizar sus propiedades.

Palabras clave Parche cutáneo; Andamiaje; Hidrogel; Bacteriófagos; Dispositivo de liberación controlada; Curcumina; Fibroblastos; Queratinocitos; Modelo de epidermis humana reconstruida; Citotoxicidad; Irritación cutánea.

Resum

Les ferides cròniques, les cremades i altres diverses condicions cutànies, com la dermatitis atòpica o la psoriasi, estan estretament associades a la inflamació crònica i les infeccions recurrents. En un context de resistència antibiòtica, on els tractaments antibacterians tradicionals ja no aconsegueixen eliminar les bacteries multiresistents emergents, l'ús de bacteriòfags com a teràpia és un dels principals focus. Els fagos, juntament amb curcumina nanoencapsulada per a proporcionar activitat antiinflamatòria i antioxidant, representen una alternativa prometedora als tractaments convencionalment utilitzats per a aquests trastorns. A més, la presència d'un hidrogel que incloguera els agents actius, dins d'un embalat elastomèric, proporcionaria les propietats hidratants i mecàniques necessàries. Amb aquest objectiu, i després de seleccionar diverses formulacions, es va dur a terme l'avaluació preclínica d'aquests dispositius. Primerament, es van realitzar assajos de citotoxicitat amb MTT en cultius de fibroblasts humans BJ i queratinòcits HEKn. D'altra banda, es va utilitzar un model d'epidermis humana reconstruïda ($SkinEthic^{TM}$RHE), com a alternativa validada a l'experimentació animal, per a avaluar si els pegats generaven irritació cutània, així com possibles canvis en la morfologia, arquitectura o proliferació del teixit. Finalment, cap de les formulacions de curcumina o bacteriòfags va generar una resposta cel·lular negativa. De manera similar, la majoria de les composicions d'hidrogel i embalat es van considerar segures per al seu ús cutani, excepte PVA/HACC. Aquest hidrogel de quitosà quaternitzat va provocar efectes qüestionables en les cèl·lules que, encara que únicament foren negatius en el monocultiu de HEKn, s'afegiren a la seua menor estabilitat estructural i a una alliberació inferior de curcumina. A més, els dos embalats de PGS estudiats van resultar innocuos, però la capacitat de Strat-T per a prevenir la migració cel·lular dins del pegat el posiciona com l'opció preferible. Aquests resultats suggereixen que és possible desenvolupar una formulació que siga funcional i biocompatible per al seu ús terapèutic, encara que seran necessaris estudis addicionals per a optimitzar les seues propietats.

Paraules clau Pegat cutani; Embalat; Hidrogel; Bacteriòfags; Dispositiu d'alliberament controlat; Curcumina; Fibroblasts; Queratinòcits; Model d'epidermis humana reconstruïda; Citotoxicitat; Irritació cutània.

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Contents

List of Abbreviations

AD	Atopic dermatitis
AMR	Antimicrobial resistance
CUR	Curcumin
DAB	3,3′-diaminobenzidine
DAPI	4′,6-diamidino-2-phenylindole
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco's Phosphate-Buffered Saline
ECM	Extracellular matrix
EE	Encapsulation efficiency
EMA	European Medicines Agency
EPS	Exopolysaccharide
EtOH	Ethanol
FDA	Food and Drug Administration
FBS	Fetal bovine serum
FESEM	Field emission scanning electron microscopy
FITC	Fluorescein isothiocyanate
GTMAC	Glycidyltrimethylammonium chloride
HACC	Hydroxypropyltrimethyl ammonium chloride chitosan
HES	Hematoxylin-eosin-safranin
HSE	Human skin equivalents
HRP	Horseradish peroxidase
MDR	Multi-drug resistant
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NAM	New approach methodology
NLC	Nanostructured lipid carrier
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAD	Peripheral artery disease
PGS	Poly(glycerol sebacate)
PVA	Polyvinyl alcohol
ROS	Reactive oxygen species
SA	Sodium alginate
SLN	Solid-lipid nanocarrier
TBS	Tris-buffered saline
WHO	World Health Organization
ZP	Zeta potential

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Relationship of the Project with the Sustainable Development Goals (SDG) of the 2030 Agenda.

	High	Medium	Low	Not applicable
SDG 1. No poverty				X
SDG 2. Zero hunger				X
SDG 3. Good health and well-being	X			
SDG 4. Quality education			X	
SDG 5. Gender equality				X
SDG 6. Clean water and sanitation		X		
SDG 7. Affordable and clean energy				X
SDG 8. Decent work and economic growth				X
SDG 9. Industry, innovation and infrastructure		X		
SDG 10. Reduced inequalities				X
SDG 11. Sustainable cities and communities			X	
SDG 12. Responsible consumption and production		X		
SDG 13. Climate action			X	
SDG 14. Life below water		X		
SDG 15. Life on land		X		
SDG 16. Peace, justice and strong institutions				X
SDG 17. Partnerships for the goals	X			

This final degree project is directly related to the third SDG, good health and well-being, whose purpose is to ensure healthy lives and promote well-being for all at all ages. The development and evaluation of this patch results on an increased access to efficient treatments for patients suffering from chronic wounds, who would otherwise have a worst outcome with the currently available therapies. The use of bacteriophages relates to the antibacterial effects of the patch, preventing and treating infections for *Staphylococcus aureus*, which is classified as a MDR (multi-drug resistant) bacteria, therefore decreasing the risk of complications related to infections and prolonged use of antibiotics, which are less specific than phages and can destroy the patient's microbiota.

Although initially there was no direct mention of AMR (antimicrobial resistance) in the SDG, in 2020 a new indicator addressing AMR was included in SDG 3.d.2: "percentage of bloodstream infections due to selected antimicrobial-resistant organisms". Bacteriophages and the medical devices that incorporate them are clear examples of effective strategies aligned with this goal.

The proposed technology also represents a medical innovation for the treatment of non-transmissible diseases, such as complications related to diabetic foot, aligning with goal 3.8. for the access to safe,

effective, quality and affordable essential medicines for all. Assessing the safety of the patch components is in fact one of the main objectives of this TFG.

In addition, it is related to target 3.4. which aims to reduce the premature mortality from diseases like diabetes. Some of the most common diabetes complications are chronic ulcers. If not treated, they can result in amputations and a number of infections that could, in some cases, escalate to sepsis and death.

On the other hand, it is highly related to SDG 17, partnerships for the goals. The present TFG has been developed in the CBIT investigation center in Universitat Politècnica de València, in association with the Equipe de Recherche sur les Relations Matrice Extracellulaire-Cellule in CY Cergy Paris Université. This project has been the result of active and sustained collaboration between both research groups throughout its development. In that sense, it is highly related to target 17.6. for the access to science and knowledge sharing, as well as 17.16, which aims to enhance partnerships for sustainable development and sharing of expertise, technology and financial resources to support the achievement of the SDGs.

1 INTRODUCTION

1.1 Physio-Pathological Skin Conditions with Chronic Inflammation and Infection

Multiple serious skin disorders exhibit clinical features linked to chronic inflammation and infection. The most prevalent are chronic wounds, burn wounds, psoriasis and atopic dermatitis, as illustrated by the *Global Burden of Disease Study* (2021) prevalence and incidence numbers in Figure 1.

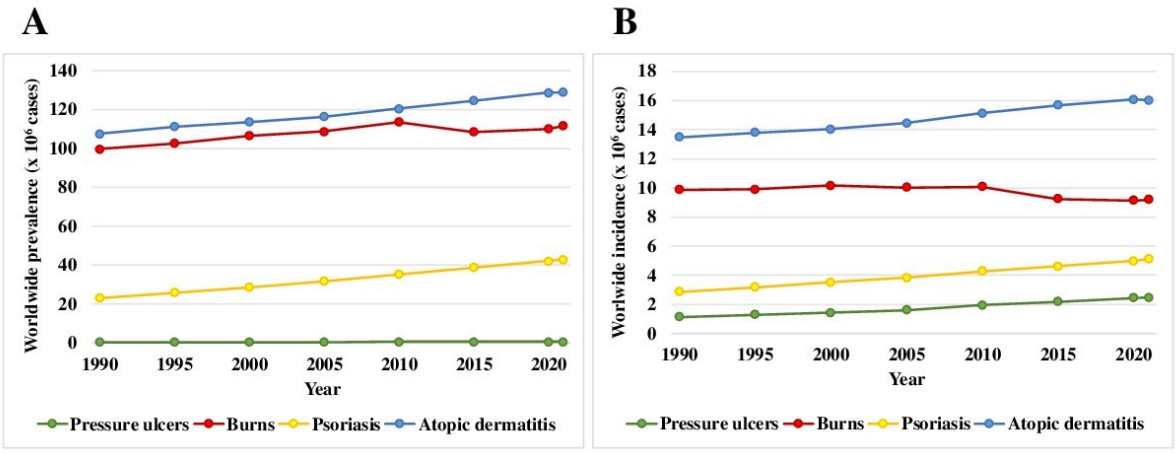

Figure 1: Prevalence (A) and incidence (B) global all-age numbers for pressure ulcers, burns, psoriasis and atopic dermatitis in years 1990 to 2021. Author's elaboration using data from the Global Burden of Disease Study 2021 (*Global Burden of Disease Study* 2021)

1.1.1 Chronic skin wounds

Wounds are considered chronic when they do not start healing within 8 to 12 weeks despite an active treatment, or when the repair process fails to restore anatomic and functional integrity after three months (T. A. Mustoe, O'Shaughnessy, and Kloeters 2006). They typically originate from a trauma or injury that, combined with an underlying condition, results in their chronification (Institute for Quality and Efficiency in Health Care (IQWiG) 2022)

Some causes include peripheral artery disease (PAD), which creates narrow arteries that compromise blood flow; venous insufficiency, causing enlarged varicose veins that accumulate blood in the lower extremities, increasing pressure and interfering with proper blood flow; mechanical pressure, regarding ulcers caused by lying motionless for long periods of time; or systemic factors such as nutritional status, weakened immune system or older age, provide further risk for infection and difficulties in healing (Azar, Rao, and Oropallo 2022; Institute for Quality and Efficiency in Health Care (IQWiG) 2022; Shamaki et al.

2022; Werdin et al. 2009).

T. Mustoe (2004) proposes a classification into three main categories: pressure sores, diabetic ulcers and venous ulcers, with a smaller fourth group for ischemia associated ulcers. In Spain, the prevalence of pressure ulcers is particularly concerning, affecting an estimated 8.25 % of the population, while leg ulcers have a prevalence of 0.16 %. Both conditions predominantly affect the elderly (Jj et al. 2006).

Current treatment strategies include the covering of wounds with a dressing and regular debridement, cleaning of dead cells and contaminated or inflamed tissue. This treatment is usually long and painful, with symptoms that aggravate upon infections and dressing changes, often found adhered to the wounds. Bacterial infections are usually treated with antibiotics along with analgesic regimen for pain relief (Institute for Quality and Efficiency in Health Care (IQWiG) 2022).

The two hallmarks of chronic wounds are infection and inflammation (Schilrreff and Alexiev 2022; Siddiqui and Bernstein 2010; Werdin et al. 2009), both of which are closely interrelated. Delayed healing in wounds has been seen to be associated to a prolonged presence of macrophages, neutrophils and monocytes, with a decrease of Langerhans cells, dendritic cells and eosinophils. In diabetic mice, elevated levels of reactive oxygen species (ROS) and proteases produced by these cells further damage the tissue and slow recovery (Joshi et al. 2020). Moreover, persistent inflammation, main responsible for impaired healing, is sustained by the chronic activation of the innate immune system, driven by interactions with the microorganisms present within the wound (Schilrreff and Alexiev 2022).

The most common identified pathogens are *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Moet et al. 2007). Additionally, chronic wounds represent a favourable environment for biofilm formation, present in around 80 % of chronic wound infections (Malone et al. 2017) and in only 6 % of acute wounds (James et al. 2008). A bacterial biofilm consists on a community of cells that are attached to each other or to a biotic or abiotic surface, while encased in a self-produced exopolysaccharide (EPS) matrix (Flemming et al. 2016). Bacteria growing in a biofilm are thought to be more resistant to antimicrobial treatments, around 10 to 1000 times more tolerant than planktonic bacteria (Mah and O'Toole 2001). Furthermore, biofilm formation facilitates not only the colonisation of the patient, but also the transmission and persistence of the pathogens in the environment, creating a public health risk (Wingender and Flemming 2011).

1.1.2 Burn wounds

Burn wounds, especially severe burns, can cause acute tissue damage and are usually chronic. Once chronified, these wounds follow a clinical course similar to those of other origins and are also associated to substantial morbidity and mortality rates (Institute for Quality and Efficiency in Health Care (IQWiG) 2022; Mason et al. 2019; Smolle et al. 2017). According to Greenhalgh (2019) in The New England Journal of Medicine, they are the fourth most frequent type of injury, with an estimated of 180.000 deaths every year (Organisation 2023b).

Severe burns often result in the development of an acute inflammatory response characterised by elevated cytokines, chemokines, acute phase proteins and the acquisition of a hypermetabolic state (Marc G Jeschke et al. 2008). Persistent or uncontrolled inflammation can lead to tissue damage, ultimately contributing to organ dysfunction and increased risk of mortality in burn victims (Marc G. Jeschke et al. 2020).

Regardless of new therapies implemented in recent years, treatment of burns still supposes a challenge with several known complications, most notably infections. Disruption of the skin barrier is a defining characteristic of burn injuries. Given the skin's critical role as a primary defence, the extent of the burn surface area is closely associated with an increased risk of both wound and systemic infections (Fitzwater et al. 2003). In fact, infection is actually considered the leading cause of mortality after burn injuries (Williams et al. 2009).

In their study, Junaid et al. (2019) presented numbers as high as 94 % for patients with severe burns that also suffered infections. As in chronic wounds, *Staphylococcus aureus* has been identified as the most frequent pathogen in burn wounds (Weinand 2024; Junaid et al. 2019), with a high prevalence of the methicillin resistant variant (Bagdonas, Tamelis, and Rimdeika 2003; Bang et al. 2002).

S. aureus, when exposed to burn serum and oxidative stress, downregulates the agr quorum-sensing system. This results in a signal cascade that leads to increased biofilm formation and cell-cell aggregation (Maslova et al. 2021). As mentioned, biofilms play a direct role in the progression of wounds and burns into a chronic state (James et al. 2008), highlighting the need for novel therapeutic strategies to effectively address this challenge.

1.1.3 Psoriasis

Psoriasis is a chronic inflammatory T-cell mediated skin disorder which manifests as skin lesions and comorbidities depending on severity, from some scaly plaques to the entire body surface. Its prevalence has been seen to increase in adults and children, globally and in Spain (Cayuela et al. 2025; Surcel et al. 2022), affecting more than 60 million people worldwide (Parisi et al. 2020).

It is a complex and multi-factorial skin condition whose causes are still unknown, highly dependent on genetic and immunological mechanisms, as well as lifestyle and environmental factors which can aggravate its symptoms (Surcel et al. 2022). Psoriasis pathophysiology is characterised by an increased and abnormal proliferation of keratinocytes and immune cell infiltration in the dermis and epidermis, mainly dendritic and T-cells (Greb et al. 2016).

Inflammation is not limited to the psoriatic skin and has been shown to affect different organ systems. Several comorbidities have been associated with psoriasis, including psoriatic arthritis, cardiometabolic disease, gastrointestinal disease, kidney disease, malignancies, infections and psychiatric disorders related to systemic inflammation (Rendon and Schäkel 2019; Takeshita et al. 2017).

Psoriasis shows characteristics of an autoimmune disease with an inflammatory background. Endogenous danger signals activate the innate immune system, in addition to T-cell driven autoimmune reactions and autoinflammatory perpetuation, depending on the case (Liang et al., 2017). The main drivers are the overactivated Th1, Th17 and Th22 with their corresponding pro-inflammatory cytokines (IL-17, IL-21, IL-22, IFN-γ), dendritic cells producing IL-20 and IL-23, among others; and keratinocytes (Sieminska, Pieniawska, and Grzywa 2024; Guttman-Yassky, Krueger, and Lebwohl 2018).

Treatments depend on the type and severity of the disease, with glucocorticoids, vitamin D analogues and phototherapy as the combination for mild psoriasis, while moderate to severe psoriasis can require systemic treatment. However, recurrence rates remain high and adverse effects and tolerance are frequently observed (S. A. Svoboda et al. 2020; Rendon and Schäkel 2019).

Streptococcal and staphylococcal bacterial species have been reported to contribute to the induction and aggravation of psoriatic lesions (Munz et al. 2010), and are thus directly implicated in their recurrence too. Additionally, (Ng et al. 2017) demonstrated that patients with psoriasis are 4 to 5 times more likely to be colonised by *Staphylococcus aureus* on the skin compared to healthy controls. In other studies, enterotoxin production, seen in 60 % of *S. aureus* infections in psoriasis patients, was seen to be significantly correlated to the severity and affected skin area of the disease (Tomi, Kränke, and Aberer 2005; Nielsen et al. 1998). Collectively, these findings emphasise the urgency for effective and safe antibacterial agents to treat psoriasis.

1.1.4 Atopic dermatitis

Along with psoriasis, atopic dermatitis (AD) is one of the most prevalent inflammatory skin diseases manifesting as recurrent, localised eczema-like eruptions that can appear in a seasonal manner as erythema, papules or exudative lesions (Sroka-Tomaszewska and Trzeciak 2021; Ständer 2021). AD shares many features with psoriasis, as both are characterised by these thickened epidermal lesions with variations in intensity and affected body surface area (Guttman-Yassky, Krueger, and Lebwohl 2018).

Affecting up to 10 % of adults and 20% of children (Laughter et al. 2021), AD is hypothesised to arise from a complex interplay among genetic predisposition, environmental factors, epidermal barrier dysfunction, microbial dysbiosis, and immune system dysregulation (Ständer 2021). The causes are complex and multi-factorial, and without a known cure, only palliative treatment is available. As a long-term disease, chronic inflammation and scratching are common, which have been seen to interfere with sleep and daily activities (Sroka-Tomaszewska and Trzeciak 2021; Chrostowska-Plak, Reich, and Szepietowski 2013).

Patients suffering from AD usually experience alternating periods of inflammation with swelling, heat and pain, and remission (Kang et al. 2025). Atopic dermatitis begins with an acute phase where Th2-, Th22-, and Th17-cell signalling is enhanced, following a chronic phase where Th1-cells are also activated (Gittler et al. 2012). The secreted cytokines, especially those by Th2 (IL-4, IL-13, IL-31) and Th22 (IL-22), are known to inhibit barrier integrity proteins such as filaggrin and loricrin, as well as downregulating terminal

differentiation and promoting hyperplasia, in the case of IL-22 (Guttman-Yassky, Krueger, and Lebwohl 2018; Gittler et al. 2012). Current therapeutic strategies involve topical corticosteroids as the first line of anti-inflammatory treatment. Nevertheless, their side effects range from skin atrophy to hypothalamic-pituitary-adrenal suppression (Sroka-Tomaszewska and Trzeciak 2021).

Complications of chronic AD include bacterial, fungal and viral superinfections, with *Staphylococcus aureus* as a frequent invader. Abnormal filaggrin expression leads to decreased urocanic acid and pyrrolidone carboxylic acid levels, so the pH rises. This, in addition to decreased skin hydration, favours *S. aureus* proliferation (V. Wang et al. 2021). Moreover, infections usually induce pruritus which can further worsen the itching (Alenazi 2023). Patients are normally prescribed topical or systemic antibiotics, with the main concern being the potential development of bacterial resistance and dysbiosis (Harkins, Holden, and Irvine 2019).

Table 1: Comparative summary of clinical features, inflammatory pathways, microbial involvement and some of the current main concerns in chronic wounds, burn wounds, psoriasis and atopic dermatitis. Author's elaboration based on data from Schilrreff and Alexiev (2022), Guttman-Yassky, Krueger, and Lebwohl (2018), Gittler et al. (2012), and Marc G Jeschke et al. (2008).

Condition/disease	Clinical features	Inflammatory pathways	Microbial involvement	Main concerns
Chronic wounds (pressure sores, diabetic ulcers and venous ulcers)	Non-healing; often painful; exudation and strong odor; inflammed and necrotic tissue.	Prolonged presence of macrophages, neutrophils and monocytes; elevated ROS, IL-1β, IL-6, IL-8, TNF-α and IFN-γ.	Most commonly *S. aureus* and *P. aureginosa* ; biofilm formation.	Delayed healing; MDR infections; amputation risk; ineffective treatment.
Burn wounds	Partial or full thickness skin loss; necrosis; chronification.	Elevated cytokines (IL-1β, IL-6, IL-7 and IL-8) chemokines and acute phase proteins; NF-κB activation.	High prevalence of *S. aureus* , especially MRSA; chronification related to biofilms.	High risk of sepsis and death; MDR infections; chronification.
Psoriasis	Chronic or pustular; scaly plaques; pruritus; itching; comorbidities like psoriatic arthritis.	Autoimmune and autoinflammatory processes; overactivated Th1, Th17 and Th22; elevated IL-17, IL-21, IL-22, IFN-γ, IL-20 and IL-23.	*S. aureus* and Streptococcus infections; enterotoxins exacerbate disease severity.	High recurrence; systemic inflammation; MDR infections.
Atopic dermatitis	Eczema-like eruptions; pruritus; seasonal cycles (swelling, pain and remission).	Overactivated Th2, Th17 and Th22; elevated IL-4, IL-13, IL-31 and IL-22.	Frequent *S. aureus* , fungal or viral superinfections; pruritus worsens symptoms.	Chronic relapses; severe pruritus; increased infection risk and MDR.

While each disease has its own particularities, they all share the chronic activation of inflammatory pathways and the frequent colonisation by *Staphylococcus aureus*, as well as some of the main concerns around them. These similarities and differences are summarised in Table 1.

1.2 Bacteriophages as an Alternative to Antibiotics

Antibiotics have been used for decades as a routine treatment upon infections, and they have surely played a critical role in the advancement of medicine and surgery (Hutchings, Truman, and Wilkinson 2019). However, their overuse has driven the emergence of antibiotic resistance in the very pathogens they were meant to target (Sengupta, Chattopadhyay, and Grossart 2013). Resistance is attributed to inappropriate prescribing, agricultural use and lack of new antibacterial therapies (Ventola 2015). Bacterial infections are once again a threat and there is an urgent demand for new therapies that overcome the resistance, as recognised by the World Health Organization (Organisation 2023a).

Bacteriophages are viruses that infect bacteria (Harper et al. 2021; Kutter and Sulakvelidze 2005). Already in the pre-antibiotic 1920s, phages started to be considered as possible therapeutic agents to treat infectious diseases, despite the limited understanding at the time (D'Herelle 1919). With the antimicrobial resistance (AMR) crisis, phage therapy has become one of the main focuses (Harper et al. 2021) as studies reveal their high efficiency to kill bacteria. In 79 % of studies performed between 2000 and 2020, as reviewed by Uyttebroek et al. (2022), there was clinical improvement in patients with difficult-to-treat infections, when phage therapy was applied, with 87 % of bacterial eradication. In another study for burn-like thermal injuries on ex-vivo porcine skin, phage treatment resulted in a significant reduction of viable *S. aureus* cells and activity, in comparison to the untreated control (Alves et al. 2018).

Notably, the bacteriophages preferred for therapy are those with a lytic and not lysogenic cycle. Both begin with the recognition and attachment of the phages to a bacterial cell with high specificity, depending on their surface receptors, initiating the insertion of the phage genome into the bacterial cytoplasm and the beginning of the corresponding cycle (Fig. 2). Lytic phages are the most interesting for therapy because they result in the rapid killing of the bacteria to release the bacteriophage progeny (Harper et al. 2021). This process is then repeated with the following susceptible cells in a "self-amplifying" process that supposes an advantage to conventional antibiotics (Kortright et al. 2019).

The nature of bacteriophage infection accounts for their specificity and low number of adverse effects as therapy. Phage recognition of gram-positive bacteria, such as *Staphylococcus aureus*, depends on the complementarity between phage adhesins and bacterial receptors. These receptors are thought to be either part of the exposed peptidoglycan layer or cell-wall associated proteins, both highly variable (Kutter and Sulakvelidze 2005). Therefore, phages tend to be specific to a certain species and strain, meaning the effects on gut microbiome are greatly reduced compared to oral antibiotics, whose use is known to cause a decrease in microbiota diversity and the consequent intestinal dysregulation (Dubourg et al. 2014). Most publications show no adverse effects in oral, local, parenteral or inhaled administration of phages (Corbellino et al. 2020; Gilbey et al. 2019; Aslam et al. 2019; Onsea et al. 2019; Fish et al. 2018).

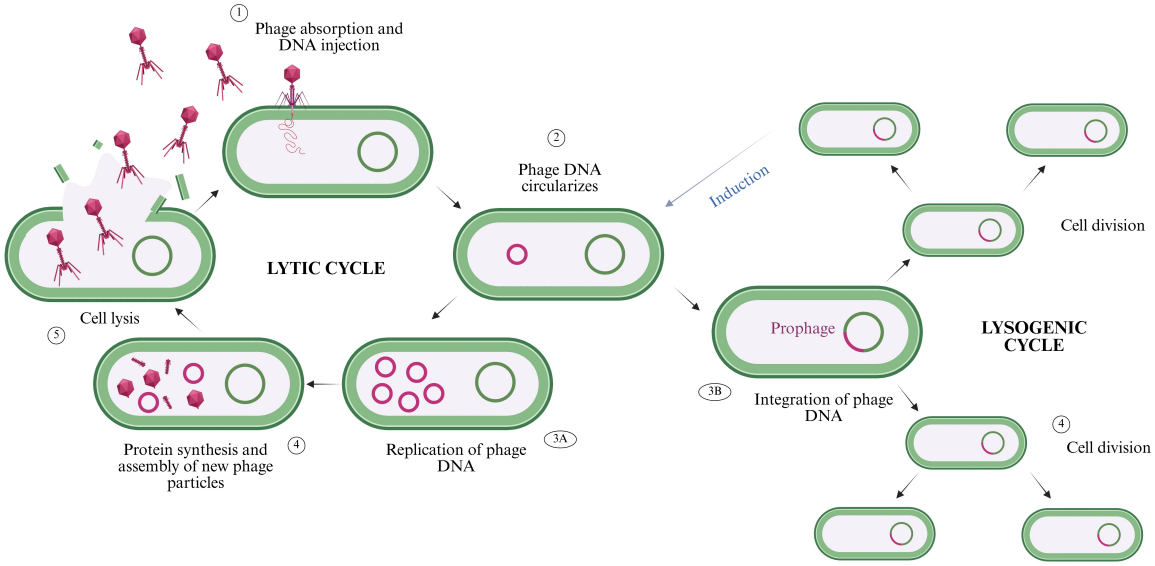

Figure 2: Bacteriophage infection pathways. After recognizing the bacterial host, bacteriophage adsorbs to the cell membrane and injects its DNA (1). The phage DNA circularizes in the bacterial cytoplasm (2), leading to one of two pathways. In the lytic cycle, phage DNA replicates (3A) and new phage particles are assembled (4), following host cell lysis (5). In the lysogenic cycle, phage DNA integrates into the bacterial genome (3B) and is transmitted to progeny cells during division (4). Under certain stresses, some lysogenic phages can excise their DNA from the host genome and initiate the lytic cycle, a process known as induction. Author's elaboration based on Harper et al. (2021). Created with Biorender.

Lastly, contrary to antibiotics, bacteriophages have been seen to be capable of degrading biofilms, especially when phage cocktails are used (Morris et al. 2019; Maszewska et al. 2018). These cocktails are composed of several types of phages that target different host receptors, thus increasing the phage activity and decreasing the chances of phage-resistance appearing (Pires et al. 2017). Kolenda et al. (2020) proved that combination therapy of bacteriophages and antibiotics could be successful against *Staphylococcus aureus* in biofilm, and Totten and Patel (2022) also showed phages were capable of infecting *S. aureus* in both planktonic and biofilm form. The presented advantages of bacteriophages make them promising alternatives to antibiotics in the current AMR crisis climate.

1.3 Managing Inflammation with Bioactive Compounds

As revealed in previous sections, inflammation is a common factor in skin disorders, especially in those with chronic nature (Schilrreff and Alexiev 2022; Guttman-Yassky, Krueger, and Lebwohl 2018; Marc G Jeschke et al. 2008). Bioactive compounds are metabolites found in nature with a potential positive effect on human health. They are classified in four categories: macronutrients, micronutrients, microbiota regulators and phytonutrients (Kussmann, Abe Cunha, and Berciano 2023).

Phytonutrients are secondary metabolites present in plants such as phenolic compounds, alkaloids or terpenes (Kussmann, Abe Cunha, and Berciano 2023). Notably, phenolic compounds have gained interest in recent years for their potential to reduce symptoms and favour healing in skin wounds (Melguizo-Rodríguez et al. 2021; Działo et al. 2016). Their core structure consists of one or more hydroxyl groups attached to an aromatic ring with the hydrogens usually replaced by a hydroxyl, methyl or acetyl group (Vermerris and Nicholson 2008).

Phenolic compounds have been seen to have anti-inflammatory and antioxidant properties thanks to their chemical structure. They can scavenge radicals with free electrons that would otherwise cause oxidative stress and damage in cell structures (Vermerris and Nicholson 2008). Skin inflammatory disorders are negatively affected by ROS, which are generated as by-products and also by NADPH oxidases, present in phagocytes and endothelial cells and thus involved in the inflammatory response (Mittal et al. 2014; Pendyala and Natarajan 2010). Although the role of ROS in this sense is not yet clear, they are known to induce inflammation and damage in wounds (Ukaegbu, Allen, and K. K. H. Svoboda 2025; Mittal et al. 2014), so the use of antioxidant molecules is an interesting approach to minimise their effects (Ukaegbu, Allen, and K. K. H. Svoboda 2025).

One of these secondary metabolites is curcumin, active component of turmeric (*Curcuma longa L.*) with chemical formula $C_{21}H_2O_6$. It is the major component of the curcuminoid group of compounds that constitute around 2 to 9 % of turmeric (Priyadarsini 2014). As depicted in Figure 3, it has three active

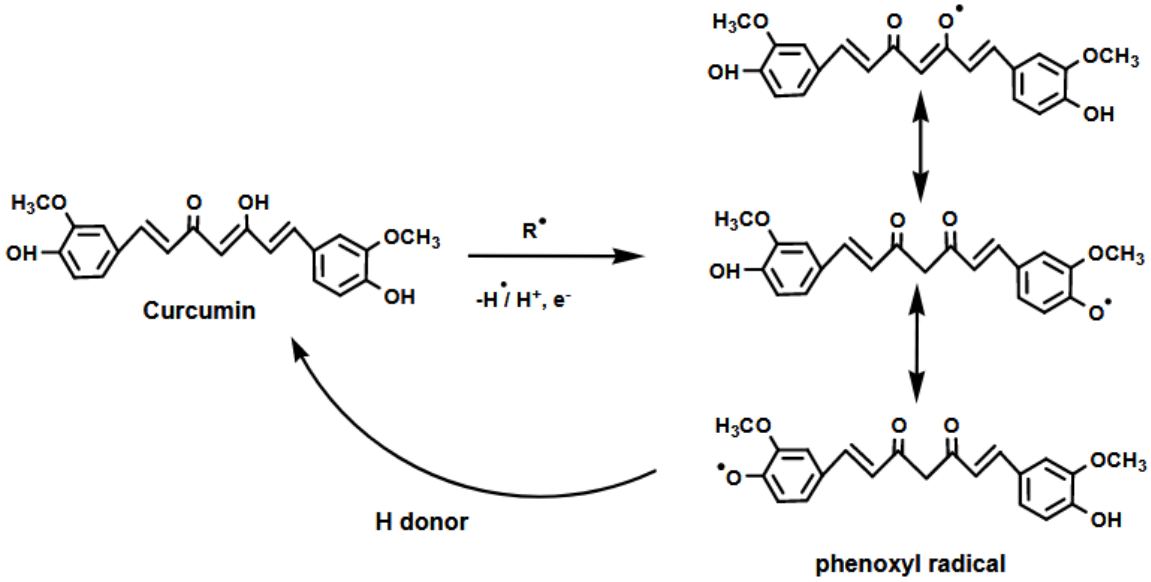

Figure 3: Chemical structure of curcumin in the enol form. Potential sites of radical attack include two phenolic hydroxyl groups and one enolic hydroxyl group. The conjugated double-bond system allows stabilization of phenoxyl radical intermediates by electron delocalization. In the presence of a hydrogen donor, the phenoxyl radical can be reduced back to neutral curcumin. Modified from Priyadarsini (2014).

sites that can undergo oxidation and has been proven an effective antioxidant (Jakubczyk et al. 2020; Priyadarsini 2014).

Curcumin also has an anti-inflammatory effect due to its involvement in several pathways, including the blocking of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Kumari et al. 2022; Merrell et al. 2009). Furthermore, curcumin increases PRAR-γ activity, therefore inhibiting angiotensin-II-induced inflammation (Kumari et al. 2022). The anti-inflammatory potential of curcumin may be useful to treat chronic skin disorders as an alternative to corticosteroids and other conventional treatments, without the known side effects of these. Research shows promising results to combat inflammation and promote tissue regeneration (Mahmood et al. 2022; Kamar, Abdel-Kader, and Rashed 2019; Li et al. 2019).

A key characteristic of curcumin is that it is highly hydrophobic and almost insoluble in water, with a 3.0 logP. For this reason, when used as therapy it needs specific delivery platforms to ensure sufficient absorption and bioavailability in the cells. Several mechanisms have been studied, mainly based on encapsulating curcumin into hydrophobic pockets (Priyadarsini 2014). Lipid nanoparticles have several advantages to liposomes, emulsions and previously used carriers, allowing for a controlled and targeted drug delivery with high physical stability. Nanostructured lipid carriers (NLC) are the newest formulation and present with higher stability and capacity loading than the alternative solid lipid nanoparticles (SLN) (Kumari et al. 2022; Garcês et al. 2018).

As illustrated in Figure 4, liposomes, SLNs, and NLCs differ in the organisation of their lipid matrices,

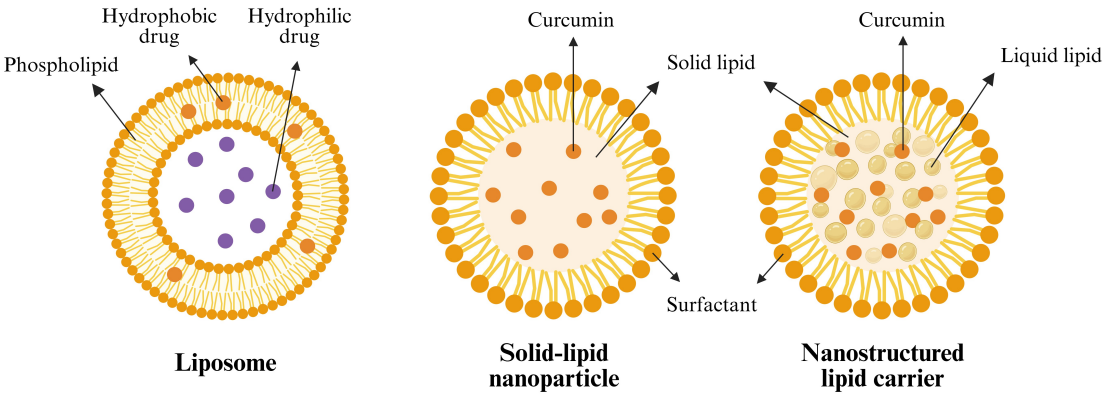

Figure 4: Structural comparison of nanoscale carriers for controlled drug release. Liposomes are formed by one or more phospholipid bilayers surrounding an aqueous core and can store hydrophilic or hydrophobic drugs. Solid-lipid nanoparticles (SLNs) are formed strictly by solid lipids and stabilized by a surfactant. The core of nanostructured lipid carriers (NLPs) consists of a mixture of solid and liquid lipids. Author's elaboration based on data from Kumari et al. (2022) and Garcês et al. (2018). Created with Biorender.

which determines their characteristics as drug carriers. Lipid nanoparticles consist of an external aqueous phase stabilised by surfactants and an internal lipid phase including solid lipids, which protect the active compound from degradation. The inner layer in the case of NLCs is composed of both liquid and solid lipids, with the latter in higher proportion (Garcês et al. 2018; Müller, Mäder, and Gohla 2000).

Curcumin-loaded NLCs facilitate the penetration and retention of curcumin in the skin in several ways. On the one hand, lipid nanoparticles adhere easily to the *stratum corneum*, enabling curcumin penetration into deeper layers. On the other hand, the nanoscale of the particles improves adhesion and maximises surface interaction to promote drug delivery (Garcês et al. 2018). Collectively, NLCs allow wounds to benefit from the anti-inflammatory and antioxidant effects of curcumin for an improved healing (Kumari et al. 2022; Priyadarsini 2014). Several studies have evaluated the effects of curcumin nanoparticles in the wounded skin with promising results (Elkhateeb et al. 2023; Mobaraki et al. 2021; Krausz et al. 2015). Sandhu et al. (2021) showed that curcumin in solid lipid nanoparticles even had a significant antimicrobial effect against *S. aureus* in both planktonic and biofilm form.

1.4 Cutaneous Delivery Platforms of Active Agents

In order to enable therapeutic application in skin wounds, the proposed delivery platform consists of a patch comprising a scaffold integrated with a hydrogel matrix, which incorporates both bacteriophages and curcumin-loaded NLCs (Fig. 5).

1.4.1 Scaffolds

Skin tissue engineering aims to heal, maintain or improve the functionality of the skin. With that purpose, biomaterials are used to promote tissue growth, most notably scaffolds (Sindhi et al. 2025; Langer and Vacanti 1993). A scaffold is a complex three-dimensional framework that is built from a biocompatible material with a skin-like porous microstructure. It is used to aid in wound healing or repairing tissue damage, by promoting cell adhesion, proliferation and differentiation (Chen et al. 2023).

Biomaterials are key in the development of scaffolds for tissue engineering. They can be of either synthetic or natural origin (Aramwit 2016). Compared to simple materials like gauze, traditionally used in the treatment of wounds, biomaterial-based dressings provide not only an ideal environment for wound healing, but also a platform for the delivery of active agents with therapeutic effects (Chen et al. 2023; Aramwit 2016).

Poly(glycerol sebacate) (PGS) is a synthetic polyester that has gained attention due to its simple, yet non-toxic synthesis, based on glycerol and sebacic acid, both FDA-approved for biomedical applications; as well as high biocompatibility, flexibility and degradability (Nasiri, Ahmadi, and Afshar-Taromi 2023; Behtouei et al. 2022).

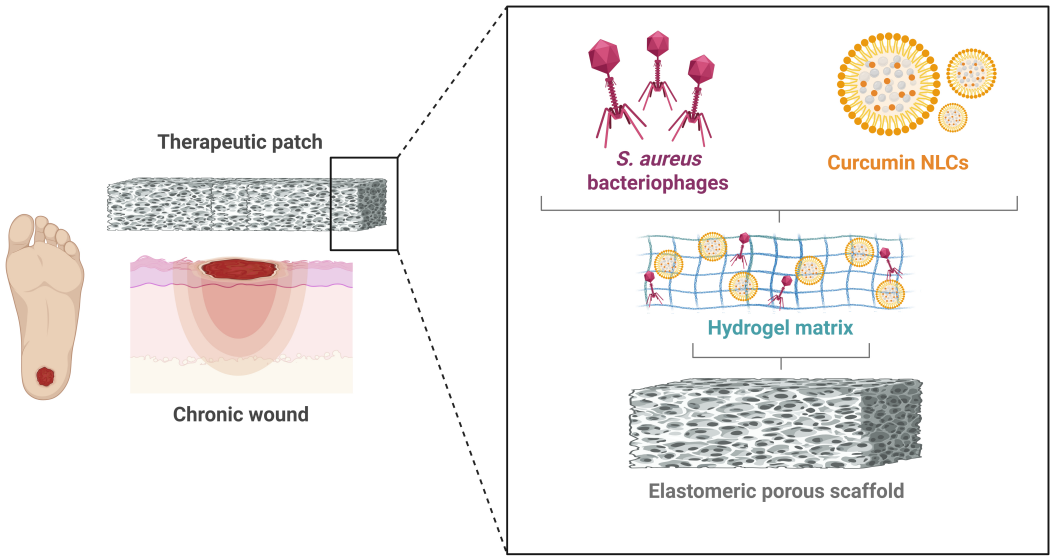

Figure 5: Components of the multi-layered therapeutic patch. The purpose of the platform is to be applied to inflammatory skin lesions such as chronic wounds. The bacteriophages against *Staphylococcus aureus* and the curcumin loaded nanostructured lipid carriers account for the antibacterial and antiinflammatory properties of the patch, respectively. Both active components are contained in a hydrogel matrix formed in the pores of an elastomeric scaffold. Author's elaboration.

The synthesis of PGS results in a covalently cross-linked three-dimensional structure that can be engineered to contain extensive pores using techniques such as salt leaching, template moulding or 3D printing. Interestingly, its degradation products can be absorbed by the body and eliminated through normal metabolic pathways, making it suitable for biomedical applications (X. Zhang et al. 2016). Furthermore, glycerol, component of PGS, has been seen to have several benefits for the skin. This includes improving skin hydration, cutaneous elasticity and protection of the epidermal barrier (Salgaonkar et al. 2022; Fluhr, Darlenski, and Surber 2008).

PGS has shown promising results in the healing of wounds (X. Zhang et al. 2016) and drug release (Heydari et al. 2018), with a linear degradation that is desirable for controlled drug delivery (Rai et al. 2012). The biocompatibility of PGS has been proved *in vitro* with rat C6 glioma cells (Behtouei et al. 2022), NIH 3T3 fibroblasts (Nasiri, Ahmadi, and Afshar-Taromi 2023), rat Schwann cells (Sundback et al. 2005), mouse retinal progenitor cells (Redenti et al. 2009) and others. *In vivo* analysis with Fisher rat (Sundback et al. 2005) and Wistar diabetic rat (Alharthi 2025) models also show the low cytotoxicity of the material.

Nasiri, Ahmadi, and Afshar-Taromi (2023) studied a PGS formulation with curcumin and bioglass to find that these active components increased cell viability, cell attachment and also PGS bioactivity in NIH 3T3 fibroblast cells. Similarly, Alharthi (2025) evaluated wound closure in diabetic rats, where a PGS/PLA-curcumin scaffold resulted in faster healing rates compared to solely PGS/PLA or curcumin. These results suggest a potential synergistic effect between PGS and curcumin in promoting tissue regeneration.

1.4.2 Hydrogels

Hydrogels are three-dimensional networks of hydrophilic polymers that interconnect through either covalent bonds or physical interactions. Their main characteristic is that they can absorb very high volumes of water or fluids without dissolving, through swelling. They have several biomedical applications, including their use within scaffolds for tissue engineering. Their hydrophilic properties, biocompatibility and controlled drug release makes for their utilisation as localised drug depots (Dong et al. 2025; El-Sherbiny and Yacoub 2013), often needing to be supported or reinforced by ancillary materials because of their poor mechanical stability.

Regarding wounds, the high percentage of water in hydrogels, exceeding 90 % in most cases, promotes hydration and creates a favourable environment to promote healing through increased angiogenesis and degradation of dead tissue (Sivaraj et al. 2021). Hydrogel dressings can absorb wound exudates while maintaining a moist environment that decreases pain levels (T. Wang et al. 2023). Depending on the specific composition of the hydrogel, additional effects may also be present.

Polyvinyl alcohol (PVA) is a biodegradable synthetic polymer and one of the most studied materials for hydrogel-based wound dressings. It has a desirable hydrophilicity and biocompatibility with good mechanical properties. However, PVA is not bioactive and it exhibits limited drug delivery ability and exudate absorption. For this reason, the focus is on PVA-based composite materials that also incorporate bioactive agents to increase wound healing rates (Jin 2022).

Alginate is an anionic natural polysaccharide that is usually obtained from the cell walls of seawater brown algae. Sodium alginate (SA), a water-soluble sodium salt of alginic acid, is widely used in several industries including food, textiles and many biomedical and engineering products (Akbar et al. 2023). SA easily forms a hydrogel through ionic cross-linking in the presence of divalent cations such as Ca^{2+} (T. Wang et al. 2023). As a biocompatible and absorbent material with some antibacterial activity, the application of sodium alginate as a chronic wound dressing has gained attention, despite its low stability in the swollen state (Kamel et al. 2023; Aderibigbe and Buyana 2018).

Multiple studies have evaluated SA to treat wounds with positive results (Bialik-Wąs et al. 2021; Abbasi et al. 2020), although the healing capacity of the dressings could be amplified with the addition of active agents (Saraiva et al. 2023). In their study, (Niranjan et al. 2019) tested a patch with PVA/SA/TiO2 and curcumin in wounds. They found that the addition of TiO2 and curcumin successfully restricted bacterial growth and enhanced tissue regeneration with faster healing compared to the PVA/SA formulation alone.

Another common polymer for hydrogel formulations is chitosan, composed by glucosamine and N-acetyl glucosamine units linked by 1-4 glycosidic bonds. Chitosan hydrogels are considered ideal to enhance wound healing because they are biocompatible, antimicrobial, biodegradable, non-toxic and also have haemostatic effects (Jin 2022; He Liu et al. 2018). They have been seen to reduce chronic inflammation and infection in wounds (He Liu et al. 2018). Chitosan, positively charged, enhances the penetration of active agents through the reversible opening of tight junctions due to its interaction with mucins and

epithelium cells to increase paracellular permeability (Amidi et al. 2010; Schipper et al. 1997).

However, chitosan has low solubility in basic pH, which limits its utilisation. In order to improve this, many chitosan derivatives have been developed (Xu et al. 2024; Amidi et al. 2010). Quaternised chitosan is obtained by the introduction of quaternary ammonium groups, which confer a positive charge to the molecule even at neutral pH. Hydroxypropyltrimethyl ammonium chloride chitosan (HACC) or N-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride is obtained by the reaction of chitosan and glycidyl trimethyl ammonium chloride (GTMAC). HACC is a water-soluble chitosan derivative which has been reported to have antibacterial properties against *S. aureus* and *E. coli*, with an increased effect following an increased degree of quaternisation (P. Liu et al. 2015).

Chitosan/PVA composite hydrogels have been widely studied as wound dressings (R. Liu et al. 2014; Nacer Khodja et al. 2013) with positive results. Quaternised chitosan and PVA have been seen to interact with good mechanical properties and promotion of their antibacterial properties (Eren Belgin and Delibalta 2023; Min et al. 2020). However, literature regarding the combination of HACC and PVA for biomedical applications is relatively limited, most likely due to the novelty of this formulation. Nevertheless, a hydrogel that combined the biocompatibility and biodegradability of PVA (Jin, 2022) with the antibacterial activity and permeability-enhancing properties of HACC (P. Liu et al. 2015; Amidi et al. 2010) could represent a promising formulation that warrants further investigation.

1.5 Animal-Free Pre-Clinical Evaluation

Although the use of animal models has been the gold standard in the discovery and development of drugs and medical devices, the emergence of alternative methods now raises the possibility of decreasing or even replacing their use. Over 50 years ago, the basic principles for animal research were established: the so-called “3Rs” for Replacement, Reduction and Refinement. According to this framework, animal experimentation should use the minimum number of animals, causing the least possible harm or pain, and only in cases where their utilisation is necessary (Russell and Burch 1959).

In 2013, the European Union banned both animal testing for cosmetic products and the marketing of cosmetic products or ingredients that had been tested on animals (European Parliament and Council of the European Union 2009). In recent times, the ethical concerns and costs of animal research in biomedicine have led regulatory authorities to consider other alternatives (Imran et al. 2024). In fact, both the FDA and the European Medicines Agency (EMA) encourage the use of validated new approach methodologies (NAMs), including *in vitro* and *in silico* models, as an alternative to animal testing in the pre-clinical assessment of new medicines and medical devices (Agency 2025; Administration 2025).

Among the most widely applied NAMs are cell culture systems. Two-dimensional (2D) cell cultures are the simplest skin model, widely used in early-stage screening to assess cytotoxicity, biocompatibility and molecular responses to biophysical or biochemical cues (Quílez et al. 2024). They are efficient models that

consist of cells growing in a monolayer with growth medium; with homogeneous growth and proliferation (Duval et al. 2017). Although 2D models are useful representations of several cellular mechanisms, they are not accurate regarding cell-to-cell and cell-to-matrix communication, as well as for the structural organisation of the tissue (Kapałczyńska et al. 2018). This accounts for the development of the three-dimensional (3D) models (Fig. 6).

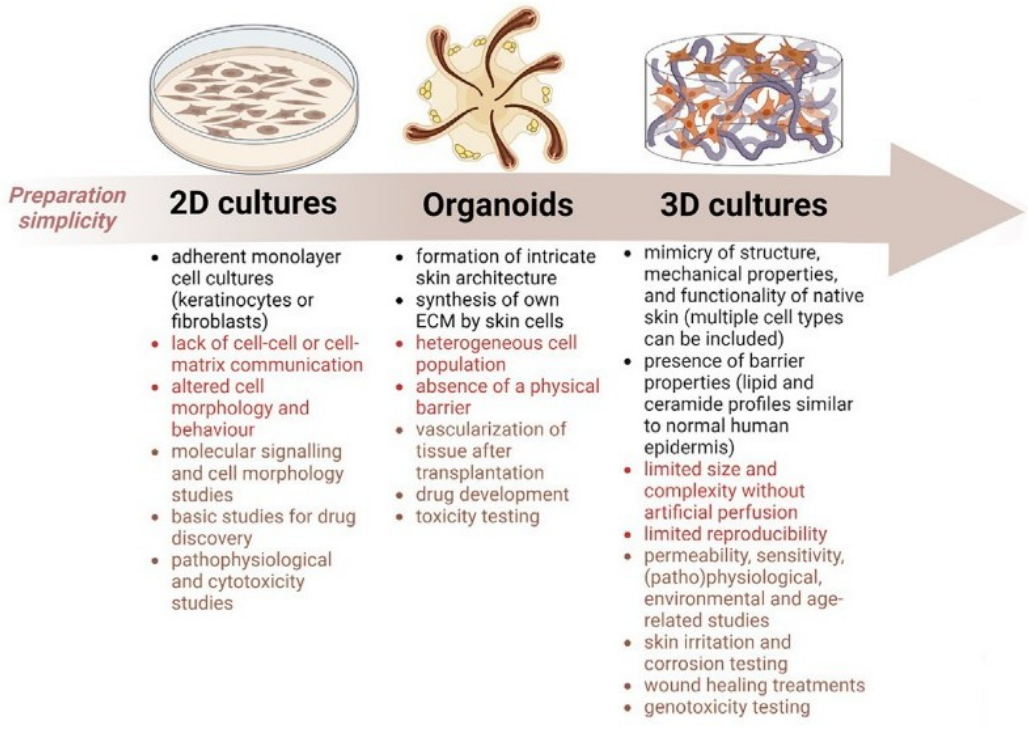

Figure 6: Comparison of major *in vitro* human skin models. Features (black), drawbacks (red) and applications (brown) are indicated for each strategy. Two-dimensional (2D) cultures are the simplest and the least physiologically relevant. In organoids, cells grow with extracellular matrix (ECM), forming self-organized clusters that differentiate and mimic structure and functions of mainly internal organs. Three-dimensional (3D) cultures, such as human skin equivalents (HSEs), provide the most accurate representation of human skin. Modified from (Quílez et al. 2024).

Multiple types of cells are co-cultured in 3D structures, that also include extracellular matrix (ECM), therefore mimicking the parental tissue more accurately than 2D representations (Kapałczyńska et al. 2018). The most used 3D skin models are the 3D human skin equivalents (HSE).

In the present, there are several available HSEs for drug screening, toxicity tests or skin sensitivity tests (Quílez et al. 2024). The French company Episkin offers several *in vitro* human skin models, including a reconstructed human epidermis ($SkinEthic^{TM}$ RHE). It is composed of human keratinocytes growing on an inert polycarbonate filter, histologically equivalent to the *in vivo* human epidermis. This epidermis model is validated under ISO 9001:2015, which guarantees its quality and reproducibility to assess the safety and efficacy of products (*EPISKIN* 2025a).

The $SkinEthic^{TM}$ RHE has multiple applications, including testing for skin sensitisation and irritation, the latter being validated under the OECD Test Guideline 439. This certification establishes this model as a scientifically reliable and regulatory-accepted alternative to animal testing for this specific endpoint (OECD 2025; EPISKIN 2025b). The use of these 3D *in vitro* models aligns with the principles of "reduction" and "replacement", as they represent a promising approach towards a future with minimal animal testing.

2 OBJECTIVES

The main objective of this project is to evaluate some of the most important features of a bi-functional patch meant to be used as a therapeutic strategy for chronic wounds, burns and other skin conditions implicating chronic inflammation and infection. With that aim, the present work focuses on the pre-clinical studies of the patch and its individual components, in order to guarantee their biocompatibility and functionality, potentially identifying the most suitable formulation among those tested. To achieve this general objective, the following specific objectives were established:

- To develop the composite platform comprising all necessary materials and active agents, and to evaluate its structure using field emission scanning electron microscopy (FESEM).
- To calculate the encapsulation efficiency of curcumin in nanostructured lipid carriers (NLCs) after purification in a size-exclusion chromatography.
- To determine the non-cumulative curcumin release from the loaded patches and to compare results obtained with different hydrogels.
- To evaluate the uptake of raw curcumin and CUR-NLCs in human BJ fibroblasts using fluorescence microscopy.
- To assess cytotoxicity of the individual components in human cell culture models, specifically BJ fibroblasts and HEKn keratinocytes.
- To measure potential skin irritation in a reconstructed human epidermis model after application of the patches.
- To verify if the studied platforms could induce changes in tissue morphology, proliferation and differentiation of reconstructed human epidermis models, using both a histological staining and immunohistochemical assays.

These studies were developed within the framework of the *Therapatch* project (PID2021-128213OB-I00), a collaborative initiative aimed at the development of a novel therapeutical bandage for the treatment of inflammatory skin disorders using selected biomaterials and bioactive agents. The experiments were carried out at the Center for Biomaterials and Tissue Engineering in Universitat Politècnica de València, in collaboration with the Equipe de Recherche sur les Relations Matrice Extracellulaire-Cellule in CY Cergy Paris Université.

3 MATERIALS AND METHODS

3.1 Reagents

Precirol® ATO5 (glyceryl palmitostearate) and Labrafac® lipophile WL 1349 (caprylic/capric triglycerides) were purchased from Gattefosse (Nanterre, France). Curcumin (CUR, purity 75 % HPLC), Tween® 80 (polyoxyethylene 20 sorbitan monooleate), Poloxamer 407 (polyoxyethylene-polyoxypropylene copolymer), sodium alginate from brown algae, sodium citrate, citric acid, sebacic acid (99 %), glycerol (99 %), calcium chloride hexahydrate (≥ 99 %), Dulbecco's Phosphate-Buffered Saline (DPBS), Dulbecco's Modified Eagle Medium (DMEM), paraformaldehyde, eosin Y, safranin O, Triton X-100 and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (Milan, Italy).

Polyvinyl alcohol (MW 133,000, 99 % hydrolysed) and paraffin wax were acquired from Polysciences (Warrington, USA). Chitosan Quaternary Ammonium Salt (HACC, substitution 90 %) was purchased from MarkNature (Changchun, China). Fetal bovine serum (FBS) from Labclinics (Barcelona, Spain) and EpilifeTM medium from Thermo Fisher Scientific (Waltham, USA) were used. Dichloromethane (DCM) and ethanol (EtOH) were purchased from VWR Chemicals (Fontenay-sous-Bois, France). MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was obtained from Deltaclon (Madrid, Spain), and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) from Roche (Basel, Switzerland).

Pure isopropanol and xylene were bought from Scharlab (Barcelona, Spain), 1 % Mayer's hematoxylin from Fluka (Buchs, Switzerland) and Tris-buffered saline (TBS) from Nzytech (Lisbon, Portugal). Anti-Filaggrin and Anti-Ki67 antibodies and Fluoroshield mounting medium with DAPI were purchased from Abcam (Cambridge, UK), as well as the rabbit-specific HRP/DAB IHC detection kit. All aqueous solutions were prepared using ultrapure water (resistivity of 18.2 MΩ cm), obtained with the Milli-Q® Direct Water Purification System (Merck KGaA, Darmstadt, Germany).

3.2 Preparation of the Materials

3.2.1 Curcumin-loaded NLCs

Unloaded (blank-NLCs) and curcumin-loaded (CUR-NLCs) nanostructured lipid carriers were prepared according to the method by Calderon-Jacinto et al. (2022), modifying parameters when necessary. Two phases were mixed at 80 ºC using high-shear homogenisation with an Ultra-Turrax (IKA, Staufen, Germany).

The organic phase was prepared by melting Labrafac™ at 0.67 % (w/v), Precirol® at 3 % (w/v), Tween® 80 at 0.4 % (w/v) and curcumin at 0.1 % (w/v), at 80 ºC. For blank-NLCs, curcumin was not added to the organic phase. Separately, the aqueous phase containing 3 % Poloxamer 407 and purified water was also heated to the same temperature. After an initial homogenisation at 10,000 rpm for 2 minutes, the aqueous phase was slowly added to the lipid phase while increasing the rotational speed until reaching 20,000 rpm. Once the complete aqueous phase was added and the speed reached, the mixing was sustained for 30 minutes at 80 °C. The preparation was cooled down at room temperature and stored overnight at 4-8 ºC. The next day, after centrifugation at 5,000 rpm for 30 minutes to remove any free molecules, the NLC samples were subjected to a size-exclusion chromatography in a Bio-Gel® P-10 (Bio-Rad, Marnes-la-Coquette, France) column with ultrapure water as the eluent.

3.2.2 Bacteriophage stocks

Two distinct bacteriophage species, specific to Staphylococcus aureus, were employed as model systems: GRCS, classified within the Podoviridae family, and ISP, belonging to the Myoviridae family. The respective recommended bacterial hosts for these phages are methicillin-resistant *S. aureus* (MRSA) strains: S. aureus HFH-29994 (BEI Resources, NR-10187) for GRCS, and *S. aureus* 812 (BEI Resources, NR-46338) for ISP. All bacteriophages used were obtained from the internal collection of CY Cergy Université, where they were previously purified to increase phage concentration, using a polyethylene glycol precipitation followed by cesium chloride density-gradient centrifugation.

3.2.3 Hydrogels

Sodium alginate was dissolved in ultrapure water at a concentration of 1 % (w/v) under continuous stirring for 3 h at room temperature. Once a homogeneous mixture was obtained, a 250 mM calcium chloride ($CaCl_2$) solution was added to initiate hydrogel formation.

To prepare the PVA/HACC hydrogel, PVA was dissolved in water at 3.2 % (w/v) by heating at 80 °C overnight. In parallel, a 4 % (w/v) solution of HACC was prepared by dissolving the polymer at 37 °C overnight. Subsequently, the two solutions were combined and stirred for 1 h to obtain final concentrations of 1.6 % (w/v) PVA and 0.3 % (w/v) HACC. The resulting mixture was subjected to a freeze–thaw process, consisting of freezing at −20 °C overnight, thawing at 5 °C for 2 h, and equilibrating at room temperature for an additional 2 h. An equivalent protocol was employed to obtain hydrogels containing 2.5 %, 0.5 %, and 0.4 % (w/v) HACC, with a constant 1.6 % (w/v) PVA concentration.

3.2.4 Scaffolds

The poly(glycerol sebacate) prepolymer was synthesised using an equimolar amount of sebacic acid and glycerol in an oil bath at 130 ºC with a nitrogen atmosphere while stirring for 24 hours. Once the prepolymer was prepared, it was mixed with sodium chloride (NaCl) (particle size: 180–215 µm) at 90 ºC. The resulting composite was cast into Teflon moulds and thermally cured at 130 °C for another 48 h. After curing, the porogen salt was removed in successive baths of distilled water until complete salt dissolution was achieved, in a process known as salt leaching. The scaffolds were then air-dried at room temperature for 48 h. Depending on the size of the salt crystals used, two types of scaffolds were obtained. The first, referred to as 1:4M and formed with the 215 µm salt, exhibited uniformly large pores throughout the structure. The second, designated Strat-T, featured a bilayered architecture comprising one layer with macropores like 1:4M and another one with micropores as a result of 180 µm sized NaCl particles.

3.3 Production and Characterisation of the Composite Platform

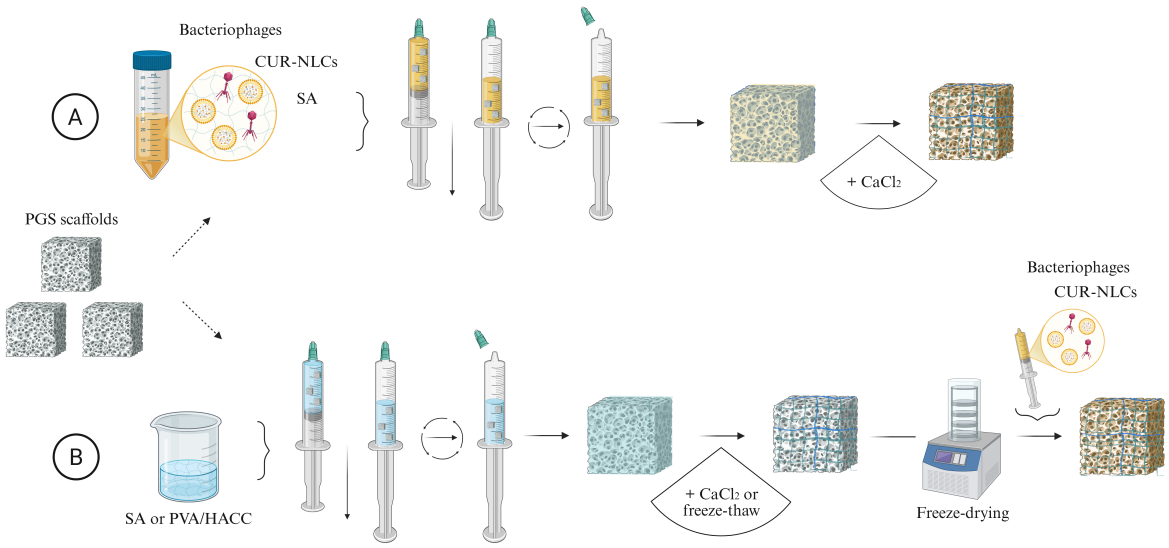

Figure 7: Schematic representation of the preparation of the composite platforms. Two strategies are illustrated: the infiltration in the scaffolds, using a syringe to create manual negative pressure, of the bioactive agents in the polymer solution, prior to gelation (A), and the loading of the scaffold solely with the polymer solution, following hydrogel formation, freeze-drying and lastly swelling with a solution containing bacteriophages and CUR-NLCs (B). Both approaches are applicable to sodium alginate–based platforms, whereas PVA/HACC hydrogels are prepared exclusively according to strategy B. Author's elaboration. Created with Biorender.

In order to incorporate all of the components into a composite platform, several steps were followed. Moreover, two different procedures were tested to load the bioactive agents in the hydrogels, represented in Figure 7.

In the first approach, the polymer solution was mixed with the CUR-NLCs and bacteriophages prior to loading. Concentrations were approximately 700 µM of curcumin, 20 mg/mL of NLCs and 10^{10} PFU/mL of bacteriophages. The infiltration in the scaffold was then performed with the mixture solution, by applying repeated cycles of manual negative pressure using a capped syringe, thereby facilitating homogeneous penetration of the liquid into the porous matrix. Gelation was subsequently achieved by the addition of 250 mM calcium chloride for the SA hydrogel.

In the second approach, scaffolds were loaded only with the hydrogel solution, then gelated and subjected to a lyophilisation process using a bench-top freeze dryer (Cryotec, Lunel Viel, Francia) for 24 h. After dehydration, the solution of bacteriophages and CUR-NLCs was incorporated into the scaffold using the same vacuum-assisted loading method previously described. Finally, the loaded scaffolds were incubated 1 h in the same solution to ensure hydrogel swelling.

Only the second strategy was used in the case of the PVA/HACC, where the active agents were introduced in the scaffold only after freeze-drying, while for the SA hydrogels both procedures were employed.

3.3.1 Material characterisation by FESEM imaging

In order to characterise the patches, field emission scanning electron microscopy (FESEM) was performed using the Zeiss Ultra 55 microscope in Universitat Politècnica de València. The samples were freeze-dried and subsequently sputter-coated with gold. A scale bar was manually added to each image using Fiji (Fiji Is Just ImageJ) and the microscope software and used for subsequent measurements.

3.3.2 Quantification of NLCs and encapsulation efficiency

After purification with SEC, quantification of the blank and curcumin NLCs was performed using a gravimetric method. For this assay, 200 µL of the purified NLC suspension was lyophilised for 24 h and the resulting dry residue was weighted. The difference in mass was used to calculate the concentration of NLCs, while the absorbance at 734 nm was utilised to obtain the concentration of curcumin. Finally, the encapsulation efficiency (EE) was calculated as the encapsulated curcumin divided by the initial CUR concentration, which was 1 mg/mL, following:

$$EE(\%) = \left(\frac{CUR_{NLC}}{CUR_{free}}\right)(\mu M) \times 100$$

3.3.3 Curcumin release assay

The released amount of curcumin was quantified by liquid–liquid extraction into an organic phase. The NLC-loaded PGS-hydrogel platforms were incubated in DPBS to evaluate curcumin release at different time intervals. Release samples were then mixed at a 1:1 ratio with an ethanol/dichloromethane mixture (EtOH/DCM, 60:40 v/v) to solubilise the NLCs and facilitate phase separation. The absorbance of the dichloromethane phase was subsequently recorded at 427 nm using a UV–Vis spectrophotometer. Curcumin concentrations were calculated from a calibration curve prepared in the organic phase under identical conditions, taking into account both the initial CUR concentration in the swelled scaffolds and subtracting the signal corresponding to blank-NLCs. The non-cumulative release percentages at t times were calculated using the equation:

$$\textit{Released CUR (\%)} = \left(\frac{\mathrm{CUR}_t}{\mathrm{CUR}_0}\right)(\mu M) \times 100$$

3.4 Biological characterisation of the patches

3.4.1 Curcumin cell uptake by fluorescence microscopy

The cell uptake of curcumin was evaluated using human BJ fibroblasts that were seeded at a density of 10,000 cells/cm² onto sterile glass coverslips in a 48-well plate. Cells were incubated for 24 h at 37º C in phenol red-free DMEM supplemented with 10 % FBS. The conditions studied included raw curcumin and curcumin-loaded NLCs at 25 µM and two negative controls corresponding to the blank NLCs and cell medium; all either in the presence or absence of bacteriophages (GRCS and ISP).

For each condition, cells were incubated with the solutions for 30 minutes at 37 ºC in the dark. After incubation, cells were triple-washed with DPBS to remove excess curcumin and nanoparticles, fixed with 4 % paraformaldehyde in PBS for 10 minutes at room temperature and washed again with DPBS. For the nuclear stain, coverslips were mounted with a 4′,6-diamidino-2-phenylindole (DAPI)-containing medium. The resulting images were obtained using a Zeiss Axiovert 200M fluorescence microscope (Oberkochen, Germany) with the 40X objective and DAPI and FITC filters.

3.4.2 Cytotoxicity evaluation

The cytotoxicity of each component of the platform was evaluated in human BJ fibroblasts and human epidermal keratinocytes (HEKn) using a MTS assay. BJ fibroblasts were seeded in 48-well plates at a density of 10,000 cells/cm² in DMEM supplemented with 10 % FBS, whereas HEKn cells were seeded at 7,500 cells/cm² in EpiLife™ medium containing 60 µM calcium. After 24 h of incubation, cells were

exposed to the individual platform components for an additional 24 h. In the direct exposure approach, materials were placed in contact with the cells, whereas in the indirect approach, cells were treated with conditioned medium obtained from a 24 h incubation of the materials at 37 °C. Following exposure, metabolic activity was assessed by adding MTS solution (10 % v/v in phenol red-free medium) to each well and incubating for 40 min (BJ fibroblasts) or 4 h (HEKn) at 37 °C.

Absorbance was measured at 490 nm using a microplate reader, and cell metabolic activity was expressed as a percentage relative to untreated control cells cultured in the corresponding medium, with a threshold of 70 %, in accordance to ISO 10993-5 (ISO 2009). The linearity between cell density and absorbance at 490 nm after MTS treatment was previously evaluated through a calibration curve for each cell line employed, up to 50,000 $cells/cm^2$.

3.5 Animal-free Pre-clinical Validation

3.5.1 Skin irritation assay using a RHE models

The potential skin irritation of the platform was evaluated using the $SkinEthic^{TM}$ RHE model by Episkin. This assay was performed following the skin irritation protocol for medical devices provided by Episkin (EPISKIN 2019).

Upon arrival, the tissues were incubated in maintenance medium for 24 h at 37 °C in 5 % CO_2. The components were put in contact with the epidermal surface for 24 h under the same incubation conditions. After exposure, tissues were thoroughly rinsed with DPBS to remove residual material and the biocompatibility was then evaluated with MTT. Tissues were incubated with the MTT solution, at 1 mg/mL in maintenance medium, for 3 h at 37 °C, followed by extraction of the resulting formazan crystals with pure isopropanol. The absorbance was measured at 570 nm using a microplate reader. Tissue viability was expressed as a percentage relative to the untreated negative control. Following ISO 10993-23 and OECD Test Guideline 439, a cell viability of less than 50 % was concluded to be an irritant response (OECD 2025)

3.5.2 Histological and immunohistochemical analysis of the RHE models

To further study the skin's response to the patches, a 72 h direct contact assay was employed for all compositions in growth medium, followed by a histological and immunohistochemical analysis to establish potential changes in the RHE, with respect to the untreated controls. Once incubation with the materials was finished, tissues were fixated, dehydrated and embedded in paraffin according to the protocol provided by Episkin.

Firstly, each tissue was freed from its insert and cut in half. A 4 % paraformaldehyde solution was used to soak two surgical sponges and the tissues were placed in between and within a histological cassette. This construct was then immersed in fixative for 1 h.

After fixation, the cassettes were transferred into solutions with increasing concentrations of ethanol, following the order: ethanol 70º, 95º (twice) and 100º (twice), each for 15 minutes. Once finished, the cassettes were immersed twice in xylene 100 % for 15 minutes. All previous steps were performed at room temperature. For the paraffin inclusion, two subsequent melted paraffin baths were used to replace the xylene, at 56-60 ºC for 30 minutes, and finally the tissue samples were embedded in fresh paraffin to form the histological blocks. They were allowed to solidify for a minimum of 15 minutes. Cuts were performed using a Microm HM 355S paraffin microtome (Waltham, USA). Sections of 5 μm thickness were mounted in treated glass slides (SuperFrost® Plus, Epredia, USA) and dried overnight.

The hematoxylin, eosin and safranin (HES) staining was employed to assess the structural integrity of the tissues. In this regard, the sections were deparaffinised (60 ºC, 1 h) and rehydrated with xylene, EtOH (100º and 95º) and water. They were incubated for 2 minutes with 1 % hematoxylin, triple-washed with water, and 3 minutes with 1 % eosin. Following the protocol, 1 % safranin was applied for 5 minutes. After treatment with EtOH 100º and xylene, mounting medium (Eukitt®, Sigma-Aldrich, USA) was used to prepare the sections for the microscope and they were dried overnight. Images were taken with a fluorescence microscope with bright field illumination at 40X.

The immunohistochemical assay followed the same initial preparation steps; however, after rehydration, antigen retrieval was performed using citrate buffer (10 mM) at 95 °C for 20 min. The tissues were permeabilised with 0.4 % TBS-Triton X-100 (2 x 5 min) and the endogenous peroxidase activity was blocked with hydrogen peroxidase (10 min) from the Abcam rabbit-specific HRP/DAB IHC detection kit. The protein blocking agent (Abcam kit) was added for 30 minutes.

Afterwards, sections were incubated 1 h at room temperature with anti-filaggrin (1:1000) and anti-Ki-67 (1:1000) as primary antibodies. The biotinylated goat anti-rabbit secondary antibody (Abcam kit) was added to all sections for 10 minutes, following incubation with HRP-labelled streptavidin (10 min, Abcam kit) and development of the DAB chromogen (40 s-1 min, 1:50 in DAB substrate, both in Abcam kit). After counter-staining with hematoxylin (2 min) and dehydrating, samples were mounted with Eukitt® and observed with a bright field fluorescence microscope.

4 RESULTS AND DISCUSSION

The following results correspond to the main findings in the pre-clinical studies of the *Therapatch* device and its individual components. Nonetheless, additional analyses, not directly related to the purpose of this work, have been performed in a simultaneous manner. That includes the chemical and mechanical characterisation of the platform, using Fourier transform infrared (FTIR) spectroscopy and rheometry, and assays regarding the formation and swelling of the hydrogels, in addition to the evaluation of the antioxidant capacity of curcumin.

4.1 Microstructural Characterisation of the System

The cross-section morphology of the composite platform was examined by FESEM to evaluate the loading of the porous scaffolds with the hydrogel solutions and to compare the two scaffold types, 1:4M and Strat-T. In both cases, a highly porous structure was observed, as shown in Figure 8. The 1:4M scaffolds display large and consistent pores of around 150-200 μm (Fig. 8A-D), while the Strat-T is divided in two distinct layers: the upper-layer, with micropores (20-50 μm); and the lower-layer, with pores of 150-200 μm (Fig. 8E-J).

Both hydrogels used to load these scaffolds were subjected to the freeze-drying protocol (Fig.7B, and certain differences can be observed in their morphologies after rehydration. SA appears to have a softer gel consistency in both 1:4M (Fig. 8B) and Strat-T (Fig. 8G), while PVA/HACC has a more heterogeneous texture with some granule-like appearance (Fig. 8D and 8J).

The structures observed here are consistent with those reported for salt-leached PGS by X. Zhang et al. (2016): open pores with extensive interconnectivity, whose size is determined by the salt crystals employed. The differences observed between 1:4M and Strat-T are particularly relevant for their use in wounds, since pore size highly influences cell infiltration and oxygen and nutrients exchange. Some studies recommend pores of 100-200 μm for scaffolds meant for skin tissue engineering (Han et al. 2014; Haifeng Liu et al. 2007). High porosity is also advised for these applications, achieved in both PGS formulations, to allow controlled absorption of wound exudates while maintaining a moist healing environment (Negut, Dorcioman, and Grumezescu 2020).

On the other hand, the Strat-T formulation could minimise damage during dressing changes, being that the presence of smaller pores in contact with the skin may limit cell transfer and tissue ingrowth (Hargis et al. 2024). Otherwise, skin cells, typically around 20-40 μm (Ismail et al. 2011), could infiltrate and start growing within the patch, hindering removal, causing pain and discomfort and potentially delaying healing, as is common with traditional wound dressings (Institute for Quality and Efficiency in Health Care (IQWiG) 2022).

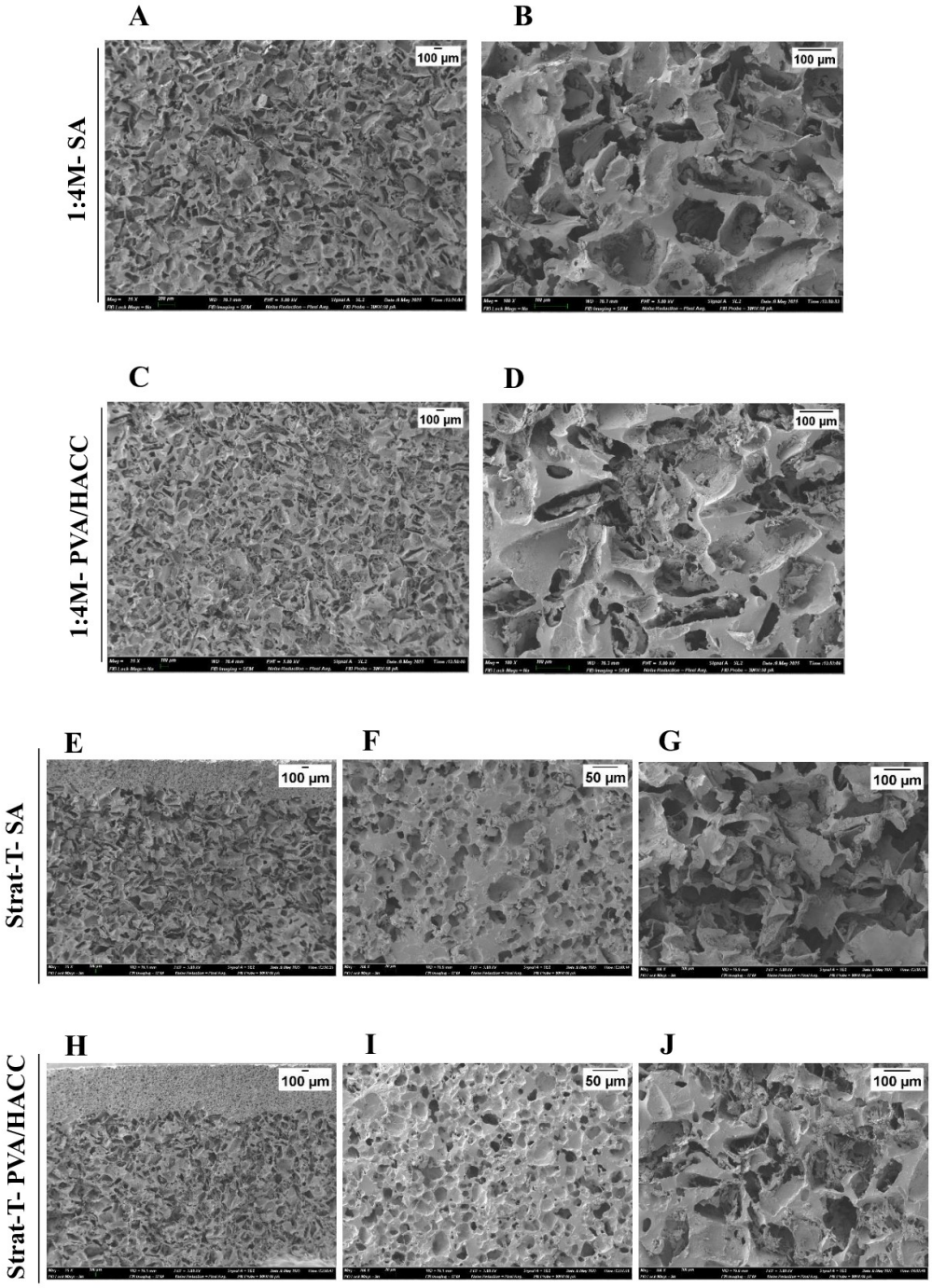

Figure 8: Cross-sectional FESEM images of PGS scaffolds loaded with hydrogels. Figures A-D correspond to the 1:4M formulation with SA at 25x (A) and 100x (B) and PVA/HACC at the same magnitudes (C, D). Figures E-H represent the Strat-T scaffold and its distinct double-layer morphology, also loaded with SA (E-G) and PVA/HACC (H-J) at 25x, 200x and 100x, respectively. Author's elaboration.

The differences between the two hydrogels may be attributed to their chemical structures and relative hydrophilicity. While SA is an anionic polysaccharide that forms hydrogels by ionic cross-linking with cations, HACC gelifies with PVA due to intra- and inter-molecular hydrogen bonds and electrostatic interactions (Aderibigbe and Buyana 2018; Y. Zhang et al. 2015). During freeze-drying, rearrangement of PVA and HACC chains may complicate their re-association and the recovery of the initial structure (Y. Zhang et al. 2015), in addition to potential swelling difficulties due to the hydrophobic carbon backbone in HACC.

4.2 Curcumin Encapsulation Efficiency

Table 2 shows results for the NLC and curcumin quantification and encapsulation efficiency, after preparation using the hot-homogenisation method and purification with SEC. Similar values for the NLC concentrations were obtained in both blank and curcumin nanoparticles, demonstrating the reproducibility of the method used.

As expected, the encapsulation efficiency was approximately 55 %, with similar results to a previous study with identical materials (Armal et al. 2025). Calderon-Jacinto et al. (2022) also reported EE of approximately 84 % and 54 %, before and after SEC, respectively. This variation may be explained by the elimination of free, non-encapsulated curcumin during purification, as well as the potential loss of CUR-NLCs during that same process.

Table 2: NLC and curcumin concentrations and encapsulation efficiency of curcumin, after SEC purification. Data for blank-NLCs and curcumin-loaded NLCs is shown. Author's elaboration. *EE was calculated corresponding to an initial CUR concentration of 1 mg/mL.

Parameter	Blank-NLCs (mean ± SD, n=4)	CUR-NLCs (mean ± SD, n=4)
[NLCs] (mg/mL)	44.28 ± 6.07	44.86 ± 5.57
[CUR] (mg/mL)	-	0.55 ± 0.07
EE* (%)	-	54.5 ± 6.35

4.3 *In Vitro* Release of Curcumin

The curcumin release from the two freeze-dried and rehydrated 1:4M formulations was evaluated using UV-Vis spectrophotometry, as depicted in Figure 9. Concentrations of released curcumin were determined using a standard calibration curve ($y = 0.139x - 0.0162$, $R^2 = 0.997$; Fig.A1.2 in Annex 1) at the wavelength of 427 nm, selected according to its absorption spectra (Fig.A1.1) along with previous studies.

Both composite platforms exhibit a burst release with higher rates during the first 6 h of the experiment. Previous works showed a similar curcumin release profile with an initial burst, followed by a gradual and sustained liberation (Nasiri, Ahmadi, and Afshar-Taromi 2023). Notably, scaffolds incorporating SA have a higher overall release than PVA/HACC, with approximately 40 % after 6 h and 58 % after 72 h compared to the 14 % and 19 % of the quaternised chitosan over the same periods.

In wounds, curcumin acts as an anti-inflammatory agent with certain antibacterial properties. However, its hydrophobic character may limit its release from the therapeutic platform, even when encapsulated into NLCs. Therefore, the liberation assay provides important insight into the potential therapeutic efficacy of these scaffolds. The scaffolds containing SA exhibited a higher percentage of release, despite having an initial CUR concentration after swelling that was approximately 1.8 times lower than that of the PVA/HACC scaffolds (data not shown).

In contrast with the present findings, other studies had reported that the extent of curcumin diffusion was directly proportional to its initial concentration, when comparing formulations of the same material (Nasiri, Ahmadi, and Afshar-Taromi 2023). This suggests a strong interaction between CUR-NLCs and the PVA/HACC hydrogel matrix, resulting in a slow release of the active agent. This may, in part, be due to the negative zeta potential (ZP) of CUR-NLCs, around -17 mV after SEC, as reported by (Calderon-Jacinto et al. 2022) for similar lipid and surfactant compositions. The electrostatic attraction between cationic HACC and the NLCs with a negative ZP, in addition to the presence of a denser hydrogel network, likely contributed to a reduced diffusional release.

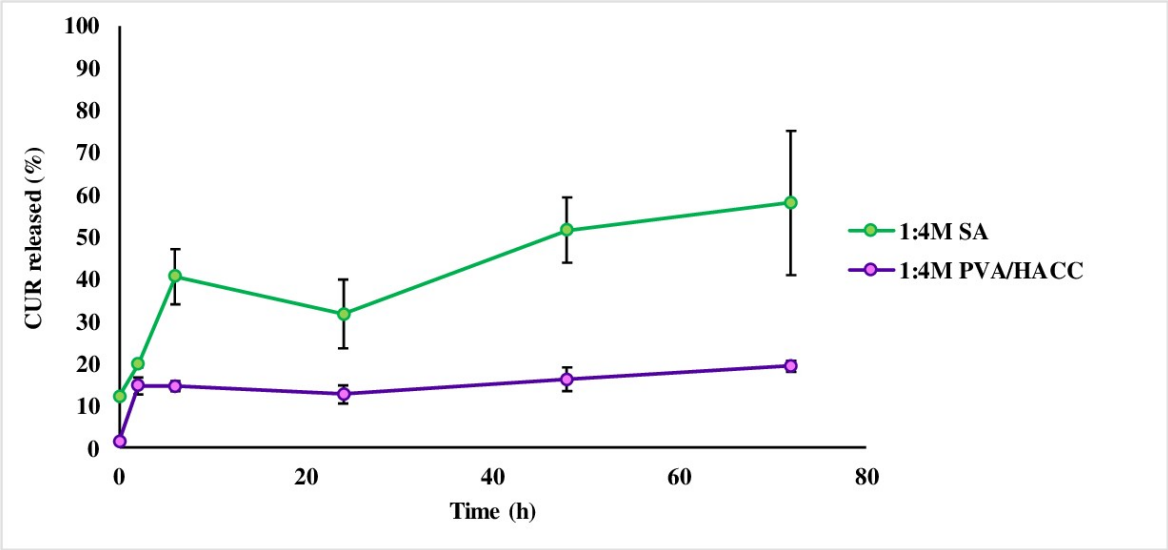

Figure 9: Non-cumulative curcumin release (%) from NLCs in 1:4M SA and 1:4M PVA/HACC. Scaffolds were freeze-dried and rehydrated with a CUR-NLC solution. The release in DPBS medium was measured at 0, 2, 6, 24, 48 and 72 h (n=3). Author's elaboration.

4.4 Curcumin Cell-Uptake

Curcumin displays intrinsic fluorescent characteristics. The qualitative uptake of 25 μM CUR-NLCs and raw curcumin was observed in BJ fibroblasts using fluorescence microscopy after treatment for 5, 15, 30 and 60 min. Figure 10 displays results for the 30 min assay, as previous literature indicated it was the optimal time for curcumin cell uptake (Kunwar et al. 2008), as well as earlier investigations performed.

Free curcumin appears to be internalised in a similar way to NLC-encapsulated curcumin, with a strong cytoplasmic presence. Cell nuclei also exhibited some fluorescence in both cases, suggesting partial nuclear internalisation of curcumin. Some curcumin aggregates are observed as well. As predicted, there is no green fluorescence in the case of blank-NLCs, only blue for the DAPI-stained nuclei. Notably, no difference in the subcellular localisation of curcumin is observed in the presence (Fig. 10D-L) or absence of phages.

The literature on the subcellular distribution of curcumin is heterogeneous, with some studies reporting preferential membrane association (Kunwar et al. 2008), while others indicate predominant cytoplasmic distribution (Del Prado-Audelo et al. 2019). Curcumin, being a hydrophobic molecule, may be expected to be localised in the cell membrane. However, its strong presence in the cytoplasm suggests that cells can easily internalise it, most probably through endocytosis. Results obtained were similar to prior research (Del Prado-Audelo et al. 2019; Lee et al. 2014). Lee et al. (2014) also reported that, in certain cell lines, curcumin uptake was enhanced when in nanoparticles. Although, in this assay, quantification of the fluorescence intensity was not performed, a visual comparison of Figures 10C and 10K could support this hypothesis, as the cytoplasmic signal appears slightly stronger in the latter, with a significant increase regarding curcumin presence in the nuclei.

While the primary goal of curcumin encapsulation is to increase skin penetration of the active agent through improved hydrophilicity and stability, the observed cell uptake increase with CUR-NLCs compared to free curcumin represents an additional advantage to their use.

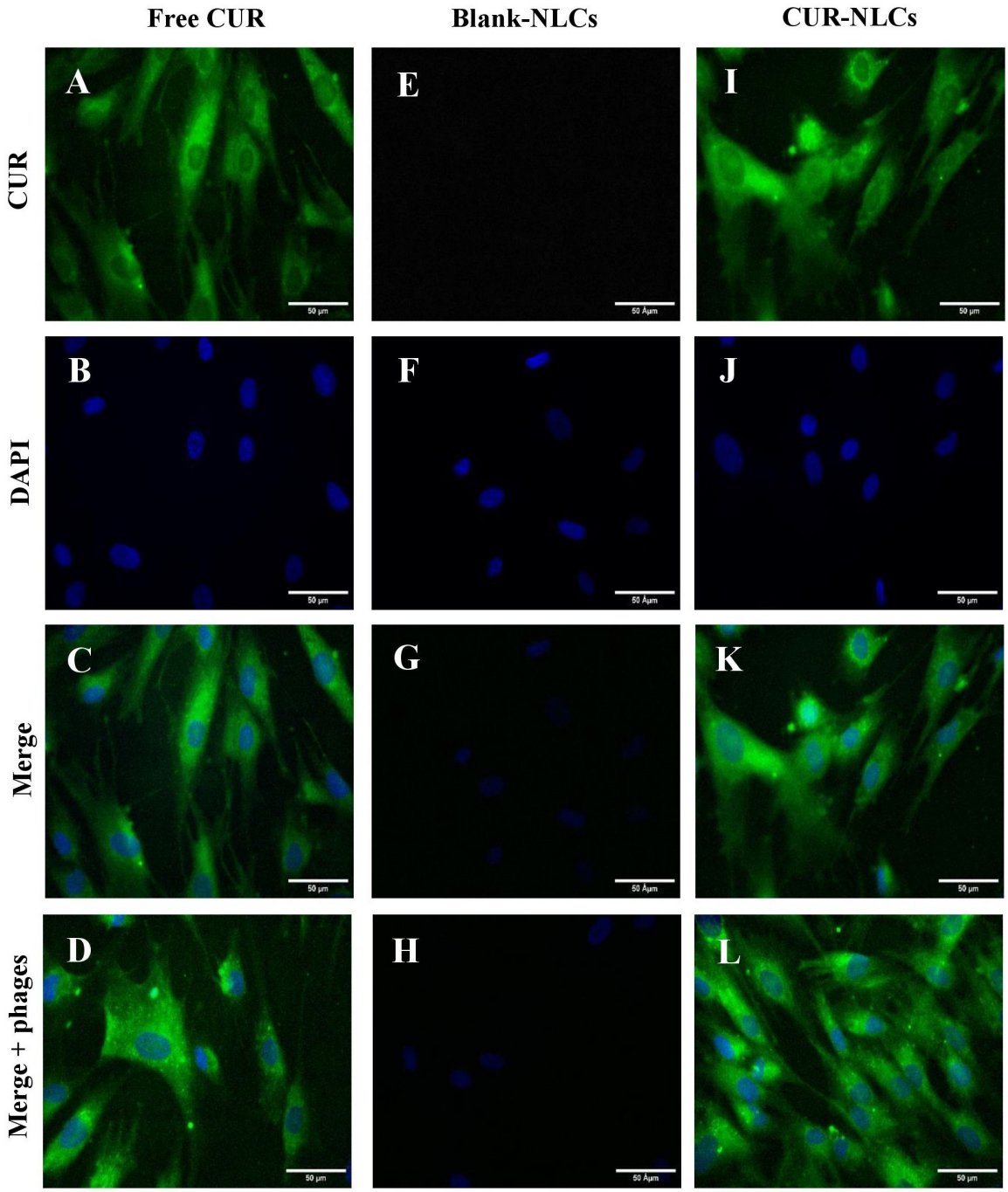

Figure 10: Cell uptake after 30 min of raw curcumin (A-D), blank-NLCs (E-H) and CUR-NLCs (I-L) in BJ fibroblasts evaluated by fluorescence microscopy. For images D, H and L cells were incubated in identical conditions with the addition of a bacteriophage culture (GRCS and ISP). Cellular nucleus stained with DAPI are shown in blue. Samples were observed at 40X. Author's elaboration.

4.5 Cytotoxicity Measurements in Human Cell Lines

The potential cytotoxicity of the studied components was established with a MTS assay in human BJ fibroblasts and HEKn keratinocytes. Figure 11 presents the results for the patch materials, expressed as a percentage of metabolic activity, considering the negative control as 100 %. According to ISO 10993-5 for the cytotoxicity evaluation of medical devices, a component is deemed cytotoxic when viability is reduced to <70 % of the untreated cells (ISO 2009).

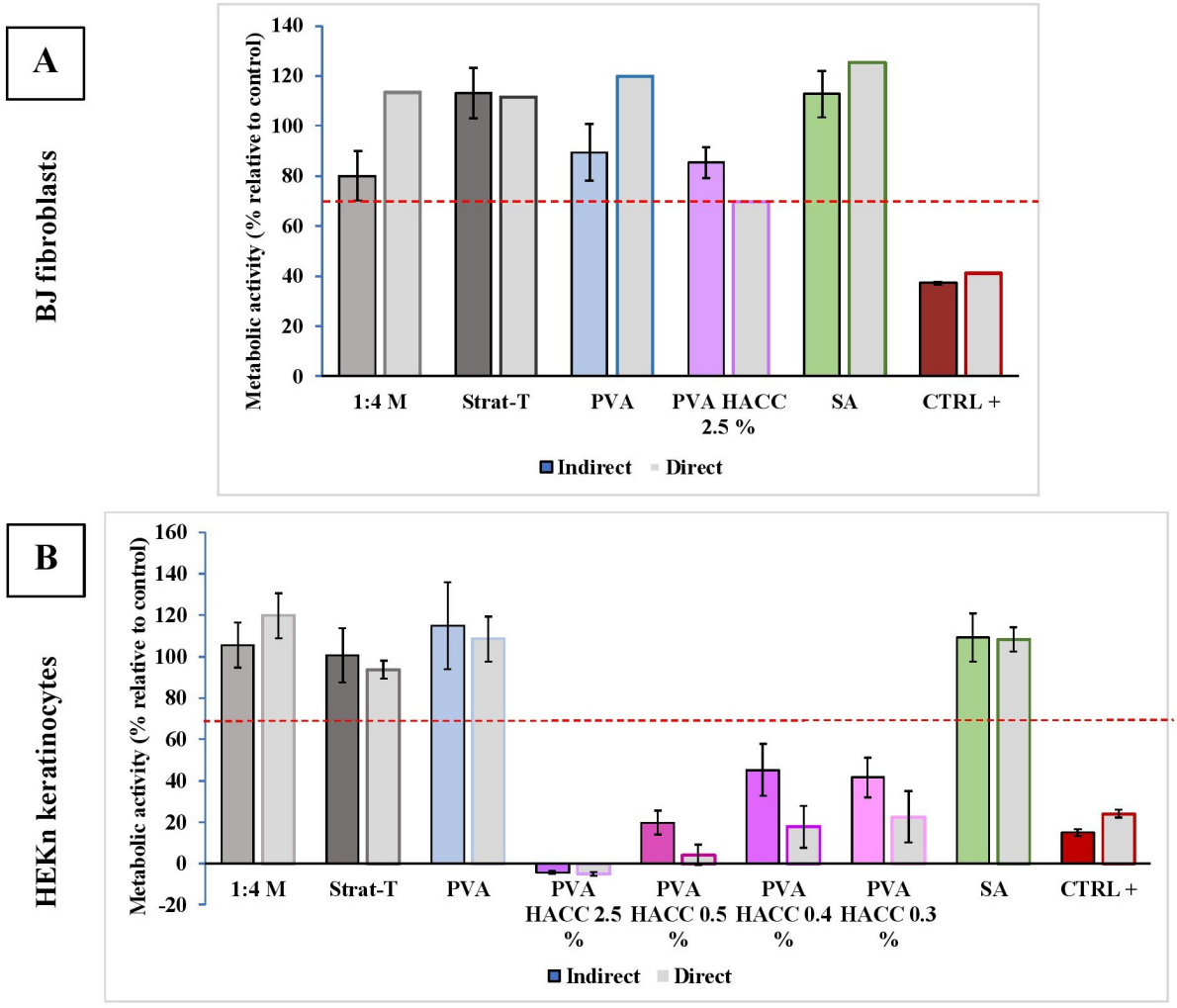

Figure 11: Cytotoxicity evaluation of 1:4M, Strat-T, PVA, PVA/HACC and SA in human BJ fibroblasts (A) and HEKn keratinocytes (B), assessed using the MTS assay. Cells were exposed either directly to the materials for 24 h or to conditioned medium obtained after 24 h of material incubation (indirect approach). The cytotoxicity threshold was established according to ISO 10993-5, with latex as positive control (n=3). Author's elaboration.

In the case of the BJ fibroblasts (Fig. 11A), both PGS formulations (1:4M and Strat-T) as well as PVA and SA were not proved cytotoxic, with activities around 80-120 % upon their direct and indirect application. Notably, the direct use of PVA/HACC resulted in a decreased cell metabolism, with an average activity of 69 % that is just below the threshold, although it was not the case for the indirect application of that same hydrogel (82 %).

An equivalent test was performed for HEKn keratinocytes (Fig. 11B). As expected, cell cultures with 1:4M, Strat-T, PVA and SA exhibited very high metabolic activity. On the other hand, PVA/HACC hydrogels were studied in more conditions, including lower concentrations of quaternised chitosan while maintaining a 1.6 % PVA concentration. A relatively high cytotoxicity was observed in all HACC formulations, including 2.5, 0.5, 0.4 and 0.3 %, in both direct and indirect assays.

It should be noted that the method of application may influence the evaluation, since materials tend to appear more cytotoxic when placed directly onto the cell monolayer. This reflects not only chemical effects but also mechanical stress and restricted gas exchange. This could possibly explain results found within the fibroblast assay.

However, our findings in keratinocytes indicate a likely cytotoxic effect of HACC, which contrasts with prior reports (Min et al., 2020) and our earlier observations in mouse fibroblasts, highlighting the need for testing medical devices and materials in several cell lines to correctly determine their toxicity. Still, additional evaluations might be recommended. These findings are in line with previous results in the present study, where quaternised chitosan hydrogels has demonstrated inferior characteristics compared to other formulations, such as sodium alginate.

With respect to the bioactive agents, which were applied exclusively by direct contact, the resulting cellular responses are represented in Figure 12. In BJ fibroblasts (Fig. 12A), high metabolic activities in all of the studied conditions were observed, including the two bacteriophages (GRCS and ISP), blank and curcumin-loaded NLCs, and raw curcumin at three concentrations of 5, 25 and 50 μM. Furthermore, no significant changes in cell morphology were identified. As with the fibroblasts, most of the agents did not alter cell metabolism in HEKn (Fig. 12B), with the exception of 50 μM raw curcumin. Previous reports had found that an increase in curcumin concentration was associated to an increase in cytotoxicity, most notably in specific cell lines that were more sensitive to its effects (Kunwar et al. 2008). Additionally, free curcumin has been seen to be more toxic than complex curcumin formulations like lipid nanoparticles inside a hydrogel (Mobaraki et al. 2021). Still, curcumin has been seen to maintain its antioxidant potential even in concentrations lower than 50 μM (Calderon-Jacinto et al. 2022), meaning that, if needed, its concentration in the patch could be decreased. Nevertheless, the platform should include exclusively CUR-NLCs, being that the encapsulation of curcumin has also been reported to enhance its antioxidant activity by up to eight-fold, with improved ability to scavenge free radicals (Elkhateeb et al. 2023).

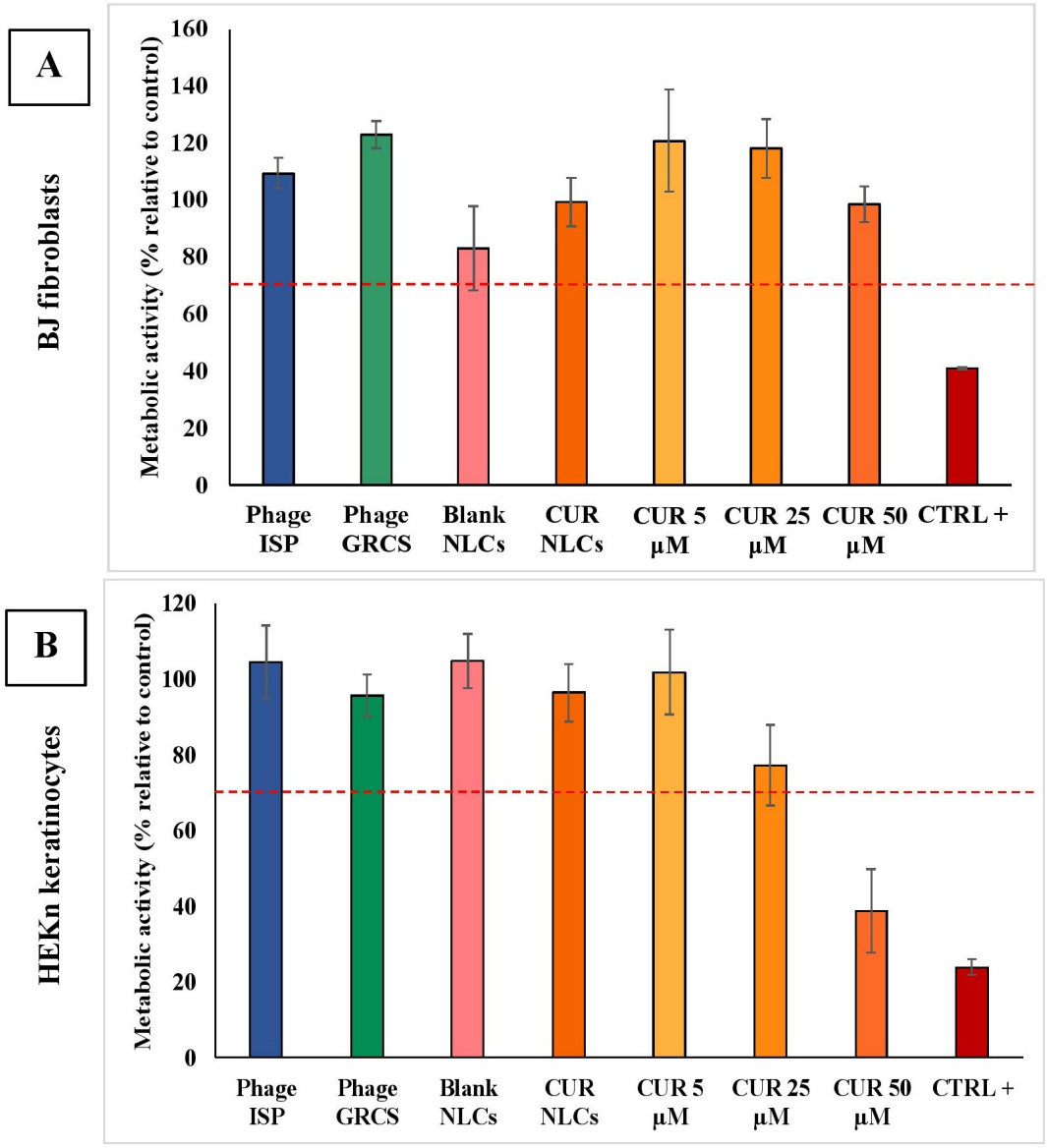

Figure 12: Cytotoxicity evaluation of phage ISP, phage GRCS, blank-NLCs, CUR-NLCs and raw curcumin at 5, 25 and 50 µM in human BJ fibroblasts (A) and HEKn keratinocytes (B), assessed using the MTS assay. Cells were exposed directly to the materials for 24 h. The cytotoxicity threshold was established according to ISO 10993-5, with latex as positive control (n=3). Author's elaboration.

4.6 Pre-Clinical Evaluation with RHE Models

4.6.1 Skin irritation

Although the aforementioned assay provides important information for the *in vitro* cytotoxicity, single-cell models lack some of the properties found in human skin, such as tissue architecture or communications between cells and with a matrix (Kapałczyńska et al. 2018). In this sense, a skin irritation test was performed using the reconstructed human epidermis model by Episkin and the protocols provided by the manufacturer, by means of measuring tissue viability after treatment with the test substances in a MTT assay.

For a substance to be considered a skin irritant, it should cause a reversible inflammatory reaction in the skin (Carlin et al. 2025). Figure 13 represents the viability of the RHE models as a percentage relative to the untreated negative control, upon application of the composite platforms, including in all cases both bacteriophages and CUR-NLCs. As shown, none of the devices induced an irritant reaction since the viability reduction was below the threshold given by the OECD Test Guideline 439 (tissue viability: ≤50 %) (OECD 2025). The protocol was validated meeting Episkin's criteria: negative control OD ≥ 1.2 and SD ≤ 18 %; positive control < 40 % and SD ≤ 18 %. There was a small reduction in the observed viability when formulations with SA and PVA/HACC were compared, with the latter being lower. Still, both platforms with PVA/HACC hydrogels were within range to be classified as non-irritant.

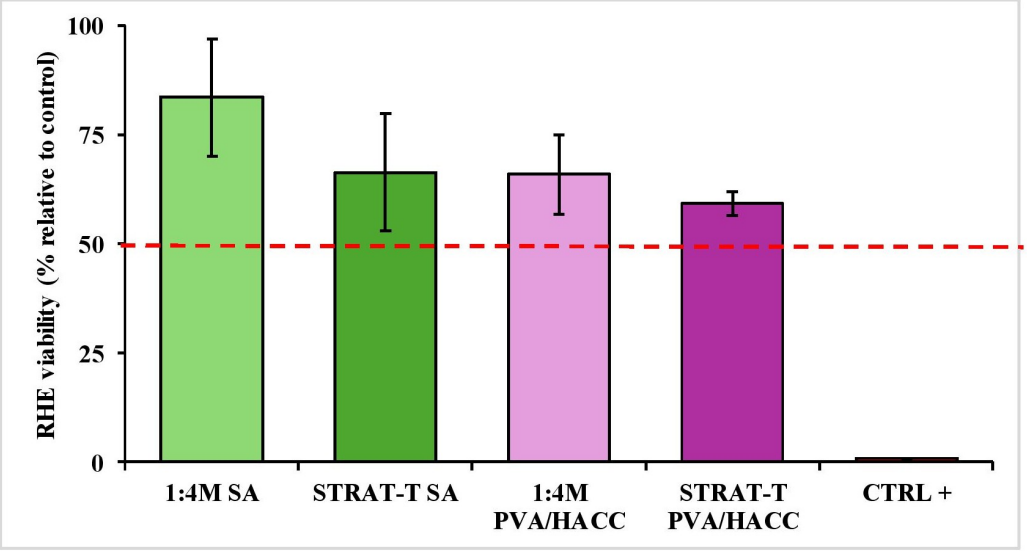

Figure 13: Skin irritation assay of the composite platforms using MTT in SkinEthic™ RHE. All samples include CUR-NLCs and bacteriophages in a hydrogel matrix (SA or PVA/HACC 0.3 %) embedded in an elastomeric scaffold (1:4M or Strat-T). Irritation was predicted with the 50 % threshold given by the OECD TG 439, with SDS 1 % as positive control (n=9). Author's elaboration.

The 3D epidermis models enable the formation of intercellular junctions and provide an accurate representation of tissue integrity and barrier function, in the case of skin models. These properties make them more reliable and predictive than 2D cell cultures (Carlin et al. 2025). In this sense, it is also possible that the presence of the curcumin nanoparticles hindered potential epidermal inflammation, thus decreasing irritant cell response compared to the study of the hydrogels alone, although no direct evidence has been reported to validate this. Therefore, further studies should be conducted to determine the effect of HACC and HACC-containing preparations in skin cells and models.

4.6.2 Histological and immunohistochemical analysis

In order to further evaluate the potential negative effects of the platforms in the skin integrity, a series of histological and immunohistochemical assays of the RHE models were employed, after direct contact with the materials for 72 h. Figure 14 represents the results for the hematoxylin-eosin-safronin staining, as well as the immunohistochemistry regarding filaggrin and Ki67.

HES stainings are employed to assess whether a specific compound or material could induce changes in the morphology of a given tissue. The present findings show no cell or tissue damage upon the application of 1:4M-SA (Fig. 14A), Strat-T-SA (Fig. 14B), 1:4M-PVA/HACC (Fig. 14C) or Strat-T-HACC/PVA (Fig. 14D); as demonstrated by the similarities of the treated tissues and the untreated negative controls (Fig. 14E). Therefore, tissue integrity is maintained upon the use of the different scaffold and hydrogel formulations, with hematoxylin-stained purple cell nuclei, mostly in the *stratum basale*, and eosin and safranin-bound cytoplasmic proteins, especially keratin in the upper layers. In contrast, the positive SDS control appears with generalised eosinophilia, reflecting extensive tissue damage and protein accumulation across all layers, as well as certain breakage in the lower areas (Fig. 14F). These results were similar to those obtained by (Pellevoisin et al. 2018) when assessing with HES the RHE models' response upon contact with different compounds, including cytotoxic agents.

Additionally, the immunohistochemistry of the tissues for filaggrin and Ki67, markers of tissue differentiation and proliferation, respectively, validated the results obtained for HES. As illustrated by Figure 14, all of the conditions resemble the negative control. The filaggrin-brown staining of the *stratum granulosum* is well-defined and visible around the central part of the tissue cuts. Similarly, in the second immunohistochemical assay, a light brown colour is observed for the actively proliferating cells with high presence of Ki67. Negative controls are shown with o *stratum granulosum* and no proliferating cells, attributable to a complete loss of tissue functionality. Both filaggrin and Ki67 immunohistochemistry show similar patterns to the images provided by Episkin as reference and publicly available (*SkinEthic RHE Reconstructed Human Epidermis* 2025).

Based on these results, none of the devices, including those containing PVA/HACC, induced detectable tissue damage. No difference was identified in the use of sodium alginate or quaternised chitosan hydrogels, nor between 1:4M and Strat-T scaffolds. Episkin's RHE models also demonstrated high reproducibility, with consistent results across the three replicates and two controls that validated the protocols.

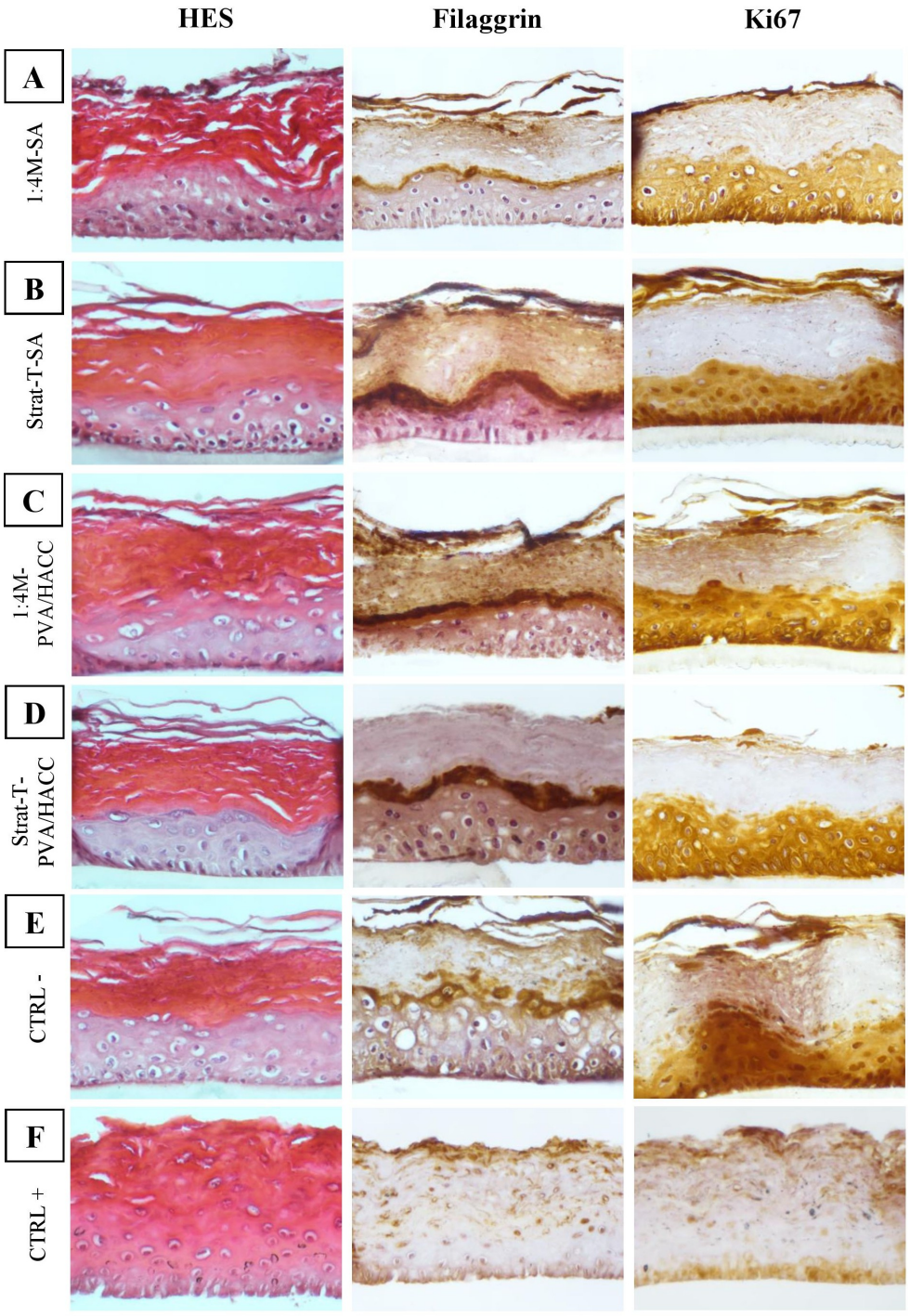

Figure 14: Histological and immunohistochemical assays of RHE models after 72 h of contact with the patches. Images correspond to formulations: 1:4M-SA (A), Strat-T-SA (B), 1:4M-PVA/HACC (C), Strat-T-HACC/PVA (D), DPBS (positive control, E) and SDS (negative control, F). In the first column, tissues were stained with hematoxylin-eosin-safranin, while the second and third correspond to two immunohistochemical assays for filaggrin and Ki67, respectively. Samples were observed at 40X (n=3). Author's elaboration.

5 CONCLUSIONS

This study has successfully characterised and pre-clinically evaluated the biocompatibility of composite patches integrating bacteriophages and CUR-NLCs embedded in a hydrogel inside a porous scaffold. When SA and PVA/HACC hydrogels were compared for their use within the device, sodium alginate demonstrated superior qualities to PVA/HACC, with significant differences in their structure stability after freeze-drying and rehydration.

In another respect, given the estimated encapsulation efficiency of curcumin and the known CUR-NLC concentration included in the patches, the corresponding activity of curcumin is considered sufficient to provide antioxidant and anti-inflammatory relief to chronic wounds or whichever inflammatory skin condition is treated.

However, a key aspect in this context is the ability of the selected materials to liberate curcumin. Notably, in all studied formulations, this release was presented with an initial burst that was later stabilised and reached a plateau around the 48-72 h time-point. These findings suggest that, for an optimal therapeutic utilisation, the use of each patch should be limited to two or three days, time after which it should be changed for a new one. Thus, the skin condition would be able to benefit appropriately from the agents included.

In addition, curcumin cell uptake was also evaluated to find that this agent is internalised in both the cytoplasm and nuclei, in either raw or nanoparticle form. Furthermore, the encapsulation of curcumin, whose main purpose was to increase skin penetration, did not hinder curcumin internalisation. On the contrary, CUR presence in the nuclei was greatly increased.

While cytotoxicity studies in individual human cell lines might have pointed to PVA/HACC as a cytotoxic material, there were significant differences in the response of the RHE models employed, where no skin irritation was observed. On the other hand, results regarding bacteriophages and curcumin formulations were equivalent in the 2D and 3D models and none of them were proved dangerous for topical use. Interestingly, keratinocytes did present with a slight sensitivity to raw curcumin in high concentrations, suggesting that NLC encapsulation could represent an additional advantage for the safety of the device.

Additionally, no significant alterations to tissue morphology, differentiation or proliferation were observed, with the use of any of the complete patch formulations. Not having observed any major differences in cell response to either 1:4M or Strat-T, the use of the bilayered scaffold is preferred in order to prevent cell infiltration into the dressing and its associated complications.

The presented findings highlight not only the need to test materials in several cell lines to provide an accurate cytotoxicity measurement, but also the considerable gap that exists between 2D and 3D models in terms of human skin representativeness and how the presence of a stratified structure with differentiated cells, proteins and a functional barrier can provide different results in comparison to a monolayer culture.

In summary, the recommended composition of the patches would consist of a sodium alginate hydrogel, loaded with curcumine-loaded NLCs and bacteriophages (GRCS and ISP), inside a Strat-T porous scaffold. While quaternised chitosan may still be considered, its questionable effects on the skin warrant further investigation.

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Annex 1: Curcumin absorption spectra and calibration curve

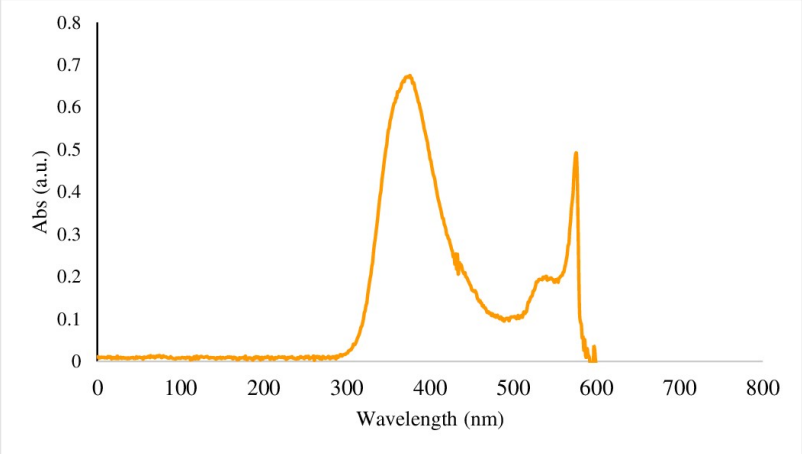

Figure A1.1: UV-Vis absorption spectra of curcumin in ethanol.

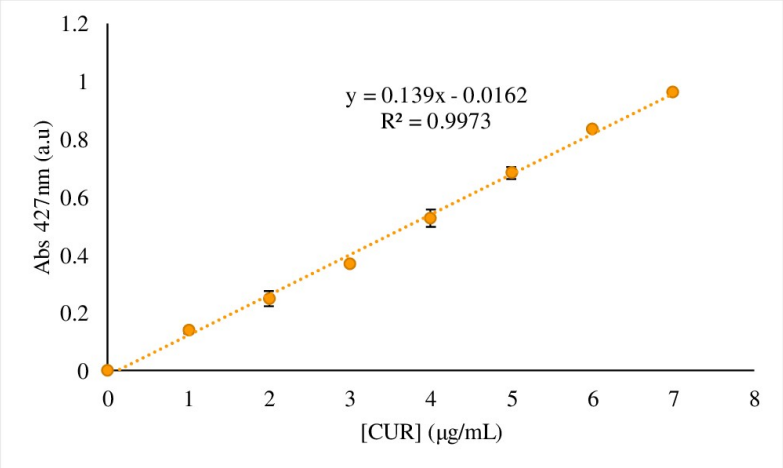

Figure A1.2: Calibration curve of curcumin in dichloromethane. Curcumin solutions (1-7 µg/mL) were prepared in DPBS, mixed with equal volume of EtOH/dichloromethane solution and after centrifugation the organic phase of curcumin and dichloromethane was recovered.