

Collagen Binding Polymer-Cytokine Conjugates for Applications in Local Extracellular Matrix Engineering

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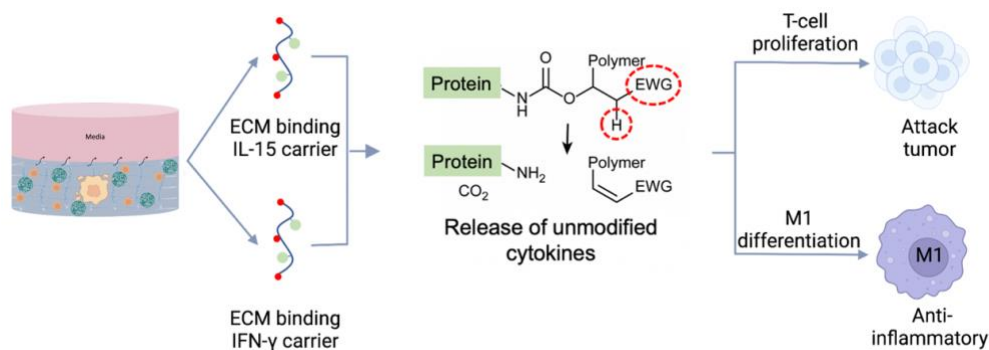
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Abstract

The therapy suppressive tumour microenvironment (TME) continues to hinder anti-cancer therapies. Local delivery of therapeutic proteins, including potentially toxic factors, is increasingly needed to enhance immunotherapeutic bioactivities and minimize systemic toxicity. To this end, we are developing vehicles that immobilize to extracellular matrix (ECM) components upregulated in TME for localization of polymer-grafted bioactive cytokines with tunable degradation rates to control cytokine clearance. The grafted cytokine would be bioactive, and the length of the therapy would be governed by the degradation kinetics of the hydrolytic linker between the cytokine and polymer. The cytokines were expressed and purified, and their biological activity was confirmed. Click chemistry was used to graft the therapeutic proteins and collagen-binding peptides to the copolymer. Production of the therapeutic carriers was confirmed by SEC and fluorescent measurements. Biolayer interferometry and tracking immobilization inside collagen gel confirmed the binding affinity between carriers and collagen type 1. In vitro studies confirmed the bioactivity of the carriers in the presence of T-cells and macrophages. In summary, ECM binding vehicles for local sustained protein release will aid in the local delivery of therapeutic proteins to alter TME and promote immunotherapies. Screens will be conducted in multicellular spheroid models to identify bioactive formulations.

1.1 Table of Content/Abstract Graphics. Created with BioRender.com.



Summary

Rationale. Many cancer immunotherapies have limited efficacy against solid tumours because of their immunosuppressive microenvironment.¹ To improve efficacy, combination therapies with cytokines that target macrophages or T cells can be delivered to alter local environments from therapy suppressive to therapy supportive.² Although several cytokines have been identified as promising therapeutics for solid tumours (e.g., IL-12, IL-15, IFN-gamma), their high systemic toxicity limits their use. Local delivery strategies with tunable clearance mechanisms for implantation at the tumour site can potentially incorporate cytokines into combination therapies with established immunotherapeutic (e.g., bispecific, checkpoint inhibitors, CAR T cells).

Although Glioblastoma (GBM) is the target for cytokine delivery in this study, this new local delivery system can potentially be used for several local tissue engineering applications. Blood-brain barrier (BBB) protection for the central nervous system makes treating brain diseases challenging, contributing to the lack of FDA-approved treatments for GBM.³ Local delivery of therapeutics help avoiding the BBB. Therefore, more studies have explored additional methods to increase therapeutic efficacy, including drug delivery nanosystems. One of the most recent ones the FDA has approved is Gliadel wafers. Since then, there has been much development for implant-type approaches; however, no common delivery system with high efficiency has yet been found.

Goal. To improve clinical translation and localize cytokines at the injection site, we are developing extracellular matrix (ECM) binding polymer conjugates that sustain cytokine hydrolytic release to control the action duration for several days. We are particularly interested in developing delivery vehicles that can be easily incorporated with the local injection of immunotherapeutics, such as CAR T cells, and, therefore, design soluble formulations which allow for simple combinations.

The ECM binding polymers will localize the polymer-grafted cytokines, which will be released upon hydrolysis of tunable linkers.

Chapter 1

Background information

2.1 Cytokines and Their Inhibitors as Therapeutics in Clinic

Cytokines are small proteins that have a crucial role in controlling the growth and activity of the immune system.⁴ They are substances such as interferon, interleukin and growth factors secreted from some immune cells and influencing others.⁵ The primary function of cytokines in the body is the regulation of inflammation. They are the signals released by the immune cells in times of threat and define how to repair the injuries or fight the danger. When an immune cell detects a harmful substance in the body, it starts releasing cytokine. The cytokines travel through the body systematically and find the appropriate receptor on the targeted immune cells. Based on the signal, the receptor cell acts on them. These signals can cause cell activation, cell proliferation, and cell differentiation. The immune response, inflammation, and hematopoiesis are three physiological processes regulated by cytokines.⁶ All these crucial roles can make cytokines a great candidate for different medical applications.

Rheumatoid arthritis (RA) is one of the diseases that are currently treated by cytokines. RA is an autoimmune disease resulting from the immune system attacking the healthy cells, mainly affecting the joints.⁷ The main symptom of RA is pain and swelling, and it can potentially decrease the quality of life and increase the mortality rate. In 1990, researchers found the function of the cytokine involved in the signalling process, tumour necrosis factor (TNF)- α .⁸ Macrophages produce TNF- α , which activates the inflammatory response. Therefore, delivery of TNF- α inhibitors reduces signs and symptoms of RA. Some examples of disease-modifying antirheumatic

drugs (DMARDs) that are currently prescribed for patients are infliximab (IFX), etanercept (ETN), and golimumab (GOLI).⁹

Cytokines have a crucial role in the immune response against the infectious disease.¹⁰ They have been commonly used for treating Hepatitis B and C,¹¹ Herpes Infections,¹² and HIV/AIDS.¹³ The most recent common usage of cytokine was for COVID-19.¹⁴ After the virus infection, the body's severe response towards the virus could cause severe inflammation and tissue damage. To avoid that, tocilizumab, an interleukin-6 (IL-6) inhibitor, was used. Other common medical contexts for using cytokine are wound healing and vaccination. This study uses cytokines such as interleukins and interferons to fight cancer.

2.2 Rational of Cytokine Delivery as Part of Cancer Treatment

The second most significant cause of death globally is cancer.¹⁵ Surgery, radiation and/or chemotherapy are the common treatment methods for the patients. Standard procedures can usually cure the primary tumours and do not prevent the metastatic spread of the disease effectively through disseminated tumour cells. Metastatic spread can be avoided to some extent by leveraging the immune system, using the immune system to detect and target cancer cells. This method of treatment is called immunotherapies.

Currently, immunotherapy is used as an effective method of treatment in treating advanced melanoma.¹⁶ Pembrolizumab, used for melanoma, treats cancer cells by avoiding immune response suppression. Pembrolizumab is an immune checkpoint inhibitor made of an antibody that can bind to PD-1 receptors on T cells. This way, T cell inhibition of action would not occur, and

the immune system continues fighting cancer. Nivolumab and atezolizumab are the other checkpoint inhibitors approved by the FDA.¹⁷ These are commonly used for certain types of non-small cell lung cancer (NSCLC) treatment and could successfully increase the survival of some patients. So, more and more cytokine-related therapies are being approved by the FDA as cancer therapeutics.

Although immunotherapy is currently being used to treat a wide variety of cancers,¹ it still lacks efficacy against solid tumours. A previous study examined the effect of anti-PD(L)1, a checkpoint inhibitor therapy, to determine the potential cause of this phenomenon. The study proved the presence of a correlation between the tumour mutational burden and objective response rate. It also confirms that immunotherapy has a significantly low efficiency against solid tumours, specifically Glioblastoma (GBM), due to the lack of adaptive response and an immunosuppressive tumour microenvironment (TME). The focus of this study is overcoming immunosuppressive TME.

TME includes immune cells, stromal cells, blood vessels, and extracellular matrix.¹⁸ Components of the TME support cancer cell survival, local invasion, and metastatic dissemination. Immune cells are the crucial portions of the TME and can either suppress or promote tumour growth. The two main populations of immune cells in TME are identified to be T cells and tumour-associated macrophages (TAMs).¹⁹ T cells kill cancer cells through adaptive immunity, in which each T cell develops a T cell receptor that recognizes specific antigens on the tumour cell surface. Additionally, T lymphocytes inhibit angiogenesis by secreting interferon-gamma (IFN- γ).²⁰ Macrophages, which IFN- γ activates, fight cancer through innate immunity, including pathogen

phagocytosis and antigen presentation. They can comprise up to 50% of the tumour mass and either promote or inhibit tumour progression.²¹ Preclinical models demonstrated that the immune system could facilitate the rejection of established cancers. However, there is still limited direct human evidence for this in predominantly immunogenic tumours like melanoma. A better understanding of TME allows us to modify suppressive TME through the delivery of cytokines.

Although Glioblastoma (GBM) is chosen as the target cancer in this study, the developed carriers can potentially be used for any solid tumour. GBM is an aggressive and common tumour that begins in astrocytes.²² It can occur in the brain and spinal cord. The patients' 1- and 3-year survival rates are 20.7% and 4.4%, respectively.²³ The median survival time with GBM is 15 to 16 months in people who receive the available treatments, including surgery, chemotherapy, and radiation. The low survival rate is due to the 100% recurrence rate, with the median time for tumour recurrence after surgical elimination of the tumour being 9.5 months.²⁴ The GBM initiating cells (GIC) are resistant to chemo and radiotherapy, making only some treatment options to increase survival, and most focus on quality-of-life improvement.²⁵ Immunotherapy's effect on increasing GBM patient survival has been proven previously.²⁶ For instance, surgery following usage of the Neoadjuvant (T cell up regulator) causes more prolonged survival of the patients (overall increased survival of 8 months).²⁷ In addition, it has been demonstrated that checkpoint inhibitors increase the survival of mouse models with GBM.²⁸ However, the lack of efficiency of the available immunotherapy treatments prevents the option from becoming universal. To improve efficiency, it is required to overcome immunosuppressive TME.²⁹ To overcome the immunosuppressive TME in GBM, the TAMs and T cell activity should be modified through cytokine delivery.³⁰

Interleukin 15 (IL-15) plays a crucial role in T cells' development, differentiation, and survival.³¹ T cells fight cancer cells in direct and indirect ways and are one of the primary immune defences against tumour cells. IFN- γ modulates immune cells of both the innate and adaptive immune systems and plays a crucial role in activating the macrophages.³² Monocytes can potentially differentiate into two major macrophage types with opposing activities. M1 macrophages that IFN- γ classically activate are responsible for inhibiting cell proliferation. They are known as anti-tumorigenic TAMs. The M1/M2 ratio can be increased by delivering IFN- γ in TME, potentially shrinking solid tumours.³³ Therefore, delivering cytokines such as IL-15 and IFN- γ into solid tumours such as GBM would potentially modify T cells and macrophage activities toward killing cancer cells.

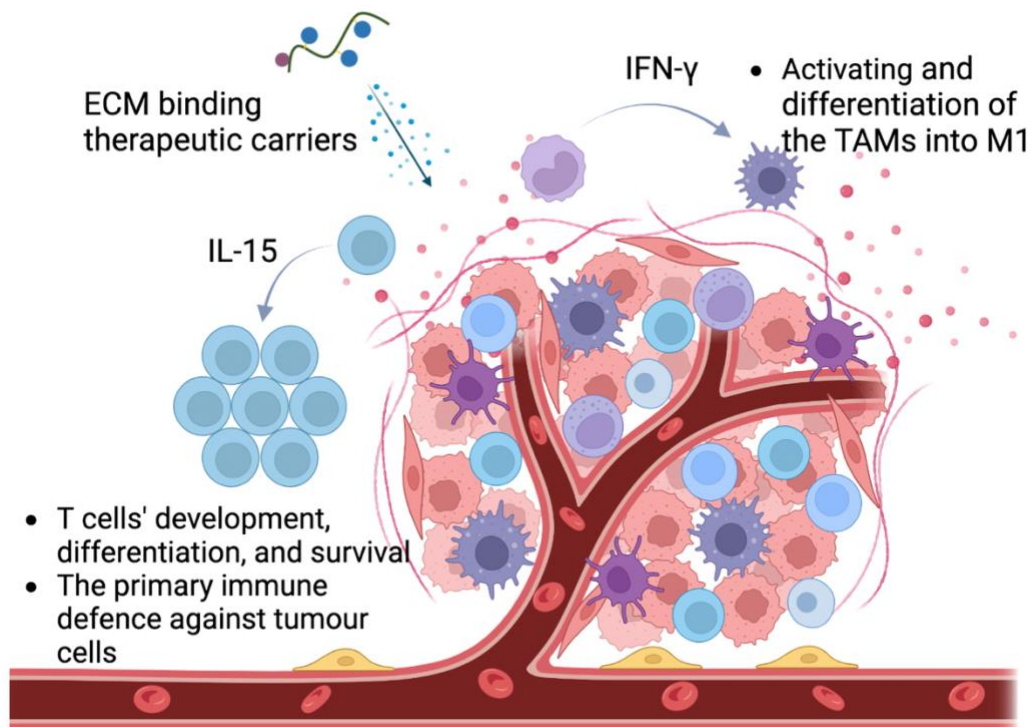


Figure 1.1. ECM binding vehicles will deliver IL-15 and IFN- γ into the solid tumour microenvironment to modify T cells' and macrophages' proliferation and activity, respectively. Created with BioRender.com.

Although cytokine can provide many benefits in cancer treatment, harmful side effects can be seen if the cytokine balance in the body is disturbed.³⁴ This is called cytokine release syndrome, which can be fatal. That is why it is crucial to deliver the cytokines locally instead of administering them systemically. Therefore, dose-limiting toxicities which prevent cytokine treatments from fulfilling the promise of efficiency seen in the preclinical trial can be overcome through localized and sustained delivery systems.³⁵

2.3 Localize Delivery

Localization of cytokine delivery maximizes the therapeutic efficiency while minimizing the potential side effects. Many different methods have been used for localized delivery of cytokines. One of the methods is injecting cytokines directly into the tissue or region of the body that needs treatment.² This method, called intra-articular and intra-tumoral injections, is often used to treat inflammatory joint conditions or tumours.³⁶ Since this method usually requires frequent administration, there is a chance of skin pigmentation, atrophy, and damage to the ligament or tendon.³⁷ Another localization method is used for localized delivery to skin or mucous membrane.⁴ In this method, cytokines are mixed in a gel or cream and administered directly on the wound or place of skin disorder. Interleukin-1 (IL-1) family cytokines have an important role in skin immunopathology. Some of their functions are anti-microbial response and host barrier maintenance through inflammatory response. Anakinra is a recombinant, slightly modified version of the human IL-1 receptor antagonist protein used topically to treat RA.³⁸ Administering cytokines topically has complications because passing through skin layers of hydrophilic keratin barrier remains challenging for drug development.³⁹ Other methods for localization are intracavitary injections and intravesical delivery, which had been previously used for the delivery

of IL-2 and IL-12 respectively.^{40,41} It should be considered that the target of this study is TME and the most common and efficient localization delivery method for tumors has been the development of biodegradable implants or delivery devices.

2.3.1 Biodegradable Delivery Devices for Cancer Treatment

Since cytokine treatment has promising results for cancer immunotherapy, research for developing biodegradable material that provides sustained and localized delivery has become popular.⁴² Polymeric nanoparticles (PNPs) are one of the examples of these materials. PNPs are particles made of polymer within the 1 to 1000 nm size range that can deliver cytokines through absorption, encapsulation, or conjugation either within or on the surface of PNPs.⁴³ These particles can potentially provide target cytokine delivery to the tumour site by having ligands, which are small molecules, peptides, or antibodies. This way, these particles provide site-specific target delivery. Poly(lactic-co-glycolic acid) (PLGA) is the most effective polymeric biomaterial for controlled drug delivery methods.⁴⁴ It has been used to deliver IL-12 and IL-6 for diabetic retinopathy and cardiovascular disease (CVD) treatment, where increased patient compliance, target performance, and duration of effect of medicines were observed.^{45,46} PLGA are FDA-approved and are generally considered safe. However, these particles demonstrate autocatalysis, which can potentially cause pH changes and drug instability.⁴⁷ In between PNPs, there are some natural examples as well. Chitosan nanoparticles, which are derived from chitin, are considered safe for dietary use. The FDA approves it for some specific applications like wound healing. In a study by Chen, Y. et al., chitosan nanoparticles encapsulated in recombinant pcDNA3.1-dsNKG2D-IL-15 were developed.⁴⁸ Administration of these particles demonstrated shrinking in the size of the tumour over time. These particles stimulate tumour cells' endocytosis to deliver the

dsNKG2D-IL-15 gene, eventually increasing tumour-killing cell activities. Specifically, an increase in IL-15 level was observed after the treatment, which could potentially increase T cell activity at TME.⁴² PEG-based NP, gold NP, and polymeric micelles are the other types of PNPs for drug delivery. While this field continues to grow, there are still some concerns about their clearance mechanism and the chance of their accumulation in the circulatory system.⁴⁹ As a result, ongoing preclinical and clinical studies examine the safety of these particles.

Hydrogels are another type of localized delivery system for cytokines. Hydrogels are made of cross-linked hydrophilic polymeric networks which swell due to water absorption.⁵⁰ These materials can be implanted in the TME and deliver cytokine.⁵¹ Their three-dimensional network provides sustained and controlled release of cytokine, which minimizes the risk of systemic toxicity and increases TME targeting specificity. Natural polymers, synthetic polymers, polymerizable synthetic monomers, and a combination of natural and synthetic polymers can be used to synthesize hydrogels. Multiple mechanisms for controlled drug release can be utilized through this delivery system. Sometimes, the cytokine is attached to a particle, which allows it to be entrapped within the hydrogel, or the cytokine is attached to the polymer backbone through degradable interactions. These drugs can either dissociate from the trapped particle or polymeric hydrogel backbone and diffuse through the gel, or the particle itself can be released over time due to the swelling of the gel.⁵² Hydrogels have been used to deliver dendritic cells as therapeutic due to their highly porous network structure, which mimicked ECM. For delivering cytokines, some success was seen when combined with thermosensitive hydrogels.⁵³ Since these hydrogels can be injected, they can be administered through local injection instead of surgical implantation. In a study, IL-2 and IFN- γ were co-delivered through injectable thermosensitive polypeptide hydrogels

of poly(ethylene glycol)/poly(γ -ethyl-L-glutamate) (PEG/PELG) block copolymers for local treatment of melanomas.⁵⁴ B16F10 xenograft mouse models were used to monitor the therapy effect, and it was seen that melanoma was significantly suppressed in these models. In another study, GM-CSF was co-delivered using a hydrophobic chitosan-based thermosensitive hydrogel, activating induced tumor-antigen-specific CD8⁺ T cells.⁵⁵ Although many promising results are obtained from hydrogels as the local delivery system, their design complexity limits their access. Currently, researchers are studying various hydrogel conditions to optimize the therapeutic outcomes.

Liposomes are another localized delivery system for cytokines. Liposomes are spherical lipid carriers with a 50-500 nm size range synthesized through emulsifying natural or synthetic lipids.⁵⁶ Liposomes demonstrate high biocompatibility, are easy to synthesize and demonstrate high drug-loading efficiency. Liposomes, the most explored nanocarriers, have been widely used to deliver cytokines to TME. In a study, transforming growth factor- β (TGF- β) inhibitor and IL-2 were co-delivered using nanoscale liposomal polymeric gels (nanolipogels; nLGs).⁵⁷ TGF- β is an immunosuppressive factor released by the tumour cells. Delivering a small hydrophobic inhibitor simultaneously with IL-2 using nLGs delayed the tumour growth and increased survival. Recent development has focused on enhancing liposome targeting ability by using ligands to keep the cytokines within the TME selectively.⁵⁸ Despite all these great results, their massive production is still impossible due to their high production cost.⁵⁹ There are also concerns about leakage and fusion of cytokines. Considering all these factors, new delivery vehicles with fewer steps, are easy to access and have high solubility might increase drug delivery efficiency.

2.3.2 Using Extracellular Matrix Binding for Cytokine Localization

The extracellular matrix (ECM) is the noncellular component of the TME. It consists of macromolecules and minerals which support the cells and define the mechanical properties of tissue.⁶⁰ Besides providing the essential physical scaffolding, it affects initiating essential biochemical cues required for differentiation and hemostasis.⁶¹ Previously, ECM has been chosen as a binding target for localized drug delivery to specific tissues. This way, scientists could increase the specificity. There are many different targets, such as gelatin, collagen, fibrin, and glycosaminoglycans, including hyaluronic acid, chondroitin sulphate, and heparin for cancer drug delivery and wound healing.⁶²

Hyaluronic acid (HA) is an essential part of ECM that controls tissue responses during injury, healing, and regeneration.⁶³ The primary receptor for HA that is also overexpressed on cancer cells is CD44. CD44 is involved in cancer cell growth, survival, and metastasis.⁶⁴ Following CD44 and HA binding, a conformational change would occur, favouring the binding of adaptor molecules, leading to cell proliferation and migration. Researchers have used this knowledge to conjugate drugs such as doxorubicin to HA and keep the therapeutic local in the TME.⁶⁵ HA has also been used as a ligand on liposomes that target cancer cells.⁶⁶

Many different specialized proteins are present in the ECM that can be used as a target for drug delivery. For example, cellular fibronectin is expressed by cancer cells, and its presence allows the invasion of cancer cells into healthy tissues.⁶⁷ It is made of two isoforms called the EDA or EDB domains, which have restricted presence in normal tissue. These domains have been commonly used as cytokine, chemotherapy, and radioisotope delivery targets. It should be

considered that not all tumours produce high levels of fibronectin. That is why the usage of antibody-targeting EDA is still under investigation.

Another target in ECM for drug delivery is collagen. Collagen is primarily a scaffold of adhesion for cells in the TME.⁶⁸ It has been proven that collagen type 1 is overexpressed in the TME of solid tumours. Therefore, collagens type 1 can be used as a scaffold for therapeutic carrier immobilization. This way, gel synthesis is not required, reducing the number of synthetic steps. To this end, by using collagen binding domains to target Collagen Type 1 or MMP-degraded collagen, the vehicles will be localized near the injection site and tumour to minimize systemic toxicity.⁶⁹ Collagen-binding drugs that convert the tumour stroma into chemotherapy have been developed.⁷⁰ For example, doxorubicin-collagen binding domain-serum albumin (Dox-CBD-SA) is a therapy that uses this technique to localize the delivery.⁷¹ In this drug, SA is the carrier for Dox, a type of chemotherapy, to the TME. It has been proven that Dox-CBD-SA treatment significantly suppressed tumour growth compared to Dox-SA and doxorubicin treatment in a mouse model of breast cancer.

Recent studies have developed new delivery systems involved in wound healing by targeting collagen type 1. Previous work of Hubbell's research laboratory developed biomaterials that bind to fibrin, a protein involved in blood clotting.⁷² They then designed and developed a collagen-binding, serum albumin-fused IL-4 variant (CBD-SA-IL-4) for non-healing wounds in mouse models with type 2 diabetes.⁷³ These CBD-SA-IL-4 materials modulate the wound microenvironment by promoting angiogenesis and M2 macrophage activation. CBD in the carrier has been proven to accelerate wound healing significantly compared to native IL-4 and HA.

Therefore, making carriers with CBD components for delivering cytokines to TME might increase the drug efficiency while lowering the systemic toxicity risk.

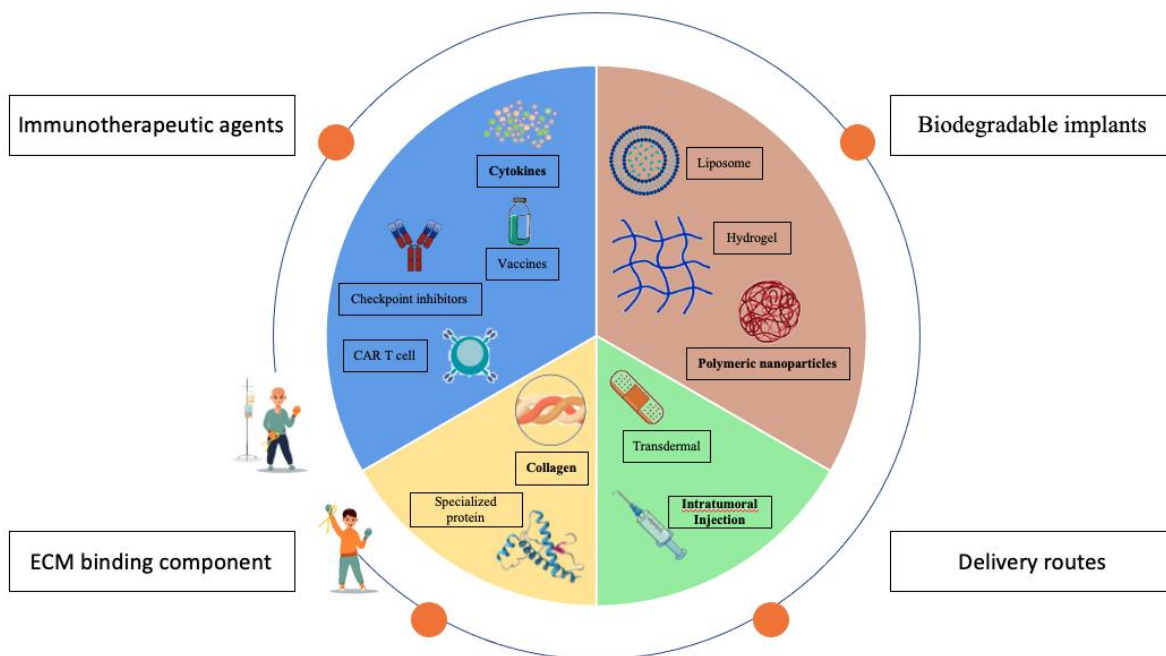


Figure 1.2 Summary of the factors that need to be considered for immunotherapy. The factor that is chosen for this study is bolded. Inspired by Abdou P. et. al.⁷⁴

2.4 Bioactive Polymers for Localization of Cytokines within ECM

In this study, a new collagen-binding cytokine delivery vehicle is designed, synthesized and characterized for the inclusion of administered cytokines into the TME with tunable degradation kinetics toward controlling the duration of action. Briefly, the collagen binding polymer component will increase the residence time of the carriers after intratumoral injection, the grafted bioactive cytokine will then contribute towards altering the TME. After hydrolysis of the cytokine/polymer linker, the cytokine will be cleared to control the duration of action. Poly(carboxybetaine-co-3-aminopropyl)methacrylamide (pCB-APMA) was chosen as the

copolymer for the backbone. PCB is common for drug delivery applications due to its high biocompatibility, antifouling properties, and low protein adsorption.⁷⁵ Also, pCB has demonstrated low immunogenicity, which means it does not provoke the immune response, which makes it a great candidate for drug delivery as the efficiency of the drug would not be compromised. In a study run by Jiang et. al., proteins were conjugated to pCB, and the result was compared with conjugation to PEG, a common method to increase the stability of the protein in vivo.⁷⁶ It was found that conjugation to pCB increases the delivered protein's stability and retains or improves the bioactivity. That is why choosing pCB as the polymer avoids the need to compromise between stability and affinity.

Strain-promoted Azide - Alkyne Click Chemistry (SPAAC) reaction is used to conjugate the cytokines and anti-collagen peptide to the copolymer. SPAAC requires minimal activation energy and does not require any catalyst, making it an appropriate choice for biological applications.⁷⁷ This reaction occurs under mild reaction conditions and has high efficiency and selectivity.⁷⁸ Dibenzocyclooctyne (DBCO) was first conjugated to the APMA primary amines, while anti-collagen peptides and cytokines were functionalized with azide groups.

Many possibilities for anti-collagen peptides that bind to collagen type 1 were available. The peptide with the RRANAALKAGELYKSILYGC sequence and K_D value of 0.86 μM was chosen because of the high literature support for this sequence.⁷⁹ The intended therapeutic carrier was synthesized after conjugating the cytokines and anti-collagen peptides on the pCB-APMA. This carrier has a relatively small number of synthesis steps, is relatively low-cost to make, and will be in the liquid phase.

We would incorporate a controlled clearance mechanism for the cytokines to control the duration of action, as continuous signalling may need negative side effects. Sustained and tunable clearance of the cytokines would be obtained by altering the linker's electronics.⁸⁰ A tunable releasing rate maintains therapeutic concentrations and minimizes systemic toxicity. Moreover, the released therapeutics would be unmodified, and their activity would not be reduced due to the conjugation. Therefore, a lower concentration is required to achieve the necessary TME modification. The linkers are degraded through the β -elimination reaction in which the release rate depends on the pKa value of the C-H bond.⁸¹ The more electron-withdrawing the modulator, the higher the degradation rate, which can vary from hours to less than a year. O-[1-(4-chlorophenylsulfonyl)-7-azido-2-heptyl]-O'-succinimidyl and O-[1-(4-methylphenylsulfonyl)-7-azido-2-heptyl]-O'-succinimidyl are chosen as the linker for the first generation of the carriers since their half-lives are 36 h and 150 h respectively, which is suitable for research purposes.

In summary, this introduction has provided a comprehensive overview of using cytokine as therapeutics for cancer treatment. To avoid systemic toxicity, researchers have developed different localization methods. The most common one is covered in the introduction section. Despite the success that are seeing in the pre-clinical trials, a universal method for delivery of cytokines to solid tumors has not yet been found. This thesis aims to design and develop a new delivery vehicle that is easy to synthesize, affordable and has the potential to be used with some FDA approved therapeutics such as CAR T cells. The following section would cover the methodology that the main results obtained.

Chapter 2

Goals and Objectives

Goals

Design polymer-cytokine conjugates that sequester cytokines within collagen matrices with tunable cytokine release to control the duration of action, providing a tool for the alteration of cytokine activities with extracellular matrices.

Objectives

1. Synthesis of therapeutic carriers comprised of a polymer-protein conjugate composed of a low-fouling polymer, collagen-binding domain, and therapeutic proteins grafted with tunable hydrolytic linkers to release unmodified proteins.
2. Evaluate the binding affinity of the therapeutic carrier through Biolayer Interferometry (BLI) and diffusion studies within collagen hydrogels.
3. Evaluate the biological activity of the carriers with appropriate cells (E.g., T cells for IL-15) and in the presence of BT935 cancer cells.

Chapter 3

Introduction

During the past decades, researchers have demonstrated the key role of the tumour microenvironment and the complex interactions within it in the cancer research field.⁸² Tumour microenvironment (TME), consisting of immune cells, the extracellular matrix, blood vessels and other cells, like fibroblasts, is the ecosystem surrounding the tumour cells and contributes to tumour progression.^{83,82} After abnormal and older cells grow and multiply when they shouldn't; they start to secrete cytokines and chemokines, which would efficiently attract stromal cells, immune cells and vascular cells. Following that, these recruited cells release growth factors⁸⁴, VEGF⁸⁵, Matrix Metalloproteinases (MMPs)⁸⁶, extracellular matrix (ECM) components that promote cell division and remodel tissue structure.⁸⁵ Ultimately, TME gives all the nutrients a tumour needs and provides the space needed for tumour expansion. All these would result in enhancement in proliferation and metastatic capability.⁸² These unique features at TME demonstrate the presence of an opportunity to treat cancer through immunotherapy, specifically through cytokine administration that promotes inflammation response instead of immunosuppressive response.² Through this work, we developed a polymer-conjugate that modulates TME by delivering cytokines that can be used for solid tumors.

Within cancer immunotherapy interleukins and interferons have been studied extensively and have some promising results. Interleukins (IL) are a cytokine produced by immune cells and are involved in activation and differentiation of them.⁸⁷ Interleukins are a broad class of proteins that bind to high-affinity receptors on the surface of cells to cause a variety of responses in those cells and tissues. Some examples of Interleukins used for cancer immunotherapy are IL-2, IL-12

and IL-15. IL-2 promotes T and NK cells' development, proliferation, differentiation and activation. IL-12 causes the induction of Th1 cells and enhances the production of interferon-gamma. IL-15's main effect is on the proliferation of T cells, B cells, NK cell memory and CD8+ T cells. T cells kill cancer cells through adaptive immunity, in which each T cell develops a T cell receptor that recognizes specific antigens on the tumour cell surface.⁸⁸ IL-2 promotes differentiation of CD4+ T cells into Th1 cells, giving it a greater potential than IL-12, which is a stronger inducer of IFN- γ production.⁸⁹ This is while IL-15 supports the development of central memory T cells and NK cells, which would provide longer immunity against cancer cells through both the innate and adaptive immune systems.⁹⁰ Interferons are the other type of cytokines that can regulate inflammatory response against tumor cells. Macrophages, which IFN- γ activates, fight cancer through innate immunity, including pathogen phagocytosis and antigen presentation.²¹ They can comprise up to 50% of the tumour mass and either promote or inhibit tumour progression. As a result, in this work, we are trying to deliver IL-15 and IFN- γ to overcome immunosuppressive TME and provide long-term immunity against tumour recurrence. Despite The promising results, cytokines are still not widely used due to their systemic toxicity effect and limited efficiency. Due to their short half-life and in vivo enzymatic proteolytic breakdown, high dosages must be given frequently which would not be possible due to the cytokine release syndrome danger.⁹¹ That is why localized delivery methods are required for using cytokine as treatment. Localization of cytokine delivery maximizes the therapeutic efficiency while minimizing the potential side effects.

The most common and efficient localization delivery method for tumours has been the development of biodegradable implants or delivery devices. Polymeric nanoparticles (PNPs) are one of the examples of these materials. PNPs are particles made of polymer that can deliver

cytokines through absorption, encapsulation or conjugation either within or on the surface of PNPs.⁴³ Poly(lactic-co-glycolic acid) (PLGA) is the most effective polymeric biomaterial when it comes to controlled drug delivery methods.⁴⁴ In a study run by Chen, Y. et al. chitosan nanoparticles encapsulated in recombinant pcDNA3.1-dsNKG2D-IL-15 were developed. Administration of these particles demonstrated shrinking in the size of the tumour over time.⁴⁸ While this field continues to grow, there are still concerns about their clearance mechanism and the chance of their accumulation in the circulatory system.⁴⁹ Hydrogels are another type of localized delivery system for cytokines. Hydrogels are made of cross-linked hydrophilic polymeric networks which swell due to water absorption.⁵⁰ These materials can be implanted in the TME and deliver cytokine.⁵¹ In a study, IL-2 and IFN- γ were co-delivered through injectable thermosensitive polypeptide hydrogels of poly(ethylene glycol)/poly(γ -ethyl-L-glutamate) (PEG/PELG) block copolymers for local treatment of melanomas.⁵⁴ B16F10 xenograft mouse models were used to monitor the therapy effect, and it was seen that melanoma was significantly suppressed in these models. Although many promising results are obtained from using hydrogels as the local delivery system, their design complexity limits their access. Liposomes are another localized delivery system for cytokines. Liposomes are spherical lipid carriers synthesized through emulsifying natural or synthetic lipids.⁵⁶ Liposomes demonstrate high biocompatibility, are easy to synthesize and demonstrate high drug-loading efficiency. In a study, transforming growth factor- β (TGF- β) inhibitor and IL-2 were co-delivered by using nanoscale liposomal polymeric gels (nanolipogels; nLGs).⁵⁷ TGF- β is an immunosuppressive factor released by the tumour cells. Delivering a small hydrophobic inhibitor simultaneously with IL-2 using nLGs delayed tumour growth and increased survival. Despite all these great results, their massive production is still impossible due to their

high production cost. Considering all these factors, new delivery vehicles with fewer steps, are easy to access and have high solubility might increase drug delivery efficiency.

Previous studies demonstrated that the targeted delivery of checkpoint inhibitor (CPI) antibodies and the cytokine interleukin-2 (IL-2) using a collagen-binding domain (CBD) could keep the therapeutics local.⁹² Collagen is primarily a scaffold of adhesion for cells in the TME.⁷⁰ It has been proven that collagen type 1 is overexpressed in the TME of solid tumours. Therefore, collagens type 1 can be used as a scaffold for therapeutic carrier immobilization. In this study, a new cytokine delivery vehicle with a collagen-binding domain is designed, synthesized and characterized. These vehicles are a co-polymer conjugate to IL-15 and IFN- γ that binds to collagen type 1 via anti-collagen peptide. The linker between the cytokines and copolymer is biodegradable, which provides a clearance mechanism for these carriers. Ultimately, a tunable releasing rate that maintains therapeutic concentrations and minimizes systemic toxicity would be obtained by using these carriers. Therefore, a lower concentration is required to achieve the necessary TME modification, which would avoid systemic toxicity side effects seen in cytokine therapies.

Poly(carboxybetaine-co-3-aminopropyl)methacrylamide (pCB-APMA) was chosen as the copolymer for the backbone. pCB is common for drug delivery applications due to its high biocompatibility, antifouling properties, and low protein adsorption. A tunable functional group on this polymer was needed to graft cytokines and anti-collagen peptides. For that purpose, a pCB-APMA copolymer was synthesized. N-(3-Aminopropyl)methacrylamide (APMA) has a pH-responsive primary amine group and a functional group for conjugation. Strain-promoted Azide - Alkyne Click Chemistry (SPAAC) reaction is used for conjugating the cytokines and anti-collagen

peptide to the copolymer. This therapeutic vehicle has the potential to improve clinical translation and localize cytokines at the injection site. Additionally, by using a degradable linker the duration of action of the cytokines is controlled in this technique. These delivery vehicles can be easily incorporated with the local injection of immunotherapeutics, such as CAR T cells, and, therefore, design soluble formulations which allow for simple combinations.

Results

3.1 poly(carboxybetaine-co-3-aminopropyl)methacrylamide (pCB-APMA) Synthesis

The procedure was adapted from literature methods.⁹³ CBMAA (1.0 g, 4.4 mmol) and APMA-HCl (87 mg, 1.1 mmol) were dissolved in acetate buffer (1 M, 4.72 ml, pH 5.2). Separately, 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid (6 mg, 22 μ mol) was dissolved in dioxane (944 μ l) and added to the CBMAA-APMA solution for a combined 1 M monomer concentration in an acetate-dioxane (5:1) buffer. The solution pH was adjusted between 3 and 4, followed by adding ACVA (3.1 mg, 11 μ mol) to the reaction solution. The reaction solution was transferred to a Schlenk flask and degassed with three freeze pump thaw cycles under N₂. Polymerization was initiated and conducted by placing the Schlenk flask in a 70 °C oil bath overnight. The reaction was quenched upon exposure to air, dialyzed (MWCO 12–14 k) against acidic water (pH ~3) for 4 d, and lyophilized to yield pCB-APMA. CB and APMA mole fractions in pCB-APMA were determined to be 87:13 by ¹H NMR analysis in D₂O. Gel permeation chromatography (GPC) confirmed that the size and polydispersity of the copolymer are 31 kDa and 1.2, respectively.

3.2 pCB-APMA-DBCO Synthesis

The procedure was adapted from literature methods.⁸⁰ pCB-APMA (30 mg) was dissolved at 30 mg mL⁻¹ in dry methanol (1 mL) and reacted with NHS-DBCO (9 mg, 1.2 eq. relative to amines) and triethylamine (9 uL, 3 eq.) overnight at room temperature. Polymers were purified by dialysis (MWCO 12 -14k) against water at pH ~3 and lyophilized to yield white powders. ¹H NMR confirmed production of pCB-APMA-DBCO (**Figure 3.1**).

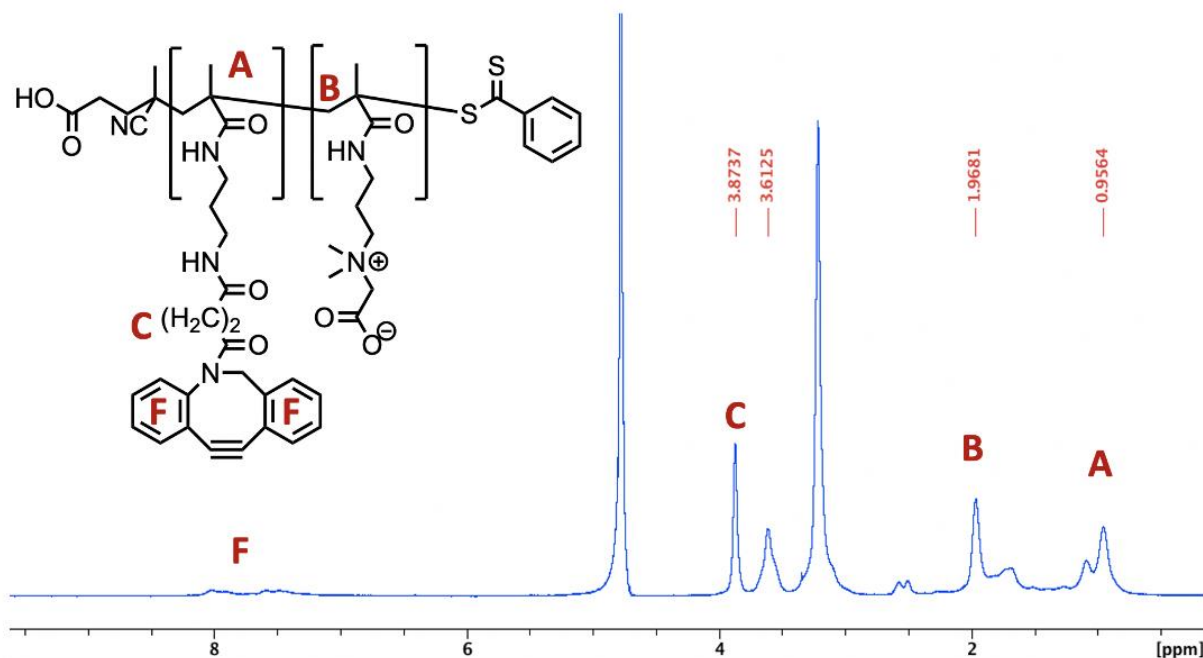
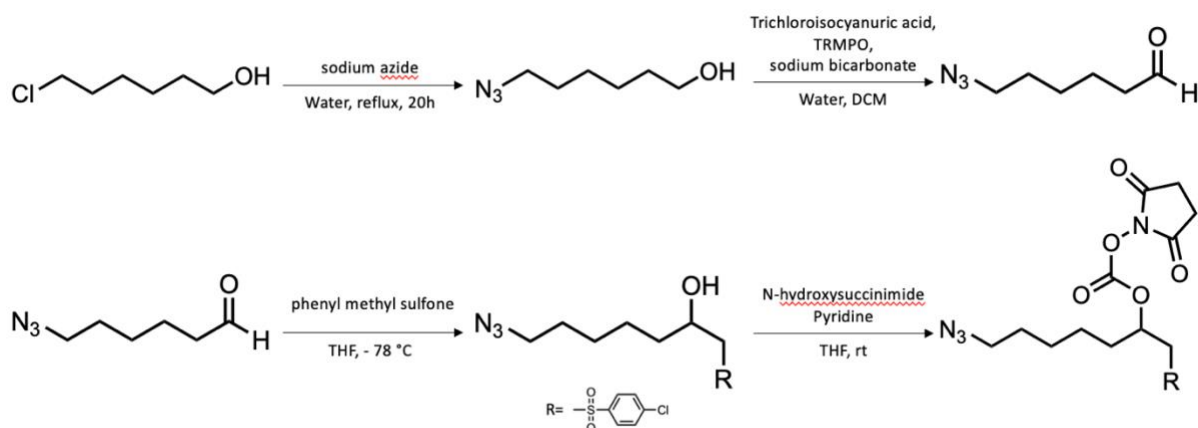


Figure 3.1. NMR spectrum of the pCB-APMA-DBCO. The NMR confirms the presence of DBCO in the final structure.

3.3 1-(4-Chlorophenylsulfonyl)-7-azido-2-heptanol synthesis

The procedure was obtained from literature methods.⁸¹ It is a 4-step reaction, which is demonstrated in scheme 1. The first three reactions of the sequence have been done successfully. The final product is colourless oil (1.49 g, 4.5 mmol, 90% yield) ¹H-NMR (400 MHz, d₆-DMSO):

δ 7.90 (2H, d, J = 8.8 Hz), 7.70 (2H, d, J = 8.8 Hz), 4.83 (1H, d, J = 6 Hz), 3.86 (1H, m), 3.39 (2H, m), 3.29 (2H, t, J = 6.8 Hz), 1.2 ~ 1.5 (8H, m).



Scheme 3.1. The reaction sequence for synthesis of O-[1-(4-chlorophenylsulfonyl)-7-azido-2-heptyl]-O'-succinimidyl carbonat

3.4 IL-15 Expression and Purification

Expression

The procedure was adapted from literature methods.⁹⁴ The IL-15 expression plasmid was transformed into chemically competent BL21 (DE3) star cells. The standard procedure for induction of the target protein using isopropyl thiogalactoside (IPTG) was followed. The selected colony was inoculated in a 100 ml Luria Broth (LB) media and grown overnight at 37 °C. Transformed cells were used to inoculate 1 L culture until the A600 reached 0.6. IPTG was then added to 0.4 mM, and incubation continued overnight. The cells from the 1 L culture were harvested by centrifugation and stored as frozen pellets at 80 °C.

Purification of IL-15 material from insoluble protein

The procedure was adapted from literature methods. The isolation of the protein was based on the literature procedure. The clarified solution containing the isolated IL-15 was loaded onto a 25 ml Ni-NTA column (Qiagen) at room temperature, previously equilibrated with 6 M GuHCl, 100 mM NaH₂PO₄, 10 mM Tris (Buffer A). After adding the sample to the column, 4 x 20ml of wash using Buffer A was done. This step was followed by washing the resins with 1 x 15 ml 6M GuHCl, 200 mM Glacial Acetic Acid (Buffer F) and eluting it. An aliquot from the collected buffers F was taken and analyzed by SDS- PAGE. SDS-PAGE confirmed the size of the expressed protein to be about 13 kDa, which is the expected size of IL-15.

Refolding of IL-15 protein

The literature review demonstrated that 100mM Tris-HCl, 0.5 M glycine, 1 mM oxidized glutathione, 10 mM reduced glutathione, pH 8.0 at 4 °C were the best conditions for folding the denatured IL-15. 1 ml of the solution with a concentration of 4.7 mg/ml was dropwise added to 46 ml of folding buffer to reach the 0.1 mg/ml concentration. The mixture was slowly shaken overnight at 4 °C. The precipitate was separated from the solution using a centrifuge. Then, the protein was concentrated using a spin column by centrifugation at 2400 g, 4 °C to reduce the volume to 4 ml.

T-cell assay

Folded IL-15 was diluted to the desired concentration in the PBS buffer, and cells previously resuspended in fresh media were added to a final concentration of 50,000 cells/well in

a final assay volume of 200 μL /well. After 24 h at 37 $^{\circ}\text{C}$ in 5% CO_2 /95% humidity, cell viability was detected using Alamar Blue. The response curve demonstrates that IL-15 increases T cell proliferation. The highest activity was seen at 0.1 nM concentration of the IL-15 (**Figure 3.2**).

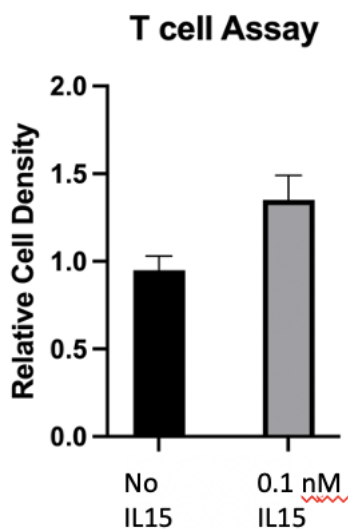


Figure 3.2. Relative T cell concentration after running T cell assay. Adding 0.1 nM Folded IL-15 increased T cell proliferation by about 33%. Cell density $< 5 \times 10^4$ cells/well. The study was conducted in triplicate.

The folding step has a low yield. Therefore, IL-2 and IL-12 folding buffers were examined as alternative buffers to increase the reaction yield. None of the tested buffers formed the correct conformation of the IL-15, which was confirmed using a T-cell assay.

3.5 IFN- γ Expression and Purification

Expression

The procedure was adapted from literature methods. BL21 E. coli were transformed with the recombinant vector. For recombinant protein expression, 1.8 L cultures of E. coli were grown

in 47.6 g/L Terrific Broth and 100 mg/mL ampicillin (Bioshop) at 37 °C to an optical density of 0.8. Expression was induced with the addition of 190 mg/mL IPTG. After 4 h, the cells were harvested.

Purification and refolding

The procedure was adapted from literature methods. Harvested *E. coli* were centrifuged, and the pellet was resuspended in denaturation buffer (6 M GuanidineHCl, 100 mM NaH₂PO₄, 10 mM Tris, 10 mM imidazole, pH 8.0) and rocked overnight at RT. Standard Ni-column purification was used to isolate the protein. The resulting protein solution was placed in 3000 Da MWCO dialysis membranes for renaturation. Recombinant IFN- γ was first dialyzed against 0.02 M TrisBase, 0.15 M NaCl, 0.2 M GuanidineHCl, 0.5 M arginine at pH $\frac{1}{4}$ 7.5 overnight at RT, followed by dialysis against 0.05 M TrisBase, 0.15 M NaCl pH $\frac{1}{4}$ 7.5 for 4 h at RT. The solution was then concentrated. The biological activity of the synthesized protein and IFN- γ carriers were examined simultaneously.

3.6 Therapeutic Carrier Synthesis

Labelled IFN- γ -azide synthesis

A 1 ml portion of 0.15 mg mL⁻¹ IFN- γ in PBS was reacted overnight at 4 °C with 90 μ L of 1 mg mL⁻¹ NHS-azide dissolved in DMF. IFN- γ -azide was then purified by dialysis at 4 °C over two days with three exchanges. IFN- γ -azide was further modified with Alexa 488 NHS ester dye. Briefly, 20 μ L of 10 mg mL⁻¹ Alexa-fluor 488 NHS ester dissolved in DMF was added to IFN- γ -azide (0.15 mg mL⁻¹) and incubated for 24 h at 4 °C in the dark. The reaction mixture was dialyzed against PBS (MWCO 12–14k) in the dark at 4 °C over 3 days with four exchanges.

Labelled anti-collagen peptide (AP) synthesis

Briefly, 6 μL of 10 mg mL^{-1} Alexa-fluor 647 NHS ester dissolved in DMF was added to anti-collagen peptide (0.05 mg mL^{-1}) and incubated for 24 h at 4 $^{\circ}\text{C}$ in the dark.

Conjugation of the components to pCB-APMA-DBCO

A 150 μL portion of 5.2 mg mL^{-1} of pCB-APMA-BDCO in PBS was reacted with 800 μL of the 0.15 mg mL^{-1} IFN- γ overnight at 4 $^{\circ}\text{C}$. The following day, a 75 μL portion of 0.67 mg mL^{-1} anti-collagen peptide in PBS was added to the reaction mixture and the mixture was shaken overnight at 4 $^{\circ}\text{C}$. The reaction mixture was purified using HPLC. The first peak demonstrated the presence of both fluorescent labels (**Figure 3.3**). The vials corresponding to that peak were isolated, and the mixture was concentrated using VivaSpin20 10 kDa falcon tubes.

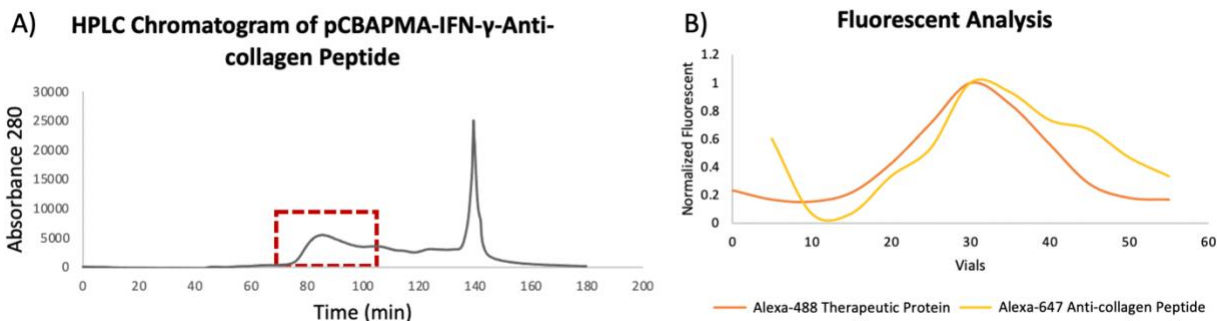


Figure 3.3. After grafting, HPLC and Fluorescent analysis results of the reaction mixture demonstrate completion of the conjugation reaction between pCB-APMA, IFN- γ and anti-collagen peptide. A) the first peak demonstrated the most extended size. Therefore, those vials must contain the vehicles. B) Fluorescent analysis demonstrates the presence of both Alexa 488 and 647 that labelled therapeutic proteins and anti-collagen peptides, respectively.

3.7 Biological Activity

IL-15 carriers

IL-15 carriers were diluted to a series of desired concentrations in the PBS buffer, and cells previously resuspended in fresh media were added to a final concentration of 50,000 cells/well in a final assay volume of 200 μ L/well. After 24 h at 37 $^{\circ}$ C in 5% CO₂/95% humidity, cell viability was detected using Alamar Blue. The response curve demonstrates that IL-15 increases T-cell proliferation (**Figure 3.4**). The higher the concentration of the carriers, the higher the cell proliferation.

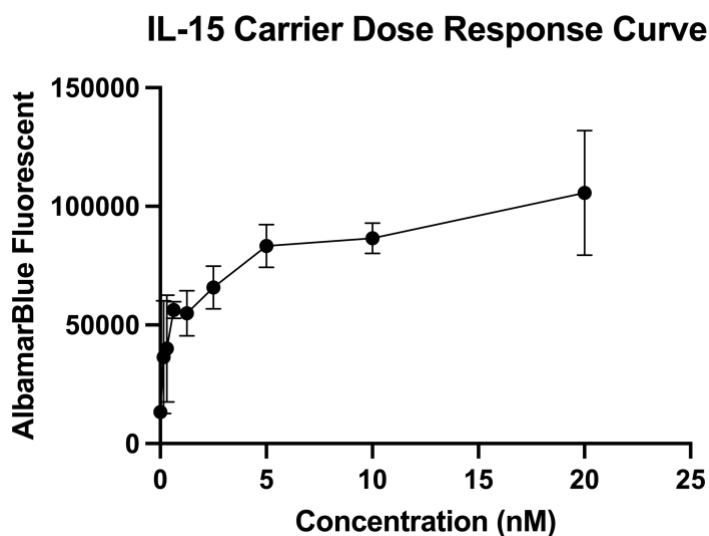


Figure 3.4. Dose-response curve for the pCB-APMA-IL-15-anticollagen peptide carriers. The higher the concentration of the carriers, the higher the T-cell proliferation.

IFN- γ carriers

The THP1 differentiation to M1 macrophage procedure was adapted from literature methods.⁹⁵ Briefly, THP 1 cells were collected, centrifuged, and resuspended in RPMI media.

PMA dissolved in DMSO (100 nM) was added to THP 1 cell at 2×10^5 cells/mL. The cells were allowed to differentiate for three days. Differentiated cells left the cell suspension and adhered to the plate. After differentiating with PMA, a mixture of IFN- γ (50 ng/ml) in media was created, and the cells were treated for two days. The phenotype of the cells was examined and compared with the literature (**Figure 3.5**). The phenotype comparison confirmed the differentiation of M0 to M1 macrophages. Despite phenotype comparison, cytokine concentration in the cell media was analyzed and compared with the literature. It is known that M1 macrophages secrete inflammation-promoting mediators such as IL-1 β and TNF, while M2 macrophages secrete anti-inflammatory cytokines such as IL-10 and IL-13.⁹⁶ Eve technology analysis demonstrated increased IL-1 β , TFN, IL-10 and IL-13 concentrations (**Figure 3.6**). Therefore, it is most likely that both M1 and M2 macrophages are present in the final sample. Therefore, further analysis by flow cytometry is required to confirm the M1/M2 ratio.

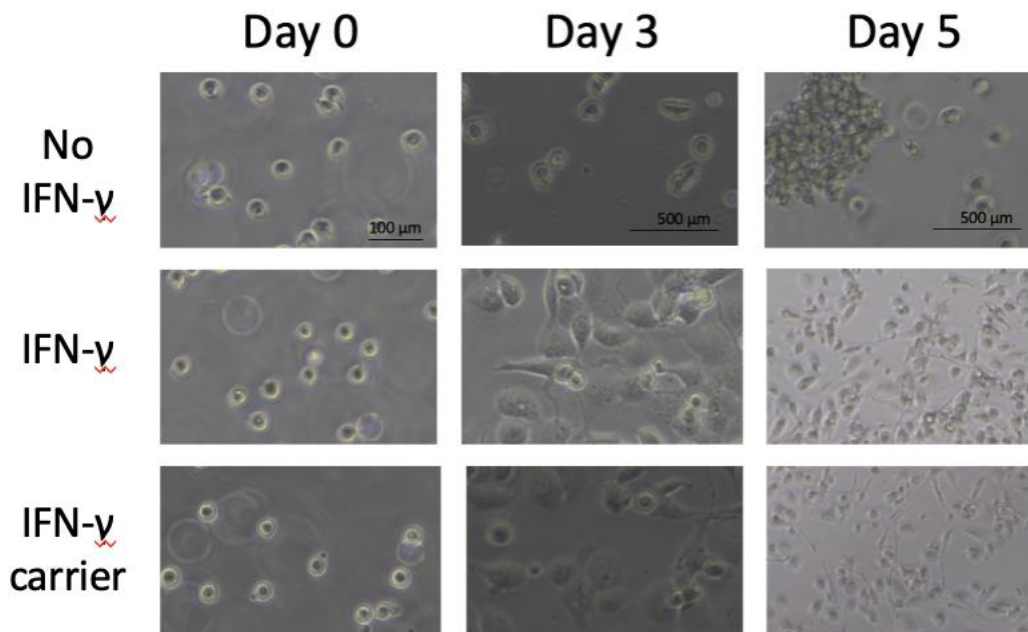


Figure 3.5. Microscopy images of the differentiated and proliferated THP1. On day 0, 100 nM PMA was added to 2×10^5 cells. On day 3, 50 ng/ml of expressed IFN- γ , purchased IFN- γ

(positive control) and IFN- γ carriers were added to the cell culture, Scales from left to right 100 μ m, 500 μ m and 500 μ m. Cells on day 5 demonstrate M1 phenotypes.

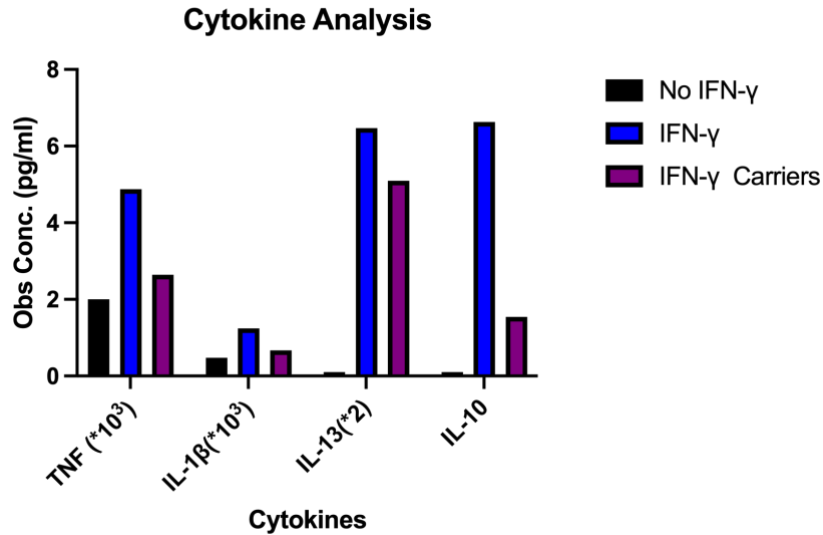


Figure 3.6. Cytokine analysis of proliferated vs non-proliferated macrophages. M1 macrophage releases IL-1 β and TNF.

3.8 Collagen Binding

Thin Cuvette

To check the binding of the carriers to collagen type 1. First, collagen (2.55 mg/ml) was cast in a thin cuvette. Then, the carriers were injected on top of the gels. Fluorescent images were obtained after 2h, 24h, and 72h. The fluorescent images showed the homogenous distribution of the carriers, which confirmed the lack of binding of the carriers (**Figure 3.7**). Next, the binding of the peptide alone was analyzed. After 72h of peptide injection dissolved in PBS (40 μ M), the gel demonstrated higher fluorescent intensity (**Figure 3.7**). This confirmed the binding of the peptide to collagen type 1.

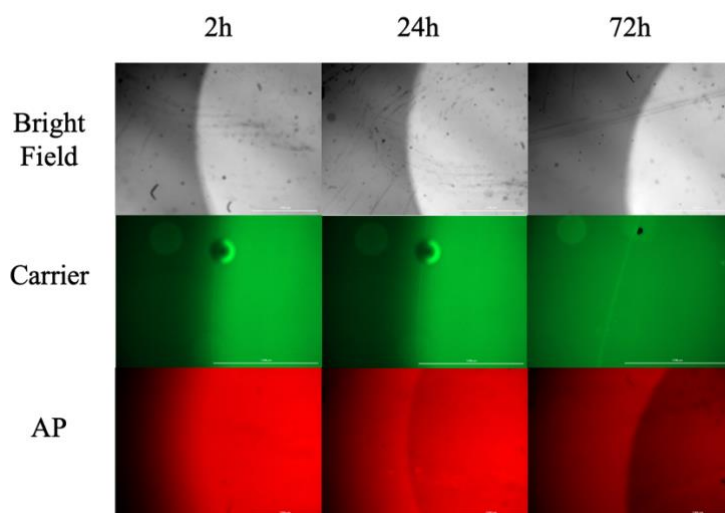


Figure 3.7. Thin cuvette comparison for the pCB-APMA-IFN- γ -anticollagen peptide carrier and anticollagen peptide (AP) binding. After 72 hours, the collagen gel demonstrates higher fluorescent intensity than the solution in the presence of AP. As a result, AP binds to collagen type 1, while the first generation of the carrier does not demonstrate binding properties.

Bio-layer interferometry (BLI)

Bi-layer Interferometry (BLI) measures the molecular interactions' kinetics and affinity.⁹⁷ In this experiment, collagen type 1 is immobilized to a Dip and Read Biosensor and its binding to anti-collagen peptide, either conjugated or free, is measured. The software would determine the K_D value by examining the shift of the interference patterns of white light reflected from a biosensor tip's surface. Using BLI binding assay, we confirmed that anti-collagen peptide (AP) binding to the collagen type 1 and the K_D value (K_D (M) = 1.08×10^{-6}) is close to the literature review value (K_D (M) = 0.86×10^{-6}) (**Figure 3.8**). However, BLI demonstrated that the carriers do not bind to collagen type 1 (**Figure 3.8**). One of the reasons for that could be the steric hindrance due to the presence of therapeutic proteins. As a result, it was hypothesized that adding PEG8 and PEG12 in between peptide and copolymer might decrease the K_D value and provide better binding.

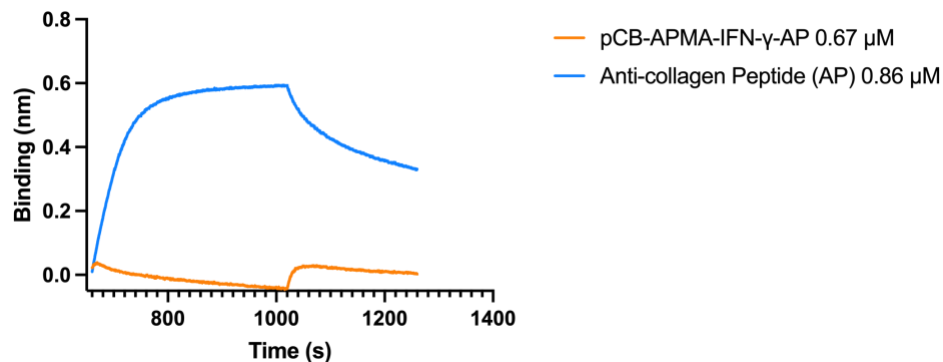


Fig 3.8. Measurement of pCB-APMA- IFN- γ -AP carrier and anti-collagen peptide (AP) binding to collagen type 1 using BLI. BLI confirmed the first generation of carriers does not bind to collagen type 1, while anti-collagen peptide (AP) binds to collagen type 1 with K_D (M) = 1.08×10^{-6} .

Addition of PEG to increase binding affinity

The procedure is the same as carrier synthesis, except NHS-PEG8/12-DBCO was used for pCB-APMA functionalization instead of NHS-DBCO. The samples containing anti-collagen peptide (AP) and PEG demonstrated precipitant formation after 24h. NMR confirmed that precipitants were the peptides. As expected, BLI shows the lack of binding of the carrier containing PEG (**Figure 3.9**).

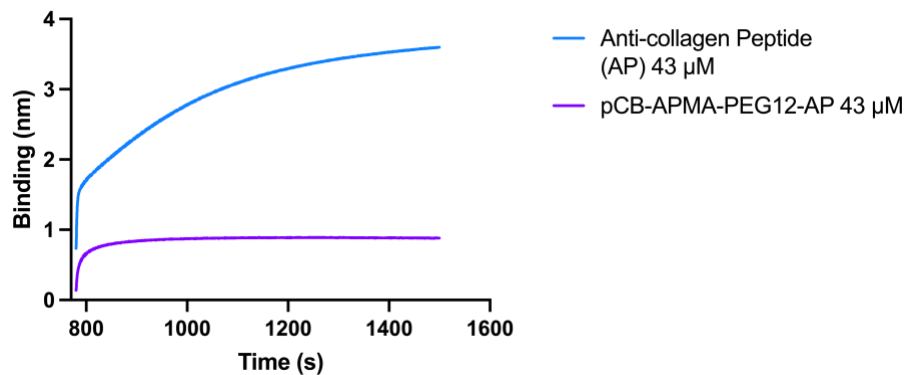


Figure 3.9. Comparison of binding of pCB-APMA-PEG12-AP and AP. The association phase of the BLI measurement confirmed the lack of binding of the pCB-APMA-PEG12-AP.

The lack of binding should be because of precipitant formation. The same experimental setup was run with a different peptide with a similar size range to AP. This standard peptide (SP) did not form any precipitant after the completion of the reaction. However, pCB-APMA-PEG-SP still did not bind to collagen, while pCB-APMA-SP did bind (**Figure 3.10**). This confirms that the presence of PEG8 or PEG12 diminishes the binding of the carrier compared to those without PEG. As a result, adding PEG was not a suitable solution for making the carriers bind to collagen type 1.

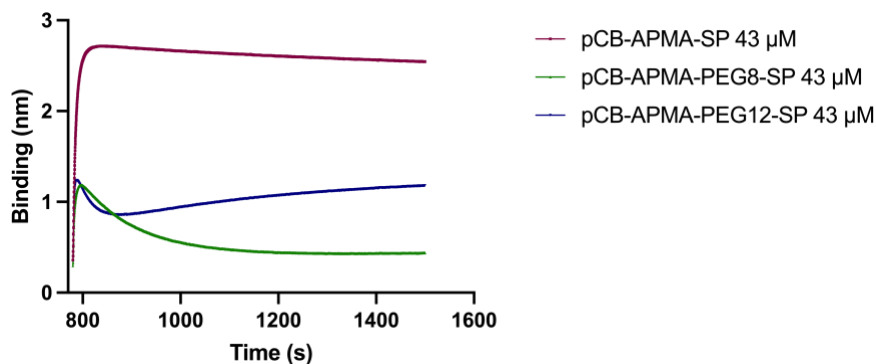


Figure 3.10. Comparison of copolymer functionalized with PEG and without PEG.

Copolymers with PEG 8 and PEG 12 as the linker between the standard peptide (SP) and the

copolymer did not bind to collagen type 1, while the copolymers without PEG did bind. Standard peptide (SP) was used for all samples.

Copolymer Size effect analysis

To examine the effect of the copolymer size on the K_D value, 14 and 31 kDa pCB-APMA copolymers were functionalized with DBCO. Then, the click reaction between the copolymer and AP was performed. The reaction mixture was purified through dialysis for three days and six exchanges at 4 °C. HPLC was used to test the purity of the samples, and it confirmed the lack of presence of free peptide. BLI was used to get the K_D value for the samples. There was no significant difference between K_D values of 14 and 31 kDa copolymers (**Figure 3.11**). The 31 kDa demonstrated the lowest K_D value. That is why it is used for future experiments.

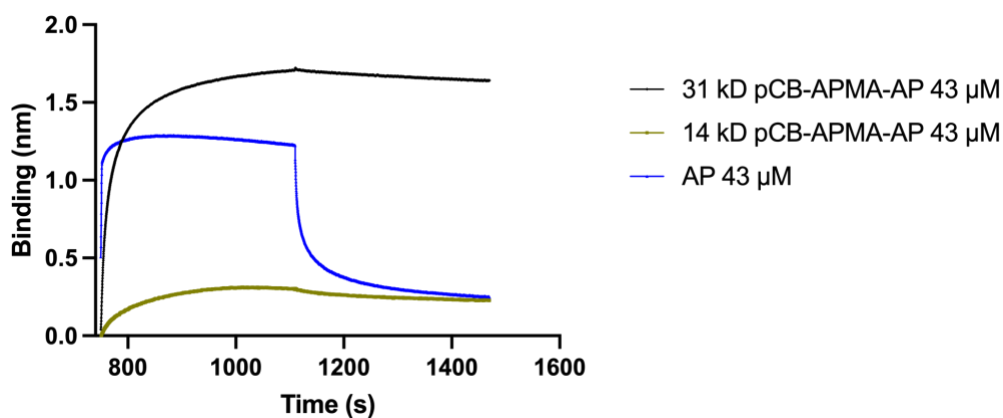


Figure 3.11. The effect of copolymer size on the binding property of the carriers. 31 kD copolymer demonstrated the lowest K_D value compared to the 14 kD copolymers. The concentration of the anti-collagen peptides (AP) was based on the UV-Vis absorbance measurement at 280 nm. All samples were standardized to have the same concentration of AP.

Table 3.1. Comparing the K_D , K_{on} , and K_{dis} values for pCB-APMA-AP made of 14 and 31 kDa copolymers

Samples	K_D (M)	k_{on} (1/Ms)	k_{dis} (1/s)
31 kD	$5.02 \times 10^{-7} \pm 0.2$	$1.46 \times 10^3 \pm 0.05$	$7.35 \times 10^{-4} \pm 0.1$
14 kD	$2.54 \times 10^{-6} \pm 0.05$	$3.71 \times 10^2 \pm 0.04$	$9.41 \times 10^{-4} \pm 0.1$
AP	$3.72 \times 10^{-7} \pm 1$	$2.69 \times 10^4 \pm 0.7$	$1.00 \times 10^{-2} \pm 0.04$

Anti-collagen peptide saturation rate effect.

To examine if the amount of the AP can provide any difference in K_D values, the pCB-APMA copolymer was saturated to different percentages with the AP. 100%, 50%, 25% and 12.5% saturation were prepared. After running BLI for all samples, it was found that the 100% saturation has a significantly lower K_D value (**Figure 3.12**). Therefore, it is hypothesized that a higher AP will provide a lower K_D value.

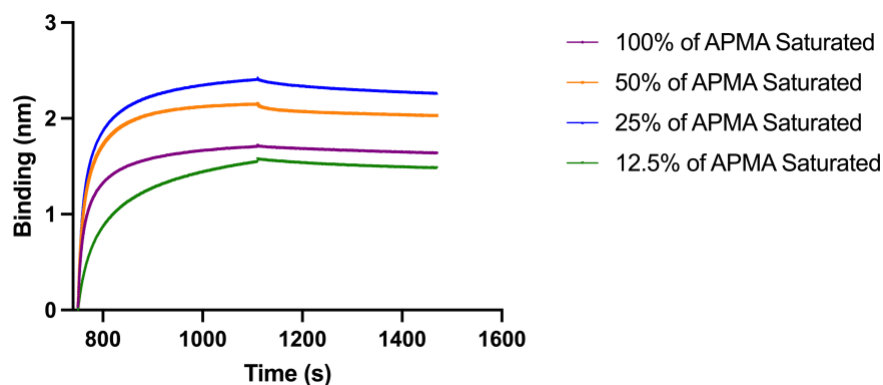


Figure 3.12. The different percentage saturation of amine groups on the copolymer effect on the binding properties. There is a significant difference in the binding properties and K_D values between different saturation percentages. 100% saturation demonstrates the lowest K_D value.

Table 3.2. Comparing the K_D , K_{on} , and K_{dis} values for pCB-APMA-AP saturated with different percentages of anti-collagen (AP) peptide

Samples	K_D (M)	$k_{on}(1/Ms)$	$k_{dis}(1/s)$
100%	$<1.0 \times 10^{-12}$	$8.84 \times 10^2 \pm 0.01$	$<1.0 \times 10^{-7}$
50%	$7.99 \times 10^{-8} \pm 1$	$9.75 \times 10^2 \pm 0.1$	$7.79 \times 10^{-5} \pm 0.9$
25%	$4.92 \times 10^{-8} \pm 1$	$9.13 \times 10^2 \pm 1$	$4.49 \times 10^{-5} \pm 1$
12.50%	$1.22 \times 10^{-8} \pm 3$	$3.38 \times 10^2 \pm 0.04$	$4.11 \times 10^{-6} \pm 0.1$

Carrier Binding

Considering all the results obtained from the previous experiment, a new batch of carriers was synthesized. The same procedure was followed, except 10X more anti-collagen peptide was added to the reaction mixture. Also, the final reaction mixture was dialyzed against the kinetic buffer before running BLI. The average signal of the first 160 s measurement obtained by BLI demonstrated a higher value than the negative control, confirming the carriers' binding. It was found that the K_D is equal to 0.204 nM (**Figure 3.13**).

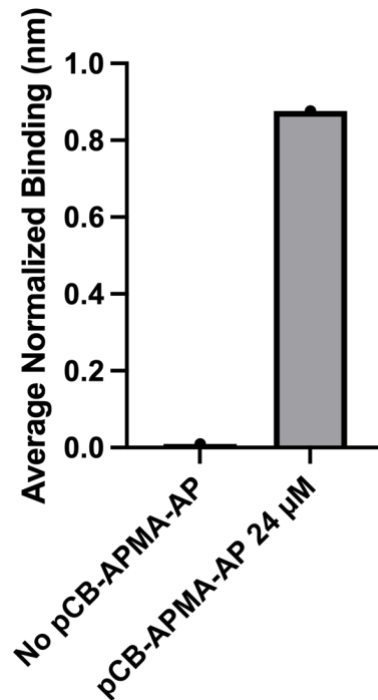


Figure 3.13. BLI demonstrates the binding of therapeutic carriers to collagen type 1. A 24 μM carriers solution sample was compared with negative control (no pCB-APMA-AP). In the presence of a carrier, the sensors demonstrate a peak rise corresponding to the therapeutic carriers' binding to the sensors.

3.9 Drug Release Assay

Conjugation of IFN-gamma to pCB-APMA-DBCO using Degradable Linker

0.205 mg of CH3 degradable linker was added to 200 μl of DMF and was mixed with 1 ml of 0.15 mg mL^{-1} IFN-gamma overnight at 4 $^{\circ}\text{C}$ with high-speed shaking. To avoid hydrolysis of the linkers and purify the reaction mixture from excess linkers, the reaction mixture was dialyzed against PBS pH 4.8 overnight with three exchanges. Then, 0.78 mg of pCB-APMA-DBCO was dissolved in 200 μl PBS and mixed with the reaction mixture to react overnight at 4 $^{\circ}\text{C}$.

Degradation

Based on the literature review, degradation starts as soon as the pH of the reaction mixture increases. The pH was increased to 7.4, and HPLC was run for the mixture immediately. The reaction mixture composition was monitored for three days by running HPLC daily while incubating at 37 °C. The first peak corresponded to the carrier's decrease over time, while the second peak increased over time (**Figure 3.14A**). This observation is confirmed after calculating the second peak area using Excel (**Figure 3.14B**).

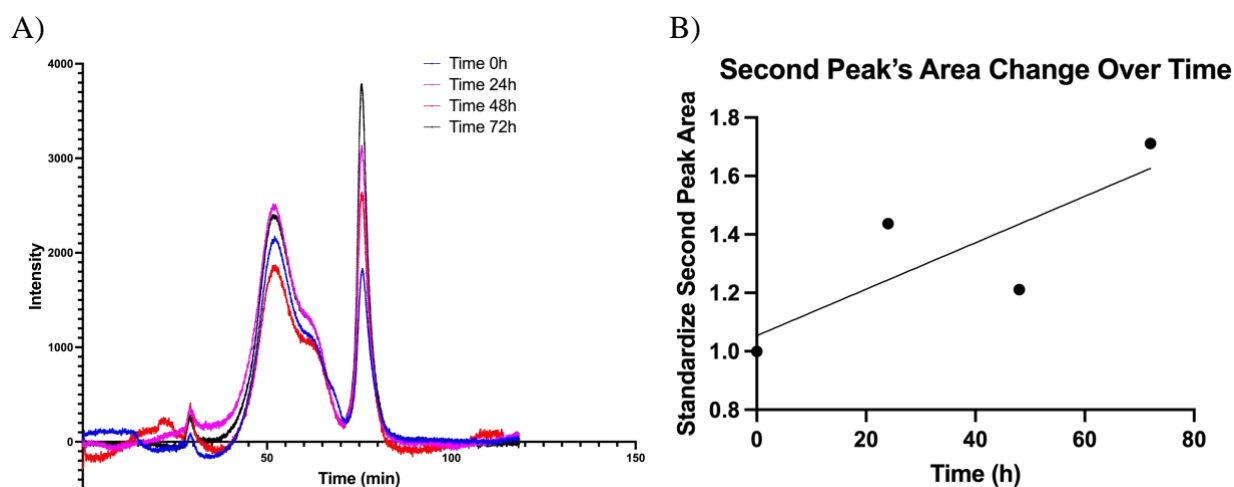


Figure 3.14. HPLC analysis of the carriers daily. A) The reaction mixture's pH was raised to 7.4. The combination was immediately subjected to an HPLC run. For the next three days, the reaction mixture's composition was tracked by executing a daily HPLC run while it was incubated at 37 °C. As time passes, the second peak rises. B) The second peak's area analysis demonstrates a positive trendline.

Discussion

Current methods of treatment for cancer treatment cannot prevent metastasis. Previous research demonstrated that the delivery of cytokines can improve the immune response toward cancer killing. Because IV injection of cytokines broadly distribute to tissues and induces systemic

side effects, local delivery was attempted to improve their pharmacokinetics and biodistribution. Hydrogel-based or nanoparticle-formulated delivery systems demonstrate improvement in pharmacokinetics and accumulation within the tumours. However, the lethal effect of normal cells and lack of knowledge about their clearance could potentially lead to neural toxicity and myelosuppression.⁹⁸ On the other hand, it has been proven that ECM in TME is very different from healthy tissue. Thus, using copolymers that actively target ECM in TME may compensate for the limitations of previously developed carriers. In this study, a pCB-APMA carrier model for cytokines was developed. Unlike previous carriers, these carriers are in the liquid phase and have the potential to degrade over time. These carriers have both cytokine and anti-collagen peptides attached to them. Subsequently, the peptide binds to the collagen type 1 after injection into the TME and converts the tumour stroma into a reservoir for chemotherapeutics. These cytokine carriers could successfully bind to collagen type 1 and significantly increase T cell proliferation. Using the degradable linker, sustained cytokine release into TME was achieved while systemic toxicity was avoided.

Regarding the component synthesis, we synthesized the pCB-APMA, degradable linker, IL-15, and IFN- γ . Although polyethylene glycol (PEG) is one of the most common polymers used for biological modification, recent human clinical trials have shown that anti-PEG antibodies can be elicited by injecting PEGylated drugs.⁹⁹ It has also been proven that PEG lowers the therapeutics' biological activity, which can be due to their potential hydrophilic networks.¹⁰⁰ However, poly(carboxybetaines) bears cationic and anionic groups, providing unique and specific properties such as antifouling, antimicrobial, vital hydration and good biocompatibility. All these properties made these polymers one of the popular choices in the biological and medical fields.¹⁰¹

The O-[1-(4-chlorophenylsulfonyl)-7-azido-2-heptyl]-O'-succinimidyl carbonat and O-[1-(4-methylphenylsulfonyl)-7-azido-2-heptyl]-O'-succinimidyl carbonat degradable linkers with 36h and 150 h half-life respectively were chosen due to their biocompatibility. However, the Cl-linker carriers could not be characterized over time, and they demonstrated material aggregation over time. That is why the linker with the methyl group was chosen for further experiments. This linker had been previously used in our lab to develop biodegradable degradable hydrogels.¹⁰² All the components were successfully synthesized, and their production was confirmed through NMR and biological assays (**Figure 3.1,3.2,3.5**). The best characterization tool available for carrier synthesis was HPLC since it separates the particles based on their size. We expected the largest particles within the reaction mixture to be the carriers that corresponded to the first peak (**Figure 3.3**). Due to the polydispersity of the co-polymer, the carrier peak was broader compared to the cytokine peak.

The biological activity of the carriers was examined using the therapeutic carriers that did not contain the degradable linker yet. Thus, IL-15 increased the proliferation of T cells while it was still attached to the copolymer (**Figure 3.4**). The IL-15 mechanism of action needs to be reviewed to understand the reason for this phenomenon. It is known that ligation of IL-15 to its receptors activates three distinct pathways: the Ras-MAPK, PI3K-AKT, and JAK-STAT pathways. Strong proliferative signals are generated through the Ras/MAPK and JAK/STAT signalling pathways, and activation of the PI3K pathway prevents cell death by raising anti-apoptotic proteins like Bcl-2 and Bcl-xL and lowering pro-apoptotic proteins like Bim.¹⁰³ Therefore, IL-15 does not require internalization within the cell, confirming the experimental results obtained. Although IFN- γ also activates the JAK-STAT pathway through interaction with

cell surface receptors, all the monocytes did not differentiate into M1 macrophages (**Figure 3.6**).¹⁰⁴ This could be explained by previous studies demonstrating that the differentiation of monocytes to M1 macrophages is concentration dependent. Although the same concentration as the literature review was used, IFN- γ being attached to the copolymer might need some adjustment regarding the concentration. Further experiments with different concentrations of carriers need to be run to determine the appropriate condition for the differentiation of monocytes to M1 macrophages using the therapeutic carriers.

Regarding the binding of the carriers to the collagen type 1, the effect of the size of the copolymer, saturation rate of the copolymer with anti-collagen peptide and addition of PEG on the binding affinity were examined to figure out the best possible formula with the lowest K_D value. Based on the results, the lowest K_D value was obtained when the copolymer size was about 31 kDa (Table 1). This could be due to the avidity of these carriers compared to the 14 kDa copolymers. The larger the copolymer, the more anti-collagen peptide is on the surface. Therefore, there is a higher chance of the multi-interaction of the carriers with collagen type 1. The addition of PEG prevented the binding of the carriers to collagen type 1 based on the BLI result (**Figure 3.10**). This was confirmed by using different peptides as well. That could be potentially due to water molecules interacting with PEG through the hydrogen bond network, potentially preventing other potential interactions.¹⁰⁵ The saturation rate of the APMA with different percentages of anti-collagen peptide demonstrated that the K_D value is significantly higher when all of the APMA functional groups are bound to anticollagen peptide (Table 3.2). As a result, adding 10X more peptide to the reaction mixture formed carriers that eventually did bind to anti-collagen type 1 (**Figure 3.13**). This could be due to steric hindrance resulting from the presence of relatively big cytokines.

Having more anti-collagen peptides on the co-polymer surface could potentially overcome this limitation.

In the drug release assay, the first peak corresponds to the carrier, while the second peak demonstrates the presence of free IFN- γ . Over time, as expected, the first peak's area decreased while the second peak increased due to the degradation (**Figure 3.14A**). Analysis of the peak area values demonstrated that the second peak overall trend has a positive slope, which confirms the release of IFN-2 over time (**Figure 3.14B**). This experiment will be further investigated over a more extended period.

In conclusion, ECM-binding vehicles for local sustained cytokine release will allow for the delivery of inflammatory factors needed to enhance cancer immunotherapies. In this project, polymer-cytokine conjugates, which bind to collagen type 1 and control the release of therapeutic proteins, were synthesized. These carriers alter local environments by utilizing the native extracellular matrix (ECM) as a drug depot for applications in cancer therapy. The synthesized carriers demonstrated T cell proliferation, which can potentially decrease the tumour size. A platform for screening the ECM binding cytokine delivery vehicles would be developed (**Figure 3.15**). This platform, made of media and the collagen layer, tracks cytokine-induced activities as a function of dosage. The bottom layer contains cancer spheroid and T cells. The size of the cancer spheroid would be monitored using microscopic images. Therefore, it allows the cancer growth to be tracked as a function of T cell proliferation and TAM activities.

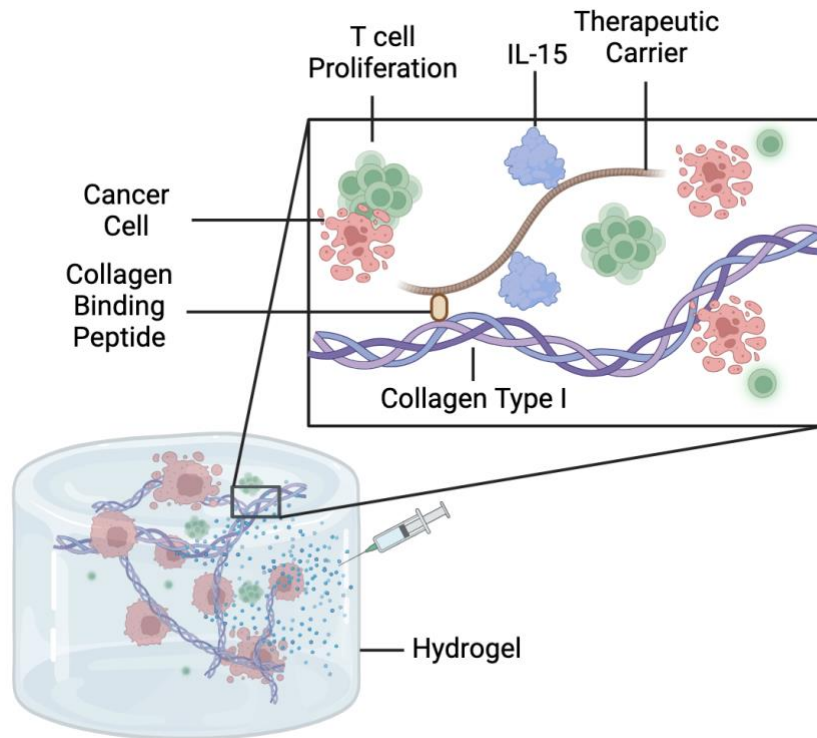


Figure 3.15. the intended platform for analysis of cytokine effect on cancer spheroids in vitro.

Spheroids and T cells would be mixed, and IL-15 carriers would be injected into the collagen gel.

The size of the spheroid over time would be monitored using a microscopic imaging tool. Created

with BioRender.com.

Chapter 4

Future directions

Previous attempts in developing local delivery biodegradable devices have focused on delivering an external substance in the cavity resulting from tumour resection. This could limit their usage, as surgery is only sometimes possible for all tumours due to their location. Additionally, cytokine usage has only been tested for untreatable tumours due to their limited clinical success and toxicity concerns. Here we developed new delivery vehicles for delivering cytokines which would stay dissolved in the solution. This allows it to be injected even into the tumours that surgery cannot remove. It can also be administered as a co-therapy with some FDA-approved therapies, such as CAR T cells. Using this technology as a carrier improves the pharmacokinetics, safety, and efficacy of the cytokines used as therapeutics.

So far, these carriers demonstrated biological activity in cell culture experiments and the potential to bind collagen type 1. As the final step before publishing the data, we want to make a 3D model in which these therapeutics are mixed with spheroid and T cells in collagen type 1. The media would be exchanged regularly on top of the gel to mimic the in vivo clearance. We expect to see higher efficiency when we are using these carriers compared to when we will be using cytokines or copolymer-cytokines without anti collagen peptides as controls.

Looking into the big picture, these carriers provide a novel tool for examining co-therapy effects. These vehicles allow the incorporation of multiple cytokines within TME, enabling combination therapy studies. Looking into the research paper, the list of cytokines examined for cancer immunotherapy is extensive; most have shown some success. In addition, co-therapy

improved efficiency most of the time. However, each one of the tumour cells has different properties, and choosing which cytokines combination would be most effective is challenging. Using these carriers in the 3D Model studies can be run in which different combinations of therapies can be used for the same cancer spheroid under similar conditions. These studies can fill the gap in cytokine words and provide a better vision of their interaction in a more complex environment.

Chapter 5

Conclusion

The design of injectable collagen binding copolymers with grafted cytokines provides a tool to alter the cytokine levels and activities at diseases when used as part of local therapies. This study demonstrated that pCB copolymers modified with anti-collagen peptides can bind collagen, and the grafted cytokines studied remain active after immobilization. Thus, a polymer-cytokine conjugate was synthesized that binds to collagen type 1 by incorporating anti-collagen peptides and controls the lifetime for the grafted cytokine through degradable linkers. Synthesis of these carriers was confirmed by running SEC analysis. T cell assays demonstrated T cell proliferation, which confirms these carriers' potential in reducing the tumour's size. BLI verified that they could be bound to collagen type 1. The release of the cytokines was shown by monitoring the carriers while using degradable linkers as a clearance technique. As the next step, a platform would be built to screen the ECM binding cytokine delivery vehicles in a 3D model in the presence of spheroids. In the next steps, a media-rich platform which contains collagen type 1, T cells and cancer spheroid in the lower stratum would be prepared to track the size of the cancer spheroid in the presence of therapeutic carriers through microscopic imaging. It is expected that these carriers will have the potential to be used for any solid tumour that cannot be removed due to their critical location. They can also be used for FDA-approved therapies such as CAR T cells.

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