Thesis for the Degree of Master of Engineering

Alboostumin:

An Engineered Albumin as a Modular Scaffold for Drug Delivery

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August 2021

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알부스투민: 약물 전달을 위한 모듈형 알부민 스캐폴드

Advisor: Prof. Sung In Lim

by

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A thesis submitted in partial fulfillment of the requirements
For the degree of

Master of Engineering

in the Department of Chemical Engineering, The Graduate School,

Pukyong National University

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August 27, 2021

Dedication

This thesis work is dedicated to my parents, who have been a constant source of support and encouragement during graduate school and life challenges. I am truly thankful for having them in my life.

Acknowledgment

First and foremost, praises and thanks to God, the Almighty, for the showers of blessings throughout my research work to complete it successfully.

I would like to express my deep and sincere gratitude to my research supervisor, Prof. Sung In Lim, for giving me the opportunity to do research and providing invaluable guidance throughout this project. His dynamism, vision, sincerity, and motivation have deeply inspired me. It was a great privilege and honor to work and study under his guidance.

I greatly appreciate the support received through the collaborative work undertaken with Dr. Dae-Hee Lee and Lee Jeong Eun during the cell assay section of this project.

I would like to thank my committee members, Professor Dr. Yiseul Park and Dr. Sang-ho Lee, for their time and contribution.

Special thanks to my family. Words cannot express how grateful I am for having them in my life. I would not be the person I am today if I didn't receive their love and support.

I would like to extend my thanks to my lab mates and friends, especially to Dr. Abbas Hajizade, for his technical guidance, comments, and suggestions throughout my research work.

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Alboostumin: An Engineered Albumin as a Modular Scaffold for Drug Delivery

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Abstract

Promising therapeutic proteins often fail to advance in the clinical evaluation due to poor half-life in the circulation. Thus, it is of great value to develop a drug carrier that can improve in vivo half-life of a payload without compromising its therapeutic activity. Serum proteins like immunoglobulin G (IgG) and albumin have been employed as long-acting drug carriers to exploit their innate ability to survive sustainably in the blood. Several Fc or albumin fusion proteins have demonstrated their potential in clinical trials. However, some proteins have complex folding pathways or conformation incompatible with genetic fusion to such carriers due to poor manufacturability or inefficaciousness. To provide a solution for this problem, this research was conducted by engineering human serum albumin (HSA) into a modular drug carrier by substituting its second domain with a self-assembling coil domain. The result indicated that the engineered HSA (eHSA) could self assemble with a payload protein modified by a complementary partner coil. Isothermal titration calorimetry (ITC), Size Exclusion Chromatography (SEC), and NATIVE-PAGE techniques have revealed the eHSA could tightly bind the payload with high affinity. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has an apoptotic effect on cancerous cells, and when it gets trimerized, its apoptotic activity would be upgraded. Cloning of trimerized TRAIL to the complementary coil as a more functional therapeutic compound and investigation by NATIVE-PAGE proved this big therapeutic molecule can still bind to eHSA with good affinity. Its interaction with FcRn by Microscale thermophoresis (MST) verify the eHSA would have a long half-life in the bloodstream.

Keywords: Albumin, self-assembling coil domain, TRAIL, Anticancer

Abbreviation

HSA- Human Serum Albumin

eHSA: Engineered Human Serum Albumin

WT: Wild Type

GFP: Green Fluorescent Protein

AB: Antibiotic

NA: Nucleic Acid

CV: Column Volume

OD: Optical Density

IPTG: Isopropyl-thio-β-d-galactoside

Kd: dissociation constant

kDa: Kilodalton

ml: Milliliter

M: Molar

PAGE: Polyacrylamide gel electrophoresis

SDS: Sodium dodecyl sulfate

RT: Room Temperature

Rpm: Round per minute

ChapterI
Introduction

1.1 Drug Delivery System

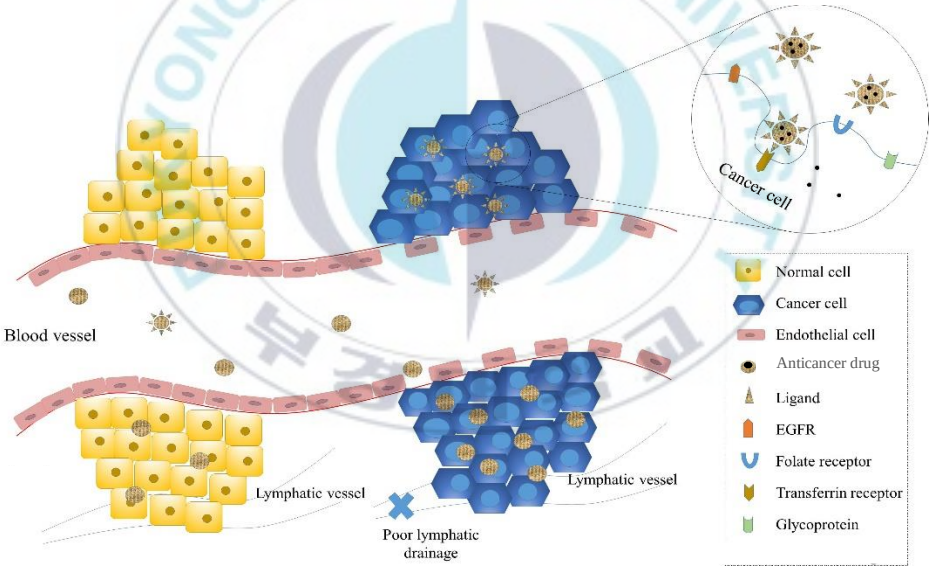
A drug delivery system (DDS) is a formulation to enhance a drug's safety and effectiveness by regulating the rate, time, and location of drug release into the body. The history of drug delivery commenced in the 1950s and has been in existence. The first generation had challenges with physicochemical properties, and the second generation tried to address the biological barrier. However, the third and novel generations focus on addressing poor water solubility of drugs, the small size of peptide and protein drugs, difficulty controlling the drug release kinetics, and the short half-life of a therapeutic compound in the bloodstream [1]. The first FDA-approved drug delivery technology was liposomal amphotericin B for treating fungal infections [2]. Application of liposome as a drug carrier could enhance the therapeutic index and provide a longer drug circulation time than the free form of the drug.

Over the years, many organic and inorganic carriers have been designed to improve the potency, selectivity, and pharmacokinetics of drugs. The most critical aspect of choosing a suitable carrier is biocompatibility. The source of the carrier should be entirely biological. It shouldn't induce any immunogenic reaction upon administration with the drug [3]. Biocompatibility and Biodegradability are essential criteria of a carrier to consider in the drug formulation.

Most scaffolds need to be designed with specific ligands and moieties to direct the migration of a drug molecule into the body. Some overexpressed receptors and molecules

in the cancerous or inflamed sites make the target site recognizable (Fig. 1). A drug-loaded carrier with targeting abilities enables the drug release at a specific and limited spot in the body.

Targetting moieties identify the diseased site selectivity and maximize the drug accumulation in the desired area. As shown in Fig. 1, the biodistribution of a drug molecule can be addressed by conjugating specific ligands targeting specific moieties and receptors in the cancerous area.



"Front. mol. Biosci, 7 (2020):193"

Figure 1. Drug targeting to the cancerous cells.

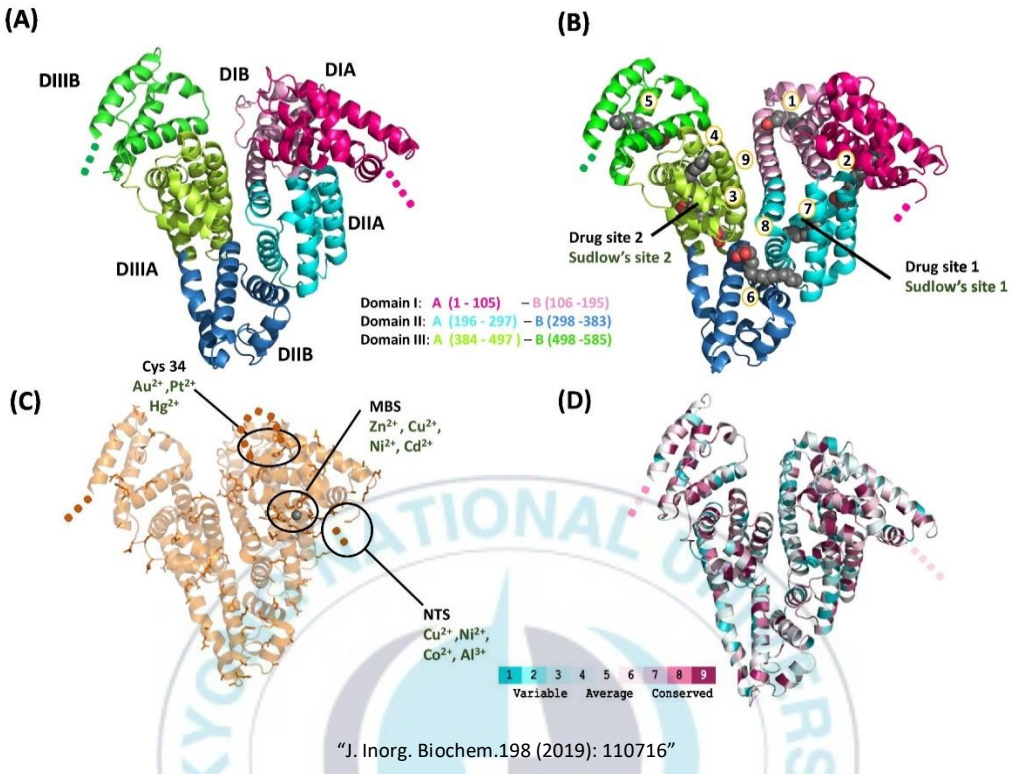
A long half-life is another important privilege that diminishes the need for drug administration multiple times and reduces the prescribed drug dosage [4]. Hemlibra is a bispecific Ab that has been approved to treat and manage hemophilia symptoms [5]. Hemlibra's elimination half-life is 30 days in the bloodstream, whereas another therapeutic hemophilia medicine (factor VIII) has an in vivo half-life of 8-12 hours [6, 7]. Providing prolonged action formulation offers sustained drug exposure with infrequent dosage. This property can dramatically elevate the satisfaction and patients' quality of life. Drug delivery could be much more functional if it can meet mentioned criteria. Albumin will be introduced as a promising scaffold in the following sections.

1.2. Albumin-based drug delivery

Albumin is one of the essential proteins in plasma with a concentration of around 40 g/L and 66.5 kDa molecular weight. It is synthesized by hepatocytes and released into the vascular space. Albumin is a carrier of a wide variety of endogenous and exogenous compounds such as fatty acids, steroids, and different drugs due to hydrophobic binding pocket on its tertiary structure [8]. It has unique characteristics as a carrier that can significantly improve the therapeutic payload's pharmacological action. Albumin-based drug delivery can be an ideal drug delivery mechanism because of its intrinsic biochemical and biophysical properties.

1.2.1. Albumin tertiary structure

Albumin consists of three homologous alpha-helical domains. Each domain comprises two subdomains, A and B (Fig. 2). Human albumin, a single non-glycosylated polypeptide chain of 585 amino acids, has a heart-shaped protein structure with 67% α-helix, no β-sheet, and 17 disulfide bonds. There are seven fatty acid-binding sites which are asymmetrically distributed in this protein. The free thiol at the cysteine-34 residue and Sudlow's sites I and II, which bind several hydrophobic drugs, are also critical binding sites [9, 10]. Aromatic carboxylates like ibuprofen and diazepam bind to a spot in DIIIA known as the major drug-binding site II, or Sudlow's site II, while bulky heterocyclic anions like warfarin bind to a site in the center of DIIA known as the major drug-binding site I or Sudlow's site I (Fig. 2) [11, 12]. Albumin is soluble in aqueous solutions around neutral pH due to its high amount of charged amino acids, 83 basic (lysine and arginine), and 98 acidic (glutamic acid and aspartic acid). HSA is highly resistant to pH changes, heat, and denaturing solvents [13].



"J. Inorg. Biochem.198 (2019): 110716"

Figure 2. Crystal structure of Human Serum Albumin, A shows different domains by color, B shows Sudlow's sites, and numbers on HSA exhibit another drug binding sites. Metal-binding sites are shown in the C. variable, and conserved residues are indicated by highlight in D.

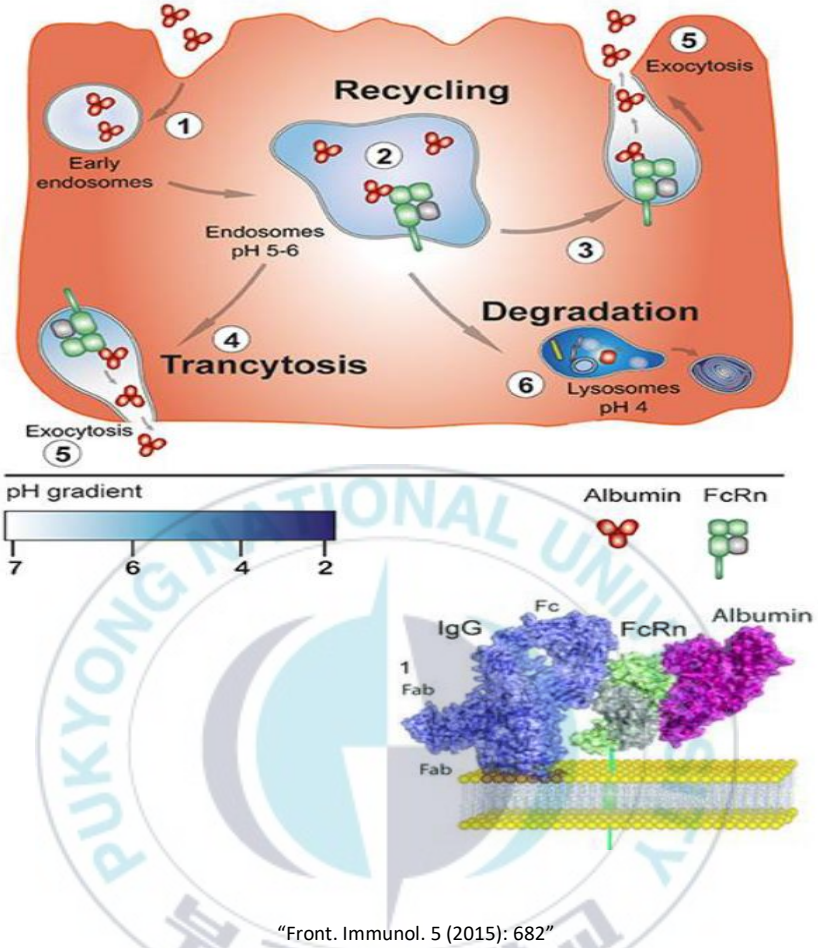
1.2.2. Long half-life

As an emerging opportunity in drug delivery, albumin can increase drug half-life by up to 3 weeks. Enzymes in the bloodstream can degrade or deactivate drugs such as peptides, proteins, and nucleic acid drugs and shorten their in vivo half-lives. Also, if the drug's

molecular weight and diameter are less than 60 kDa or 6 nm, it will be quickly removed from the systemic circulation by renal filtration [14]. A short therapeutic half-life reduces drug application in the clinical stage. Patients must prescribe higher doses more often to maintain the desired therapeutic effect, which is cumbersome and risks undesirable side effects. So prolongation of plasma half-life is a prerequisite for numerous drug candidates in clinical use. Drug carriers can increase the half-life of small-molecule drugs in plasma by increasing their adequate size to more than the renal filtration cutoff. HSA's long half-life is associated with FcRn recycling and resistance to kidney filtration due to the larger size of HSA and negative charge repulsion [15]. The glomerular basement membrane contains proteoglycans, which function as an anionic barrier, prevent negatively charged macromolecules like albumin from passing through.

Albumin's long half-life makes it an attractive carrier for boosting the pharmacokinetic properties of drugs. The neonatal Fc receptor (FcRn), an intracellular receptor that protects albumin from degradation, is primarily responsible for this long half-life [16]. This receptor is found all over the body and has been shown to prolong the half-life of serum albumin and IgG [17, 18]. The FcRn works by binding these proteins in the acidic endosome and diverting them away from the degradative lysosomal pathway. Exocytosis of the FcRn and its attached albumin to the extracellular space allows the ligand to be released from the FcRn at physiological pH. The strong binding affinity at the endosome's low pH indicates that this receptor interaction is pH-dependent. Details of FcRn mediated recycling are

elaborated in Fig. 3. Several engineered HSA variants exhibit stronger affinity to FcRn at endosomal pH and consequently survive longer than native HSA in the bloodstream [19, 20].



"Front. Immunol. 5 (2015): 682"

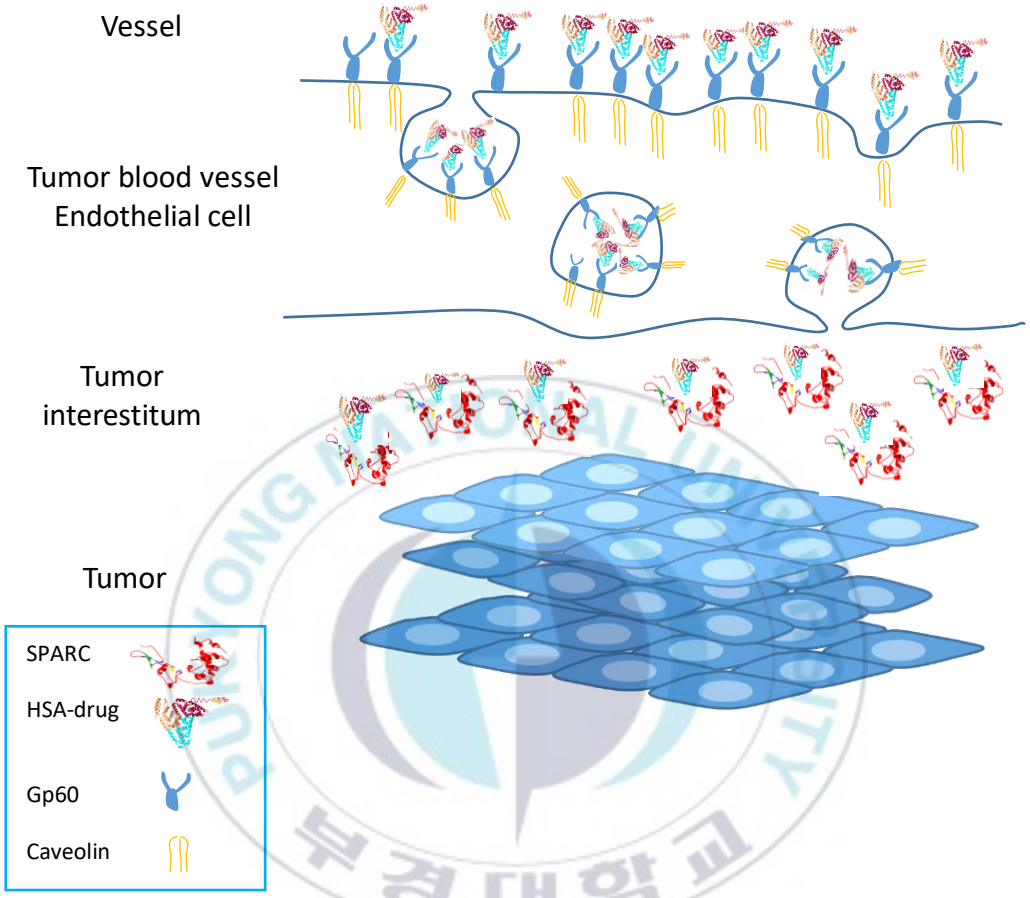
Figure 3. Mechanism of the FcRn-mediated recycling pathway. 1) HSA-drugs are taken up from the bloodstream by pinocytosis. 2) FcRn on acidified endosomal membranes binds HSA-drugs, and the complex is directed to the recycling pathway. 3,4,5) At the neutral pH of the bloodstream, the interaction between FcRn and HSA is lost and releasing HSA-drug back to the bloodstream.6) HSA-drugs don't bound FcRn would degrade in lysosomes.

1.2.3. Specificity

Passive and active targeting are two techniques that can be utilized to target the desired drug release site. Passive targeting is the accumulation of the therapeutic compound in the diseased area due to the enhanced vascular permeability like inflamed and cancerous areas. Another approach is active targeting which needs functionalization of drug carriers with ligands [21]. Ligand-receptor interaction is highly selective, and some special receptors are overexpressed in the diseased area. This could allow more precise targeting of the site of interest. As a carrier that meets both concepts of specificity, albumin can target inflamed and cancerous sites specifically.

Hypoalbuminemia is common in patients who are suffering from inflammation. The lower HSA concentration is thought to be due to increased vasodilation in inflamed tissues and a higher fractional catabolic rate [22, 23]. In terms of drug delivery, inflamed sites could serve as an albumin "sink" in which an HSA-coupled payload could be localized and allow for targeted delivery. Although increased capillary permeability and a lack of successful lymphatic drainage in the tumor microenvironment (TME) cause HSA to be passively localized, HSA-specific membrane receptors found in cancerous cells actively recruit HSA to TME as a source of growth and energy [24]. Gp60 and SPARC are two HSA receptors that have been thoroughly studied in terms of their physiology and functions in HSA-based drug delivery [25]. The receptor GP60, also known as albondin, is responsible for this

process. Albumin binds to GP 60, causing albumin-GP 60 clusterings at the cell surface and interaction with Cav-1, the main caveolae-forming protein. Cav-1 causes the internalization of a vesicle made up of the surface membrane that surrounds these clustered albumin-GP 60 receptors as well as albumin-bound compounds. Cav-1 is then transferred to the basolateral membrane and fused with it to complete transcytosis. SPARC presence in the tumor environment would concentrate the albumin-fused drug and enhance its therapeutic effect (Fig. 4).



"Biotechnol Bioproc E 25, (2020): 985–995"

Figure 4. HSA-drug accumulation in tumor site by usage Gp60, SPARC, and Caveolin in the tumor site.

1.2.4. Albumin binding strategies

As mentioned in the last section, drug molecules can be explicitly incorporated into HSA for medication. Let's take a look briefly at how a therapeutic molecule can be accompanied by an HSA molecule. There are three primary methods for binding drug entities to albumin: covalent, fusion, and nanoformulation.

1.2.4.1. Covalent binding

Residues such as lysines, tyrosines, and the free SH-group on the cysteine-34 of albumin are the most common residues for covalent conjugation and drug binding. Covalent conjugation of methotrexate (MTX) to Lys residue of HSA showed improved pharmacokinetic profile and anticancer efficacy. It showed tumor accumulation and the prolonged half-life of conjugated MTX compared to free MTX [9].

Maleimide also offers thiol conjugation chemistry to albumin with high specificity and reactivity toward the Cys34. This approach can be utilized for in situ attachment to endogenous albumin after intravenous administration of maleimide-activated prodrug [26]. Aldoxorubicin (CytRx) is a thiol-reactive targeted prodrug in Phase III clinical trials to treat soft tissue sarcoma. The linkage is done by acid-labile N-ε-maleimidocaproic acid hydrazide (EMCH). EMCH is proposed to facilitate in situ attachment to the free thiol of endogenous albumin. This linkage will be cleaved and in the acidic tumor environment and

release doxorubicin [27]. As shown in Fig. 5, modified drugs with natural or artificial binding moiety can also bind to endogenous albumin upon administration [28]. Covalent binding would offer selective drug binding to HSA, but a limited site is available for drug conjugation on albumin structure.

1.2.4.2. Genetic fusion

Genetically engineered recombinant fusion HSA consists of a drug connected to either the C, N, or both termini of human serum albumin (Fig. 5). It allows recombinant expression of a fusion protein or a single drug built with a regulated albumin-to-drug ratio [28]. Albumin fusion technology is a simple and technically less challenging method of combining therapeutic proteins with HSA. Idelvion, recombinant FIX-albumin fusion is FDA approved for the treatment of hemophilia. It has a short cleavable linker susceptible to cleavage during the coagulation cascade. It exhibited a prolonged half-life compared to coagulation Factor IX, which needs to be administrated every 2-3 days to prevent spontaneous bleeding. This recombinant product reduces dosing frequency, thereby significantly improving patients' compliance with therapy [29]. The importance of a peptide linker in optimizing the fusion strategy can not be overstated. The versatility and length of a peptide linker significantly impact intramolecular and intermolecular interactions, thus on fused proteins' biological activity and physicochemical stability in vivo. The FcRn binding site of albumin should be considered that the fused drug doesn’t

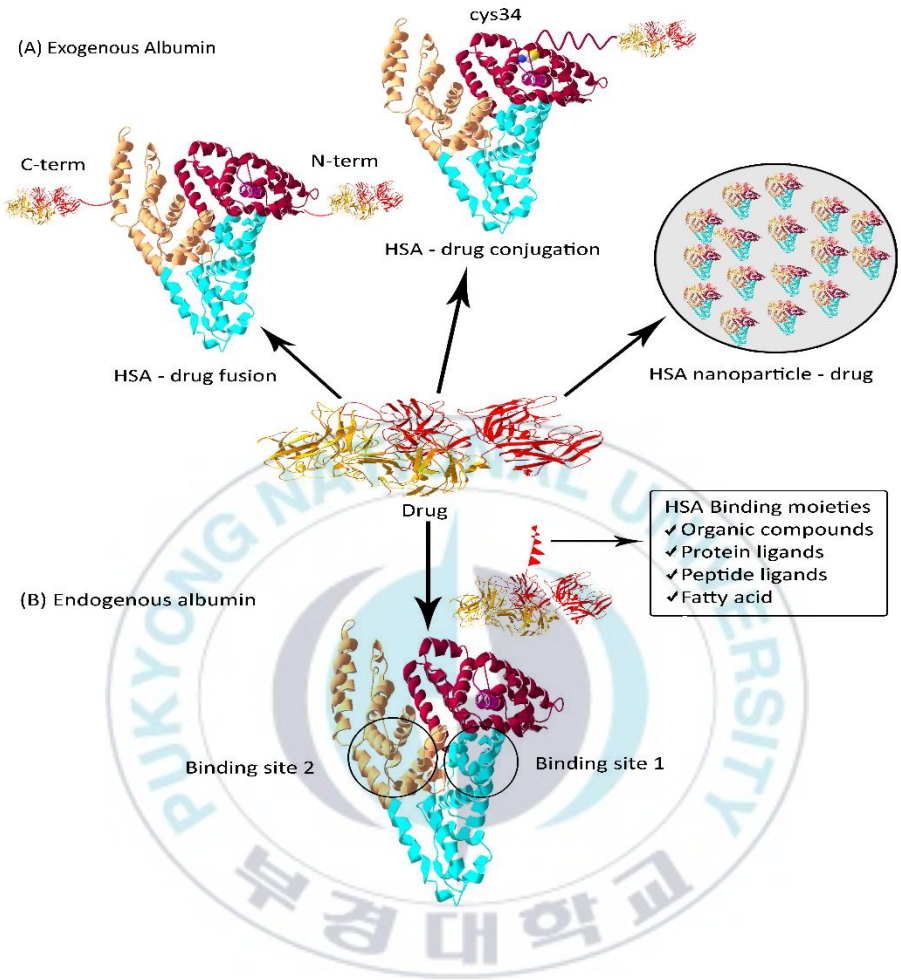
have any steric interference with FcRn engagement. Albiglutide is a long-acting glucan-like peptide 1 (GLP-1) analog that genetically connects two tandem copies of GLP-1 to HSA. This construct provides a long half-life and better potential to treat type-2 diabetes mellitus (T2DM) than conventional drugs in clinics with modest weight reduction [30].

The C-terminal domain of HSA consists of primary residues to bind FcRn moiety. Domain I is also important in this interaction; thus, genetic fusion can impede and reduce binding affinity to FcRn [31]. Although this method can be a straightforward technique to incorporate drugs on the HSA construct, it may impair the FcRn binding site.

1.2.4.3. Nanoformulation

As a drug carrier, nanoparticles have gotten significant attention. A nanoparticle delivery system can significantly increase solubility and target specificity of therapeutic agents with poor bioavailability (Fig. 5). Desolvation, thermal gelation, emulsification, and self-assembly are some synthesis methods for generating albumin nanoparticles [32, 33]. The most well-known albumin nanoparticle is the FDA-approved nab-paclitaxel, also known as Abraxane. It is used to treat non-small cell lung cancer (NSCLC), breast cancer, and pancreatic cancer and is studied in other cancers, too [34]. It showed a significant advantage rather than the free form of paclitaxel and also exhibited more potent antitumor activity when associated with radiotherapy and chemotherapy [35]. To provide additional

specificity to cancer-associated receptors, albumin nanoparticles can be decorated with various targeting ligands. Though albumin nanoformulation offers a higher drug loading to albumin ratio and the ability to transit across tissue and cellular barriers, it needs harsh conditions like sonication and cross linker like glutaraldehyde for synthesis and drug encapsulation [28]. It would dramatically affect the therapeutic activity of drug molecules.



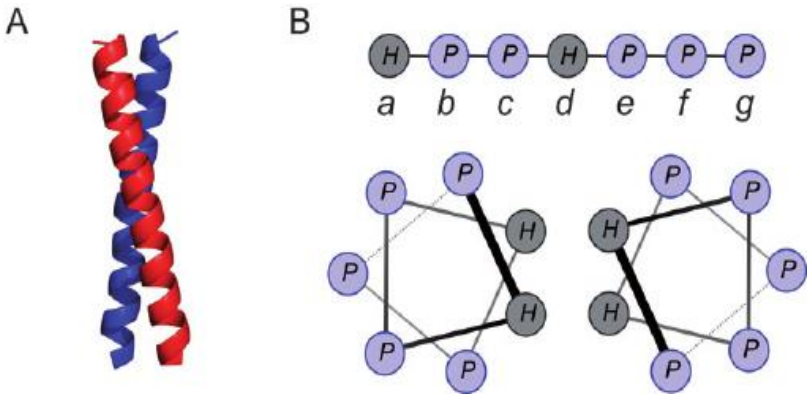
"Biotechnol Bioproc E 25, (2020): 985–995"

Figure 5. Albumin-based strategies for drug delivery. (A) Genetically fusion of drug to the N and C terminal of HSA, covalent conjugation to Cys34, and drug encapsulation in HSA nanoparticle. (B) Modification of the desired drug by binding moieties and in situ bindings upon administration.

As explained, while all albumin binding techniques improve pharmacokinetic activity, each method has its own set of drawbacks. FcRn binding inhibition in genetic fusion, limited binding sites in conjugation approaches, and harsh conditions during albumin nanoparticle synthesis require developing a new and superior albumin format. The use of a self-assembly coil in the albumin structure can result in modular construction with more extra binding sites while leaving the FcRn binding site unaffected.

1.3. Self-assembly domain

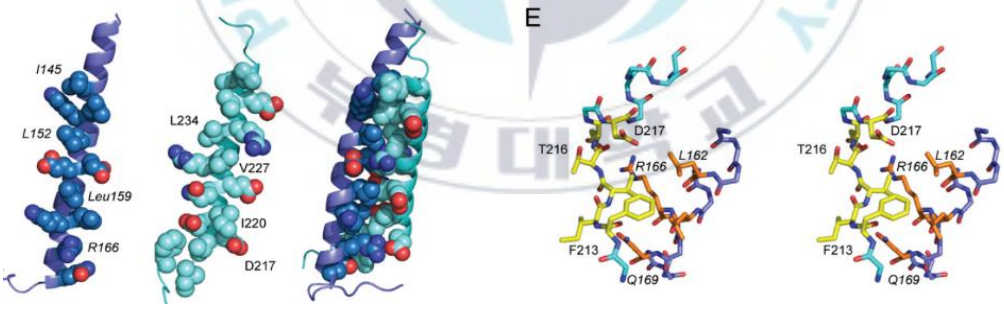
Application of coiled coils for generating drug carriers would also be feasible. The coiled-coil domain is a common motif found in 10% of eukaryotic proteins. This motif can be identified by a regular seven amino acid repetition of hydrophobic and charged residues [36]. The hydrophobic residues are situated in *a* and *d* positions, and others present polar residues (Fig. 6). These seven residues make two helix turns and wraps around another helix to form a "coil of coil" domain. This Coiled-coil domain can produce a unique home- or hetero-oligomeric complex with 2–7 helices arranged in parallel or anti-parallel patterns, which is critical for various cellular processes, either alone or as part of larger protein complexes [37].



"Faraday Discuss. 143 (2009): 305-317"

Figure 6. Self-assembly coiled-coil domain.

A coiled-coil interaction between the methyl-cytosine binding domain 2 (MBD2) and p66α is part of a protein complex critical for DNA methylation. The antiparallel, heterodimeric coiled-coil interaction between MBD2 and p66 has a high affinity (Fig. 7) [38].



"PNAS, 108.18 (2011): 7487-7492"

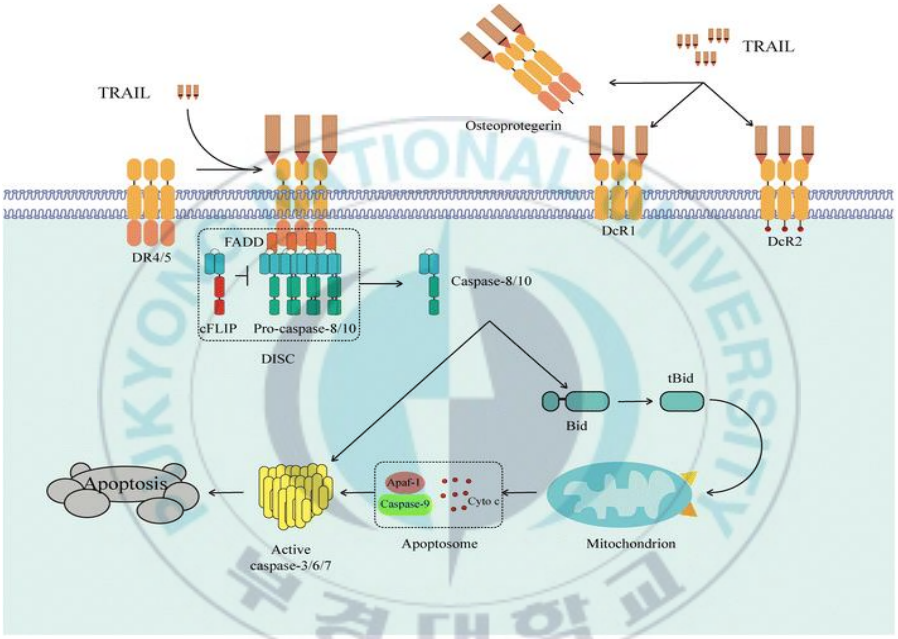
Figure 7. The coiled-coil interaction between MBD2 homologues and p66 α.

1.4. TNF-related apoptosis-inducing ligand (TRAIL)

For over decades, the goal of cancer immunotherapy has been the development of promising treatments for various types of cancer. The hallmarks of cancerous cells are uncontrolled growth, angiogenesis, and apoptosis evasion. One of the favorable therapies is promoting the effective elimination of cancer cells by apoptosis. Apoptosis is natural programmed cell death which has an essential role in development and homeostasis [39].

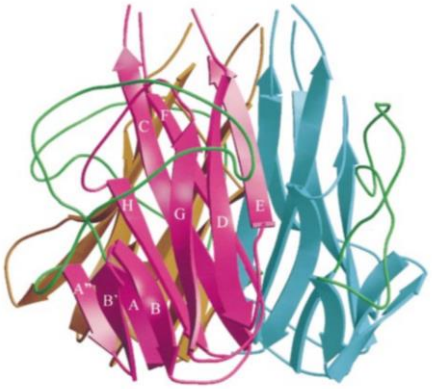
Two signaling pathways lead to apoptosis: the intrinsic (mitochondrial) pathway and the extrinsic (death receptor) pathway. The death receptor is activated by ligands such as tumor necrosis factor (TNF)-related apoptosis-inducing ligand to start the extrinsic apoptosis pathway. TRAIL is a member of the TNF superfamily, which can effectively and selectively induce apoptosis in various primary tumor cells [40]. There are currently two TRAIL receptors with a functional death domain (DR4 and DR5) that induce apoptosis [41]. DR4 and DR5 are transmembrane proteins sharing 58% sequence homology with a cytoplasmic or death domain that engages apoptosis signaling molecules to induce cell death. As shown in Fig. 8, DR1 and DR2 lack the functional death domain and are therefore unable to transmit the apoptotic signals [42]. Apoptosis signals cause some intrinsic changes inside the cell; it leads to trimerization of DR4 and DR5 and initiation of signaling cascades. Trimerization of TRAIL receptors activates caspases. It leads to the cleavage of cellular components required for normal cellular function, such as cytoskeletal and nuclear

proteins. Apoptotic cells begin to shrink and undergo plasma membrane changes that signal the macrophage response. The molecular mechanism of TRAIL action is described in Fig. 8. Targeting apoptosis is the most successful non-surgical treatment, and TRAIL-based therapies can improve the therapeutic efficacy dramatically [43].



"Cancer Metastasis Rev, 37.4 (2018): 733-748"

Figure 8. The TRAIL apoptosis pathway. FADD, caspase-8/-10, and cFLIP are recruited to death receptors DR4 and DR5 in response to TRAIL stimulation, forming the death-inducing signaling complex (DISC), activating caspases, and apoptosis pathway. Three decoy receptors: DcR1, DcR2, and osteoprotegerin are unfunctional receptors.



" Immunity 11.2 (1999): 253-261"

Figure 9. The ribbon structure of the TRAIL trimer.

As mentioned, TRAIL belongs to the tumor necrosis factor (TNF) family. TRAIL is a 20 kDa protein that contains many antiparallel β sheets that form a β sandwich as a core scaffold and interacts with the adjacent subunits in a head-to-tail fashion to create a bell-shaped homotrimer (Fig. 9).

1.5. Objective of this study

The advance of this field would be designing a biocompatible carrier and customizing its structure for drug delivery. Enhancement of the DDS to minimize toxicity and improve the efficacy of existing treatment can offer significant benefits for patients and open up new markets for pharmaceutical companies. In this project, we want to create an engineered form of albumin with the self-assembly coil domain to create a modular scaffold. Modularity offers this condition that each therapeutic compound quickly and efficiently

can be accompanied and incorporated into the platform. Each therapeutic entity on this platform would retain its therapeutic activity, and this modular construct would have a long half-life.

Chapter II
Materials and Methods

This study works with Genetically Modified Organisms (GMO) which are considered biohazards. Bacterial Culture, DNA, and proteins derived from GMOs should be destroyed and removed carefully. General guidelines should be notified when working in the molecular lab.

2.1. Materials

In this work, materials and reagents were purchased from Qiagen, Samchun, Bioneer, Biosesang, Enzymonics, Thermo scientific, Takara, and Sigma. Primers were designed with genome compiler software. Order primers and Sequencing of DNA fragments were performed by Bionics.

Table 1. Media, buffers, and reagents

name	components	application
LB media for 1 L	5 gr Trypton, 2.5 yeast extract, 5 gr NaCl	microbial growth medium
LBA for 1 L	5 gr Trypton,2.5 gr yeast extract, 5 gr NaCl, 6.5 gr Agar	microbial growth medium
2xYT for 1 L	31 gr 2xYT powder	microbial growth medium
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4 pH:7.4	Protein buffer
DNA loading dye	10 mM Tris-HCl 0.03 % Bromophenol blue, 60 % Glycerol, 60 mM EDTA pH:7.6	Agarose gel electrophoresis
TAE buffer	2M Tris, 1 M Acetic acid, 50 mM EDTA pH: 8.3	Agarose gel electrophoresis
Glycerol	30% glycerol in D2O	Stock preparation
Lysis buffer	50 mM NaH2PO4, 300mM NaCl, 10 mM Imidazole pH:8	Protein purification
TBST buffer	20 mM Tris, 150 mM NaCl, 0.1% Tween pH:7.4	Wash buffer (Western blot)
Skim milk	5% Skim milk in wash buffer	Blocking buffer (Western blot)
Transfer buffer	25 mMTris, 192 mM Glycine, 10% Ethanol pH:8.3	Transfer buffer (Western blot)
Wash buffer	50 mM NaH2PO4, 300 mM NaCl, 20 mM Imidazole, pH 8.0	Ni-NTA purification
Elution buffer	50 mM NaH2PO4, 300 mM NaCl, 250 mM Imidazole, pH 8.0	Ni-NTA purification
Destaining solution	Methanol: Acetic acid: Deionized water (3:2:1)	SDS-PAGE
Protein electrophoresis buffer	25 mMTris, 192 mM Glycin, pH:8.3	SDS-PAGE
Buffer A	20 mM Bis-Tris	Ionic Exchange Chromatography
Buffer B	20 mM Bis-Tris, 1M NaCl	Ionic exchange Chromatography
Sample buffer 2X	63 mM Tris, Glycerol 25 %, Bromophenol Blue 0.0025 % pH 6.8	Native-PAGE

Table 2. List of antibiotics in this study.

Name	Working Concentration
Ampiciline	100 µg/mL
spectinomycin	100 µg/mL
tetracyclin	10 µg/mL

Table 3. The bacterial strains were used in this project.

Name	Source
DH5α	Enzymonics
BL21 (DE3)	Enzymonics
SHuffle	Enzymonics
Origami	Enzymonics

Table 4. Vector information.

Vector name	Gene	Antibiotic resistance
pET21	GFP-coil	Amp
pET21	monoTRAIL-coil	Amp
pET28	monoTRAIL	Amp
PQE-T7	HSA	Amp
PQE-T7	eHSA	Amp
PQE-T7	3TRAIL-coil	Amp

2.2. Construction of plasmid and expression hosts

To produce each construct (HSA, eHSA, TRAIL, TRAIL-coil, 3TRAIL-coil), the protein-coding sequence was cloned into a suitable expression vector and transferred into a bacteria host. The cloning of each DNA fragment was done based on the below flowchart (Table 5).

Table 5. Flowchart of cloning.

step	Method
1	Primer design by genome compiler
2	Amplification of gene sequence by PCR
3	Purification of PCR product
4	Pls prep
5	Infusion reaction
6	DNA adenine methylase incubation (Dam)
7	Transformation to DH5α by heat shock
8	Select some positive colonies for analyzing by colony PCR
9	Pls prep from positive colonies
10	Send recombinant vector to Bioneer company for sequencing
11	Prepare glycerol stock from DH5α containing the recombinant vector

All primers were designed by genome compiler software. The designed primers in Table 6 were synthesized by Bioneer and Cosmogenetech companies.

Table 6. Primers used in this project.

Primer name	sequence
TRAIL mono FWD	5‘GGCGGCGGAGGCAGCGTGAGAGAAAGAGGTCCTCAGAG 3’
TRAIL mono REV	5’CGAGTTAATGATGATGATGATGGCCAACTAAAAAGGCCCCG 3’
Lin GFP-p66 alpha FWD	5’CATCATCATCATCATCATTAACTCGAGCAC 3’
Lin GFP-p66 alpha REV	5’ GCTGCCTCCGCCGCCACTGC 3’
REVTRAILcoilT7	5’ GTGGTGGTGGTGGTGTTTTTGGGCCGTCGCCTC 3’
HSA1C3-pQE-FWD	5’ CTCAGGGGGAGGATCAGGAGGTGGGTCAAAGGCTTTCATTGTCACGGAT3’
HSA1C3-pQEter	5’ CAAGCTAGCTTGGATTCTCACC3’

Gene amplification was performed by Pfu PCR pre-mix according to the manufacturer's protocol.

Initially, colony PCR was used to confirm cloning, and then the pls were sent to Bioneer Company for sequencing to ensure the accuracy of the cloned DNA into the vectors.

2.3. Protein expression

After cloning verification by sequencing, the desired vector was transferred to the expression host for protein expression. The expression of albumin and engineered albumin was examined in the various E.coli strains (DE3, origami, and SHuffle).

2.3.1. Transformation by heat shock

The transformation was performed by heat shock at 42°C. To keep new recombinant bacterial cells for a long time, glycerol stock was prepared. The glycerol cell stock should be kept at -80°C.

2.3.2. Optimization of recombinant protein expression

To check recombinant protein expression, the procedure was done based on the below protocol. First, we should optimize protein expression by finding a suitable host, desired temperature and time, and inducer concentration. The below protocol was used for optimizing the expression of HSA, eHSA, GFP-coil, TRAIL, TRAIL-coil, and 3TRAIL-coil.

- **Preculture**: Forming colonies were inoculated into the LB and 2xYT media containing the desired antibiotic. BL21 (DE3) and SHuffle should be incubated at 37°C and 30°C, respectively, for 12-16 hours.

- **Main culture**: After bacteria growth, 1 ml of bacteria was inoculated in100 ml of new media containing selective AB. Optical density was monitored till 0.7 at 600 nm, 1 ml of culture was taken as before induction, and other parts were induced by 1 and 0.5 mM concentration of IPTG. After induction, the culture was incubated at various temperatures; 37°C, 42°C, and 28°C.

- **Harvest**: bacteria cells were collected at various time intervals by centrifugation (30 minutes at 7500 rpm at 4°C).

- **Analyze**: cell pellet and supernatant were investigated on SDS-PAGE.

2.3.3. Batch of desired protein (production)

After finding an optimized condition for protein expression, a larger batch was run to produce enough protein for further analysis.

- A single colony of transformants or 50 µl of glycerol stock was taken and inoculated into 5 ml of culture media containing desired AB based on vector resistance (table 4). the culture grew overnight.

- 500 ml of LB media (including antibiotics) was inoculated with 5 ml of the overnight cultures and grew in the incubator (30°C for SHuffle host, 37°C for DE3) with vigorous shaking 180-200 rpm until the OD600 was 0.7.
- Protein expression was induced by the addition of 1 mM IPTG. Before induction, 1 ml of culture was taken as noninduced control for analysis of protein expression.
- The cultures grew in incubator till OD reach near 3. (Expression of HSA(WT) and eHSA done at 30°C for 16 h, GFP-coil at 37°C for 5 h, TRAIL-coil at 26°C for 6 h)
- The cell was harvested by centrifugation for 30 minutes at 7500 rpm, and the supernatant was discarded.
- Cell pellets were freezed at -20°C.
- After freezing, the cell pellet was thaw at RT. It could increase lysis efficacy.
- Cell pellet was resuspended in lysis buffer and lysozyme. (rotating for 30 minutes on rotator at RT).
- The cell was lysed by sonication on ice (ULTRA SONICATOR BKUP-250N). Sonication was performed for 7 minutes with 10s burst at 50% power with a 10s cooling period between each shot. (sonicator should be equipped with a microtip)
- The lysate was centrifuged for 20–30 min at 7500 rpm to remove cellular debris and the supernatant was transferred to a fresh tube.

2.4. Protein Purification

To purify the desired protein from a crude mixture of bacterial proteins, chromatography with Ni-NTA column and anionic exchange chromatography were performed.

2.4.1. Ni-NTA purification

The cleared lysate supernatant containing the 6xHis-tagged protein was mixed with Ni-NTA resin for 1 hour at 4°C.

1. The mixture was loaded on the column.
2. The column was washed with 10 CV with wash buffer.
3. The desired protein was eluted with five CV elution buffers (collect fractions in separate microtubes)
4. The concentration of each elution fraction was measured by Biodrop at 280 nm.
5. The resin was washed with alcohol 30 % and stored at 4°C.
6. Note: Used column can be reused if appropriately kept at 4°C for 4 to 5 times, and the resin column can be recharged with Ni-sulfate [44].

2.4.2. FPLC

Ion exchange chromatography was performed with Äkta prime automated liquid chromatography using Hi Trap Q HP column. At the pH above the protein's isoelectric point, protein will negatively charge and bind to the anion exchanger column (Q column).

2.4.2.1. Buffer exchange

Buffer exchange was done by the PD-10 desalting column.

1. At the first usage, the bottom cap should be Cut off to pour off excess liquid.
2. The Buffer Reservoir was put on top of the PD-10 column, and the column was equilibrated with 25 ml buffer A (pH of buffer A was 1 unit lower than the isoelectric point of protein).
3. The flow-through was discarded, and 2.5 ml of the sample was loaded at the top of the resin.
4. The first flow-through was discarded.
5. The protein sample was collected in 3.5 ml of buffer A.

2.4.2.2. Anionic exchange chromatography

Q sepharose column is a strong anion exchanger. This technique is based on a reversible binding of charged protein to the opposite changed group of an insoluble matrix.

All lines, tubes, and columns should get equilibrated with buffer B and then with buffer A (table1).

* Before equilibration, column, buffers, and sample should be at RT.

Sample loading should be done with 1 ml/min. The sample was in buffer A, and after loading the whole sample, buffer A was injected into the instrument until the lines stabilized. (The pressure limit is 0.5 Mpa).

UV, pH, and conductivity should monitor.

After the lines stabilized, autozero was done, and the gradient was started (Buffer B).

Falcon tube should be placed in the fractionize position.

Whenever the UV graph detected the start of a peak, the feed tube was pressed.

The collected fractions were analyzed on SDS-PAGE (2.4.3.1).

2.4.3. Calculation of the batch yield

The concentration of purified proteins after purification and buffer exchange were measured by Biodrop at 280 nm. Based on the volume of each production set (bacteria culture medium), the yield of each batch was calculated.

2.5. Protein characterization

To verify protein expression, they were analyzed on SDS-PAGE and Western Blot.

2.5.1. SDS-PAGE gel electrophoresis

SDS-PAGE is an analytical method to separate proteins based on their MW in discontinuous gel electrophoresis. Protein separation by SDS-PAGE was applied to estimate the protein expression and relative protein molecular mass. 12% SDS gel prepared based on the below table (Table 7).

Table 7. 12% SDS_PAGE components.

	Stacking gel 4%	**Resolving gel 12%**
30% Acrylamide/bis	1.98 ml	6ml
0.5M Tris/HCl, pH:6.8	3.78 ml	-
1.5M Tris/HCl, pH:8.8	-	3.75ml
10% SDS	150 µl	150 µl
dH2O	9 ml	5.03 ml
TEMED	35 µl	30 µl
APS 20%	85 µl	85µl

2.5.1.1. Sample Preparation

To analyze protein samples on SDS gel, they were mixed with loading dye 1x thoroughly and boiled for 10 min at 95°C. Each protein sample's secondary and tertiary structure would be disrupted, and all would be at their primary structure on the gel.

2.5.1.2. Electrophoresis

Electrophoresis of protein samples was done at 150 V for 90 minutes.

2.5.1.3. Staining and destaining

After the electrophoresis was finished, the gel was stained to visualize protein bands. Gel staining was done with Coomassie Brilliant Blue R-250 for 1 hour on the shaker at RT. Afterward, the gel was covered with the destaining solution and allowed the gel to destain with gentle agitation on the shaker. It is better to be changed it 2 times; the destaining solution was removed two times by pouring a new destaining solution until protein bands got visible on the transparent background.

2.5.2. Western blot analysis

Western blot or "immunoblot" is applied to detect a specific protein in a crude protein mixture. This method is based on antibody-antigen interaction. It refers to the electrophoretic transfer of proteins from SDS-PAGE gel to a nitrocellulose membrane, followed by immunodetection of proteins using antibodies with chemiluminescent detection.

2.5.2.1. Gel electrophoresis

At first, the protein sample was analyzed on the electrophoresis gel as mentioned in section 2.5.1.

2.5.2.2. Blotting to membrane

Following electrophoresis, the protein was transferred from the gel to a membrane. The electrophoretic transfer would transfer protein bands with electrophoretic mobility from gel to membrane. This process places a protein-containing polyacrylamide gel in contact with a piece of nitrocellulose membrane in a sandwich structure. The process involves the use of porous sponges and filter paper to facilitate the transfer. The protein bands move out of the polyacrylamide gel onto the surface of the membrane in the presence of an electric field.

The transfer cassette was placed in a transfer tank filled with transfer buffer for 2 hours at 80V with an ice pack inside the tank. I ran two gels simultaneously, one was stained with coomassie blue, and another one was kept for immunoblotting.

2.5.2.3. Detection

Following the transfer, the nitrocellulose membrane was stained with ponceau to visualize proteins and subsequently destained in deionized water and TBST. Nitrocellulose

membrane was kept in 5% skim milk for 1 hour to block non-specific binding sites. Following blocking, the primary antibody was diluted in TBST and incubated with the nitrocellulose membrane for 2 hours on a shaker at RT. Following incubation with primary antibodies, the nitrocellulose membrane was washed in TBST 3 times for 10 minutes each time. The nitrocellulose membrane was then incubated with the secondary antibody conjugated to horse-radish peroxidase (HRP) in TBST for 1 hour at RT. Following incubation, the nitrocellulose membrane was again washed in TBST 3 times for 10 minutes each time.

2.5.2.4. Imaging

Proteins were then visualized by addition substrate and mix for 1 minute. After visualizing bands, the substrate was washed with d_2o and removed from the membrane.

2.6. Investigation of the interaction between two constructs

2.6.1. NATIVE-PAGE

Native-PAGE is a method that qualitatively measures the binding of two protein constructs to each other. The affinity of eHSA and its complementary coil, which was genetically fused to GFP or therapeutic drug first analyzed on NATIVE-PAGE. The gel was prepared based on table 8. Samples were mixed with loading dye which doesn't have SDS and

mercaptoethanol, to help proteins remain in their tertiary structure. It doesn't need to boil samples. Electrophoresis was done in cold temperatures and 90V.

Table 8. NATIVE-PAGE components.

Reagents	Main gel	Stacking gel
dH2O	4.11 ml	6.2 ml
1.5 M Tris pH:8.8	2.5 ml	-
0.5 M Tris pH:6.8	-	2.5 ml
30%Bis-Acrylamide	3.33 ml	1.33 ml
20% APS	50µl	50µl
TEMED	20µl	20µl

2.6.2. HPLC-SEC

SEC (size exclusion chromatography) is a type of HPLC that uses porous particles in the column to separate molecules in solution based on their size. Size exclusion chromatography was performed by Shimadzu Prominence 20 HPLC System. The chromatography column was Superdex 200 Increase 10/300 G.

2.6.3. Isothermal Titration calorimetry (ITC)

Isothermal Titration calorimetry (ITC) is based on the measurement of heat released or absorbed due to biomolecular interaction. During the ITC screening, the macromolecule of the reaction is put in a temperature-controlled cell, and the ligand is gradually injected into

the system. When binding occurs, heat will be released or absorbed. This heat change is proportional to the amount of binding.

2.6.3.1. Sample preparation

To study the interaction between eHSA and GFP-coil, samples were prepared at a particular concentration and volume. The concentration of ligand should be 10 to 20 times higher than the macromolecule. Protein samples were prepared in standard buffer (phosphate buffer, pH:7.4), and binding was analyzed with Auto-ITC200 Microcalorimeter (GE Healthcare). A total of 19 injections (2.5 μl per injection) of the GFP-coil (27μM) did into 4.4 μM of eHSA inside the calorimetric cell.

2.6.4. MST

To investigate the binding affinity of FcRn to recombinant HSA, MicroScale Thermophoresis (MST) with Monolith. NT115 was done. The MST method is based on the directed movement of molecules along a temperature gradient, an effect termed "thermophoresis." The motion of molecules is detected and quantified using covalently attached fluorophore dyes. One of the constructs should be labeled by fluorophore molecule. This instrument allows monitoring the directed movement of fluorescent molecules through microscopic temperature gradients in μl-volumes.

2.6.4.1. Labeling

The standard curve for Flamma 648 sulfo NHS ester as labeling molecule at 648 nm drew. Labeling of FcRn did base on the following procedure.

- The buffer of 140 µl of FcRn (4 µM) was changed to sodium bicarbonate pH: 8.5 by Vivaspin column.
- FcRn was mixed with Flamma 648 sulfo NHS ester with a 1:5 molar ratio. 1 mM Dye prepared in DMSO.
- The mixture was mixed on the rotator for 1 hour at RT.
- The labeled FcRn buffer was changed to phosphate buffer pH: 5.5.
- The protein concentration at 280 nm and dye at 648 nm were measured to check the labeling rate.

2.6.4.1.1. Analysis of labeling

To measure labeling, the different concentrations of the labeled FcRn were prepared and loaded into the capillary and scan with the instrument. We wanted to know which concentration of labeled protein was optimum to be scanned by instrument.

2.6.4.2. Prepare samples

Samples should be prepared for 16 different concentrations by serial dilution. 10 µl of ligand buffer (pH: 5.5) was added from 2 to 16 wells. 20 µl of the nonlabeled construct HSA(WT), eHSA, and commercial HSA was transferred to the first well, then 10 µl was taken and added from 2 to 16 to prepare serial dilution. Finally, 10 µl labeled protein (FcRn) was added to wells (1 to 16) and mixed thoroughly. The capillaries were dipped into each tube, and the mixture was aspirated automatically by capillary force. All samples were put into 1 to 16 of the device tray. All capillaries were scanned by instrument, and the interaction was investigated by affinity assay.

2.7. Cell assay

Cell assay analysis was performed by Beckman Coulter-CytoFLEX LX Flow Cytometer. The study was done by EzWay™ Annexin V-FITC Apoptosis Detection Kit. It's based on the binding of Annexin V to exposed phosphatidylserine on the apoptotic cells. Cell assay was investigated on a human colorectal carcinoma cell line (HCT116). The analysis was conducted by 5 ng per ml of protein concentration.

Chapter III
Results

3.1. Design of the new albumin construct (Alboostumin)

As shown in Fig. 10, Alboostumin as a new albumin scaffold was created by replacing the second domain with the self-assembly coil domain. This construct was cloned in a bacterial expression vector.

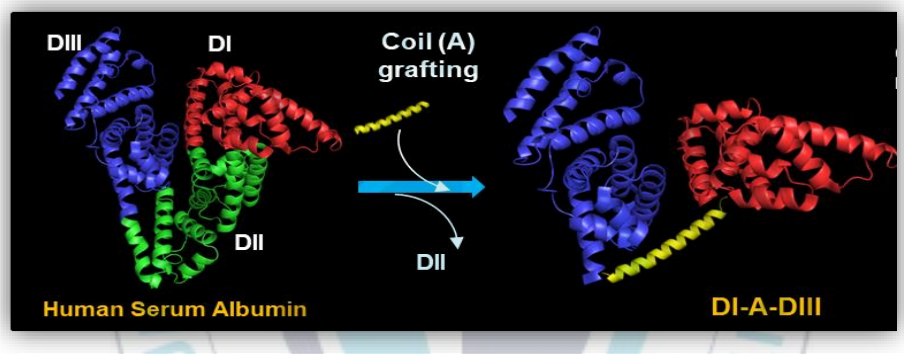


Figure 10. Alboostumin, the engineered Human Serum Albumin.

3.2. Recombinant expression of the native and the engineered HSA

Following the confirmation of cloning of native and engineered albumin by sequencing analysis, the expression profile was investigated by transferring the verified vector into the DE3, Origami, and SHuffle strains of E.coli. It showed SHuffle is a better strain compared to origami and BL21 (DE3). The design of the experiment by taking into account various parameters such as host (BL21, origami, SHuffle), the role of media (2XYT and LB),

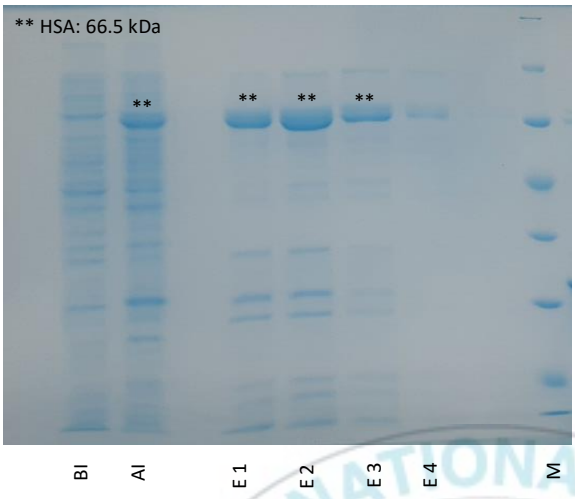
temperature, and time was performed. It exhibited 16 h at 30°C in 2 XYT media in SHuffle strain would be the optimal condition for HSA and eHSA expression (data not shown).

3.3. Result of protein purification

3.3.1. Ni affinity chromatography of the native and the engineered HSA

After expression analysis, purification was done by the Ni-NTA column. Purified HSA and eHSA are shown on coomassie stained SDS-PAGE, and the concentration of each fraction is mentioned in Fig. 11.

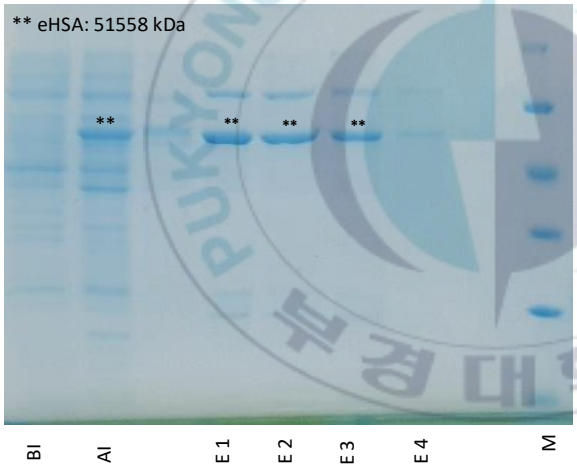
A



B

fractions	Con. mg/ml
Elution 1	1.8
Elution 2	2.4
Elution 3	1.13
Elution 4	0.3
Elution 5	0.11

C



D

fractions	Con. mg/ml
Elution 1	0.7
Elution 2	0.54
Elution 3	0.196
Elution 4	0.157

Figure 11. Purification of HSA and eHSA by Ni-NTA column. A, C) Samples are analyzed by coomassie stained SDS-PAGE. (BI: Before Induction, AI: After Induction, E: Elution, M: Markers are in kDa). B, D) The concentration of elution fractions was measured at 280 nm.

3.3.2. Ionic exchange chromatography of the native and the engineered HSA

In order to have higher purity of HSA and eHSA, buffers of fraction 1, 2 and 3 were exchanged to buffer A with pH 7.5. Purification was performed with anionic exchange chromatography column with a gradient length of 50 ml and target 100%. Fig. 12 shows purified proteins after ionic chromatography.

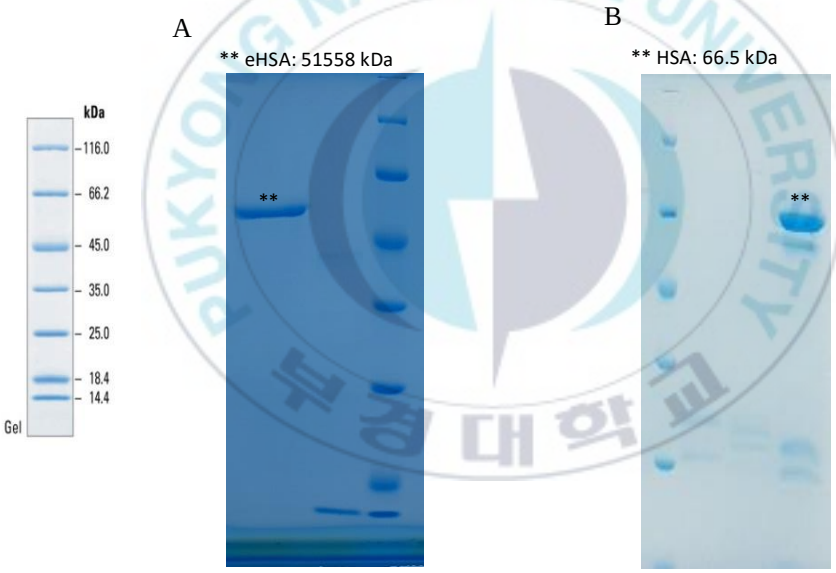
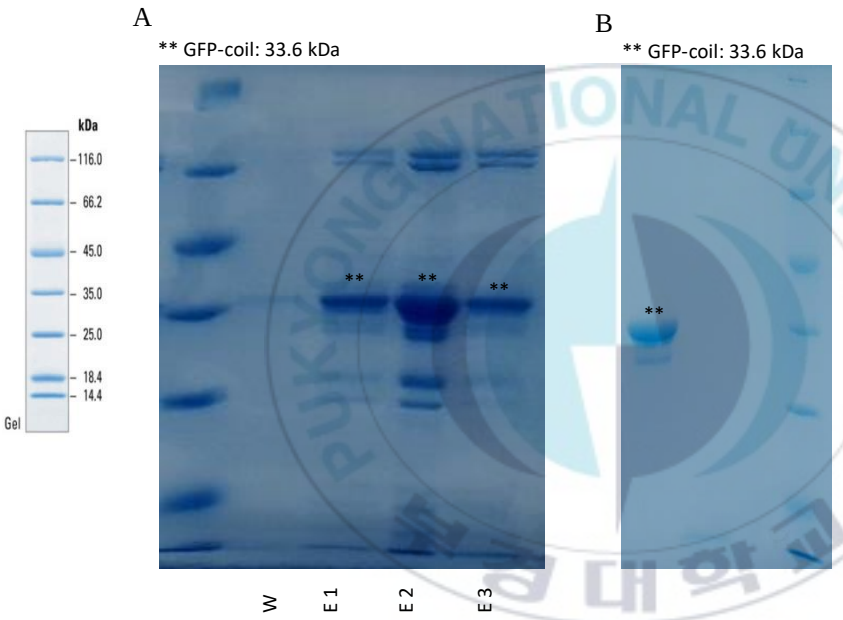


Figure 12. Coomassie-stained SDS-PAGE of purified eHSA and HSA after anionic exchange chromatography.

3.3.3. Expression & purification of the GFP fused coil as payload

This construct was designed as p66alphaGFP and ordered for synthesis by Enzymonics. The expression was done in BL21(DE3) in the incubator at 37°C for 5 hours. Purification was carried out by Ni-NTA and anionic exchange chromatography (Fig. 13).



C

fractions	Con. mg/ml
Elution 1	4
Elution 2	5.5
Elution 3	1.99

Figure 13. A) Coomassie-stained SDS-PAGE of GFP-coil by Ni affinity chromatography.W: Wash, E: Elution, B) purified GFP-coil after ionic exchange chromatography. C) The concentration of elution fractions was measured at 280 nm.

3.3.4. Expression & purification of the TRAIL

Following verification of cloning, the transformation of the verified vector containing TRAIL to BL21 (DE3) was done by heat shock. Expression of TRAIL in DE3 was performed at 37 for 5 hours. The purified band in Fig. 14 indicates TRAIL after Ni affinity chromatography. This sample was expressed and purified as the positive control. Buffer exchange of purified protein to PBS was done, and the final sample was aliquoted in multiple microtubes and stored at -20°C.

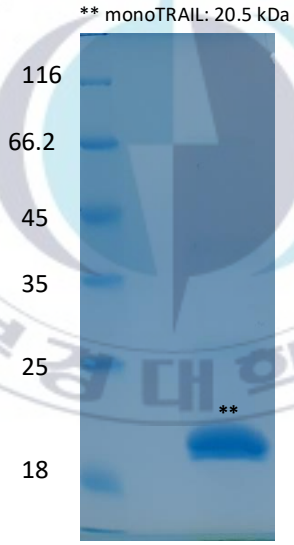
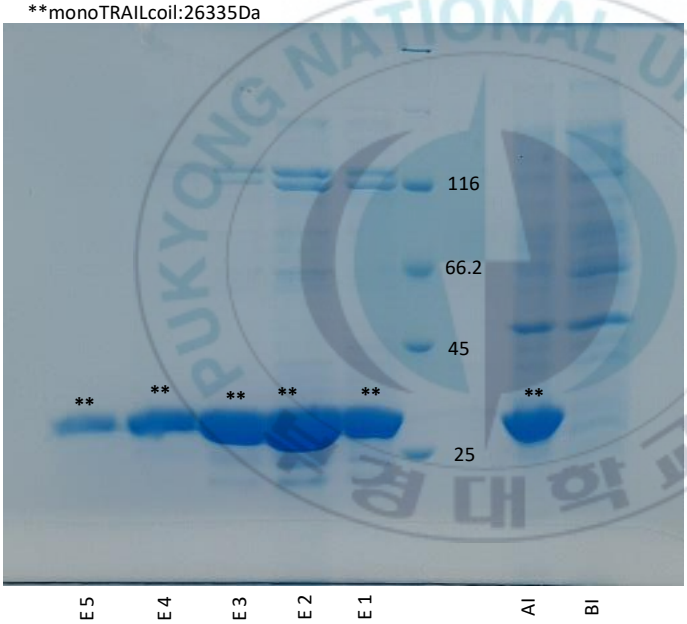


Figure 14. Coomasie stained SDS-PAGE Of the purified TRAIL. Markers are in kDa.

3.3.5. Expression & Purification of the monoTRAIL-coil as payload

Following verification of TRAIL fused to the coil by cloning, expression was done in BL21 (DE3). Fig. 15 shows the purification of the monoTRAIL-coil after Ni affinity chromatography. The yield of each batch was 12 mg of purified protein from 500 ml of bacteria culture.

A



B

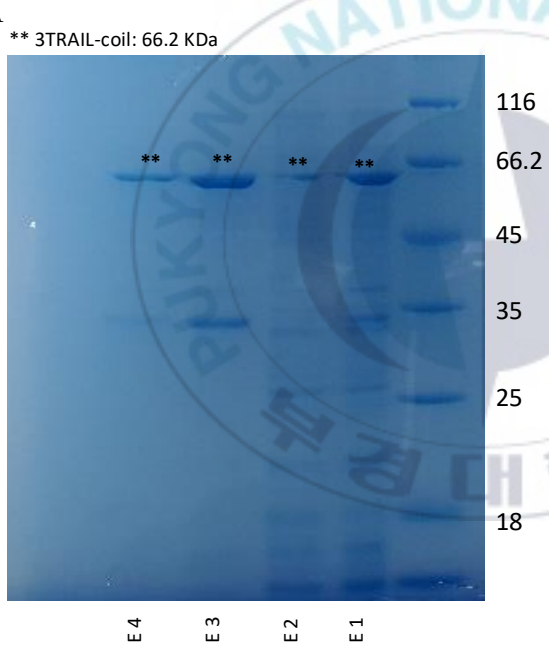
fractions	Con. (mg/ml)
E1	2.18
E2	4.4
E3	1.3
E4	0.57
E5	0.27

Figure 15. A) Purification of TRAIL-coil with Ni-NTA column analyzed on Coomassie-stained SDS-PAGE. (BI: Before Induction, AI: After Induction, E: Elution, Markers are in kDa) B) Concentration of elution fractions were measured at 280 nm.

3.3.6. Expression & purification of the 3TRAIL-coil as payload

Expression analysis of 3TRAIL-coil in various hosts, in the presence of chaperones, and different media showed BL21 (DE3) is a better host. In the presence of a chaperone, 3TRAIL-coil was visible in the soluble fraction rather than insoluble. Due to the high isoelectric point of TRAIL, the molecular weight of the complex was shown a bit lower than expected (Fig. 16).

A



B

fractions	Con. (mg/ml)
E1	0.8
E2	0.6
E3	0.72
E4	0.5

Figure 16. A) Purification of 3TRAIL-coil by Ni-NTA on Coomassie stained SDS-PAGE. B) Concentration of elution fractions was measured at 280 nm.

3.3.7. Western Blot

In order to verify TRAIL expressions, monoTRAIL-coil and 3TRAIL-coil were subjected to western blotting. Following separation of proteins by SDS-PAGE, transfer to nitrocellulose membrane and immunoblot against AntiTRAIL were carried out. The visible band on the nitrocellulose membrane at Fig. 17 indicates TRAIL protein.

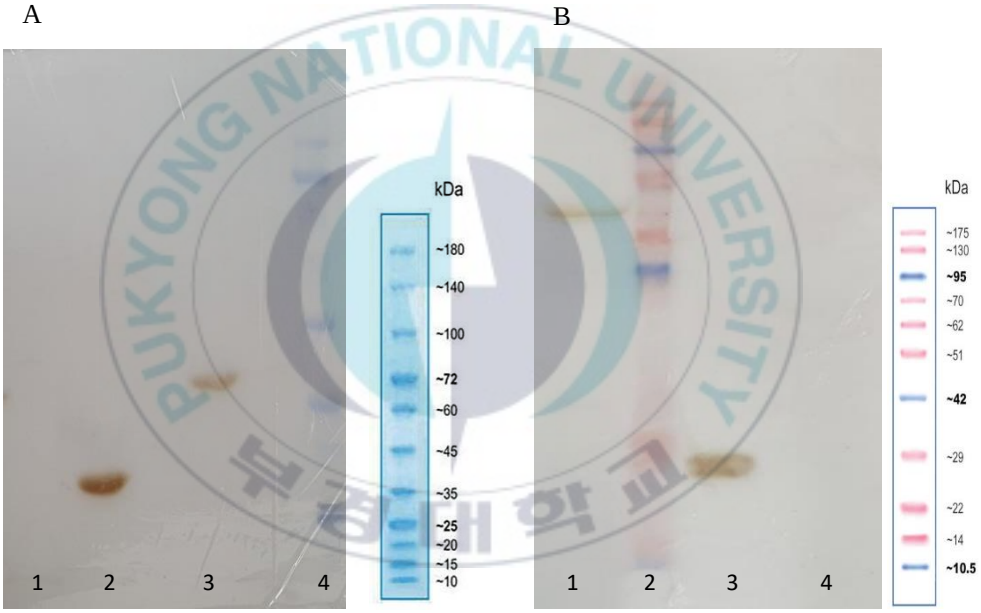


Figure 17. Immunoblotting analysis of monoTRAIL-coil and 3TRAIL-coil. A1) negative control, A2) positive control (TRAIL), A3) monoTRAIL-coil, A4) ladder. B1) 3TRAIL-coil, B2) ladder, B3) positive control (TRAIL), B4) negative control.

3.4. Result of the interaction Investigation

3.4.1. NATIVE-PAGE of the eHSA and the GFP-coil

In order to assess whether MBD2 in HSA construct (eHSA) can interact with p66 alpha fused to GFP, they were put in the reaction mixture at different time intervals, and their interaction was investigated on NATIVE-PAGE. Interaction of eHSA and GFP-coil was performed by stoichiometry 1:1. Interaction between HSA (WT) and GFP-coil was considered negative control. Fig. 18 confirms eHSA and GFP-coil can interact with each other specifically.

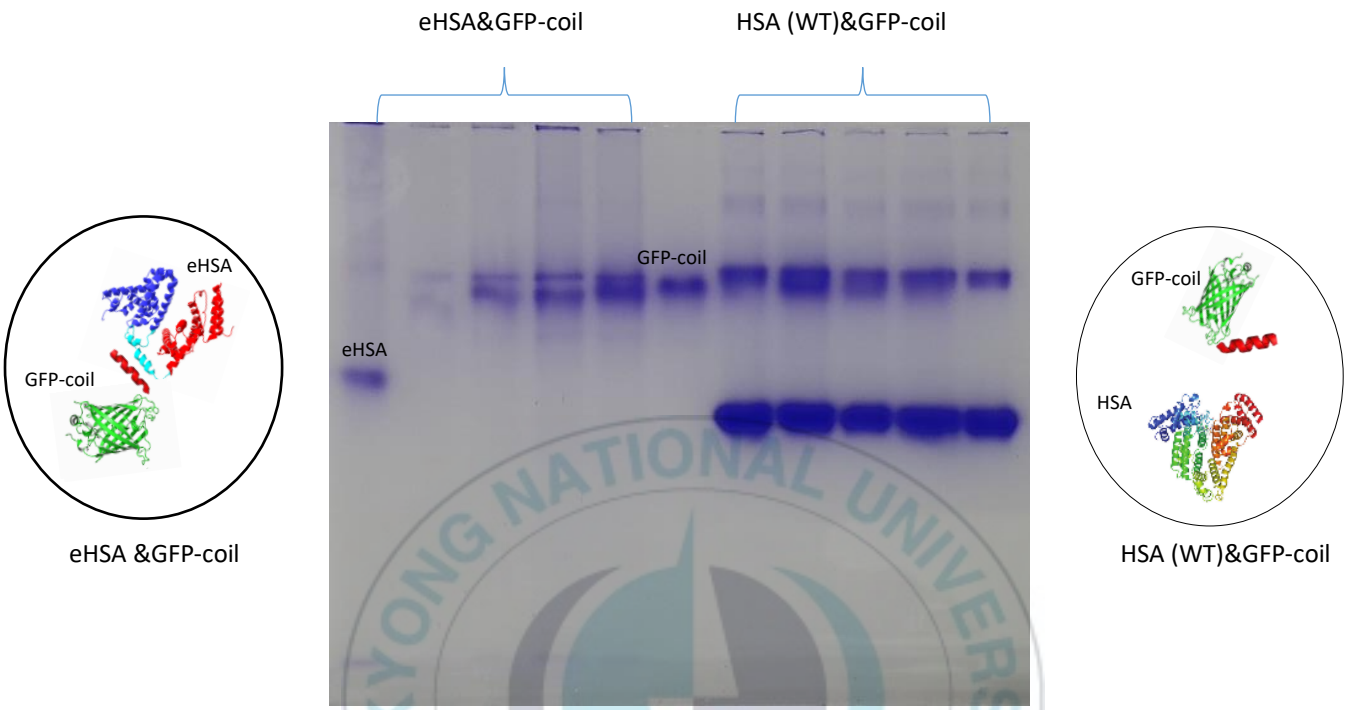


Figure 18. NATIVE-PAGE of interaction between eHSA and GFP-coil. The right side shows the interaction between HSA (WT) and GFP-coil, and the left side interaction between eHSA and GFP-coil.

3.4.2. HPLC-SEC of the eHSA and the GFP-coil

To check the interaction between eHSA and GFP-coil, they were loaded on SEC column. As the brown peak shows in Fig. 19, all eHSA molecules interact with GFP-coil and elute from the column early.

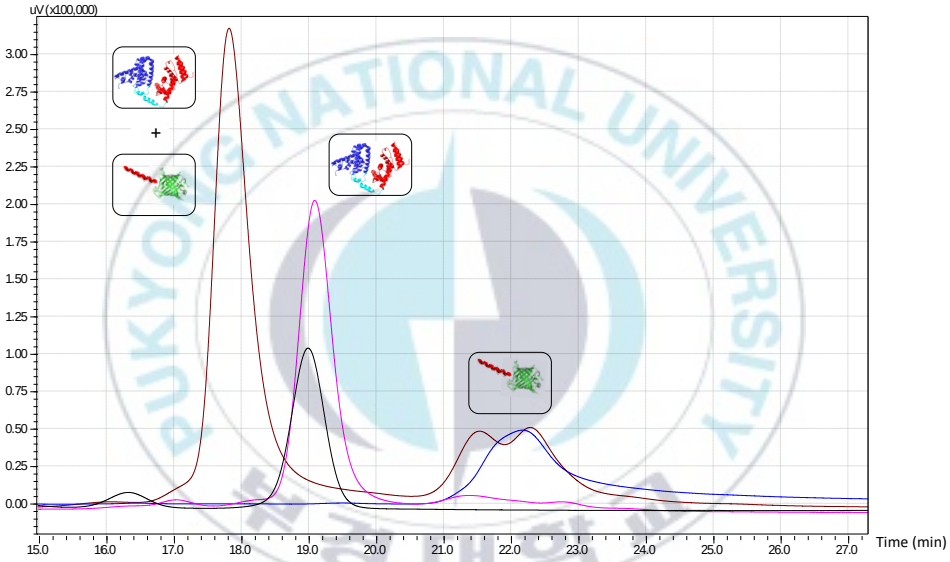
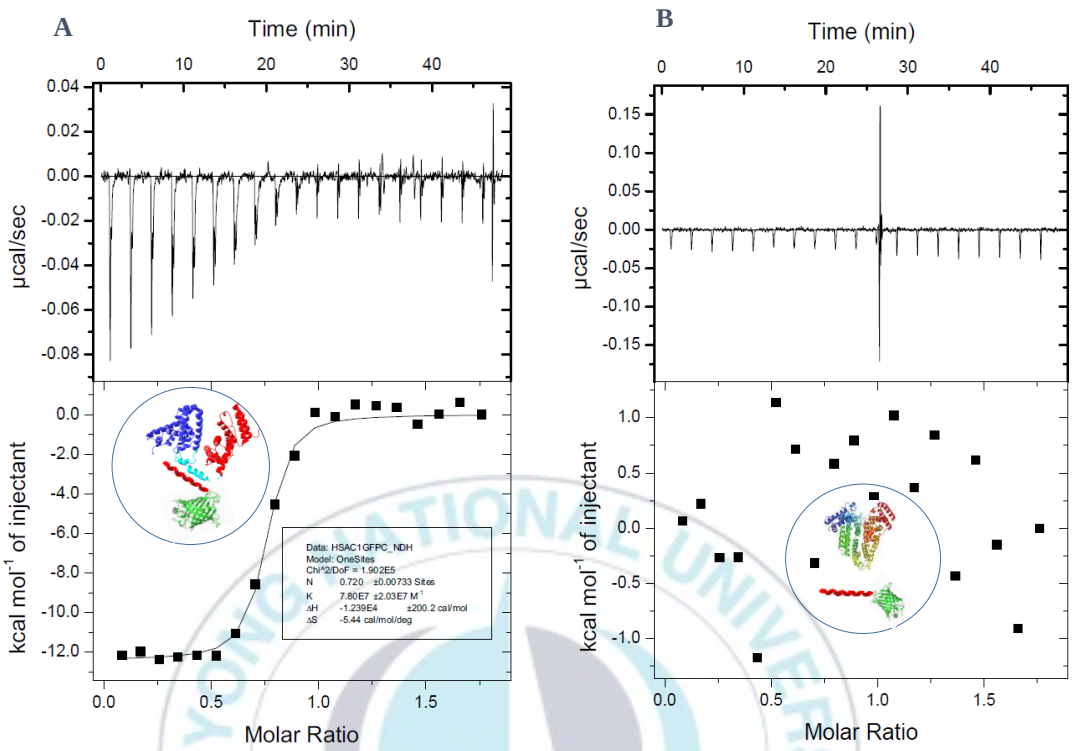


Figure 19. Size Exclusion chromatography to investigate the interaction between eHSA and GFP-coil.

3.4.3. ITC of the eHSA and GFP-coil

In order to confirm the preliminary result of NATIVE-PAGE and quantitatively measure affinity between eHSA and GFP-coil, Isothermal Titration Calorimetry was done. As expected and shown in Fig. 20, eHSA and GFP-coil could interact with each other by low Kd and high affinity (negative control didn't show any binding).



C

parameters	n	Kd (nM)	ΔH (kcal/mol)	-TΔS cal/mol/deg
value	0.7	12.8	- 12.4	5.44

Figure 20. ITC result of interaction between eHSA and GFP-coil. A) interaction between eHSA and GFP-coil. B) interaction between GFP-coil and HSA(WT) as a negative control.

3.4.4. NATIVEPAGE of the eHSA and monoTRAIL-coil

To verify the interaction capability of the complementary strand fused to therapeutic compound (TRIAL-coil) with eHSA, they put an interaction mixture in two different pHs (acidic and neutral). As demonstrated in Fig. 21, the binding could persist in acidic pH. Interaction between eHSA& GFP-coil was considered as a positive control.

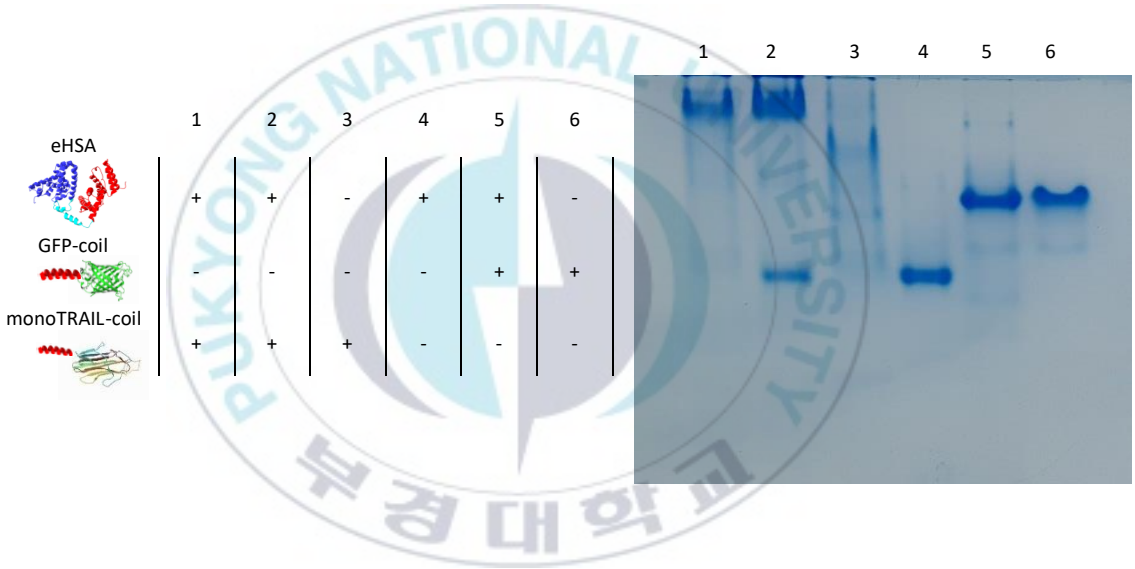


Figure 21. NATIVE-PAGE of interaction between eHSA and TRAIL-coil. The intercalants name of each well mentioned in the table. Interaction between eHSA and monoTRAIL-coil were performed in neutral and acidic pH in well 1 and 2 respectively.

3.4.5. NATIVE-PAGE of the eHSA and 3TRAIL-coil

The interaction capability of complementary strand fused to large therapeutic compound (3TRAL-coil) to eHSA was examined using NATIVE-PAGE. As it is evident in Fig. 22, the migration pattern of eHSA changed after interaction with 3TRAIL-coil.

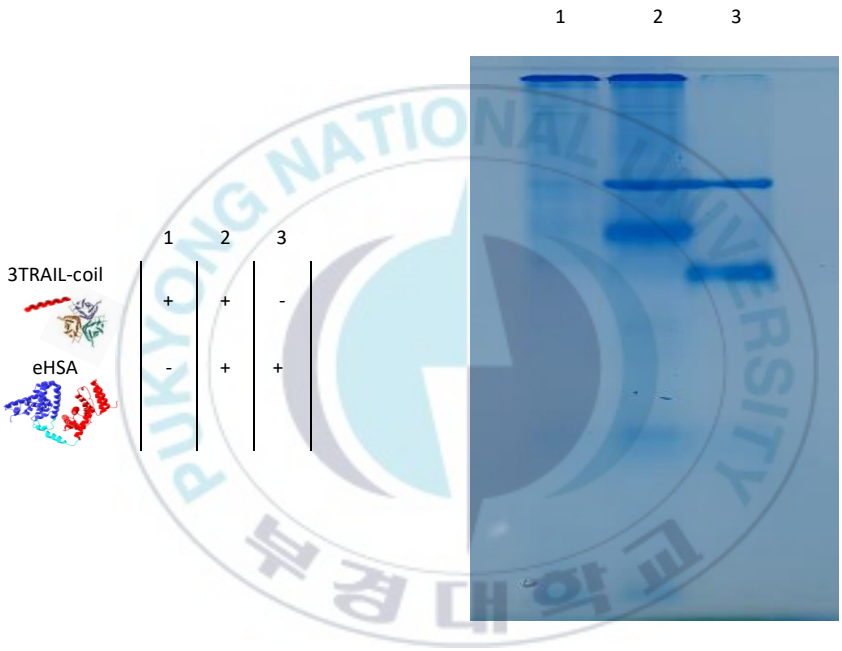


Figure 22 NATIVE-PAGE of interaction between eHSA and 3TRAIL-coil.

3.5. HPLC-SEC of the monoTRAIL-coil

In order to check the ability of monoTRAIL-coil to multimerize, it was loaded on size exclusion chromatography column in the purified and native state. HSA, eHSA, and IgG were considered controls to compare and check different monoTRAIL-coil sizes. Fig. 23 proves the monoTRAIL is in the separate multimerized states.

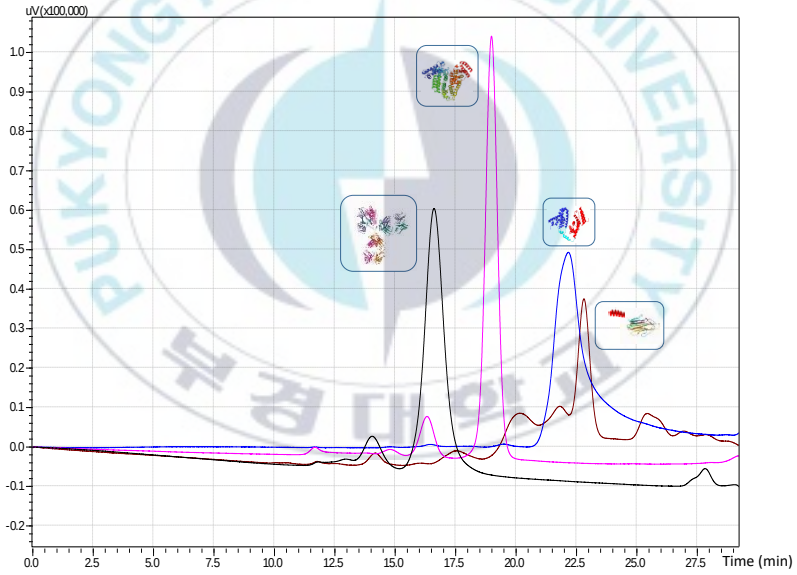


Figure 23. SEC of monoTRAIL-coil

3.6. Result of the FcRn binding (MST)

3.6.1. FcRn labeling

To scan labeling, 4 capillaries were loaded with different concentrations of labeled FcRn and put inside the MST instrument to examine. As shown in Fig. 24, FcRn labeling did efficiently, and 100 nM was chosen as optimal concentration for interaction analysis.

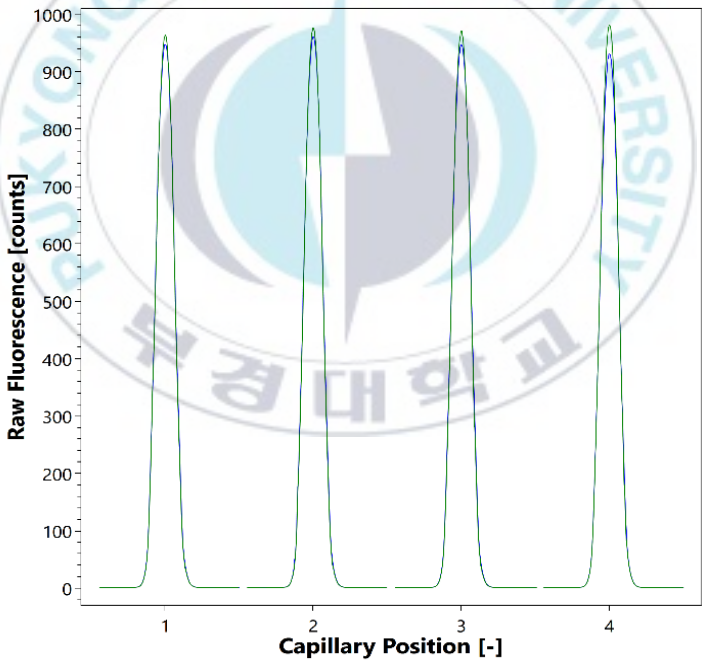


Figure 24. Capillary scanning of labeled FcRn

3.6.2. FcRn-HSA (WT)

At first, MST analysis was performed for wild-type albumin. This sample was considered a positive control to show the functionality of FcRn. It should be mentioned all FcRn binding interactions did in the acidic environment. Fig. 25 proves FcRn binds effectively to native HSA.

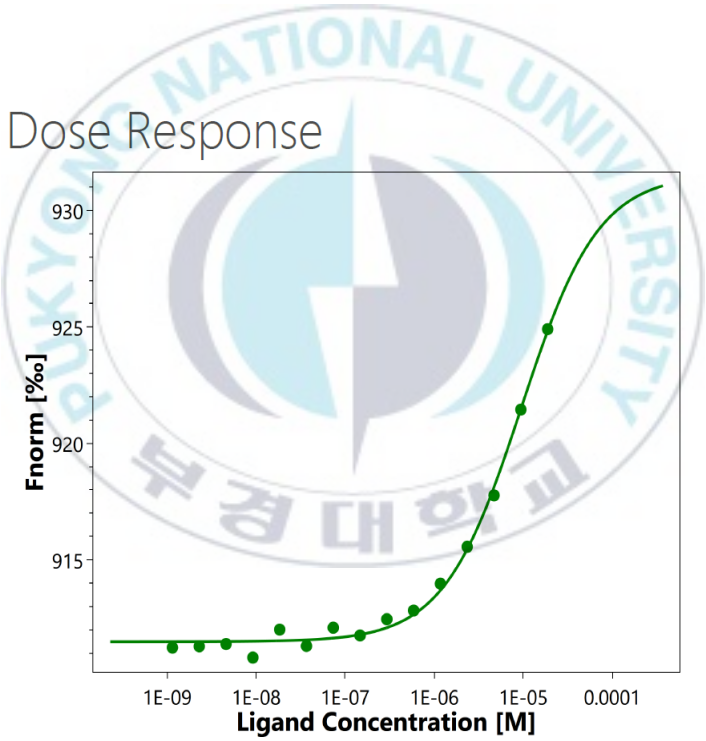


Figure 25. FcRn binding to HSA (WT).

3.6.3. FcRn-eHSA

To check the interaction of FcRn to the new albumin construct (eHSA), MST method was applied. To investigate the interaction, thermophoresis scanning of 16 capillaries containing serial dilution of eHSA and labeled FcRn with the infrared (IR) laser was performed. The result shows eHSA can still bind to FcRn strongly (Fig. 26).

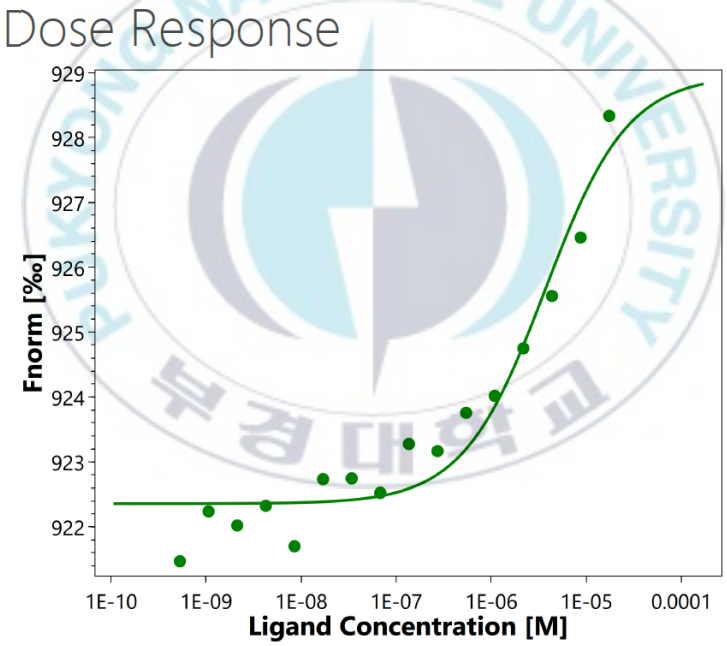


Figure 26. FcRn binding to the engineered HSA (eHSA).

3.6.4. FcRn-eHSA-payload

To check eHSA still has a binding affinity to FcRn, monoTRAIL-coil was also added to the reaction mixture. As the graph in Fig. 27 shows, monoTRAIL-coil doesn't have any inhibitory effect on FcRn binding to eHSA.

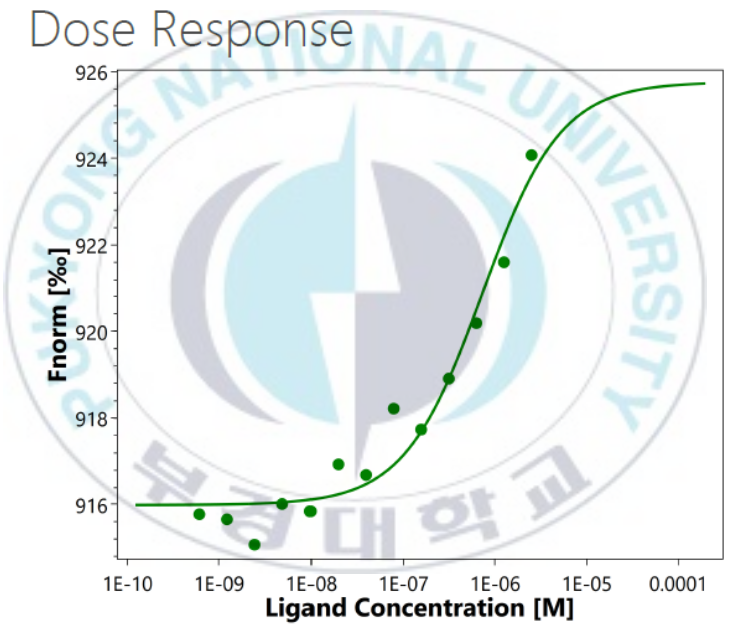


Figure 27. FcRn binding to engineered HSA (eHSA) in the presence of TRAIL as payload.

Table 9. Binding affinity to FcRn.

	Kd at pH 5.5 (μM)	Kd at pH 7.4 (μM)
HSAwt	9.5	-
eHSA	3.4	-
eHSA/TRAIL	7.2	-

3.7. Result of cell assay

Analysis of the apoptotic activity of monoTRAIL-coil and 3TRAIL-coil on cancerous cell lines showed that 3TRAIL-coil has more apoptotic activity than monoTRAIL-coil. Also, coil fusion doesn't have any inhibitory effect on TRAIL trimerization and activation of death receptors on the cancerous cell lines. TRAIL-coil company on eHSA platform won't diminish its biological activity. (Fig. 28, 29). TRAIL R&D was considered as a positive control.

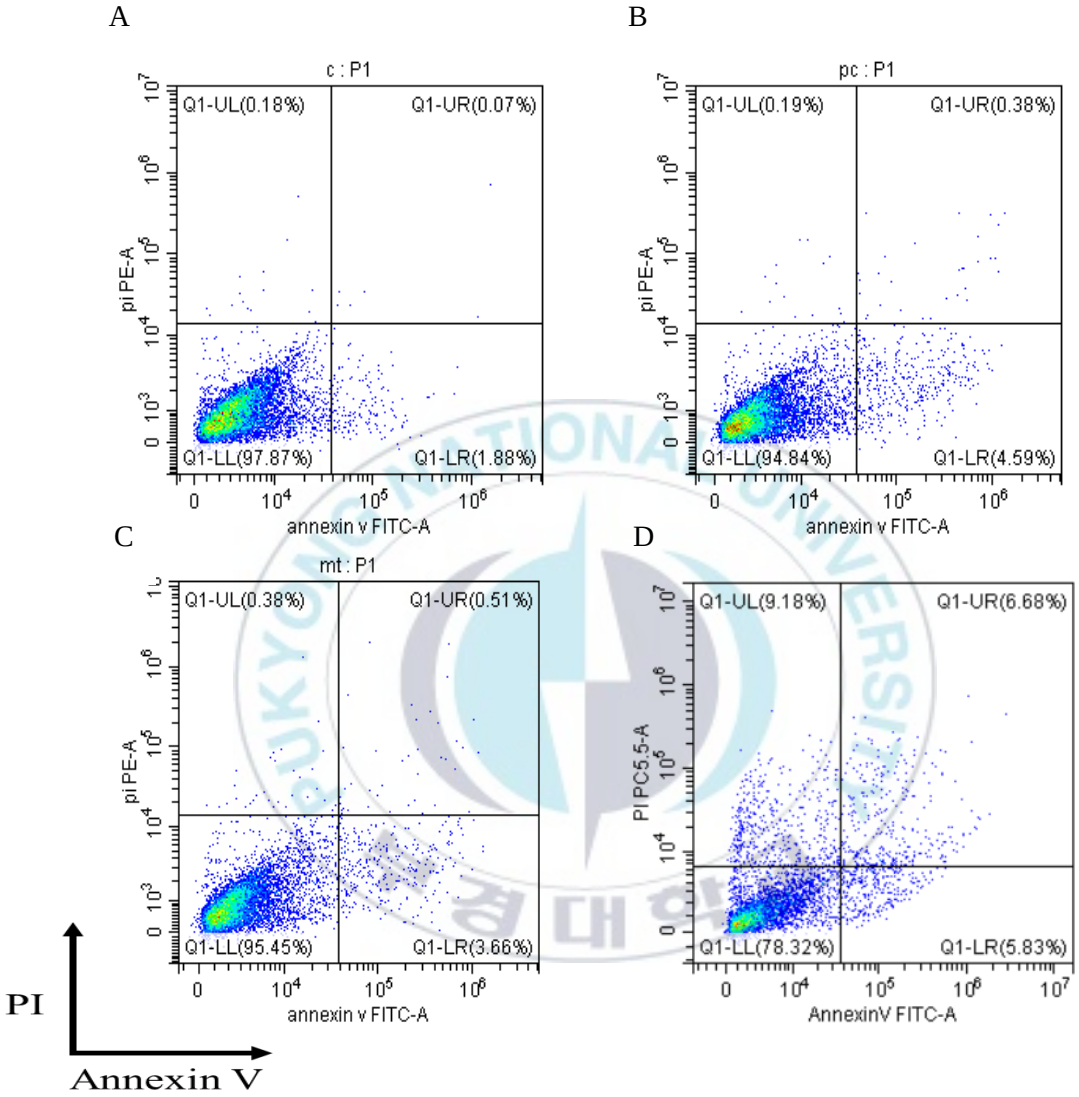


Figure 28. Appoptotic activity of TRAIL.

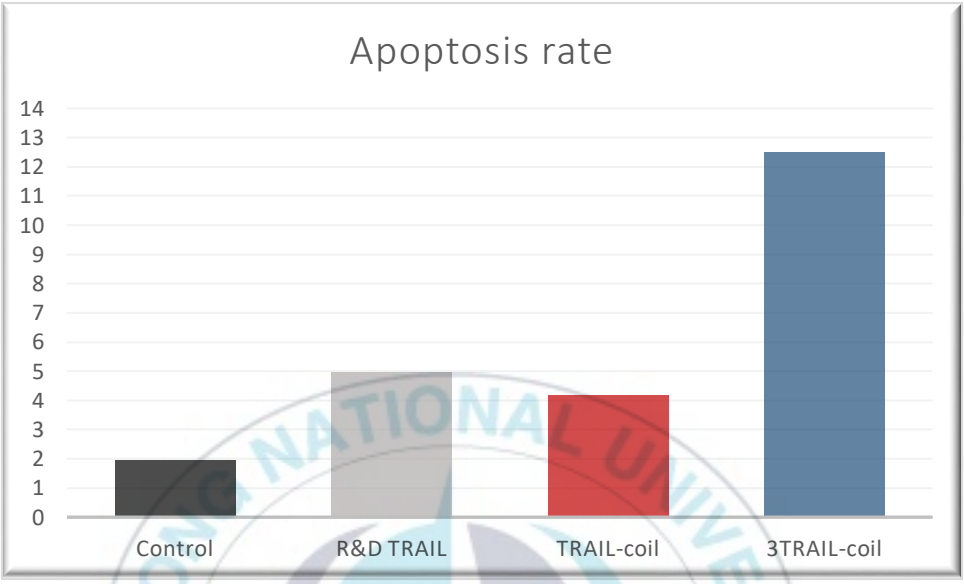


Figure 29. Rate of cellular appoptosis in the presence of TRAIL (HCT116).

Chapter IV
Discussion

Chemotherapeutic drugs are typically administrated as a cocktail of highly toxic drugs. Because of high toxicity and severe side effects, the dosage and frequency of their administration should be limited. DDS can have a significant therapeutic impact as a biopharmaceutical innovation by localizing chemotherapeutic compounds in the diseased area and reducing off-target toxicity in tissues that exhibit undesirable drug responses [45, 46].

The application of therapeutic proteins to treat human disorders also shows considerable growth, but there are some obstacles to their usage in the clinical market. It's due to quick removal from circulation by proteolysis and short serum half-life. The conventional method for extending the circulation lifetime of a therapeutic peptide is conjugation with polyethylene glycol (PEG). There are various concerns highlighted with PEG molecules, including their immunogenicity, accumulating in the body due to poor degradability, and loss of therapeutic peptide activity upon conjugation. Therefore, new approaches to prolonging the serum half-life of therapeutic proteins and peptides are needed.

Alboostumin, an engineered variant of albumin, utilizes a self-assembling coil domain in its tertiary structure. The long half-life and specificity of albumin toward tumor cells make it a feasible carrier for targeted drug delivery. Hydrophobic binding pockets, conjugatable thiol residue, and N- and C-termini in HSA inherently serve as suitable spots for carrying various peptidyl and non-peptidyl drugs. The self-assembly coil domain in the albumin

tertiary structure creates another binding site with a modular capability on the eHSA structure. This modular scaffold creates a situation in which one therapeutic component can be easily accompanied on the eHSA platform.

The most convenient residue for site-selective conjugation on HSA is Cys 34. Chemical conjugation of therapeutics needs functionalization by chemical compounds like maleimide or N-hydroxysuccinimide (NHS) [47], which may affect the therapeutic activity of a drug molecule [48]. A study by Schmøkel et al. illustrated site-selective conjugation of an anticoagulant aptamer to the Cys 34 of recombinant albumin leads to a 25% reduction of anticoagulant activity [49]. They attributed this reduction to the buried position of Cys34 in the deep shallow crevice and the steric hindrance of the conjugated aptamer. Lack of molecular flexibility interferes with the correct three-dimensional conformation of therapeutics and reduces their activity [50]. Another frequent method to load a drug molecule on an albumin carrier is to fuse a therapeutic peptide to the N- or C- terminus of albumin. Fusion of interferon-gamma to albumin by Bing Li et al. demonstrated a significant reduction of in vitro activity of IFNγ. It would be due to steric hindrance, affecting interferon dimerization, correct folding, and proper conformation of the therapeutic drug [51]. Fusion and conjugation of big therapeutic molecules compromise target recognition and therapeutic efficacy [52].

Mentioned challenges can be addressed by designing a new and an engineered form of albumin.

coiled-coil as structural domain forms from heptad repeat and found in a diverse array of human proteins [53]. Creating a novel albumin platform by taking advantage of the coiled-coil domain provides an additional site for the fusion or conjugation of various types of drugs. The coiled-coil domain as a drug carrier showed enhanced cytotoxicity and drug selectively toward cancerous cells in liposomal drug delivery [54]. Coiled-coil domain also can be easily expressed and purified in the prokaryotic system.

Since bacteria is a cost-effective and straightforward host for recombinant protein production, optimizing the expression of therapeutic compounds in the prokaryotic system can be beneficial. Manufacturability and scale-up are much easier in the bacteria to be optimized [55]. Low yield, low stability, low batch consistency, and high cost are common drawbacks of recombinant drug production in mammalian cells [56]. Most organic and protein-based carriers like antibodies have a challengeable process to produce on a large scale. Expression of protein such as albumin with multi disulfide bonds in bacteria also needs to be optimized. Utilizing the genetically engineered SHuffle E.coli strain with an oxidative cytoplasm would offer disulfide bond formation conditions. Due to deletion mutations in the thioredoxin reductase (trxB) and glutathione reductase (gor) genes, it dramatically enhances disulfide bond formation in the cytoplasm [57]. Albumin and engineered albumin can be produced with a suitable yield in the SHuffle strain (Fig. 11).

The new albumin construct has a modular structure. All interaction analysis among eHSA and various payloads (GFP-coil, TRAIL-coil, 3TRAIL-coil) shows eHSA can interact

specifically with different payloads. High binding affinity between coil in the middle of HSA and coil fused GFP illustrate that coil assembly domain can form very strong and specifically (Fig. 20). The capability of TRAIL fused-coil to interact with eHSA was examined on NATIVE-PAGE and size exclusion chromatography. Visible band and different migration patterns compared to eHSA on PAGE proved the interaction and formation of eHSA as a modular scaffold. The investigation on NATIVE-PAGE was carried out at two different pH levels, and the analysis shows that the interaction can still occur at acidic pH level (Fig. 21).

These results verify the functionality of the eHSA platform as an effective modular scaffold in DDS. Substitution of any other big and complex therapeutic compound like 3 TRAIL also showed the possibility of engineered HSA as a carrier to deliver complex drug configuration. In this structure, critical binding sites on domains 1 and 3 will remain intact, and there isn't FcRn binding hindrance anymore. It's just the new binding site on the complementary coil, which can easily be modified with therapeutic entities. Drugs fused to the complementary coil could be produced separately with high yield and just react with eHSA to create an effective DDS and administrate into the body. As shown in Fig. 30, there are fusion sites at both ends of eHSA and complementary coil for protein and peptide drugs, free Cys 34 as a conjugation site for binding various drugs. This also shows the potential of eHSA as a multispecific scaffold, which other binding sites can also be loaded with other therapeutics.

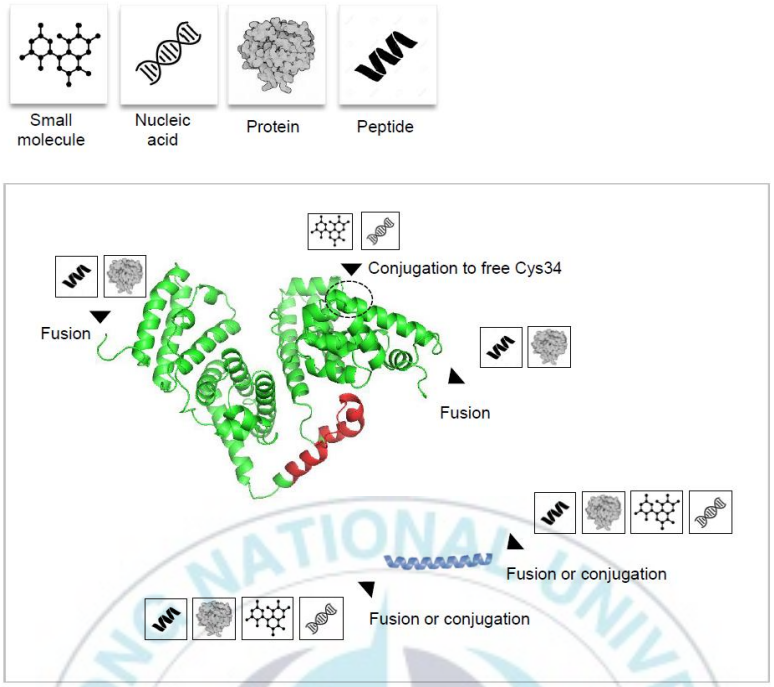


Figure 30. Available conjugation and fusion sites on eHSA .

Oligomerization of TRAIL leads to higher apoptotic activity in cancerous cells. As discussed in chapter 1 and Fig 8, TRAIL's apoptotic signaling pathway is activated by trimerized TRAIL binding to DR4 and DR5, which causes the receptors to homotrimerize, allowing the death-inducing signaling complex (DISC) to form [58]. Other research studies also showed trimerized TRAIL would have a more favorable therapeutic activity and pharmacokinetic profile [59, 60]. As the result of the cell assay shows, trimerized TRAIL has higher apoptotic activity toward cancerous cells than monoTRAIL. (Fig. 30), and also, TRAIL fusion to the complementary coil won't affect its therapeutic activity.

Higher therapeutic efficacy and successful eradication of cancerous cells are achieved with a high enough drug dosage over a sufficient period of time. But drug binding to HSA due to binding to FcRn can increase the capability of surviving the therapeutic compound in the blood over 3 weeks.

In a process called FcRn-mediated recycling, human serum albumin binds to FcRn in acidic environments of the endosome (Fig.3). Then it is transported outside cells and released to the neutral environment of the bloodstream [61]. Binding to FcRn in this study was estimated by microscale thermophoresis. This analysis was performed at acidic and neutral pH. We didn't observe any FcRn binding in neutral pH. Maintainance the pH-dependent binding require for intracellular transport of albumin and recycling by hFcRn. Fusion of human lactoferrin to the C-terminal of HSA by KeisukeUeda et al. demonstrated the inability of albumin to bind FcRn at acidic pH [62]. Other studies also proved that domain 3 and intact C-terminal are crucial for albumin's long half-life [31, 63]. We demonstrated that deletion of domain 2 and substitution by coil domain wouldn't negatively affect FcRn binding capability.

A research study revealed covalent attachment of cargo to Cys34 resulted in a 2-3 fold lowered hFcRn affinity of albumin. It may be due to slight conformational change in DI after drug conjugation and disruption of the interactions between DI and III, which is important for FcRn binding [64]. Another study also proved that DI and D III of HSA are

vital to maintaining optimal hFcRn binding and a long plasma half-life [65]. Two domains of albumin (DI and DIII) harbors the main binding sites for FcRn. DIII contains the main FcRn binding region, whereas DI modulates receptor binding. [31, 66].

Investigation into acidic pH revealed that eHSA can bind to FcRn more strongly than wild type. Our study showed the interaction between FcRn and engineered HSA is more than 2 times stronger than FcRn and native albumin. It confirms this new platform has the potential to increase drug circulation time much more effectively, and the therapeutic compound would effectively detach from FcRn and release the neutral pH of the bloodstream.

The binding affinity of eHSA in drug presence is still higher than HSAwt (Table 10). It illustrated that fusion of the drug to the complementary coil won't negatively impact FcRn binding. A study by Maja ThimLarsen upon fusion of payload to the albumin showed fusion to the C-terminal of wild-type albumin diminish binding to FcRn receptor. They had to put some extra mutation in domain 3 of albumin to maintain FcRn binding engagement [63]. Our study shows engineered albumin with just simple substitution with coil domain would offer a prolonged half-life for the fused drug.

알부스투민: 약물 전달을 위한 모듈형 알부민 스캐폴드

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요약

전도유망한 치료 단백질은 종종 혈액 순환의 낮은 반감기로 인해 임상 평가에서 발전하지 못한다. 따라서, 치료 효과를 저해하지 않고 페이로드의 생체 내 반감기를 개선할 수 있는 약물 운반체를 개발하는 것이 매우 중요하다. 면역글로빈 G (IgG)와 알부민과 같은 혈청 단백질은 혈액에서 지속 가능한선천적능력으로 장기 작용을 위한 약물 운반체로 사용되어 왔다. 여러 Fc 또는 알부민 융접 단백질은 임상 시험에서 그 가능성을 입증했지만, 일부 단백질은 낮은 제조 가능성 또는 비효율성으로 인하여 그러한 운반체에 대해 유전적 융합과 양립할 수 없는 복잡한 접힘 경로 또는 형질을 가지고 있다. 이 문제에 대한 해결책을 제공하기 위해 본 연구는 인간 혈청 알부민(HSA)을 모듈식 약물 운반체로 엔지니어링하여 두 번째 도메인을 자가 조립 코일 도메인으로 대체함으로써 수행되었다. 결과는 엔지니어링된 HSA(eHSA)가 보완 파트너 코일에 수정된 치료 단백질과 자체 조립할 수 있음을 보여주었다. 등온 적정 열량계 (ITC), 크기 배제 크로마토그래피 (SEC) 및 Native-PAGE 기술은 eHSA가 높은 친화력으로 페이로드를 단단히 결합할 수 있음을 보여준다. 종양 괴사 인자와 관련된 세포사멸 유도 리간드(TRAIL)는 암세포에 세포사멸 효과가 있고, 그것이 삼합체를 형성하게 되면, 세포사멸

활성도가 증가될 것이다. 보다 기능적인 치료 화합물로서 TRAIL 을 보완 코일에 융합하고 Native-PAGE에 의한 조사를 통해 이 큰 치료 분자가 양호한 친화력으로 eHSA 에 결합할 수 있다는 것이 입증되었다. MST(Microscale Thermophoris)에 의한 FcRn과의 상호 작용은 eHSA가 혈류에서 긴 반감기를 가질 것을 검증한다.

키워드: 알부민, 자가 조립 코일 도메인, TRAIL, 항암제

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