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New strategies for the
prevention and treatment of
complications associated with
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TESIS DOCTORAL

NEW STRATEGIES FOR THE PREVENTION AND TREATMENT OF COMPLICATIONS ASSOCIATED WITH DIABETES MELLITUS

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RESUMO

No panorama actual mundial da saúde, a diabetes mellitus emerxeu como unha das afeccións crónicas máis estendidas e desafiantes do século XXI, alcanzando o status de pandemia global debido á súa alarmante prevalencia. Esta enfermidade, xunto coas súas múltiples complicacións, exerce un impacto profundo na evolución clínica dos pacientes, deteriorando de maneira significativa a súa calidade de vida. Factores como o sedentarismo e as dietas pouco saudables xogan un rol crucial no incremento da incidencia da diabetes, especialmente nas nacións máis desenvolvidas. Dentro das variantes desta enfermidade, a diabetes mellitus tipo 1 e a diabetes mellitus tipo 2 sobresaen, sendo esta última a que presenta unha maior taxa de prevalencia a nivel mundial.

A diabetes mellitus é unha enfermidade que transcende a mera presenza de niveis elevados de glicosa en sangue; desencadea unha serie de complicacións tanto microvasculares como macrovasculares que comprometen severamente a calidade de vida dos afectados, incrementando de forma notable a morbilidade e mortalidade asociadas a esta condición. As complicacións microvasculares, que afectan os vasos sanguíneos de menor calibre, poden desencadear patoloxías graves como a retinopatía diabética, a cal compromete a saúde ocular e pode levar á cegueira se non se administra o tratamento adecuado, a nefropatía diabética, que dana os riles debido á exposición prolongada a altos niveis de glicosa, e a neuropatía diabética, que resulta do dano aos nervios e afecta a sensibilidade e funcionalidade sensorial, motora e autónoma. Doutra banda, as complicacións macrovasculares inciden nos vasos sanguíneos de maior tamaño, elevando o risco de desenvolver enfermidades cardiovasculares. Entre estas complicacións inclúese a aterosclerose, que conleva ao estreitamento das arterias por mor de acumulacións de graxa, e a enfermidade arterial periférica, que pode reducir significativamente o fluxo sanguíneo cara ás extremidades e, en situacións críticas, levar á amputación.

Ademais, observouse que as persoas que padecen de diabetes teñen un risco significativamente maior de experimentar infeccións causadas por unha variedade de axentes patóxenos, incluíndo bacterias, virus e fungos. Un exemplo palpable desta vulnerabilidade é a onicomíose, que ilustra de maneira contundente como as complicacións asociadas á diabetes poden impactar de maneira negativa nas extremidades

inferiores. Este aumento na susceptibilidade ás infeccións débese á interacción de varios factores críticos asociados coa diabetes. Por unha banda, a inmunidade comprometida, resultado directo do descontrol glicémico, impide que o sistema inmunolóxico responda de maneira eficaz ante a invasión de microorganismos patóxenos. Doutra banda, a mala circulación sanguínea, característica frecuente en pacientes diabéticos, limita a chegada de células inmunitarias e nutrientes esenciais ás extremidades, dificultando a curación de feridas e aumentando o risco de infeccións. Adicionalmente, a neuropatía diabética, que reduce a sensibilidade nos pés, pode facer que as feridas pasen desapercibidas e inféctense sen que o paciente déase conta ata que a infección avanzou significativamente.

Estes factores, cando interactúan, crean un terreo fértil para que as infeccións por fungos, como a onicomíase, non só instáuren senón que prosperen, levando a complicacións aínda máis graves. As infeccións non controladas poden derivar en úlceras nos pés, que son difíciles de curar e poden infectarse profundamente, provocando infeccións cutáneas extensas e celulites. Se estas condicións non se manexan adecuadamente, poden conducir a consecuencias devastadoras, incluíndo a necesidade de amputacións nos escenarios máis extremos. Esta cadea de eventos subliña a importancia dun manexo coidadoso e proactivo da diabetes para previr infeccións e as súas complicacións asociadas.

Neste contexto, faise evidente a imperiosa necesidade de salientar a prevención eficaz e o manexo integral da diabetes mellitus e as súas diversas complicacións como aspectos cruciais para atenuar os impactos negativos que esta enfermidade pode ter na saúde e o benestar xeral dos afectados. A consecución deste obxectivo fundamental apóiase no control óptimo dos niveis de glicosa sanguínea, a realización regular de exames médicos e a adopción dun estilo de vida saudable, que incorpore unha dieta balanceada e a práctica constante de actividade física.

A **introdución** desta tese doutoral dedícase a unha revisión exhaustiva e detallada da diabetes mellitus e as súas complicacións, proporcionando unha visión ampla sobre a escala e o carácter do desafío que representa esta enfermidade no ámbito da saúde pública global. Ao longo do texto, delinéanse e examinan detalladamente os aspectos fundamentais que serán abordados nos capítulos subseguintes, onde se introducen e discúten as diversas estratexias e enfoques terapéuticos destinados ao tratamento da enfermidade e as súas complicacións asociadas. Préstase especial atención á administración subcutánea de fármacos, analizando tanto as limitacións inherentes a esta

vía de administración como as novidades e avances no desenvolvemento de formas farmacéuticas de liberación prolongada que buscan superar ditas limitacións. Ademais, profúndase no tratamento oral específico para a diabetes mellitus, proporcionando unha revisión exhaustiva dos tratamentos actuais dispoñibles para afeccións como a retinopatía diabética, así como os progresos recentes no desenvolvemento de formas farmacéuticas oculares de liberación estendida e a aplicación de técnicas avanzadas como o uso de CO₂ supercrítico.

A introdución tamén se estende á cobertura do tratamento oral da diabetes mellitus e aborda desde unha perspectiva farmaco-técnica o manexo da onicomiose, destacando as innovacións en técnicas mecánicas deseñadas para incrementar a permeabilidade dos medicamentos a través da uña. Por último, leva a cabo unha discusión ao redor dos estudos preclínicos e os retos que actualmente se enfrontan no proceso de desenvolvemento de formulacións farmacéuticas que poidan ser empregadas no tratamento das complicacións asociadas a diabetes, que estean adaptados as distintas vías de administración. Neste sentido, introdúcese a impresión tridimensional (3D) como unha metodoloxía revolucionaria para a creación de modelos *in vitro*, *in situ* e *ex vivo* especializados, o cal representa un avance significativo que permite a optimización do desenvolvemento de investigacións preclínicas. Este enfoque innovador non só abre novas vías para a investigación farmacéutica, senón que tamén promete mellorar a eficacia e a precisión na elaboración de medicamentos adaptados ás necesidades específicas dos pacientes.

O **capítulo 1** desta tese salienta a crecente tendencia cara á adopción da administración subcutánea de biofármacos, presentándoa como unha opción terapéutica efectiva e cada vez máis preferida sobre as tradicionais infusións intravenosas por parte dos pacientes. Esta preferencia débese en gran medida aos beneficios que ofrece, particularmente para aqueles sometidos a tratamentos de longo prazo, como é o caso dos individuos con diabetes mellitus. Observouse que esta vía de administración non só mellora significativamente a adherencia do paciente ao tratamento, senón que tamén contribúe á redución dos custos asociados á atención sanitaria. Con todo, a pesar destes avances positivos, aínda se enfrontan diversos desafíos que inclúen a necesidade de progresos tecnolóxicos adicionais, a cuestión dunha biodisponibilidade incompleta e as preocupacións ao redor da inmunoxenicidade que poden xurdir con este tipo de administración.

Neste contexto, ponse especial énfase na diabetes mellitus, resaltando a súa crecente prevalencia a nivel mundial, o significativo impacto económico que representa e a urgente necesidade de optimizar a administración de medicamentos para esta condición, especialmente cando se trata de combinacións complexas de fármacos. A análise histórica do desenvolvemento de medicamentos baseados en proteínas, coa insulina como caso emblemático, senta as bases para comprender a evolución cara á administración subcutánea. A discusión avanza cara aos agonistas do receptor do péptido similar ao glucagón tipo 1 (GLP-1) e os miméticos da amilina, resaltando a súa aprobación regulatoria e a súa eficacia comprobada no manexo da diabetes tipo 2. Con todo, a pesar destes avances, os agonistas GLP-1 enfrontan limitacións, como a súa curta vida media que resulta na necesidade de múltiples inxeccións diarias e os efectos adversos asociados. De maneira similar, os miméticos da amilina, exemplificados pola pramlintida, presentan un gran potencial terapéutico, pero o seu uso vese limitado polas súas desafiantes propiedades farmacocinéticas.

A revisión continúa salientando a importancia de avanzar na xestión da diabetes mellitus, dada que as formulacións farmacéuticas dispoñibles na actualidade aínda requiren de administración frecuente. O obxectivo primordial é ofrecer unha panorámica comprensiva dos progresos recentes no desenvolvemento de sistemas de administración subcutánea que sexan tanto seguros como efectivos para o tratamento da diabetes tipo 2, poñendo un enfoque particular nos medicamentos non insulínicos e os agonistas GLP-1. Analízase a correlación entre as reaccións no sitio de inxección e a administración de volumes elevados de formulacións que conteñen agonistas GLP-1, xunto cos desafíos relacionados co seu inmunoxenicidade.

Ademais, profúndase no uso de modelos *in vitro* e *ex vivo* para a investigación preclínica no desenvolvemento destes biofármacos. Examínase a investigación actual sobre sistemas de administración prolongada de medicamentos e rutas de administración alternativas para os agonistas GLP-1. En particular, explórase o potencial das formulacións orais e bucais como alternativas menos invasivas ás inxeccións subcutáneas, e examínanse avances en formulacións para a administración nasal, incluíndo liposomas, hidroxelos termosensibles, sprays e solucións. Considéranse tamén rutas alternativas como a pulmonar e a transdérmica, destacando innovacións como os parches de microagullas. Finalmente, discútese o papel emerxente das nanopartículas e micropartículas no panorama de tratamento para a diabetes mellitus, subliñando o seu

potencial para mellorar a eficacia e a experiencia do paciente na xestión desta complexa enfermidade.

O **capítulo 2** desta tese doutoral está dedicado a explorar o desenvolvemento innovador dun implante intravítreo, deseñado especificamente para abordar e tratar enfermidades crónicas que afectan á retina, entre elas, a retinopatía diabética e o edema macular diabético, patoloxías oculares de alta prevalencia na diabetes mellitus. Estas enfermidades son recoñecidas a nivel mundial por ser causas significativas de morbilidade asociada á saúde ocular, así como da redución na agudeza visual e a perda de visión en pacientes afectados por estas condicións. Tradicionalmente, a abordaxe terapéutica para enfrontar estas patoloxías da retina implicou a administración intravítrea de fármacos que actúan inhibindo o factor de crecemento endotelial vascular, coñecidos como tratamentos anti-VEGF, ou ben, a utilización de corticosteroides. Con todo, estas modalidades de tratamento presentan certas limitacións, entre elas, os altos custos asociados aos medicamentos e procedementos, así como a necesidade de aplicar inxeccións de maneira repetitiva e frecuente, o que representa un desafío considerable para o manexo eficaz e sostido destas enfermidades oculares.

Ante estes desafíos, faise patente a necesidade de innovar e avanzar no desenvolvemento de sistemas de liberación intraocular de medicamentos que sexan capaces de ofrecer unha acción prolongada. O obxectivo principal detrás deste esforzo de investigación e desenvolvemento é facilitar unha redución significativa na frecuencia das inxeccións necesarias para o tratamento destas afeccións oculares crónicas. Ao lograr estender a duración da efectividade do tratamento mediante estes sistemas de liberación prolongada, poderíase mellorar substancialmente a calidade de vida dos pacientes, minimizando as molestias e complicacións asociadas aos réximes de tratamento máis tradicionais e frecuentes. Este capítulo, por tanto, céntrase en detallar o proceso de investigación, deseño, e potencial implementación do devandito implante intravítreo, subliñando a súa importancia no contexto dun manexo máis eficiente e menos invasivo da retinopatía diabética e o edema macular diabético.

Neste contexto, menciónase o lanzamento ao mercado de Ozurdex[®], un implante intravítreo que libera dexametasona durante varios meses como un avance significativo. Con todo, os métodos convencionais de fabricación destes implantes adoitan requirir o uso de altas temperaturas e solventes orgánicos, o que pode comprometer a seguridade e a eficacia do tratamento. Fronte a este desafío, propónse a adopción da tecnoloxía de

fluídos supercríticos, en particular o uso de CO₂ supercrítico, como unha alternativa máis amigable co medio ambiente. Esta tecnoloxía permite fabricar implantes poliméricos porosos sen expoñer os fármacos a condicións potencialmente prexudiciais. A técnica de espumado supercrítico facilita a creación de estruturas porosas cunha porosidade que pode ser finamente axustada para modular a taxa de liberación do medicamento.

Este capítulo propónse investigar a viabilidade de producir implantes intravítreos utilizando acedo poliláctico-co-glicólico (PLGA) mediante o proceso de espumado supercrítico. Para iso, utilizáronse moldes personalizados fabricados mediante impresión 3D especificamente deseñados para este fin. Seleccionouse a dexametasona como o composto modelo e o PLGA como material base para o implante, dada a súa coñecida biodegradabilidade e biocompatibilidade. Nesta fase do traballo levouse a cabo unha avaliación exhaustiva de como as diferentes taxas de despresurización afectan as características morfolóxicas dos implantes e, polo tanto, o perfil de liberación do fármaco *in vitro*. Ademais, o capítulo aborda a posibilidade de integrar medicamentos biolóxicos dentro destes implantes, incluíndo un estudo específico sobre a incorporación do bevacizumab, un anticorpo monoclonal que pode ser especialmente sensible á degradación baixo condicións de fabricación adversas.

No **capítulo 3** desta tese abórdase a creación e avaliación dun innovador sistema de administración deseñado especificamente para potenciar a capacidade de penetración dos medicamentos antifúnxicos nas uñas afectadas por onicomíose. A onicomíose representa a patoloxía ungueal máis frecuentemente diagnosticada a nivel global, impactando ata o 90% das uñas dos pés e aproximadamente o 50% das uñas das mans. Esta condición maniféstase a través de varios problemas nas uñas, incluíndo cambios na cor, engrosamento, aumento da fragilidade e, nalgúns casos, dor significativa. A prevalencia desta enfermidade é particularmente alta entre as persoas de idade avanzada, así como entre aqueles pacientes que padecen enfermidades crónicas como diabetes mellitus, psoríase ou VIH, xa que a súa situación inmunolóxica os situá nun risco elevado de contraer onicomíose. A abordaxe terapéutica para as infeccións fúnxicas ungueales presenta numerosos desafíos, tales como a tenacidade da afección, unha elevada probabilidade de recorrencia e certas limitacións inherentes ás terapias dispoñibles na actualidade. Aínda que os médicos adoitan preferir os tratamentos antifúnxicos orais debido á súa efectividade, estes poden desencadear efectos secundarios sistémicos adversos, o que intensificou a procura de alternativas tópicas máis seguras e efectivas.

Con todo, a eficacia dos tratamentos tópicos vese comprometida pola dificultade de penetrar na compacta estrutura da placa ungueal, o que á súa vez contribúe ao desenvolvemento de resistencia aos antifúnxicos.

A estrutura intrincada da placa ungueal, composta por densas capas de queratina e proteínas, xoga un papel crucial na limitada permeabilidade dos fármacos. Os enfoques mecánicos existentes, como a abrasión ungueal, son técnicas invasivas que non garanten unha mellora na efectividade do tratamento. Fronte a este panorama, propónse a aplicación de unha nova técnica segura, económica e menos invasiva para facilitar a penetración dos medicamentos nas capas profundas da uña. Para este estudo, seleccionouse DexULac[®], unha laca antifúnxica, como o composto modelo para ser administrado mediante a técnica desenvolvida. Para realizar esta avaliación empregáronse diversas metodoloxías analíticas avanzadas, incluíndo a microscopía confocal, a microscopía electrónica de varrido, a espectroscopía Raman e a difracción de raios X, así como estudos de permeación ungueal *ex vivo* co fin de obter unha comprensión detallada da eficacia do sistema de administración proposto. Os resultados mostran que a aplicación da nova tecnoloxía permite un incremento substancial da velocidade de penetración e da distribución do fármaco na matiz ungueal. Esta técnica permite o acceso do fármaco en pouco tempo incluso as capas mais profundas da uña, onde o acceso a partir de lacas medicadas está mais restrinxido. Polo tanto a técnica desenvolvida poderá ser utilizada nos tratamentos relacionados coas patoloxías ungueais relacionadas cos pacientes diabéticos para favorecer a súa curación.

No **capítulo 4** desta tese doutoral, abórdase o desenvolvemento dun recubrimento inerte especial para a súa aplicación en materiais acrílicos. As limitacións atopadas durante o traballo experimental en fases anteriores da tese deron pé ao estudo en maior profundidade dun prototipo para a realización de ensaios de liberación *in vitro*. Este traballo permitiu a creación de avanzados sistemas mediante o uso de tecnoloxía de impresión 3D, especificamente deseñados para levar a cabo estudos de liberación de medicamentos baixo condicións tanto *in vitro* como *ex vivo*. Estes sistemas representan unha ferramenta esencial no ámbito farmacéutico, xa que as células de difusión son empregadas cumpridamente para investigar como os fármacos se liberan a través de membranas que simulan barreiras tanto artificiais como biolóxicas. Este tipo de estudo é fundamental en múltiples etapas do proceso de desenvolvemento de medicamentos, empezando pola formulación inicial, seguindo coa optimización da mestura de compostos

activos e concluindo coa validación da uniformidade e calidade dos lotes fabricados. Aínda que as células de difusión, como as células de Franz e as de configuración lado a lado, foron tradicionalmente ferramentas predilectas para estas investigacións, non están exentas de limitacións, incluída a súa susceptibilidade a danos pola súa fraxilidade e o alto custo que implica a súa adquisición e mantemento ou a dificultade para desenvolver formas e esquemas mais axeitados para simular e adaptarse a certas vías de administración de medicamentos como é o caso da administración intraocular ou a administración nas uñas.

En resposta ás limitacións identificadas, este capítulo dedica unha atención especial á conceptualización e desenvolvemento de células de difusión innovadoras, as cales son producidas mediante o uso de tecnoloxías de impresión 3D. O propósito desta iniciativa é ofrecer solucións alternativas que non só sexan máis resistentes e duradeiras, senón que tamén resulten ser máis económicas e accesibles desde unha perspectiva de custos e de deseño adaptado as necesidades da vía de administración. Para materializar esta visión, optouse por empregar a técnica de estereolitografía (SLA), unha modalidade avanzada de impresión 3D, co fin de crear modelos de alta precisión e definición. Un desafío crítico que se aborda detalladamente no transcurso deste estudo é a problemática asociada coa tendencia dos materiais acrílicos, utilizados na fabricación 3D, a reter medicamentos na súa estrutura. Este fenómeno presenta un risco potencial de comprometer a exactitude e fiabilidade dos estudos de liberación de fármacos. Como estratexia para contrarrestar esta dificultade, levou a cabo unha exploración exhaustiva sobre a posibilidade de aplicar unha capa de recubrimentos inertes ás células de difusión impresas en 3D. O obxectivo primordial desta intervención era reducir significativamente, ou mesmo eliminar por completo, a capacidade de absorción e retención dos fármacos por parte do acrílico, asegurando así a integridade e precisión dos resultados obtidos nos ensaios de liberación.

A avaliación da efectividade dos recubrimentos inertes aplicados sobre os materiais acrílicos levouse a cabo empregando fármacos de diferente natureza, tanto no referente o seu tamaño molecular, lipofilia ou natureza (obtidos por síntese química ou de natureza proteica. As células recubertas validáronse mediante unha serie de probas de liberación in vitro, seleccionando como modelo de estudo un colirio de voriconazol. Este enfoque permitiu determinar a capacidade dos recubrimentos para manter a súa funcionalidade e eficacia en condicións simuladas de uso real. Adicionalmente, realizouse unha análise detallada e rigorosa da durabilidade e a capacidade de reutilización destes recubrimentos,

someténdoos a probas que avaliaron a súa resistencia ante a exposición a diversos solventes, a fluctuacións no pH e a variacións nas condicións de temperatura. Esta metodoloxía exhaustiva de avaliación buscou non só confirmar a viabilidade dos recubrimentos como unha solución para mellorar a precisión e a fiabilidade nos estudos de liberación de fármacos, senón tamén explorar o seu potencial para revolucionar a fabricación de células de difusión. Ao demostrar a súa capacidade para soportar condicións adversas sen degradarse nin perder efectividade, estes recubrimentos inertes perfílanse como unha ferramenta clave para a creación de sistemas de difusión personalizados, os cales poden ser adaptados especificamente ás esixencias particulares de investigacións farmacolóxicas variadas como as que nos ocupan no desenvolvemento de sistemas de liberación intraoculares e ungueais. Este avance representa un paso significativo cara á optimización dos procesos de desenvolvemento e avaliación de medicamentos, ofrecendo unha vía máis eficiente, custo-efectiva e adaptable para a realización de ensaios preclínicos con múltiples aplicacións biomédicas.

No seu conxunto, esta tese no ámbito da Tecnoloxía Farmacéutica recolle un traballo que abarca o desenvolvemento de diferentes sistemas de liberación de fármacos dirixidos a facilitar o tratamento de patoloxías relacionadas coas complicacións da diabetes mellitus. Este resumo proporciona unha visión xeral da investigación levada a cabo na tese doutoral, utilizando metodoloxías innovadoras (implantes elaborados mediante tecnoloxía de fluídos supercríticos, modelos en impresión 3D, e lacas aplicadas mediante unha nova técnica mecánica), e diferentes principios activos (corticoides, fármacos biolóxicos e antifúnxicos). En resumo, esta tese doutoral demostra a importancia de adoptar un enfoque multidisciplinar para abordar retos complexos desde o punto de vista do desenvolvemento de terapias innovadoras, co obxectivo xeral de obter formulacións de medicamentos seguras e efectivas a través de distintas vías de administración e para o tratamento de diferentes enfermidades relacionadas coas complicacións da diabetes mellitus.

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OBJECTIVES

OBJECTIVES

Diabetes mellitus (DM) is a challenging chronic disease with global health implications. Unhealthy diets, obesity, and sedentary lifestyles contribute to DM's high prevalence with 700.2 million expected in 2040, with over 90% falling under Type 2 Diabetes Mellitus (T2DM). DM high prevalence not only poses health significant health risks but also carries socioeconomic implications, making DM a major public health concern. The complications associated with DM are severe and diverse, ranging from nervous system damage, cardiovascular issues, stroke, peripheral vascular disease, blindness, limbs amputation, to an increased risk of infections.

Given the high prevalence of the disease and its severe complications, the general objective of this thesis is to enhance the quality of life for diabetic patients. This involves the reduction of complications associated with DM, improving existing pharmaceutical treatments, and ultimately to lower healthcare costs linked to these therapies. To achieve this goal, the following specific objectives have been established:

1. To review and discuss the challenges associated with the subcutaneous administration of biotherapeutics, alongside recent advancements in the field. (Chapter I)
2. To develop biocompatible polymeric implants for intravitreal administration using supercritical carbon dioxide (scCO₂) foaming, specifically to address diabetic retinopathy. (Chapter II)
3. Chapter III: restricted due to an ongoing patent application.
4. Chapter IV: restricted due to an ongoing patent application.

INTRODUCTION

1 INTRODUCTION TO DIABETES MELLITUS

Diabetes mellitus (DM) poses a significant and intricate challenge to global healthcare. With distinct causes, including genetic and environmental factors, various forms of DM exist, all leading to chronic hyperglycaemia due to impaired insulin function. The complexity of DM necessitates comprehensive research for global mitigation (1).

Two common types of DM are Type 1 (T1DM) and Type 2 DM (T2DM), with gestational and monogenic forms less frequent. T1DM involves the autoimmune destruction of pancreatic beta cells and is often diagnosed in childhood or adolescence. On the other hand, T2DM is characterised by insulin resistance and insufficient production, linked to lifestyle and genetic factors. The prevalence of T2DM is on the rise, particularly in lower middle-income countries and among youth globally (2).

Unhealthy diets, obesity, and sedentary lifestyles are major contributors to the prevalence of DM, currently at 9.3% and expected to rise to 10.9% by 2040. Projections indicate a staggering 700.2 million cases in 2040, with over 90% being T2DM (3). The high prevalence of T2DM poses significant health and socioeconomic risks, making it a major public health concern. Complications, such as nervous system damage, cardiovascular issues, stroke, and peripheral vascular disease, underscore its significance (4).

2 TYPES OF DIABETES MELLITUS

2.1 Type 1 diabetes mellitus

T1DM is an autoimmune disorder characterised by the destruction of insulin-producing beta cells in the pancreas. Genetic and environmental factors trigger immune-mediated attacks on these cells, with T lymphocytes playing a key role in causing progressive beta cell loss and insulin deficiency. The absence of insulin prevents cells from taking up glucose, resulting in persistent high blood sugar. This disruption in metabolic processes lead to increased glucose production and decreasing utilisation, preventing glucose storage as glycogen and resulting in elevated blood glucose levels. Classic symptoms of DM, such as polyuria, polydipsia, and weight loss ensue, become apparent. In T1DM, chronic hyperglycaemia can give rise to long-term microvascular and macrovascular complications (5).

2.2 Type 2 diabetes mellitus

T2DM is a metabolic disorder characterised by insulin resistance and impaired insulin secretion, arising from a combination of genetic, lifestyle, and environmental factors. Insulin resistance hinders glucose uptake by peripheral tissues, and over time, compensatory pancreatic insulin production fails, resulting in chronic hyperglycaemia. The condition is exacerbated by dysregulated lipid metabolism and chronic low-grade inflammation. Increased free fatty acids impair insulin action, while cytokines and adipokines contribute to worsening insulin resistance. Factors such as obesity and inactivity further exacerbate insulin resistance in T2DM (1).

2.3 Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is a temporary form of diabetes that occurs during pregnancy, characterised by insulin resistance due to the metabolic demands of pregnancy. Similar to T2DM, placental hormones hinder insulin action, leading to elevated blood sugar levels. While the pancreas compensates with increased insulin production, in GDM, this compensation often falls short, causing persistent hyperglycaemia. Risks associated with GDM include preeclampsia, caesarean delivery, and potential complications for the child, such as macrosomia (excessive birth weight), as well as an increased risk of future obesity and T2DM. Effective management involves dietary adjustments, physical activity, and sometimes insulin therapy to maintain healthy blood glucose levels, reducing risks for both the mother and the baby (6).

3 DIABETES MELLITUS COMPLICATIONS

DM goes beyond hyperglycaemia, causing intricate complications. Microvascular issues like retinopathy, nephropathy, and neuropathy damage small blood vessels, impairing vision, causing kidney failure, and resulting in nerve damage (7). On a macrovascular scale, problems like cardiovascular diseases and stroke elevate morbidity and mortality among individuals with diabetes (8). Moreover, DM increases susceptibility to infections, delays wound healing, and increases the risk of cancer, underscoring the importance of a comprehensive understanding of the disease and its diverse complications (9).

3.1 Microvascular complications

Microvascular complications in DM stem from small blood vessel damage, significantly impacting disease morbidity.

3.1.1 Diabetic retinopathy

Diabetic retinopathy is a severe eye disorder occurring in individuals with DM, causing damage to the retinal blood vessels located at the back of the eye. Chronic elevated blood sugar levels gradually weaken and damage these tiny vessels, leading to vessel blockage or leakage. If untreated, this condition progresses, resulting in impaired vision and potential blindness. Diabetic retinopathy often advances silently, underscoring the importance of regular eye screenings, especially among DM patients (10).

As diabetic retinopathy advances, patients may develop diabetic macular oedema (DME), which involves retinal thickening in the macular area. Effective management, involving controlled blood sugar levels, regular medical examinations, and a healthy lifestyle, substantially lowers the risk of diabetic retinopathy and associated vision-related issues (11).

3.1.2 Diabetic nephropathy

Diabetic nephropathy, a severe complication of DM, damages the kidneys due to prolonged high glucose levels. Elevated blood sugar weakens the kidneys' filtration, causing essential proteins to leak into urine. With progression, kidneys lose efficiency, leading to toxin buildup. If left untreated, diabetic nephropathy may result in chronic kidney disease, requiring dialysis or transplantation. Mitigating risks involves controlling blood sugar, maintaining a low-sodium diet, and monitoring blood pressure. Regular medical check-ups and early intervention prevent complications (12).

3.1.3 Diabetic neuropathy

Diabetic neuropathy, a complication of DM, results from high blood sugar damaging nerves, impacting sensory, motor, and autonomic functions. Sensory neuropathy manifests as numbness, tingling, or pain, usually starting in the feet and progressing upward. Motor neuropathy weakens muscles, impeding movement, while autonomic neuropathy disrupts internal organ function, affecting digestion, heart rate, and blood pressure. This condition significantly diminishes quality of life, impairing mobility and causing discomfort. Crucial measures for prevention and management include tight blood sugar control, regular foot exams, and managing risk factors (7).

3.2 Macrovascular complications

Macrovascular complications, stemming from prolonged high blood sugar, damage larger blood vessels, elevating the risk of cardiovascular diseases. Atherosclerosis, characterised by artery narrowing, results from glucose-induced fatty deposits, hindering blood flow and heightening the risks of coronary artery disease, heart attack, angina, and cerebrovascular diseases. DM accelerates atherosclerosis, making individuals more susceptible to arterial blockages (13).

Peripheral arterial disease (PAD) is a significant complication that restricts blood flow to the limbs. This limitation results in weakened vascularisation of the legs and arms, causing pain, ulcers, and, in severe cases, gangrene that may require amputation. DM increases the risk of aneurysm, involving weakened areas in vessels prone to life-threatening internal bleeding. Hypertension exacerbates complications by stressing weakened vessels, escalating atherosclerosis, and amplifying the risks of heart disease and stroke. DM-related inflammation and oxidative stress further damage blood vessels, intensifying complications. These complications not only decrease overall life quality but also elevate mortality, involving mechanisms such as inflammation and endothelial dysfunction (8).

3.3 Infection susceptibility

Diabetic patients face heightened infection risks due to elevated blood sugar levels, promoting bacterial and fungal growth while impairing immune responses. Poor circulation impedes immune cell delivery, leading to a delayed healing. Neuropathy, reducing sensation, poses a challenge in detecting injuries promptly, making diabetics prone to severe infections. The vulnerability to skin, urinary tract, respiratory infections, and complications from foot ulcers not only poses immediate health threats but also complicates DM management (9).

3.3.1 Onychomycosis in diabetic patients

Onychomycosis, a common fungal infection affecting nails, arises from fungi, yeasts, or moulds thriving in warm, moist environments. It causes discoloration, thickening, brittleness, and, in advanced stages, deformity or loss of the nail platter. Beyond its cosmetic concerns, onychomycosis significantly impairs the quality of life due to physical discomfort and social stigma. Moreover, it can lead to bacterial infections, exacerbating nail damage and compromising skin integrity (14).

Millions worldwide suffer from onychomycosis, with factors like aging, impaired immunity, DM, vascular diseases, and trauma heightening the risk. Public facilities such as pools and gyms further amplify the chances of infection. Despite its prevalence, onychomycosis is often underdiagnosed due to its slow progression and lack of early discomfort (15).

In individuals with DM, onychomycosis is intricately linked with foot complications. The compromised immunity, poor circulation, and neuropathy common in DM make the feet particularly vulnerable. Neuropathy reduces sensation, leading to a lack of awareness of injuries or infections, including onychomycosis, which can develop in the toenails. Onychomycosis stands as one of the most significant predictors of foot ulcers (16).

The thickened and brittle nails characteristic of onychomycosis can create pressure points within shoes, potentially causing ulcers in diabetic patients. Additionally, the presence of onychomycosis can exacerbate existing foot problems, increasing the risk of more severe complications such as foot ulcers, skin infections, cellulitis, or, in extreme cases, amputation. The combination of neuropathy, reduced blood flow, and fungal infections makes regular foot inspections, proper nail care, and prompt treatment of onychomycosis essential for diabetic individuals. Managing onychomycosis effectively is not just a matter of cosmetic concern but a critical aspect of diabetic foot care to prevent complications and maintain overall foot health (17,18).

4 DIABETES MELLITUS TREATMENT

4.1 Subcutaneous administration of drugs

Subcutaneous injections have revolutionised the administration of biotherapeutics, offering targeted drug delivery to optimise patient outcomes with convenience and minimal discomfort. Subcutaneous injections are administered beneath the skin's surface and provide efficient drug absorption thanks to the well-vascularized tissue matrix. Unlike oral medications, they bypass the gastrointestinal tract, ensuring consistent drug absorption and avoiding first pass metabolism. Compared to the intravenous route, subcutaneous injections can be self-administered by the patients, promoting independence and treatment adherence. Innovative technologies like prefilled syringes ensure accurate dosing, minimising injection-related fears (19). These injections enable sustained release of therapeutic agents, valuable in managing chronic conditions requiring stable medication levels (20). Various therapeutic areas benefit from subcutaneous

injections. In autoimmune disorders like rheumatoid arthritis, disease-modifying antirheumatic drugs (DMARDs) are administered via subcutaneous injections. Pain management, hormone replacement therapy, and vaccinations leverage subcutaneous injections for their effectiveness and ease of administration (21–24).

In T1DM management, insulin therapy remains central, and its subcutaneous administration provides precise control over blood glucose levels (25). Multiple daily subcutaneous insulin injections or insulin pumps mimic natural insulin release (26). Modern insulin formulations focus on improved glycaemic control, utilising rapid-acting insulins for mealtime spikes and long-acting insulins for basal coverage (27). Continuous glucose monitoring (CGM) systems provide real-time glucose data, enhancing precision in insulin dosing. Ongoing research explores advanced technologies such as closed-loop systems, offering autonomous insulin adjustments based on CGM readings (28). Additionally, studies explore immunotherapies and beta-cell transplantation to preserve and restore pancreatic function, ultimately enhancing patients' quality of life (29,30).

For T2DM management, glucagon-like peptide 1 (GLP-1) receptor agonists and amylin mimetics represent a promising advancement (31,32). These injectable medications mimic the actions of GLP-1 or amylin, regulating insulin secretion and glucose levels. GLP-1 agonists offer glucose-lowering effects, weight loss, and reduced hypoglycaemia risk. They slow stomach emptying, reducing post-meal blood sugar spikes (33).

4.1.1 Subcutaneous administration limitations

The use of subcutaneous injections for antidiabetic medications has transformed DM management, ensuring precise glucose control. However, this approach is not without limitations. The requirement for frequent injections, ranging from daily to weekly, can lead to injection fatigue and reduced adherence. Patients often find it uncomfortable, causing anxiety and impacting their overall quality of life. The psychological impact, especially in paediatric patients or those with needle phobia, is significant, affecting emotional well-being and potentially hindering treatment outcomes (34).

Common injection-site reactions like redness, swelling, or pain deter consistent medication administration. Meticulous injection techniques and site rotation are crucial to prevent complications like lipodystrophy, where repeated injections cause uneven fatty tissue under the skin. Subcutaneous injections may not fully replicate the body's natural

insulin or GLP-1 secretion, leading to suboptimal glucose control, especially post-meals. Other challenges of subcutaneous administration of biotherapeutics include immunogenicity issues, a lack of appropriate preclinical models, the administration of high drug concentration formulations, or unsteady bioavailability (35). Despite advancements in needle design and devices, addressing these limitations is vital for enhancing the patient experience and treatment effectiveness in DM management.

4.1.2 Sustained release delivery systems for subcutaneous administration

Sustained release drug delivery systems offer controlled and sustained release of medications, ensuring therapeutic levels in the bloodstream. Unlike immediate-release formulations, they gradually release drugs, reducing dosing frequency and improving patient compliance. Technologies like microparticles, nanoparticles, and implants allow customised drug release tailored to medical needs. These systems find applications in chronic conditions such as cardiovascular diseases, pain management, and DM minimising side effects and optimising outcomes (36).

Microparticles and nanoparticles are essential in sustained release drug delivery. Microparticles encapsulate drugs and proteins, enabling gradual release, while nanoparticles, smaller in size, target specific cells, facilitating precise drug delivery. Both technologies protect drugs, improving stability, and enable tailored release kinetics. Their versatility allows encapsulation of various drugs, offering treatments for several diseases (37). As these technologies advance, these systems promise more effective and personalised therapies.

Implants, another pharmaceutical form that allow sustained release of drugs, are usually composed of biodegradable materials. They release medication gradually after implantation, eliminating frequent dosing, enhancing compliance, and ensuring stable drug release. Implants are valuable for continuous drug administration, like hormone replacement therapy and pain management. They prevent fluctuations in drug concentration, providing stable outcomes. The effectiveness and tailored drug release offered by implants hold promise for improving patient outcomes and quality of life (38).

Biocompatible materials, especially polymers like poly(lactic-co-glycolic acid) (PLGA) or poly(caprolactone) (PCL), are well suited for sustained release drug delivery. These polymers encapsulate drugs providing controlled release with a tuneable release pattern by adjusting their composition. These polymers are biocompatible, minimising

adverse reactions and tissue damage. They also play a crucial role in developing sustained release drug delivery systems, enhancing efficacy, safety, and patient compliance (39,40).

In DM management, sustained release drug delivery systems address challenges with biotherapeutics administration. For example, Bydureon[®], containing the GLP-1 receptor agonist exenatide, was approved to treat T2DM. It contains PLGA microparticles encapsulating the drug and allows once-weekly subcutaneous administration (31). The sustained release maintains consistent drug levels, ensuring continuous glycaemic control and reducing injection frequency. These systems enhance patient compliance, stabilise blood glucose levels, and reduce side effects, making DM management convenient and less burdensome.

4.2 Oral administration

Oral medications play a crucial role in T2DM management, providing diverse options for individual patient needs. Biguanides (e.g., metformin), are the first-line therapy for T2DM treatment. Metformin improves insulin sensitivity and reduces liver glucose production. Sulfonylureas (e.g., glyburide, glipizide, glimepiride), stimulate insulin secretion from pancreatic beta cells, regulating blood sugar levels. Thiazolidinediones (e.g., pioglitazone, rosiglitazone), enhance insulin sensitivity in muscles and fat tissues, optimising glucose utilisation. Dipeptidyl peptidase 4 (DPP-4) inhibitors (e.g., sitagliptin, alogliptin), extend incretin hormones' activity, stimulating insulin secretion and reducing glucagon release to lower blood glucose levels (41).

The advent of oral GLP-1 receptor agonists, like Rybelsus[®] (semaglutide), marks a significant DM management advancement (42). These drugs mimic incretin hormones, boosting insulin secretion in response to high glucose levels and curbing appetite, benefitting many patients. Sodium-glucose transport protein 2 (SGLT2) inhibitors (e.g., empagliflozin, canagliflozin), aid glucose control by excreting excess glucose in urine, thereby reducing blood sugar levels (41).

Combination of oral antidiabetic is a common practice in DM management (43). Healthcare providers assess factors like overall health and comorbidities, tailoring treatments by combining oral medications to optimise glycaemic control and minimise side effects. Guidelines like NICE, specify drug combination based on patient characteristics (44). In advanced stages, insulin injections may be necessary for optimal blood glucose control (45).

Regular blood glucose monitoring, coupled with lifestyle changes, enhances medication effectiveness, improving DM management and reducing complications. Patient education and adherence empower individuals, enabling active participation in their DM care and overall well-being (46).

5 DIABETES MELLITUS COMPLICATIONS TREATMENT

Various strategies aim to prevent, alleviate, and slow down the progression complications associated with DM, such as cardiovascular diseases, nephropathy, retinopathy, neuropathy, and foot problem. Pharmaceutical interventions focus on optimising blood glucose levels, blood pressure, and cholesterol levels using medications. Angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB) protect kidneys, while statins manage cholesterol and reduce cardiovascular risk (47,48). Medications for neuropathic pain and topical treatments for foot ulcers contribute to improve the quality of life for affected individuals (49,50).

5.1 Diabetic retinopathy treatment

In recent years, the pharmaceutical management of diabetic retinopathy, a common complication of DM with the potential to cause blindness, has experienced significant advances. Traditional eye drug delivery, although effective for some conditions, often fails to reach therapeutic levels in the retina, a crucial part of the eye responsible for vision (51). Intravitreal drug delivery has emerged as the gold standard for treatment of retinal diseases. Intravitreal injections deliver therapeutic agents directly into the vitreous cavity and this, bypassing ocular barriers and ensuring high drug bioavailability in the vitreous humour (51). The simplicity of this method lies in injecting carefully measured dosages of therapeutic agents, creating a drug reservoir in the vitreous for sustained release. This approach minimises the need for frequent administrations, enhancing patient compliance and significantly improving visual outcomes in conditions like age-related macular degeneration and diabetic retinopathy. However, challenges, including infection risks and drug-resistant strains, require careful consideration (52).

Targeted therapies aim to preserve vision and enhance the quality of life for diabetic patients. Anti-vascular endothelial growth factor (Anti-VEGF) intravitreal injections, including bevacizumab, ranibizumab, and aflibercept, are at the forefront of revolutionary treatments. These medications block abnormal blood vessel growth and leakage, reducing retinal swelling and preventing further damage (53). Corticosteroid injections (e.g.,

dexamethasone), can also be used to reduce inflammation (54). Traditional laser therapies, such as focal/grid laser and panretinal photocoagulation, are employed to seal leaking blood vessels and shrink abnormal ones (55). Moreover, controlling systemic factors like blood pressure and glucose levels is crucial. Strict glycaemic and hypertension control can slow retinopathy progression. Regular eye screenings play a vital role in early detection, enabling timely intervention to preserve vision (56).

5.1.1 Intravitreal administration of sustained release delivery systems

Sustained release drug delivery systems represent an advancement in the treatment of retinal diseases, offering enhanced efficacy and convenience (57). For example, Ozurdex[®] is an intravitreal PLGA implant loaded with the corticosteroid dexamethasone, which is administered by intravitreal injection using a minimally invasive procedure. It provides a sustained release of the drug, reducing the need for frequent injections (58). Ozurdex[®] effectively targets inflammation and vascular abnormalities associated with DME among other ocular diseases by maintaining consistent dexamethasone levels within the vitreous cavity (59). This leads to improved treatment outcomes and reduces the treatment burden for patients by minimising discomfort and anxiety linked to frequent injections (60).

The development of sustained release anti-VEGF delivery systems is becoming a hot topic in the pharmaceuticals field, and diabetic patients would greatly benefit from such advances. Anti-VEGF drugs are the first-line treatment in diabetic retinopathy, administered through intravitreal injection to reduce the abnormal blood vessel growth and oedema in the retina. However, the need for frequent intravitreal injections poses challenges for both patients and healthcare professionals. Research in the field aims to address these challenges by the development of anti-VEGF sustained release delivery systems, including biodegradable microparticles, nanoparticles, or implants. These systems could be implanted via minimally invasive procedures, and provide a controlled anti-VEGF release in the eye with consistent therapeutic levels and reduced injection frequency. These innovative systems have the potential to enhance the diabetic patient compliance and comfort while preserving their eye health (61).

However, many of the techniques employed in the manufacturing of sustained drug delivery systems involve the use of harsh conditions, such as high temperatures and organic solvents. High temperatures can cause thermal degradation of thermosensitive

drugs and organic solvents pose toxicity concerns and environmental hazards. Moreover, these harsh conditions may limit the range of drugs and excipients that can be used, thereby restricting the versatility of these delivery systems, particularly for drugs like anti-VEGF agents (62,63). Current research is focused on developing innovative and environmentally friendly manufacturing methods that minimise or eliminate the use of such harsh conditions.

5.1.2 Supercritical CO₂ technology

Supercritical fluids have gained significant attention in various industries, including pharmaceuticals, due to their unique properties. In particular, supercritical carbon dioxide (scCO₂) foaming has emerged as a transformative technique, offering a versatile and eco-friendly approach to create lightweight materials with tailored properties (64). Traditional methods of foaming, often employing chemical blowing agents or physical foaming agents, come with environmental concerns and limitations regarding the control over material properties. In contrast, supercritical CO₂ foaming represents a green alternative, leveraging CO₂'s inherent advantages such as low toxicity, non-flammability, and its ability to readily dissolve in a wide array of polymers and materials.

The foaming process is based on the dissolution of CO₂ within the polymer matrix. Under high pressure and controlled temperature, supercritical CO₂ impregnates the polymer matrix, creating a supercritical solution. Upon depressurisation, the dissolved CO₂ expands, forming a multitude of small bubbles within the material structure, resulting in a lightweight, porous, and highly versatile foam (65).

The applications of scCO₂ foaming are diverse, including areas such as polymer science, biomedical, nutrition, or nanotechnology among others. In the polymer science area, it has enabled the development of lightweight and high-strength materials for applications in automotive components, packaging materials, and insulation. In the food industry, it has been employed to produce foamed products with enhanced texture or flavour retention (66).

In the biomedicine, scCO₂ foaming enables the development of biocompatible porous scaffolds for tissue engineering and drug delivery systems designed for sustained release. This technology allows precise control over pore size, distribution, and interconnectivity while avoiding the use of organic solvents and high temperatures, making it advantageous for drug delivery (67). The resulting porous materials are

biocompatible and provide a larger surface area, facilitating efficient drug loading and controlled release (68). This method is environmentally friendly and compatible with drugs prone to degradation.

5.2 Onychomycosis pharmacological treatment

The standard treatment for onychomycosis involves antifungal medications administered orally or topically (14). Oral antifungals like terbinafine and itraconazole are often considered more effective systemic delivery but require a longer course and regular monitoring due to potential side effects (69). Topical treatments, usually in the form of nail lacquers or creams, are more convenient but may be less effective for severe cases (70). In some instances, physical interventions like laser therapy are explored, and the choice of treatment depends on the severity of the infection, the patient's overall health, and their preferences. Given the persistent nature of onychomycosis, the adherence to the prescribed treatment and the hygienic measures are essential for successful outcomes (14).

One of the most significant challenges in topical drug delivery to the nail is the drug low permeation rate. The nail dense keratin structure acts as a hermetic barrier, limiting the penetration of drugs and making it difficult for topical treatments to reach the fungal infection site. This low permeation rate hampers the efficacy of antifungal agents and leads to prolonged treatment durations, hindering treatment compliance and worsening therapeutic outcomes (70). Research in the area is focused on new strategies to enhance drug permeation, such as the development of novel formulations, penetration enhancers, and advanced drug delivery systems like nanoparticles (71). Overcoming this challenge is critical for optimising the efficacy of onychomycosis treatments, improving patient outcomes, and enhancing the overall management of this disease (72).

Innovative mechanical methods have emerged as promising solutions to overcome the challenge of low drug permeation rates in the nail. Techniques such as microneedles, which involve tiny, painless punctures in the nail plate, facilitate the direct delivery of medications beneath the nail surface (73). These microneedles create micro-channels that enhance drug penetration, allowing antifungal agents to reach the infection site effectively. Another approach involves nail abrasion or filing, where the outer layer of the nail is removed, making it more porous and receptive to topical treatments (74). Moreover, ultrasound technology has been explored to disrupt the nail's structure

temporarily, enabling improved drug penetration (75). The lack of suitable preclinical models for the development of new transungual formulations or drug delivery systems hinders the innovation in this field, and new models mimicking the complex environment of an onychomycotic nail are necessary (76).

6 PRECLINICAL TESTS

6.1 Traditional preclinical testing

In pharmaceutical research and development, preclinical models such as *in vitro* and *ex vivo* testing methods are essential for studying drug behaviour outside living organisms (77). These controlled laboratory tests provide insights into drug formulation, aiding formulation development by helping in the better understanding of release mechanisms and permeation properties in an environment resembling *in vivo* characteristics. Diffusion cells are essential tools in these studies, consisting of two compartments separated by an artificial membrane in *in vitro* studies or a living tissue in *ex vivo* studies, simulating biological barriers (78). Diffusion cells provide information about drug permeation rate, tissue permeability, absorption kinetics, and formulation effectiveness. These data guide the development of formulations for several routes of administration including transdermal, intravitreal, subcutaneous, or ungual (79).

The use of diffusion cells in preclinical testing is fundamental for pharmaceutical research, reducing the need for *in vivo* studies and minimising animal use (80). However, the current diffusion cells do not replicate the environment of the administration site accurately. This limitation hampers formulation development for administration routes which require a complex environment simulation, where numerous factors influence drug behaviour.

6.2 Three-dimensional printing technology for preclinical testing

Three-dimensional printing (3DP) technology, also known as additive manufacturing, creates 3D objects from digital models in a layer-by-layer manner. It enables rapid and precise prototyping, saving time and resources in product development, especially in the pharmaceutical sector. Researchers use 3D printing to create detailed prototypes of drug delivery systems, medical devices, and laboratory material for feasibility studies and concept refinement before mass production (81).

3D printing allows personalised device production in pharmaceutical testing. Tailoring devices to specific experimental or patient requirements enhances accuracy in laboratory experiments. Personalised drug delivery testing prototypes mimic specific administration pathways, improving the precision of pharmaceutical research and reducing the need for animal testing. Additionally, 3D printing enables the production of intricate structures like microfluidic devices and tissue models (82). These custom-made devices, used in pharmaceutical laboratories, accurately simulate physiological conditions. They aid researchers in studying drug behaviour at the cellular level, accelerating drug discovery and development.

In summary, 3D printing technology transforms prototyping and personalised device production in pharmaceutical research. Its rapid fabrication and customisation capabilities enhance accuracy, efficiency, and innovation in drug development and healthcare solutions.

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CHAPTER I

Fighting type 2 diabetes: Formulation strategies for peptide-based therapeutics

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CHAPTER I**Abstract**

Diabetes mellitus is a major health problem with increasing prevalence at a global level. The discovery of insulin in the early 1900s represented a major breakthrough in diabetes management, with further milestones being subsequently achieved with the identification of glucagon-like peptide-1 (GLP-1) and the introduction of GLP-1 receptor agonists (GLP-1 RAs) in clinical practice. Moreover, the subcutaneous delivery of biotherapeutics is a well-established route of administration generally preferred over the intravenous route due to better patient compliance and prolonged drug absorption. However, current subcutaneous formulations of GLP-1 RAs present pharmacokinetic problems that are related to adverse reactions and treatment discontinuation. In this review, we discuss the current challenges of subcutaneous administration of these drugs and provide an overview on the formulations available for the different routes of administration, also evaluating emerging strategies for the development of GLP-1 RAs formulations with improved bioavailability that require a lower frequency of administration.

Keywords

Type 2 Diabetes Mellitus; Glucagon-like peptide-1 receptor agonists; Exenatide; Subcutaneous administration; Amylin mimetics; Drug delivery systems; Biotherapeutics; Peptide delivery

I INTRODUCTION

Subcutaneous delivery of biotherapeutics has attracted increasing attention across many disease areas and has shown to be effective, well-tolerated and generally preferred by patients and healthcare workers over other administration routes (1). This route of administration has been indicated in providing an alternative for intravenous infusions, which involve invasive and time-consuming procedures that represent an economic burden for healthcare systems. Indeed, since the first subcutaneous formulations were approved by regulatory bodies, significant progress has been made towards developing therapies for several disease areas, including rheumatoid arthritis, multiple sclerosis and diabetes mellitus (DM) (2).

Particularly, a considerable number of these subcutaneous medications can be administered at home by patients or caregivers. Given the positive impact that home administration had on patient adherence to treatment and on reducing costs and resources, it became evident the benefits of switching from intravenous infusions to subcutaneous injections. However, significant developmental issues and knowledge gaps remain that hamper the progression of subcutaneous biotherapeutic formulations (3). In 2020, the Subcutaneous Drug Delivery and Development Consortium identified several major challenges that need to be addressed before the complete implementation of the subcutaneous route to deliver biotherapeutics. Among others, the need for technological advances to successfully deliver high-dose/volume formulations, the incomplete bioavailability of subcutaneous formulations and the concerns about the higher immunogenicity of the subcutaneous route compared to the intravenous route can be cited (4).

Patients subjected to chronic treatment regimens that require multiple administrations, such as diabetics, particularly benefit from the subcutaneous drug administration strategy. DM is a metabolic disorder characterized by pancreatic β -cell dysfunction and insulin resistance that has become a major global health problem (5). In 2019, the International Diabetes Federation (IDF) estimated that nearly 500 million people have DM, and the number of cases is expected to increase rapidly in the upcoming years. DM not only causes premature mortality (DM was the direct cause of approximately 4 million deaths in 2019) and reduces patient's quality of life, but also represents a high economic burden for any health system; in 2019, DM investment

reached USD 760 billion (capital expenditure increased by 4.5% since 2017), and the IDF expects it to continue growing (6). Although major milestones have occurred in the development of alternative drug delivery systems, DM management can be improved further, especially for patients who have failed common DM therapies and need complex drug combinations.

Since the discovery of insulin in the early 1920s, the first peptide to be isolated and administered therapeutically, other protein-based drugs have been developed (7,8) (Figure 1). Particularly, insulin paved the way for the employ of the subcutaneous route as an option for the administration of proteins. Although other routes (e.g. pulmonary and oral) have been explored for the administration of peptides, the subcutaneous route remains the route of choice (8).

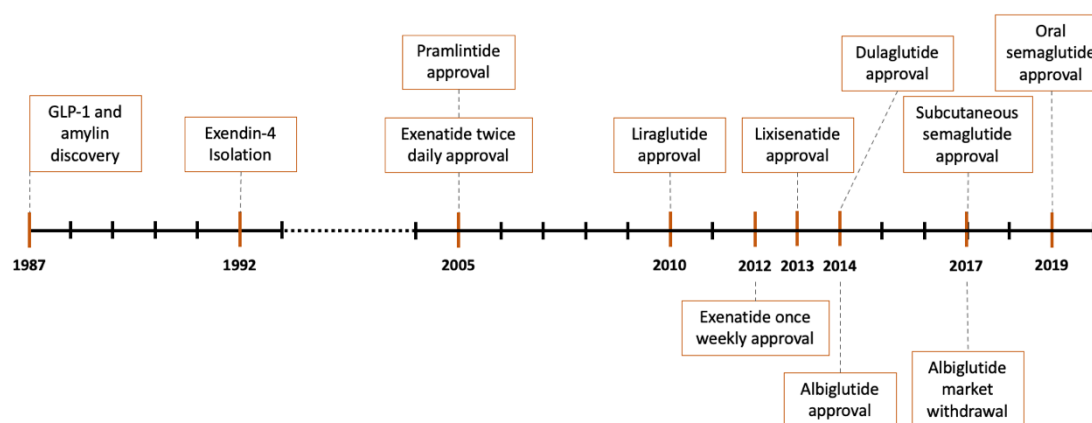


Figure 1. Milestones in GLP-1 and amylin therapy development since their discovery.

In 2005, exenatide (Byetta®), a glucagon-like peptide-1 receptor agonist (GLP-1 RA), was approved by the Food and Drug Administration (FDA) for type 2 diabetes mellitus (T2DM) treatment (9). GLP-1 RAs are administered in combination with other antidiabetic drugs (10). Besides a proven efficacy in glycaemic control, additional benefits of GLP-1 RAs therapy include reduced cardiovascular risk, lower risk of hypoglycaemia and good tolerance by the patient (11). However, due to its short half-life (2.4 hours in humans) (12) multiple injections are required, with gastrointestinal adverse events and injection-site reactions being the most common adverse effects derived from this repetitive administration (13).

In the same year, another antidiabetic drug, pramlintide (Symlin®), a synthetic amylin analogue, was approved by the FDA as an adjunctive treatment for patients with T1DM

and T2DM in whom glycaemic control was not achieved with insulin therapy (14,15). Amylin mimetics are a promising class of antidiabetic drugs that have also been shown to be useful for weight loss, especially in combination with other agents, such as leptin (16). However, they are underused in clinical practice due to their poor pharmacokinetic properties (17). Amylin plays an important role in the control of food intake, and the alteration of this control could be responsible for the increase in obesity rates, so the development of other amylin mimetics with improved potency and pharmacokinetics may be a promising approach for future treatment options (16).

Although much has been accomplished in the field of DM management, with five GLP-1 RAs and one amylin mimetic already commercialized for subcutaneous administration (Figure 1) (13,14), it is still necessary to improve and develop novel formulations with longer circulation times to decrease the frequency of administration (18–20). This review aims to provide an overview of the current situation and recent advances in the production of safe and effective systems for the subcutaneous administration of T2DM drugs (non-insulin), in particular, GLP-1 RAs. Additionally, ongoing research for new drug delivery systems and alternative routes for GLP-1 RAs administration will be discussed.

2 SUBCUTANEOUS ADMINISTRATION OF BIOTHERAPEUTICS: GENERAL CONSIDERATIONS

As alluded, the development of novel peptide and protein formulations for subcutaneous delivery as an alternative to the conventional intravenous infusions is of primary importance to improve therapy effectiveness. The increment in the half-life of the drugs would reduce the frequency of injections, which together with the possibility of home administration would positively impact patient compliance with medication and, ultimately, could reduce healthcare costs.

Despite extensive clinical use of the subcutaneous route, the specific mechanism underlying the subcutaneous absorption of peptides and proteins remains to be fully understood. Many academic papers have described the anatomy and components of the subcutaneous tissue and how its particular characteristics can affect the absorption of drugs administered by this route (21–24). Briefly, the subcutaneous injection administers the formulation into the hypodermis – between the muscle and the dermis – which is composed of loose connective tissue (areolar tissue) and adipose tissue. This site of

injection is also irrigated by blood and lymphatic capillaries, which play a fundamental role in the absorption of large peptides (>16 kDa) (1,25). The extracellular matrix (ECM) is produced by fibroblasts and is mainly composed of collagen and elastin fibres and glycosaminoglycans (23). Collagen and elastin are two structural proteins that determine the mechanical properties of the subcutaneous tissue, while glycosaminoglycans are responsible for the viscoelasticity of the ECM (26), controlling its hydraulic conductivity (21). The difficulty for water to diffuse through the subcutaneous tissue prevents the rapid spread of the formulation from the administration site (21).

The limited proteolytic activity of the subcutaneous tissue makes it ideal for the administration of biotherapeutics. However, the unsteady bioavailability of proteins after subcutaneous administration is a major concern; whereas intravenous injections introduce the drug directly into the systemic circulation, a drug administered subcutaneously has to be absorbed from the subcutaneous tissue to the systemic circulation by a combination of vascular and lymphatic vessels (4). In this sense, several factors can affect bioavailability. On the one hand, the person-to-person variability regarding subcutaneous tissue thickness, tissue pH, temperature, or hydrostatic and osmotic pressure within the tissue. On the other hand, the characteristics of the formulation (e.g. volume and viscosity), the charge and hydrophobicity of the drug, and ECM binding interactions or excipient interactions could also affect the bioavailability of the protein or peptide (1,4,21,23). Other factors, such as the patient's physical activity and the application of heat or massage at the injection site can also affect absorption (3,4). Thus, several challenges should be addressed before obtaining an ideal subcutaneous formulation (Figure 2).

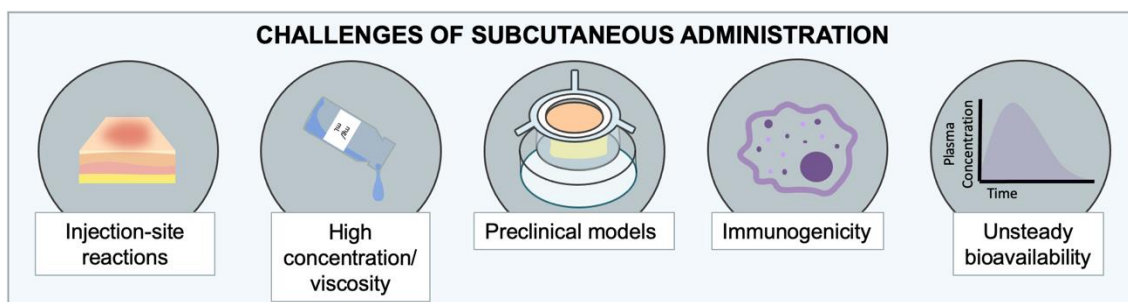


Figure 2. Challenges of subcutaneous administration of biotherapeutics.

The immunogenicity incidence of this route of administration is another concern that should be studied in depth. A more detailed understanding of the behaviour of the immune system in the subcutaneous tissue is needed, especially in the case of interactions between

the immune system and the drug. In addition, more immunogenicity data for drugs used both subcutaneously and intravenously are required to address specific issues, such as the greater immunogenicity of subcutaneous than intravenous administration, which remain controversial (4).

Furthermore, there is a lack of appropriate *in vitro* and *in vivo* models to help better understand and predict the absorption and bioavailability of subcutaneously administered biotherapeutics. Although various *in vitro* and *ex vivo* models have been proposed, these exclude variables that should be considered, such as immune cell interactions (1,21,22). *In vivo* animal models based on rodents, pigs or monkeys are usually employed during the preclinical development of peptide and protein therapies. However, no animal model could perfectly reproduce human characteristics and the collected data must be handled carefully (1). The absence of data prior to clinical trials adds uncertainty and if during pharmacokinetic studies the bioavailability results do not turn out as expected, the project is terminated, resulting in a waste of resources (4).

The administration of formulations with high protein loads is another major challenge in the field of subcutaneous administration. Some biotherapeutics, such as monoclonal antibodies, require high doses, leading to a choice between developing high-volume or high-viscosity formulations (21). The maximum volume for subcutaneous injections is approximately 1 to 2.5 mL. Larger volumes are associated with injection pain, injection site adverse effects (inflammation, bleb formation, or induration), and injection site leakage (4,21,25). Furthermore, high protein concentrations are commonly associated with high viscosities, difficulties with protein solubility, protein aggregation, and decreased protein stability (27). Injection back pressure is also increased in large subcutaneous injection volumes and high viscosity formulations, increasing injection forces, chances of drug leakage, pain, and tissue deformation (21,28).

Different strategies have been applied to achieve higher volumes at the site of injection. Permeation enhancers, like hyaluronidase, are already being used for commercial products and allow injection of volumes from 5 to 100 mL (3). Other strategy is the employ of technologies to reduce the viscosity of high concentration formulations. For example, EXCELSE™ technology allows injections with a volume of 1 mL with concentrations up to 250 mg/mL without increasing the viscosity (29). It uses a mixture of amino acids that covers regions of the drug without modifying it to avoid aggregation and stability problems. Arestat® is a technology that also allows switching between

intravenous to subcutaneous administration of biotherapeutics among other possibilities, however, there is little information available on how it works (30). Finally, new trends are emerging such as the formulation of proteins as physical protein complexes, microparticles, spherical microbeads, or paste formulations for their successful sustained release after subcutaneous administration (4).

3 GLUCAGON-LIKE PEPTIDE-1 RECEPTOR AGONISTS AND AMYLIN MIMETICS

3.1 Glucagon-like peptide-1 receptor agonists pharmacology and current state

Glucagon-like peptide-1 (GLP-1) is a 30-amino acid peptide that acts as an incretin hormone. It is produced by the epithelial endocrine L-cells in response to food intake and is responsible for various effects throughout the body, as GLP-1 receptors are distributed in the pancreas, gastrointestinal tract, brain, heart, and the kidneys (31). The main function of GLP-1 is to strictly stimulate insulin secretion when sugar levels rise (incretin effect) and to inhibit glucagon secretion helping to lower glucose levels. Moreover, GLP-1 enhances β -cell functions and inhibits β -cell apoptosis, delays gastric emptying thereby reducing postprandial glucose levels and regulates appetite and energy intake by activating GLP-1 receptors in the Central Nervous System (13,31,32). Despite its potent anti-diabetic effect, GLP-1 is rapidly metabolized by the enzyme dipeptidyl peptidase IV (DPP-4) (its half-life is <1 min) (9) and, therefore, its clinical application is limited.

To overcome the bioavailability problems of GLP-1, some peptide analogues with a longer half-life have been formulated. Exenatide, with homology to GLP-1 of approximately 50% (the same for lixisenatide), was the first to be commercialised. It was developed from exendin-4, a molecule isolated from Gila monster venom. Other GLP-1 RAs, such as liraglutide, dulaglutide, or semaglutide, were directly developed from GLP-1 and their homology is >90% (11). GLP-1 half-life extension strategies included amino acid sequence modification (exenatide and lixisenatide), fatty acid attachment (liraglutide and semaglutide), sustained-release microparticles (exenatide), fusion with human serum albumin (albiglutide), or fusion with the fragment crystallizable (Fc) region of a monoclonal antibody (dulaglutide) (13).

Five GLP-1 RAs are currently commercialized: exenatide (Byetta[®], Bydureon[®]), liraglutide (Victoza[®]), lixisenatide (Adlyxin[®] in US and Lyxumia[®] in EU), dulaglutide

(Trulicity®) and semaglutide (Ozempic®). GlaxoSmithKline (GSK) marketed albiglutide (Tanzeum®) from 2014 to 2017 but stopped marketing the drug due to its limited prescription (33).

The mechanism of action of GLP-1 RAs is similar to that of GLP-1. As they are glucose-dependent, when blood glucose levels increase, GLP-1 RAs lower blood glucose levels by binding and activating GLP-1 receptors that induce insulin secretion and inhibit glucagon secretion (9,11,13,32). GLP-1 RAs differ from each other in molecular size, molecular structure, and pharmacokinetics (34). Thus, their half-lives depend on the molecule and the delivery system. On this basis, the administration can be twice a day, once a day or once a week (11).

Common adverse reactions of this class of drugs include gastrointestinal events (e.g. nausea, diarrhoea, vomit, decreased appetite, dyspepsia and constipation), injection-site reactions, hypoglycaemia (when combined with insulin or an insulin secretagogue), macrovascular outcomes, acute pancreatitis, acute kidney injury, thyroid C-cell tumours, immunogenicity and hypersensitivity (35–41).

Real-world data from T2D patients initiating GLP-1 RAs treatment suggest high discontinuation rates. In an 18 months real-world study, Exenatide Once Weekly (Bydureon®) was discontinued by 42.5% of patients of which 16% was due to adverse reactions. The average time to Bydureon® treatment discontinuation was 6 months for adverse reactions reasons and 12 months for general reasons. Other real-world data studies agree on the low GLP-1 RAs treatment (liraglutide, exenatide once weekly and exenatide twice daily) persistence for 12 months or more. More data would be needed to conclude the reason for this discontinuation or change in treatment.

3.2 Relation between GLP-1 RAs administration volume and injection-site reactions

Table 1 lists the data on the dose/volume relationship for each commercialized GLP-1 RA. As shown, volumes range between 0.02 mL for Byetta® and 0.75 mL for Ozempic®, and doses range between 5 µg for Byetta® and 50 mg for Tanzeum®. Although all administration volumes are within the maximum range for subcutaneously administered volumes, injection-site reactions continue to be a common side effect of GLP-1 RAs. In particular, Bydureon® has shown severe injection-site reactions with or without subcutaneous nodules, such as abscesses, granulomas, cellulitis, and necrosis.

Subcutaneous nodules form more frequently with Bydureon® therapy than with other GLP-1 RAs (77% of subjects experienced at least one subcutaneous nodule during treatment in clinical trials) due to the use of a microsphere-based formulation (36). These nodules were temporary and generally, no medical intervention was needed (32). Additionally, Bydureon®-treated patients have a higher incidence of injection-site reactions (17.1%) than Byetta®-treated patients (12.7%) (42). In both Bydureon® and Byetta®, injection-site reactions are associated with high titers of anti-exenatide antibodies (36,37), and Bydureon® shows a higher percentage of antibody-positive patients than Byetta® (43). In the placebo-controlled trials of Tanzeum®, 18% of treated patients experienced injection-site reactions (including hematoma, erythema, rash, hypersensitivity, haemorrhage, or pruritus) that led to discontinuation of treatment within 2% of participants (39). As with exenatide, injection-site reactions caused by albiglutide appear to be mediated by the immune system (44).

Table 1. Dose/volume relationship in GLP-1 RAs.

Administration	GLP-1 RA	Tradename	Dose	Volume	Concentration
Twice daily	Exenatide	Byetta® (37)	5 µg	0.02 mL	250 µg/mL
			10 µg	0.04 mL	
			0.6 mg	0.1 mL	
Once daily	Liraglutide	Victoza® (41)	1.2 mg	0.2 mL	6 mg/mL
			1.8 mg	0.3 mL	
	Lixisenatide	Adlyxin® (US),	10 µg	0.2 mL	50 µg/mL
		Lyxumia® (EU) (35)	20 µg	0.2 mL	100 µg/mL
Once weekly	Exenatide (Controlled release)	Bydureon® (36)	2 mg	0.65 mL	3.08 mg/mL
	Albiglutide	Tanzeum® (39)	30 mg	0.5 mL	60 mg/mL
			50 mg	0.5 mL	100 mg/mL
	Dulaglutide	Trulicity® (40)	0.75 mg	0.5 mL	1.5 mg/mL
			1,5 mg	0.5 mL	3 mg/mL
	Semaglutide	Ozempic® (38)	0.25 mg	0.1875 mL	1.34 mg/mL

0.5 mg	0.375 mL
1 mg	0.75 mL

However, the percentage of injection-site reactions for the remaining analogues was lower: 0.2% for Ozempic[®], 0.5% for Trulicity[®], 2% for Victoza[®], and 4% for Adlyxin[®] (35,38,40,41). According to Table 2, the administration volume of Ozempic[®], Trulicity[®], Victoza[®], and Adlyxin[®] is less than 1 mL, and their concentrations are low (≤ 6 mg/mL). Both low volume and concentration may be responsible for the greater acceptance of these formulations compared to the other analogues. Byetta[®], Bydureon[®], and Tanzeum[®] show a higher proportion of injection-site reactions, which may be related to the higher immunogenicity of these formulations. In addition, Bydureon[®] includes 50:50 poly(D, L-lactide-co-glycolide) (PLG) polymer microspheres in its formulation (36), which may account for the higher proportion of injection-site reactions and nodules formation (32,42). Tanzeum[®] was used in a small volume (0.5 mL) but in high concentrations (60 and 100 mg/mL). High protein concentrations are associated with protein aggregation and protein particle formation (21), which can increase immunogenicity (45) and thus injection-site reactions.

3.3 Immunogenicity issues

In general, protein and peptide pharmaceuticals are considered potentially immunogenic. Subcutaneous immunogenicity is suggested to be higher than IV, mainly because of the higher exposure to the lymphatic system and to antigen-presenting cells (1,4). Immunogenic effects may alter pharmacokinetics, produce safety risks (injection-site reactions, hypersensitivity, or anaphylactic reactions), and attenuate therapeutic effects (1,46).

The immunological effects of GLP-1 RAs were evaluated in several clinical trials. Studies with Byetta[®] (add-on to either metformin or a sulfonylurea, or both) showed that 38% of treated patients had anti-exenatide antibodies, of which 6% had high antibody titers. Glycaemic control failed in 3% of them (37). On the other hand, the immunogenicity data were higher for Bydureon[®]. In various clinical trials, 43-49% of patients had antibody formation. Moreover, 6% of patients showed an attenuated glycaemic response (36). In the case of Adlyxin[®], 70% of the patients tested positive for antibodies and 2.4% of them showed an attenuated glycaemic response. Allergic reactions and injection-site reactions were more likely to occur in antibody-positive patients (35). For Victoza[®], immunogenicity ranged from 0.9% to 8.6%, depending on the trial.

However, antibody formation was not associated with reduced efficacy of Victoza[®] or with adverse effects related to immunogenicity (41). Immunogenicity for the remaining GLP-1 RAs was lower: 5.5% for Tanzeum[®], 1.6% for Trulicity[®] and 1.0% for Ozempic[®] (38–40). For these, immunogenicity was not related to the neutralization of glycaemic control.

As the data suggest, human GLP-1 derived GLP-1 RAs appear to be associated with lower antibody titers than exendin-4 derivatives (Byetta[®], Bydureon[®], and Adlyxin[®]) (46). However, these data should be interpreted carefully because antibody detection depends on several factors (assay sensitivity and methodology, sample taking time and processing, accompanying medications and underlying conditions). For these reasons, data obtained from different trials or with different GLP-1 RAs cannot be directly compared with each other (35–41).

3.4 In vitro and ex vivo models for subcutaneous injection

The subcutaneous drug delivery market size is expected to grow by 9.7% between 2020 and 2027 (47). This fact, coupled with the growing trend to minimize the use of experimental animals, makes the development of a reliable *in vitro* or *ex vivo* model an urgent task. These models should make it possible to predict the pharmacokinetic parameters of the drug, the interactions of the formulation components with the subcutaneous environment, the immune response and the toxicity. In addition, there is a strong need for models capable of conducting long-term, yet stable, studies to evaluate controlled release drug delivery systems.

Recently, some companies have developed *in vitro* and *ex vivo* skin models for different purposes. Particularly, Genoskin has launched Hyposkin[®], the first *ex vivo* skin model to test subcutaneous injections (48). This model contains a real human skin biopsy that can last at least 7 days. Basically, it is a culture insert that contains donated human skin from abdominal surgery and a patented matrix and culture medium (Figure 3).

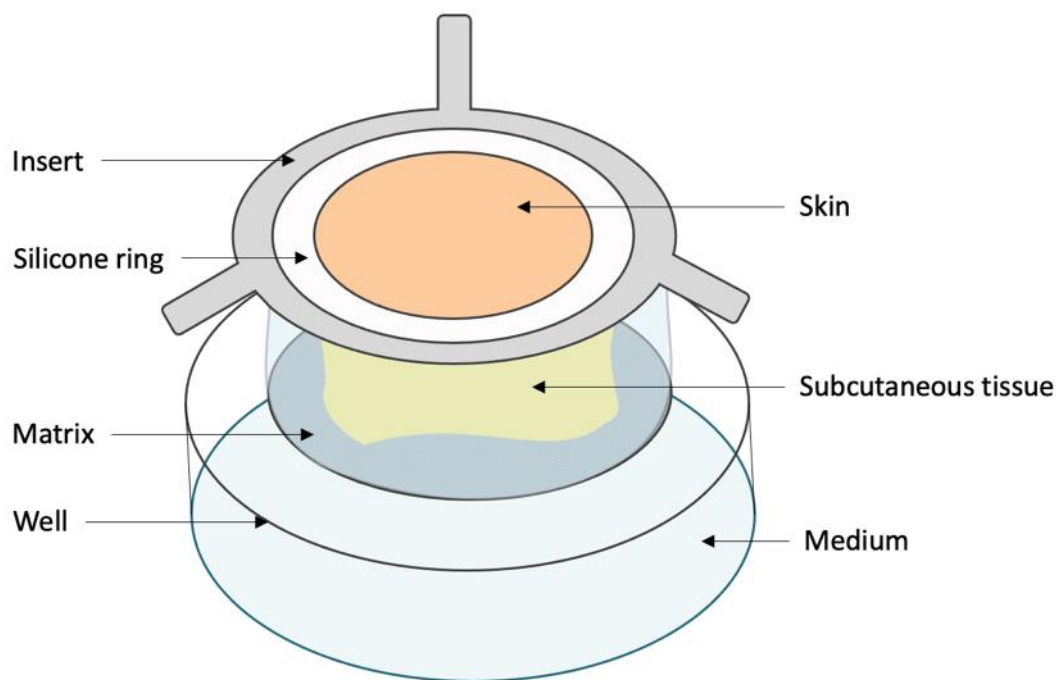


Figure 3. HypoSkin[®] scheme.

Another company, Straticell, has developed several skin models, including skin cells in monolayer cultures, *in vitro* reconstituted human epidermis, and *ex vivo* skin explants (49). However, none of the models is specifically designed for subcutaneous injection.

Another *in vitro* model, called Scissor (Subcutaneous Injection Site Simulator), uses a dialysis-based injection chamber with acellular ECM components immersed in a container of a bicarbonate-based physiological buffer to emulate the movement of the injected biotherapeutic from the subcutaneous tissue to the systemic circulation (22). It allows real-time monitoring of the drug and the medium (ECM components, pH, ionic composition, interstitial pressure and temperature). Scissor is now marketed by Pion Inc (50) and some studies have been performed to predict the bioavailability of eight monoclonal antibodies injected subcutaneously with a strong correlation with data obtained *in vivo* in humans (51).

While the latter model focuses on pharmacokinetic prediction, other researchers have turned their attention to *in vitro* models for immunogenicity prediction (52). However, each of the models described above has its limitations. There is not yet available a model of subcutaneous administration that allows continuous drug and medium monitoring, emulation of subcutaneous tissue properties and interactions, prediction of immunogenicity and pharmacokinetics and feasibility for long-term studies.

3.5 Amylin mimetics

Amylin is a 37 amino-acid peptide produced by the β -cells that acts as a glucoregulatory hormone and an energy metabolism regulator. It is co-secreted with insulin in response to blood sugar levels (53). However, its physicochemical properties make it easily precipitable, so its clinical application is troublesome. Pramlintide, an analogue of human amylin, was approved by the FDA in 2005. It replaces amino-acids in positions 25, 28, and 29 of human amylin with proline, improving the problem of precipitation (17). Also, it is used in combination with insulin in both T1DM and T2DM (16). Even though it was approved in 2005, its clinical use is quite limited due to its inconvenient administration; pramlintide requires 3 to 4 subcutaneous injections daily and does not appear to adequately mimic the natural release profile of amylin. The wide variations in plasma levels throughout the day lead to dose-related gastrointestinal adverse reactions (16,17,53). Moreover, Astra Zeneca has just launched it in the US as Symlin[®] (14), but other amylin analogues with improved pharmacokinetics are under development, especially for obesity treatment (16).

4 FORMULATION DESIGN TO IMPROVE THE PHARMACOKINETICS AND BIOAVAILABILITY OF GLP-1 RAS

Along with attempts to improve the subcutaneous administration of GLP-1 RAs, many efforts are being made in the development of new formulations with enhanced pharmacokinetic properties and, above all, with characteristics that could improve patient acceptability. To this aim, very different approaches are being taken, both by academy researchers and pharmaceutical companies. This section reviews the different drug delivery systems proposed for GLP-1 RAs: oral tablets, buccal formulations, liposomes, nasal formulations, transdermal formulations, pulmonary formulations, nanoparticles, and microparticles (Figure 4).

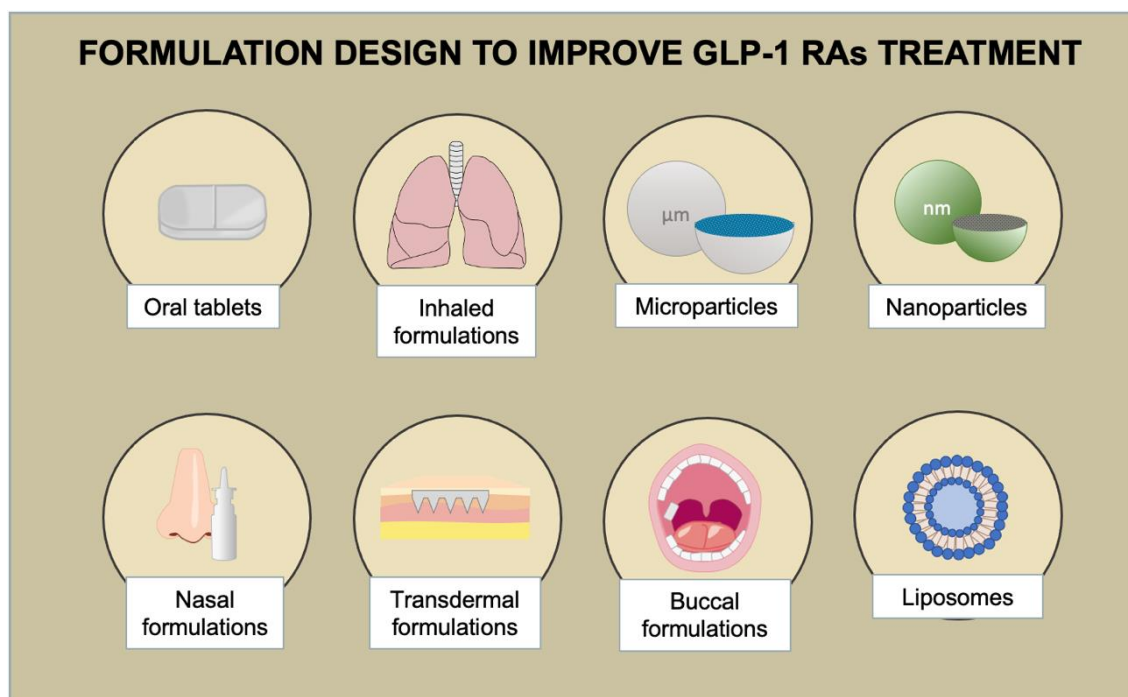


Figure 4. Formulation strategies for the administration of GLP1-RAs.

4.1 Oral tablets

Until 2019, GLP-1 RAs were only available as an injectable treatment, so despite their efficacy, they were underused. In 2019, Rybelsus[®], the first oral GLP-1 RA, was approved by the FDA and marketed by Novo Nordisk. Rybelsus[®] is an oral semaglutide formulation (tablets of 3, 7, or 14 mg) that is administered once a day (54). It uses Eligen[®] technology (Emisphere Technologies, Inc) to enhance the semaglutide absorption in the stomach. Eligen[®] technology uses a small fatty acid derivative, sodium N-(8-[2-hydroxybenzoyl] amino) caprylate (SNAC), to prevent degradation of semaglutide and improve its oral bioavailability (Figure 5) (55). SNAC allows semaglutide to utilize passive transcellular transport of the gastric epithelium and its effect depends on time and concentration (56). The bioavailability of semaglutide is estimated to be approximately 0.4 to 1% after administration of Rybelsus[®] (54).

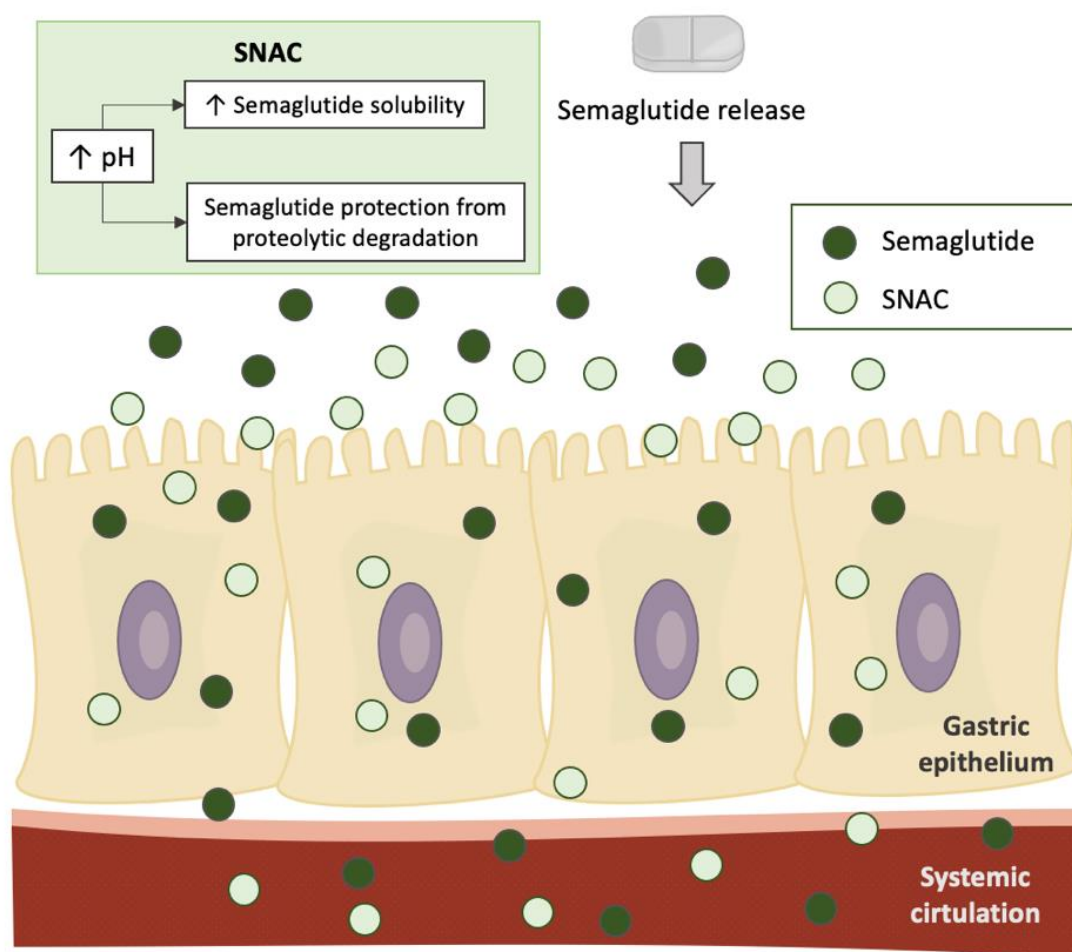


Figure 5. Effect of SNAC effect on the oral absorption of semaglutide. SNAC enhances the transcellular absorption of semaglutide by increasing the pH.

Another company, Oramed Pharmaceuticals Inc, has been developing ORMD-0901, an oral exenatide formulation based on the company's PODTM technology (57). Although the company has presented some promising results, there is little information on the product, and it is still in the company's pipeline.

4.2 Buccal formulations

The oral mucosa allows the drug to directly access the systemic circulation, thereby avoiding first-pass metabolism. Moreover, the buccal route of administration is much more convenient than the subcutaneous route and causes fewer problems than the nasal, transdermal, vaginal or pulmonary routes (58). The main drawback of the buccal route is the low bioavailability of larger molecules that require the use of several strategies to be absorbed, such as the use of absorption enhancers or enzyme inhibitors to improve their pharmacokinetics (59).

Despite the efforts that have been made to develop an effective formulation for buccal administration of peptides, this remains a challenge. Some attempts have been made for the buccal delivery of insulin (an oromucosal spray and a dissolvable film with embedded gold nanoparticles) but they failed to reach the market due to its low efficacy and variable pharmacokinetics (60).

For instance, ArisCrown technology (Arisgen SA) has been used for the buccal administration of exenatide (ARG011). This technology uses biodegradable cyclic compounds (crowns) to selectively and reversibly mask (with non-covalent interactions) peptide functional groups. The modified peptide is then included in a lipid formulation optimised to maintain the properties of the peptide (61,62). Preclinical studies have been conducted with buccal exenatide in mice and monkeys. In mice, buccal administration of exenatide controls blood glucose levels in a manner equivalent to an intraperitoneal injection of the unformulated peptide (63). In monkeys, the buccal formulation of exenatide included in a buccal patch controls blood glucose levels in a manner equivalent to the subcutaneous formulation (64). Despite the promising results, the development of this formulation was cancelled because Arisgen SA is currently closed.

4.3 Liposomes

GLP-1 was formulated in liposomes to improve its pharmacokinetics and pharmacological effect (65). Anionic, cationic, and non-ionic liposomes were prepared and characterized and 130-210 nm liposomes with moderate size homogeneity and high dispersibility were obtained. Anionic liposomes showed the highest encapsulation efficiency (80,2%) and the best pharmacokinetic parameters, as well as an evident improvement in pharmacological effects (Figure 6).

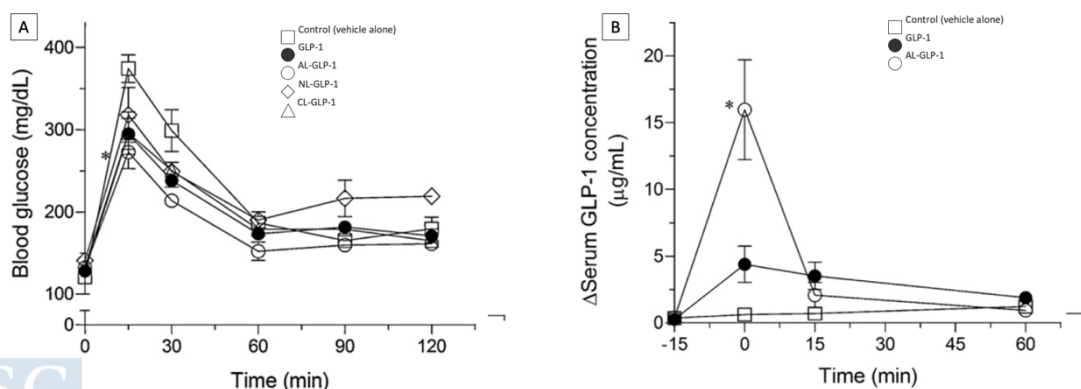


Figure 6. (A) Activity of GLP-1 formulations in an intraperitoneal glucose tolerance test in rats. (B) Serum GLP-1 concentrations in rats after intravenous administration of GLP-1 formulations. Figure reproduced and modified with permission from (65).

Although anionic liposomes have shown great promise, intravenous administration requirements are a significant drawback for their future development and commercialisation.

Exendin-4-loaded liposomes coated with chondroitin sulfate-g-glycocholic acid (EL-CSG) were also developed for oral delivery (66) and the role of bile acid transporters in the absorption of exendin-4 was evaluated. The average size of liposomes was 230 nm and their loading efficiency was 77%. Experiments in rats showed a relative bioavailability of 19.5% (versus subcutaneous administration). The antidiabetic effects (HbA1c, body weight, blood lipid concentration) were evaluated for four weeks and were found to be equivalent to those obtained with the subcutaneous administration of free exendin-4.

4.4 Nasal formulations

The nasal route of administration, in addition to being non-invasive, allows avoiding the first-pass metabolism. Nasal delivery of small lipophilic molecules is feasible as they can reach therapeutic levels in the bloodstream. However, peptides and proteins must be co-administered with a nasal absorption promoter due to their usual low nasal absorption (67).

4.4.1 Nasal Microparticles

A nasal formulation consisting of a capsule filled with approximately 60 μm particles of recombinant human GLP-1 amide bound to calcium carbonated and coated with corn starch was developed and tested in a double-blind placebo-controlled study (68). Each capsule contained 1.2 mg of recombinant human GLP-1 and a device for intranasal delivery of drugs was used for the GLP-1 microparticles delivery.

GLP-1 treated patients did not develop any serious adverse reaction after nasal administration. The C_{max} was 47.2 pmol/L and the T_{max} was 8.1 min. GLP-1 induced insulin secretion, inhibited glucagon secretion and improved glycaemic control markers in the medium term. However, the short study period (2 weeks) makes long-term trials necessary to evaluate the safety, efficacy and tolerability of this treatment. Comparison

with other GLP-1 RAs formulations also may be desirable. Moreover, the addition of a nasal absorption promoter should be considered to improve the bioavailability of the formulation and to reduce associated costs.

4.4.2 Nasal thermosensitive hydrogels

Other approach to formulate exenatide for nasal administration was based on the creation of exenatide-loaded chitosan-based thermosensitive hydrogels (69). Chitosan was shown to activate protein kinase C transduction pathways by opening tight junctions in epithelial cells and increasing permeability. The chitosan/glycerophosphate thermogelling systems were lyophilised for storage and then redissolved in the presence of a metal salt (CaCl_2 or MgCl_2). The hydrogel formulation redissolved with MgCl_2 , compared to that redissolved with CaCl_2 , preserved the stability of the exenatide, increased transport through Calu-3 cell monolayers, and increased the bioavailability of exenatide in rats after nasal administration (Figure 7).

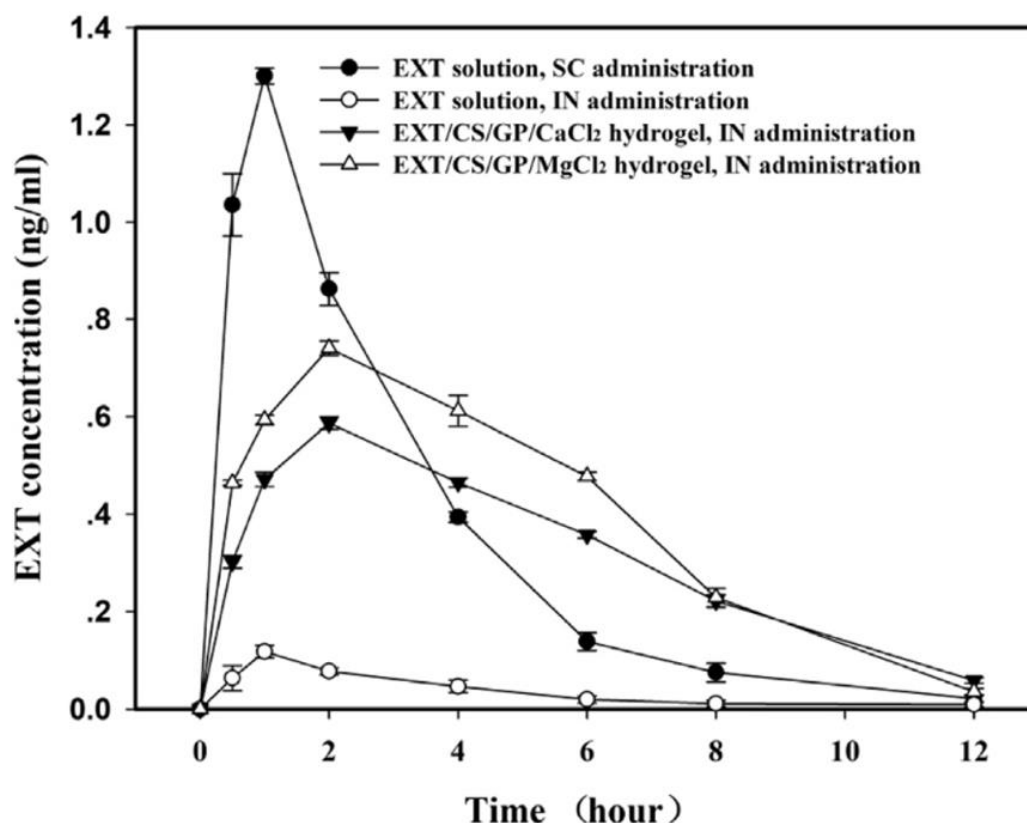


Figure 7. Exenatide blood levels vs. time profiles after subcutaneous (SC) or intranasal (IN) administration of exenatide (EXT) in solution or included in CaCl_2 or MgCl_2

hydrogels. Values are expressed as the mean $\pm$ SD ($n = 5$). Figure reproduced and modified with permission from (69).

This formulation significantly decreased food intake and body weight in high-fat-fed rats compared to an exenatide solution and appears to be suitable for nasal administration of exenatide for fat reduction, but further studies are needed to evaluate its efficacy in DM treatment, its safety and its tolerability.

4.4.3 Nasal solution with a cell-penetrating peptide

Recently, it has been suggested that Alzheimer's disease could be considered type 3 diabetes (70), and it has been proposed that insulin and GLP-1 RAs could improve learning and memory by increasing glucose uptake in the neuronal cells of the hippocampus. For this reason, it could be interesting to develop nose-to-brain drug delivery systems for exendin-4 and GLP-1. Solutions with different concentrations of exendin-4 or GLP-1 and penetratin (L- or D- penetratin) were formulated and administered intranasally to anaesthetised ddY mice (71). Intranasal coadministration with L-penetratin increased the systemic absorption of both peptides, and in the case of exendin-4, its concentration in the olfactory bulb increased significantly as shown in Figure 8. The concentration in the whole brain was much lower than in the olfactory bulb. However, the distribution in the brain showed a significant increase in exendin-4 levels in the hippocampus. Exendin-4 plays a role in the hippocampus by mediating memory and learning. According to this study, L-penetratin could act as an absorption enhancer, but further research is required to improve this approach.

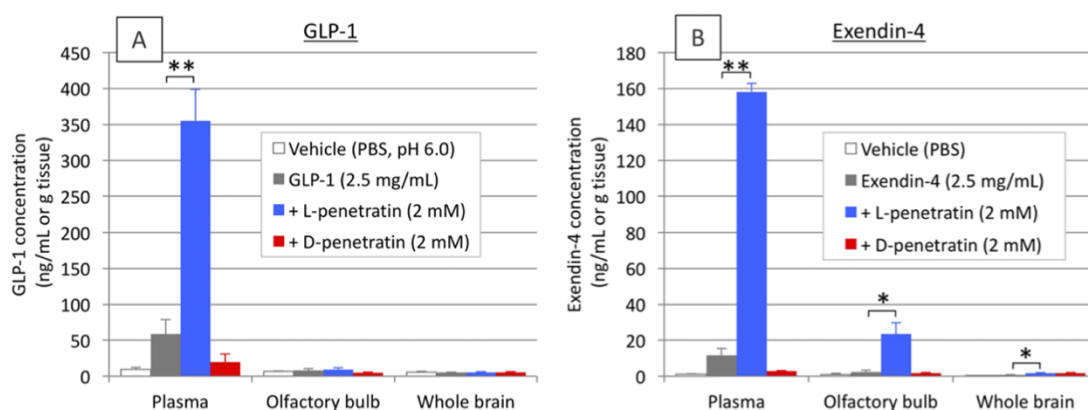


Figure 8. Systemic absorption and brain transport of GLP-1 and its analogue exendin-4, after single intranasal administration of GLP-1 and exendin-4 with or without L- or D-penetratin (2.0 mM) to male ddY mice. Each data point represents the mean $\pm$ SEM of n

= 3–4. * $p < 0.05$, ** $p < 0.01$ indicate significant difference with the control group receiving GLP-1 or exendin-4 solution. Figure reproduced and modified with permission from (71).

4.4.4 Nasal spray

Other approaches have been undertaken to develop a nasal formulation of exenatide. For example, MDRNA Inc. (previously Natestch Pharmaceutical Company Inc.) and Amylin Pharmaceuticals developed an exenatide nasal spray that reached phase II trials (72). The exenatide spray formulation comprised a viscosity enhancer, methyl-3-cyclodextrin, a surfactant, tartrate buffer for pH control, and a chelating agent for cations (73). However, its development was interrupted, and it was never approved.

In 2014, Aegis Therapeutics LLC (now Neurelis Inc.), offered a license to develop, market and sell a patented exenatide nasal spray that used its Intravail® technology (74). Intravail® is a group of absorption enhancers belonging to a class of compounds called alkylsaccharides (sugars and one or more alkyl chains covalently bound together) (75). Although the company has commercialised other nasal formulations, development exenatide product has been interrupted.

4.5 Transdermal formulations

The transdermal administration of exenatide could have some advantages compared to current treatments. Transdermal delivery of drugs is non-invasive and painless, and patients can self-administer the drug. In addition, this route is usually well tolerated. However, achieving skin penetration of proteins and biotherapeutics is a major challenge. The problems faced by the drug when penetrating through the skin can be solved by permeabilizing the outermost layer of the skin, the *stratum corneum*. This can be achieved by creating microchannels using microneedles or by temperature modulation using radiofrequency. These strategies allow for painless drug absorption for the patient (76).

4.5.1 Microneedle patches

Exenatide dissolving microneedles patches were created using low-molecular-weight sodium hyaluronate and using the micromould casting method (77). Dissolving microneedles showed a high loading capacity, and likewise, they did not leave sharp biohazard residues. Materials selection and geometry shape are particularly relevant in dissolving microneedles due to their weak mechanical properties. In this case, sodium

hyaluronate was selected for its biocompatibility and high hydrophilicity, and pyramidal microneedles were chosen for its high mechanical strength. The patches showed similar pharmacokinetics and hypoglycaemic effect to subcutaneous delivery. Exenatide was released and absorbed almost completely from the patch within 2 minutes.

Despite the potential of this system, it still has some drawbacks. For instance, the high hydrophilicity of sodium hyaluronate may be a problem in high humidity environments because the stability of exenatide and the mechanical strength of the formulation could be compromised. Storage should be strict to ensure long-term stability, patches should be stored in waterproof packaging with a dry nitrogen atmosphere and the addition of sucrose or trehalose should be considered. In any case, administering the patches twice a day could be inconvenient for the patients and their safety profile should be further evaluated.

Another formulation consisting of dissolving microneedle arrays was developed employing carboxymethyl cellulose and using the centrifugal lithography method (78). Three fundamental factors in the manufacture of microneedles were optimised (temperature during manufacture, pH and concentration of the polymer) to ensure the activity of the drug once encapsulated in the microneedles and stored. The formulation showed no reduction in activity after eight weeks of low temperature storage.

In other study, indissoluble alginate microneedle array patches loaded with dual mineralized particles containing exendin-4 and glucose oxidase were prepared (Figure 9) (79).

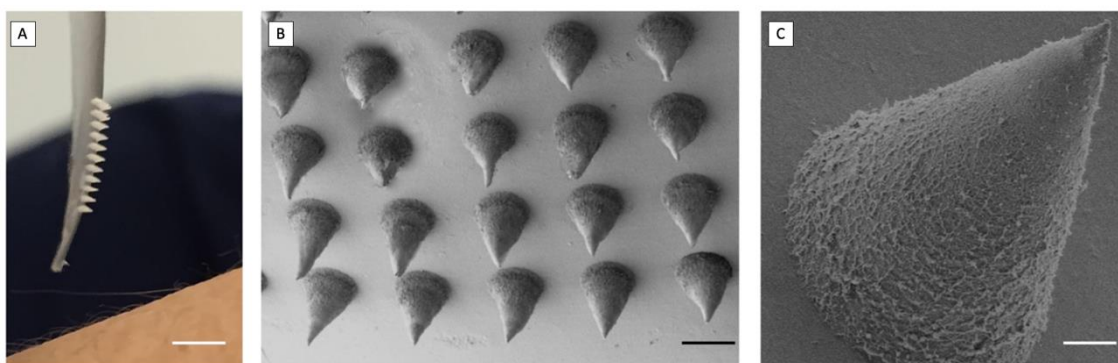


Figure 9. (A) Photograph of the patch with a microneedles array. Scale bar, 0.5 cm. (B) SEM image of the microneedles array. Scale bar, 500 µm. (C) Single microneedle. Scale bar, 100 µm. Figure reproduced and modified with permission from (79).

These patches act as a closed-loop system, releasing exendin-4 while immobilizing glucose oxidase in a glucose-responsive manner. Two different mineralised particles were used; copper phosphate encapsulates glucose oxidase and calcium phosphate encapsulates exendin-4. In the hyperglycaemic state, glucose oxidase reacts with glucose and lowers the pH. Calcium phosphate responds to the decrease in pH by releasing exendin-4, while copper sulphate prevents the release of glucose oxidase. The mineralised particles improved the mechanical resistance of the microneedles by crosslinking with the alginate, promoting skin penetration. These microneedle patches achieve exenatide release on-demand, without patients having to monitor their blood glucose.

Despite the positive results obtained in preclinical studies, the patches must be redesigned for clinical translation, and dose adjustment, as well as the length and morphology of the microneedles and the frequency of application, must be adapted for human administration.

4.5.2 Transdermal drug delivery through temperature modulation

TransPharma Medical Ltd. developed a product called ViaDor[®], which has now reached phase 1. It uses a radio frequency-based physical heating technique to enhance drug diffusion through the skin (80). However, limited information is available, and its development has been interrupted.

4.6 Pulmonary delivery

Several formulations have been developed to deliver inhaled GLP-1 and GLP-1 RAs (81). For instance, Afrezza[®] is an inhaled insulin approved by the FDA in 2014 using Technosphere[®] technology (82). Technosphere[®] is a powder formulation composed of fumaryl diketopiperazine (FDKP) microparticles where the peptide is encapsulated. The size of the microparticles ranges from 2 to 5 μm . The Technosphere[®] microparticles dissolve due to the physiological pH of the alveoli, releasing the peptide for absorption (83).

The pharmacokinetics and pharmacodynamics of Technosphere[®] GLP-1 (7-36) amide (MKC253) were studied in healthy and diabetic humans. When administered, MKC253 is rapidly absorbed (peak concentrations within 5 min) and rapidly degraded (baseline values within 30 min). In addition, it stimulates insulin and C-peptide secretion but hardly reduces glucagon secretion (84). As this study was a proof-of-concept, further studies are required for the optimisation of the formulation and its inhalation device.

Furthermore, the negative experience with Afrezza[®] in the market (inadequate insurance coverage, new adverse effects and safety concerns and the emergence of new therapeutic alternatives) should be thoroughly analysed before developing new formulations (85).

4.7 Nanoparticles

GLP-1 RAs nanoparticles (NPs) have been extensively investigated in recent years, especially for oral administration. Table 2 shows a summary of studies since 2017 that have designed and tested GLP-1 RAs NPs. At the moment these studies are just proof of concepts, and none are intended to be marketed in a short period of time. However, the development of this technology in the coming years must be taken into account.

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Year	Molecule	Formulation	Purpose	Method	Loading Efficiency	Route of administration	Relative Bioavailability	Insulinotropic effect	Hypoglycaemic effect	Other Findings	References
2020	Exenatide	Two formulations of zein NPs (COM and DIS NPs) with absorption enhancers (cholic acid, phosphatidylcholine)	Stable blood glucose control and β -cell repair of T2DM mice	Anti-solvent precipitation method	COM NPs - 79.7% DIS NPs - 53.6%	Oral	COM NPs - 10.9% DIS NPs - 5.6%	Both formulations increased insulin secretion by >60%	Comparable to free exenatide administered subcutaneously	β -cell proliferation by more than 120% after 7-week administration	(86)
2019	Exenatide	DEX-PLGA-NPs (DPs) and CSK-DEX-PLGA-NPs (CDPs)	Improve its mucus penetration and intestinal absorption	Double emulsion solvent evaporation method	DPs - 76.23% CDPs - 71.64%	Oral	DPs - 5.7% CDPs - 9.2%	Higher for the CDP group than for DP group	After 4 h, DPs reduced from 100% to 85.2% and CDPs to 75%	CDPs can target small intestinal goblet cells improving exenatide absorption	(87)
2019	Liraglutide	PLGA NPs	Design and stabilize Liraglutide loaded PLGA NPs by applying 7-factor 2-level Plackett-Burman screening design	Double emulsion solvent evaporation method	51.81%	Oral	-	-	-	-	(88)
2019	Exenatide	Large pore dendritic silica NPs	Achieve high peptide loading and improve intestinal	Bi-continuous microemulsion approach	Between 40% - 18% depending on pore size and volume	Oral	-	-	-	Potential improvement in permeability and in vitro relative bioavailability	(89)

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			permeation of exenatide							
2019	Exenatide	Lipid-based nanocapsules (LNC)	Increase endogenous secretion of GLP-1 and improve exenatide absorption into the circulation	First exenatide was encapsulated within reverse micelles and then further encapsulated within LNC	~85%	Oral	4.32%	LNC significantly reduced the insulin resistance index	LNC completely normalized the glycaemia	LNC trigger endogenous GLP1 secretion by themselves when presenting a size of ~200nm (90)
2019	Liraglutide	PLA (polylactide acid)- NPs coated with a cyclic, polyarginine-rich cell penetrating peptide (cyclic R9-CPP)	Enable oral delivery of liraglutide	Modified double emulsion solvent evaporation method	70 - 77% depending on the amount of liraglutide	Oral	4.5-fold higher than free liraglutide	-	-	Absence of toxicity in the target concentrations on Caco-2 cell assays (91)
2019	Exenatide	Anionic silica NPs	Enhance intestinal permeability	Commercial NPs	-	Oral	Silica NPs - 10% Non-silica NPs - 1.4%	-	-	The permeation- enhancing effect of the NPs was reversible, non- toxic, and attributable to the binding to integrins on the surface of epithelial cells (92)

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2019	Exenatide	Low molecular weight protamine-dextran-poly(lactic-co-glycolic acid)-NPs (LDPs)	Overcome the mucus barrier and improve intestinal targeting efficiency	Double emulsion solvent evaporation method	DPs - 75.73% LDPs - 68.94%	Oral	DPS - 5.3% LDPs - 8.4%	-	DPS - 20% reduction LDPs - 70% reduction after 6 h	-	(93)
2019	Exendin-4	Tannic acid/Fe (III) NPs	Provide sustained release of exendin-4 <i>in vivo</i>	Flash nanocomplexation method	~100%	Intraperitoneal	AUC 7.2-fold higher than the free exendin-4 injection	-	Rapid reduction of the blood glucose level to the normal range within < 12 h and maintained the same level for 72 h following a single intraperitoneal dose	-	(94)
2019	Liraglutide	Ternary liraglutide/tannic acid (TA)/Al ³⁺ NP system	Improve blood glycaemic control and reduce cardiovascular complications in a mouse model of T2DM	Flash nanocomplexation method	~100%	Intraperitoneal	-	-	Lowered the high blood glucose level in mice to the normal range within 6 h	-	(95)

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2019	Exendin-4	Injectable hydrogel depot system using exendin 4 (Ex-4) interactive and complex forming polymeric ionic-NPs	Long-term treatment of T2DM	Mixing and incubation method	High loading efficiency without any loss of protein content	Subcutaneous	Improved bioavailability compared with exendin-4 solution	-	Significantly prolonged glucose-lowering effect; the lowered blood glucose levels (under 200 mg/dL) were maintained for 4 days	Reduced food uptake and body weight in DM (db/db) mice model	(96)
2018	Exenatide	Oral self-emulsifying drug delivery system (SEDDS)	Develop an oral self-emulsifying drug delivery system for exenatide and evaluate its <i>in vivo</i> efficacy	Exenatide was lipidized via hydrophobic ion pairing with sodium docusate (DOC) and incorporated in SEDDS	-	Oral	14.62%	Insulin response was significantly increased	Significant 20.6% decrease in AUC values of blood glucose levels	-	(97)
2018	Exenatide	Fc-modified exenatide-loaded PEG-PLGA NPs	Improve hypoglycaemic effects in mice	Double emulsion solvent evaporation method	PEG5000-PLGA28000 - 59.5% PEG5000-PLGA20000 - 83.5%	Oral	Improved oral bioavailability	-	Significantly high hypoglycaemic effects, comparing with the orally administered exenatide solution	NPs-Fc stayed in the gastrointestinal tract for a longer time in comparison with the unmodified NPs,	(98)
2018	Liraglutide	Chitosan coated calcium-alginate nanocapsules	Oral sustained delivery of liraglutide	Modified two-step method (Rajaonarivony et al. (99)) for preparing	92.5%	Oral	-	-	-	-	(100)

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alginate-poly-l-lysine NPs											
2018	Exenatide	PEG-PLGA-low molecular weight protamine NPs	Enhance oral absorption of exenatide	Double emulsion solvent evaporation method	71.3%	Oral	7.44%	Stimulates insulin secretion	25% reduction after 4 h and 57% reduction after 6 h	-	(101)
2018	Liraglutide	Poly-N-(2-hydroxypropyl) methacrylamide/chitosan NPs	Effective oral administration of peptides	Double emulsion solvent evaporation method	~80%	Oral	10.12%	-	-	-	(102)
2018	GLP-1	NPs induced by embedding self-assembling cassette into glucagon-like peptide 1	Improving <i>in vivo</i> stability	Formulation of self-assembling GLP-1 derivatives	-	Subcutaneous	-	Cadyglp1e and cadyglp1m were clearly capable of inducing insulin secretion	Cadyglp1e and cadyglp1m exhibited long-acting glucoregulatory activity	-	(103)
2018	Exenatide	NPs embedded in gastro-resistant microparticles	Overcome exenatide's inability to cross the enterocytes and increase its gastrointestinal stability	Albumin and dextran NPs cross-linked using sodium trimetaphosphate / Eudragit L and HPMC MPs prepared by spray drying	77%	Oral	77% relative to Byetta® injection	Raised insulin levels between day 1 and 30 almost 30% (p<0.01)	Decrease AUC about 600 mg h/dL	Reduced HbA _{1c} by ~2% and prevented natural weight gain in mice	(104)

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2017	Exenatide and liraglutide	Nanostructured lipid carriers (NLC)	Stimulation of GLP-1 secretion and delivery of GLP-1 agonists	High-pressure homogenization technique	Liraglutide - 95.4% Exenatide - 87.5%	Oral	-	-	No significant hypoglycaemic effect	NLCs were mainly confined to the mucus-layer covering the intestinal cells	(105)
2017	Exenatide	Mesoporous silica NPs	Control de sustainability of exenatide	Hydrothermal method	15.2%	Subcutaneous	Significantly improved versus exenatide solution	-	Exhibited long-lasting and remarkable glucose-lowering effects	-	(106)
2017	Exenatide	Exenatide-loaded linear poly(ethylene glycol)-brush poly(L-lysine) block copolymer	Study the implications on diabetic nephropathy	Mixing and dialyzing	12.11%	Subcutaneous	-	-	Reduces blood glucose levels to 5.8 mmol/L after 2 h. Then maintained low blood glucose levels for 7 days	Significantly alleviated diabetic nephropathy	(107)
2017	Three analogues of GLP-1 (7-37) with C-terminal modified region	Gold NPs	Study the potential application of gold NPs for GLP-1 analogues delivery	Reducing H ₂ AuCl ₄ with sodium citrate, 1:4 molar ratio (I. Olmedo et al. (108))	-	Intraperitoneal	-	Maintain insulinotropic activity	Greater reduction of glycemia after 2 h of administration than that reached with the free peptide	-	(109)

Table 2. Properties of GLP-1 RAs nanoparticles and modified GLP1 from selected studies between 2017 and 2020

4.8 Microparticles

Biodegradable microparticles based on PLGA polymers have proven to be useful drug delivery systems in other biomedical applications such as adjuvant/vehicle for vaccines, tissue engineering, sustained-release systems or cancer treatment (110–114).

In 2012, the FDA approved Bydureon® for the treatment of DM (36). Bydureon® uses Medisorb® technology, which is based on PLGA microparticles with a size of 60 μm that provide an extended-release of exenatide when administered subcutaneously (32). Drug release occurs in three steps: an initial burst release, a lag phase, and a drug diffusion phase. The burst release phase, shown in Figure 10, is mainly caused by surface and easily accessible drug molecules, and can transitorily increase drug concentrations in the blood, which can cause adverse effects.

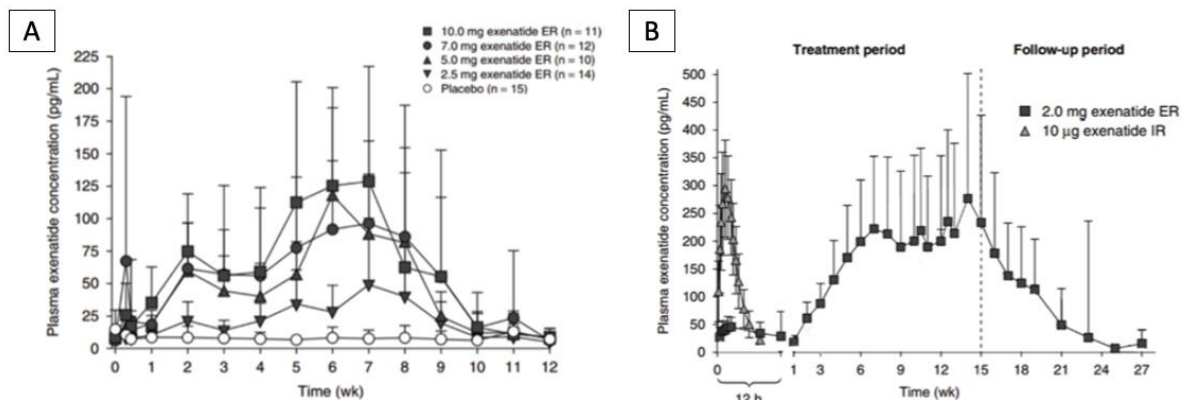


Figure 10. (A) Plasma concentrations of exenatide after a single extended-release exenatide injection (0 to 12 weeks) (B) Plasma concentrations of exenatide after repeated weekly exenatide injection (0 to 27 weeks). Figure reproduced and modified with permission from (13).

Since the approval of Bydureon®, research on GLP-1 RAs microparticulate systems has focused on improving the pharmacokinetic and pharmacodynamic properties of Bydureon®. To this end, various approaches have been developed for the release of GLP-1 RAs.

4.8.1 PLGA microparticles

Most of the published works on GLP-1 RAs microparticles have focused on the use of PLGA as an encapsulating polymer and exenatide as a model drug. After Bydureon® release, several efforts have focused on improving the original formulation. For instance, several batches of microspheres with polymers of different molecular weight and composition have

been prepared by different methods and employing different solvents (115). According to this study, microspheres prepared with 65 KDa 50:50 PLGA using a water-in oil-in oil (W/O/O) emulsion-solvent extraction method showed the highest encapsulation efficiency ($98.0\% \pm 9.4$) and loading efficiency ($4.53\% \pm 0.44$). Besides, by using heptane as the only hardening solvent, the microspheres had a round shape, smooth surface and good dispersion. All microspheres prepared by the W/O/O emulsion-solvent extraction method provided low burst release *in vitro* (Figure 11), attributable to the non-aqueous processing medium (silicon oil).

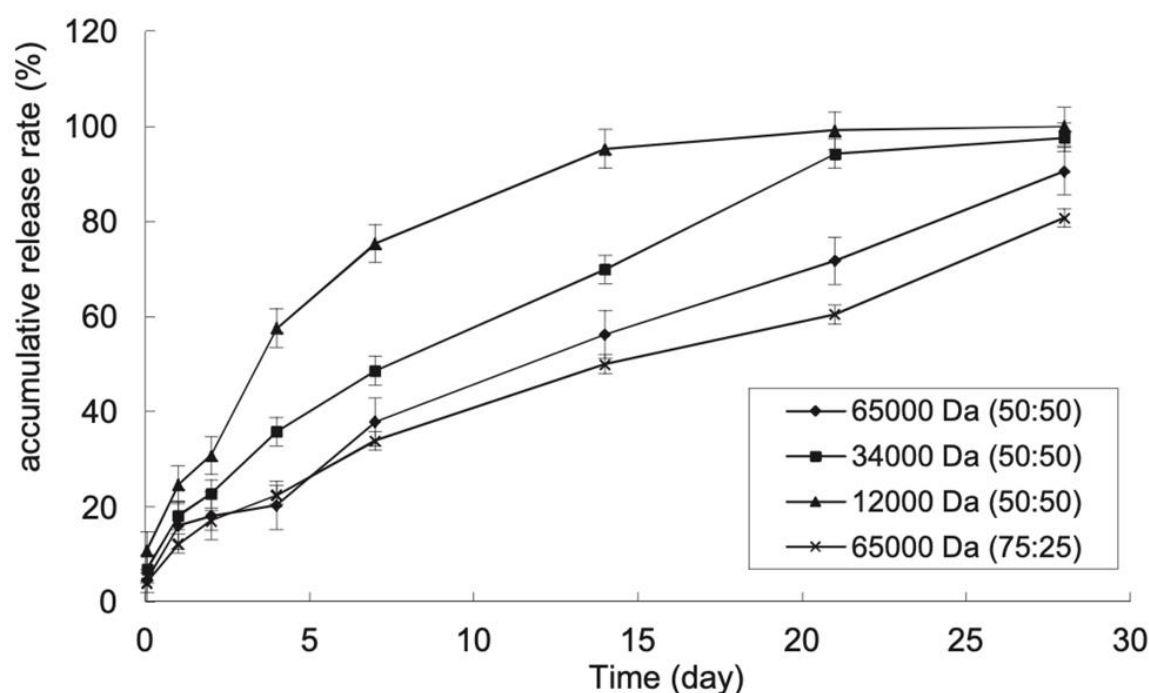


Figure 11. In vitro release profiles of exenatide loaded PLGA microspheres prepared with PLGA of different molecular weights and different copolymer compositions in HEPES buffer pH 7.4. Figure reproduced and modified with permission from (115).

However, it was observed that the use of organic solvents such as silicon oil, dichloromethane, heptane, or ethanol in the process was not appropriate for obtaining microparticles. Also, mechanical dispersion methods were not effective in controlling the size distribution of the microparticles, which led to problems of inconsistency in the results. In another study, exenatide-loaded PLGA microspheres of uniform size were fabricated using the Shirasu Porous Glass premix membrane emulsification technique combined with water-in oil-in water (W/O/W) emulsion solvent extraction method. Microspheres of approximately 20 μm

were obtained with high encapsulation efficiency. (116). Moreover, exenatide-loaded PLGA microparticles prepared by spray drying were compared to those prepared by the ultrafine particle processing system (UPPS) based on the disk rotation principle (117) (Figure 12). This technique provided a simple and scalable alternative to reproducibly manufacture microparticles under mild preparation conditions.

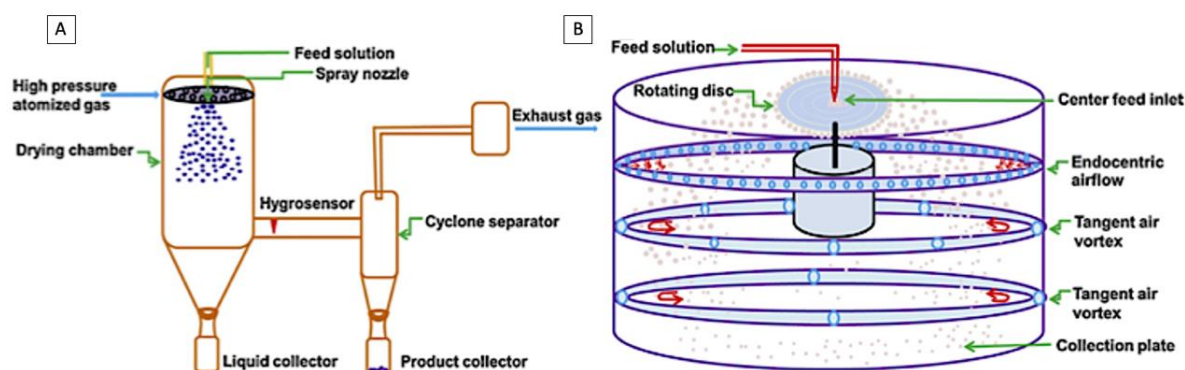


Figure 12. Schematic illustration of spray dryer (A) and ultrafine particle processing system (UPPS) (B) for the manufacture microparticles. Figure reproduced and modified with permission from (117).

The UPPS microparticles were larger in size and showed higher encapsulation efficiency than the microparticles obtained by spray-drying. In *in vitro* studies, UPPS microparticles released exenatide in a stable and sustained manner, whereas in the *in vivo* studies in rats, the antidiabetic effect was observed for one month.

Using the same technique, dimpled exenatide-loaded PLGA microparticles were prepared to investigate the mechanism of formation and release characteristics (118). The microparticles showed high encapsulation efficiency ($91.50 \pm 2.65\%$) and a sustained drug release for two months with a reduced initial burst. Additionally, effective drug concentrations were maintained for three weeks after a single injection.

In other work, hollow microparticles loaded with GLP-1 were compared to solid microparticles prepared by a modified oil-in-water (O/W) emulsion solvent evaporation technique (119). The purpose of the study was to reduce the accumulation of acidic products of PLGA degradation and to reduce polymer-peptide interactions. It was concluded that in the hollow microparticles 93% of the extracted peptide was active, while in the solid microparticles only 58% of the peptide was active. Thus, the *in vitro* release of the hollow microparticles on

day 14 was $88 \pm 8\%$ compared to $33 \pm 6\%$ for solid microparticles. In the same year, a novel GLP-1 analogue with a longer half-life was synthesised and encapsulated in PLGA microspheres by the double emulsion-solvent evaporation method (120). Zinc was incorporated to slow initial burst release and achieve uniform drug distribution.

Also for the purpose of reducing the burst release, PLGA microspheres containing exenatide-loaded lecithin NPs were prepared by a modified solid-in oil-in-water (S/O/W) emulsion technique (121). The microparticles released the drug for more than 60 days and showed reduced burst release. An encapsulation efficacy of $66.33\% \pm 3.75$ was obtained, and the size of the microparticles was $5.29 \pm 0.98 \mu\text{m}$ with a Span value of 1.32 ± 0.01 .

In a more recent study, exenatide-loaded inside-porous PLGA microspheres with outside layers were fabricated using a W/O/W emulsion method with a microfluidic technique (122). To form the porous microspheres, NH_4HCO_3 was chosen as the porogen. The preparation of the microparticles is shown in Figure 13. Briefly, to form the microparticles, an emulsion was first prepared with W_1 (exenatide and NH_4HCO_3 solution) and O (PLGA in dichloromethane), then this emulsion was mixed with W_2 (PVA and NaCl solution) through the microfluidic cross chip. Solid microspheres were obtained after stirring to volatilize the dichloromethane.

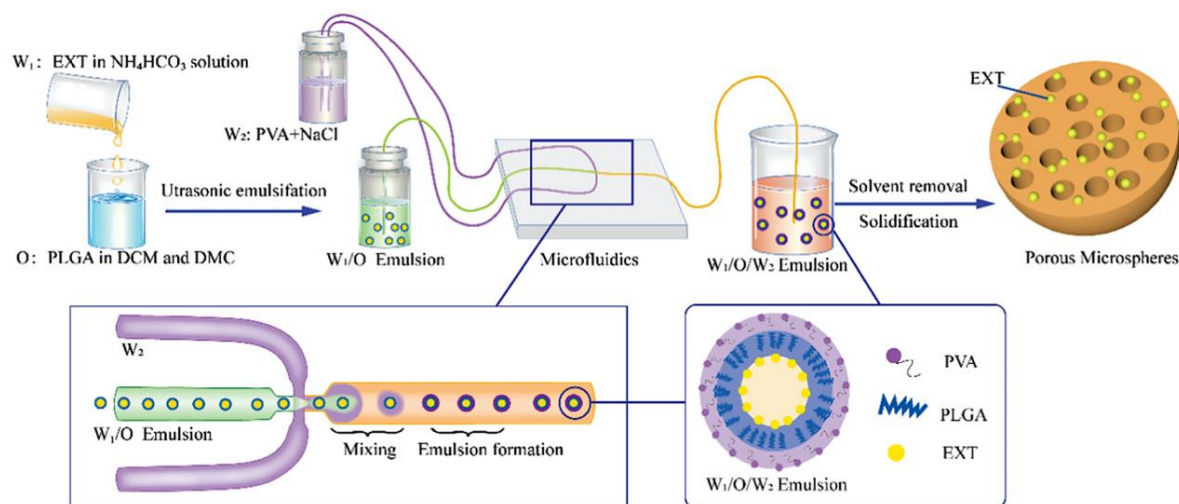


Figure 13. Synthetic process for the exenatide-loaded inside-porous PLGA microspheres. Figure reproduced and modified with permission from (122).

The porous microspheres had low burst release and absence of a lag phase with high encapsulation efficiency, improving the release profile of existing exenatide-PLGA microparticles.

More studies have been published to evaluate various parameters of PLGA microparticles that were affecting the encapsulation of GLP-1 RAs (123–125). Conversely, only a few authors have proposed different polymers to PLGA to encapsulate GLP-1 RAs to overcome its severe limitations in the pharmacokinetic profile of the drug, such as minimizing initial burst release or extending the time between administrations. In addition, there has not been much innovation nor have many alternatives been proposed in the methodology used to encapsulate these drugs, which are usually techniques based on the emulsion-solvent evaporation method. This method has several drawbacks, such as the presence of organic solvent residues in the final formulation, a wide particle size distribution or being hardly scalable for the industry.

4.8.2 Hydrogel microparticles

Elaboration of hydrogel drug delivery systems for the administration of exenatide has also been proposed. For instance, a drug delivery system based on [Gln²⁸]exenatide (a more stable analogue of exenatide) covalently bound to hydrogel microspheres has been developed by means of a self-cleaving β -eliminative linker (126–128). For preparing the microparticles, a large pore tetra-PEG hydrogel polymer was used. This system can be administered once a month and has already been tested in cats for feline DM with positive results.

In another study, a gel matrix microsphere gel deposition system was developed by encapsulating exenatide-loaded hydrogels in PLGA microspheres encapsulated into blank hydrogels (129,130). A 46 days release *in vitro* without burst release was achieved also obtaining stable blood glucose for 20 days *in vivo*.

5 CHALLENGES AND FUTURE PERSPECTIVES

To date, it is estimated that 9.3% of the world population (aged 20-79 years) suffers from DM and it is expected that his percentage will continue to increase until reaching a global prevalence of more than 10% in the next 10 years, which makes DM one of the most prevalent chronic diseases in the world (6). Improving efficacy, safety and patient compliance continue to be the primary goals of DM management. Advances are likely to come from the development of novel subcutaneous treatments capable of effectively controlling glycaemic levels and convenient to ensure patient's adherence to therapy. Commercialised formulations of GLP-1 RAs and amylin mimetics still present pharmacokinetic problems that limit the effectiveness of

the therapy and cause frequent adverse effects (16,131). In addition, the high cost of these treatments means that their prescription is limited to specific cases.

The obtention of longer acting GLP-1 RAs and amylin mimetics that allow a prolonged and constant release of the drug is an area of ongoing research. Novel drug encapsulation methods must be developed that employ biocompatible polymers and avoid the use of organic solvents or high temperatures. These methods must not only be industrially scalable and allow a loading efficiency close to 100% to avoid the loss of drugs as expensive as biotherapeutics, but also capable of producing formulations that allow a constant drug release without major fluctuations in blood drug levels.

Alternatives to injectable formulations such as transdermal, nasal and oral routes have also been proposed. These alternative routes could be especially convenient for patients with motor or visual impairments, very common in DM. In particular, there is a growing interest in making oral delivery of GLP-1 RAs and insulin a reality. The recent marketing of Rybelsus[®] is a proof of this (54). However, oral peptide therapeutics still have to face several challenges, such as poor oral absorption, pH and enzymatic instability along with food effect and pharmacokinetic variability. Significant investments should be made in formulation design and development to provide sufficient oral bioavailability before oral GLP-1 RAs and insulin assume an important role in the DM management (10).

Regarding transdermal drug delivery, development work is focused mainly on minimally invasive microneedle patches. Conventional approaches to fabricate microneedle patches include micromoulding, lithography and coating techniques (76). However, these techniques are usually time-consuming and difficult to scale-up. Innovative technologies such as 3D printing could offer an alternative for the manufacturing of these devices. Stereolithography (SLA) 3D printing has already proven capable of creating microneedle patches with high degrees of complexity and reproducibility in a fast and cost-effective manner (132,133). The use of biocompatible resins helps to circumvent toxicity problems; however, regulatory and technical challenges still remain before the adoption of 3D printing in the clinical practice (134).

GLP-1 RAs and other protein and peptide drugs also represent interesting targets for intranasal administration. However, these molecules are charged, hydrophobic and usually present high molecular weights, and thus are poorly permeable through lipid barriers such as the nasal. Some pharmaceutical strategies have been applied to overcome the low permeability

as well as the physicochemical instability in the nasal mucosa to increase drug bioavailability, such as the use of nano and microparticulate systems, enzyme inhibitors and permeation enhancers. Of particular interest is the inclusion of GLP-1 RAs in chitosan-based thermosensitive hydrogels. Chitosan is well recognised as a polymeric absorption enhancer capable of opening tight junctions in epithelial cells and thereby increasing permeability (135). Despite the potential showed by these formulations, further research and investment is needed before optimised intranasal therapies can reach the market.

Microparticulate systems have also shown great promise for GLP-1 RAs delivery. Since PLGA is well accepted by regulatory bodies and has been shown to be effective in delivering biotherapeutics, many of the microparticle formulations approved by the FDA to date are PLGA based (136). One example is found in Bydureon[®], the polymer microsphere formulation of exenatide, a once-a-week product (36). Once injected subcutaneously, the microspheres form a matrix drug reservoir *in situ* and exenatide is released in a discontinuously, with an initial low burst release in the first 48h and two peaks of drug release in the second and seventh week. This discontinuous rate of drug release is due to the properties of PLGA, the main drawback of these formulations being the impossibility of releasing the drug at a steady rate (13).

Aside from the development of improved formulations, the establishment of *in vitro* and *in vivo* models for preclinical testing of such formulations is almost equally important. The lack of *in vitro* dissolution tests for biotherapeutics and the high variability between subcutaneous tissues from different animal models are major obstacles during the development of new biotherapeutic formulations (137). Although novel methods, such as the *in vitro* methodology called Scissor for Subcutaneous Injection Site Simulator (22), represent important advances to achieve better reproducibility and more suitable experimental conditions to test the formulations, none of the proposed models completely mimic the human subcutaneous environment. Future preclinical models should allow long-term studies of the pharmacokinetics, bioavailability and immunogenicity of sustained-release formulations.

Finally, another key consideration is the efficient management of diabetic patients through optimal monitoring of blood glucose levels. Current monitoring of the disease is mainly based on the use of glucometers and glycohemoglobin test kits, which rely on invasive lancet approaches that hinder patient compliance. Non-invasive glucose monitoring systems are not widely employed due to their uncertain measurement accuracy and weak blood glucose

correlation (138). Immense research has been carried out to develop low-cost, robust and reusable systems for monitoring and controlling diabetes (139). In particular, considerable advances have been made in the creation of wearable biosensors for non-invasive continuous monitoring of glucose levels. Some examples are found in the development of skin-like (138) and contact lens biosensors (140). The joint use of improved subcutaneous formulations with longer circulation times that allow more separate administrations and adequate glycaemic monitoring is forecast to be a game-changer on DM management as well as on patient's quality of life.

6 CONCLUSIONS

GLP-1 receptor agonists and amylin mimetics have the potential to become a key treatment for diabetes mellitus. This review article summarises the current challenges of subcutaneous administration of these drugs as well as the emerging strategies for the development of GLP-1 receptor agonists formulations with improved bioavailability. Also, a broad overview of currently marketed dosage forms is provide, from oral nanoparticles to subcutaneous injections for the controlled release of biotherapeutics. Although all these new strategies can greatly benefit diabetes mellitus therapy, subcutaneous drug delivery systems still have many challenges to face. Several problems, including the lack of reliable preclinical models, immunogenicity issues, the development of high-dose/volume formulations, or relatively high costs need to be addressed to enhance the development of future diabetes mellitus formulations.

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CHAPTER II

Intravitreal implants manufactured by supercritical foaming for treating retinal diseases

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CHAPTER II**Abstract**

Chronic retinal diseases, such as age-related macular degeneration (AMD), are a major cause of global visual impairment. However, current treatment methods involving repetitive intravitreal injections pose financial and health burdens for patients. The development of controlled drug release systems, particularly for biological drugs, is still an unmet need in prolonging drug release within the vitreous chamber. To address this, green supercritical carbon dioxide (scCO₂) foaming technology was employed to manufacture porous poly(lactic-co-glycolic acid) (PLGA)-based intravitreal implants loaded with dexamethasone. The desired implant dimensions were achieved through 3D printing of customised moulds. By varying the depressurisation rates during the foaming process, implants with different porosities and dexamethasone release rates were successfully obtained. These implants demonstrated controlled drug release for up to four months, surpassing the performance of previously developed implants. In view of the positive results obtained, a pilot study was conducted using the monoclonal antibody bevacizumab to explore the feasibility of this technology for preparing intraocular implants loaded with biologic drug molecules. Overall, this study presents a greener and more sustainable alternative to conventional implant manufacturing techniques, particularly suited for drugs that are susceptible to degradation under harsh conditions.

Keywords

Supercritical foaming utilizing CO₂; PLGA intravitreal implants; dexamethasone; monoclonal antibodies; age-related macular degeneration (ADM); three (3D)-dimensional printing.

I INTRODUCTION

Chronic retinal diseases, including age-related macular degeneration (AMD), diabetic retinopathy (DR), or diabetic macular edema (DME), are a leading cause of ocular morbidity and global visual impairment. Notably, AMD stands as the primary cause of blindness in developed countries, particularly among the elderly, and its prevalence is expected to increase due to the exponential aging of the population [1, 2]. In the realm of AMD treatment, intravitreal anti-vascular endothelial growth factor (anti-VEGF) therapy stands as the current gold-standard approach. Among the first-line anti-VEGF drugs are bevacizumab, aflibercept, and ranibizumab, which are administered via intravitreal injections every 1 to 3 months, depending on the patient's clinical response [3]. In cases where anti-VEGF drugs fail as effective therapy or are not well-tolerated, intravitreal corticosteroids like dexamethasone or fluocinolone have emerged as viable secondary treatment options [4]. The cost associated with providing care for AMD is considerably high due to the treatment burden that entails frequent visits to ophthalmology departments and the expenses related to anti-VEGF drugs [5]. Furthermore, patients who adhere to a regular treatment regimen tend to achieve better visual outcomes compared to those who follow an irregular schedule [6]. Therefore, future treatments for chronic retinal diseases should strive to enhance the longevity of drugs within the vitreous chamber, thus reducing the frequency of injections [7]. Significant progress has been made in this regard since the approval of Ozurdex® (Allergan) in 2014 [8] for the treatment of DME. Ozurdex® is an intravitreal implant composed of a poly(lactic-co-glycolic acid) (PLGA) matrix containing 700 µg of dexamethasone, capable of releasing the drug over several months [9]. The implant is placed into the vitreous cavity of the eye through a small incision in the sclera, which is created by an ophthalmologist using a specialised instrument, after applying local anaesthesia.

Recent research has been dedicated to the development of long-acting drug delivery systems that offer extended release of drugs within the vitreous chamber [5, 10]. However, current manufacturing techniques for implantable devices often involve harsh fabrication conditions, such as high temperatures or the utilisation of organic solvents [11-16]. One prevalent technique, material extrusion, necessitates elevated temperatures that can lead to structural damage and a decrease in therapeutic efficacy for heat-sensitive drugs, including biologics. To address these limitations, alternative approaches such as solvent casting have been

explored, which employ organic solvents as substitutes for high temperatures. Nevertheless, organic solvents are generally recognised as non-thermodynamically stable and toxic substances that pose risks to both human health and the environment. Consequently, endeavours in the field are focused on the development of greener technologies that are both safe for organisms and employ mild processing conditions [17-20].

Supercritical fluid technology, utilising supercritical CO₂ (scCO₂), has emerged as a versatile processing platform for manufacturing highly customisable, drug-loaded structures without the need for organic solvents or high temperatures [21-23]. The mild conditions at the critical point of CO₂ (7.38 MPa and 304 K) enable the processing of polymeric structures that efficiently incorporate thermolabile drug molecules, particularly biologics, with high yields [24, 25]. One key application of this technology is supercritical foaming, which involves using scCO₂ as a porogen to produce polymeric implantable devices loaded with drugs and with adjustable shapes and textural characteristics, including pore size distribution and interconnectivity. The process of supercritical foaming occurs in two stages. In the first stage, CO₂ is absorbed and dissolved into the polymeric matrix under high pressure, resulting in polymer swelling. In the second stage, the system is depressurised, leading to the formation of a porous structure with controlled porosity [24, 25]. The porous structure of the medicated implant plays a crucial role in regulating the drug release profile and can be optimised by adjusting various operating parameters, such as temperature, pressure, contact time, and depressurisation rate [26-30]. These parameters can be fine-tuned to achieve desired release kinetics and enhance therapeutic efficacy.

Given the current need to develop long-acting intravitreal drug delivery systems and alleviate the treatment burden associated with existing therapies, this study aims to investigate the viability of producing PLGA-based intravitreal implants using supercritical foaming. Customised moulds with specific geometries were created through stereolithography (SLA) 3D printing to facilitate the manufacturing process [31]. The use of 3D printing to produce the moulds is primarily advantageous due to its simplicity and cost-effectiveness, which is particularly beneficial in a research setting. Moulds can be adjusted in terms of size, shape, and composition, and their reusability further contributes to their practicality. Dexamethasone, a commonly used corticosteroid for treating chronic retinal diseases, was chosen as the model drug. The selection of PLGA as the matrix material was based on its biodegradability, biocompatibility, and favourable foaming properties. The study primarily focused on assessing

the impact of slight modifications in depressurisation rates [32, 33] on the morphological properties of the foamed implants and their subsequent in vitro drug release. In addition, comprehensive characterisation of the implants was performed to evaluate their physicochemical properties, biocompatibility, and cytotoxicity. In the light of the results obtained from the initial batch of implants, further experiments were performed to explore the potential of incorporating biologic drugs into the implants. To achieve this, a proof-of-concept study involving the monoclonal antibody bevacizumab was conducted. Bevacizumab was chosen as the model drug due to its susceptibility to degradation under harsh processing conditions such as high temperatures and pH changes [34].

2 MATERIALS AND METHODS

2.1 Materials

Resomer[®] RG 503H (RES) (Mw: 10 kDa; lactide:glycolide = 50:50) was purchased from Evonik (Essen, Germany), Purasorb[®] PDLG 5002A (PUR) (Mw 16 kDa; lactide:glycolide = 50:50) was purchased from Purac (Gorinchem, The Netherlands), dexamethasone (DEX) base was purchased from Acofarma (Barcelona, Spain), bevacizumab (BEV) (Avastin[®]) was purchased from Roche (Basel, Switzerland). Hydroxypropyl- β -cyclodextrin (HP β CD) was purchased from Roquette (Lestrem, France). The clear 3D printing resin (Anycubic resin UV wavelength 405 nm) was obtained from Shenzhen Anycubic Technology Co. Ltd., (Shenzhen, China). Culture media, FBS and all supplements were purchased from Biological Industries (Cromwell, CT, USA).

2.2 Methods

2.2.1 Manufacturing of 3D printed moulds

Moulds were designed using Autodesk Fusion 360 (Autodesk, CA, US). The design consisted of two halves, with a funnel shape in the upper part and a tubular shape in the lower part (Figure 1). Both halves were securely attached using a pressure screw system, which prevented powder leakage during processing. The CAD file was exported in .stl format and processed in the Photon Workshop (Shenzhen, China) slicer software. The slice parameters were set to 0.05 mm layer thickness, 5 s normal exposure time, 0.05 s off time, 30 s bottom exposure time, and 4 bottom layers. The sliced mould file was then loaded into the SLA 3D printer (Anycubic Photon Mono X 6K, Shenzhen Anycubic Technology Co., Ltd., Shenzhen,

China). The 3D printer was filled with clear 3D printing resin and a batch of 20 moulds was printed. The 3D printed moulds were then washed with isopropanol in an ultrasonic bath and cured in a UV light cabinet for 1 min on each side.

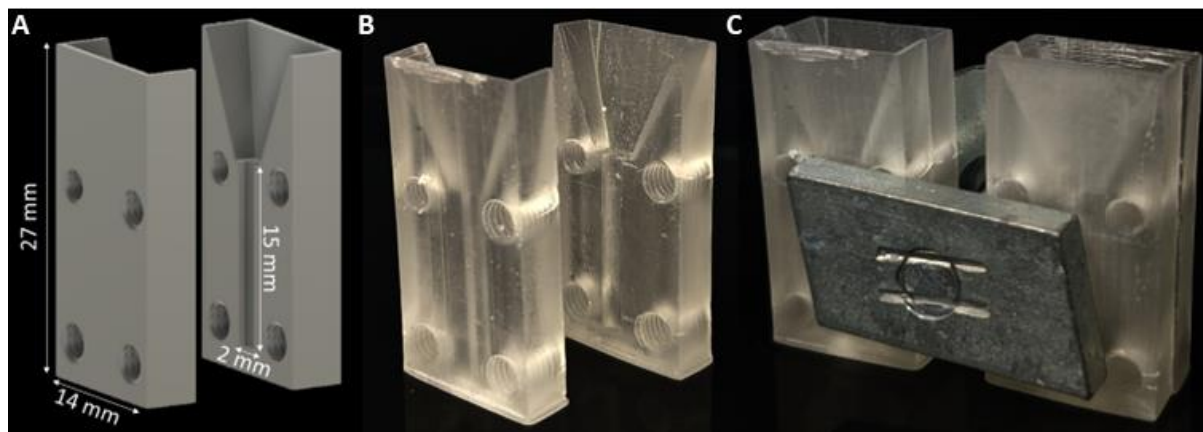


Figure 1. (A) CAD design of the intravitreal implant mould with the desired dimensions. (B) Gross view of a 3D-printed mould with a funnel shape to facilitate powder loading. (C) View of the moulds assembled with a pressure screw system.

2.2.2 Manufacture of implants by supercritical foaming

A preliminary study was conducted to select the optimal blend of PLGAs for the foaming process. Several blends were tested, including RES:PUR (50:50), RES:PUR (25:75), RES:PUR (75:25), and RES:PUR (40:60) and (60:40). Additionally, moulds with varying sizes of the tubular part (5, 10, 15, and 20 mm) were tested to assess their impact on the foaming process. The effect of covering the inlet funnel with Parafilm[®] M sealing film, silicone, or leaving it uncovered was also evaluated. The components of the implants, in the form of dry powders, were weighed according to the proportions specified in Table 1, physically mixed in a mortar, and ground with a pestle. The dose of each drug was selected to match the doses administered in clinical practice.

Table 1. Implant composition % (w/w) and depressurisation rates (g/min) of implants containing PLGA, DEX, BEV and HP β CD processed by supercritical CO₂ foaming.

USC Implant UNIVERSIDADE DE SANTIAGO DE COMPOSTELA	Depressurisation	RES	PUR	DEX	BEV	HP β CD
	(g/min)	%(w/w)	%(w/w)	%(w/w)	%(w/w)	%(w/w)

1.8DEX	1.8	46.7	46.7	6.6	-	-
3.6DEX	3.6	46.7	46.7	6.6	-	-
7.2DEX	7.2	46.7	46.7	6.6	-	-
3.6BEV	3.6	41	41	-	10	8

For BEV implants, a previous purification step of the commercial formulation of bevacizumab (Avastin[®]) was necessary. Avastin[®] was purified using an Amicon[®] Ultra-2 mL centrifuge filter (NMWL 30 kDa) (Millipore[®], Burlington, Massachusetts, USA) to remove the excipients included in the commercial formulation. The purified BEV solution was then resuspended in Milli-Q[®] water and 8% (w/w) hydroxypropyl- β -cyclodextrin (HP β CD) was added to the solution to act as an antiaggregant and cryoprotectant [35-37]. HP β CD was found not to be toxic or irritating for ocular use [38]. The resulting solution was then freeze-dried to obtain the monoclonal antibody in powder form. The solutions were frozen at -80°C and then freeze-dried using a LyoQuest freeze-dryer (Telstar, Terrassa, Barcelona) with a final condenser temperature of -53°C.

A second-derivative ultraviolet spectrum was carried out using a UV-Vis spectrophotometer (Agilent Cary 60 UV-Vis, Santa Clara, California, USA) to verify the integrity of the BEV structure in Avastin[®] solution, freeze-dried Avastin[®] and purified and freeze-dried Avastin[®] with 8% (w/w) HP β CD before and after the processing with supercritical CO₂ [39].

The powder blends were loaded into the 3D-printed moulds and compacted with a compression rod. The moulds were then placed in a 100 mL-high-pressure stainless steel autoclave (Thar Technologies, Pittsburgh, PA, USA). The supercritical foaming was carried out following a pressurisation-soaking-single depressurisation stepwise protocol. CO₂ was pressurized into the autoclave at 5 g/min until a pressure of 100 bar was reached, and the temperature was maintained at 35°C. The system was left under stirring at 700 rpm in batch mode for 1 h (soaking period). Subsequently, the autoclave was depressurised at a venting rate of 1.8, 3.6 or 7.2 g/min for DEX implants and at 3.6 g/min for BEV implants until atmospheric

pressure was reached. The implants were stored overnight at 4°C to allow complete CO₂ desorption.

2.2.3 Structure analysis

The dimensions of the implants, including diameter and length, were measured with a digital Vernier calliper. The mass of each implant was determined using an analytical balance (Sartorius Quintix 35-1S). The morphological analysis of the implants was conducted by observing the external structure of the implants through a stereomicroscope with an electronic camera (Olympus® SZ-CTV/Olympus® SC100). The surface topography and internal structure of the implants were examined using field emission scanning electron microscopy (FESEM) (ZEISS EVO LS15). The sample surface was imaged at 20kV using the backscattered electron detector (BSD) under variable pressure (VP) conditions. The implants and their transversal cuts were fixed on 25 mm aluminium stubs using self-adhesive carbon discs prior to the analysis.

The microstructure of the implants was analysed by microcomputed tomography (Micro-CT) using a Skyscan 1272 high resolution X-ray 3D micro-CT equipped with an 11 MP X-ray detector (Bruker, Kontich, Belgium). The implants were imaged at 2 µm pixel resolution, 50 kV and 200 µA without filter. Projections were collected with a rotation step of 0.5° for 180° with an exposure time of 900 ms, and then reconstructed and analysed using NRecon and CTvox software (Bruker, Kontich, Belgium), respectively. For 3D analysis, slices were converted into binary images using a threshold of 70–255 and a volume of interest (VOI) of 53 mm³ was chosen to evaluate total porosity, pore size and interconnectivity.

2.2.4 Texture analysis

Texture analysis was performed using a precision Universal Testing Machine AGX-X series (Shimadzu, Kyoto, Japan), equipped with a 50N load cell and Trapezium X software (Shimadzu, Kyoto, Japan). For this purpose, a blade was secured in the upper support, while the implant was positioned on the lower plate. The blade was then gradually lowered at a speed of 1 mm/min until the implant ruptured. The force-displacement curve was subsequently recorded, from which three key material parameters were derived: rupture strength, failure work, and elasticity.

Breaking strength was determined as the maximum force observed at the point of material fracture, corresponding to the highest force recorded on the force-displacement curve. Failure

work was calculated as the area under the curve (AUC) between force and displacement until the breaking strength was reached. Elasticity was assessed using the modulus of elasticity, known as Young's modulus, which is the ratio between force and deformation obtained from the slope of the force-displacement curve.

2.2.5 Drug distribution in the implant

The distribution of DEX within the implants was analysed using energy dispersive X-ray analysis (EDX) and Raman spectroscopy. For EDX, a semi-quantitative microanalysis and distribution mapping of the elemental composition of the surface and the core of the DEX implants was conducted using an EDS Ultim[®] Max (Oxford Instruments, Abingdon, United Kingdom) controlled by INCA software (Oxford Instruments, Abingdon, United Kingdom). The acquisition conditions were 20Kv and a working distance of 8.5 mm for a more precise analysis. The same analysis was performed on the DEX implants after the *in vitro* release assay.

Raman spectra were recorded at room temperature on a Raman confocal microscope alpha300 R (WITec, Ulm, Germany) using a 532 nm laser with a power of 7 mW. The implants were sliced and placed in a sampler holder to examine the core of the implant. A total area of 70x70 μm was recorded with a lateral resolution of 1 spectrum every 300 μm , for a total of 19600 spectra. Project Five 5.3 (WITec, Ulm, Germany) was used for data processing and imaging.

2.2.6 Crystallinity

The crystal structure and thermal stability of DEX, PLGA powders, implants, and the physical mixture of PLGA and DEX were identified and characterised using X-ray powder diffraction (XRD) and differential scanning calorimetry (DSC). XRD measurements of the powdered samples were measured with Bragg-Brentano geometry using a Bruker D8 Advance diffractometer (Bruker, Massachusetts, USA) (40 kV, 40 mA, theta/theta) equipped with a Cu sealed X-ray tube ($\text{CuK}\alpha 1$ ($\lambda = 1.5406 \text{ \AA}$) and a LYNXEYE XE-T detector). The diffractograms were obtained in the 2θ angle range $3^\circ - 40^\circ$ with a step of 0.02° and a counting time of 2 s per step. The thermal properties of DEX, PLGA powders, implants, and the physical mixture of PLGA and DEX were determined using a TA Instruments[®] Q1000 DSC/TGA/IR analyser (TA Instruments, Newcastle, USA). Samples were heated from 0°C to 400°C at a rate of $5^\circ\text{C}/\text{min}$ under nitrogen atmosphere.

2.2.7 Drug content

To measure the drug loading of the DEX implants, 3 mg of each implant were dissolved in 5 mL of acetonitrile (n=3). Subsequently, DEX concentrations were measured using a Cary 60 UV-Vis spectrophotometer (Agilent, Santa Clara, California, USA) at a UV wavelength of 241 nm.

2.2.8 *In vitro* drug release

DEX and BEV release patterns were recorded by placing loaded implants (3 replicates of each processing condition) in glass vials with 3 mL of phosphate-buffered saline (PBS) at physiological pH (7.4). Vials were kept in an orbital shaker at 37°C under constant orbital movement (100 rpm). At predetermined times, 0.5 mL aliquots were withdrawn from the medium and replaced with the same volume of fresh medium. DEX concentrations were measured using the method described above (2.2.6. Drug content) and the pH of the samples was measured with a Hanna® HI5522 pH meter (Hanna Instruments, Eibar, Spain) at each time point.

Samples of BEV solutions were filtered through 0.22 µm PVDF filters (Millipore®, Burlington, Massachusetts, USA) and the concentration of the drug was determined with high-performance liquid chromatography (ACQUITY UPLC H-Class Plus, Waters, Milford, Massachusetts, USA) with an FTN injector and PDA detector. The stationary phase was a BioResolve RP mAb Polyphenyl, 450Å column, 2.7 µm, 2.1×50 mm (Waters, Massachusetts, USA), and the mobile phase was a gradient from 15% water 0.1% trifluoroacetic acid (TFA) and 85% acetonitrile 0.1% TFA to 85% water 0.1% TFA and 15% acetonitrile 0.1% TFA. The flow rate was set to 0.8 mL/min, with an injection volume of 1 µL, a column temperature of 80°C, and a UV wavelength of 280 nm. The elution time of BEV was approximately 2.4 min. Data were processed using Empower 3 software (Waters, Milford, Massachusetts, USA).

2.2.9 Biocompatibility assessment of the implants

2.2.9.1 Hen's egg test on the chorioallantoic membrane (HET-CAM)

The HET-CAM test was used to assess whether the implants would cause ocular irritation, following the method previously described by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) [40]. Fertile Broiler chicken eggs were placed in an incubator YZ-56S for 9 days at 38 ± 0.5°C and 65% relative humidity. On day 9, the

eggshell was carefully removed from the widest end of the egg to expose the CAM. Afterwards, 300 μ L of drug release medium (n=3) from day 135 and 5 of the DEX and BEV implants, respectively, were applied to the CAM. Three possible reactions, if applicable, were monitored for 5 min: haemorrhage, vascular lysis, or coagulation of the vessels. 0.9% NaCl and 0.1 N NaOH solutions were used as negative and positive controls, respectively.

2.2.9.2 Cytocompatibility studies with the ARPE-19 cell line

The ARPE-19 cell line was used due to its morphological and functional characteristics similar to adult retinal pigment epithelium (RPE) cells. ARPE-19 cells (ATCC[®], American Type Culture Collection, Manassas, VA, USA) were cultured in F-12/DMEM medium supplemented with 10% inactivated Foetal Bovine Serum (FBS), 2 mM L-alanyl-L-glutamine and 1% of an antibiotic/antimycotic mixture (10,000 units/ml penicillin G, 10 mg/ml streptomycin and 10 mg/ml neomycin). Cell culture was maintained at 37°C, under a 5% CO₂ atmosphere with 95% relative humidity, in 25 and 75 cm² T-Flasks. For maintenance, sub-confluent cultures (80%) were split using 0.25% trypsin/EDTA.

To determine the viability of ARPE-19 cells in the formulations, an assay based on the metabolization of a tetrazolium salt (XTT) was used. The tetrazolium salt is metabolised by viable cells leading to a water-soluble formazan salt that is quantifiable spectrophotometrically at 450 nm [41]. XTT sodium salt (1 mg/mL) in phenol-red free F12/DMEM and phenazine methosulfate (PMS) in PBS (3 mg/mL) solutions were prepared and stored at -80°C. Immediately before performing the assay, XTT and PMS solutions were mixed at a ratio of 1:400 to obtain the activated XTT reagent.

ARPE-19 cells (10,000 cells/well) were seeded in 96-well plates and maintained at 37°C with 5% CO₂. The BEV and DEX implants were sterilised under UV light and incubated in F12/DMEM low serum cell culture medium (37°C; 100 rpm). Samples from BEV implants were taken on days 1 and 7 while samples from DEX implants were taken on days 1, 7, 14 and 21. At one-week intervals, ARPE-19 cells were co-incubated with 100 μ L extracts obtained from the implants. After incubation, 50 μ L of activated XTT reagent was added to each well and the plate was incubated at 37°C protected from light for 4 hours. The absorbance at 450 nm was determined and the number of viable cells was calculated according to a calibration curve in the range of 15,000-234 cells per well. The results were expressed in terms of cell viability

percentage with respect to the negative control, which corresponds to ARPE-19 cells cultured in regular growth medium without any treatment. The experiments were performed in triplicate.

3 RESULTS AND DISCUSSION

3.1 Manufacturing of dexamethasone-loaded implants

In this work, a novel approach for manufacturing intravitreal implants using supercritical foaming was investigated. Customised implants with specific dimensions (10 mm x 2 mm) were prepared by designing and 3D printing moulds with precise geometries using an SLA 3D printer, aiming for future clinical applications. The high-resolution capability of the 3D printing technique enabled the production of moulds with accurate geometries [31]. The selected 3D printing resins were acrylic polymer-based [42], and preliminary findings indicated their suitable permeability to supercritical CO₂, making them appropriate for use in the supercritical foaming process to obtain porous structures. The moulds were composed of two parts, which when assembled, created a funnel and a tube. The function of the funnel was to facilitate the dosing of the powder, ensuring homogeneous batches with precise drug doses, which is particularly relevant for expensive drugs such as monoclonal antibodies and other biopharmaceuticals. The tube securely held the powder in place, shaping the implant during the supercritical CO₂ processing.

The two types of PLGA utilised in this study were chosen based on their molecular weight, as it is a crucial factor in determining degradation kinetics. Low molecular weight PLGAs with low inherent viscosity were selected to achieve a release duration of less than 3 months [43, 44]. On the other hand, higher molecular weight polymers possess longer polymeric chains and consequently higher intrinsic viscosity. This results in a slower degradation rate, which is not desirable for the objectives of this study since complete polymer degradation was intended to coincide with the completion of drug release. Furthermore, the 50:50 lactic/glycolic acid ratio and the presence of free carboxylic acid groups enhance the hydrophilicity of the polymer [45].

Supercritical CO₂ foaming parameters were set at moderate pressures (100 bar), mild temperatures (35°C), and short soaking times (1 h). These processing conditions were chosen because they are suitable for thermolabile compounds, such as monoclonal antibodies, as they aim to achieve adequate polymer foaming while maintaining the drug's activity. When using PLGA blends with a RES:PUR ratio exceeding 50:50, uncontrolled foam expansion was

observed, while below this threshold, the polymer blend exhibited low expansion rates (data not shown).

Therefore, the optimal blend for further foaming testing was selected as a 50:50 ratio. In terms of mould optimization, the best results were achieved with uncovered 15 mm moulds. This approach allowed the production of PLGA implants with high porosity, a smooth and non-porous surface, and excellent pore interconnectivity.

3.1.1 Dexamethasone crystalline state in the implants

XRPD and DSC analyses were conducted to examine the physical state of DEX in the implants. Previous literature has described two polymorphic crystalline forms of DEX [46], known as Form A and B, which exhibit similar physical and dissolution properties. The XRPD pattern of pure DEX exhibited prominent peaks at $2\theta = 8.1, 12.6, 13.7, 14.6, 15.15, 16.25,$ and 17.85 (Figure 2), corresponding to the polymorphic crystalline Form A of DEX. The PLGA material displayed a characteristic broad peak at $2\theta = 10-25^\circ$. In the implants containing DEX and PLGA, the distinct peaks of pure DEX powder were observed, indicating the presence of DEX in the crystalline Form A. Conversely, these distinct dexamethasone peaks were not detected in the blank implant sample.

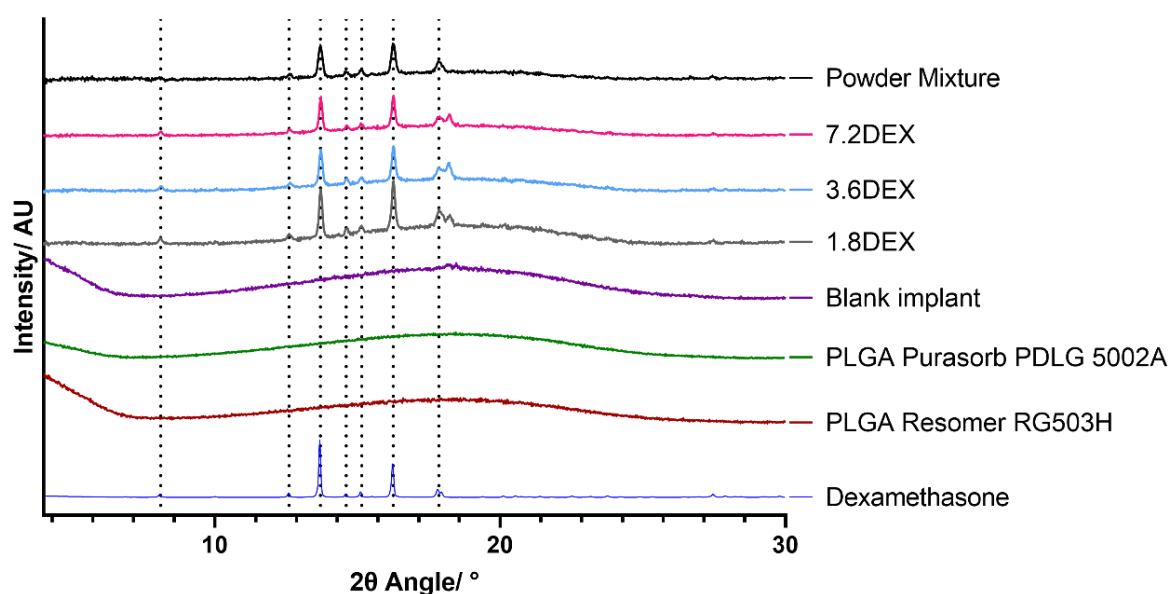


Figure 2. X-ray powder diffractograms of the drug DEX, PLGA powders, implants, and the physical mixture of PLGA and DEX.

DSC results revealed that DEX exhibited a melting endotherm with an onset temperature of 250 °C (Figure 3). This value was slightly higher than the previously reported onset temperature for the polymorphic crystalline Form A, which was 242 °C [46]. The melting endotherm of DEX is complex, involving a melting/recrystallization process between Form A and Form B, resulting in a broader endothermic peak [46]. RES PLGA exhibited two distinct thermal events: a glass transition temperature (T_g) of 49.5 °C and a degradation endotherm starting at 300 °C. On the other hand, PUR PLGA showed a barely observed T_g event at around 40 °C, and the thermal degradation began at higher temperatures compared to RES PLGA. This can be attributed to the lower molecular weight of PUR PLGA (17000 Da) compared to RES PLGA (24000-38000 Da). The implants, prepared using a blend of both PLGAs, displayed a thermal event around 50 °C, corresponding to the T_g of the polymer.

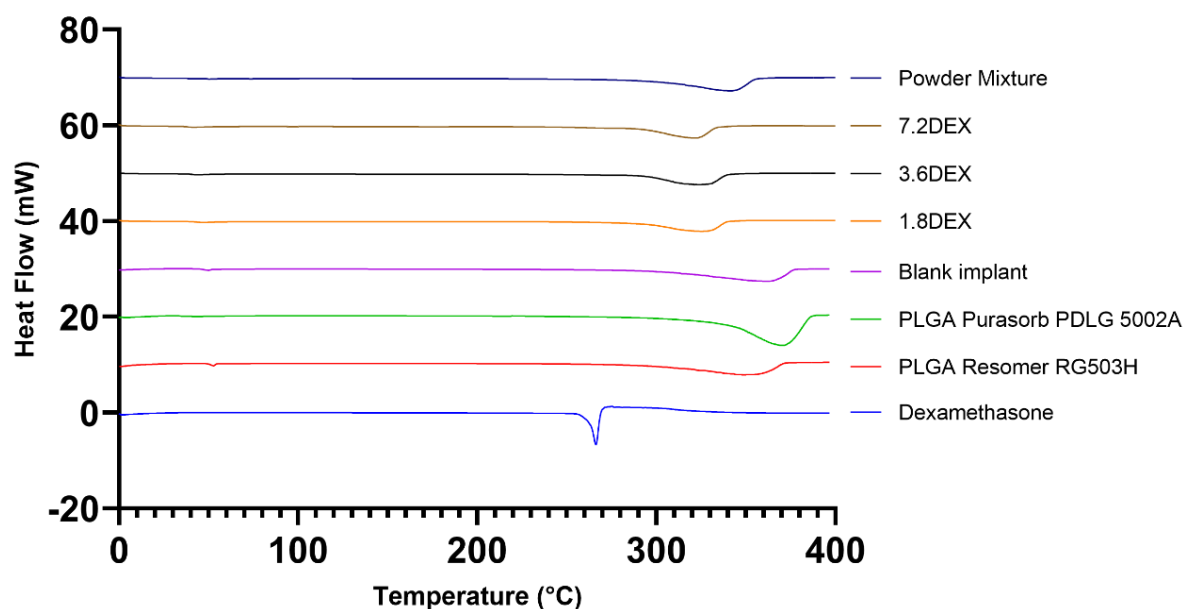


Figure 3. DSC curves of DEX, PLGA powders, implants, and the physical mixture of PLGA and DEX (Exo Up).

The melting peak of DEX was not detected in the DEX implants or the physical mixture of DEX and PLGA. However, XRPD analysis indicated the presence of some degree of crystallinity in the DEX implants, similar to that observed in the physical mixture of PLGA and DEX powders. The absence of a drug melting endotherm in the DSC analysis could be attributed to the dissolution of the drug in the melted PLGA, even if drug crystals are present in the implants. One possible explanation for the presence of DEX in a crystalline state within

the implant could be the recrystallization of DEX occurring during storage prior to the assessment of crystallinity [47].

3.1.2 Morphological characterisation of dexamethasone-loaded implants

The implants exhibited satisfactory handling properties and a smooth surface. They also demonstrated good uniformity in their physical dimensions, as indicated in Table 2, and no noticeable macroscopic morphological differences were observed between the formulations, as shown in Figure 4. The inclusion of DEX led to a slight increase in the weight and length of the implants, which was determined to be statistically significant ($\alpha < 0.05$); however, this increase was considered negligible.

Table 2. Physical properties of dexamethasone implants (n=3).

Implant	Weight $\pm$ SD (mg)	Length $\pm$ SD (mm)	Diameter $\pm$ SD (mm)
Blank	9.97 $\pm$ 0.27	8.51 $\pm$ 0.07	1.73 $\pm$ 0.21
1.8DEX	10.35 $\pm$ 0.28	9.54 $\pm$ 0.19	1.88 $\pm$ 0.01
3.6DEX	10.35 $\pm$ 0.18	8.47 $\pm$ 0.09	2.02 $\pm$ 0.04
7.2DEX	11.14 $\pm$ 0.46	9.37 $\pm$ 0.35	1.85 $\pm$ 0.00

SEM images of the surface and core of the DEX implants are depicted in Figure 4. The implants exhibited a porous core enveloped by a non-porous outer skin layer measuring 100-140 μm in thickness, which is a typical characteristic of scCO₂ foamed materials [48]. The core displayed pore sizes ranging from 20 to 150 μm , and interconnected pores were observed within larger pore cavities. Notably, no drug crystals were detected in the SEM images.

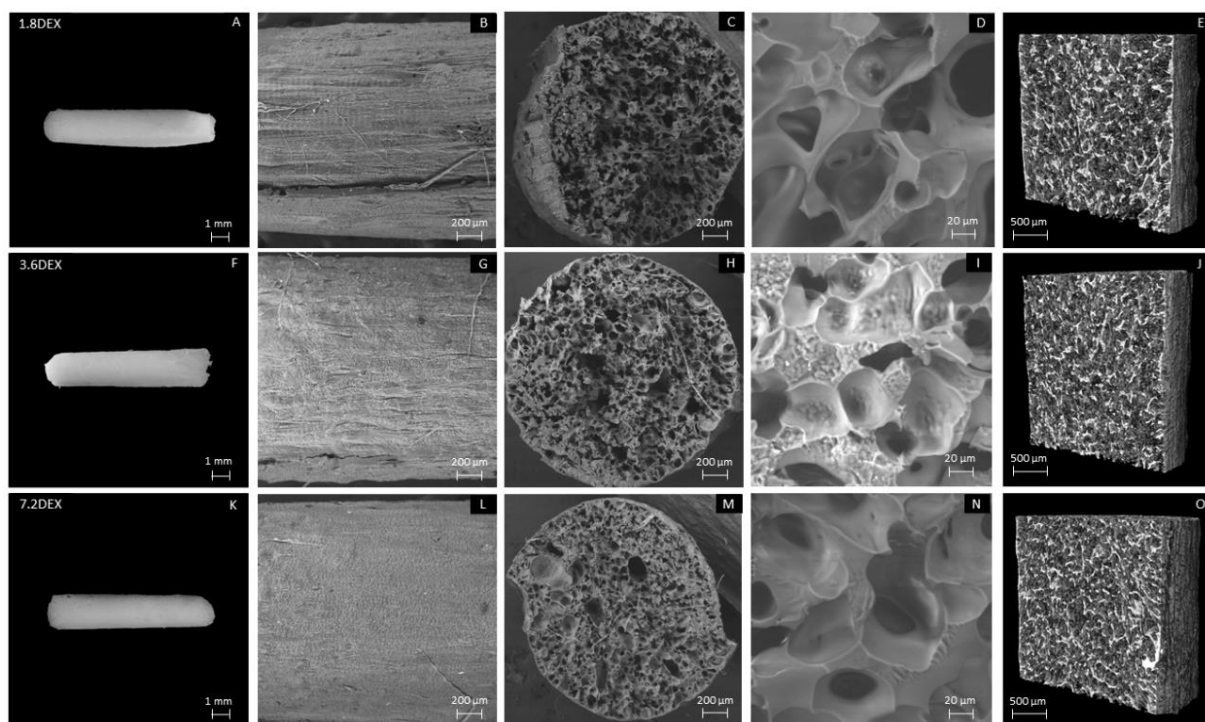


Figure 4. Stereomicroscopy pictures of the implants (A,F,K). SEM images of the surface and cross-sections of the implants (B,C,D,G,H,I,L,M,N). 3D reconstructions with Micro-CT computed tomography data of the 1.8DEX, 3.6DEX and 7.2DEX implants (E,J,O).

The morphological properties of the DEX-loaded implants were subjected to additional analysis using Micro-CT and 3D reconstruction techniques, as illustrated in Figure 4. Porosity data obtained from the implants are summarized in Table 3. It was observed that all the implants exhibited a highly porous structure, with overall porosity values exceeding 68%. However, significant differences were observed between different formulations.

Table 3. Micro-CT porosity data analysis of DEX implants (n=3).

Implant	Pore diameter (μm)	Pore throat (μm)	Open porosity (%)	Overall porosity (%)
1.8DEX	77.33 ± 11.38	31.13 ± 4.01	71.73 ± 2.73	71.74 ± 2.73
3.6DEX	53.99 ± 2.97	25.81 ± 1.01	68.60 ± 2.88	68.61 ± 2.89
7.2DEX	54.44 ± 5.09	24.65 ± 1.16	69.08 ± 1.78	69.09 ± 1.78

The highest pore diameters ($77.33 \pm 11.38 \mu\text{m}$) were achieved in the implants processed at a slower depressurisation rate of 1.8 g/min ($\alpha < 0.05$). Higher depressurisation rates of 3.6 and

7.2 g/min resulted in smaller pore diameters ($53.99 \pm 2.97 \mu\text{m}$ and $54.44 \pm 5.09 \mu\text{m}$, respectively) as shown in Table 3 and Figure 5A. An increasing trend was observed for the pore throat sizes in implants processed with longer depressurisation rates, as shown in Figure 5B. The homogeneity of the implants was also affected, with narrower pore size distributions observed in implants processed under higher depressurisation rates [30]. These aspects are illustrated in Figure 5A and B. The findings of this study align with the principles of classical nucleation theory [49]. Specifically, for a fixed temperature and soaking time, rapid depressurisation promotes nucleation overgrowth (pore formation) and results in a greater number of pores with smaller diameters. Conversely, a decrease in the depressurisation rate leads to larger pores due to increased pore growth and coalescence. This phenomenon also influences the degree of interconnectivity within the structures.

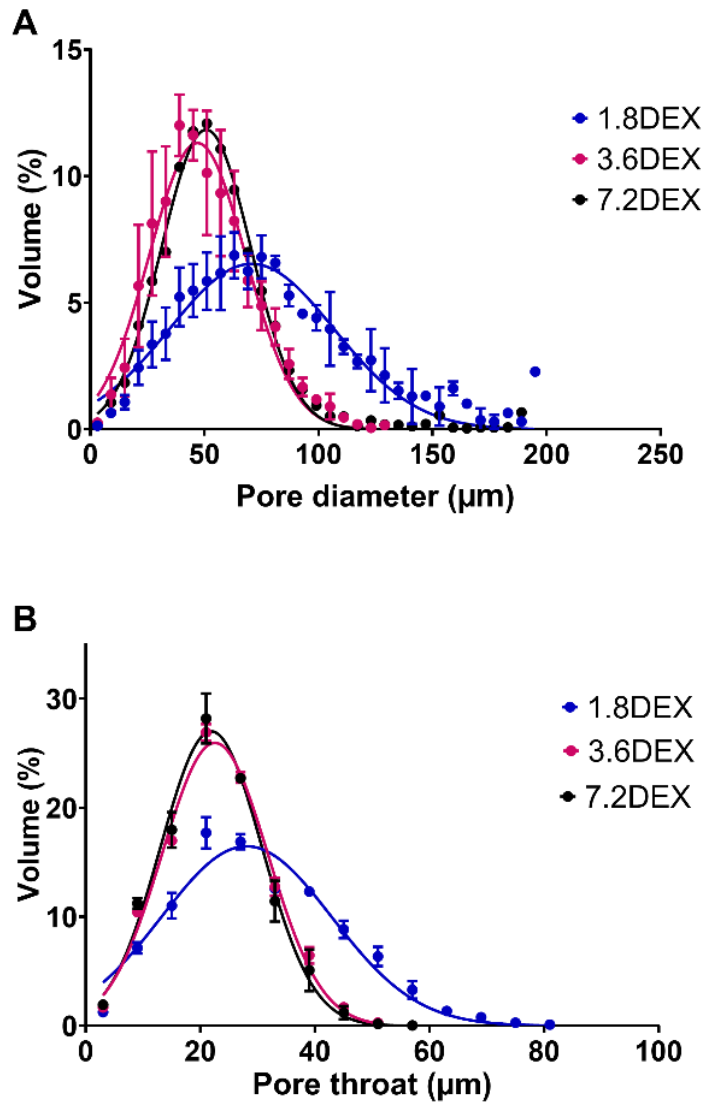


Figure 5. (A) Pore size and (B) pore throat distributions of the DEX implants calculated through Micro-CT.

3.1.3 Texture analysis

Figure 6 shows the key parameters related to the texture analysis of the implants. An evident linear relationship exists between rupture strength, elasticity, and failure work, and the depressurisation rate employed during the process. These variations in the analysed parameters can be attributed to the porous structure of the implants achieved under different conditions. Notably, implants produced at a depressurization rate of 1.8 g/min, which exhibited larger pore diameters, demonstrated reduced mechanical strength.

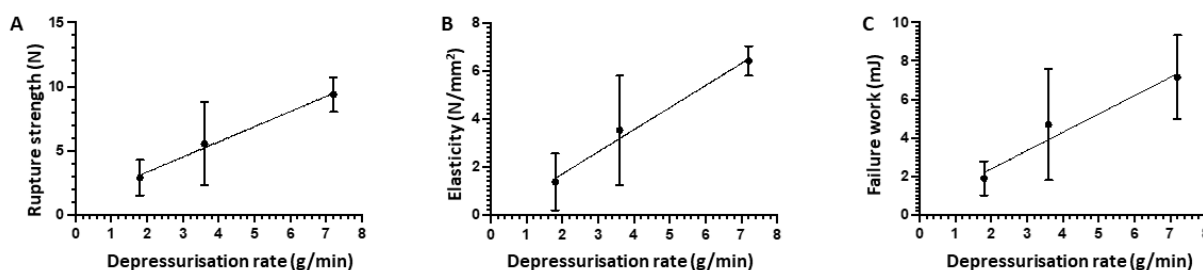


Figure 6. Texture analysis parameters of the implants, including rupture strength, elasticity, and failure work.

The rupture strength values were similar to, or even higher than, those reported in a previous study [50] where intracochlear PLGA implants were produced using hot melt extrusion. The intracochlear implants were compared to Ozurdex[®] implants in terms of breaking force, resulting in values of 5.33 N for intracochlear implants prepared with 90% PLGA and 10% dexamethasone, and 2.70 N for Ozurdex[®] implants.

3.1.4 Dexamethasone distribution in the implants

Figure 7 displays the EDX mapping images of the surface and core of the DEX-loaded implants, as well as the released implants. The distribution of the drug within the implant was determined by the presence of the fluorine atom in the DEX molecule, which is absent in the PLGA molecule. Consequently, a higher concentration of DEX was observed on the surface of the implants, while the core exhibited a lower concentration.

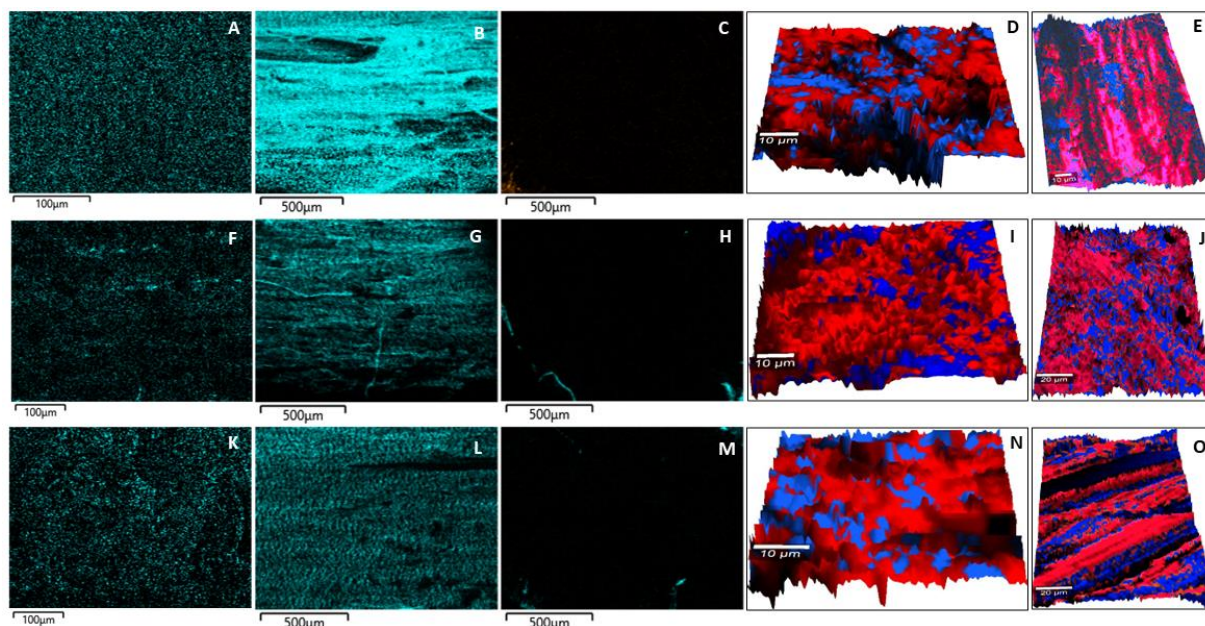


Figure 7. EDX mapping and Raman confocal microscopy images of the DEX-loaded implants. EDX images reveal the surface of 1.8DEX (A, B), 3.6DEX (F, G), and 7.2DEX (K, L) implants, as well as the core of 1.8DEX (C), 3.6DEX (H), and 7.2DEX (M) implants. The blue dots indicate the distribution of fluorine atoms. Raman confocal microscopy images depict the distribution of the DEX in the core of 1.8DEX (C, E), 3.6DEX, and 7.2DEX (N, O) implants. Red areas correspond to PLGA and blue areas correspond to DEX.

Table 4 summarises the element distribution for each implant and location. Furthermore, the presence of fluorine was still observed on the surface of the analysed sample even after the *in vitro* release study, indicating the presence of residual DEX in the implant.

Table 4. Elemental analysis data of the DEX implants core, surface and DEX released implant.

Implant	Distribution	Element (%)		
		Carbon	Oxygen	Fluor
1.8DEX	Core	50.9	47.8	1.2
	Surface	40.0	14.5	45.1
	Released implant surface	32.46	3.13	62.76
3.6DEX	Core	51.4	47.9	0.8
	Surface	35.8	12.8	51.4
	Released implant surface	33.69	11.59	45.73

	Core	50.9	48.3	0.8
7.2DEX	Surface	44.8	19.9	35.0
	Released implant surface	32.2	2.47	64.62

However, Raman confocal microscopy images obtained from longitudinal sections of the implants at higher magnification (Figure 7) revealed the presence of DEX within the core of the implants processed under all three depressurisation rate conditions. In all cases, aggregates smaller than 10 μm were observed dispersed in the PLGA matrix. These results suggest that DEX is present in higher concentration on the surface than in the core of the DEX-loaded implant. During the depressurisation process, the DEX that is solubilised in the supercritical CO_2 might tend to distribute towards the surface of the implant while the foam is created.

3.1.5 Dexamethasone content and release from the implants

The drug loading of the DEX implants was assessed, yielding values of $98.41 \pm 5.05\%$, $97.76 \pm 4.34\%$, and $98.13 \pm 4.17\%$ for 1.8DEX, 3.6DEX, and 7.2DEX, respectively. These findings serve as confirmation of the remarkable drug loading capacity achieved through supercritical foaming.

Figure 8 illustrates the release profiles of DEX from the implants, demonstrating a controlled release of the drug for a minimum of 60, 75, and 120 days for 1.8DEX, 3.6DEX, and 7.2DEX implants, respectively. To validate the role of the PLGA matrix in regulating drug release, DEX base powder (700 μg) was dissolved in the same volume of medium, and samples were collected and analysed at predetermined time points. Consequently, DEX completely dissolved in the medium within 8 days, highlighting the effective control exerted by the polymeric matrix on drug release. Following this controlled release stage, the release of the drug reached a plateau or even decreased for 1.8DEX and 3.6DEX implants. In the case of 7.2DEX implants, the drug release was extended until the recorded drug concentration levels in the release medium matched those obtained with the DEX solution. On day 135, a one-way ANOVA statistical test was conducted, indicating that the amount of drug released by 7.2DEX was significantly higher ($\alpha < 0.05$) compared to the amount released by 1.8DEX and 3.6DEX, with no statistically significant differences observed between the latter two (α n.s.).

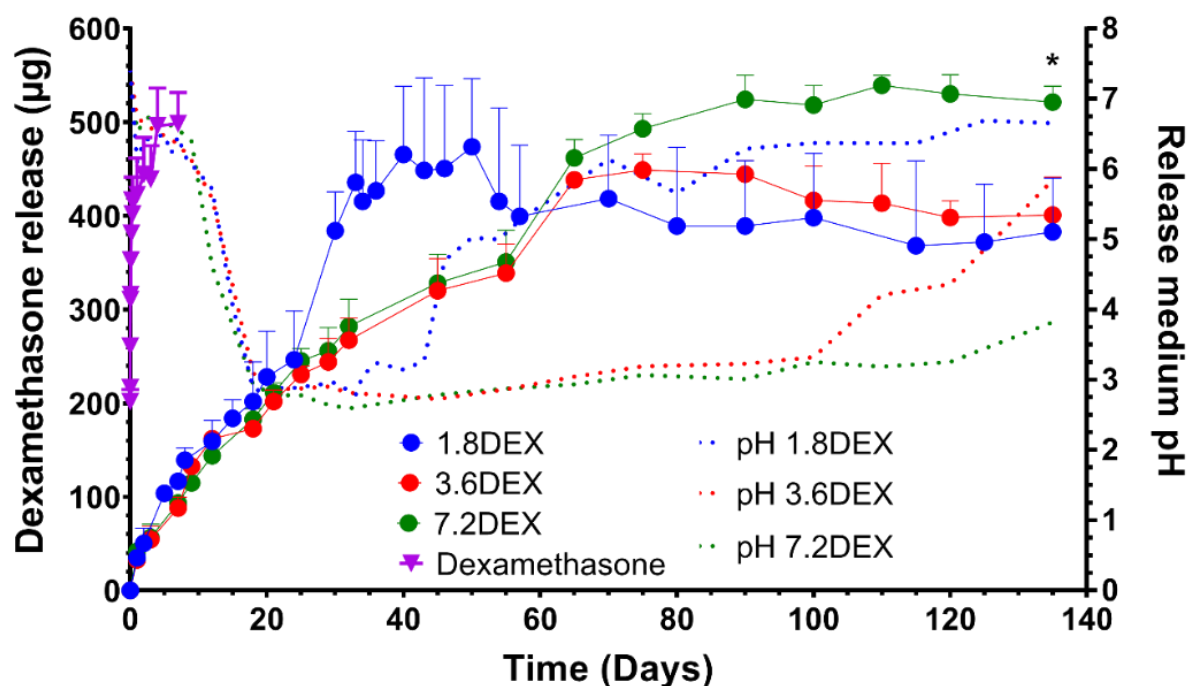


Figure 8. *In vitro* release profiles of DEX base powder, 1.8DEX, 3.6DEX and 7.2DEX implants and pH changes during DEX release in phosphate buffer pH 7.4 (n=3).

According to Figure 8, a noticeable decline in the pH levels of the release medium was observed, reaching below pH 3 after day 10. This acidic environment is a result of the hydrolysis of the PLGA, causing the degradation of polymer chains and the production of lactic and glycolic acids. These acids are highly acidic and water-soluble, classified as short-chain fatty acids [51]. Additionally, despite the intended pH maintenance at 7.4 through the use of PBS, the pH drop can be attributed to the infrequent medium fluid volume renewals and the extended intervals between sampling [52].

The pH exerts a notable influence on the stability of DEX in solution, with the drug exhibiting greater stability under acidic pH conditions and decreased stability as the pH increases. In a study, the stability of DEX in pediatric formulations was evaluated and accelerated stability tests were conducted at varying pH levels [53]. At pH 7.85 and 60°C, over 90% of the drug degraded within 42 hours. However, when the pH was reduced to 2.13, no degradation of DEX occurred within the same study duration. Moreover, DEX degradation has been observed during *in vitro* release in PBS with a pH of 7.4, as demonstrated in certain studies conducted on intravitreal implants [54, 55]. These studies employed liquid chromatography-mass spectrometry (LC-MS/MS) analysis to predict the amount of DEX that underwent

degradation during *in vitro* release. However, due to the requirement of LC-MS/MS analysis, this particular model could not be applied to the present study.

Implants produced through supercritical foaming exhibit a non-porous external layer and a porous core. In the initial 30 days of the study, the release of DEX from the implants was controlled by the PLGA matrix forming the non-porous external layer. However, subsequent to this period, the pH profile emerged as the primary factor influencing DEX release. In the case of 1.8DEX implants, the release medium's pH increased more rapidly compared to 3.6DEX and 7.2DEX implants, aligning with the pattern of DEX release from the implant. Subsequently, when the pH of the medium exceeded 5-6, a decline in DEX concentrations was observed due to increased drug degradation. In the case of 3.6DEX implants, the pH elevation took longer, resulting in continued concentration increase up to 75 days, followed by a decline at 90 days, coinciding with the rise in medium pH. Notably, no significant pH increase was observed for the 7.2DEX implants, which released a greater amount of DEX over an extended duration without a decrease in drug concentration. These findings indicate that DEX remains stable under low pH conditions but undergoes degradation at higher pH values.

The transition in release patterns for 3.6DEX and 7.2DEX implants occurred at a later stage compared to 1.8DEX implants. The variations in release behavior and drug degradation rates can be attributed to differences in microstructure, hardness, and the distribution of DEX within the implants. Specifically, among the implants, 1.8DEX exhibited the lowest mechanical strength and the largest pore diameters and throats, facilitating quicker access to the release medium and promoting faster degradation of the polymeric matrix. Additionally, 1.8DEX implants displayed the highest surface concentration of the drug. In contrast, 7.2DEX and 3.6DEX implants showcased smaller pore throats and diameters, along with higher mechanical strength, and a lower DEX concentration on their surface. This combination contributed to a more favorable drug release pattern.

The results obtained in this study can be compared to previous research that investigated the release of DEX from PLGA implants manufactured using different technologies [56]. For instance, a study examined the *in vitro* release of DEX from intact or fractionated commercial Ozurdex[®] implants in 30 mL of saline solution [57]. The drug was completely released from the implants within 28 days, and no differences were observed between the fractionated and intact implants. Another study investigated the *in vitro* release of DEX from implants produced

through hot-melt extrusion, utilizing 50:50 acid-terminated or ester-terminated PLGAs in 5 mL of PBS [58]. The complete release of the drug was observed after 4-5 weeks (28-35 days). Additionally, the *in vitro* performance of Ozurdex[®] and DEX cochlear implants, fabricated using hot-melt extrusion with PLGA Expansorb[®] 50-2A (10400 Da), was studied in 1 mL of phosphate buffer at pH 7.4 [50]. Ozurdex[®] exhibited complete drug release within 28 days, and the cochlear implants were able to release 70% of the drug within 10-20 days, reaching 90% after 45 days of testing. In comparison to these examples, the implants developed in this study demonstrated a successful release rate, enabling controlled release of the drug for up to 4 months.

3.2 Manufacturing of bevacizumab-loaded implants

Due to the promising *in vitro* performance of the DEX-loaded implants, a subsequent investigation was undertaken to incorporate thermolabile and complex compounds of interest. In this particular case, the model drug chosen was BEV. To fabricate BEV-loaded implants with an appropriate size-to-drug dose ratio, the dimensions of the moulds were optimised. BEV requires high doses to achieve efficacy (1.25 mg in 0.05 mL of solution). The commercial formulation, Avastin[®], contains significant amounts of excipients (trehalose dihydrate, sodium phosphate, and polysorbate 20) to stabilize the monoclonal antibody. Consequently, each implant necessitates large volumes of freeze-dried drug powder. The Avastin[®] solution underwent purification to eliminate the excipients, and subsequently, it was freeze-dried with 8% (w/w) HP β CD, serving as a cryoprotectant and antiaggregant (refer to Methods, Section 2.2), resulting in the obtainment of BEV in powder form.

3.2.1 Bevacizumab stability after freeze-drying

To examine and compare the structural characteristics of monoclonal antibodies, a second derivative ultraviolet absorption spectroscopy analysis was performed. Second derivative spectra enable the differentiation of spectral bands associated with aromatic amino acids, allowing for the detection of changes in the structural state of the monoclonal antibody. The microenvironment of tyrosine residues within the BEV sequence was investigated to compare the BEV structure across all the samples. To accomplish this, the a/b ratio was utilised, where "a" represents the distance between the maximum ($\sim\lambda 287$) and minimum ($\sim\lambda 283$) absorbance of the negative peak associated with tyrosine, and "b" represents the distance from a second maximum ($\sim\lambda 295$) to a minimum ($\sim\lambda 290.5$) related to tryptophan [59]. Figure 9 illustrates the

a/b ratios observed in the tested samples. No significant differences were observed in the a/b ratios among all the samples (α n.s.), suggesting comparable protein structures between the resuspension of freeze-dried BEV, the processing with supercritical CO₂, and the commercial solution (Avastin®).

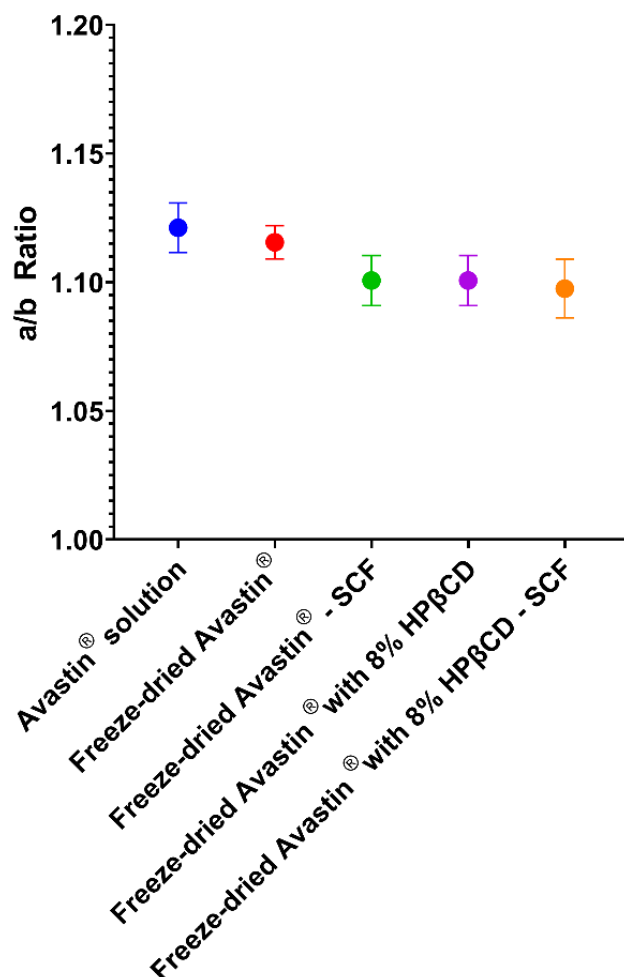


Figure 9. a/b ratio for Avastin® solution, freeze-dried Avastin® and freeze-dried Avastin® with 8% (w/w) HPβCD.

3.2.2 Morphological characterisation of bevacizumab-loaded implants

The BEV-loaded implants displayed slightly rough surfaces while maintaining satisfactory handling properties. The results presented in Table 5 demonstrate consistent physical dimensions among the implants, indicating good uniformity. However, the inclusion of lyophilised BEV led to a significant disparity in implant weight ($\alpha < 0.05$), while no notable variations were observed in terms of length or diameter.

Table 5. Physical properties of BEV implants (n=3).

Implant	Weight $\pm$ SD (mg)	Length $\pm$ SD (mm)	Diameter $\pm$ SD (mm)
Blank	9.97 $\pm$ 0.27	8.51 $\pm$ 0.07	1.73 $\pm$ 0.21
3.6BEV	11.90 $\pm$ 0.35	9.73 $\pm$ 1.07	1.91 $\pm$ 0.10

SEM images of the BEV-loaded implant are shown in Figure 10. The surface of the implant exhibited an irregular pattern with noticeable macropores, while the inner structure appeared non-porous. In contrast to DEX-loaded implants, the level of porosity within the core of the BEV-loaded implants was lower. Additionally, patches of drug or excipient crystals were observed within the polymeric matrix of the implant core, preventing the formation of a porous structure. This observation could be attributed to the larger volume of drug and excipient powder required for BEV, which impeded the effective foaming of the polymer.

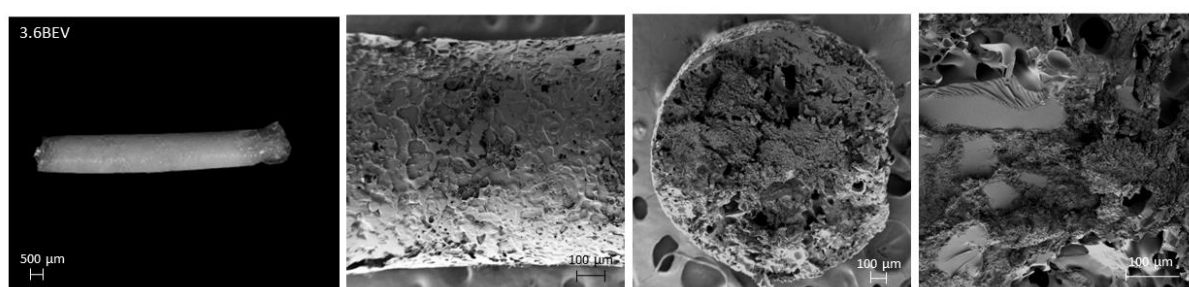


Figure 10. Representative stereomicroscopy and SEM images of the surface and cross-sections of BEV implants produced by supercritical foaming.

3.2.3 Bevacizumab release from the implants

The release profile of the 3.6BEV implants is shown in Figure 11, showcasing the results. These implants were loaded with 1.25 mg of BEV powder, which corresponds to the clinically used dosage, and the release kinetics were assessed over a period of 5 days. Notably, the release of BEV was exceptionally rapid, with a release rate of $91.41 \pm 10.64\%$ observed on the first day, and complete release was achieved by day 5. In contrast to the porous inner structure observed in DEX-loaded implants (Figure 4), the drug-excipient patches present in the BEV-loaded implants functioned similarly to porogen agents, facilitating the accelerated release of the drug. When in contact with the pH 7.4 phosphate buffer medium, the BEV-HP β CD patches rapidly

dissolved, resulting in almost complete drug release on the first day. The pH of the release medium was measured and found to decrease from its initial value of pH 7.4 to pH 6.75 by day 5.

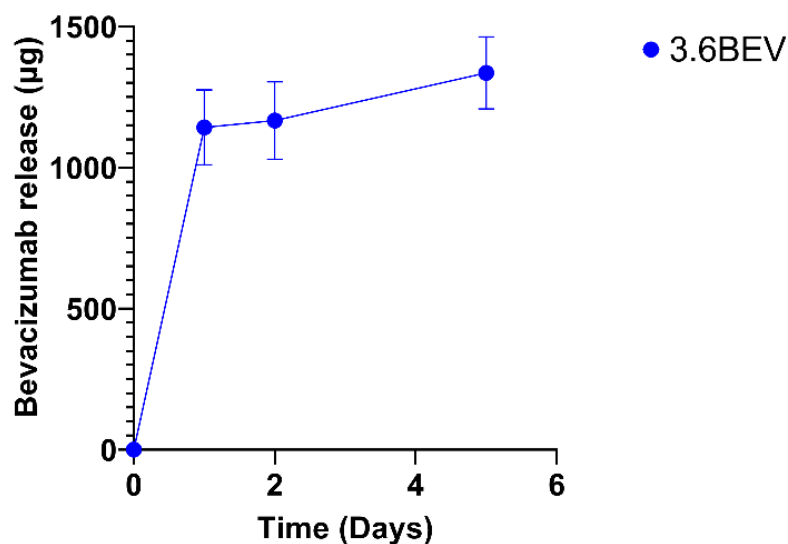


Figure 11. *In vitro* release profile of BEV from 3.6BEV implants in PBS pH 7.4 (n=3).

Hydrophilic compounds, such as proteins or monoclonal antibodies, do not dissolve in CO₂, which is a non-polar molecule [60]. In contrast, DEX, which is non-polar in nature (logP = 1.83), quickly dissolves in CO₂ during the supercritical foaming process. The CO₂ then dissolves in the polymer, resulting in polymer swelling, which facilitates the embedding of the drug into the polymer matrix and ultimately the formation of the implant.

Supercritical foaming has previously been used successfully to incorporate hydrophilic drugs into polymeric matrices [61]. The hydrophilic/hydrophobic properties of the polymer matrix have a significant impact on hydrophilic drug loading. Polymer blends with a higher proportion of hydrophilic domains are more suitable for embedding hydrophilic compounds. RES and PUR PLGA polymers were initially selected for this study due to their acidic end groups, which make them more hydrophilic than PLGA polymers with ester end groups. However, this selection resulted in an extremely rapid release of the drug. To achieve the desired release profile, it would be necessary to select another PLGA combination, alter the polymer-BEV ratio, or eliminate additional excipients.



This work showcases the successful integration of biological drugs, such as BEV, into a PLGA matrix using supercritical foaming. It emphasizes the importance of meticulous material

selection and precise processing conditions when developing drug-loaded implants, as these variables greatly influence the release profile and effectiveness of the implant. Innovative techniques like three-dimensional (3D) printing have demonstrated promising capabilities in fabricating drug-loaded implants with tailored sizes and drug dosages [62, 63]. However, many of these methods require elevated temperatures or organic solvents [13, 15, 64], which present the aforementioned drawbacks.

Supercritical foaming allowed the manufacturing of the implants without harsh conditions, which is ideal for processing thermolabile compounds. However, further optimisation of the implants dimensions and release profiles is required in view of clinical application. The creation of 3D printed moulds allowed the manufacturing of implants with the desired size. Implant dimensional modifications can be achieved simply by adjusting the size of the 3D printed moulds. The next steps would involve the use of PLGA polymers with different lactide/glycolide ratios to better fit BEV properties and better characterise BEV stability in the processed implant. This approach can also be applied to other monoclonal antibodies/proteins, as they may interact differently with the PLGA matrix, resulting in different release profiles. Through careful modulation of polymer composition and depressurisation rates, the implant can be customized to accommodate specific bioactive molecules.

Additionally, the capability of sterilising the implants directly after production, as demonstrated in a previous study [65], brings these implants closer to clinical implementation. In a prospective scenario, ophthalmologists could receive sterilised implants of the desired size and dosage, ready for administration. This approach offers a convenient and efficient solution for personalised treatment, with the potential to optimise the manufacturing process and facilitate widespread clinical adoption.

3.3 Biocompatibility assessment of the implants

3.3.1 Hen's egg test on the chorioallantoic membrane (HET-CAM)

Figure 12 shows the results of the HET-CAM test. The images show that there was no haemorrhage, lysis, or coagulation after 300 s of contact between the release medium of the implants and the chorioallantoic membrane (Figure 12E-F, G-H, I-J, K-L). The irritation score was null, indicating no difference between the negative control (Figure 12A-B) and the formulations, in contrast to the positive control (Figure 12C-D). The absence of significant changes in the membrane, as indicated by HET-CAM results, suggests that the implants have a

reduced likelihood of causing eye irritation or damage. This observation implies a greater level of safety for ocular use.

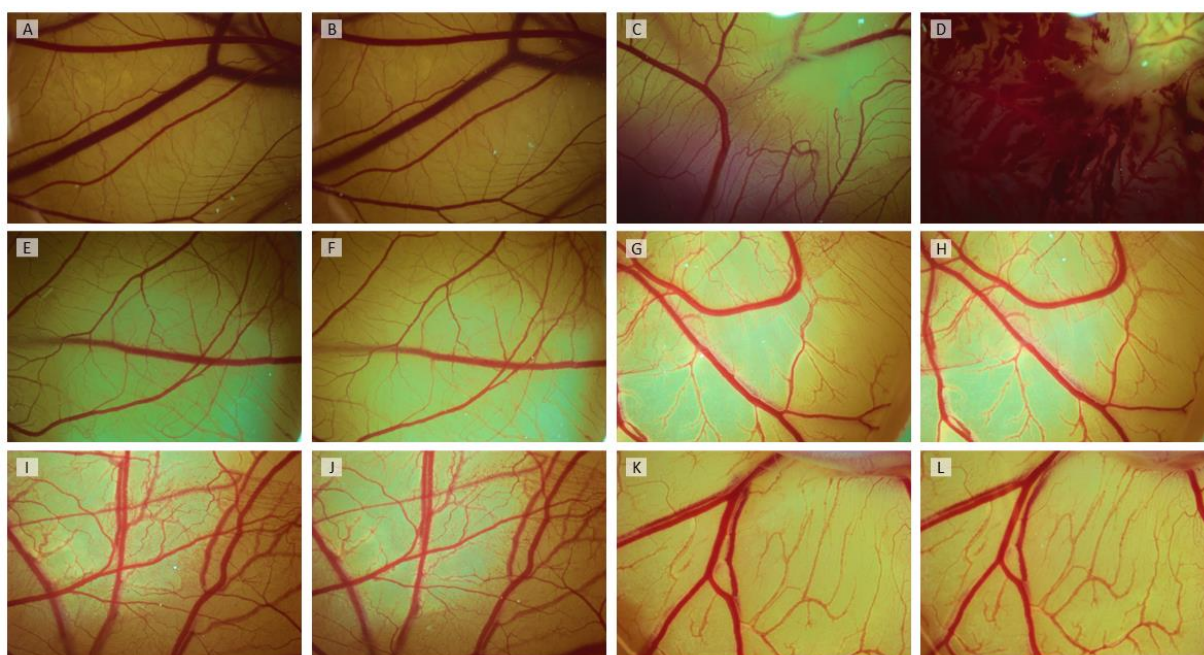


Figure 12. Images of the chorioallantoic membrane before (A, C, E, G, I, K) and after 300 s of contact with negative control (B), positive control (D), 1.8DEX (F), 3.6DEX (H), 7.2DEX (J), and 3.6BEV (L) release medium.

3.3.2 Cytocompatibility studies with the ARPE-19 cell line

To assess the cytocompatibility of DEX-loaded implants, ARPE-19 cells were co-incubated with the release medium from the implants and a DEX solution containing the highest dose levels (1.08 mg/mL). A blank PLGA implant served as a control, and as shown in Figure 13, the percentage changes in cell viability closely resembled those of untreated cells. PLGA is widely recognized as a biocompatible material and finds applications in various drug delivery systems and biomedical fields, including microspheres, tissue engineering scaffolds, and sutures [66, 67].

The exposure of RPE cells to DEX-loaded implants containing different drug levels, as well as the DEX solution with the highest dose, did not have a significant impact on cell viability when compared to untreated cells. These findings indicate that DEX-loaded implants, within the clinically relevant intravitreal dosage range of 0.2-1.08 mg/mL, do not exhibit toxicity towards ARPE cells.

Nevertheless, there is still a concern regarding the influence of pH on the stability of DEX over an extended period of incubation. Despite the progressive degradation of PLGA over time, resulting in a decrease in pH as documented in this study and previous research [52, 67], the pH of the cell culture medium remained consistently stable throughout the entire testing period (pH 7.2-7.4) owing to its effective buffering capacity. Multiple studies have demonstrated a decrease in cell viability for ARPE-19 cells when exposed to DEX for more than one day, despite an initial boost in cell proliferation within the initial day of incubation [68]. Interestingly, DEX-loaded implants did not display any signs of toxicity, even at doses exceeding those used in clinical practice, within the first 24 hours. However, it is worth noting that the observed lack of toxicity might be attributed to the degradation of the drug in the cellular medium at normal physiological pH, as previous research has indicated that DEX exhibits greater stability under acidic pH conditions [53]. Hence, these findings confirm the cytocompatibility of PLGA and suggest that clinical doses of DEX are seemingly well-tolerated by cells.

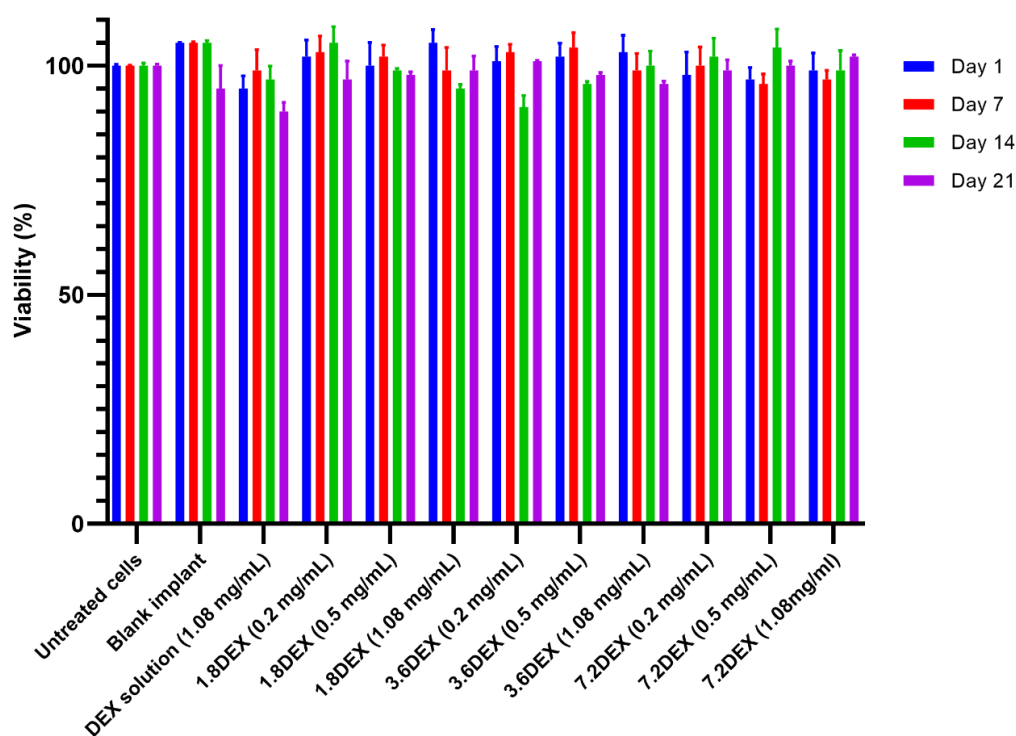


Figure 13. Percentage changes in cell viability following co-incubation with cell culture medium (untreated cells), blank implants release medium, DEX solution, and DEX-loaded implants release medium for days 1, 7, 14 and 21.

The cytocompatibility of BEV-loaded implants was also evaluated by comparing three different concentrations (0.047 mg/mL, 0.141 mg/mL, and 0.235 mg/mL) of BEV obtained from the release medium of the implants and from the commercial solution (Avastin®). To achieve equivalent concentrations of the implant release medium, the drug was diluted serially in a low serum culture medium. A blank PLGA implant served as a control, and as shown in **Figure 14**, the cell viability percentage was comparable to that of untreated cells. High percentages of live cells were observed after co-incubation with the release medium on days 1 and 7, coinciding with complete drug release. The changes in cell viability percentage exhibited significant differences among all the groups for the three BEV concentrations ($p < 0.05$). Both BEV from the commercial solution and BEV released from the implants on day 1 stimulated more pronounced cell growth in ARPE-19 cells. However, the viability percentage for BEV released from the implants on day 7 approached values similar to the control group, albeit with a concentration-dependent impact on cell growth.

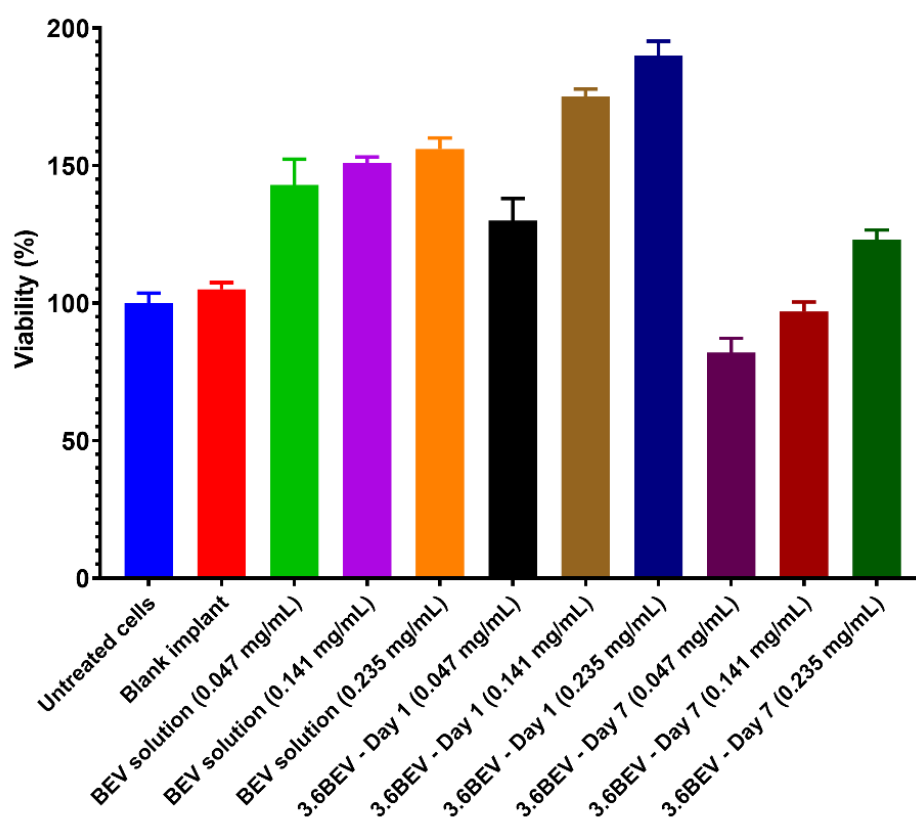


Figure 14. Percentage changes in cell viability following co-incubation with varying concentrations of BEV from the implant release medium and the BEV commercial solution.

The observed effect appears to diminish over time, which may be attributed to the degradation of BEV in the cell culture medium or its potential adsorption to components within the medium [69]. In other investigations, ARPE-19 cells exposed to more concentrated BEV solutions exhibited similar viability values (close to 100%) as the control group [70, 71]. These discrepancies could arise from variations in experimental conditions, cell culture protocols, or the specific assays employed to assess viability. While the precise mechanisms underlying the effects of BEV on ARPE-19 cells remain to be fully elucidated, it is important to note that no morphological changes were observed in these cells.

4 CONCLUSIONS

In this study, supercritical foaming technology was employed to prepare intravitreal PLGA implants loaded with dexamethasone. By manipulating the depressurisation rates during the foaming process, implants with desirable characteristics and customisable release patterns were successfully obtained. Notably, the dexamethasone implants exhibited improved release performance compared to previous studies [50, 56-58], demonstrating their ability to release the drug in a controlled manner for up to 4 months. In view of these positive outcomes, the feasibility of incorporating bioactive molecules into the implants was explored, using the monoclonal antibody bevacizumab as a model drug. Through the same supercritical foaming process, a proof-of-concept investigation was conducted with bevacizumab-loaded implants. Further investigations will focus on optimising the implant dimensions and release patterns, with the ultimate goal of future clinical applications for the treatment of chronic retinal diseases. This study underscores the potential of supercritical foaming technology in the development of biocompatible intravitreal implants with precise release patterns and properties for the controlled delivery of drugs.

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CHAPTER III

Content in this section is restricted due to an ongoing patent application.

In Chapter 3 of this thesis, the creation and evaluation of an innovative drug delivery system designed specifically to enhance the penetration capability of antifungal medications into nails affected by onychomycosis is addressed. Onychomycosis represents the most frequently diagnosed nail pathology worldwide, impacting up to 90% of toenails and approximately 50% of fingernails. This condition manifests through various nail issues, including changes in color, thickening, increased fragility, and in some cases, significant pain. The prevalence of this disease is particularly high among elderly people, as well as among those patients suffering from chronic diseases such as diabetes mellitus, psoriasis, or HIV, since their immunological status places them at an elevated risk of contracting onychomycosis. The therapeutic approach to fungal nail infections presents numerous challenges, such as the condition's tenacity, a high probability of recurrence, and certain limitations inherent to the therapies currently available. Although oral antifungal treatments are often preferred by doctors due to their effectiveness, these can trigger adverse systemic side effects, which has intensified the search for safer and more effective topical alternatives. However, the efficacy of topical treatments is compromised by the difficulty in penetrating the compact structure of the nail plate, which in turn contributes to the development of antifungal resistance.

The intricate structure of the nail plate, composed of dense layers of keratin and proteins, plays a crucial role in the limited drug permeability. Existing mechanical approaches, such as nail abrasion, are invasive techniques that do not guarantee an improvement in treatment effectiveness. Against this backdrop, the application of a new safe, economical, and less invasive technique to facilitate the penetration of medications into the deep layers of the nail is proposed. For this study, DexULac®, an antifungal lacquer, was selected as the model compound to be administered using the developed technique. Various advanced analytical methodologies were employed for this evaluation, including confocal microscopy, scanning electron microscopy, Raman spectroscopy, and X-ray diffraction, as well as ex vivo nail permeation studies to obtain a detailed understanding of the efficacy of the proposed delivery system. The results show that the application of the new technology allows for a substantial increase in the speed of penetration and distribution of the drug within the nail matrix. This technique enables the drug to access even the deepest layers of the nail in a short time, where access from medicated lacquers is more restricted. Therefore, the developed technique could be utilized in treatments related to nail pathologies associated with diabetic patients to favor their healing.

CHAPTER IV

Development of inert coatings to prevent drug retention in 3D-printed diffusion cells

CHAPTER IV

Patent application ES202331049. Method of manufacture of a laboratory object suitable for experiments with active pharmaceutical or cosmetic active (2023).

Abstract

Diffusion cells play a crucial role in the pharmaceutical and cosmetic fields by assessing the release and permeation of active pharmaceutical ingredients across membranes, whether synthetic or biological. However, commercially available glass-based devices, such as Franz diffusion cells, are expensive and require delicate handling due to their fragility. Fortunately, the emergence of three-dimensional (3D) printing technology offers an innovative solution, enabling the creation of diffusion cells using various cost-effective polymeric materials and resins with exceptional precision and custom geometries. Nonetheless, there are still challenges to address with this technology, as the materials used are non-inert and tend to interact with most drug molecules. This work aimed to develop inert coatings for 3D-printed diffusion models. Diffusion devices were designed and 3D-printed with a stereolithography (SLA) 3D printer, and different coatings were applied to the 3D-printed devices. Then, two model drugs were used to evaluate drug retention by 3D-printed coated devices compared to glass vials. Among the tested coatings, one of them showed great potential in preventing drug retention and was extensively characterised and selected for subsequent experiments with different model drugs and conditions. Finally, voriconazole eyedrops were used as a representative pharmaceutical formulation to confirm the viability of 3D-printed Franz diffusion cells as an *in vitro* drug release diffusion model. The favourable results obtained with the coating promote the use of 3D printing as a cost-effective manufacturing technology, capable of producing diffusion cells in desired shapes tailored to specific study requirements, facilitating and reducing the costs associated with the development of new drug delivery systems.

Keywords



3D printing; Stereolithography; Drug retention; Franz diffusion cells; *In vitro* release tests; Inert

coatings

I INTRODUCTION

Diffusion cells are commonly used for the study of drug release and permeation of active pharmaceutical ingredients through polymeric or biological membranes. Pre-formulation studies include *in vitro* drug release studies (i.e., *in vitro* release tests (IVRT)) and *in vitro* permeation studies (IVPT), which are crucial for formulation development, composition optimisation and batch validation (1,2). Relevant data, such as release kinetics and drug permeation, are obtained from these studies and are essential for the characterisation of new drug formulations. *In vitro* studies are also needed to predict the *in vivo* performance of a dosage form prior to *in vivo* studies and subsequently to establish *in vitro-in vivo* correlations (IVIVC). These correlations describe the relationship between the *in vitro* properties of the dosage form (e.g., eye drops or transdermal skin formulations) and the *in vivo* response (3,4). Moreover, IVRT performed during pre-formulation development allow to discard formulations that do not meet optimal drug release requirements, thereby saving time and reducing costs. More importantly, properly conducted IVRT and IVPT allow to reduce the number of animals needed to test new formulations in future *in vivo* studies, helping to comply with the 3Rs principle in animal experimentation (5,6).

The most commonly used devices for testing drug release, diffusion and penetration of drugs across biological and synthetic membranes are glass Franz cells and Side-Bi-Side cells (7,8). Franz and Side-Bi-Side cells primarily consist of a donor part (donor chamber) and a recipient part (receptor chamber), which are separated by a membrane or tissue, with a sampling port leading from the receptor chamber. A magnetic stirrer or an orbital shaker can be used to provide agitation to the medium and maintain homogeneity in both chambers. Additionally, to keep the temperature constant in the diffusion cells, a water jacket around the cell body (jacketed diffusion cells), an orbital incubator, or a thermostatic bath can be employed (7). These static devices are used to evaluate compound uptake into or across a membrane and concentration in the receptor chamber. On the other hand, to mimic *in vivo* behaviour more closely, continuous flow cells (in-line cells) are also available. Continuous flow cells simulate blood flow, allowing to also determine the drug clearance rate (8).

Although diffusion cells are used in pharmaceutical R&D laboratories worldwide, they are not without their drawbacks (9,10). Since they are made of glass, they are very fragile and prone to breaking easily. Depending on the type and the size, its price can go up to \$500, which makes

them very expensive, especially for small laboratories and academic institutions (11). In addition, different types of diffusion cells are needed based on the formulation and intended route of administration, requiring adaptation to the desired membrane or tissue type, sample volume, receptor medium, and type of agitation. For instance, some studies have focused on the development of models to mimic the physiology of the eye or the skin for *in vitro* modelling (12–15). Therefore, the manufacturing of more robust, affordable and personalised diffusion cells that meet the same pharmaceutical requirements as currently commercialised glass cells is an area of ongoing development (16–19).

Additive manufacturing technologies, namely three-dimensional (3D) printing, have proven to be an excellent tool for the production of spare parts and small pieces of laboratory equipment (16,20,21). The affordability and design flexibility offered by this technology along with the wide variety of low-cost materials that can be used make 3D printers essential in any research laboratory (22–25). Among the available 3D printing technologies, stereolithography (SLA) 3D printing creates objects in a layer-by-layer fashion by selectively solidifying a liquid resin composed of monomers and a photoinitiator. This technology is highly adequate to 3D print small parts with complex geometries at very high resolutions ($< 10 \mu\text{m}$) and very short times (26,27). Even more interesting, completely solid objects can be created, thus avoiding the risk of leakage when liquids are introduced inside, this being very relevant if the object to be printed are diffusion cells.

However, the main problem with this technology lies in the use of photocurable resins, mainly composed of acrylates (28). Acrylic materials are permeable to various substances, including solvents and solutes (e.g., drugs). In addition, these materials can absorb water and other solvents, and depending on their degree of cross-linking, they can even exhibit swelling (29). As a result, drug surface adsorption issues may arise when in contact with a pharmaceutical formulation (16). Ideally, this problem could be solved by applying a coating that prevents the drug from adhering to the wall of the printed device. Although some research has been carried out to explore materials that can be used as inert coatings for the interior of 3D-printed objects to prevent drug retention, none of them have been successful so far (16).

The aim of this study was to develop inert coatings for 3D-printed diffusion models. Initially, a basic hollow cylinder 3D model was created using CAD software and subsequently printed using an SLA 3D printer. Subsequently, various coatings were applied to the 3D-printed

hollow cylinders to assess their ability to retain two model drugs in comparison to glass vials. Among the coatings tested, one exhibited promising potential and was chosen for further experimentation with diverse drugs and model conditions. To validate the model, an *in vitro* release test was conducted on 3D-printed coated Franz cells, as well as commercially available glass Franz cells, utilising a voriconazole eye drop formulation. The selected coating was also subjected to additional characterisation, evaluating its reusability and resistance to solvents, pH, and temperature. Finally, the chosen opaque coating was compared to both a translucent coating with a similar composition and an in-house made coating based on the main polymer of the opaque and translucent coating.

2 MATERIALS AND METHODS

2.1 Materials

405 nm Clear UV Resin was purchased from Anycubic (Shenzhen, China), ciclopirox olamine and dexamethasone base were obtained from Acofarma (Barcelona, Spain), voriconazole was purchased from Normon (Madrid, Spain), bevacizumab (Avastin[®]) was purchased from Roche (Basel, Switzerland), insulin was purchased from Merck (Darmstadt, Alemania), hydroxocobalamin acetate was obtained from HealthTech BioActives (Barcelona, Spain), 2-hydroxypropyl- β -cyclodextrin (HP β CD) (Kleptose[®], 0.65 M substitution ratio, MW 1399 Da) was purchased from Roquette Laisa S.A. (Valencia, Spain), and hyaluronic acid (HA) was obtained from Acofarma (Barcelona, Spain). Polytetrafluoroethylene (PTFE) powder spray coating (A), Plioway[®] resin-based coating (B), acrylic and self-crosslinking elastic emulsion coating (C), epoxy-polyamidoamine resin-based coating (D), bisphenol A epoxy resin-based opaque coating €, and bisphenol A epoxy resin-based translucent coating (F) were purchased in the local hardware store. Poly(Bisphenol A-co-epichlorohydrin), glycidyl end-capped was purchased from Merck (Darmstadt, Alemania).

2.2 3D printing process and leak testing

Two types of 3D models were designed and printed: hollow cylinders and Franz diffusion cells. The 3D hollow cylinders were used for the preliminary tests to facilitate the work, as they are easier to manipulate. The CAD software Fusion 360 V2.0.16265 (Autodesk Inc., USA) was used to design the 3D models. The 3D model of the vertical Franz cell was designed from measurements obtained with a digital Vernier calliper of a common glass vertical Franz cell. The resultant .stl files were sliced using the slicer software Photon Workshop V2.1.29

(Anycubic, Shenzhen, China). The printing parameters consisted of a layer height of 0.05 mm and a UV light (405 nm) exposure time of 2 sec. The final objects were printed using a Photon Mono X 6K 3D printer (Anycubic, Shenzhen, China). The 3D printed devices were then washed with isopropanol in an ultrasonic bath and cured in a UV light cabinet for 1 min on each side. Figure 1 shows the 3D model designs of the hollow cylinder and the vertical Franz diffusion cell used for the studies.

Liquid leakage assessment was conducted by filling a batch of 3D-printed coated devices and 3D-printed coated vertical Franz diffusion cells with a predetermined volume of water and placing them at 4°C to prevent water evaporation. At different times, the devices were weighed and visual inspected to evaluate any weight loss or presence of any external water leakage.

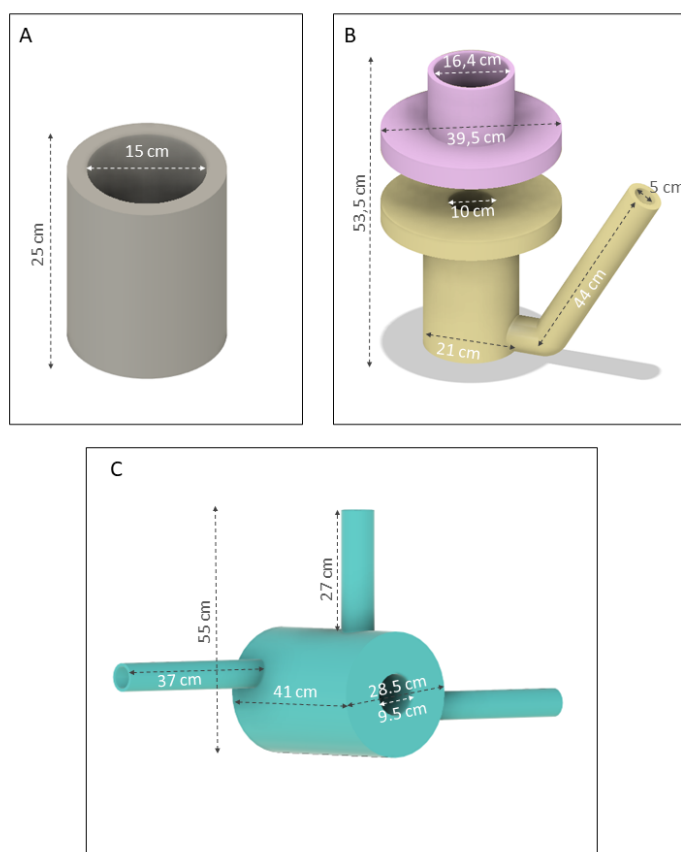


Figure 1. (A) 3D model design of a hollow cylinder with base. (B) 3D model design of a vertical Franz diffusion cell (C) 3D model design of one of the parts of a Side-Bi-Side diffusion cell.

2.3 Selection and application of coatings

Several types of coatings were tested along with uncoated 3D-printed cylinders and glass vials. The selection of coatings was based on their potential to prevent drug retention. The following coatings were evaluated: PTFE powder spray coating (A), Plioway[®] resin-based coating (B), acrylic and self-crosslinking elastic emulsion coating (C), epoxy-polyamidoamine resin-based coating (D), and bisphenol A epoxy resin-based opaque coating (E). More detailed information can be provided upon individual request.

Coating A was applied by spraying the interior of the 3D-printed cylinders 5 times, with a 30-minute dry period between applications. The remaining coatings were applied by filling the inside of the 3D-printed cylinders and removing excess material 30 minutes later. The cylinders were left to dry for 48 hours.

2.4 Preliminary screening of the coatings

The coatings were tested with two model drugs, ciclopirox olamine and dexamethasone base. A 25 µg/mL dexamethasone base solution (pH 7.25) and a 50 µg/mL ciclopirox olamine solution (pH 8.5) were prepared. The 3D-printed cylinders were coated according as described in Section 2.3 and subsequently filled with the corresponding solution, covered with Parafilm[®] M sealing film, and maintained at 4°C to avoid evaporation. Drug retention was assessed in comparison to uncoated cylinders and borosilicate glass type I vials by measuring drug concentration at different time points (1, 2, 5 and 8 days) using ultra high-performance liquid chromatography (UHPLC) (ACQUITY UPLC H-Class Plus, Waters, Milford, Massachusetts, USA) with an FTN injector and PDA detector.

For ciclopirox olamine the stationary phase was a ACQUITY UPLC BEH 1.7 µm C18 column, 2.1×50 mm (Waters, Massachusetts, USA), and the mobile phase was an isocratic elution of 60% orthophosphoric acid 20 mM and 40% acetonitrile pumped at a flow rate of 0.5 mL/min. The injection volume was 50 µL, and column temperature was set to 30°C with UV-wavelength of 300 nm. The elution time of ciclopirox olamine was approximately 0.85 min.

For dexamethasone base the stationary phase was a ACQUITY UPLC BEH 1.7 µm C18 column, 2.1×50 mm (Waters, Massachusetts, USA), and the mobile phase was an isocratic elution of 65% water 0.1% trifluoroacetic acid (TFA) 35% methanol pumped at a flow rate of 0.7 mL/min. The injection volume was 5 µL, and column temperature was set to 35°C with

UV- wavelength of 241 nm. The elution time of dexamethasone base was approximately 1.9 min.

Chromatograms were processed using Empower 3 software (Waters, Massachusetts, USA). Drug recovery (Eq. 1) in each time point was calculated with the following equation:

$$\text{Drug recovery (\%)} = \frac{[\text{Drug}] \text{ in the coated 3D printed cylinder}}{[\text{Drug}] \text{ in the glass vial}} \cdot 100 \quad (\text{Eq. 1})$$

2.5 Preparation of formulations with selected model drugs

Coated and uncoated 3D-printed cylinders and glass vials were subjected to a preliminary drug retention test using dexamethasone base and ciclopirox olamine solutions. One of the coatings was selected for subsequent tests. To validate the chosen coating, drug model solutions were prepared, including human insulin, bevacizumab, voriconazole with 0.1% (w/v) 2-hydroxypropyl-β-cyclodextrin (HPβCD), and hydroxocobalamin acetate. Drug selection was made based on their different physicochemical properties, which are contained in Table 1.

Table 1. Physicochemical properties of the model drugs.

Drug	Molecular weight (Da)*	logP (o/w)*	pKa/Isoelectric point*	Aqueous solubility (mg·mL ⁻¹ , 25 °C)*
Dexamethasone base	392.5	1.83	1.89 and 6.18	0.89 at pH 7.0
Ciclopirox olamine	268.35	2.3	6.84	1.41 at pH 7.0
Human insulin	5808	-13.1	5.4	2 at pH 2-3
Bevacizumab	149000	-	8.3	-
Voriconazole	349.31	1.75	1.76	2.7 at pH 1.2
Hydroxocobalamin acetate	1346.35	-14	1.81	>15 at pH 7.0

* Data extracted from PubChem (30).

2.6 Drug retention studies with the selected coating

Given the positive results obtained with coating E, further drug retention studies were conducted using 100 µg/mL human insulin (pH 2.3), 25 µg/mL bevacizumab (pH 6.15), 50 µg/mL voriconazole with 0.1% (w/v) 2-hydroxypropyl-β-cyclodextrin (HPβCD) (pH 7.12), and

50 µg/mL hydroxocobalamin acetate (pH 5.71) solutions. Drug recovery was evaluated using various chromatographic methods, as described in Section 2.4.

For insulin the stationary phase was an ACQUITY UPLC BEH 1.7 µm C18 column, 2.1×50 mm (Waters, Massachusetts, USA), and the mobile phase was an isocratic elution of 65% potassium phosphate 0.1 M pH 3.1 buffer, 27% acetonitrile, and 8% methanol. The flow rate was set to 0.6 mL/min, with an injection volume of 10 µL, a column temperature of 25°C, and a UV wavelength of 214 nm. The elution time of insulin was approximately 0.98 min.

For bevacizumab the stationary phase was a BioResolve RP mAb Polyphenyl, 2.7 µm 450Å column, 2.1×50 mm (Waters, Massachusetts, USA), and the mobile phase was a gradient from 15% water 0.1% trifluoroacetic acid (TFA) and 85% acetonitrile 0.1% TFA to 85% water 0.1% TFA and 15% acetonitrile 0.1% TFA. The flow rate was set to 0.8 mL/min, with an injection volume of 1 µL, a column temperature of 80°C, and a UV wavelength of 280 nm. The elution time of bevacizumab was approximately 2.4 min.

For voriconazole the stationary phase was a ACQUITY UPLC BEH 1.7 µm C18 column, 2.1×50 mm (Waters, Massachusetts, USA), and the mobile phase was an isocratic elution of 70% 10 mM ammonium acetate buffer and 30% acetonitrile. The flow rate was set to 0.5 mL/min, with an injection volume of 10 µL, a column temperature of 25°C, and a UV wavelength of 256 nm. The elution time of voriconazole was approximately 2 min.

For hydroxocobalamin acetate the stationary phase was a CORTECS UPLC 1.6 µm C8 column, 2.1×100 mm (Waters, Massachusetts, USA), and the mobile phase was an isocratic elution of 90% 10 mM ammonium formate and 10% acetonitrile. The flow rate was set to 0.4 mL/min, with an injection volume of 10 µL, a column temperature of 40°C, and a UV wavelength of 351 nm. The elution time of hydroxocobalamin acetate was approximately 0.85 min.

2.7 *In vitro* release study of a voriconazole eye drop in vertical glass Franz cells and 3D-printed coated Franz cells

Firstly, a voriconazole hydrogel eye drop formulation developed in a previous study (10) was prepared by adding 0.4% (w/v) hyaluronic acid to a 1% (w/v) voriconazole solution with a 20% (w/v) HPβCD. An *in vitro* release study was then conducted to compare the release profiles of the voriconazole eye drop formulation in 3D-printed coated vertical Franz cells and

glass vertical Franz cells. 0.5 mL of formulation was added to the donor compartment, while the receptor compartment was filled in with 6 mL of phosphate-buffered saline (PBS) pH 7.4. Visking[®] dialysis membranes (Medicel[®] membranes Ltd.) with a 12–14 Kda cut-off (0.784 cm² available surface area) were placed between donor and receptor compartments. The Franz cells were kept thermostated at 37°C and the medium homogenised by placing them in an orbital shaker incubator (Unimax 1010 and Inkubator 1000, Heidolph, Schwabach, Germany) at 100 rpm during the assay. Samples of 1 mL were withdrawn at pre-determined time points and replaced with fresh PBS medium. The concentration of voriconazole was determined by the method previously described in Section 2.6.

2.8 Morphological and physicochemical characterisation of the selected coating

2.8.1 Scanning electron microscopy

The morphological properties of the E coating surface were examined by scanning electron microscopy (SEM). SEM images of the coated and uncoated devices were taken with a ZEISS EVO LS15 microscope at 20Kv using a BSD detector (backscattered electron detector)/SE (secondary electron detector), under conditions of variable pressure.

2.8.2 Energy dispersive X-ray analysis

Elemental microanalysis of the coating E was performed by energy dispersive X-ray analysis (EDX) with an EDS Ultim[®] Max detector (Oxford Instruments) controlled by INCA software. The acquisitions conditions were 20Kv and a working distance of 8.5 mm for a more precise analysis.

2.8.3 X-ray diffraction

X-ray diffraction (XRD) measurements were carried out using an Empyrean diffractometer (Malvern Panalytical, UK). The X-rays were obtained from a sealed tube with a Cu anode ($\lambda(K_{\alpha 1}) = 1.5406\text{\AA}$) which was collimated, prior to incidence on the sample, with an optic including a W/Se type bilayer mirror. The radiation emitted by the sample was obtained with a “PIXcel3D” type solid-state detector. The diffractograms were taken in an angular range from 2° to 40° with a step of 0.02° and a time per step of 2 sec. Phase identification of the obtained diffractograms was performed using HighScore Plus 3.0d software (Malvern Panalytical, UK).

2.8.4 Raman spectroscopy

Raman spectroscopy was performed on the coated and uncoated 3D-printed devices using a Bruker Raman FT Raman Scope. The Raman spectra provided information on the functional groups of the materials, allowing comparison of the chemical composition of coated and uncoated devices. Raman spectra were recorded at room temperature on a Raman confocal microscope alpha300 R (WITec) using a 532 nm laser with a power of 1.3-2.1 mW, 0.3 s of integration time, 50 accumulations, and a lens of 50x LD. Project Five 5.3 (WITec) was used for data processing and imaging.

2.9 Evaluation of the reusability of coated devices

The 3D-printed cylinders coated with coating E, which were used in sections 2.6 and 2.7, were cleaned with a neutral detergent or a neutral detergent and alcohol. The cylinders were then analysed using EDX, XRD, and Raman spectroscopy. The Scherrer's formula (Eq. 2) was used as a measure of the crystallinity of coating E before and after washing:

$$Dp = \frac{K \cdot \lambda}{B \cdot \cos \theta} \quad (\text{Eq. 2})$$

Where Dp is the average crystallite size (nm), K is the Scherrer constant (0.94 for spherical crystallites with cubic symmetry), λ is the X-ray wavelength (for mini XRD, $\text{Cu K}\alpha = 1.541787 \text{ \AA}$), B is the full width at half maximum (FWHM) of XRD peak and θ is the XRD peak position.

Comparisons with data from unused coated devices from Section 2.8 were made to examine whether any drug residue was retained. Subsequently, a 25 $\mu\text{g/mL}$ of dexamethasone base solution was loaded into the 3D-printed coated recycled cylinders, and dexamethasone retention was assessed according to the procedure described in Section 2.4.

2.10 Solvent resistance of the selected coating

The 3D-printed cylinders coated with coating E were exposed to different solvents including acetone, ethanol, isopropanol, neutral detergent, methanol, or liquid paraffin for 24 hours. After exposure to the solvents, the cylinders were dried and analysed using SEM as described in Section 2.8 to assess any potential changes in the surface morphology of the coatings. Solvents were checked visually for any turbidity after the test period and the coatings were inspected for any structural changes such as pinholes or blemishes.

2.11 Temperature and pH resistance

The resistance of the coating E to temperature was assessed by exposing the 3D-printed cylinders to different temperature conditions. Conditions tested included 4°C, 25°C and 37°C. A 25 µg/mL dexamethasone base solution was added to the 3D-printed coated cylinders and drug retention was assessed following the method described in Section 2.4.

The resistance of the coating E to changes in pH was investigated by exposing the 3D-printed cylinders to neutral, acidic and basic pH buffers for 24h. Buffers consisted of pH 7.5 and pH 9.0 phosphate buffer solution, and pH 1.2 simulated gastric fluid without enzyme (31). Subsequently, the buffers were removed, and a 25 µg/mL dexamethasone base solution was added to the 3D-printed coated cylinders. Drug retention was assessed following the method described in Section 2.4.

2.12 Comparison between the opaque coating (E), the translucent coating (F), and the in-house made coating (G).

The coating G was elaborated by dissolving 10% (p/v) poly(bisphenol A-co-epichlorohydrin), glycidyl end-capped, in ethyl acetate. The coatings F and G were applied by filling the inside of the 3D-printed cylinders and removing excess material after 30 minutes. Subsequently, the cylinders were left to dry for 48 hours. Both coatings were analysed using SEM, EDX, XRD, and Raman spectroscopy as detailed in Section 2.8.

Coating F retention was assessed by adding a 25 µg/mL dexamethasone base solution to the coated 3D-printed cylinders. Drug retention was analysed following the method described in Section 2.4.

Coating G retention was characterised in depth by adding the following solutions: 100 µg/mL human insulin, 25 µg/mL dexamethasone base, 50 µg/mL voriconazole with 0.1% (w/v) 2-hydroxypropyl-β-cyclodextrin (HPβCD), 50 µg/mL hydroxocobalamin acetate, and 50 µg/mL ciclopirox olamine. Drug recovery was evaluated using various chromatographic methods, as described in Sections 2.4 and 2.5.

3 RESULTS AND DISCUSSION

3.1 Design, manufacture, coating, and leak testing of 3D-printed devices

For the design of the diffusion cells, the measurements of a standard glass diffusion cell were replicated to achieve a comparable internal volume. The donor and receptor chambers

were 3D-printed separately and positioned upside down to eliminate the need for supports. The resulting components exhibited smooth walls without requiring additional sanding or post-processing (Figure 2). The chosen layer height of 0.05 mm enabled the production of 3D-printed diffusion cells with excellent resolution. In addition, the durability of the 3D-printed devices was assessed through drop and shock tests (data not shown), which demonstrated superior robustness in comparison to glass diffusion cells. To facilitate the studies, 3D printed cylindrical vials were employed for preliminary investigations.

The high level of adhesion between layers achieved through SLA printing ensures the production of watertight 3D-printed devices. The leak detection study performed on the 3D printed devices resulted in no liquid leakage observed and no significant change in the weight of the devices during the studied period (α n.s.). However, one of the challenges associated with using SLA technology for manufacturing pharmaceutical equipment lies in the utilisation of acrylic resins, which are not inert and can potentially undergo chemical or physical interactions with many drugs (16). Consequently, various coatings were selected based on their ability to prevent drug retention and were applied to the components of the devices that come into contact with the drug solution.

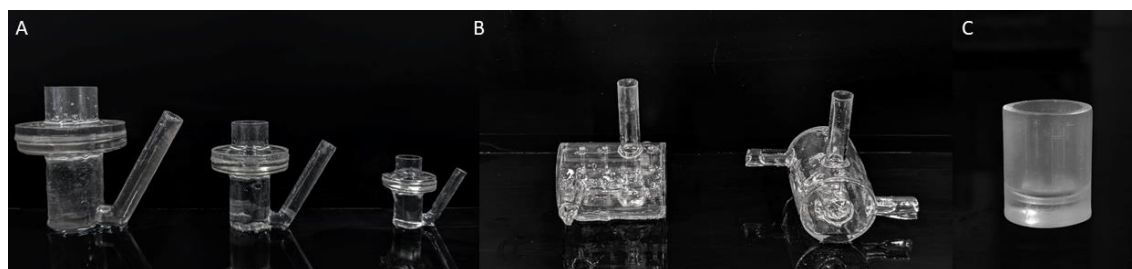


Figure 2. (A) 3D printed vertical diffusion Franz cells in different sizes. (B) 3D printed Side-Bi-Side cell. (C) 3D printed cylinders.

3.2 Preliminary screening of the coatings

An initial evaluation of the coatings was conducted through a drug retention study, involving two drugs with distinct chemical properties: dexamethasone base and ciclopirox olamine. Figure 3 shows the results of drug recovery (%) from the preliminary test. On day 8, the uncoated resin device retained $33.58 \pm 0.21\%$ of dexamethasone base and $65.43 \pm 4.95\%$ of ciclopirox olamine. Similarly, the retention of dexamethasone base for coatings A, B, C and D on day 8 was $40.93 \pm 2.33\%$, $20.90 \pm 1.41\%$, $47.94 \pm 16.39\%$, and $8.30 \pm 0.27\%$, respectively.

For ciclopirox olamine, the retention on day 8 was even higher, with values of $69.35 \pm 2.90\%$, $70.74 \pm 9.65\%$, $86.89 \pm 5.30\%$, and $88.86 \pm 10.93\%$ for coatings A, B, C, and D, respectively. Contrarily, coating E exhibited complete recovery with no retention observed for either drug at any time point. There were no significant differences found between time points for dexamethasone base (α n.s.). However, the retention of ciclopirox olamine in the glass container increased with contact time ($\alpha < 0.05$). The recovery of ciclopirox from coating E reached approximately 105% on day 8. Based on these promising results, coating E was further tested with drugs of different chemical properties to assess its drug retention.

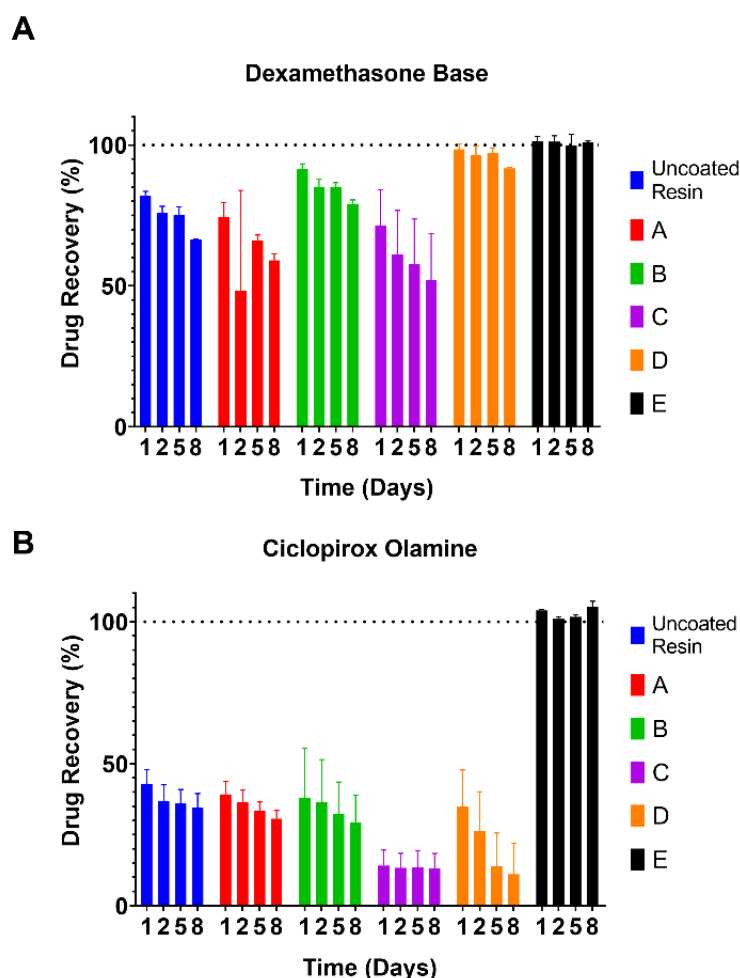


Figure 3. Drug recovery (%) from coated and non-coated 3D printed vials compared to glass vials. (A) Dexamethasone base recovery (%). (B) Ciclopirox olamine recovery (%).

3.3 Drug retention with the selected coating

Coating E was subjected to additional drug retention testing with drugs of diverse characteristics, including hydroxocobalamin acetate, voriconazole, human insulin, and bevacizumab. The latter two are biomacromolecules—a protein and a monoclonal antibody, respectively—becoming increasingly relevant for clinical applications (32,33). Certain studies suggest that proteinaceous molecules can bind to borosilicate glass type I containers. Therefore, one of the objectives of this study was to evaluate the potential binding to coating E (34).

Drug solutions tested included human insulin, bevacizumab, voriconazole and hydroxocobalamin acetate. Figure 4 shows the percentage of drug recovery for each drug at different time points. No significant differences were observed between time points for hydroxocobalamin acetate, voriconazole, and insulin (α n.s.). However, in the case of bevacizumab, the retention in glass vials was higher compared to the coated devices ($\alpha < 0.05$). Extensive studies have been conducted on the retention of proteins and monoclonal antibodies in glass containers (34,35), and the findings of this study align with the existing literature. Consequently, coating E demonstrates the potential to prevent interactions between protein-based drugs and their primary container.

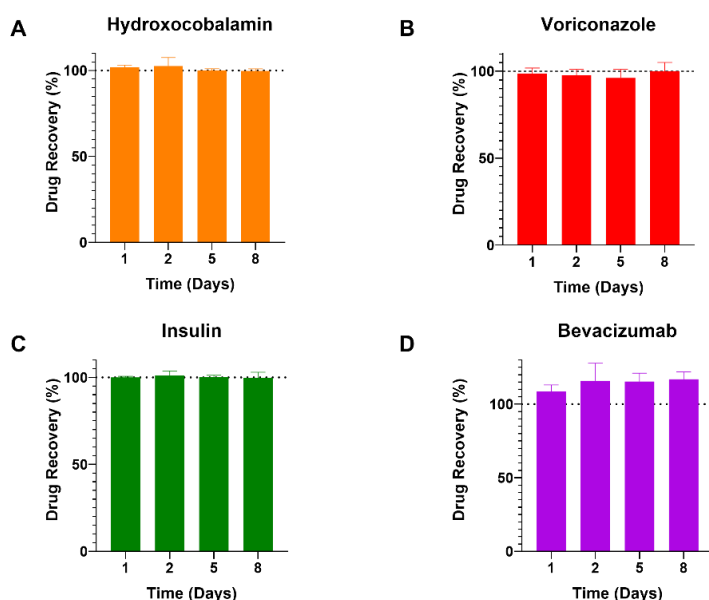


Figure 4. Drug recovery (%) from 3D printed cylinders coated with coating E compared to glass vials. (A) Hydroxocobalamin acetate recovery (%). (B) Voriconazole recovery (%). (C) Human insulin recovery (%). (D) Bevacizumab recovery (%).

3.4 *In vitro* release study using voriconazole eye drops in 3D-printed coated Franz cells and glass vertical Franz cells

To compare the suitability of 3D-printed coated Franz cells and conventional glass Franz cells for *in vitro* drug release tests, an *in vitro* release study was performed using voriconazole eye drops. The results of the study are shown in Figure 5. No significant differences were observed in the release of voriconazole from the eye drops between the two types of Franz cells (α n.s.).

Some studies have conducted *in vitro* investigations with different drugs, comparing the drug retention achieved with 3D-printed diffusion cells and conventional glass Franz cells. For instance, in one study, the compatibility of different drugs with 3D-printed diffusion cells using SLA technology was evaluated (16). The results revealed the occurrence of drug retention, which was attempted to be addressed by post-processing coating of the devices. However, even with the use of hydrophobic coatings, they proved ineffective in reducing drug retention. In a subsequent study, caffeine was used as a hydrophilic model drug in uncoated SLA 3D-printed devices (17). Although the use of this hydrophilic drug improved the compatibility with the SLA acrylic resin, the hydrophobic compounds still did not exhibit compatibility with the proposed model.

In another study, fused deposition modeling (FDM) was used to manufacture vertical diffusion cells using polypropylene (PP) filament (19). However, it has been shown that PP is susceptible to drug adsorption, thus rendering it unsuitable as an inert material for contact with drug solutions (36). On the other hand, polylactic acid (PLA) has also been employed in other studies for 3D printing diffusion cells using FDM technology (18). Nonetheless, PLA exhibits drug adsorption as well (37). In addition, SLA offers certain advantages over FDM technology. For example, FDM is more prone to production errors and the resulting devices have a higher risk of fluid leakage. In contrast, SLA is significantly and capable of producing several devices simultaneously (26,38). FDM filaments typically possess an opaque or slightly translucent nature, whereas SLA resins provide clear and transparent materials (39,40). Therefore, the coating presented in this study demonstrates more promising outcomes in comparison to those observed in previous studies conducted to date.

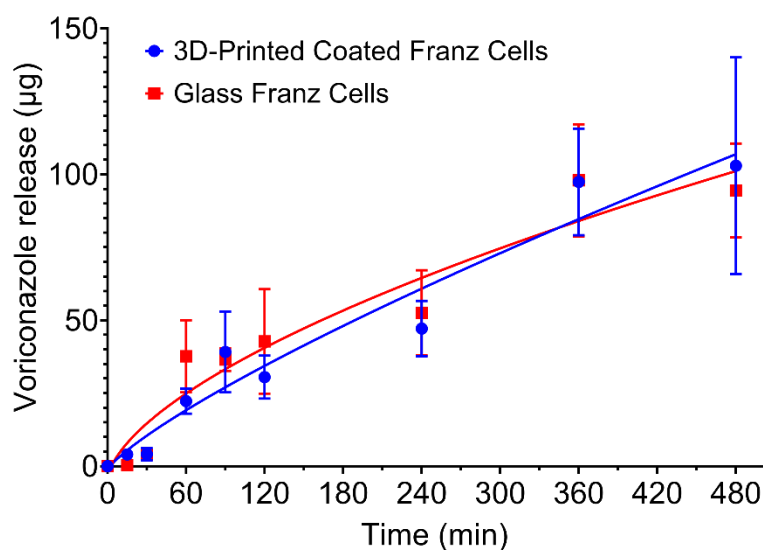


Figure 5. *In vitro* release of voriconazole eye drops in 3D-printed coated Franz cells and in glass Franz cells.

3.5 Morphological and physicochemical characterisation of the selected coating

Figure 6 shows the SEM images of both the uncoated and coated resins surfaces. The uncoated resin surface appears rough and exhibits small fragments of resin adhered to its surface. These resin residues are often deposited during the 3D printing process and their subsequent removal is challenging due to their UV light curing. However, upon the application of coating E, the surface becomes smooth, devoid of significant pores or flaws. The side view of the coated device's surface clearly exhibits the noticeable contrast between the uncoated surface and the E-coated surface. The coating includes small crystals within its composition, a finding confirmed by XRD analysis. The thickness of the coating layer was approximately 350 μm .

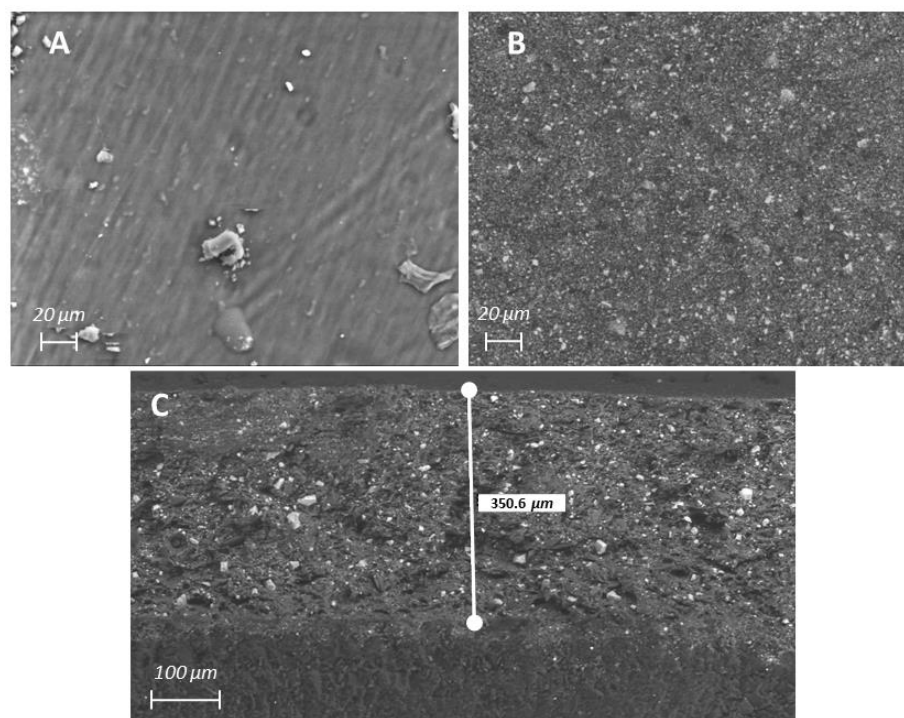


Figure 6. SEM images of (A) the uncoated resin surface, (B) the coated resin surface, and (C) the side view of the coating E applied to the resin material.

The surface elemental composition of both the uncoated resin and the coated resin was analysed using EDX. The results of the analysis are presented in Table 2. The acrylic resin primarily consisted of carbon and oxygen, whereas coating E contained a higher proportion of carbon and oxygen, along with trace amounts of titanium, barium, sulphur, aluminium, silicon, phosphorus, zinc, magnesium, and sodium. These findings suggests that coating E incorporates additional components beyond the bisphenol A-based epoxy resin. XRD and Raman analyses were conducted to further investigate the coating E.

Table 2. Surface elemental analysis of the resin and the coating E.

Element	Resin (%)	Coating E (%)
Carbon (C)	68.4	57.0
Oxygen (O)	31.5	26.8
Titanium (Ti)	-	8.6
Barium (Ba)	-	3.3
Sulphur (S)	-	0.9
Aluminium (Al)	-	0.8
Silicon (Si)	-	0.7
Phosphorus (P)	0.1	0.6

Zinc (Zn)	-	0.6
Magnesium (Mg)	-	0.6
Sodium (Na)	-	0.2

Figure 7 shows the XRD diffractogram of the uncoated resin and the coated resin. The resin did not exhibit any diffraction peak as it is in an amorphous state. In contrast, the coated resin, as observed in the SEM images, displayed a micro- or nano-crystalline structure with detectable crystals. The phase identification analysis revealed that the crystalline peaks corresponded to different components, including chlorite (containing Mg, Al, and Si), barite (BaSO_4), and rutile (TiO_2), which are essential raw materials in coating and paint industry used to provide resistance, opacity, and colour to the coating. In this case, chlorite was used as a filler and rutile (Pigment White 6 or CI 77891) and barite (Pigment White 21 or CI 77120) were used as white pigments in the coating composition (41,42).

The results of the previous EDX elemental analysis align with the findings from the XRD analysis, as these elements (Mg, Al, Si, Ba, S, Ti, and O) were part of the elemental composition of coating E. It should be noted that these peaks do not indicate the concentration of these compounds in the final composition of coating E, but rather indicate their crystallinity.

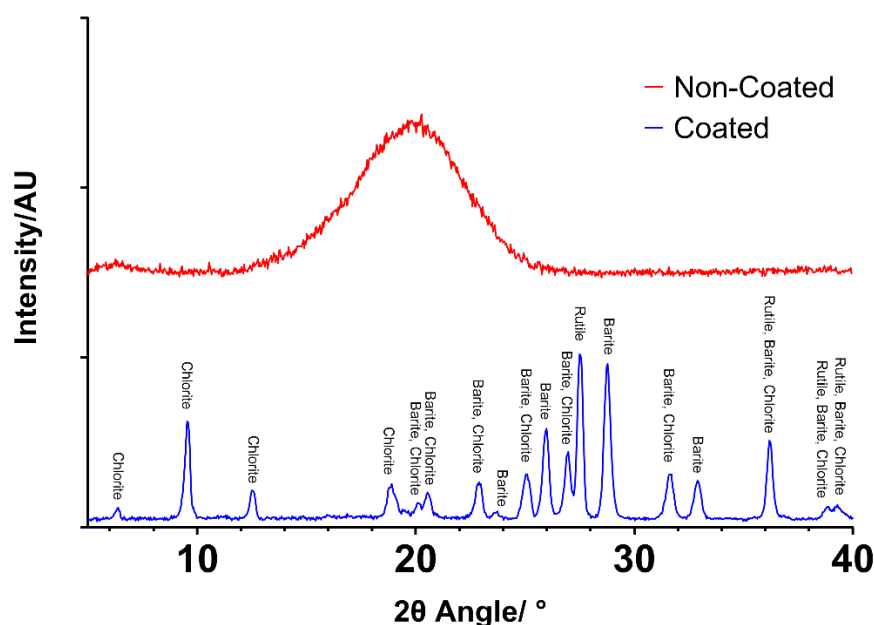


Figure 7. XRD diffractograms of the surface of coated and uncoated devices.

Figure 8 presents the Raman spectra of the surface of coated and uncoated devices. Although performing a comprehensive molecular analysis was challenging due to the complex composition of the resin and coating E, notable variations in the Raman spectra were observed. The Raman spectra of the uncoated device exhibited characteristic features of an acrylic polymer, with a peak at 1715 cm^{-1} corresponding to the C=O bond vibration in the acrylate groups, as well as intense peaks in the $2800 - 3000\text{ cm}^{-1}$ region corresponding to C-H bond vibrations of the CH_2 and CH_3 groups of the polymer main chain (43).

On the other hand, the Raman spectra of the coated device displayed weak peaks in the epoxy band ($1150\text{-}1350\text{ cm}^{-1}$) at 1257 cm^{-1} and 1186 cm^{-1} , corresponding to the epoxy ring breathing vibration and the C-O-C backbone vibration, respectively (44). The intense peaks observed at 448 cm^{-1} and 612 cm^{-1} are associated to O-Ti bonds from the TiO_2 used as an opacifier in the coating composition, as confirmed by the EDX and XRD results (45).

Based on the Raman results, it was observed that the coating was uniformly applied to the resin surface. The components of the coating did not react with those of the resin. Additionally, only the spectrum of coating E was detected in the analysed area, indicating the absence of pores that could expose the resin to contact with the drug. These results suggest that the coating E could be used with different types of resin or 3D printing materials.

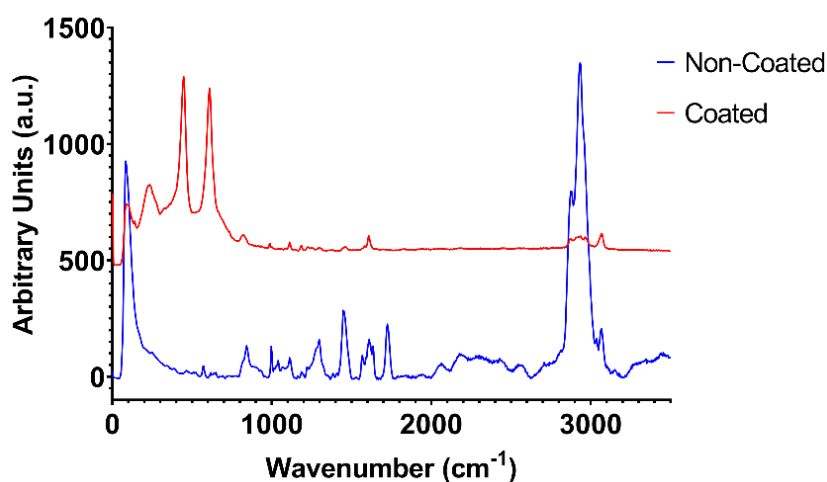


Figure 8. Raman spectra of the surface of coated and uncoated devices

3.6 Evaluation of the reusability of the coated devices

The 3D-printed cylinders coated with coating E were cleansed using a neutral detergent or a combination of neutral detergent and alcohol. An EDX analysis was performed to detect any

potential changes in the elemental composition of the coating that could have resulted from drug retention during contact. The analysis focused on identifying atoms specific to the drugs that had come into contact with the coating, including nitrogen (ciclopirox olamine, bevacizumab, human insulin, and voriconazole), cobalt (hydroxocobalamin), and fluorine (dexamethasone base and voriconazole). The EDX analysis did not detect the presence of any target atoms, indicating that the drugs did not adhere to the coating's surface. Furthermore, there were no significant differences in composition between the unwashed devices, those washed with neutral detergent, and those that were washed with neutral detergent and alcohol.

A XRD analysis was performed to examine potential changes in the coating's crystallinity after washing and to detect any peaks corresponding to drug crystallisation. Figure 9 shows the diffractograms of all the tested devices. All observed peaks corresponded to the coating matrix, which confirms the absence of drug retention within the coating. While there were variations in the crystallinity of the phases after cleansing, all phases remained present. A slight broadening of the peaks was observed after washing, suggesting a minor increase in crystallinity.

The Scherrer's formula was employed to assess the change in coating crystallinity before and after washing. The crystallite sizes of barite, chlorite and rutile were determined (Table 3) and compared across the different devices. The washed coated devices exhibited larger crystallite domain sizes for all components, regardless of the washing method ($\alpha < 0.05$). Some components of coating E may have dissolved during washing with water or ethanol. During the drying process, these compounds could crystallise, leading to the formation of larger crystals with different conformations.

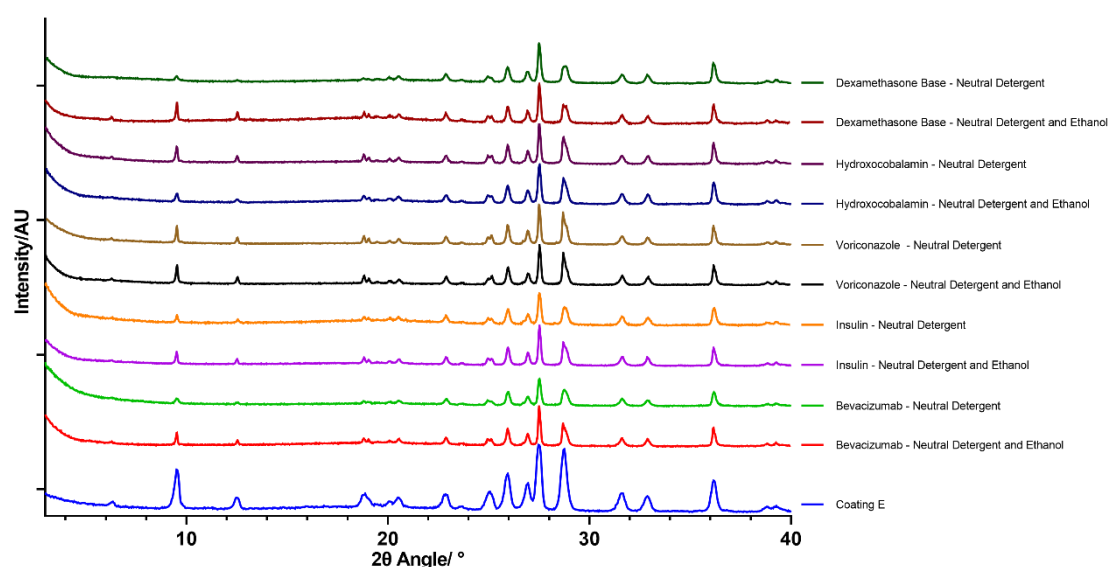


Figure 9. XRD diffractograms of the washed devices and the coating E

Table 3. Scherrer's formula results from washed and unwashed coated devices.

Device	Barite crystallite size (nm)	Chlorite crystallite size (nm)	Rutile crystallite size (nm)
Coating E	39.5	40.6	29.7
Bevacizumab – Neutral Detergent	43.4	37.3	50.0
Bevacizumab – Neutral Detergent and Ethanol	77.2	37.8	61.1
Insulin – Neutral Detergent	63.1	35.7	45.7
Insulin – Neutral Detergent and Ethanol	79.4	33.2	59.4
Voriconazole – Neutral Detergent	81.7	83.2	65.3
Voriconazole – Neutral Detergent and Ethanol	77.2	67.0	58.2
Hydroxocobalamin – Neutral Detergent	68.9	72.0	50.9
Hydroxocobalamin – Neutral Detergent and Ethanol	81.7	62.1	61.5
Dexamethasone – Neutral Detergent	46.6	86.6	53.1
Dexamethasone – Neutral Detergent and Ethanol	92.6	86.6	59.8

Raman spectroscopy analysis was performed to assess potential changes in the functional groups present on the surface of the washed coated devices. Figure 10 shows the Raman spectra of the washed devices compared to coating E. The peaks associated with Ti-O bonds exhibited reduced intensity in the washed devices, suggesting that the cleaning process might remove some TiO₂ particles from the surface of the coating. Moreover, the XRD results suggested an increase in rutile crystallite size. Consequently, the analysed area may possess larger crystals, albeit in lesser quantity than before washing, which could explain the diminished intensity of the Ti-O bonds peaks. However, no new peaks were observed in the Raman analysis for any of the drugs, indicating that coating E did not cause drug retention.

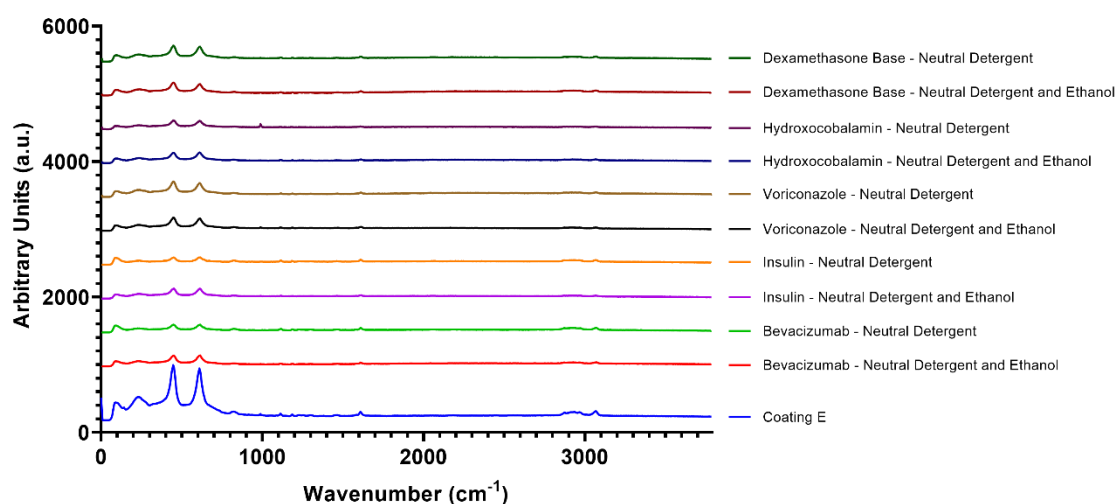


Figure 10. Raman spectra of the washed devices and the coating E.

The recycled 3D-printed coated cylinders underwent drug retention testing by introducing a 25 µg/mL solution of dexamethasone base after cleansing and drying. Figure 11 illustrates that the recycled devices exhibited complete recovery of dexamethasone base, with no drug retention observed at any time point. No significant differences were found between time points for dexamethasone base (α n.s.). These results demonstrate that the coated devices are reusable, since the cleaning process did not impact drug retention.

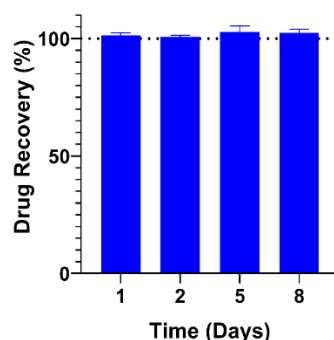


Figure 11. Dexamethasone base recovery (%) from recycled 3D printed cylinders coated with coating E.

3.7 Solvent resistance of the selected coating

The coated cylinders were exposed to a variety of commonly used solvents, including liquid paraffin, acetone, ethanol, isopropanol, neutral detergent, and methanol. SEM images of the cylinders after exposure to the solvents are shown in Figure 12. Prior to exposure, the solvents were visually examined for any signs of turbidity, and no visual disturbances were observed after contact with the coating. SEM images were inspected to identify possible changes in the morphology of the coating surface, such as pores or imperfections, but no significant alterations were observed for any of the solvents. These results indicate that coating E is compatible with the tested solvents.

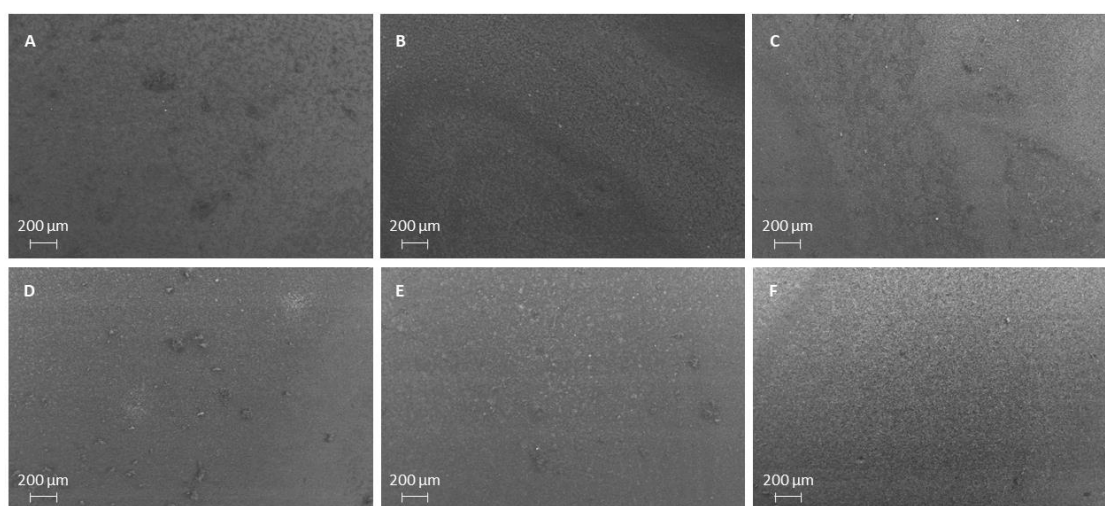


Figure 12. SEM images of the coating surface after exposure to (A) liquid paraffine, (B) acetone, (C) ethanol, (D) isopropanol, (E) neutral detergent, (F) methanol.

3.8 Temperature and pH resistance

A dexamethasone base retention test was performed to assess the thermal stability of the coated devices. The temperatures tested were 4°C, 25°C, and 37°C. A 25 µg/mL dexamethasone base solution was added to the devices to assess drug retention. On day 8, the recovery of the dexamethasone base was approximately 100% for all tested temperatures, except for 37°C, where recovery was $104.72 \pm 2.31\%$ (Figure 13). This slight increase in recovery at 37°C was likely due to evaporation of the solvent in the 3D-printed cylinders, which were covered with Parafilm® M sealing film instead of a cap like the glass vials.

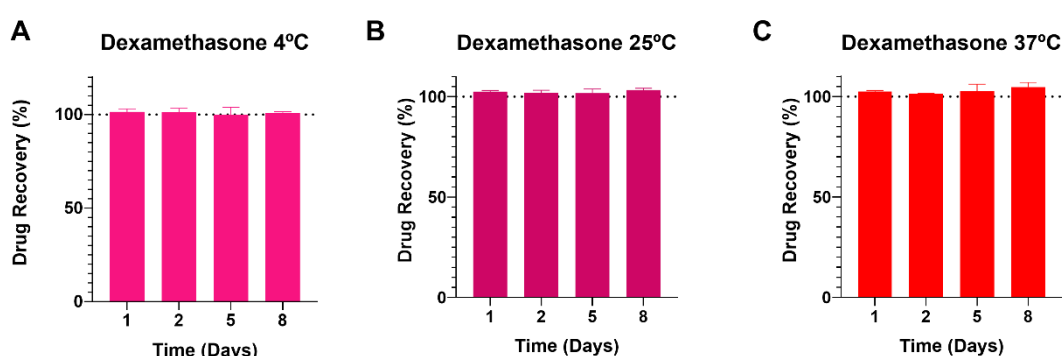


Figure 13. Recovery (%) of dexamethasone base at different temperature values.

Although the previously tested drug solutions (Section 3.2 and 3.3) covered a wide range of pH levels, a specific test was carried out to investigate the potential influence of pH on drug retention. The coated devices were filled with various buffers of different pH's (pH 1.2, 7.5 and 9). After 24 hours, the buffers were removed, and a drug retention test was performed using a 25 µg/mL dexamethasone base solution. The coated devices showed complete recovery of dexamethasone base with no drug retention at all time points (Figure 14). On day 8, the recovery of the dexamethasone base was approximately 100% for all pH values tested.

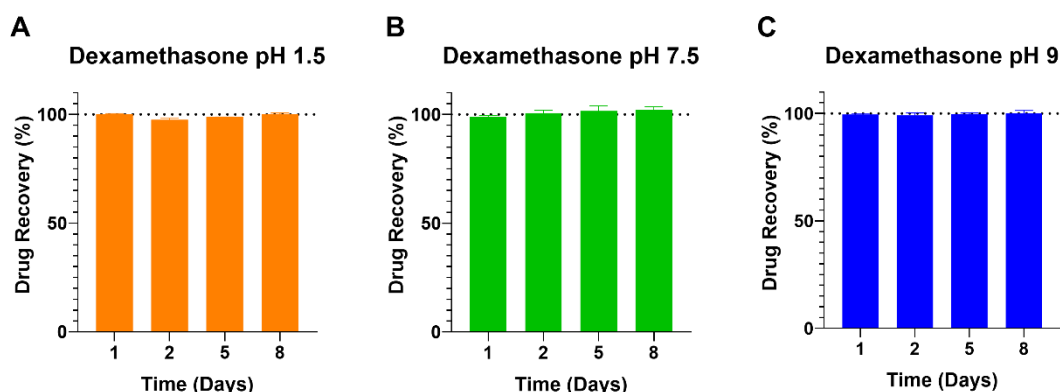


Figure 14. Recovery (%) of dexamethasone base after exposure of coating E to buffers of different pH's.

3.9 Comparison between the opaque coating (E), the translucent coating (F), and the in-house made coating (G).

The microscopic appearance of the surface of coatings F and G is shown in Figure 15. As with coating E, no large pores or defects were observed. This indicates that the coatings F and G cover the entire surface of the resin. Small crystals are visible on the surface of the coating F, the presence of which was confirmed by XRD analysis (Figure 16).

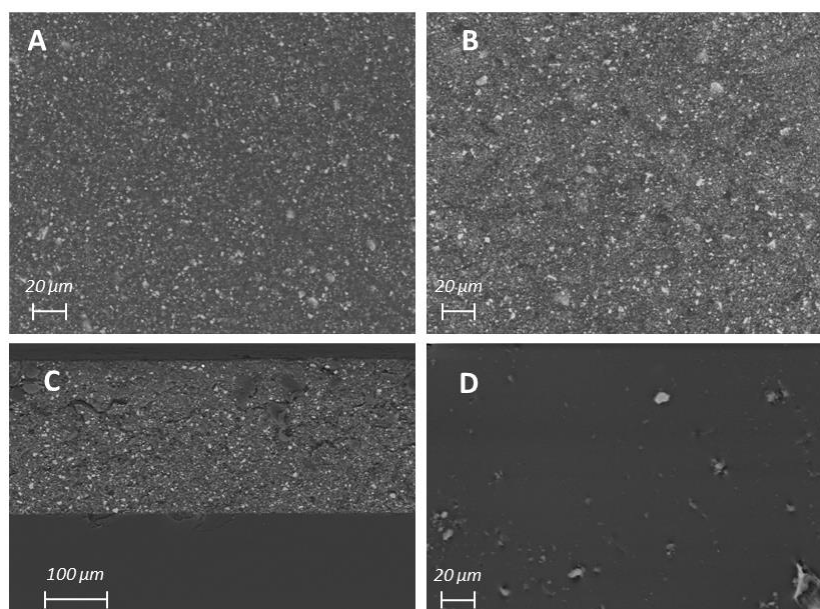


Figure 15. SEM images of (A) coating F, (B) coating E, (C) the side view of the coating F applied to the resin material, (D) coating G.

The elemental composition of the surface of coatings F and G was analysed by EDX and compared with that of coating E. Table 4 presents the comparison of the elemental composition between coating E, F, and G. Coating G is primarily composed of C and O, aligning with its expected composition. Coating E includes Ti from rutile molecules, serving as a white pigment. Moreover, coating E contains chlorite, incorporating Mg, Al, and Si elements. Coating F, on the other hand, shows a lower proportion of these elements, suggesting the absence of chlorite. The presence of Ba suggests that barite is present in both coatings, although it appears to be in a higher proportion in the coating F.

Table 4. Elemental analysis of the surface of coating E, F, and G.

Element	Coating E (%)	Coating F (%)	Coating G (%)
Carbon (C)	57.0	66.1	81.2
Oxygen (O)	26.8	18.9	18.5
Titanium (Ti)	8.6	-	-
Barium (Ba)	3.3	7.4	-
Sulphur (S)	0.9	1.8	-
Aluminium (Al)	0.8	<0.1	-
Silicon (Si)	0.7	<0.1	-
Phosphorus (P)	0.6	<0.1	0.2
Zinc (Zn)	0.6	-	-
Magnesium (Mg)	0.6	0.1	-
Sodium (Na)	0.2	<0.1	-
Calcium (Ca)	-	5.2	-
Chloride (Cl)	-	-	0.1

Coating F also contains calcium (Ca). To determine to which compound the calcium belongs to, an XRD analysis was performed. Figure 16 shows the diffractograms of coatings E, F, and G. The previously analysed Ca corresponds to calcium carbonate or calcite (CaCO_3) molecules, probably included to act as filler material to provide hardness and flexibility to the coating polymer (42). As can be seen, the peaks corresponding to rutile are not visible, demonstrating that rutile is not part of the composition of Coating F. Coating G shows an XRD pattern characteristic of an amorphous state due to its polymeric composition.

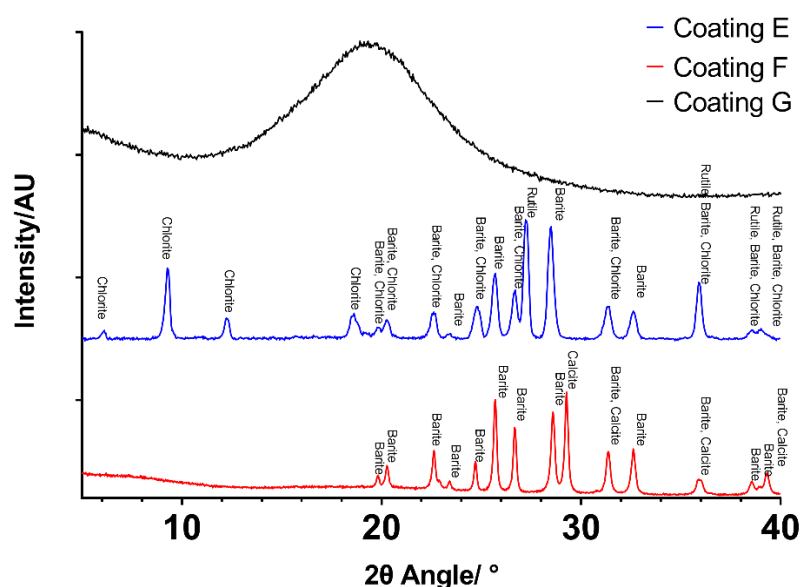


Figure 16. XRD diffractogram of the surface of coating E and coating F.

The Raman spectra confirmed the absence of TiO_2 in the coating F, since the peaks corresponding to the Ti-O bonds were missing. Despite this difference, the spectra of both coatings were similar, suggesting a similar composition. Similarly, coating G showed only the characteristic peaks of the polymer. Notably, the Raman spectra did not reveal any peaks associated with the acrylic resin beneath the coating, emphasising the effectiveness of the coating process.

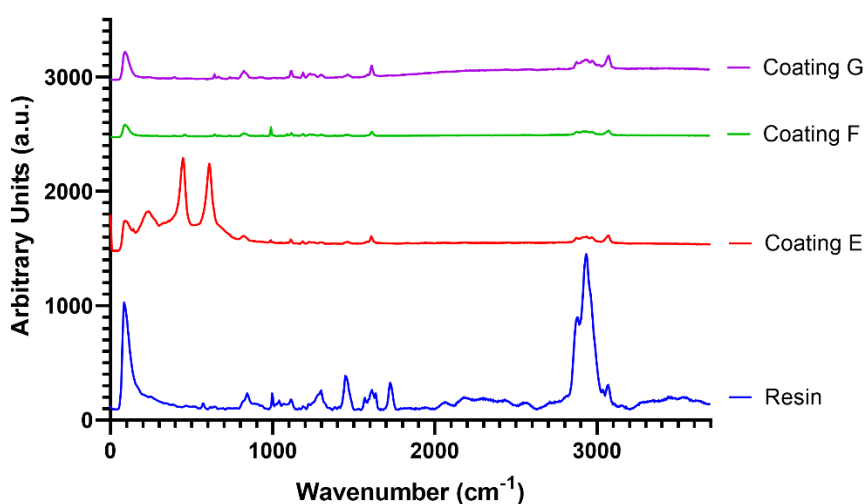


Figure 17. Raman spectra of the surface of coating E and coating F.

Coating F was tested for dexamethasone base retention by introducing a 25 µg/mL solution into F-coated 3D-printed cylinders. Figure 18 shows that coating F exhibited complete recovery of dexamethasone base with no drug retention at all time points, with no significant difference between time points being found for dexamethasone base (α n.s.). In addition, no significant differences were found between dexamethasone base retention in coating E and F (α n.s.). These results suggest that coating F would not cause drug retention, although further studies with more types of drugs are required.

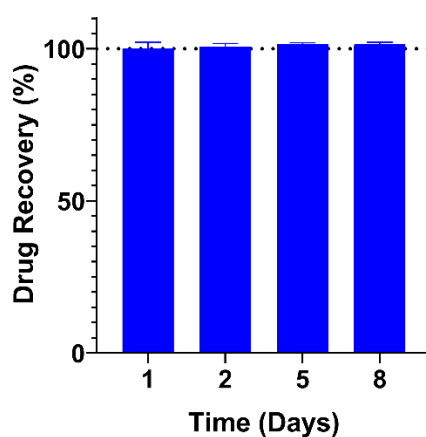


Figure 18. Dexamethasone base recovery (%) from 3D-printed cylinders coated with coating F.

Coating G was tested for drug retention by introducing 100 µg/mL human insulin, 25 µg/mL dexamethasone base, 50 µg/mL voriconazole with 0.1% (w/v) 2-hydroxypropyl- β -cyclodextrin (HP β CD), 50 µg/mL hydroxocobalamin acetate, and 50 µg/mL ciclopirox olamine solutions into G-coated 3D-printed cylinders. Figure 19 shows that coating G exhibited complete recovery of all the drugs, with no drug retention at any time point.

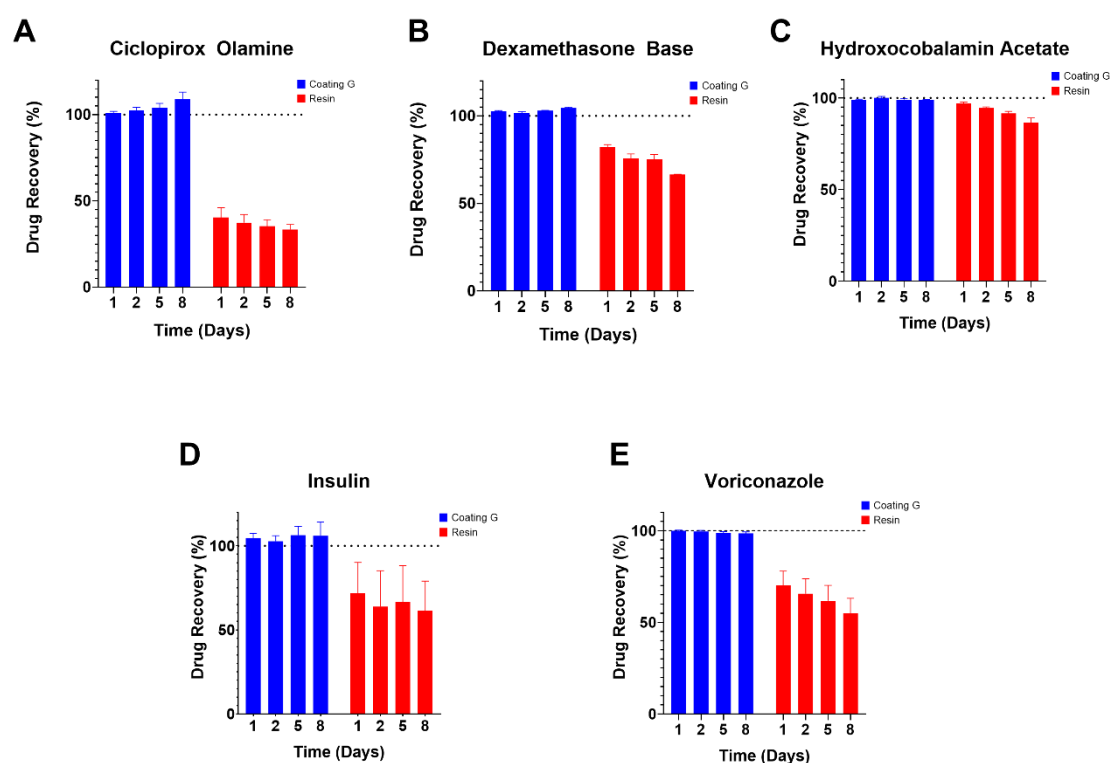


Figure 19. Drug recovery (%) from 3D printed cylinders coated with coating G and non-coated cylinders compared to glass vials.

4 CONCLUSION

For the first time, the development of an inert coating specifically designed for 3D printed diffusion models has been achieved. This coating effectively prevents drug retention during *in vitro* drug release studies, encompassing small molecules as well as biologics from pharmaceutical formulations. The application of this coating to the 3D-printed models ensured their leak-free performance and demonstrated excellent chemical resistance to solvents. Furthermore, the coating's opacity renders these 3D models highly suitable for light-sensitive drugs. Leveraging the versatility of 3D printing, these models can be tailored to accommodate formulations intended for various routes of administration, providing adaptability in terms of geometries and volumes. Ultimately, the coatings presented in this study will facilitate the integration of 3D-printed diffusion models into pre-formulation pharmaceutical studies, paving the way for the use of sustainable and reusable devices that are both robust and cost-effective to produce.

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GENERAL DISCUSSION

Diabetes mellitus (DM) affects approximately 9.3% of the global population aged 20 to 79 years. Projections indicate this prevalence will exceed 10% within the next decade, underscoring DM's prominence as a global chronic disease. The management of DM focuses on enhancing treatment efficacy, safety, and patient compliance. The development of innovative subcutaneous therapies is anticipated to significantly advance DM management by facilitating better glycaemic control and improving patient adherence (1).

The critical review carried out for the Chapter I of this thesis has emphasised the current pharmacokinetic limitations and challenges of commercial formulations of glucagon-like peptide-1 receptor agonists (GLP-1 RAs) and amylin mimetics (2). These challenges diminish treatment effectiveness and contribute to frequent adverse effects. Additionally, the high cost of these treatments restricts their application to certain cases. Efforts are underway to develop longer-lasting GLP-1 RAs and amylin mimetics (3). The goal is to achieve extended and stable drug release through novel encapsulation techniques that utilise biocompatible polymers. These techniques should avoid organic solvents and high temperatures, be scalable, ensure nearly complete drug loading, and provide consistent release.

Exploration of alternative delivery routes, including transdermal, nasal, and oral pathways, is progressing. Such alternatives could benefit DM patients with motor or visual impairments. Despite challenges such as poor absorption and instability in the gastrointestinal tract, oral delivery of GLP-1 RAs and insulin has seen advancements, as evidenced by Rybelsus® (4). However, formulation improvements are necessary for oral delivery to become a significant component of DM management. Transdermal delivery is focusing on the development of minimally invasive microneedle patches (5). Traditional manufacturing techniques face scalability issues, but 3D printing offers a promising solution for producing complex, reproducible, and cost-effective patches. The field of intranasal administration is also exploring strategies to improve drug bioavailability, including the use of nanoparticulate systems and absorption enhancers (6).

Diabetes mellitus is a metabolic disorder associated with a myriad of pathologies, including hypertension, diabetic retinopathy, diabetic neuropathy, renal and cardiac disorders, as well as skin and nail disorders. Improving the management of diabetes mellitus also involves optimising treatment for its associated conditions, with diabetic retinopathy and diabetic macular edema being particularly significant (7).

Diabetic retinopathy and diabetic macular edema are complications of diabetes that affect the posterior area of the eyes. Elevated blood glucose levels in diabetes can obstruct the blood vessels that supply the retina, leading to a disruption in the natural blood flow. This results in the formation of abnormal new vessels, leakage of fluid, and swelling. Symptoms may include blurred vision, floaters in the eye, patches of dark vision, and overall visual impairment (8). Macular edema typically occurs in people suffering from diabetic retinopathy in its more advanced (proliferative) stages when fluid leaks from the retinal blood vessels (9). Early-stage diabetic retinopathy can be managed through intensified diabetic treatment, while more advanced stages may necessitate specific interventions such as intravitreal anti-VEGF injections, intravitreal release of corticosteroids, photocoagulation, and ultimately vitrectomy (the removal of blood and scar tissue from the retina) (10).

Intravitreal injections of anti-VEGF drugs such as ranibizumab, aflibercept, bevacizumab, brolucizumab, or faricimab, as well as corticosteroids like triamcinolone or dexamethasone, are commonly utilised treatments for age-related macular degeneration, diabetic retinopathy, and diabetic macular edema. These treatments require repeated injections into the eye, with injection frequencies varying depending on the specific drug, and they are associated with numerous complications (11).

To mitigate the need for frequent intravitreal injections, pharmaceutical companies are developing various implants and devices designed to extend the time between administrations. One such product is Ozurdex[®], a dexamethasone implant marketed by Allergan for treating diabetic macular edema and inflammatory conditions at the back of the eye. Ozurdex[®] is composed of a 50:50 mixture of PLGA ester and acid, containing 700 micrograms of dexamethasone, produced via a hot melt extrusion process, ensuring its effect lasts for 4 to 5 months (12).

However, hot melt extrusion processes for biodegradable polymers like PLGA typically require temperatures exceeding 100°C. While dexamethasone remains stable during the manufacturing of intravitreal implants, the thermal stress involved in this process makes it less suitable for producing more sensitive drugs such as monoclonal antibodies and fusion proteins used as anti-VEGF agents. One alternative is the fabrication of implants via casting, but this method necessitates the use of organic solvents to dissolve the polymers, potentially affecting the drugs incorporated into the implants (13).

In this thesis, we have developed a production method based on the use of supercritical fluids technology, a green approach that avoids the use of organic solvents and high temperatures, for creating intravitreal implants for the controlled release of dexamethasone and bevacizumab (14). This study underscores the integration of biological drugs like bevacizumab into a poly lactic-co-glycolic acid (PLGA) matrix using supercritical foaming, emphasising the critical role of material selection and processing conditions in determining the release profile and efficacy of the drug-loaded implant.

Supercritical foaming presents a milder alternative for fabricating implants without the need for high temperatures or organic solvents (15). Nevertheless, optimising implant dimensions and release profiles is essential for clinical application. Although pharmaceutical 3D printing offers promising solutions for creating drug-loaded implants with specific sizes and dosages, it often requires conditions unsuitable for thermolabile compounds (16). The use of 3D printed moulds facilitates the manufacturing of implants with precise dimensions, and adjusting these moulds allows for easy dimensional modifications. Future efforts should focus on selecting PLGA polymers with lactide/glycolide ratios that match bevacizumab properties and further characterizing bevacizumab stability within the implant.

Applying this technique to other monoclonal antibodies or proteins could yield different interactions with the PLGA matrix and, consequently, diverse release profiles. Customising the implant to specific bioactive molecules requires fine-tuning the polymer composition and depressurization rates. The possibility of sterilising implants post-production enhances their clinical applicability, envisioning a scenario where ophthalmologists receive sterilised, ready-to-use implants tailored to individual patient needs (17). This method promises to streamline the manufacturing process and encourage the clinical adoption of personalised treatments, marking a significant advancement in drug-loaded implant technology.

The skin and nails also are organs significantly impacted in patients with diabetes mellitus. Conditions such as diabetic foot, diabetic dermopathy, and infections of the skin and nails are highly prevalent among diabetic patients (18). Due to their condition, diabetics are more susceptible to various bacterial and fungal infections affecting the skin and nails, increasing their likelihood of experiencing onychomycosis (19).

Onychomycosis, a prevalent fungal infection affecting the nail plate, presents therapeutic challenges due to the limited efficacy of current treatments, leading to prolonged therapy and a

high relapse rate. The highly cross-linked keratin network of the nail plate acts as a barrier, impeding effective drug delivery. While dermatophytes are the primary cause, non-dermatophytes and yeasts also contribute. *Tinea pedis plantaris* and *tinea pedis interdigitalis*, fungal infections commonly associated with humid environments and occlusive footwear, pose additional risk factors for developing onychomycosis (20).

Treatment of onychomycosis consists of eradicating the causative pathogens so that the healthy nail can be restored to prevent recurrence or spread of infection. The therapeutic options are multiple, but the rates of therapeutic failure and recurrence are not negligible (21). Nail topical drug delivery systems to treat onychomycosis are well accepted by patients and preferred to avoid the adverse effects of systemic antifungal. But its main drawback is the low drug permeation in the nail plate due to the limited permeability of the nail structure which reduces drug access in the nail bed. Transungual delivery possess serious challenges regarding drug permeation across the nail.

To improve the efficacy of topical nail treatments, several alternatives involving the use of chemical promoters or physical methods have been proposed. The development of aqueous or hydroalcoholic formulations has been a major advance over medicated organic nail lacquers. Significant improvements have been observed in the penetration of antifungals and other drugs into the nail. However, in cases where the deeper layers of the nail are affected, the drugs take time to reach effective concentrations, delaying healing. To improve the rate of penetration, different physical methods have been proposed to modify the permeation resistance of the nail plate. Methods such as filing the nail, the use of microneedles, or iontophoresis have been used for this purpose. In the most severe cases, partial or total ablation of the nail is also used (22–24).

To improve the efficacy of nail lacquers we have developed a new physical technique that represents a novel approach for treating onychomycosis, offering a simple and direct method to deliver antifungal treatments. Unlike complex and often less effective drug delivery systems, the developed method allows to apply antifungal lacquer directly to the affected nail areas, ensuring targeted treatment and potentially enhancing treatment effectiveness, especially for proximal nail infections. The developed technique overcomes the challenge of drug penetration through the nail plate, a major limitation of topical therapies, and does not require new drug formulations, making it a practical and promising option for onychomycosis treatment. Future

research will focus on its application with various antifungal agents, aiming to advance this method toward clinical studies.

The development of new drugs for the treatment of diabetes and its associated diseases requires the availability of preclinical methods *in vitro*, *ex vivo* or *in situ* to evaluate the kinetics of drug release and predict its bioavailability. The availability of suitable *ex vivo* and *in situ* models to study the behaviour of subcutaneous injections of GLP-1 RAs or to determine the ocular or nail permeability of new drugs is essential during their preclinical development. It is essential to simulate the environment of the administration site, including the use of simulated biological fluids, media volumes, pH, temperature, ionic strength, viscosity, as well as maintaining similar hydrodynamic characteristics (25,26). These systems must allow to place in the system fragments of tissues that constitute the biological barriers or to simulate them, in order to evaluate the absorption capacity of the drugs.

Biologic and synthetic skin substitutes have been developed for transdermal drug delivery. Hoof bovine model and human nails can be used for transungual delivery. Whole eyes and corneas from cattle, swine, rabbits, sheep or goats have been used for drug release and ocular toxicity studies. All these studies require the availability of diffusion cells adapted to the conditions of each administration site. As example microparticulate systems, especially those utilizing PLGA, are promising for GLP-1 RA delivery. Bydureon[®] exemplifies this approach but also highlights the difficulty of achieving a consistent drug release rate.

The development of new formulations requires reliable preclinical testing models that accurately mimic the human subcutaneous environment. This is a significant challenge in the current development process. Future models should facilitate long-term studies on pharmacokinetics, bioavailability, and immunogenicity. Similarly, the development of new intravitreal implants or studies of nail permeation of medicated lacquers require systems adapted to study the release and *in vivo* penetration capacity of the drugs they carry.

Examples of such systems are the vertical Franz cells useful for dermal and transdermal release studies, side-by-side diffusion cells, more commonly used for intestinal permeability studies, or more developed models such as the PK-eye model developed (27) for new ocular drug delivery systems development or the OcuFlow *in vitro* eye model to evaluate drug delivery from medicated soft contact lenses (25).

Due to the versatility and advances in the field of 3D printing and graphic design software, this technology can present great advantages when developing these systems adapted for the preclinical study of drugs. The availability of CAD for cell design and the precision of some printing techniques such as SLA allow the rapid construction and optimisation of this type of model at reduced economic costs. The availability of printers with excellent resolution and different resins with adequate mechanical and chemical resistance are also an additional advantage.

One of the main problems of SLA printing resins is that they give rise to materials with certain permeability and adsorption capacity of some molecules. In the last chapter of the thesis, it is shown that the acrylic resins used in this type of printer allow obtaining very elaborate models of the diffusion cells, being able to vary their shape and size on demand. However, it also shows the capacity of absorption and adsorption of drug molecules present in the solutions used for release and penetration studies.

Experiments performed with drugs with different chemical structures, different lipophilicity, and size show how these acrylic materials can incorporate these molecules by removing them from the solution. This process has already been described by other researchers and constitutes a great handicap for the use of these technologies and materials for the development of *in vitro* and *ex vivo* models useful in the development of new drugs, including those used in the treatment of diabetes and its complications (28,29).

Therefore, the last chapter of this doctoral thesis marks a significant advancement in pharmaceutical research through the development of an inert coating designed specifically for 3D printed diffusion models. For the first time, this coating has been shown to prevent drug retention effectively during *in vitro* drug release studies, applicable to both small molecules and biologics. The application of this innovative coating ensures that 3D-printed models are leak-free and possess excellent chemical resistance to solvents, an essential feature for conducting reliable pharmaceutical formulations studies.

Furthermore, the versatility of 3D printing technology allows for the customisation of these models to accommodate a wide range of formulations and routes of administration. This adaptability, in terms of geometries and volumes, underscores the potential of 3D-printed diffusion models to simulate real-life application scenarios more accurately, thereby improving the predictive value of *in vitro* studies.

The introduction of this coating technology represents a step forward in the integration of 3D-printed models into pre-formulation studies. It opens the door to the development of sustainable and reusable devices that are not only robust but also cost-effective to produce. Such advancements are poised to enhance the efficiency and sustainability of pharmaceutical research, facilitating the rapid screening and optimisation of drug formulations.

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CONCLUSIONS

The comprehensive analysis of results derived from the various chapters of this doctoral thesis leads to the following conclusions:

GLP-1 receptor agonists and amylin mimetics have emerged as promising treatments for diabetes mellitus. The challenges associated with subcutaneous administration are reviewed, highlighting the need for improved formulations. Despite their potential benefits, obstacles such as preclinical model reliability, immunogenicity, formulation issues, and cost remain, demanding collaborative efforts for future advancements in diabetes therapy.

The utilisation of supercritical foaming technology in Chapter II has resulted in intravitreal PLGA implants loaded with dexamethasone, exhibiting desirable characteristics and customisable release patterns. The controlled release for up to 4 months demonstrated enhanced performance compared to previous studies. Further exploration of bioactive molecule incorporation, particularly in a proof-of-concept investigation with bevacizumab-loaded implants, further emphasises the technology's potential for precise drug delivery in treating chronic retinal diseases.

Chapter III: restricted due to an ongoing patent application.

Chapter IV pioneers the development of an inert coating for 3D-printed diffusion models, ensuring leak-free performance and chemical resistance. These coated models, adaptable for various formulations and routes of administration, offer a sustainable and reusable solution for pre-formulation pharmaceutical studies. The introduced coatings enhance the integration of 3D-printed diffusion models, providing robust and cost-effective devices for research purposes.

APPENDIX

CONFLICT OF INTEREST

The doctoral student declares that there is no conflict of interest regarding the doctoral thesis.

EXPERIMENTS WITH HUMAN NAILS

Finger and toenail clipping samples were donated by healthy volunteers of both sexes, aged between 25 and 65 years, after signing informed consent. The nail donation protocol was approved by the Ethics Committee of Galicia (Project identification code: 2018/099, Date of approval: 22 February 2018).

LIST OF PUBLICATIONS

CHAPTER I

TITLE: Fighting type 2 diabetes: Formulation strategies for peptide-based therapeutics

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CHAPTER II

TITLE: Intravitreal implants manufactured by supercritical foaming for treating retinal diseases

AUTHORS: Carlos Bendicho-Lavilla, Iria Seoane-Viaño, Víctor Santos-Rosales, Victoria Díaz-Tomé, María Carracedo-Pérez, Asteria Luzardo-Álvarez, Carlos A. García-González, Francisco J. Otero-Espinar

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FIGURES AUTHORSHIP

The author declares that all unreferenced figures of this thesis are of his authorship.

Diabetes mellitus has emerged as one of the most prevalent chronic diseases of the 21st century with serious complications worsening its progression. This doctoral thesis is divided into four chapters focused on improving the treatment of complications associated with diabetes mellitus. In the first chapter, the subcutaneous administration of biopharmaceuticals is addressed as an effective and patient-preferred alternative to intravenous infusions. In the second chapter, supercritical foaming technology was utilised to manufacture PLGA implants loaded with dexamethasone and bevacizumab. The third chapter develops a drug delivery system to enhance the penetration of antifungal drugs into nails with onychomycosis. The fourth chapter develops an inert coating for in vitro testing with 3D printed devices.