A collagen-based formulation for sustained release of aflibercept

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A thesis submitted in partial fulfilment of the requirements of the University of East London for the degree of Master Research in Bioscience

MARCH 2022

Abstract

Angiogenesis is a chronic condition in wet- age-related macular degeneration (AMD) disease that is currently considered the leading cause of irreversible blindness in the elderly population. Vascular endothelial growth factor (VEGF) is a pro-angiogenic cytokine which its inhibition has revolutionised treatment of wet-AMD.

Aflibercept is anti-VEGF Fc-fusion protein, which is currently considered as a golden therapy for treatment of AMD and retinal neovascularization. Aflibercept inhibits VEGF from binding to VEGF-receptor and has a binding affinity towards all isoforms of VEGF-A family member. Intravitreal injections of Aflibercept are given every 4 weeks, followed by the regular injection every 8 weeks for several years. The frequency of injection is not convenient for the elderly patient. Hence, there is a recognised need to increase the residence time of aflibercept in the vitreous to decrease the frequency of intravitreal injection.

Different strategies to prolong the vitreous residence time of therapeutic proteins have been suggested, but these must address the challenges to maintain protein stability and ocular tolerability. One strategy is to use collagen drug delivery method to sustain the release of therapeutic protein. We have used In situ polymerized collagen (IPC) to formulate aflibercept with the aim to sustain the release of aflibercept once it's injected to the vitreous. This could potentially lead to improving patient compliance and enhanced efficacy for drug. The IPC solution could rapidly form a dense fibril structure when exposed to physiological conditions (pH 7.4, 37°C) and physiological fluids such as PBS. Initially, when sink model (no flow) was used, an increase in aflibercept release profile from 1 day to 4-5 days for the IPC formulation was observed. Rig model connected to peristaltic pump to mimic ocular vitreous flow rate (2µL/min) and volume size (200µL), was later used to examine the release profile of collagen-based aflibercept formulation. Optimization of the aflibercept-IPC formulation was performed to extend the release profile with a proper ratio of IPC/Aflibercept and incorporating Hyaluronic acid (HA). Because of the high hydrophilicity properties of HA, cross-link HA provides a high swelling factor to maintain excellent tamponade effect by reducing the initial burst release of drugs.

The aflibercept release from HA-IPC formulation achieved a zero-order kinetics and an extended release of more than 13 days was observed. Other excipients such as polyethylene glycols and surfactants e.g., Tween-20 were also used in the formulation to extend the release of aflibercept from collagen system. Our results,

however, showed no significant difference on release profile of aflibercept when PEGs and Tween-20 were used in the formulation. While more studies need to be done to fully characterise the release profile of aflibercept, our preliminary results presented in this MRes, suggest that using HA-based IPC formulation could be a promising delivery method to prolong the action of aflibercept in the vitreous.

Declaration

I, Hamid Heidari Kashkooli confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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List of Abbreviations

AMD	Age-related macular degeneration
ASRS	American Society of Retinal Specialists
BCVA	Best corrected visual acuity
BSA	Bovine serum albumin
CNV	Choroidal neovascularisation
DI	Deionised
Dex-SH	Thiolated dextran
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
HA	Sodium hyaluronate
HA-VS	Vinyl-sulfone HA
HPLC	High performance liquid chromatography
ILM	Inner limiting membrane
IPC	In-situ polymerisable collagen
IVT	Intravitreal injection
mAb	Monoclonal antibody
PBS	Phosphate buffered saline
PCL	Polycaprolactone
PEG	Polyethylene glycol
PIGF	Placental grow factor
PLGA	Poly lactic-co-glycolic acid
PSC	Pepsin Soluble Collage
RPE	A thin layer located between the choroid and retina layers of the eye
SEC	Size Exclusion Chromatography
SD	Solid dispersion
UV-Vis	Ultraviolet-visible
VEGF	Vascular endothelial growth factor

Acknowledgements

Completing my MRes has been a course where I have gained vital skills in the scientific field and a personal level. I'd like to express my deepest thanks to the people who have been made it possible for me. First, I am extremely grateful to my research supervisor, Dr. Hanieh Khalili for her invaluable advice, continuous guidance, and patience during my MRes study. No words can express how thankful I am for her support not only to complete my academic research but making me the scientist I am today. I would also like to thank my second supervisor Dr Mukhlesur Rahman for his great advice on the direction of the project during the research meeting. My grateful thanks are also extended to Mr. Matthew Collins for his providing beneficial feedback, enthusiastic encouragement, the continuous input of ideas and help in experiments. I would also like to thank all lab members of University of East London specially Dr Helen Stanyon who provided training in the set-up of the HPLC system.

1.Introduction

Ocular drug delivery of biologic to the back of the eye is an important and rapidly developing field of research because of their applications in treatment of ocular neovascularisation disease such as age-macular degeneration (AMD). Vascular endothelial growth factor (VEGF) is a pro-angiogenic cytokine that plays a significant role in the formation of abnormal blood vessel and neovascularization leading to wet AMD (1,4) (**Figure 1**). Target angiogenic growth factor and cytokines by blocking the VEGF signalling pathway is being pursued to reach better inhibition of the blood vessels growth which support angiogenesis. A variety of anti-angiogenic agents including antibodies, peptides, aptamers, and small molecules have been extensively studied to block VEGF and its effect on angiogenesis. Some of these therapeutic agents have been approved by FDA for marketing and some are in clinical research phase. Anti-VEGF therapy has revolutionised the treatment of AMD, one of the most common causes of central visual loss in the ageing population, during the last 1-2 decades (2). To date 3 anti-VEGF antibody-based therapies have been clinically approved for intraocular use i.e., ranibizumab (Fab fragment, Lucentis®), aflibercept (Fc-fusion protein, Eylea®) and brolucizumab (scFV fragment, Beovu®), and 1 non-antibody-based therapy, i.e., pegaptanib (PEG-aptamer, Macugen®). Bevacizumab (an IgG, Avastin®) is also widely used off-label to treat intraocular neovascularization (3).

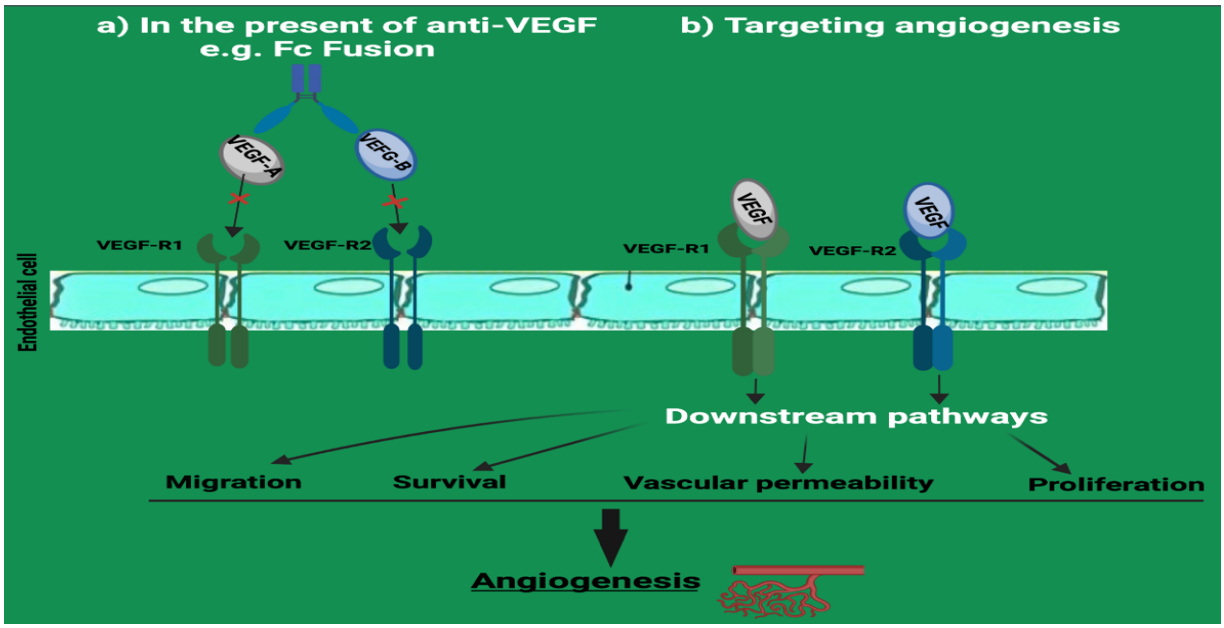

Figure 1. A) Mechanism of action of anti-angiogenesis drugs e.g., Fc-fusion to treatment of AMD disease. B) Schematic diagram demonstrating the binding of the VEGF-A/B to its VEGFR-1/2 induces downstream pathways that mediates Angiogenesis process (4).

Ranibizumab (Lucentis®, 2006) is an antibody antigen-binding fragment (Fab) that can bind to VEGF-A to inhibit binding of VEGF-A to its receptor, VEGFR2 (5). Aflibercept (Eylea®, 2011) is a Fc-fusion protein that comprises the Fc region of an IgG_1 fused to two copies of the extracellular domain-2 of VEFGR-1 linked to domain 3 of VEGFR-2 (6). Aflibercept has shown a wider binding capacity VEGF-A, VEGF-B and placental growth factor (PIGF) and higher VEGF binding affinity compared to ranibizumab (7). Brolucizumab is a unique single-chain anti-VEGF antibody fragment that inhibits all isoforms of vascular endothelial growth factor-A (VEGF-A). In comparison to a Fab fragment or full antibody, brolucizumab is a humanized single-chain antibody fragment, considered the smallest subunit of an antibody therapeutics which retains the full binding capacity to its entire target (8).

Bevacizumab (Avastin®, 2004) is a full IgG_1 that binds to VEGF-A. Brolucizumab (Beovu®, 2019) is a humanised single chain variable fragment (scFv, molecular weight of ~26 kDa) capable of binding to three isomers of VEGF-A ($VEGF_{110}$ $VEGF_{121}$ and $VEGF_{165}$) to prevent their interaction with both VEGFR-1 and VEGFR-2 (8) (**Figure 2**).

Brolucizumab has only recently been approved (10) and post marketing concerns over safety have been reported to the American Society of Retinal Specialists (ASRS) and case studies have subsequently been published (11). Hence, aflibercept, remained a current standard therapy for treatment of wet-AMD and we wish to use it in this project.

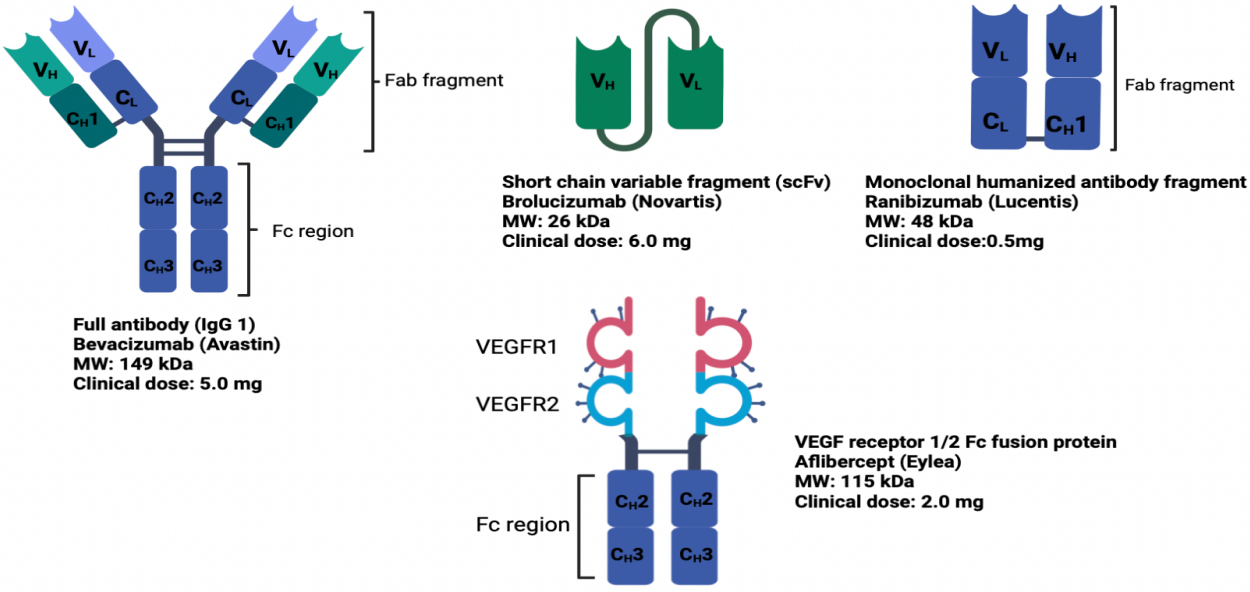

Figure 2. Different types of anti-VEGF biologics currently used for treatment of wet-AMD; bevacizumab is used off-label while other 3 antibodies are clinically approved (11).

The route of administration of these anti-VEGF antibody-based medicines are through direct injection into the vitreous cavity of the eye (posterior segment) which is called intravitreal injection (IVT). Phase III trials study of Aflibercept demonstrated that monthly injections prevent vision loss of best corrected visual acuity (BCVA) in 95% of patients. Additionally, the IV injections of Aflibercept are initiated with one injection per every 4 or 8 weeks by producing average vision gains.

The frequency of Aflibercept injection will be decrease to 4.2 injection during the second year after one year of regularly administration (13). The recommended dose for aflibercept is 2mg (0.05 mL) which is initiated with one injection per month and then carried on with injection every other month. The need for repeated intravitreal administration is difficult for patients where compliance decreases after the first year of treatment (14).

Repeated injections for all anti-VEGF antibodies carry certain risk and side effects of potential complications, including endophthalmitis, IVT haemorrhage, retinal detachment, prone to infection or local reaction and cataracts (15).

Therefore, there is an unmet need to reduce frequency of IVT injection and sustain or prolong anti-VEGF resident time in the vitreous. This clinical need will eventually enhance patient compliance and result in less side effects. There are different strategies to extend the release and prolong duration of action of the proteins in the eye. By formulating antibodies as slowly dissolving solid forms (e.g., microspheres, hydrogels, nanofibres, and nanoparticles) or suspensions, their release rate and clearance rate could be significantly reduced.

1.1. Drug encapsulation and various release strategies

1.1.1. Hydrogel-based

Hydrogel is a hydrophobic and 3D polymer network, which can maintain the large number of biological fluids and water. The biodegradable chains within the hydrogel can form a cross-linked polymer with various numbers of meshes and allow to entrap of small solute (16). The diffusion and release rate mainly depend on the mesh size and any non-covalent interaction between therapeutic agent and hydrogel. The embedded therapeutic agents (Figure 3) are entrapped within the mesh network of the hydrogel and as bulk degradation occurs, the gel is first swelled by decreasing cross-linked density, allowing the drugs to diffuse more freely.

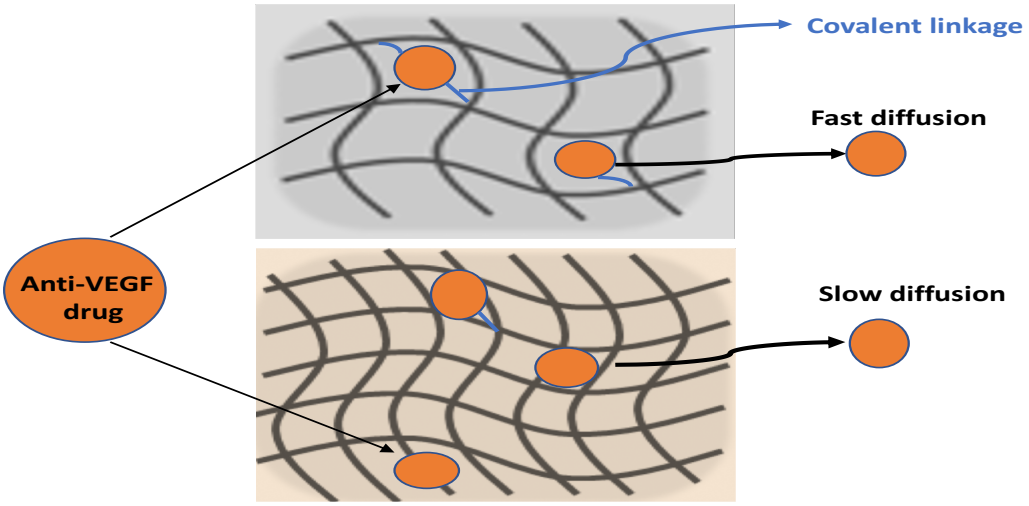

Figure 3. Schematic representation the space between polymer chains in the hydrogel network (mesh size) which can keep therapeutics via a covalent linkage. The high-water content and bigger pore sizes of gels often leads to relatively escape and rapid drug release (15).

A thermo-responsive hydrogel, such as injectable poly(N-isopropylacrylamide) based PNIPPAAm-hydrogel, encapsulated with biodegradable poly lactic-co-glycolic acid (PLGA) microspheres has been shown great promise in sustained release formulation of biologics due to their ability to change the physical state rapidly from liquid-like state to a gel above the transition temperature (~32°C) upon injection. However, the release of proteins in this model was found to be quite short (less than 2week) (17,18).

The majority of the hydrogel formulations have the rapid release issue (burst effect) due to their high-water content (>90%), lack of covalent cross-links and large pore sizes. Because the transition could be result in the expulsion of large amounts of water in the separation of the polymer phase (18). For instance, about 200 weigh percentage (%) of water expelled by heating of poly(N-isopropylacrylamide) (PNI-PAAm)-based materials from 22°C to 37°C. This rapid deswelling leads to the encapsulation of hydrophilic molecules out of the matrix which has caused a large burst release (20). As the temperature increases, the hydrophobic interaction becomes stronger, whereas the hydrogen bonding between the hydrogel networks becomes weaker. It results in precipitation of collapse within the hydrogel structure that could pose a concern for antibody stability and immunogenicity of hydrogels (21).

Hence the positive release profiles of hydrogel-based formulation, poor biodegradability, high antigenicity, and weak biocompatibility are existing as the main

limitations of most formulations. Another example is suspension of bevacizumab with thermo-responsive, injectable hydrogel by combination of vinyl-sulfone HA (HA-VS) and thiolated dextran (Dex-SH) at specific pH and temperature. The biocompatibility and slow-release profile of bevacizumab were evaluated for 6-month in vivo and in vitro. Despite having a wide range of viscoelastic, biodegradable materials such as thiol group to minimize the reactive group, but the results proved the disruption of bevacizumab molecule or inhibitory proteomic modification by native vitreous protein matrix (22).

1.1.2. Collagen-based

Collagen is the most abundant structural protein found in proportion of 80% at skin level that play critical role in maintaining of the extracellular matrix (ECM). Collagen has been considered for bio-medical use because of its biodegradability, superior biocompatibility, and weak antigenicity (23).

Generally, collagens consist of three parallel polypeptide strands (α chains), stabilized in the triple-helical structures containing (Xaa-Yaa-Gly) in repeating sequences with Proline (Pro) located at X and Y (24).

Among various types of collagens appearing in the human body, Type I collagen is the most abundant and can form large, eosinophilic fibres known as collagen fibres (25). Unique Type I Pepsin Collagen (PSC) is widely used forms of collagen-based biomaterial which is extracted from cornea and bovine dermis. The telopeptide regions of collagens contain a high degree of variation in the amino acid sequence, leads to be easily recognised by the immune system (26). Solutions of diluted acetic acid containing pepsin and natural salt precipitation are involved to purify, remove most part of telopeptide (non-helical) region and decrease the solubility of collagen (26).

Atelocollagens, however, demonstrate a good safety profile and they are commonly used in the clinic for decade e.g., Zyplast® and Zyderm® collagen implant (27). As mentioned, collagen have a superior biodegradability property which undergoes to degradation process by specific gelatinases, endogenous collagenase, and telopeptide-cleaving enzymes such as MMP-1(28).

Elongated fibrils collagen consists of at least one triple helix structure that are bound together to shape a supramolecular network. Collagen, however, have poor mechanical property which can be addressed by modification or crosslinking with other synthetic and natural polymers (29). Formulations based on collagen have shown

biodegradability and biocompatibility superior to other formulations. Majority of collagen-based drug delivery have been clinically used as film. These collagen films are experimentally prepared from reconstituted atelocollagen incorporated with some drugs, for instance, tetracycline. Drugs can be loaded into collagens by covalent bonds, hydrogen bonding or simple entrapment (30). For ocular drug delivery, cross-linked collagen hydrogels have been formulated as artificial corneas.

Some limitations exist with cross-linked collagens including the use of leachable toxic crosslinkers, a short duration of action, activation of T-cells and difficult injection of the polymerised networked materials. To address the limitations of the cross-linked collagen, an in situ polymerizable collagen (IPC) which is liquid at room temperature and form fibrils upon injection at physiological conditions (temperature and pH dependence) without chemical cross linker (31) has been suggested to use as a scaffold for drug delivery. Collagen is mostly considered as a weak antigen, and it has a low risk of collagen immunogenicity due to their purity in the preparation. Collagen also has an anti-collagenase characterise which prevent them from digestion by tissue enzyme (32).

Table 1: A summary of advantage and disadvantages of using collagen-based biomaterials (33).

Advantages	Disadvantages
Most abundance protein in mammals and easily can be purified from living organisms	Pure collagen type-I is expensive
Biocompatible and Biodegradable	Small risk of antigenicity
Biological plastic due to minimal express ability and high tensile strength	Hydrophilicity that causes more rapid release or swelling
Can be formulated into various dosage forms e.g., film	Poor mechanical properties
Easy modification by utilize it functional group	Variability in enzymatic degradation in compare to hydrolytic degradation
Compatible with synthetic polymers	Issue with handling properties
Biodegradability can be controlled by cross-linking	Variability in enzymatic reaction e.g., fibre size

According to the collagen advantages listed in the **Table 1**, designing collagen for slow release of biologic would be an attractive strategy by comparing to other hydrogel-based polymers. The global number of collagen field has grown exponentially from collagen chemistry to biomaterials for tissue engineering and cell therapy during the last 40 years. For tissue engineering, collagen compositions have been developed to act as a scaffold to deliver cells (34).

1.2. Aim of this project

To solve the limitations of the crossed-linked collagen formulations, an in situ polymerized collagen which is a liquid in the presence of EDTA and then fibrilised in physiological fluid was used in this project. IPC was prepared following the protocol optimised in the patent (35). IPC is a clear, viscous, and easily injectable (via a 27 or 29G needle) solution at neutral pH. It can potentially use to formulate with drugs to sustain their release or act as a vehicle to deliver therapeutic cells to the local tissue (36). The collagen compositions are chemically treated with EDTA, sucrose D-mannitol. The pH of collagen solution is gradually increased from pH 5.0 to pH 7.0, during stepwise dialysis over 7 days. The IPC is formed based on Type I collagen and it can form fibrils upon injection due to its interaction with external stimuli, including pH, temperature, and ionic strength (37).

This study is based on the principle that the IPC could absorb a large amount of water and could turn to a polymerised, regular system from an injectable liquid by forming fibrils upon injection due to the loss of EDTA and the induction of ions and temperature (37ºC) in body fluid. (35).

The IPC is tested to be easily injectable (via a 29G needle). In practice, the liquid form IPC before the injection is evenly mixed with the drug and then the IPC polymerises upon injection because of the loss of EDTA and the presence of ions and the temperature (37ºC) (37). In this process, collagen forms fibrils and meshes are formed between the collagen fibrils (**Figure 4**). The fibrils can encapsulate the drug in the meshes to sustain the release of the drug (35).

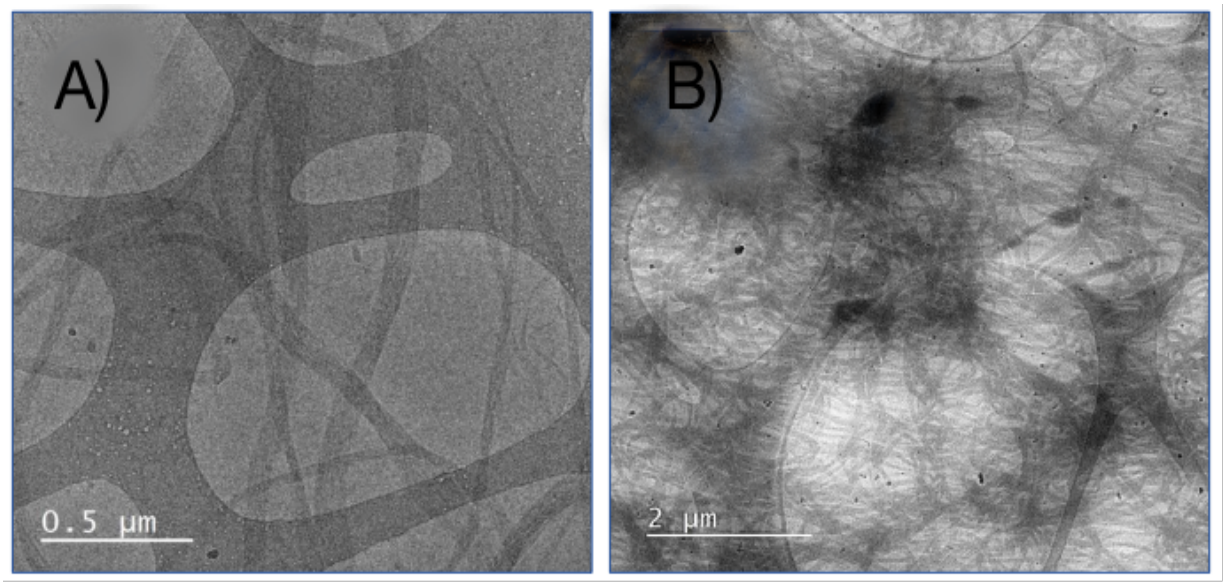

Figure 4. A) Cryo-TEM image of the fibril formation process in the IPC. and B) Fibril density and alignment Cryo-FIB-SEM image of the IPC (37).

Previous research constructed at least on layer of collagen to act as a wafer, membrane-type, and film delivery systems for use in controlled drug release (38). The IPC has not been formulated with biologics such as aflibercept which is the main aim of this project. Its usage has also not been examined yet for intravitreal injection for retinal therapy. It is a hypothesis which IPC can be conducted to act as a modified release formulation of aflibercept in sustained release system while aflibercept remain stable (38). High water content within IPC could play an important role in maintaining protein stability and avoid aggregation.

The protein could be entrapped within fibril network through mixing process or dialysis of the protein and the IPC to ensure uniform distribution of the protein in the meshes of the IPC. This could help to avoid burst release profile. Longer incubation and mixing process between IPC and protein, could result in a better encapsulation.
In addition, hyaluronic acid (HA) is proposed to use as a degradable, and safe crosslinker in the IPC/aflibercept formulation to aid with prolongation of release. HA is a predominant glycosaminoglycan which is also found in the vitreous humor and is biocompatible and biodegradable (39). High molecular weight HA could eventually reduce the penetration of biomolecules, swell in the presence of water, and increase the viscosity and tightness of the formed IPC (40). Therefore, we aim to prepare HA incorporated IPC formulation as a biodegradable delivery system to formulate aflibercept for the first time to increase its duration of action.

We assumed the mechanisms to predict the released protein from the collagen-based scaffold by using the sink and rig models. In the sink model, study is carried out without any flow rate. A constant volume of solution, released from formulation was replaced with fresh buffer solution at each collection time point and concentration of aflibercept was measured in the released solution. In a rig flow study, a flow rate of 2 µL /min (equivalent to ocular flow rate) was applied and the solution was collected into a tube every day for over 2 weeks. Then the concentration of aflibercept in both sink and rig models was determined using Size Exclusion Chromatography (SEC) system with a UV-visible detector at 280 nm.

Different types of pharmaceutical surfactant excipients such as Tween-20 and Polyethylene glycol (PEG) were also considered in the formulation to address some challenges associated with fast release of aflibercept. Tween 20 (**Figure 5**) is a nontoxic and nonionic surfactant and/or emulsifiers commonly used in pharmaceutical application to develop the solubility of the weakly soluble drugs in the certain physiological conditions, T = 37°C and pH=7.4, to enhance drug release (41). Polyethylene glycol (PEG) is a hydrophilic and synthetic polymer that is widely employed in the biomedical research due to its high hydrophilicity and anti-protein adsorption properties and biocompatibility (42).

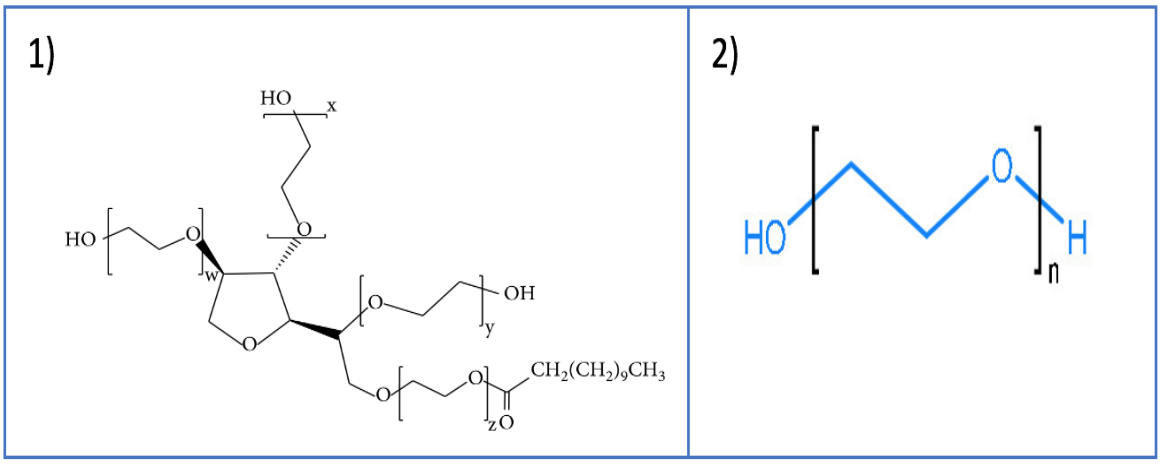

Figure 5. Chemical structure of 1) Tween20 (41) and 2) Polyethylene glycol (PEG) (42).

1.3. Research objectives

1-An IPC will be prepared following the protocol optimised in the patent (39). The collagen compositions will be chemically treated with EDTA, sucrose D-mannitol. The pH of collagen solution will be gradually increased from pH 5.0 to pH 7.0, during stepwise dialysis over 7 days.

2- Aflibercept and HA to be formulated within prepared IPC using simple mixing methods or dialysis using dialysis cassette. The mixing method need to be optimised. Also, different amount of IPC and HA to formulate aflibercept at its clinical dose (2 mg) to demonstrate zero-order kinetic release profile will be studied. The aim is to be able to sustain the release of aflibercept longer than 8 weeks.

3- Different formulation solutions to be prepared; IPC alone (control), IPC and HA alone (control), aflibercept-HA with IPC (different HA concentration) and aflibercept (without HA) formulated with IPC to study the release profile.

4- The IPC mixed with or without HA will be examined to formulate an injectable depot slow-release system for aflibercept which is designed for intraocular use.

5- Protein stability of released aflibercept to be examined using size-exclusion chromatography. If there is any aggregation or degradation, additional peak will be observed. SDS-PAGE analysis will also be used to analyse any degradation if aflibercept will show after being released from formulation.

6- Biological activity of released aflibercept will also be examined using ELISA.

2. General experimental methods:

2.1. Materials

Aflibercept (Eylea,40 mg/mL) was provided from Moorfield eye hospital, Pierce Silver Stain Kit (cat no. 24612) and 1-Step Ultra TMB-ELISA (cat no. 34028) were purchased from Thermo Scientific (U.S.A.). Sucrose (cat no. 57501), HPLC grade water (cat no. W/0106/17) and Phosphate buffered saline (PBS) tablets (cat no. BP2944) all were purchased from Fisher Scientific (UK). Ethanol (cat no. 32221), D-mannitol (cat no. M9546, MW: 182.17), ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate (cat no. ED2SS), sodium chloride (NaCl, cat no. S7653), hydrochloric acid (HCl, 1 M, cat no. 35328), disodium salt dihydrate (cat no. ED2SS), calcium chloride ($CaCl_2$, cat no. 793639), Sodium carbonate (Na_2CO_3, cat no. 13418), sodium hydroxide (NaOH, 1 M, cat no. L1980), glucose (cat no. D9434), human $VEGF_{165}$ (cat no. V7259), anti-human IgG (cat no. A0170),) and sodium hydrogen carbonate ($NaHCO_3$, cat no. 401676) were purchased from Sigma-Aldrich (UK). E Positive Displacement Pipettes (50 to 250 uL, FD1005) and Capillary Piston (50 to 250 uL, F148114 were purchased from Gilson™ Microman (USA). NuPAGE LDS sample buffer (4×, cat no. NP0007), NuPAGE 4–12% Bis-Tris gels (cat no. NP0321BOX), Collagenase (cat no. 17018-029), NuPAGE MOPS SDS running buffer (cat no. NP0001), Hank's Balanced Salt Solution (HBSS, 1×, cat no. 24020-083), and Novex sharp pre-stained protein standards (cat no. LC5800) were purchased from Life Technologies (UK). Collagen (6.0 mg/mL HCL solution, cat no. FS22004) and Type I Porcine Collagen solution (3.2 mg/mL, cat no.5169-100mL) were purchased from Collagen Solutions and Advanced Biomatrix (UK). Instant Blue (cat no. ISB1L) was ordered from Expedeon (UK). Acetic acid (cat no. A6283) was purchased from Honeywell (U.S.A.). Slide-A-Lyzer™ Dialysis Cassettes (10K MWCO, 0.5mL, cat no.10401955) was purchased from Thermo Scientific Fisher (UK). Dialysis membrane (12K MWCO) was purchased from Medicell International (UK). Hyaluronic acid sodium salt (HA, MW: 30,000-50,000, cat no. HA15M-5) was purchased from Lifecore Biomedical (U.S.A). Collagenase (lyophilized non-sterile, 500mg, cat no.17018-029) was purchased from Gibco Manufacturer's (UK). Polyethyleneglykol (M.W.3,400, cat no.20,244-4) was ordered from Aldrich (UK). Tween™ 20 Surfact (cat no.85113) was purchased from Thermo Fisher (UK). Anti-human IgG (Fab specific)-peroxidase (cat. no. A0293) was purchased from Sigma Aldrich).

2.2. Instrumentation

Ultracentrifuge machine (Eppendorf AG, 22331 Hamburg, Germany); Universal oven (Memmert GmbH, UN30, Germany); Ismatec peristaltic pump (Cole-parmer, USA); Magnetic Stirrer Hotplate (Radleys, UK); Vivaspin centrifugal (accuSpinTM Micro, Fisher Scientific, USA); The Amersham Biosciences AKTA (BOX-900, USA); HPLC Column 4 µm, 4.6X250 mm (Agilent Zorbax GF-250, USA).

2.3. Methods

2.3.1. Preparation of IPC

A neutralized, acid solubilized collagen type I (6mg/mL), that remains in solution at physiological temperatures, was used in the IPC. This soluble collagen could be extracted and purified from animal hide including bovine based on the initial method described in (38).

In order to wash a membrane, a dialysis membrane with a molecular weight cut-off (MWCO: 12-14 KDa, 10.0 cm in length) was transferred into a 500mL bottle solution which was contained of water, 2% NaHCO3 and EDTA (1.0 mM). The membrane was then stirred and heated to 80ºC within the solution. At the final stage, it was incubated for at least 30 minutes to wash the membrane. The membrane was placed into another bottle solution and it was washed 3 more times with a purified water. Then, it was kept in a container of purified water in the fridge.

Soluble Type I collagen was initially precipitated with NaCl (0.8M). To do this, 11mL of the collagen placed in the 50mL centrifuge tube and then 39mL of the NaCl (0.8mL) was added. The centrifuge tube was slowly shaken up and down until protein was flocculated in the solution and then it was centrifuged at very high speed (4600 rpm) for 30 minutes at 4 °C. The NaCl solution was slowly squeezed out and the excess NaCl solution was remained to drain from the precipitate. The solution was collected from the bottom of the tube and it was transferred into the dialysis membrane.

The solution was then dialyzed against 0.1M acetic acid to remove residual salts for 24 hours. The resulting acidic collagen solution was extensively dialyzed against disodium EDTA solutions to prevent premature aggregation or collagen fibrillogenesis. EDTA was also used to enhance the collagen formulation by inhibition of MMP enzyme which was shown as a collagen digest enzyme (38). The solution in the bottle was adjusted to pH 5.0, 5.5, 6.0, 6.5, 7.0 EDTA solution (35.0 mM, 250.0 mL) every 24h to

obtain a collagen solution with a pH value of 7.0 ±0.2. During last step of dialysis, the collagen solution was dialysed with 35mM EDTA,100mM sucrose and 3.5% of D-mannitol (**Figure 6**). Sucrose and D-mannitol were added to help the stability of viscous collagen (42). The structure of EDTA could be changed by dissociation of each hydrogen in the carboxyl group, as the pH of EDTA was increased above pH 7.0 (42).

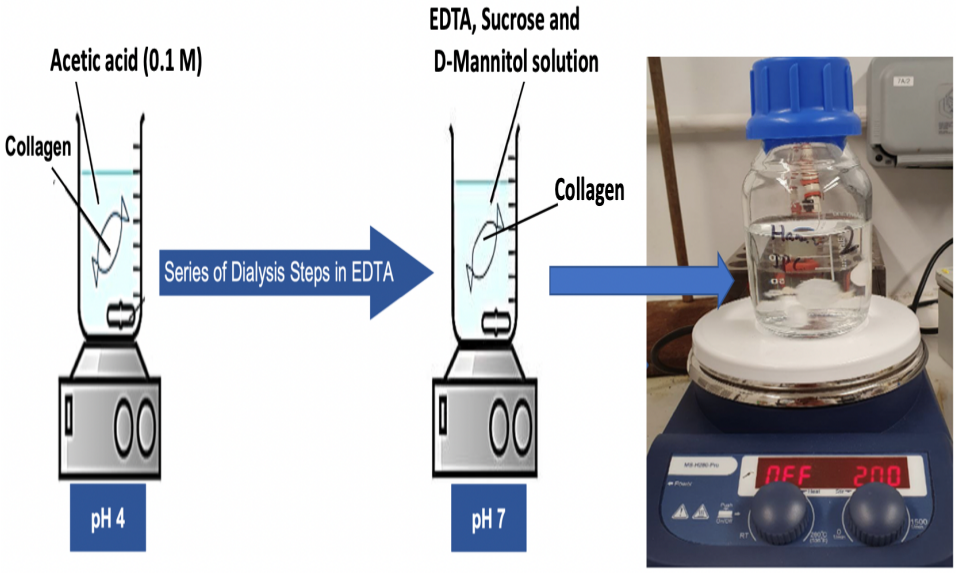

Figure 6. A schematic diagram of optimised IPC perpetration using a series of dialysis against EDTA buffer solutions by gradually increasing the pH to reach pH 7.0. The clear and transparent collagen was collected without any typical fibrillogenesis until it was contacted by physiologic stimuli.

2.3.2. Preparation of aflibercept in the IPC and IPC-HA

Initially two solutions were prepared: (1) Aflibercept with IPC, (2) Aflibercept with HA and IPC. To prepare HA solution to get mixed with aflibercept and then IPC, HA (MW: 30 kDa, 2 mg) was dissolved in EDTA buffer solution (pH 7.0, 20 µL) and allowed to stir to produce homogenous gel-like solution. Three concentrations of Aflibercept (0.24mg/mL, 0.48mg/mL and 0.96 mg/mL) were mixed with prepared HA solution using syringe-to-syringe mixing method as it shown in **Figure 7**.

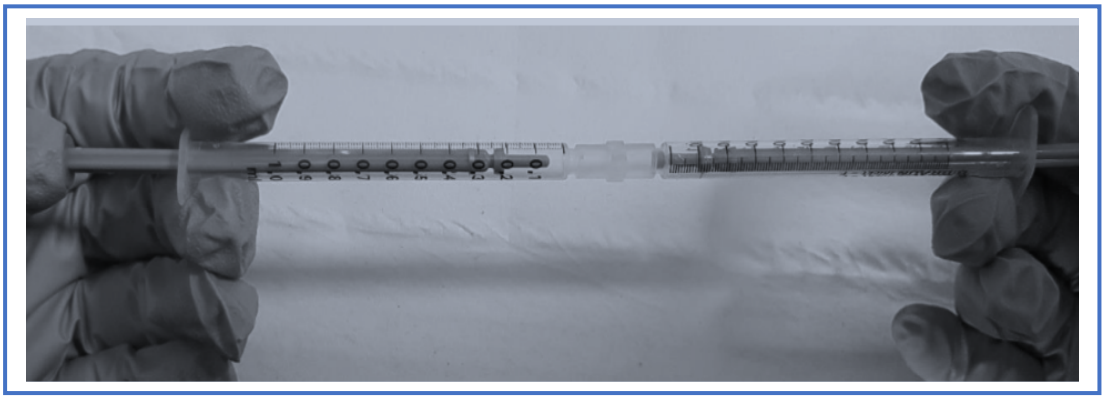

Figure 7. A visual image of the syringe-to-syringe mixing method of Aflibercept-HA by using a female luer-luer connector and two syringes.

Different amount of IPC (82 uL ,70 uL,46 uL) was then added to the combined HA and aflibercept solution to prepare overall solution of 130 uL. Dialysis was conducted using dialysis cassettes (10K MWCO, 0.5mL) against EDTA (pH 7.0), D-Mannitol (3.5%) and Sucrose (100mM) buffer solution at 4°C for 24 hours to achieve a homogenous mixture of aflibercept in IPC and HA hydrogel. After 24 hours, solutions (aflibercept in IPC/HA or aflibercept in IPC alone) were removed from dialysis cassettes and transferred into the vial. Two separate studies were conducted to analyse the release profile of aflibercept from IPC/HA delivery system; (1) Sink model; drug loaded solutions (130 µL) were transferred into the glass vial containing PBS buffer (270 µL) and stored in oven at 37^0C during experimental study. The sample (130 µL) was taken every day and replaced with fresh PBS buffer (130 µL). Solution was stirred during incubation time using magnetic stirrer.

Table 2: The sink condition was used in the investigation of aflibercept release from the HA-IPC.

Sample	**PBS buffer**		**Drug loaded HA-IPC**		
	Initial volume (µL)	**Replacement buffer volume (µL)**	**Aflibercept concentration (mg/mL)**	**IPC Volume (µL)**	**HA Amount (mg)**
Control (HA/IPC)	**270**	**130**	**0**	**114**	**2**
Tube 1 (aflibercept/IPC)	**270**	**130**	**0.24**	**80**	**0**
Tube 2 (aflibercept-IPC/ HA)	**270**	**130**	**0.24**	**60**	**2**
Tube 3 (aflibercept-IPC/ HA)	**270**	**130**	**0.48**	**48**	**2**
Tube 4 (aflibercept- IPC/ HA)	**270**	**130**	**0.96**	**24**	**2**

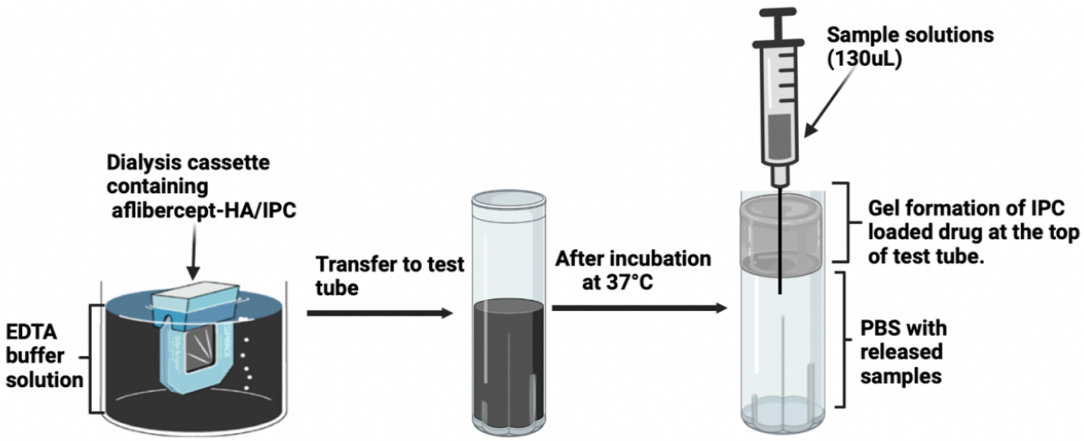

Figure 8. Simple release study of aflibercept from HA-IPC formulation using sink model.

(2) Rig model where peristaltic pump was used to apply flow rate equivalent to the vitreous flow rate (2 uL/min). In this model, rigs were kept on the heater to provide 37^0C temperature necessary for in situ forming collagen. To prepare an IPC-HA formulation based on **Table 3**, EDTA buffer (20µL, pH 7.0) was used to dissolve different HA amount of (2.0 ,4.0 and 5.0mg) and a solution of aflibercept (20 µL, 100 mg). The solution was slowly mixed with different volumes of IPC solutions (150,110,80 µL) using the syringe mixing method (**Figure 7**). The same dialysis procedure was conducted for the compositions as the sink study model. After overnight dialysis, the mixture (200 µL) of IPC-HA alone (B1), aflibercept-HA/IPC (B2), aflibercept-HA/IPC/T20 (T2 and T4), aflibercept-HA/IPC/PEG (P1) was loaded into the rig model (**Figure 9**) using a positive displacement pipette. Samples of releasing aflibercept were collected 6, 12 hours and then once daily afterwards. The samples were subsequently analysed using SDS-PAGE, Silver staining and Size Exclusion Chromatography.

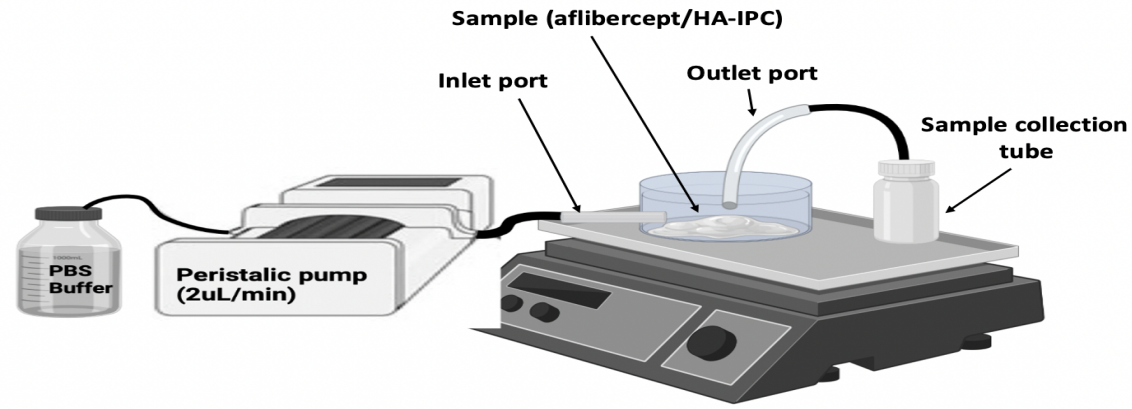

Figure 9. The rig model release study sets up. An Ismatec peristaltic pump was connected via plastic tubing to a sample cell chamber with the size of 200 µL which consist of an outlet, the well where the aflibercept-IPC samples were placed, sample collection tube.

Table 3. Preparation of aflibercept-HA with IPC for rig release study.

Formulation Optimization	Excipients						Buffer Properties	
	Batch code (n=2)	IPC (uL)	HA (mg) diluted in EDTA buffer (20uL)	Aflibercept (100mg/mL) in uL	PEG (mg)	Tween20 (uL)	PBS (uL)	Dialysis time against EDTA/ D-Mannitol and Sucrose (hours)
1-Experimental design to avoid initial burst release	B1	150	2	0	0	0	50	-
	B2	110	2	20	0	0	50	24
2- The effect of HA, Tween20 and PEGs on release rate	B1	110	4	0	0	0	70	-
	B2	110	4	20	0	0	50	24
	T2	110	4	20	0	2	48	24
	P4	110	4	20	3	0	47	24
3- Effect of dialysis time	B1	80	5	0	0	0	100	-
	B2	80	5	20	0	0	80	12
	T2	80	5	20	0	2	78	12
	T4	80	5	20	0	4	76	12

2.3.3. Drug release study using rig model

As mentioned above, rig model was used as a preferred method to study different formulation strategies to sustain the release of aflibercept from IPC solution. To mimic the retinal optical flow rate (2uL/min), we used peristaltic pump (**Figure 9**) to apply flow rate of 2 uL. The rig model was assembled by connecting the rig to an inlet and outlet. The inlet was connected directly to an Ismatec peristaltic pump that pumps PBS buffer (pH 7.0). The flow rate and dispensing volume peristaltic pump was manually calibrated and was set up at approximately 2 µL/min. The outlet was drained into a sample collection tube (7 mL vial) and sealed with parafilm to avoid evaporation at 37°C after connecting to the outlet port. The spread of the IPC solutions within the rig and then gelling formation could block the inlet and outlet ports of the rig. Thereby, the PBS solutions (**Table 3**) were immediately added to the top of the solution, when the sample (**Table 3**) was transferred into the rig.

2.3.4. Different methods used to formulate aflibercept in IPC

2.2.4.1. Formulation of aflibercept-HA/IPC to avoid initial burst release

To prevent IPC dilution, use of high concentration of aflibercept was considered in the formulation. If IPC become diluted by the protein solution, the protein release become much faster compared to when IPC is not diluted (43). The aflibercept was concentrated from 40mg/mL to 100mg/mL using a centrifugal concentrator (30.0 kDa molecular weight cut off). The experiment was carried out using centrifugal concentrators at 13,000 rpm, at 4 °C for 5minute (twice). The solution (10uL) was then diluted in PBS (990uL, 1:10) to measure and calculate the protein concentration at 280nm. The stability of antibodies was investigated using both SDS-PAGE and HPLC techniques.

2.3.4.2. The role of HA and excipients

The influence of using more HA was investigated in the formulation. Effects of excipients and surfactant were studied on development of aflibercept release. Different volume of 2uL and 4uL Tween-20 and Poly (ethylene glycol) were used in the formulation to investigate the release profile of aflibercept.

2.3.4.3. Effect of dialysis time

To ensure uniformity, the distribution of the protein in the meshes of the IPC, different dialysis time (12 hour and 24 hour) of aflibercept-HA/IPC against EDTA (pH7.0), Sucrose and D-Mannitol buffer solutions were conducted (**Table 3**) using a dialysis cassette.

2.3.5. Quantification of released aflibercept

The released aflibercept from the IPC or IPC/HA solution in the sink and the rig model were analysed using Size exclusion chromatography and ELISA techniques as described in following sections.

2.3.5.1. Size Exclusion Chromatography

Aflibercept concentration was analysed by using Size Exclusion Chromatography by constructing a standard curve for the standard aflibercept (0.125 mg/mL – 2 mg/mL). The AKTA system was set up with Column temperature (30 °C), injection volume (0.2ml/mL), wavelength (280 and 495 nm), pressure (18 MPa), Running time (8-10 minutes), mobile phase (isocratic, 100% PBS, pH 7.4) and flow rate (0.5mL/min).

2.3.5.2. SDS-PAGE

The presents of protein samples from the aflibercept-IPC formulation were monitored using SDS-PAGE. This analytical technique was established to separate and identify each component in releasing solution based on molecular weight (MW) and the migration rate. In comparison, SDS-PAGE technique of protein detection is a more sensitive qualitative test than the SEC assay (44) that can quicky evaluate multiple samples. The protein samples (20 µL) were added to the sample buffer (6 µL). The solutions were mixed using tube Rotator. Then, 10uL of each sample was loaded into precast gel. Then, the pre-stained protein (5 µL) was also loaded into the gel as a molecular weight marker. An electrophoresis tank was filled with freshly prepared running buffer (20×). The precast gel was resolved for 1 hours at 70 mA and 200 V. The gel was then stained with Coomassie blue dye for 1 hour and washed with distilled water for 1hour.

2.3.5.3. Silver Staining

Silver staining can identify any trace of protein (ng/mL) in the released solution (44). After running the samples on polyacrylamide gels, the gel was washed in ultra-pure water for 5 minutes (2×). Then, the gel was fixed in fixation solution (60% water, 30% ethanol, 10% acetic acid,) for 15 minutes, twice. It was then treated with 10% ethanol for 5minute, twice. The ethanol solution was then replaced with water for 5minute, twice. The gel was incubated in sensitizer solution (50 µL sensitizer + 25 mL water) for exactly one minute, it was then immediately washed with ultra-pure water for 1minute, twice. After this, the gel was incubated in working station buffer (500 µL enhancer + 25 mL stain) for 30 minutes. The gel was washed with pure water for 1minute, twice. The gel was then rinsed immediately with developing buffer, including 1 part of silver stain enhancer with 50 parts of silver stain developer (500 µL enhancer + 25 mL developer) for 3minute. The stopper buffer (5% acetic acid) was added for 10 minutes incubation. Finally, the gel was fixed onto polyester sheet for analysis using a Bio-Rad laboratories machine.

2.3.6. Investigation of enzymatic degradation of collagen

A collagen degradation method was primarily utilized to evaluate the biodegradability of collagen loaded with aflibercept (45). The collagen degradation assay was also used for screening the presence of aflibercept within IPC on day 7 and day 13, due to loss of detection and resolution of Size Exclusion Chromatography and SDS-PAGE. A collagenase solution was prepared by mixing 11mg of collagenase with 220uL PBS

buffer in the test tube. The collagenase solution was then mixed with the IPC solution with or without aflibercept and incubated at 4°C for 24 hours. The sample solutions were then analysed using SDS-PAGE and Size Exclusion Chromatography techniques.

2.3.7. Binding activity of released aflibercept

The amount of functionalise aflibercept that was released from IPC formulations, was quantified using an Enzyme-Linked Immunosorbent Assay (ELISA) (46). As shown in **Figure 10,** a 96 well plates were used to run the ELISA assay, which each well was coated and incubated with 100ul of VEGF165 (0.1 µg/mL) for 24 hours at $4C^0$. The VEGF solutions then were removed and immediately were replaced with 300uL blocking buffer (PBS tablet + 10 % B.S.A + 0.05 % Tween20) for 2 hours incubation at ambient temperature. The blocking buffer solutions were removed, and the wells were washed using 300uL washing buffer solutions including PBS tablets, DI water and 0.05 % Tween20. After this, 100uL of sample solutions, that were diluted with PBS buffer at different range of Concentrations, were added to each well for 2 hours incubation. After a certain incubation time, the sample solutions were removed and were washed with 300uL washing buffer for three times. Then, each well was coated with 100uL Anti-Human IgG (Fab specific)-peroxidase (1/5000 dilution) and was incubated for 1.0 hours at ambient temperature. After 1.0 hours the solutions were removed and were washed off with washing buffer for three times. The development of the blue colour was observed after 5minute by adding 100uL TMB. When the blue colour was monitored enough for each well, then 50Ul HCl (1.0 M) solution was added to make a constant yellow colour. At the final steps, the plate was read using a plate reader at 450 nm wavelength.

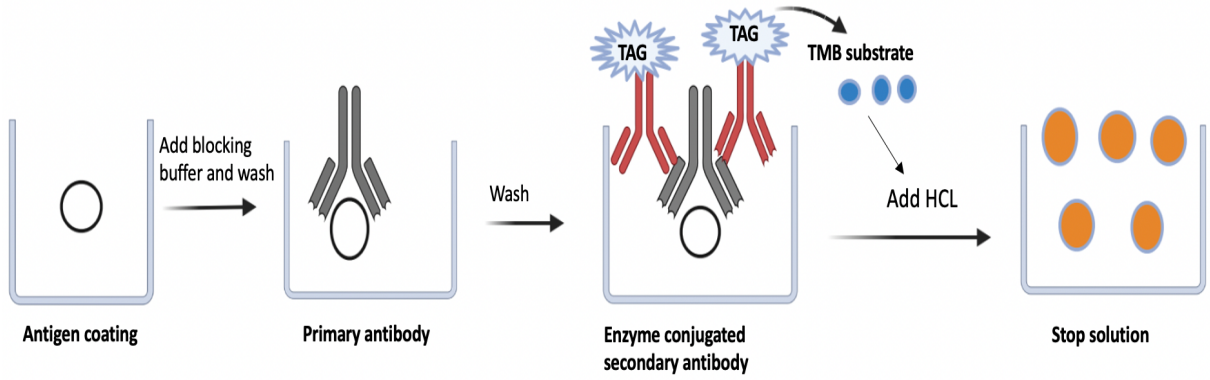

Figure 10. Schematic presentation of ELISA (Enzyme-linked immunosorbent) assay. A target antigen (VEGF 165) is immobilized on the surface of 96 well plates and incubated with the primary antibody, followed by a secondary antibody (Fab specific) against the primary antibody. The activity of the microplate well-bound enzyme (orange) is then measured after washing (46).

2.3.8. Injectability and Syringe ability test of IPC

The injectability of the aflibercept with HA/IPC application was studied using 29G needle. Syringe ability was utilized to investigate the release of IPC through the needle and injectability was facility to determine the force needed the administration via needle. The low viscosity was required low extrusion force with no significant bubble generation which allows for easy injectable administration. The required pressure for injection or any bubble generated during the test process were detected and recorded.

2.3.9. Statistical analysis results

The data sampling was carried out statically analysed as the mean and standard deviation (± STD), standard error (SEM) and data were plotted using Prism-GraphPad and Microsoft Excel 2019 for Mac.

3. Results

3.1. Aflibercept calibration curve using SEC

First serial dilution of aflibercept at different concentration (2.0 mg/mL to 0.125 mg/mL) were prepared and their concentration were measured using Size Exclusion Chromatography technique with UV-Visible spectroscopy detector at 280 nm. The calibration curve as shown in **Figure 11**, indicated a direct line with equation of Y=1233.4 X – 85.228. We then used this equation to measure the amount of aflibercept that was being released form IPC formulation.

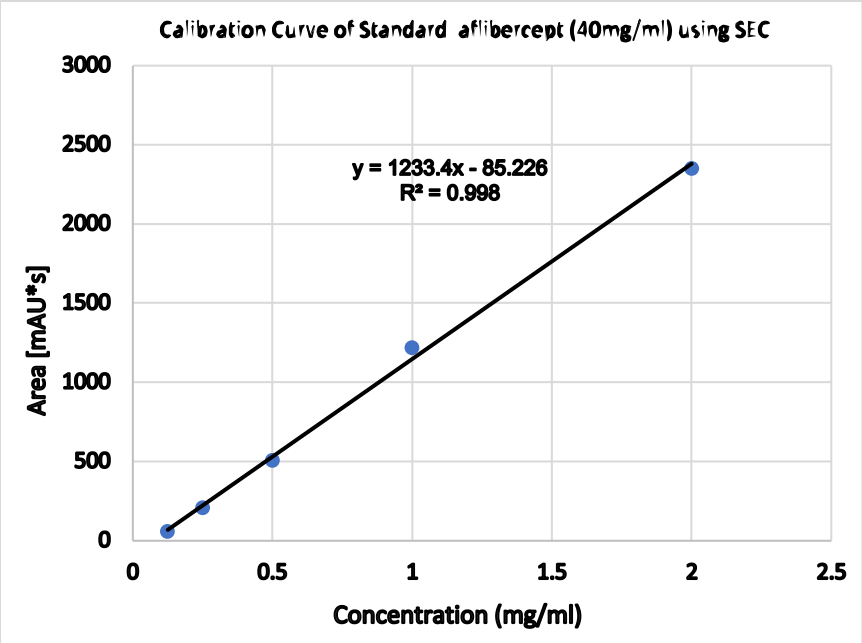

Figure 11. A calibration graph of peak area (mAU×ml) vs concentration of standard aflibercept (0.125-2mg/ml). An R^2 value of 0.998 was obtained using Size Exclusion Chromatography (280nm) at flow rate of 0.5mL/min and 0.2mL injection volume.

3.2. IPC preparation and injectability

The white collagen type I was formed after adding NaCl (0.8M) and being centrifuged (at 4600 rpm for 30 minutes) (**Figure 12)**. Dialysis was then performed against acetic acid and EDTA (pH7.0) buffer overnight, which was resulted in formation of clear, colourless gelly like solution of IPC (**Figure 12)**. Acetic acid removed the excess salt and resulted in the forming of clear IPC. The fibrillization happened after the IPC (100µL) being incubated with aqueous PBC buffer (300 µL) and incubation at 37°C. The solution changed from the clear viscous to an opaque, solid product. This was due to the presence of EDTA within the formulation of IPC, which was incorporated via dialysis to avid spontaneous polymerization. Once the EDTA molecules exposed to heat and PBS buffer, the EDTA was displaced with water molecules and caused to form banded collagen fibril.

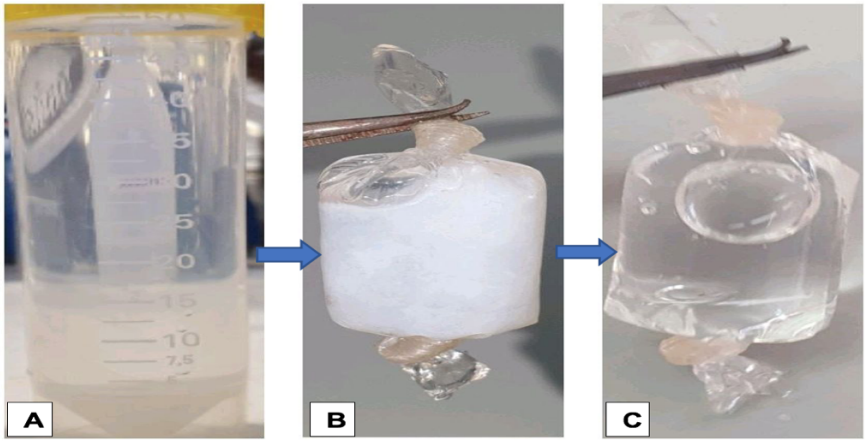

Figure 12. A) Forming a white collagen gel at the bottom of centrifuge tube after precipitation with NaCl, B) White collage form before dialysis, C) Formed IPC; transparent colourless form of IPC after dialysis against acetic acid solution and EDTA (pH7.0) buffer.

The IPC was then mixed with aflibercept to be incubated at 37 ^{0}C. The transparent form of IPC remained as a white gel while mixed with aflibercept. Due to high viscosity of IPC gel, the injectability test carried out after being mixed with aflibercept (as described in section 2.2.10). When a 29gauge needle used, no significant bubble was generated during the examination.

3.3. Aflibercept analysis released from the IPC or HA/IPC solution using Sink model

The IPC solution turned from clear and transparent form to opaque, when the solution was mixed with aflibercept and stored at 37^0C (**Figure 13**). The IPC/HA alone (control) and aflibercept-IPC/HA formulation release was analysed using the sink model (**Figure 8**). The formulation (130 µL) transferred into the vial (1mL) and mixed to the PBS buffer (270 µL). IPC started to become fibril and turned into a soft, white, semi-solid gel after 1hour incubation at 37°C (**Figure 13**).

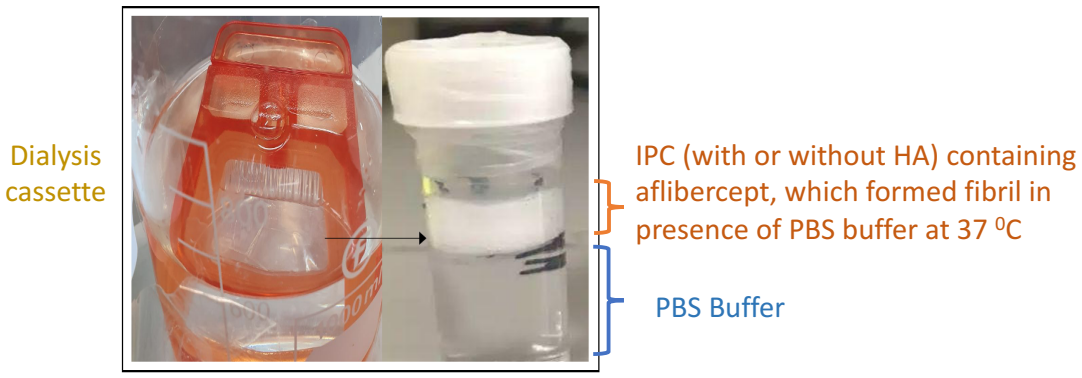

Figure 13. Formation of aflibercept-IPC with or without HA under sink model.

The first experiment was done to determine the role of HA within the aflibercept-IPC formulation. For this purpose, two different groups of 1) aflibercept (0.24 mg/mL) with IPC (80 µL) and 2) aflibercept (0.24 mg/mL), HA (2mg) and IPC (60 µL) were studied. An experimental method (as described in section 2.2.2) was used to measure released drug from IPC-HA or IPC alone formulation using sink model.

When Silver staining gel was used for protein detection (**Figure 14**), bands corresponding to the collagen molecular weight (110kDa) obtained in both the IPC control and in the release samples. Bands consistent with intact aflibercept (~115 kDa) appeared in the release samples of group 1 (aflibercept with IPC, no HA) up to day 2.

As shown in lanes 11-15, **Figure 14,** the bands to the intact aflibercept appeared for five days in the presence of HA within the aflibercept-IPC formulations. This suggested that the presence of HA within the IPC formulation had an impact on decreasing the mesh size of hydrogel and aided aflibercept to have sustained release. Using of HA in the formulation was also useful to avoid aflibercept burst release within first 24 hours.

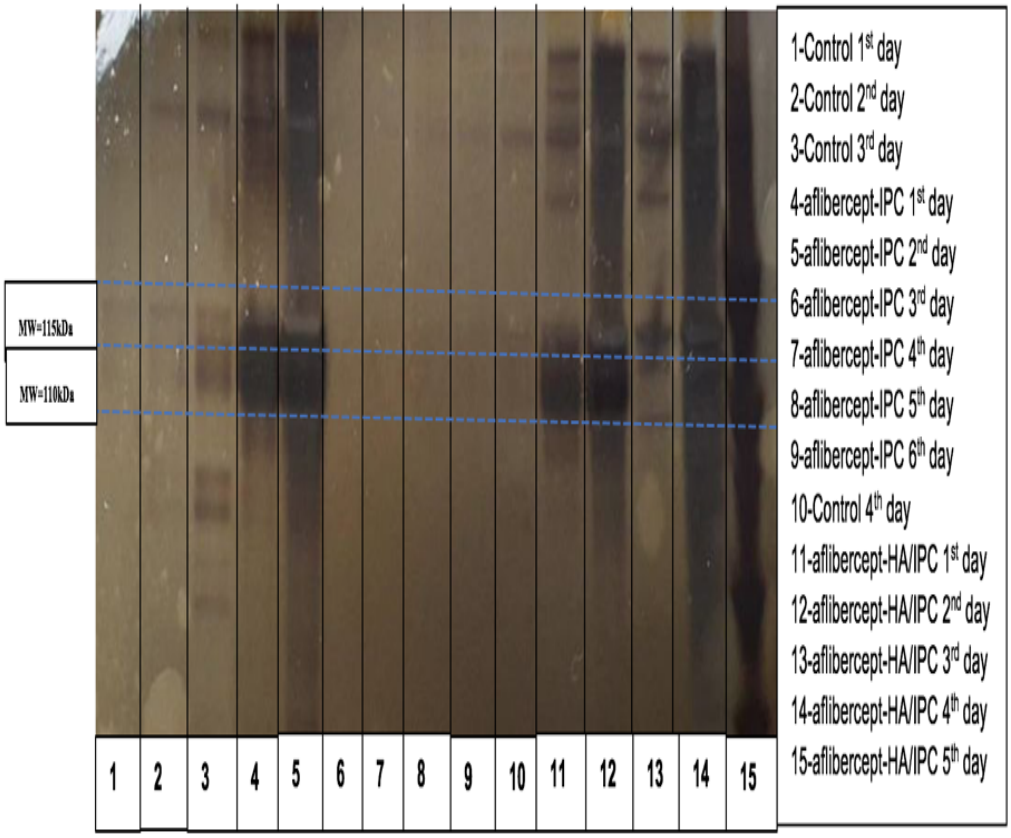

Figure 14. Silver staining loaded with aflibercept after being released from IPC or IPC-HA using sink model. Lanes1,2,3 and 10 are control (HA-IPC alone): day 1,2,3 and 4, Lanes 4-9 are aflibercept-IPC (without HA): day 1,2,3,4,5,6; Lanes 11-15 are aflibercept-IPC/HA: day 1,2,3,4,5.

The second experiment was designed to investigate whether the change of the aflibercept concentration in the HA-IPC formulation make a difference in the release profiles. Different concentration of aflibercept (0.24, 0.48 and 0.96mg/L) formulated with HA-IPC as described in **Table 2**. The released sample solution (130 µL) was taken every day and it was analysed using Size Exclusion Chromatography to quantify the concentration of aflibercept. As is shown in **Figure 15**, the main peaks were corresponded to aflibercept (elution time(et)= 2.2±0.6 min) and IPC (et= 3.2±0.5 min). Total Area Under of Curve (AUC) was used to estimate the released Aflibercept (mg/mL) in the collected samples.

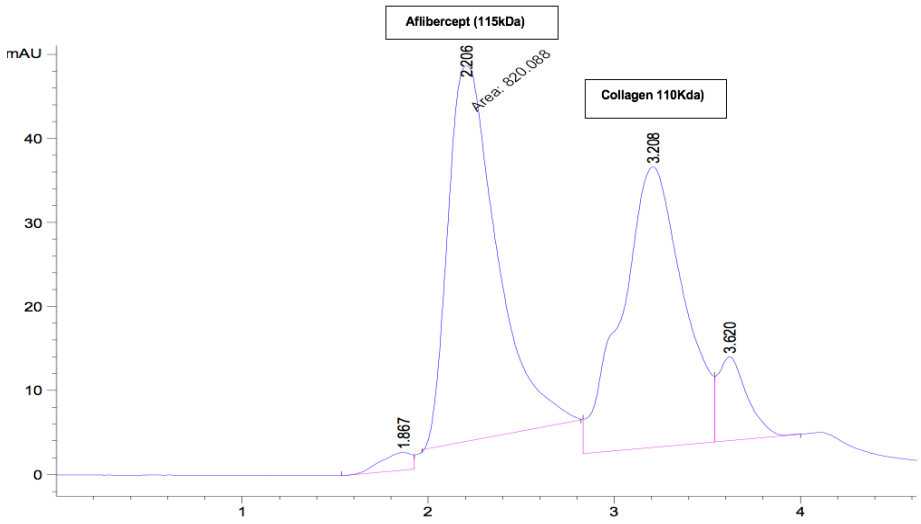

Figure 15. Chromatogram of aflibercept release from HA-IPC formulation in the 1st day using Size Exclusion Chromatography.

The cumulative percentage of released aflibercept from the sink study was estimated based on percentage (%) of three different formulations of aflibercept/HA/IPC. The cumulative released (%) aflibercept is shown in **Figure 16**. Data suggested the %92.0 ± 5 of aflibercept was released within 4th days of release study and the peak was turned flat in 5th day.

According to the data, a rapid initial burst release of aflibercept was observed within the 1st day (burst effect) when HA was not used in the formulation. It is apparent that more than 50% of aflibercept were released in the first 48hrs of sink study.

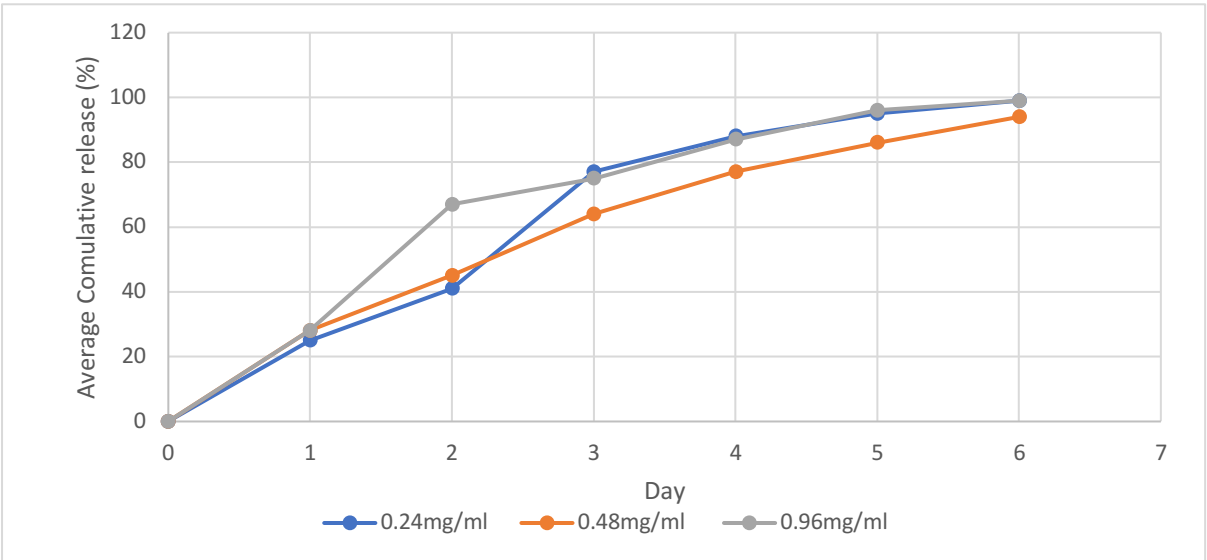

Figure 16. The increase in cumulative drug release (%) over time for aflibercept. Different concentration of aflibercept formulated within the HA-IPC. The values represent the release (%) of aflibercept from three different groups of HA-IPC formulation using sink study model.

The SDS-PAGE used to monitor the effect of drug concentration within the IPC-HA formulation. As shown in lanes 4-9, **Figure 17**, aflibercept appeared with the thick band at approximately 150 kDa in all groups of aflibercept-IPC with HA formulations. The data showed the initial burst release in the first 48h which the formulation required further optimisation to reduce the burst release and prolong duration of the release. Then, the rig study carried out to investigate the release profiles and to test the effect of the flow on the formulation.

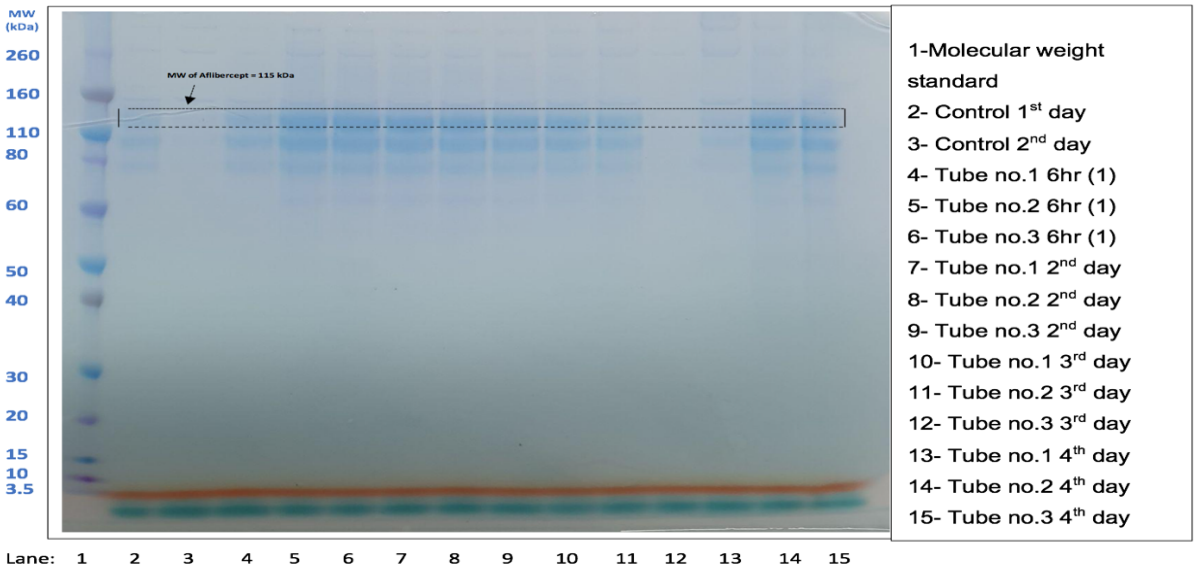

Figure 17. An SDS-PAGE image of a gel loaded with samples after being released from formulations of Control (HA-IPC alone, Lanes: 2 and 3), group1(0.24mg/mL aflibercept-HA/IPC, lanes:4,7,10 and 13), group 2 (0.48 mg/mL aflibercept-HA/IPC, lanes: 5,8,11 and 14) and group 3 (0.96 mg/mL aflibercept-HA/IPC, lanes: 6,912 and 15).

3.4. Formulation strategies to prolong release of aflibercept from IPC

In previous studies conducted in our research group, it was found that if IPC become diluted with protein solution, it could impact on protein release profile. In order to prevent IPC from dilution, we decided to reduce the volume of protein solution. To reduce down the volume of aflibercept solution to 20 uL while maintaining the amount of the protein as 2.0 mg, we needed to concentrate down the initial aflibercept solution from 40 mg/mL to 100 mg/mL. Taking 20 ul from 100 mg/mL aflibercept solution, would provide 2.0 mg protein to mix with IPC gel. Using less volume of aflibercept would also allow to use more volume of HA-IPC solution and ensure uniform distribution of the protein in the meshes of the IPC. However, protein is at high risk of aggregation when it is concentrated. It was important to study the stability of concentrated aflibercept (100 mg/mL) before being formulated with IPC.

Stability study was performed using Size Exclusion Chromatography and SDS-PAGE analysis as shown in **Figures 18** and **19**. Based on the presented data, no high molecular weight band or degradation band observed in SDS-PAGE analysis. Furthermore, no high molecular weight peak was observed in Size Exclusion Chromatogram (**Figure 19**).

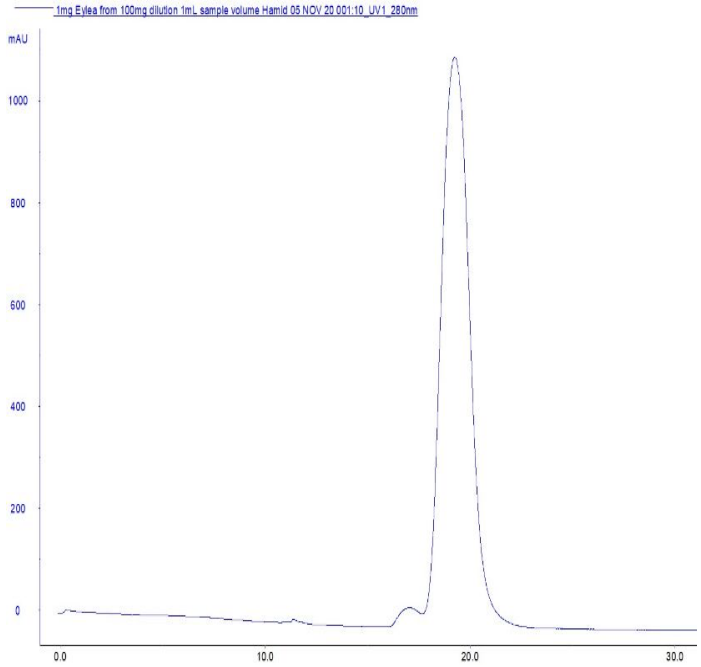

Figure 18. A chromatogram image of aflibercept (100mg) diluted with PBS buffer (1:100).

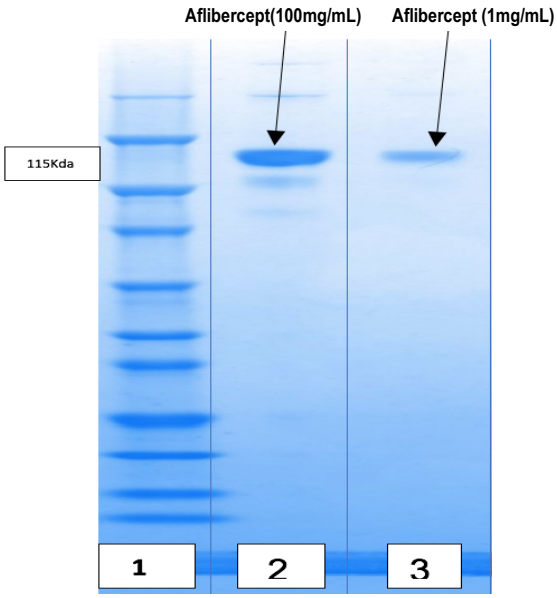

Figure 19: An SDS-PAGE image of aflibercept in the highest concentration. Lane 1: Protein marker, Lane 2: aflibercept (100mg/mL) diluted with PBS (1:100) and Lane 3: Standard Aflibercept (1 mg/mL).

After ensuring the stability of protein at high concentration, about 20µL of concentrated aflibercept containing 2.0 mg protein was added into IPC solution (110µL) and HA (2mg).

Solution was then mixed and dialysed against EDTA (pH 7.0), D-mannitol and Sucrose (**Figure 6**) for 24 h. The solutions formed a transparent liquid form in the 0.5mL dialysis cassette after overnight dialysis (**Figure 20A**). For the control group, solution of HA (2mg) and IPC (150µL) without aflibercept, was prepared and dialysed. Dialysed solutions (150 uL) were then transferred to the rig (with maximum capacity of 200 uL), as shown in **Figure 20B**, and PBS buffer (50uL) was then added (**Table 3**). The rig was then connected to the peristaltic pump with the flow rate of 2 uL/min. The inlet was buffer, and outlet was the release solution collecting into the tube for further analysis. To make a fibril, it was important to carry out the experiment at 37 ^{0}C. The transparent IPC gel turned into a soft, collagen fibril after approximately 1hour at 37°C (**Figure 20B**).

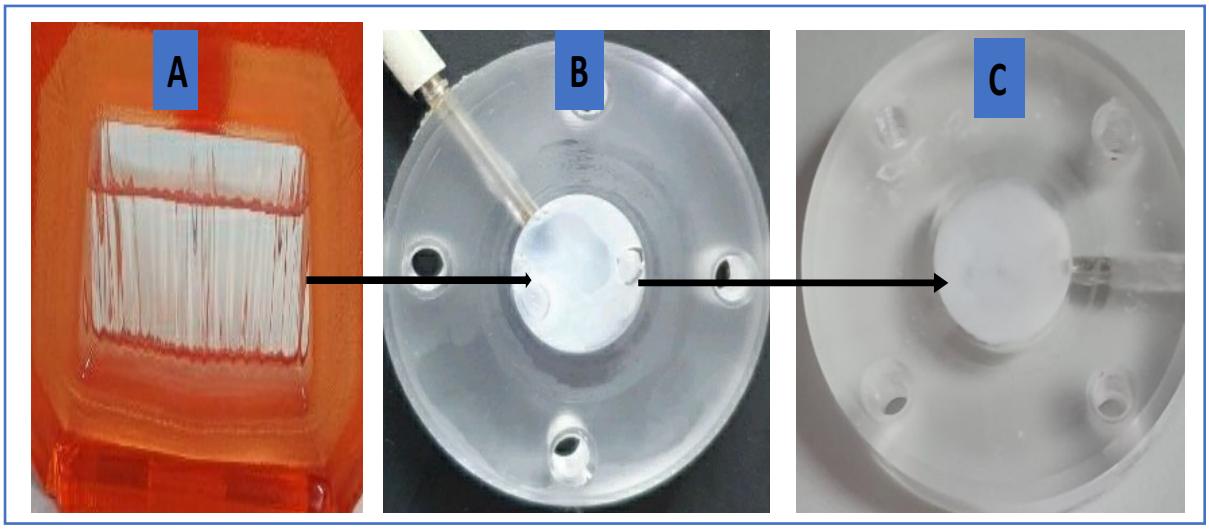

Figure 20. The IPC appearance before, after gel formulation and at the end of drug release process. **A)** A transparent form of IPC before formulation **B)** Forming an opaque collagen matrix after 1hr loading into the rig. **C)** A fully intact gel formation inside the rig after 7th day completing the drug release.

The sample collection was carried out at 6, and12 hours and then once a day for the period of 7 days (**Figure 20C**). The concentration of release samples was determined using SEC and was calculated based on the aflibercept calibration curve. The concentration of aflibercept detected after 12hour was 198± 0.03 µg/mL (n=2) which was 10% of the initial (total=2.0 mg/mL) aflibercept. No aflibercept was observed after 7 days release in SEC chromatogram. Total release percentage was about 40% with remaining of 59.2± 7% as shown in **Figure 21**.

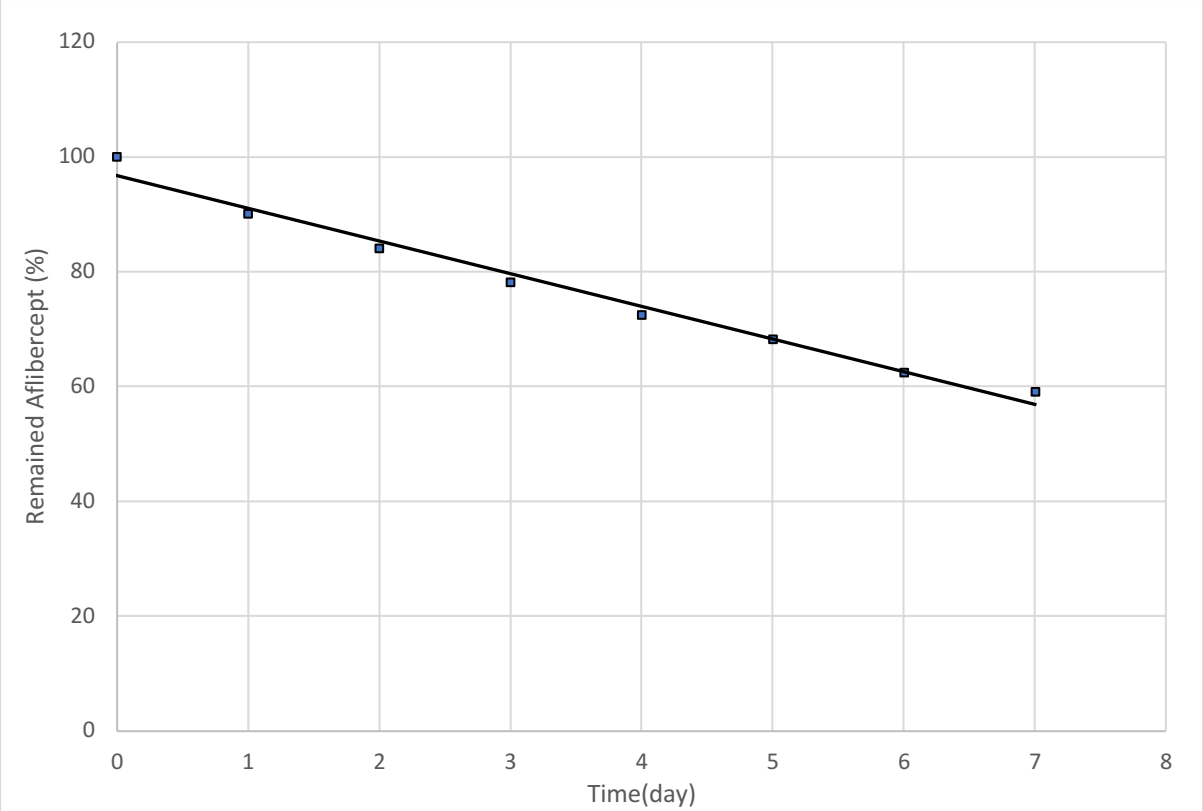

Figure 21. Release study of aflibercept-IPC with rig model within 7th days in 37°C in EDTA (pH 7.0) compositions. The data are shown as the mean (n=3) of three different rigs. The mean protein loss of 17± 1.5% was calculated by 1st day and the remained (%) aflibercept of 59±1 was seen till 7th day release profile.

Solution released from formulation was further loaded to the Size Exclusion Chromatography column to measure aflibercept quantity. For further analysis, Size Exclusion Chromatography fractions were collected at different time points and loaded to the SDS-PAGE stained with silver-staining. As shown in lanes 5-7 of **Figure 22B**, bands appeared at 110 kDa were related to the aflibercept molecular weight. The SEC fraction collected at 5 -6 min was related to aflibercept and SEC fraction collected at 6-7 min was for IPC. As it presented in the SEC fraction collected at 6 min (Lane 7, **Figure 21**), there is some evidence of aflibercept that are eluted with collagen peak. This cloud be possible for aflibercept peak to overlap into the collagen peak in the SEC chromatogram (**Figure 22**) that might effect on calculating protein concentration.

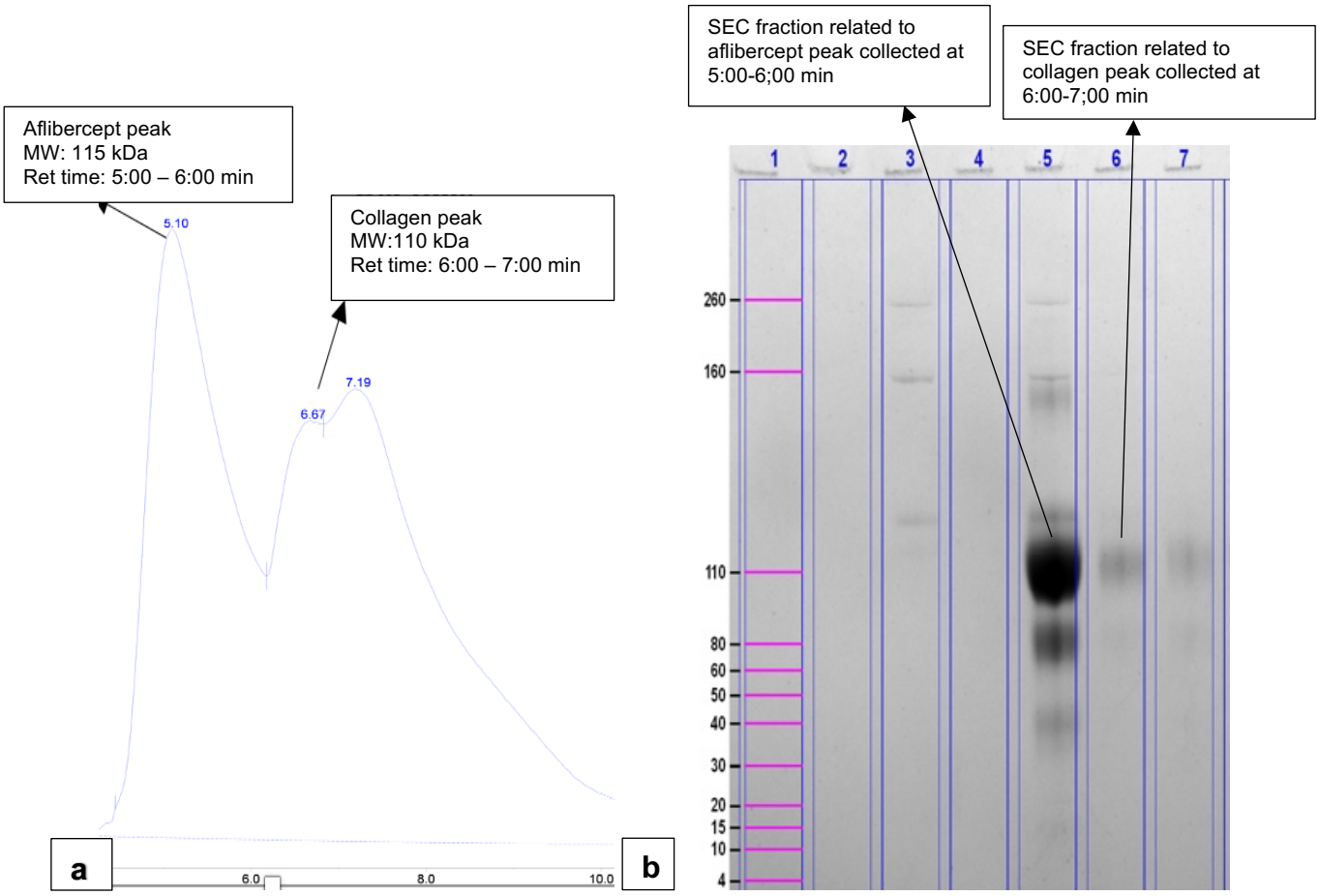

Figure 22. Showing (a) SEC chromatogram of aflibercept release from HA-IPC in the first 6hr of rig study (b) Silver staining gel loaded with Lane 1 and 2 are SEC fraction collected at 5:00-6:00 min and 6:00-700 min from EDTA dialysis solution of rig 1 (aflibercept/IPC-HA). Lane 3 is control rig (IPC/HA alone). Lane 4 is SEC fraction collected from control rig (IPC-HA) rig at 6:00-7:00 min. Lane 5 is rig 1 (aflibercept/IPC-HA). Lane 6 is SEC fraction collected at 5-6:00 min from rig 1 (aflibercept/IPC-HA). Lane 7 is SEC fraction collected at 6-7:00 min from rig 1 aflibercept/IPC-HA).

3.5. Collagen degradation

Since it was not possible to detect any aflibercept releasing from IPC/HA formulation after day 7, and only 40% of total aflibercept detected using SEC. To investigate the remaining aflibercept 3 different studies carried out. First, a study accomplished to confirm that drugs could not be able to diffuse through the dialysis membrane during overnight dialysis. As shown in lanes 2 and 3 of **Figure 22**, no bands related to the intact aflibercept observed in the gel.

Secondly, a collagenase assay used to break down IPC and to evaluate the stickiness of aflibercept to the IPC if it has entrapped. As it is well known that collagen type I has very high sticky properties to the extracellular matrix and cell adhesion (47). Thirdly, an ELISA technique performed to the calculate concentration of aflibercept released from formulation after day 7 (**Figure 10**). Because the detection limit was very low which was not possible to detect aflibercept if they release after day 7.

To evaluate if aflibercept has stuck to the IPC, it needed to degrade or break up the collagen using collagenase. For collagenase to fully degrade IPC, it was necessary to incubate collagenase with IPC for 24 hours. Figure 22, (a) shows that no degradation was observed when collagenase incubated with IPC for 1 hour and IPC remained as fibril. Total IPC degradation was, however, observed in **Figure 23**, b, after 24-hour incubation with collagenase.

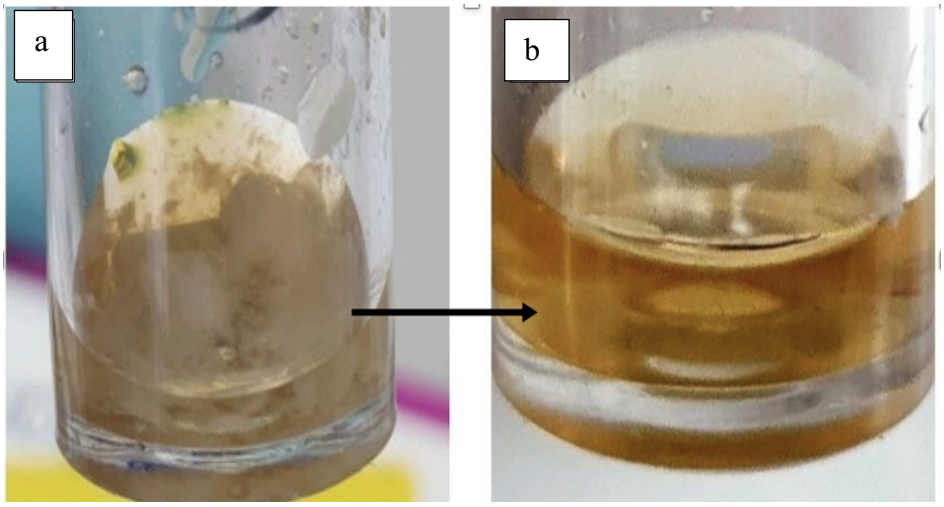

Figure 23: (a) the resistance of IPC against enzymatic degradation by collagenase after 1 h. (b) fully degradation of fibrillated IPC using collagenase (Clostridium histolyticum) after 24 hours.

The next step was to evaluate the effect of collagenase on aflibercept stability hence a simple mixing method was performed by adding collagenase to aflibercept alone (1mg/mL) and aflibercept-IPC formulation. The degradation was only monitored after 1hour incubation using SDS-PAGE analysis. Distinct bands at 50 kDa and 35kDa (lane 4, **Figure 24**) was observed for degraded aflibercept, showing denaturation of aflibercept once being incubated with collagenase after 1 hour. However, once collagenase was incubated with aflibercept formulated in IPC, no degradation was observed for aflibercept, and it remained stable (Lane 3, **Figure 24**). While this was only studied after 1hour incubation, it was thought that presence of EDTA in IPC formulation might protect aflibercept from degradation and resist the degradation by collagenase.

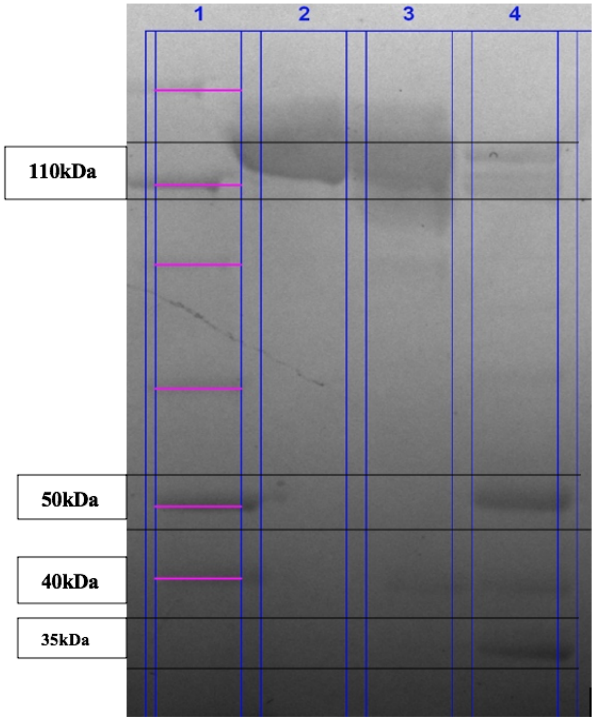

Figure 24. Non-reducing SDS-PAGE gel loaded with Lane 1: molecular weight standard and Lane 2: standard aflibercept (1mg/mL). Lane 3: aflibercept (1mg/mL)/IPC with collagenase (10mg). Lane 4: aflibercept (1mg/mL) with collagenase (10mg).

Once it ensured that collagenase did not degrade aflibercept, collagenase assay was applied on aflibercept/IPC formulation at day 8 to investigate the loss of aflibercept (40-50%) loaded HA-IPC. As shown in lanes: 2-15 of **Figure 25**, bands at 110-115 kDa appeared with some traces of bands at 50 kDa at all points of the release study. The bands under non-reducing conditions at 115kDa and 50kDa were visible with the molecular weight corresponding to intact aflibercept and degraded fragments (Lanes: 5 and 8, **Figure 25**) respectively. Whereas other bands were related to the molecular wight of collagen (250-300kDa) and its alpha II chain (110-115kDa). The SEC fractions collected for aflibercept peak at 5-6 min (aflibercept-IPC with HA) indicated that some of aflibercept still remained within the collagen fibril networks (Lanes 6 and 9, **Figure 25**), which was released after IPC being degraded.

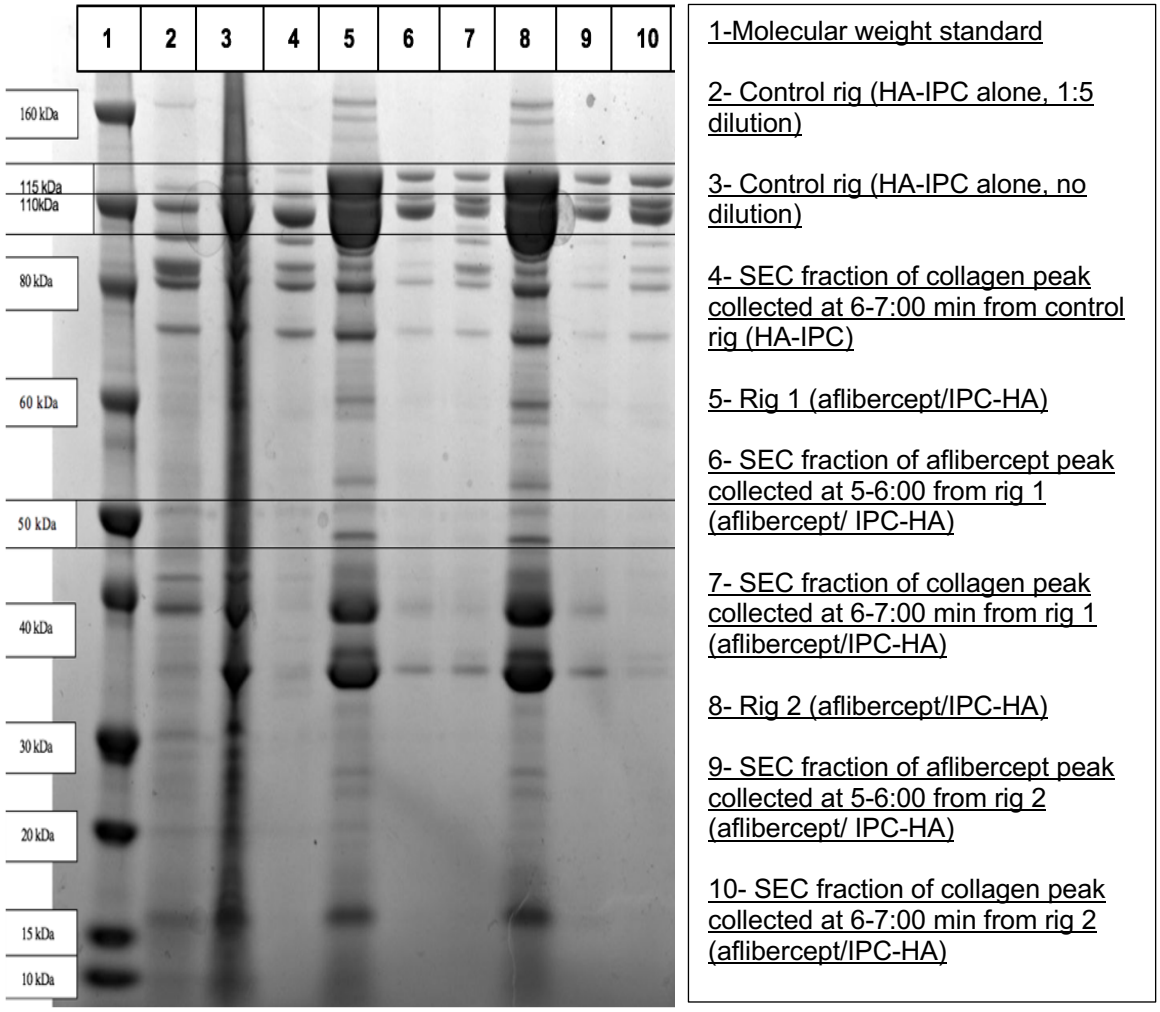

Figure25. Non-reducing SDS-PAGE gel loaded with collagenase solution with samples after being released from rigs after 7th day release studies. All the Rigs (n=2) contain aflibercept (2mg/mL), IPC (110uL), HA (2mg) and PBS buffer (50uL).

3.6. Effect of excipients on sustain release formulation of aflibercept

Hyaluronic acid (HA) is a biodegradable and biocompatible polymer that can trap water molecules and forms a viscoelastic gel solution. Using HA in our formulation was thought to reduce the mesh size and enhance protein entrapment. In this section we wanted to study if amount of HA used in the formulation increase from 2 mg to 4 mg, will we have less burst release and prolong release formulation for aflibercept. Once more HA was used in the formulation, it was important to study the injectability of overall formulation since HA could effect on the viscosity of the solution. No change in injectability was observed using syringe gauge 29 when 4 mg HA was used in the formulation.

Other excipients were also considered to use in the formulation to reduce the stickness of protein to IPC including polyethylene glycol (PEG) and Tween20. PEG is water soluble polymer which in theory could reduce the tendency of protein to stick to collagen (48) and Tween20 is a surfactant which also known to reduce adhesion properties between protein and IPC (49). As shown in table 3, four different formulations were prepared: (1) IPC (110µL) and HA (4mg) alone without aflibercept as control, (2) aflibercept (20µL, 2 mg) loaded in HA (4mg) and IPC (110µL), (3) aflibercept (20µL, 2 mg) loaded with HA (4mg), IPC (110µL) and Tween 20 (2 µL), (4) aflibercept (20µL, 2 mg) loaded with HA (4mg), IPC (110µL) and PEG (3mg). All these formulations were dialysed against EDTA, sucrose and D-mannitol buffer solutions for 24 hours prior to release study. Release study was carried out using the same method as explained before and examined by SEC for 5 days. No aflibercept was detected after day 5 using SEC analysis.

As shown in **Figure 26 and 27**, the burst release was lowest for aflibercept in IPC/HA (4 mg) solution (formulation (2)) with only 116.5ug/mL of aflibercept (equivalent to 5.9% of initial amount of aflibercept) was measured after 12hour of release study. The cumulative release was increased approximately to 23 % in the day 2 and reached to 59% by day 5. Formulation (3, aflibercept in IPC/HA with tween20) showed the highest initial burst release with 205.5 ug/mL aflibercept was measured after 12hour of release study, but only 50% of loaded aflibercept was released after five days. Formulation (4) when PEG was used in combination with IPC and HA, aflibercept released was only 35% by day 5, leading to %65 of protein remained in the formulation.

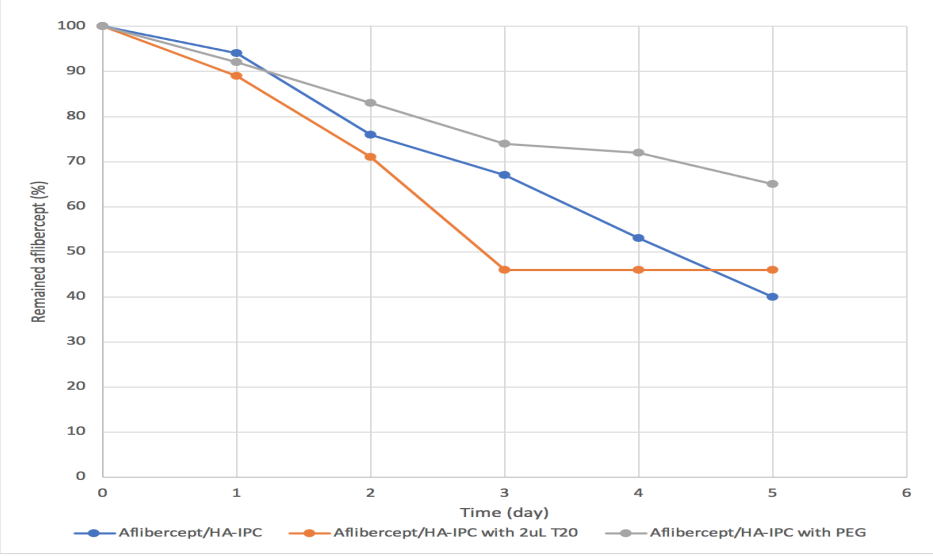

Figure 26. The slow-release profile of aflibercept from the HA-IPC formulation with and without PEG and Tween20. The values represent the mean and n=2.

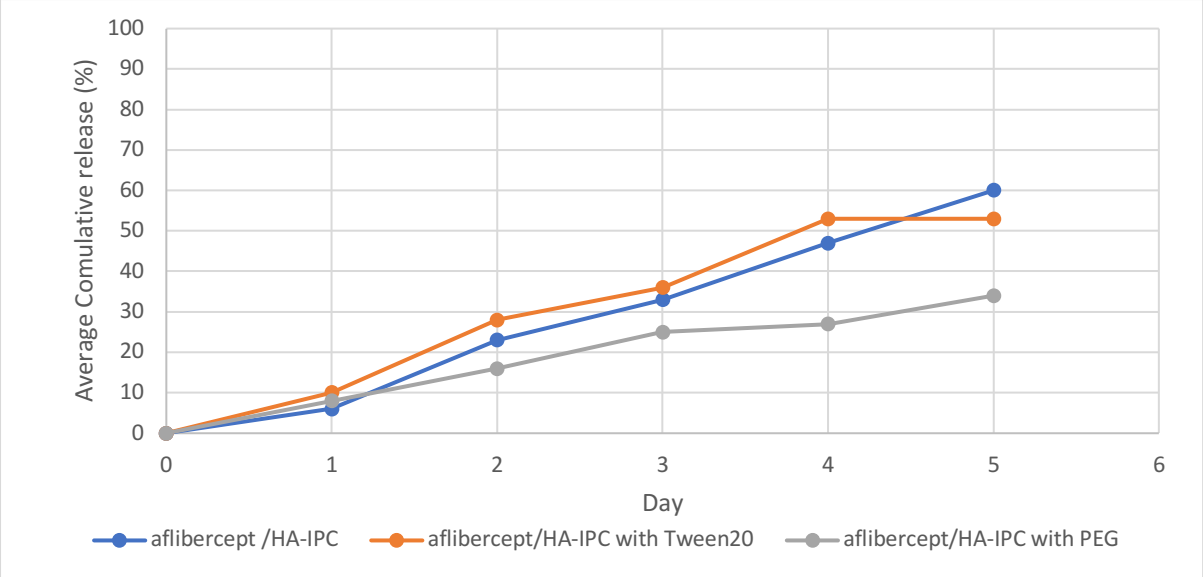

Figure 27. Cumulative release of aflibercept from HA-IPC with or without Tween20 and PEG. All data is presented as its mean (n=2).

To figure out what happened to remaining aflibercept, a collagenase assay was used to analyse the samples after 5 days and then analysed using SDS-PAGE and Silver staining methods. Silver staining was used because it can stain and detect any trace (ng) of protein if it is in the solution. **Figure 28** shows the bands for each rig in first day and after 5 days after being incubated with collagenase. Aflibercept that was stuck to IPC, released after collagenase but in the form of degradation as band at 35 kDa and 50 kDa appeared in **Figure 27**, Lanes 5,7,9,11,13 and 15. Distinct bands at 50 kDa and 35kDa were much thicker for the formulations in the present of PEG used as excipient in comparison to other formulations. Previous researches quantitively confirmed that PEG and Tween 20 have no tendency to denature protein (49 and 50). In conclusion, it generally assumed that use of PEG-based IPC could prolong release of proteins in constant rate. However, our studied have demonstrated that the use of PEG in the formulation did not lead to sustain release of aflibercept as more aflibercept was stuck to IPC compared to other formulation.

To investigate the effect of excipients on the formulation, using of alternative strategies such as shortening dialysis time or using of more volume (µL) of Tween20 were carried out to extend the release profile.

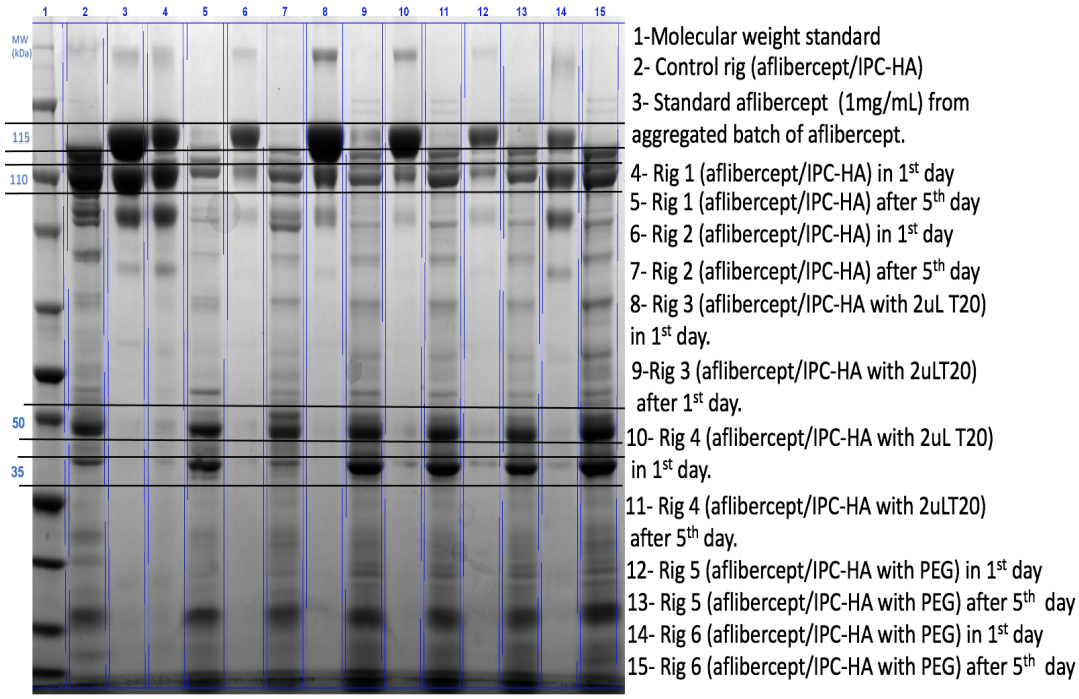

Figure 28. Non-reducing gel loaded with different samples after being released from the IPC (Lanes 2-15). Lane 1: molecular weight standard; Lanes 2 is collagenase of IPC, Lane 3 is standard aflibercept (1mg/mL), Lane 4-7 are rig no.1&2 [aflibercept (2mg)/HA(4mg)/IPC (110uL)] in the day 1 and day 5 after collagenase. Lane 8-11 are rig no.3 and rig no.4 in the present of [Tween20(2uL)/ aflibercept(2mg)/HA(4mg)/IPC] in the day1 and day 5 after collagenase. Lane 12-15 are rig no.4 and rig no.6 in the presence of [PEG/ aflibercept(2mg)/HA(4mg)/IPC] in the day 1 and day 5 after collagenase.

3.7. Dialysis time may have impact on the release profile

In this section, we examined if change of dialysis time could have effected on release profile of aflibercept from IPC/HA formulation. If aflibercept has less dialysis time, it might have less chance to stick to IPC and hence release fully from the formulation over the release study. Two different dialysis times of (1) 24 hour and (2) 12 hours tested against EDTA (pH 7.0), D-Mannitol and Sucrose solutions. The HA amount was also increased from 4mg to 5mg. Four different formulations (n=2) were prepared; (1) control group, IPC/HA (5 mg) without aflibercept, (2) Aflibercept with IPC/ HA (5 mg) without tween20, (3) Aflibercept with IPC/ HA (5 mg) and tween20 (2µL), (4) Aflibercept with IPC/ HA (5 mg) and tween20 (4µL). In all batches, the release of aflibercept was continued for 12 days, which was detected using Size Exclusion Chromatography (**Figure 29**).

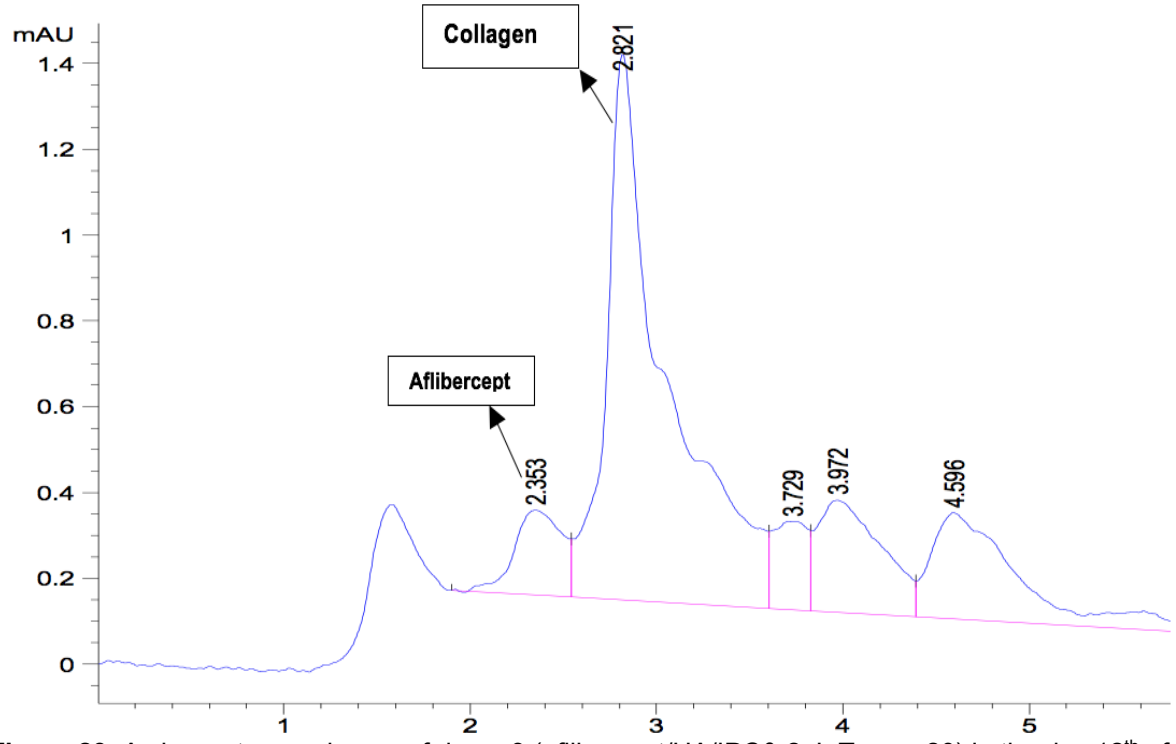

Figure 29. A chromatogram image of rig no.3 (aflibercept/HA/IPC& 2uL Tween 20) in the day 12th of release study. The retention time (RT) of the main peak representing Aflibercept was recorded at 2.3min and for the Collagen was respectively at 2.8min.

Based on the Size Exclusion Chromatgraphy analysis as shown in **Figure 29**, a cumulative (%) release (**Figure 30** and **31**) in the formulation (2) where no tween 20 was used, was approximately 62.7± 3% with the maximum concentration (C_{max}: 1.11± 0.12 mg/mL). In formulation (3) where tween-20 (2µL) was used, the total release of the drug was 47.6± 8% (C_{max} of 1.05 ± 0.3 mg/mL). However, in the formulation (4) (aflibercept-HA/IPC with 4µL Tween20), the total release of the drug was decreased with the total cumulative release of 43.3± 2% at 12th day rig study (the maximum concentration of aflibercept released was 0.88± 0.02 mg/mL). It was found that using Tween20 in the IPC formulation decreased the cumulative release (%) of aflibercept. Using higher amount of Tween20 (4µL), the cumulative release was approximately 50% which was 5% lower than the rigs with 2µL Tween 20 (cumulative release 56%). The data also showed that shortening the dialysis time from 24h to 12h and using more HA (5mg) could extend aflibercept ‘s release up to 13th day with higher cumulative release (more than 60% of drug).

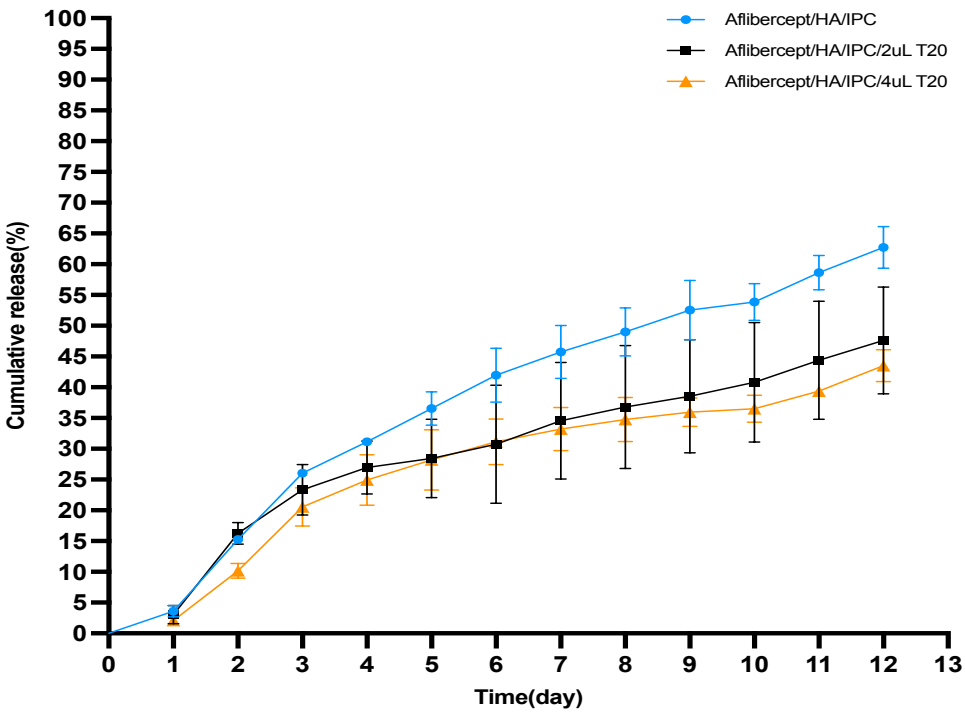

Figure 30. The slow release of Aflibercept in IPC in compared to Tween20(2uL) and further volume of Tween20 (4uL) by the presence of HA. All the value represents the mean (n=2) and SEM.

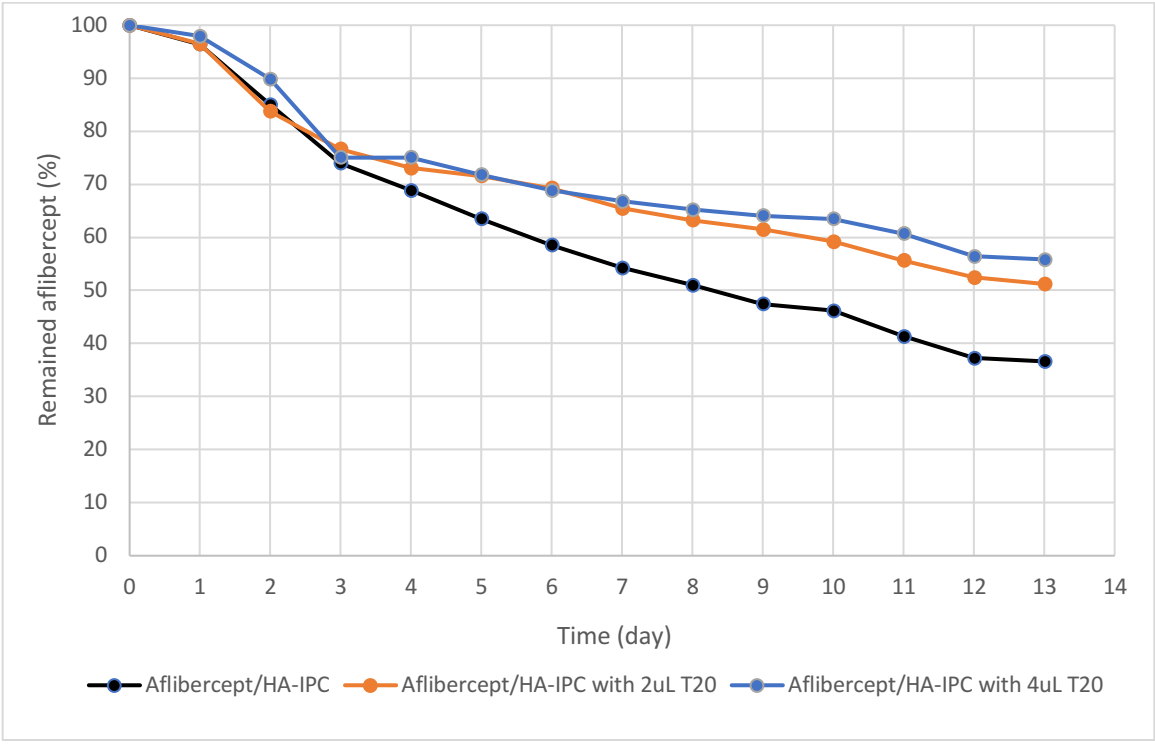

Figure 31. The decrease of total aflibercept concentration with time, which were contained 2mg/mL aflibercept in the presence of HA-IPC with 0,2and 4uL of Tween20. All the solutions were dialyzed in EDTA, mannitol and sucrose solution stored at 37 °C for at least 12hr the before rig release study. The values represent the mean (n=2).

3.8. Using ELISA to measure functional and active concentration of aflibercept

The Enzyme-linked immunosorbent assay (ELISA) is a binding activity technique which able to measure active protein concentration releasing from formulation. As explained before, one of the reasons for not being able to detect aflibercept release for more than 12-days is the SEC detection limit. ELISA is able to measure protein concentration in ng range. We first needed to perform and optimise ELISA assay for standard aflibercept using $VEGF_{165}$. A serial dilution of standard aflibercept (0.004-4000 ng/mL) were prepared (**Figure 10**) and ELISA curved was plotted (**Figure 32**). We were able to plot ELISA curve using 4-400ng/mL for aflibercept.

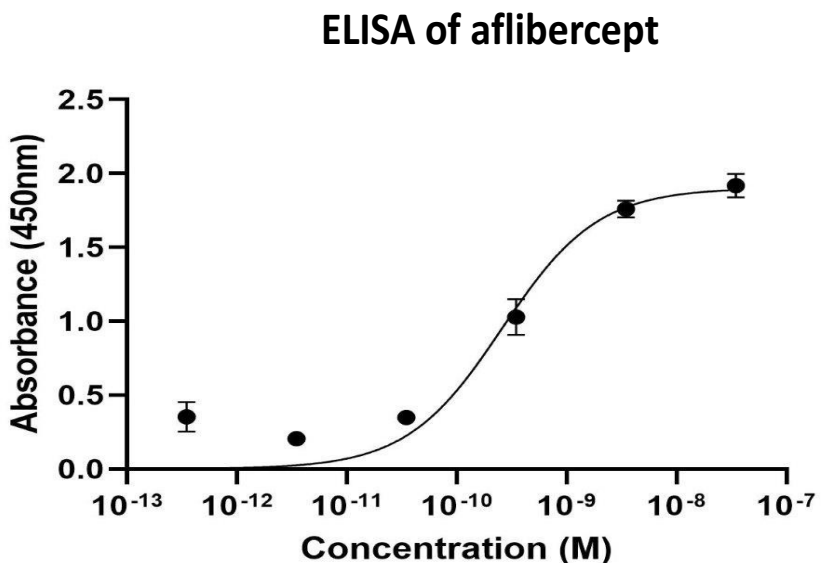

Figure 32. An ELISA curve of standard aflibercept (0.004-4000.0 ng/mL) detected at 450 nm by plate reader.

To detect how much of aflibercept remained (stuck) in the IPC formulation, the solution was first treated with collagenase and then used in ELISA assay. As shown in table 4, less than 2% of aflibercept had VEGF activity because collagenase degraded aflibercept as shown in **Figure 33**.

Table 4: The cumulative release (%) of three groups Aflibercept/HA-IPC without and with presence of Tween20, calculated according to absorbance (450nm) of fractions under UV

Samples	Absorbance(450nm)	Volume (mL)	Dilution time with PBS	Average Aflibercept amount (mg)	Cumulative release (%)
Aflibercept/HA/IPC	**2.905** **0.981**	**0.6**	**1:200**	**0.097**	**0.6**
Aflibercept/HA/IPC/ 2µL Tween20	**1.47** **2.85**	**0.6**	**1:200**	**0.024**	**1.1**
Aflibercept/HA/IPC/ 4µL Tween20	**3.6** **0.442**	**0.6**	**1:200**	**0.035**	**0.6**

No detectable band corresponding to the aflibercept molecular weight (~115kDa) appeared after using of collagenase in all rigs (Lanes: 5-10, **Figure 33**). A thick band at 35kDa in all points of release study (Lane: 5-10, **Figure 33**), represents denaturation of aflibercept, which might be resulted in loss of total proteins detected by ELISA.

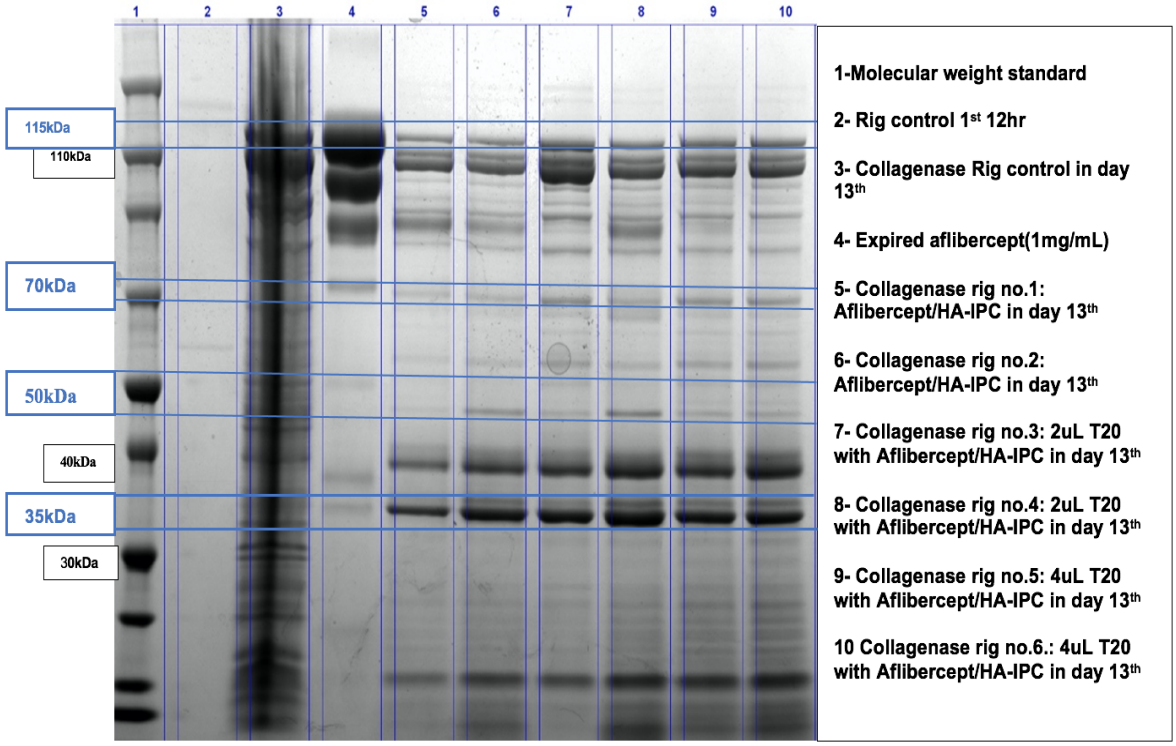

Figure 33. Non-reducing SDS-PAGE gel loaded with samples collected rigs after using collagenase in day 13th of release study. Lane M: molecular weight standard; Lanes 2 and 3, are Controls: IPC/HA. Lane 4 is expired 1mg/mL aflibercept, lane 5-10 are samples from collagenase of rigs 1,2,3,4,5 and 6 in the 13th day of release.

4. Discussion

The preparation process of the IPC was successfully carried out. Once the EDTA molecules exposed to PBS, the water molecules displaced with EDTA and allowed to form banded collagen fibrils. The polymerization (gelling) process completed after 1hour incubation at 37°C and resulted in a soft and semi-solid gel. A syringe mixing method to formulate IPC with HA-aflibercept conducted by following an injectibillity test. There was a limitation to use high amounts of HA and IPC due to the high viscosity of components.

Howerever, our injectibility and syringibility tests showed that the IPC mixed with HA (5mg) were all suitable for injection. When IPC was formulated with aflibercept alone, most of the drugs (Lanes: 4 and 5, **Figure 14**) released within 48h. However, once HA was used with IPC, (Lanes: 11-15, **Figure 14**) aflibercept's release was prolonged up to 5-day under sink condition. Because, the HA oligomers swelled in the presence of water molecules, the mesh sizes of the IPC reduced to an appropriate size to reduce the release of aflibercept from IPC. It was also observed that adding more amount of HA (5 mg instead of 2 mg) resulted in reducing of the burst release of aflibercept to less than 15% (**Figure 30**).

The aflibercept to IPC ratio was also optimised in order to prepare a sustain release depot. When diluted ratio of 1:2 (1 aflibercept and 2 IPC) was used in the sink study, approximately 92.0 ± 5% of drug was released within the first five days (**Figure 15**). This was due to losing the fibril density of collagen in the IPC, which caused to fully release of drugs. It is well known that increasing the amount of collagen could increase the mechanical stiffens and fibril density (47). The best results of aflibercept release profile from HA-IPC achieved for approximately 12-day (**Figure 30**) by changing the ratio of aflibercept to IPC (1:4) and shortening the dialysis time (12h instead of 24h). Suspending the aflibercept in the IPC with 1:4 ratio resulted more steady release (**Figure 30**), which more than 1.12mg (62%) of total loaded aflibercept released over 12 days. No further antibody was detected by SEC after 12^{th} days.

The SEC analysis measured only 62.7% of total released aflibercept up to the 12th day (**Figure 32**). Thereby, the specific binding activity of the aflibercept was also investigated by ELISA for further detection of aflibercept in the solution. However, the ELISA did not accurately measure aflibercept concentration because of the use of collagenase. It was shown in **Figure 24**, presence of EDTA in IPC formulation could avoid degradation of aflibercept from collagenase by chelating the calcium ions that

are essential for degradation process. Once the IPC decomposition was completed after 24h, the degradation of aflibercept was resulted leading to loss of its antigen-binding activity observed by ELISA. Several studies demonstrated that antibody-based medicine could undergo degradation due to some enzymatic digestion or storage conditions (48 & 49). It was not however shown in the literature that using collagenase (proteases) could degrade the proteins

While PEG may increase and maintain suitable mechanical properties of collagen during fibrillogenesis, whereas our results showed that using the PEG within the IPC-HA formulation did not help to achieve a sustainable release profile. This might be due to formation of a variety of covalent bond linkage between aflibercept and polymer which these stable covalent linkages could retain the aflibercept until the network degrades (50). The cleavable covalent linkages caused to short release of aflibercept till 5-day of release study. Thus, it seems that most proportion of loaded aflibercept retained and physically entrapped inside the mesh network of HA-IPC/PEG formulation (Lanes:13 and 15, **Figure 28**).

Although non-ionic surfactant such as Tween 20 was used as a stabilizer in protein formulation to evaluate their effect on the release rate (52). As shown in **Figure 31**, a less concentration (ug/mL) of aflibercept was observed by adding more volume of Tween 20 in the precipitating solution after 12- day. To conclude, our results revealed that using Tween 20 within aflibercept-HA/IPC formulation caused to decrease in release profile. This could be due to the influence of surfactant on the increasing of the IgG size, which leads to decrease in the release rate (52).

5. Conclusion and future direction

In conclusion, an easy-to-inject liquid IPC which fibrilizes upon interaction with biological conditions has been modified to formulate aflibercept, in which 63% of loaded drugs were slowly released up to 13-day. Antibodies were easily added to IPC-HA by mixing and then were loaded into the sink (no flow) and rig models. The presence of HA associated with reducing the mesh size and burst release of drugs, which could minimal the burst release less than 15% of total loaded drugs in the first 48h. Optimised method to prolong drug release at a predetermined rate was explored by using three main strategies in this work, including: 1-shortening dialysis time up to

12 h, 2- finding a suitable correlation between IPC/aflibercept in the ratio of 1:4 (80µL of IPC with 20µL of aflibercept) and 3- adding an affordable amount of HA (5mg).

In future work, detection methods should be optimized for extremely sensitive detection, especially in ultra-low concentration by using Biacore and Liquid chromatography–mass spectrometry (LC–MS) techniques. To avoid the air bubble formation and reduce drug losses, using a FlakTeck Speed mixer would be ideal to prepare the formulation (53). To enhance protein stability and prevent aggregation of aflibercept, incorporation of antibody may be maintained by using some excipients such as glutamate, arginine, and histidine (54). Tween 20 and PEG didn't show a sufficiently sustained release profile, but other polymers such as poly (lactic-co-glycolic acid) (PLGA) core would be evaluated in the future test to develop the hydrophobicity and reduce drug release (55).

6. References

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7. Appendices

Appendix 1: Drug analysis released from the IPC or HA/IPC solution using Rig model

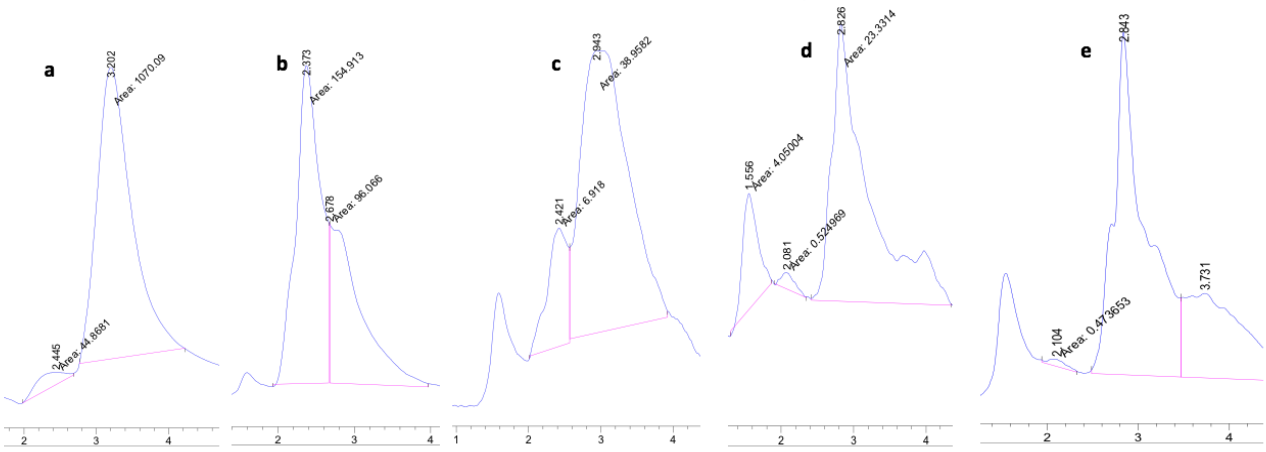

Figure A1. SEC chromatogram of aflibercept/IPC-HA in a)-e): first 12 h, day 3, 6, 9 and 12 of release study using rig model.

Appendix 2: Examples SEC report of the release study

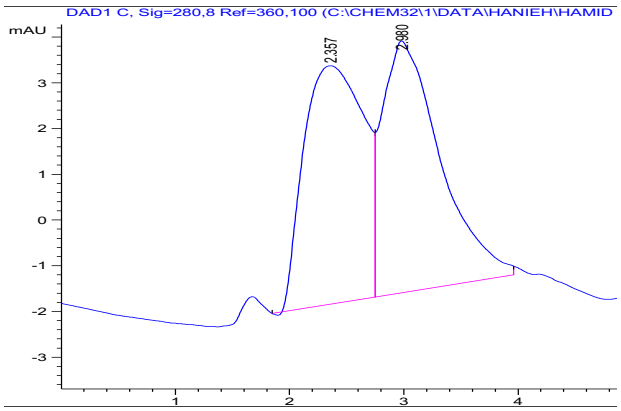

Figure A2. The SEC chromatogram peak of aflibercept and collage. a) aflibercept, retention time= 2.357 min and b) collagen, retention time= 2.980 min.

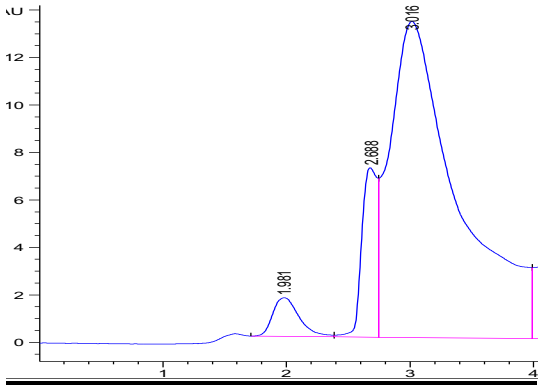

Figure A3. The SEC chromatogram peak of IPC alone (control), retention time of collagen=2.688.