Summer 8-2011

Expression and Characterization of Protein Chimera with Embedded Activated Protein C Generation

MinJeong Schneider
University of Nebraska-Lincoln, schneid92000@yahoo.com

Expression and Characterization of Protein Chimera with Embedded Activated Protein C Generation

by

MinJeong Schneider

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Chemical Engineering

Under the Supervision of Professor Anuradha Subramanian

Lincoln, Nebraska

August, 2011

Expression and Characterization of Protein Chimera with Embedded Activated Protein C Generation

MinJeong Schneider, M.S.

University of Nebraska, 2011

Advisor: Anuradha Subramanian

Increasing demand of medical implants has lead researchers to develop biomaterial surfaces that offer improved performance and lower thrombogenecity. There have been attempts to model biomaterial surfaces after the native endothelium as it represents an optimal non-thrombotic surface. Typically, when biomaterials are contacted with blood, they often invoke the activation of clotting cascades. Thus, to overcome surface mediated thrombotic events, proteins and molecules with anticoagulant attributes of the endothelium have been immobilized onto biomaterial surfaces. Protein C (PC) plays a main role in blood coagulation and acts as an anticoagulant when it is converted into activated protein C (APC). Activation of PC can be initiated only by thrombin (TR)-thrombomodulin (TM) complex with a relatively slower reaction, and is enhanced 20-fold when EPCR, the co-factor to PC, is also present. The objective of this thesis is to generate a chimeric protein that includes the functional domains of both EPCR and TM in an attempt to generate APC in-situ. The synthetic genes encoding the functional domains of EPCR (1-193) and TM (224-462) with either a myc or flag linker along with a histidine tag were first assembled by PCA/PCR method, sequenced and the synthetic genes encoding the correct sequences for chimera I, chimera II, and TM2-His were successfully expressed in yeast *Pichia* host X-33 strain. Expressed protein was analyzed by Western blotting analysis, purified using Ni-chelate chromatography, and the ability of EPCR-TM chimera to catalyze PC activation was tested in solution phase. Overall, the expression level of chimera I, chimera II, and TM2-His was low, and mature proteins were partially secreted into the medium, and His-tag purification yielded partially pure products. Chimera containing EPCR and TM was noted to generate 1.8-fold higher APC compared to standard TM at comparable concentrations.

DEDICATION

To my husband Jacob and my daughter Cinae

AUTHORS'S ACKNOWLEGEMENTS

I would like to thank Dr. Subramanian for giving me an opportunity to work on this research project and for guidance and unlimited support to achieve my goal.

A special thanks to Dr. Nicholas Whitney, Dr. Sanjukta Guha Thakurta, Dr. Jennifer Calcaterra, Dr. Karl Kador, Dr. Joel Ter Maat, Mr. Mikail Kraft, Ms. Allyson Lamb, Mrs. Trish Fenster, and Mr. Leonard Akert, whom provided their help for me to solve the problems.

Table of Contents

List of Table

List of Figures

Introduction

Biomedical implants and devices play a significant role in maintaining and improving human health. Further, the demand of medical implant devices, including heart valves, vascular grafts, stents, and catheters, has been steadily increasing over past century to save millions of patients[1-3], and a variety of synthetic polymeric materials has been developed to be more biocompatible such as Dacron®, polytetrafluoroethylene (PTFE), polyurethane (PU) and biodegradable materials [4]. Polymers such as Dacron® and PTFE have been widely used for large vessels (7-9 mm) with a 45% of patency compared to 60 to 80% patency of autologous vein grafts, however, patency of Dacron® and PTFE is far lower in smaller diameter grafts (<6 mm), 20-25% patency with 1 mm diameter microvessels[5]. The reason is that these biomaterials activate thrombus formation on its lumen while the differential compliance at the anastomotic site contributes to the formation of intimal hyperplasia (IH)[6]. Newer material, polyurethane (PU), a radially compliant polymer, has been developed to reduce the occurrence of intimal hyperplasia at the anastomotic site [7]. Since the porosity of biomaterials is a key factor for the failure in small diameter grafts due to hyperplasia, it is imperative to produce PU with porosity of 0.6 to 5.0 μm [8]. The patency rate in the pore sizes larger than 5.0 μm was 75% and that of pore sizes smaller than 2.0 μm was 50%. This study indicated that microvenous polyurethane-based prostheses with a luminal pore size larger than 5.0 μm yielded a better patency rates than prostheses with a luminal pore size smaller than 5.0 μm. Other interesting materials are biodegradable polymers such as polylactic acid (PLA), plyglycolic acid (PGA), polyhydroxyalkanotae (PHA), and plydioxanone (PDS)[6]. Even though these polymers possess the mechanical strength

comparable to that of native vessel, researchers have been focusing on the control of degradation rate to slow down absorption and allow sufficient time for arterial regeneration for a long term patency[9]. An alternative strategy to improve synthetic and degradable vascular grafts is the manipulation of proteins that compose native extracellular matrix (ECM). Collagen gels and fibers from type I collagen, a major ECM component in the blood vessel, are widely used to improve the mechanical strength of grafts by preventing critical rupture of the vascular wall[10]. To overcome the limitation of stiff collagen-based biopolymers, researchers have been exploring the elastic fibrin gels [11], which can be produced from the patients' own blood to prevent an inflammatory reaction due to the implantation[12]. These grafts remained in the vein of lambs for 15weeks and contained both collagen and elastin without any toxic degradation. The mechanical properties were comparable to that of native coronary arteries[13].

When these biomaterials are exposed to blood, they can cause numerous and complex responses, including coagulation, complement activation, platelet adhesion and activation leading to thrombosis, leukocyte adhesion, and red cell interactions that can produce hemolytic damage[14]. To overcome these complications caused by biomaterials, researchers have been focusing on resolving the early complication such as coagulation and thrombosis since they can be prevented by controlling the initial protein adsorption to eliminate undesirable response for example fibrinogen interaction with platelets. One of the two major approaches for protein adsorption control is the surface grafting with polyethylene oxide (PEO), which can keep the non-specific proteins away from a surface by steric exclusion or by binding water or by phospholipid/phospholipid-

like layers[15]. The other approach is the promotion of the selective and exclusive binding of proteins, which results in attaining a desired specific biological activity [14]. For example, heparin is immobilized on the biomaterial surface to attract antithrombin III that binds to thrombin, so that the surface can provide an anticoagulant effect [16]. Another example is using lysine to convert plaminogen or tissue plasminogen activator (t-PA) into plasmin, a clot dissolving enzyme in the body. Therefore, maintenance of hemocompatibility on the biomaterial surfaces has been one of the main issues in a long term use[3, 4], and researchers have been taking direction in the development of completely inert biomaterial surface against blood with a limited success [17, 18]. To better understand the body response against blood contacting biomaterials, it is important to review how the extrinsic and intrinsic pathways of blood coagulation contribute to generation of thrombosis.

In the traditional view of blood coagulation, the initiation phase is triggered by the extrinsic pathway, while amplification requires the intrinsic pathway[19]. At site of vessel injury, disruption of endothelium exposes platelets to collagen in the vessel wall and plasma factor VII/VIIa (FVII/FVIIa) to the integral membrane protein tissue factor (TF). When TF complexes with plasma factor VIIa in the extrinsic pathway, initiation of fibrin formation occurs. Alternatively, coagulation can be initiated through the intrinsic pathway when factor XII is activated on a charged surface by contact activation [20]. Activation of factor XII is followed by activation of factor XI and factor IX. The extrinsic and intrinsic pathway converges at the activation of factor X. Factor Xa activates prothrombin to thrombin in the presence of the cofactor factor Va, and thrombin converts fibrinogen to fibrin, forming blood clot. Thrombin is a key regulator for

coagulation pathway and triggers anticoagulant pathway by forming a complex with thrombomodulin. Thrombin-thrombomodulin complex converts protein C to activated Protein C that can form a complex with protein S to inactivate activated factor Va and activated factor VIIa. Figure 1 shows the schematic representation of blood coagulation and anti-coagulation pathway and this study is focused on the anti-coagulation pathway.

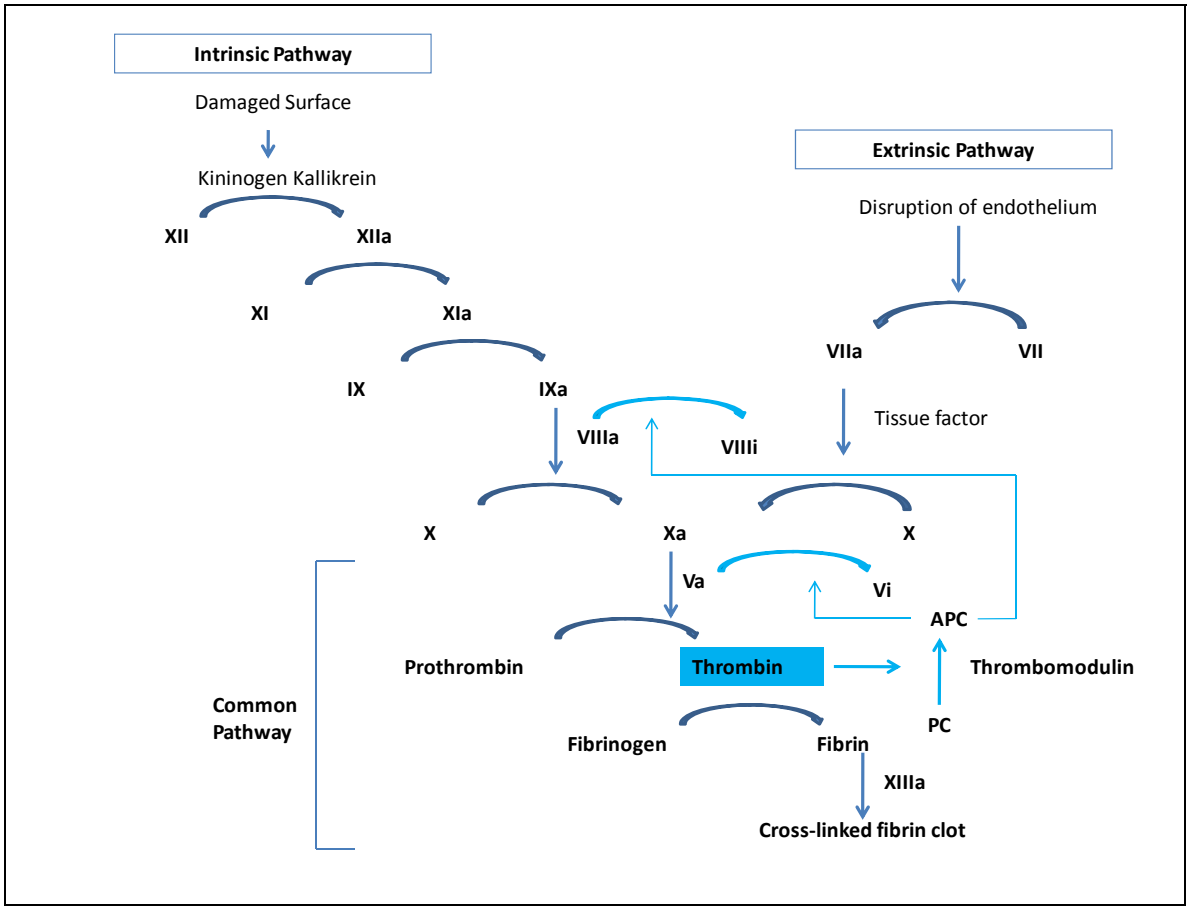


Figure 1. Schematic representation of blood coagulation and anti-coagulation pathway

To delay or reduce early clot formation on the biomaterial surface, researchers have been attempted to produce biomaterial surfaces to provide anticoagulant activity at the level of native endothelium [21-23]. A healthy endothelium demonstrates an outstanding thromboresistancy due to soluble molecules (prostacyclin, nitric oxide, and

antithrombin III) and membrane bound molecules (heparin sulphate (HS), thrombomodulin)[3]. When a biomaterial, immobilized with prostacyclin (PGI2), was contacted with blood, PGI2 started to fuse into blood stream to prevent the aggregation and release of platelets [24]. It is very powerful material, but PGI2 is an expensive and unstable product because its life time is less than 1min after hydrolysis under biological conditions. Thus, this type of biomaterial is not practical, yet. Another molecule that inhibits platelet activation is nitric oxide (NO). NO releasing peptide was added into the main chain of polyurethane to improve thromboresistance [25]. Release of NO lasted for two months to reduce platelet adhesion and smooth muscle cell growth, while NO release stimulated endothelial cell proliferation. Inhibition of platelet adhesion is important in the extrinsic coagulant pathway, whereas control of thrombin is essential in the common coagulation pathway. In this sense, heparin was coated onto polymeric materials to obtain an antithrombogenic function, which enhanced the binding of antithrombin III (AT III) and thrombin, thus preventing blood coagulation [26]. The results showed that the heparin release from Biomer/poly (NiPAAm)-coated surfaces significantly reduced thrombus formation on test surfaces in contact with venous blood by neutralization of thrombin and factor Xa. As mentioned above, thrombin is a key enzyme in coagulation pathway and also plays an important role in anticoagulant pathway[27]. For this reason, recombinant human thrombomodulin (TM), forming a 1:1 molar complex with thrombin, has been immobilized on the expanded polytetrafluoroethyelen (ePTFE) by either a direct coating or two-step binding process [28]. In this study, the two-step binding method rTM activated protein C by 6000-fold compared to the buffer control and 50-85% more than direct coating method because modified ePTFE surface immobilized rTM better to

stabilize APC. This result supports that TM has an excellent anticoagulant function on endothelium to generate activated protein C (APC) in the presence of thrombin[29]. To emphasize the roles of TM in blood coagulation, it is necessary to review previous studies on TM. These studies have explored the catalytic function of TM onto membrane-mimetic polymer film that mimics the native endothelium by immobilizing TM alone [30], a combination of TM and heparin [22] and TM, heparin and nitric oxide (NO), which is capable of generating APC to inactivate factor Va and factor VIIIa. Tseng et al designed a membrane-mimetic film containing TM by a photopolymerization process to generate actively antithrombogenic surfaces. The result demonstrated that increasing shear and TM surface concentration enhances the rate of protein C activation per unit surface area. Tseng et al also immobilized TM and/or heparin onto the same surface above to compare the thrombin production, which resulted in complete elimination of steady-state thrombin responses by surface bound TM and heparin, and the lowest thrombin generation was observed by TM-mediated activated PC than by heparin-mediated activation of ATIII. Wu et al immobilized TM alone on the outer polymer coating over the inner NO release film, and TM and heparin were immobilized on the same NO releasing film to examine the biological activities of TM and heparin by APC generation and anti-factor Xa assay. In this work, a decreased amount (19.8+/- 2.7%) of platelet adhesion was observed by employing NO releasing polymeric coating compared with polymer without NO release, while TM, anticoagulant agent, and heparin, enhancing binding affinity for ATIII and thrombin, regulate the down-stream of coagulation cascade . It is crucial to review the mechanism of anticoagulant pathway, which is initiated by thrombin (TR)-thrombomodulin(TM) complex to activate protein C (PC).

PC is a 62kDa plasma glycoprotein that a disulfide bond is holding a 42kDa heavy chain and a 21kDa light chain together [31]. The composition of PC is a gamma-carboxyglutamic acid residue (Gla)-rich domain, 2 epidermal growth factor (EGF)-like domains, a short activation peptide, and the serine protease domain (SP) [32]. The Gla domain of PC binds to calcium, negatively charged phospholipid membranes and EPCR[33]. PC circulates in blood as a zymogen form of serine protease and PC functions as an anticoagulant when it is converted to activated protein C (APC) by thrombin bound thrombomodulin complex on the endothelial cell surface [34-36]. To initiate the PC anticoagulant pathway, TR needs to bind TM with the high affinity resulting in >1000-fold increase of the rate of PC activation [37]. TM is a single chain endothelial cell surface glycoprotein with a molecular weight of 74kDa[38] functioning as a potent anti-coagulant by converting TR from a procoagulant protease to an anticoagulant. The mRNA of TM is about 4kb in length, while the cDNA is 1,728bp long and encodes a protein of 575 amino acids[39]. TM consists of several domains: an N-terminal domain followed by 6 repeated epidermal growth factor (EGF)-like domain, a Ser/Thr-rich region containing chondroitin sulfate side chain, a transmembrane section, and a short cytoplasmic tail[32]. The region the fourth, fifth and sixth EGF-like domains of TM has the ability to bind thrombin and activate protein C[40]. The fourth EGF-like domain interacts with serine protease domain of PC[32, 40] while TR binds to the fifth and sixth EGF-like domains of TM [41]. In addition, the chondroitin sulfate of TM has shown unique effects on its interaction of thrombin, including accelerated reaction of thrombin with antithrombin III and enhanced blocking of procoagulant functions[40]. Soluble TM with chondroitin sulfate demonstrated 5-10-fold increase in thrombin affinity

compare with TM lacking the chondroitin sulfate[40], however, the rate of PC activation by TR-TM complex is relatively low affinity reaction (K_d = 0.7-1.0uM)[42]. On the other hand, generation of APC is significantly increased with a high affinity reaction (K_d= 30nM)[37] when TR-TM complex is co-existed with endothelial Protein C Receptor (EPCR), a cell-specific type I transmembrane glycoprotein that has an ability to binds both PC and APC on the cell surface and was identified by Esmon and Fukudome[43, 44]. A 43kDa soluble EPCR is identified in human plasma, circulating at a concentration of 2.5nM[45]. The genomic DNA of EPCR is about 6kb [46] and the length of mRNA is about 1.4kb, while the cDNA has 717 nucleotides encoding a protein of 238 amino acids [43]. PC binding to EPCR is mediated by the vitamin K-dependent Gla domain that is able to bind to negatively charged phospholipid surface[47]. Binding of PC to EPCR is also Ca^{2+} -dependent, which helps Gla residues to position for assisting hydrogen bonding interaction with EPCR[48]. When Gla domain of PC binds to EPCR, a hydrophobic pockets of EPCR opens up to accommodate Phe4 of PC Gla domain[48]. The crystal structure of EPCR showed that the C-terminal of EPCR would be inserted into the membrane, whereas APC would bind at the opposite end of EPCR and point away from the membrane surface[48]. The Gla-domain of PC/APC interacts with EPCR[33], and the Gla-domain of PC also interacts with TM and/or TR[49] and with the phospholipid membrane. To integrate TR, TM, PC, and EPCR on a biomaterial surface providing an efficient anticoagulant activity, a multiple protein immobilization technique is required.

To immobilize multiple proteins and biomolecules, a number of methods have been developed and used for biosensors, biodiagnostics, genomincs, proteomics,

cytology, material science, and general chemistry [50]. However, challenges remain in the development of methods such as providing selectivity in biomolecule binding and positioning, preserving biological activity, and avoiding non-specific biomolecule binding on the micro-scale to nano-scale surfaces [51]. Among these techniques, dip-pen nanolithography provides a flexible nanolithographic method capable of positioning molecules on a substrate with 10 nm resolution [51, 52]. To attach proteins through the reactive group on the substrate, covalent coupling is employed by chemical reactions; however these chemical reactions can involve residues to affect the activity of the proteins [53]. Thus, more site-specific technique has been applied by using monoclonal antibodies [54]. This approach recognizes the protein of interest without affecting the function of protein, but the size of antibodies is too large to be suitable for high-resolution structured surface fabrication. Most of all these techniques is required to shield the surface of the substrate with protein compatible layers to avoid the denaturation of protein caused by the interaction with solid substrate because these techniques are limited on silica chips, gold and galss-type surfaces, which cannot be used in human body as a biomaterial [55]. From the review above, a site-specific immobilization of multiple proteins is important to make all the proteins perform their functions on the biomaterial surfaces providing an efficient anticoagulant activity. The active site of TR (5.5nm diameter)[40] bound to fourth and sixth EGF-like domain of TM is positioned about 6.5nm above the membrane[56], and the active site of APC (5.7nm diameter)[57] bound to EPCR is located about 9.4nm above the phospholipid membrane[58]. To catalyze the conversion of PC to APC by TR, the active site of TR needs to be positioned between 0.7nm and 1.0nm with respect to the activation peptide

region of PC [59, 60] In these studies, the natural anticoagulant components, EPCR and TM, of the endothelial surface were sequentially and orderly immobilized via bi-dentate moiety grafted onto a biomedical–grade polyurethane surface within a molecularly relevant physical distance, less than 10nm, to support the enzymatic reaction and the rate of PC activation from sequential immobilization was compared to that of random immobilization. To achieve properly orientated EPCR on the PU surface, RCR-2 (mAb against EPCR) was used to bind carboxyl terminal of EPCR to keep the PC-binding site, NH2 terminal, away from the material surface [61]. The attachment of TM and EPCR was controlled by the functional group at the end of the bi-dentate bridge with fast coupling reaction to attach TM via carboxyl moiety followed by the slower reaction, the nucleophilic reaction, to attach EPCR via oxirane moiety. The result showed that the amount of APC generation from co-immobilization of TM and EPCR via bi-dentate bridge is statistically higher level with $P < 0.05$ compared to that of random immobilization. A smart design of these studies provided an equivalent distribution of TM and EPCR due to the defined immobilization sites and eliminated a chemical grafting of second protein to the first protein by conducting a fast coupling reaction followed by a slower reaction, the nucleophilic addition through an oxirane surface group that is not found on proteins or biomolecules to avoid creation of the protein-protein grafting. However, this method has several limitations in immobilization of proteins: (1) It is hard to guarantee that attachment of bidentate moiety bridge on the backbone of PU surface is one hundred percent successful, which means one can have either carboxyl moiety or oxirane moiety instead of having both moiety. If the distance between two single moieties with corresponding proteins is close enough, they would activate the protein C exactly

like a bidentate bridge with both TM and EPCR does. (2) It is hard to check the distribution of bidentate moiety bridges on the PU surface, which means that bidentate bridges can be populated on a small area of PU instead of spreading out evenly on the PU surface. If one can modify the PU surface with bidentate bridge evenly, the rate of APC generation would significantly be improved. (3) It is hard to control the distance between two bidentate moiety bridges, which means that some of bidentate bridge might be too close to each other or vice versa. If the distance between two bidentate bridges is too close to each other, molecules might have a problem with serve as they should because of their limited spatial arrangement. If the distance between two bidentate bridges is too far from each other, it would not be enough to create a significant change in amount of APC generation. To ensure immobilization of two major proteins on the biocompatible surfaces within an enzymatically relevant distance without any chemical reactions, this research proposes a production of chimera protein containing both TM and EPCR. This chimeric protein created with EPCR and TM using a linker protein in between and six additional His tag were attached at the end of TM sequence. It is important to review how to design chimeric protein and the choice of the expression system.

A recombinant DNA technology with recently improved methods of DNA chemical synthesis allows design and relatively rapid synthesis of modest-sized genes, and chimeric/fusion protein techniques are widely used tools in biochemical research to increase the expression of soluble proteins and to facilitate protein purification[62]. To create chimeric/fusion proteins, parts of two or more different genes encoded for separate proteins are connected by a peptide linker[62]. An adequate cushion distance is often required when designing the linkers[63]. The length of the linkers varied from 2 to 18

residues and the average length was 5.15 residues[64]. Arai et al. showed that chimeric proteins with helical linkers were more elongated than those with flexible linkers, but flexibility of liker did not reflect the flexibility in the inter-domain distance[65]. The difference between flexible and helical liker represented the spatial separation of the two domains in the chimeric proteins, and these linkers play an important role in chimeric protein to control the distance, orientation, and relative motion of two functional domains proteins[63, 65]. Number of studies has been reported that the number of steps and the time required for purification was reduced when protein was fused with six or more additional histidine residues at the end of C- or N-terminus [66, 67]. Hu et al purified chimeric baculovirus with histidine tag using a simple immobilized metal affinity chromatography (IMAC) with a high purity of 87%. This method is far simpler than the conventional methods using multiple ultracentrifugation steps. Sonia et al used another form of a single step metal chelating chromatography to purify a His-tagged chimeric D-amino acid oxidase, and the purified protein showed a highly specific enzyme activity with 82% yield. To optimize the production of recombinant protein, a variety of expression systems has been developed and evaluated.

The development of novel protein expression systems has been a major feature and challenge in biotechnology community [68, 69]. The choice of protein expression system needs to be determined by the use of the product, not for the ease production, since recombinant proteins designed for human use require specific glycosylation to guarantee proper cellular targeting [69]. *Escherichia coli* and Chinese hamster ovary (CHO) have been the most common expression systems to produce recombinant proteins [68, 70]. Over the past three decades, E. Coli has been widely used as a cellular host for

protein expression. However, *E. coli* as a prokaryotic host is not able to fold foreign proteins properly and cannot perform other post-transitional modifications, and typically does not secrete these proteins [71, 72]. Since *E. coli* cannot mimic mammalian glycosylation to produce eukaryotic proteins, the proteins have a tendency to be misfolded and biologically inactive [73]. Thus, *E. coli* has constraint in the types of proteins that can be expressed, such as human insulin gene, which is a relatively modest-size and simple structure gene that can be incorporated into prokaryotic cells for gene expression.

On the other hand, CHO cells as a mammalian system have been most broadly used expression system for the production of complex human glycoproteins [74]. CHO cells are enable to produce glycoproteins similar to human glycosylation patterns, but the glycan structures formed from CHO cells are not truly identical to those produced in human cells, which requires a modification to assure therapeutic use [75, 76]. There are major disadvantages for using mammalian cells. The cost of maintenance and cell growth in mammalian cells is expensive, the process of screening takes longer time, and transmitting virus and prions is the issue [77].

Another useful expression system for the production of eukaryotic proteins is the use of the methylotrophic yeast *Pichia pastoris*, which has become popular recently [71]. *P. pastoris* is a eukaryote, therefore, it provides the potential for producing soluble, correctly folded recombinant proteins that have undergone all the post-translational modifications required for functionality [71]. Another benefit of the *P. pastoris* is the use of strong alcohol oxidase promoters that are able to drive the expression of a foreign

gene(s) of interest, which results in large production of the target protein(s) with relative technical ease and at a lower cost than most other eukaryotic systems[78].

In the past, a numerous chimeric proteins have been expressed in a various expression systems mentioned above and the review of three antithrombogenic chimeric proteins expressed in E. coli, CHO cell, and *P. pastoris* are followed. A chimeric protein, rscu-PA-40kDa/Hir, combining the functional properties of a fibrin –specific palsminogen activator with the inhibitory potential of a specific and potent thrombin inhibitory, was obtained by fusion of the C-terminal amino acids 53-65 hirudin (Hir) to the C-terminal of a 40-kDa fragment (Ser47—Leu411) of recombinant single-chain urokinase-type palaminogen activator (recu-PA) via a 14 –amino acid linker sequence [79]. The protein was produced by expression of the corresponding chimeric cDNA in E. coli in order to use in thrombolytic therapy of myocardial infarction and purified using silica gel column. The eluted protein was further purified by chromatography on copper chelate and carboxymethyl-cellulose matrices. The purified rscu-PA-40kDa/Hir reduced thrombin concentration to 50% at 95nM compared to 4.7nM of hirudin, and the catalytic efficacies for plasmin-meditated conversion to two-chain molecules were comparable for rscu-PA-40kDa/Hir and rscu-PA (0.63 and 0.65$pM^{-1}s^{-1}$, respectively). Thus, the intrinsic specific thrombin inhibitory activity of hirudin was not fully expressed, but the activity was compatible with that of hirugen, indicating that a higher concentrations of the chimera was required for inhibit of blood coagulation in the circulation than lysis of a fibrin clot. A chimeric protein, rscu-PA-40kDa/Hir, maintained the specific fibrinolytic and plasminogen activating activity of rscu-PA as well as its finbrinolytic potency in plasma. Another novel chimeric protein, tissue plaminogen activator (t-PA), was

constructed by fusion of t-PA without the first three domains (finger domain, growth factor domain, and kringle 1) with kringle 2 serine protease domain of t-PA via 7 amino acid, GHRP-SYQ with high fibrin affinity [80]. Deletion of first three domains in t-PA prolongs its half-life while addition of GHRP-SYQ to truncated t-PA compensates for diminished fibrin affinity due to finger domain that is a responsible for fibrin affinity. A chimeric truncated t-PA was expressed in Chinese Hamster Ovarian (CHO) cell and purified by the one-step purification procedure using HiTrap Benzamidine FF (high sub) column. The expression level of chimeric truncated t-PA was 566,917 IU/mg and 752 IU/ml with 86% of fibrin affinity of full length t-PA at fibrinogen concentration of 0.2mg/ml, based on amidolytic assay. The results demonstrated this novel chimeric t-PA could be a promising approach to treat thrombotic disease because the fibrin binding capacity of commercial form, Reteplase, was only 30% of that of full-length form [81]. The last chimeric to review is thrombin inhibitor, dipetarudin, and it is expressed in expressed in *P. pastoris* using pPIC9 vector to compare the production of the same chimera in E. coli[82]. Dipetarudin has 64 amino acids and three disulfide bonds composed of the N-terminal head structure of dipetalogastin II and the exosite 1blocking segment of hirudin, connected by a five glycine linker. Although, dipetarudin was initially expressed in E. coli, but the yield of purified protein was low with 0.3mg/l [83]. The yield of dipetarudin in *P.patoris* was 500-fold higher than the yield obtained with the E. coli system, 150mg/l and 0.3mg/l respectively, and kinetic studies showed that dipetarudin isolated from P. pastoris inhibited the thrombin catalytic activity with an apparent inhibition constant (K_i) of 399+/- 83fM[82] , which is consistent with the value of 446+/- 85fM for the inhibitor isolated from E. coli[83]. In this report, cloning of

dipetarudin in *P. pastoris* allowed a high-yield production, which can be increased more by using fermentation technology.

Upon review of the expression systems and other chimeric proteins expressed in the past, this study propose to synthesize APC generating chimeric proteins, chimera I protein (EPCR-myc-TM2-His_6) and chimera II protein (EPCR-flag-2MT-His_6), as follow. EPCR (1-193, binding of PC and APC) was fused to TM2 (4^{th}-6^{th} EGF-like domain, binding of TR) via a linker protein, myc for chimera I and flag for chimera II, followed by six additional histidine to simplify the purification steps. The sequence of TM2 in chimera II protein is reversed. Figure 2 shows the native form of EPCR and TM and the functional domains used for this study. The synthetic genes encoding functional element of EPCR and TM is expressed in yeast, *Pichia pastoris* X-33 strain. This study shows the expression, purification and biological activity characterization of chimeric protein containing EPCR and TM.

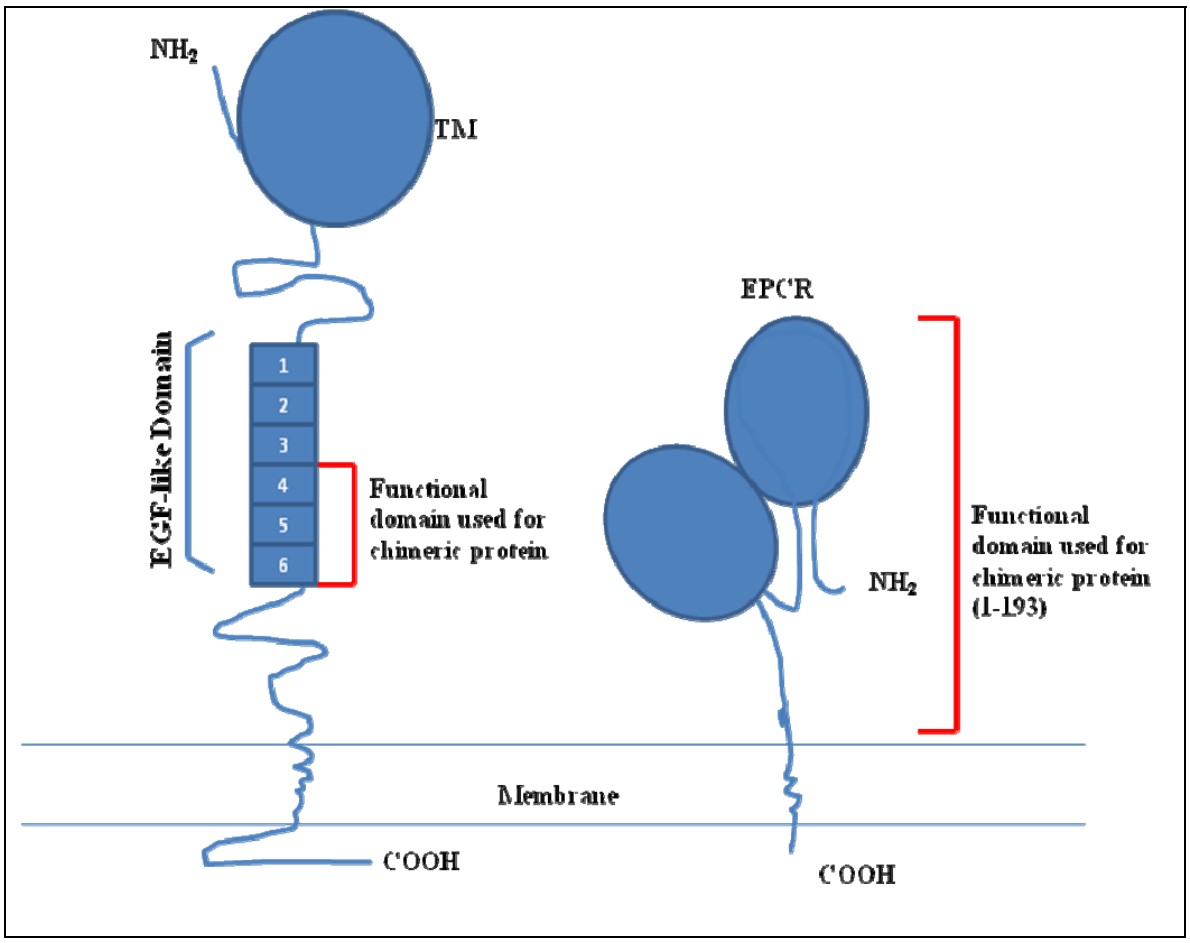


Figure 2. Schematic representation of TM and EPCR and functional domains used for chimeric proteins The 5th and 6th EGF-like domain of TM are binding site for thrombin and 4th EGF-like domain is enhancing the activation of Protein C. EPCR has N-linked glycosylation site at 32, 49, and 121 and O-linked glycosylation site at 185 and 188.

Materials and Methods

Oligonucleotide design of chimeric genes

To construct chimera I (EPCR-myc-TM-His_6) and chimera II (EPCR-flag-TM-His_6), the gene sequence of EPCR-1(residue 1-193 for chimera I and chimera II) was obtained from GenBank accession no. L35545 and the gene sequence of mutant TM-2 (residue Asp^{226} to $Lys^{466}$for chimera I and residue Ala^{224} to Cys^{462} for chimera II) was obtained from GenBank accession no. BC035602. A myc epitope (EQKLISEEDL) and a flag epitope (DYKDDDDK) were used as a linker to connect EPCR and TM for chimera I and chimera II, respectively. His-tag was added at the end of TM-2 to aid protein purification, and the amino sequence of TM-2 in chimera II was reversed. Xho I and Not I restriction sites were designed in the gene sequence to help direct insertion into pPICZ α A vector for protein expression analysis in *Pichia pastoris*.

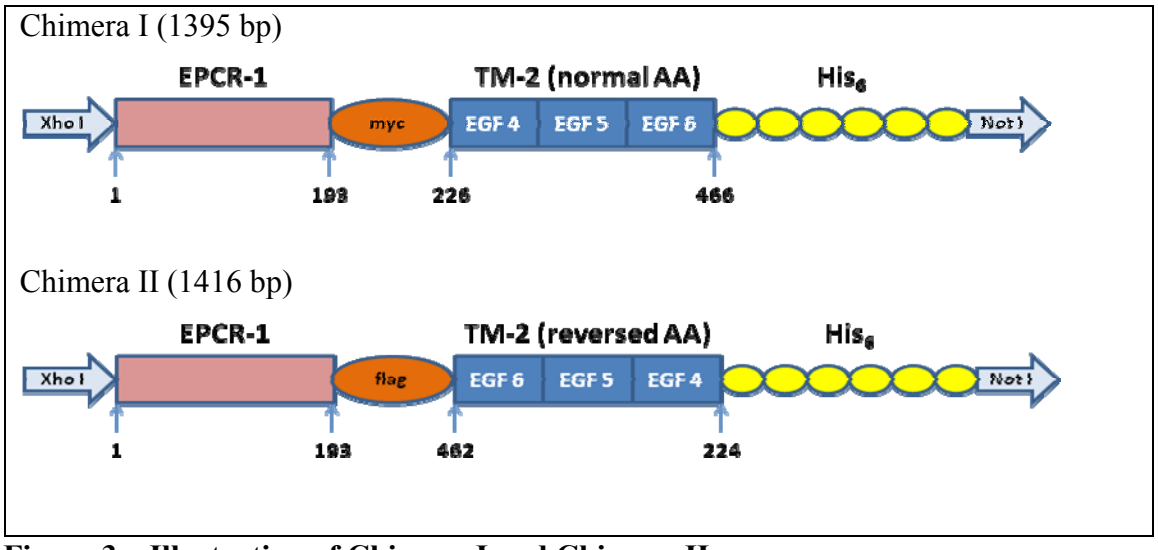


Figure 3. Illustration of Chimera I and Chimera II genes.

Oligo design for chimera I was done with the Gene2Oligo software of Rouillard and others (2004), and the oligo design for chimera II was prepared by internally developed oligo design software of Louw and others (2010). The codon optimized

oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). KOD Hot Start polymerase was obtained from Novagen (Madison, WI). Linker protein myc was purchased from Invitrogen and flag was purchased from Sigma. A gel elute extraction kit was obtained from Qiagen (CA).

The gene of TM2-His was constructed with residues from Ala^{224} to Cys^{462}. The gene of EPCR-His was constructed with residues from 1 to 193. In addition, His-tag was added to the gene sequence for helping purification. Xho I and Not I restriction sites were designed in the gene sequence to help direct insertion into pPICZ α A vector for protein expression analysis in *Pichia pastoris*.

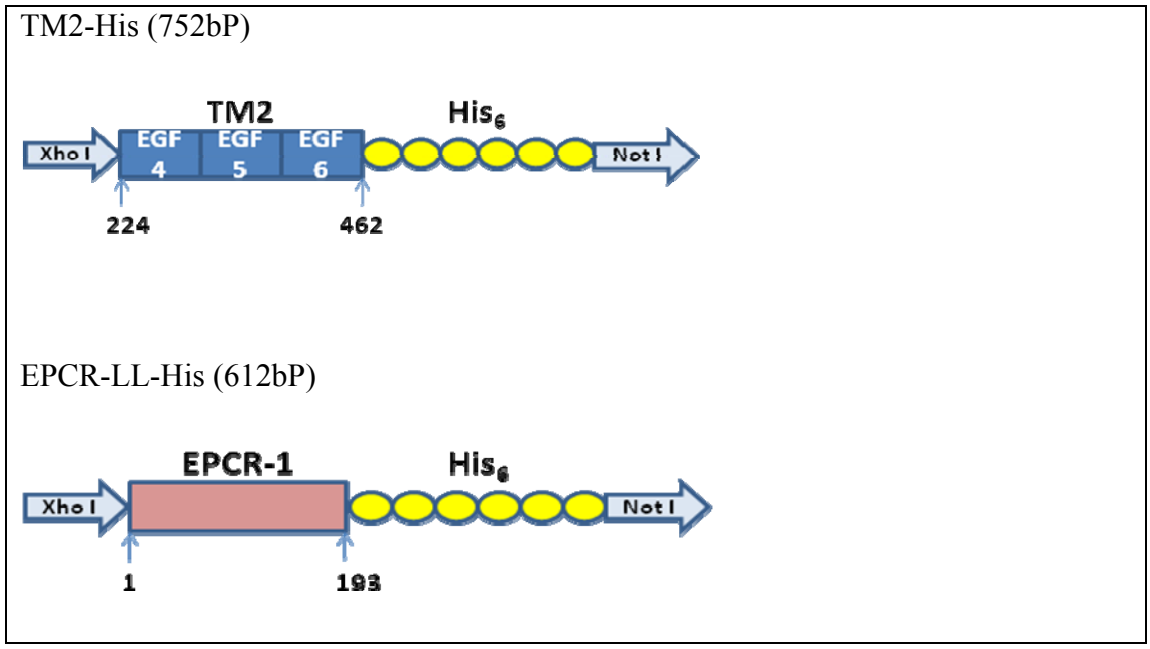


Figure 4. Illustration of EPCR-1 and TM2 chimeric genes.

Oligo design of EPCR-His was done with the Gene2Oligo software of Rouillard and others (2004), and the oligo design for TM2-His was prepared by internally developed oligo design software of Louw and others (2010). The codon optimized oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

KOD Hot Start polymerase was obtained from Novagen (Madison, WI). A gel elute extraction kit was obtained from Qiagen (CA).

PCA synthesis of chimeric genes

The PCA (Stemmer et al., 1995) product of each chimera was carried out in a 25 µl volume containing 0.1 µM of each oligonucleotide, 200 µM of each dNTP, 4 mM $MgSO_4$, 400 µg/ml non-acetylated BSA and 0.5 units KOD Hot-Start Polymerase in 1X manufacturer's buffer from Novagen (Madison, WI). 25nM of each oligonucleotide was used in the equimolar PCA reaction. PCA was performed under the following conditions: a 30 second hot start at 92°C followed by 30 cycles of 92°C for 1 second and 56°C for 10 seconds and 72°C for 10 seconds, and a final extension at 72°C for 15seconds.

Assembly PCR and amplification of chimera

2ul of PCA product of each chimera was used as template for PCR amplification with 0.7uM of forward primer and reverse primer. PCR amplification was conducted under the following conditions: 30 seconds hot start at 94°C, followed by 35 cycles of 94°C of 2 seconds and 58°C for 3 seconds and 72°C for 10 seconds, and a final extension at 72°C for 15 seconds

Vector constructions and expression of chimera genes in a *Pichia pastoris*

After amplification, gel-purified PCR products of chimera I and chimera II were cloned into TOPO vector using Zero Blunt ® TOPO® PCR cloning kit from Invitrogen (Carlsbad, CA). The DNA plasmids were transformed into chemically competent cells of *E. coli* strain TOP-10 from Invitrogen. 10-50ul from each transformation was spread on a prewarmed LB plates (1% tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% agar, pH 7.0)

containing 50ug/ml kanamycin and incubated overnight at 37°C. Ten positive clones of transformants from chimera I and chimera II were selected and transferred into 5ml of LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl pH 7.0) containing 50ug/ml kanamycin to be cultured overnight at 37°C. Then, DNA plasmids from chimera I and chimera II were isolated by using PureLink™ Quick Plasmid Miniprep kit (Invitrogen, Carlsbad, CA) according to the manufacturer's manual. The overnight cell culture was centrifuged to separate the pellet from medium. The pellet was lysed and loaded to the spin column to be eluted. Eluted DNA plasmids were sequenced at University of Nebraska-Medical Center sequencing facility with gene specific primers. Sequence analysis was performed using ChromasPro v1.5 software.

Primers	Sequence
M13 Reverse	5'-CAGGAAACAGCTATGAC-3'
M13 Forward	5'-GTAAAACGACGGCCAG-3'

Table 1. List of sequence primers used for subcloned chimera I and chimera II into TOPO vector

Sequence verified clones from chimera I and chimera II were digested with Xho I and Not I for 3hours at 37˚C and then directionally inserted into pPICZ α A vector that was digested with the same enzymes. The plasmids were transformed into chemically competent cells of *E. coli* strain TOP-10 from Invitrogen (Carlsbad, CA) according to manufacturer's protocol. 50-100ul of each transformants were spread on a prewarmed LB plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar, pH 7.0) containing 25ug/ml Zeocin and incubated overnight at 37°C. Ten positive clones from chimera I and chimera II were selected and transferred into 5ml of LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl pH 7.0) containing 25ug/ml Zeocin to be cultured overnight at 37°C. Then, DNA plasmids from chimera I and chimera II was isolated by using

PureLink™ Quick Plasmid Miniprep kit from Invitrogen (Carlsbad, CA) and the isolated DNA plasmids from positive clones were sequenced at University of Nebraska-Medical Center sequencing facility with gene specific primers. Sequence analysis was performed using ChromasPro v1.5 software.

	primer name	sequence
Chimera II	5' *Pichia*	5'-GACTGGTTCCAATTGACAAGC-3'
	3' *Pichia*	5'-GCAAATGGCATTCTGACATCC-3'
	α Factor	5'-TACTATTGCCAGCATTGCTGC-3'
	New F1	5′- CTCAGGTTACCTCTGGAGTTG -3′
Chimera I	5' *Pichia*	5'-GACTGGTTCCAATTGACAAGC-3'
	3' *Pichia*	5'-GCAAATGGCATTCTGACATCC-3'
	α Factor	5'-TACTATTGCCAGCATTGCTGC-3'
	Old F1	5′- AAGTCACCTCTGGAGTTGTC -3′

Table 2. List of primers used for sequence analysis of chimera I and chimera II gene after ligation with pPICZ α A vector

For EPCR-His and TM2-His, each PCR product was directly inserted into pPICZ α A vector that was digested with the same enzymes. The rest of procedure was as same as described above for chimera I and chimera II.

	primer name	sequence
TM2 -His EPCR-His	5' *Pichia*	5'-GACTGGTTCCAATTGACAAGC-3'
	3' *Pichia*	5'-GCAAATGGCATTCTGACATCC-3'
	α Factor	5'-TACTATTGCCAGCATTGCTGC-3'

Table 3. List of primers used for sequence analysis of TM2-His and EPCR-His gene after ligation with pPICZ α A vector

One of sequence verified clones from TM2-His were transferred into 5ml of LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.0) containing 25ug/ml Zeocin and incubated overnight at 37°C. DNA plasmids were isolated with Quigen gel extraction kit (Valencia, CA). These isolated plasmids were linearized with Pme I and transformed into electrocompetent cells of *Pichia* strain X-33 according to the

manufacturer's protocol from Invitrogen. The cells were pulsed in a 0.2cm Gene Pulser® cuvette (Bio-Rad, Hercules, CA) at 2.0 kV, 300 Ω and 25 μF and 1ml of ice-cold 1M sorbitol was immediately added. 200-300ul of transformants from TM2-His were spread on prewarmed YSDS palate (1% of yeast extract, 2% of soy peptone, 2% dextrose, 1M sorbitol, and 2% agar) containing 100ug/ml Zeocin and 500ug/ml Zeocin and incubated at 30°C for 3-10 days until colonies formed.

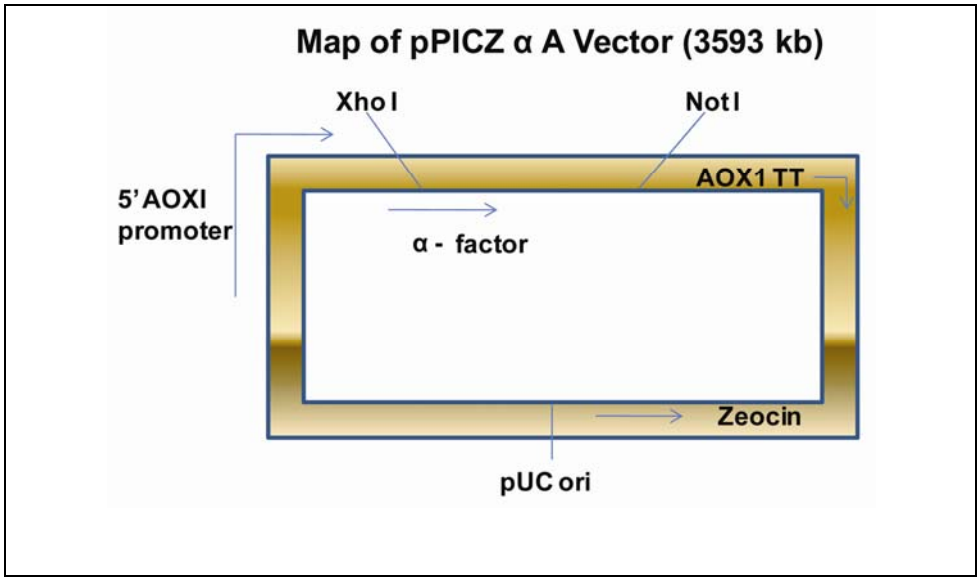


Figure 5. Map of pPICZ α A vector from Invitrogen with cloning sites. pPICZ α A vector contains 5' AOX1 promoter for methanol-inducible, high level expression of the gene interest in *Pichia*, α-factor secretion signal for efficient secretion of protein from *Pichia*, AOX1 transcription termination region for permitting efficient 3' mRNA processing, Zeocin resistance gene for selection of transformants in *E. Coli* and *Pichia* and pUC origin for replication and maintenance of the plasmid in *E. Coli*.

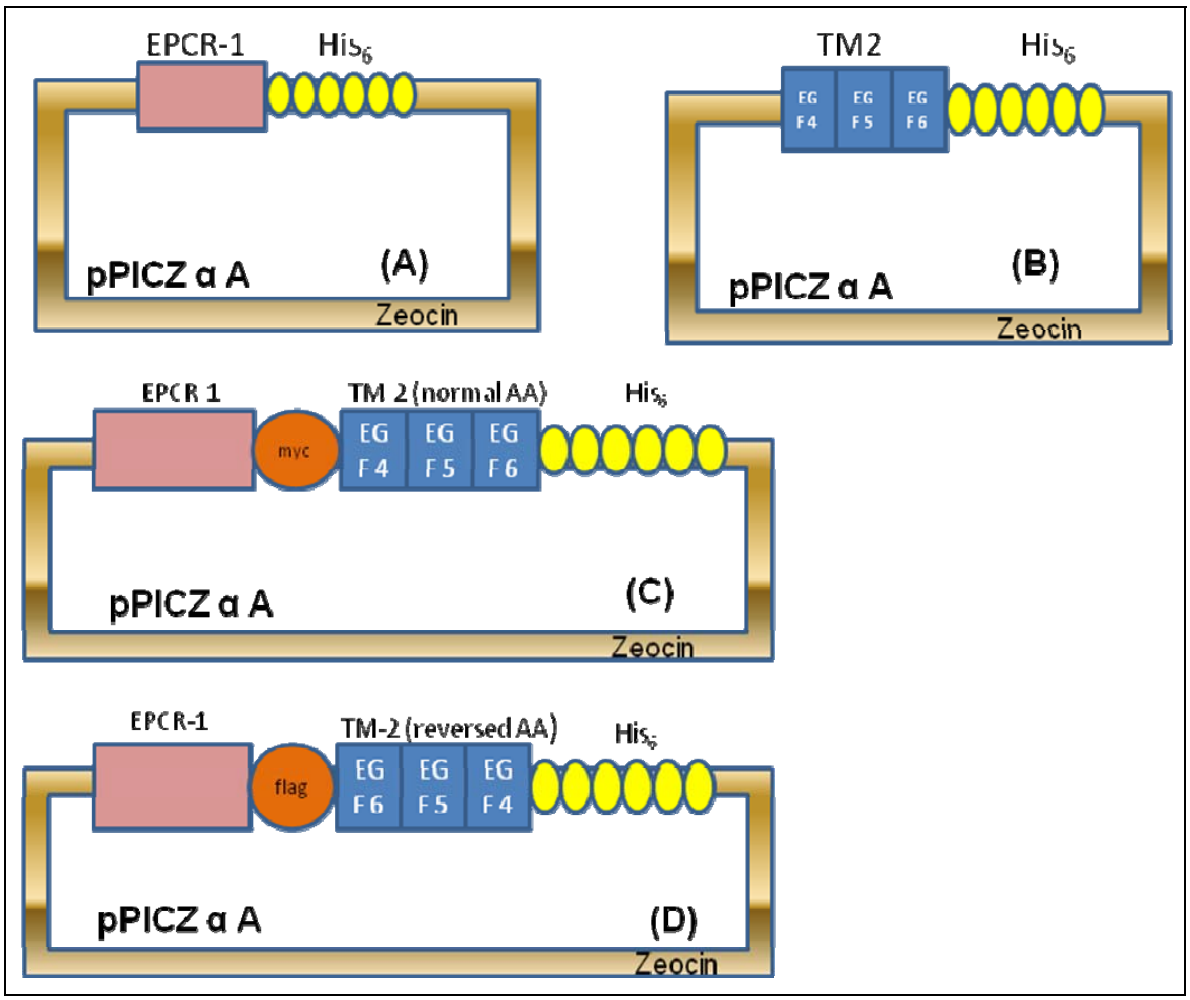


Figure 6. Illustration of ligated *Pichia* vector and chimeric genes.

Optimization of Induction Time

In order to achieve a higher expression level of recombinant proteins in *P. pastoris*, the induction time of chimera I, chimera II, and TM2-His in a shake flask was carried out. A single recombinant *P. pastoris* colony of chimera I, chimera II, and TM2-His was selected from YSD plates (1% yeast extract, 2% soy peptone, 2% Dextrose, and 2% agar) with 100µg/ml Zeocin and inoculated into 3ml of YSD medium (1% yeast extract, 2% soy peptone, and 2% Dextrose). The cell culture was incubated at 30˚C and 250rpm in a rotary shaker until OD_{600} reached to 2 -6. The overnight cell culture was transferred into a 50ml baffled flask with 15ml of BMGY medium (1% yeast extract, 2% soy peptone, 1% glycerol, 1.34% YNB without amino acids, $4x10^{-5}$% biotin, and 100mM potassium phosphate, pH 6.0) with starting OD_{600} at 0.05 and incubated at 30˚C and 250rpm until OD_{600} reached to 2 -6. Cells were harvested and resuspended in a 50ml baffled flask with 15ml of BMMY medium (1% yeast extract, 2% soy peptone, 1% glycerol, 1.34% YNB without amino acids, $4x10^{-5}$% biotin, and 100mM potassium phosphate, pH 6.0) containing 1% methanol. The cells were allowed to grow for 96 hours at 30˚C and 250rpm in a rotary shaker. To maintain induction level, 100% methanol was added to a final concentration of methanol at 1% every 12 hours. 500µl of cells were collected every 12 hours after addition of 100% methanol for western blotting analysis. The cells were harvested at 3,000xg for 5minutes at 4˚C and the supernatant and pellets were stored at -80˚C for western blotting.

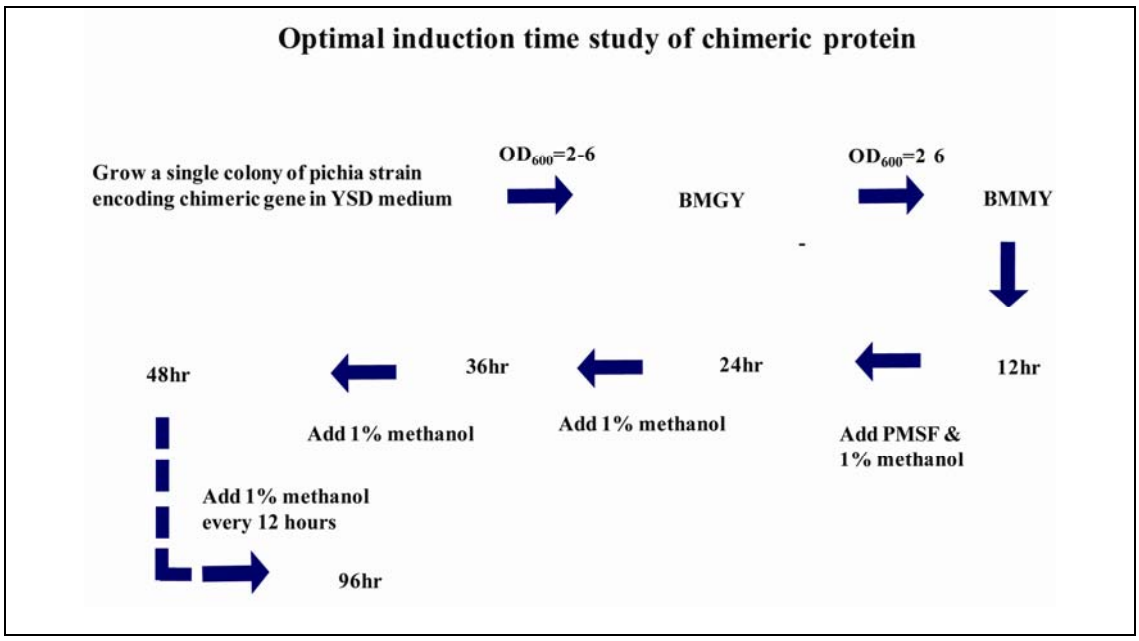


Figure 7. Schematic optimal induction time study of chimera in a shake flask. Transformed *Pichia* strains encoding chimeric gene were first grown in 5ml YSD, followed by 15ml of BMGY and 15ml of BMMY. Methanol was added to the cell culture every 12hours in order to keep the induction level and protease inhibitor (PMSF) was added after first 12hours induction. 500ul of cell culture was taken from each shake flask at every 12hours. Supernatants were separated from pellets via centrifugation and pellets were lysed. Supernatant and pellet were stored at -80C for western blotting analysis.

Large Scale Expression of Recombinant Proteins

After an optimal induction time for chimera I, chimera II, and TM2-His was determined, *Pichia* strain X-33 cells encoding chimera I, chimera II, and TM2-His gene were inoculated into a two different one liter flask. Each flask had 80ml of BMGY medium (1% yeast extract, 2% soy peptone, 100mM potassium phosphate, pH 6.0 with 1.34% YNB, $4x10^{-5}$% biotin, and 1% glycerol, pH 6.0) and the starting OD_{600} was 0.05. The cells were incubated at 30˚C and 250rpm in a rotary shaker until OD_{600} reached to 2 - 6. Cells from each flask were harvested and resuspended in a clean one liter flask with 80ml of BMMY medium (1% yeast extract, 2% soy peptone, 100mM potassium phosphate, pH 6.0 with 1.34% YNB, $4x10^{-5}$% biotin, and 1% glycerol, pH 6.0) containing 1% methanol. The cell culture was allowed to be incubated for an optimal induction time corresponding to each recombinant protein. Methanol was added every 12 hours to induce expression of the recombinant genes. *Pichia* strain X-33 cells encoding each recombinant gene were harvest by centrifugation at 3,000xg for 5min and the supernatant and pellets were stored at -80˚C for SDS-PAGE and Western blotting.

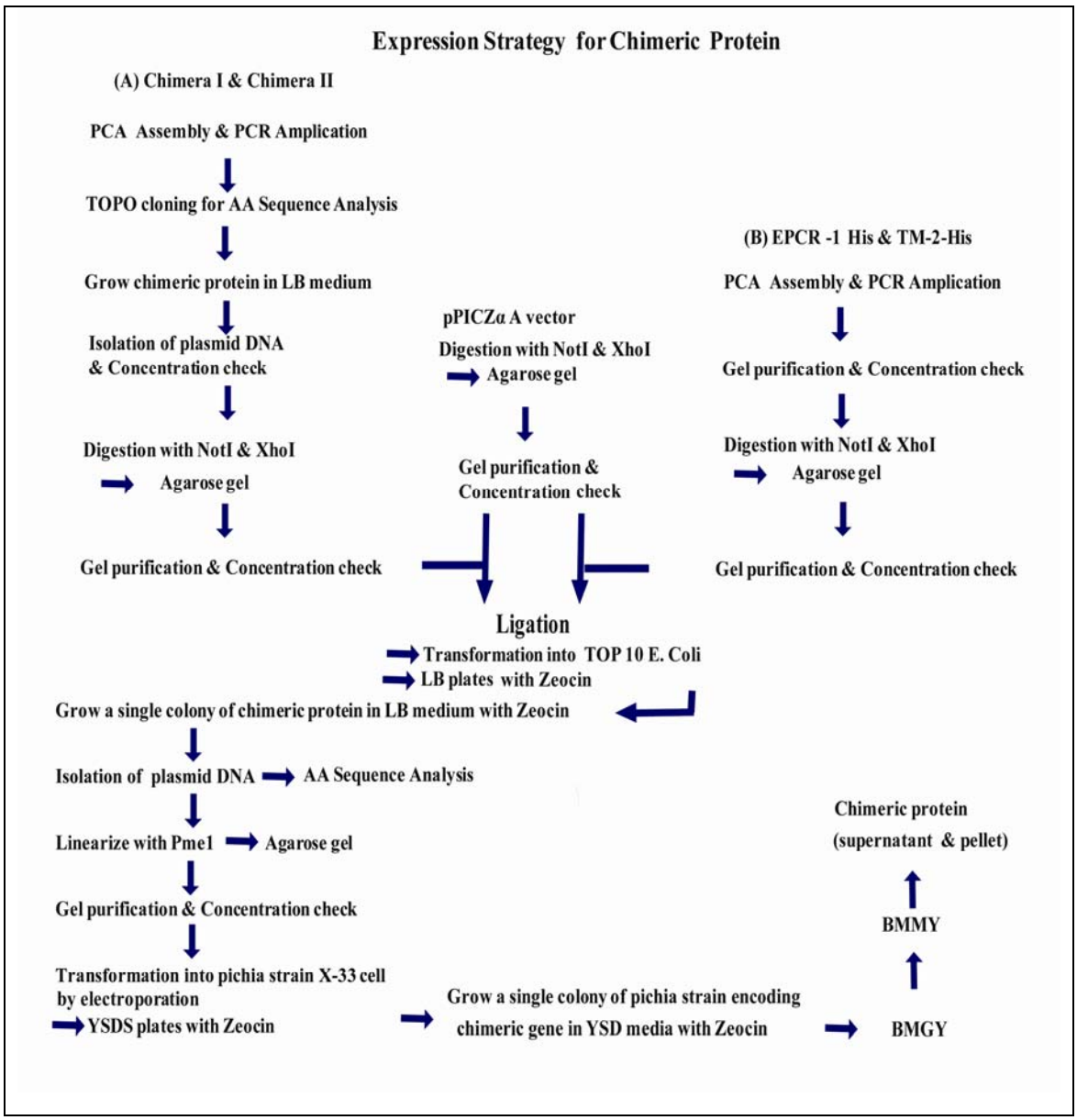


Figure 8. Schematic Expression Strategy for Chimera I and Chimera II (A) and EPCR-1-His and TM2-His (B) Chimera I and Chimera II were subcloned into TOPO vector using Zero Blunt ® TOPO® PCR cloning kit from Invitrogen (Carlsbad, CA) and transformed into *E. Coli*. Selected clones were sequenced and cloned into pPICZ α A vector, followed by transformation into *Pichia* strain X-33 cells via electroporation. Transfomants were selected by Zeocin and sequenced. Sequence verified clones were grown in YSD, BMGY, and BMMY. Supernatants were separated from the pellets via centrifugation and stored at -80˚C for further analysis (A). EPCR-His and TM2-His were directly cloned into pPICZ α A vector followed by transformation into *Pichia* strain X-33 cells via electroporation. Transfomants were selected by Zeocin and sequenced. Sequence verified clones were grown in YSD, BMGY, and BMMY. Supernatants were separated from the pellets via centrifugation and stored at -80˚C for further analysis (B).

Protein extraction and purification

For secreted proteins, the supernatants were removed from growth medium by centrifugation at 3,000x*g* for 5 min. Total volume of supernatant from chimera I, chimera II, and TM2-His was 160 ml each. Supernatant was dialyzed against five liters of dialysis buffer (10mM PBS, pH 7.0) for four hours, followed by eight hours dialysis against fresh five liters of dialysis buffer and another four hours dialysis against fresh five liters of fresh dialysis buffer at 4˚C. Dialyzed supernatant was concentrated down to 20 ml via lyophilization. Concentrated supernatant was resuspended with 20 ml of 3X binding buffer, followed by 10 ml of 1X binding buffer. The resuspended mixture was purified with Novagen His-Bind Resin (EMD Chemicals Inc., San Diego, CA) according to the manufacturer's manual. Purified proteins were analyzed by SDS-PAGE and Western blotting.

For proteins that were expressed inside the cell, pellets from each recombinant protein were lysed to extract proteins. Total mass of pellets for chimera I, chimera II, and TM2-His was 10 grams each. The pellets were resuspended with 16ml of lysis buffer (50mM Tris-HCl, 10mM MgSO4, 1mM EDTA, 10mM Potassium acetate, 1mM Dithiothreitol, and 2mM Phenylmethanesulphonyfluoride in methanol, pH 9.5). 16 ml of ice-cold glass beads (Biospec, Bartlesville, OK) were added and vortexed for 20 seconds, followed by 20 seconds of resting on the ice. The process was repeated 15 times. 54ml of 1X binding buffer was added to the proteins extracted from pellets and the mixture was purified according to the Novagen His Bind Resin manual (EMD Chemicals Inc., San Diego, CA).

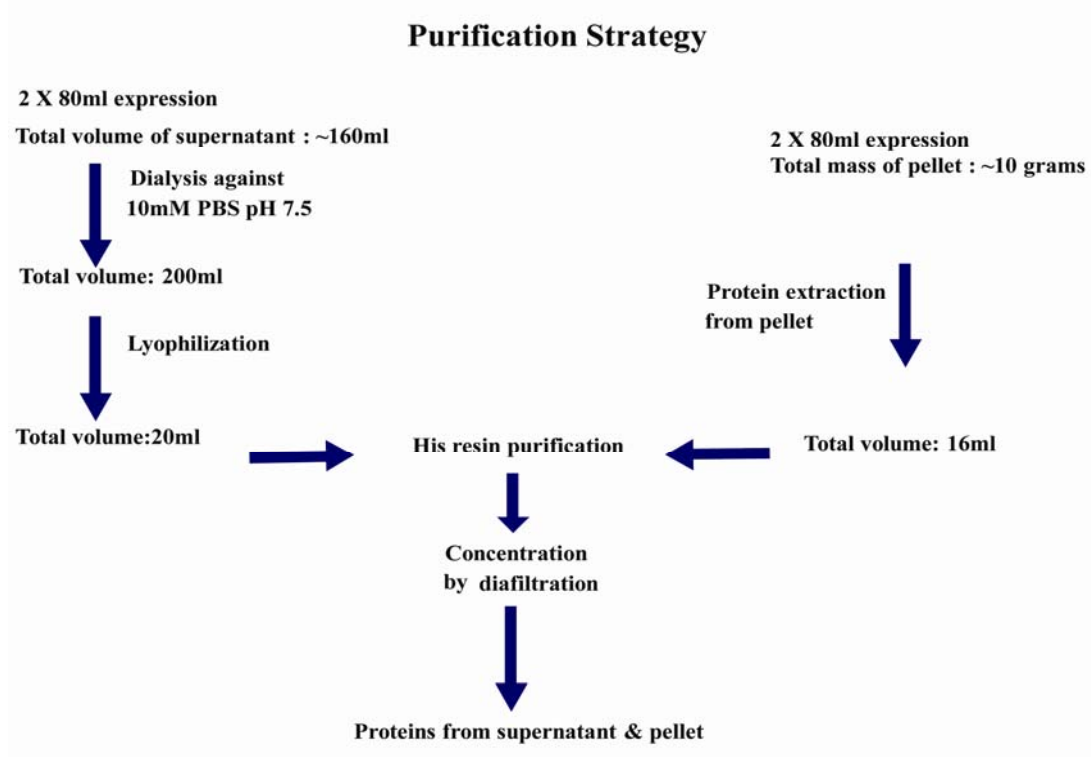


Figure 9. Schematic purification strategy of chimeric proteins. Supernatant from a large scale (2X 80mL) expression of chimeric protein was separated from pellet via centrifugation and the pellets were lysed. Total 160ml of supernatant from each chimera was dialyzed against 10mM PBS pH 7.5 and the total volume was 200ml, followed by lyophilization to bring the volume down to 20ml. 54ml of 1X binding buffer was added to lyophilized supernatant and purified with Novagen His-Bind resin (EMD Chemicals Inc., San Diego, CA) in a batch mode. Eluted proteins were diafiltered with Sartorius stedium biotech Vivaspin 4 from Fisher Scientific (Pittsburgh, PA) to be concentrated. A soluble lysate from pellets was mixed with 20ml of 3X binding buffer and 10ml of 1X binding buffer, and purified with Novagen His-Bind resin from EMD (Gibbstwon, NJ) in a batch mode. Eluted proteins were diafiltered with Sartorius stedium biotech Vivaspin 4 (Fisher Scientific, Pittsburgh, PA) to be concentrated.

Western blotting

For Western blotting, 10 ml of supernatant of chimera I, TM2-His, and EPCR-His was concentrated to 2 ml via lyophilization. 4ml of chimera II was diafiltered against 10mM PBS, pH 7.5 and concentrated to 200 µl via lyophilization. Protein samples obtained from supernatant were prepared under reducing condition. 10 µl of LDS buffer (Invitrogen, Carlsbad, CA), 4 µl of reducing agent (Invitrogen, Carlsbad, CA), and 16 µl of water were added to 10 µl of chimera I, TM2-His, and EPCR-His and 30 µl of chimera II from processed supernatant above. Protein samples obtained from pellet lysate were prepared under reducing condition. 2.5 µl of 4X LDS buffer (Invitrogen, Carlsbad, CA), 1 µl of reducing agent (Invitrogen, Carlsbad, CA), and 1.5 µl of water were added to 5 µl of chimera I, chimera II, TM2-His, and EPCR-His. These prepared samples were heated at 100˚C for 10 minutes, cooled down on the ice, centrifuged for 1 min at 13,000 rpm, and loaded on 4-12% Bis-Tris NuPAGE gels (Invitrogen, Carlsbad, CA). The proteins were separated by gel electrophoresis with NuPAGE MOPS SDS Running Buffer at 200 V for 50 minutes. Proteins were electroblotted onto a PVDF membrane (Millipore, Billerica, MA) by applying 23 volts for 35 minutes and then probed with a mouse monoclonal antibody against histidine (1:100), a rabbit polyclonal antibody against EPCR (1:1000), a mouse monoclonal antibody against human thrombomodulin (1:1000), a mouse monoclonal antibody against myc (1:5000) and a mouse monoclonal antibody against flag (1:1000). The signals were visualized with Pierce ECL Western blotting substrate (Fisher Scientific, Pittsburgh, PA) and hyperfilm ECL (Amersham Biosciences, Piscataway, NJ).

For partially pure proteins, the protein samples were prepared under reducing condition. 10ul of LDS buffer (Invitrogen, Carlsbad, CA) and 4ul of reducing agent (Invitrogen, Carlsbad, CA) was added to partially pure protein from pellet lysate of chimera I (7ul), TM2-His (10ul), and chimera II (10ul). The rest of sample volume was filled up with water to make a total volume of 40 µl. 10ul of LDS buffer (Invitrogen, Carlsbad, CA) and 4ul of reducing agent (Invitrogen, Carlsbad, CA) was added to partially pure protein from supernatant TM2-His (1.5ul), chimera I (2ul), and EPCR-His (2ul). The rest of sample volume was filled up with water to make a total volume of 40 µl. These samples were heated at 100˚C for 10 minutes, cooled down on the ice, centrifuged for 1min at 13,000 rpm, and loaded on 4-12% Bis-Tris NuPAGE gels (Invitrogen, Carlsbad, CA). The proteins were separated by gel electrophoresis with NuPAGE MOPS SDS Running Buffer at 200 V for 50 minutes. Proteins were electroblotted onto a PVDF membrane (Millipore, Billerica, MA) by applying 23 volts for 35 minutes and then probed with a mouse monoclonal antibody against histidine (1:100). The signals were visualized with Pierce ECL Western blotting substrate (Fisher Scientific, Pittsburgh, PA) and hyperfilm ECL (Amersham Biosciences, Piscataway, NJ)

Binding ability of partially pure proteins with human Protein C and human α thrombin

To check the binding ability of partially pure proteins with human protein C (hPC, Enzyme Research Labs, South Bend, IN) and human α thrombin (Enzyme Research Labs, South Bend, IN), the protein samples were prepared under reducing condition in the same manner explained above. Electroblotted proteins on a PVDF membrane was

incubated with 2 μg/ml human protein C and 2 μg /ml thrombin. The signals were visualized with Pierce ECL Western blotting substrate (Fisher Scientific, Pittsburgh, PA) and hyperfilm ECL (Amersham Biosciences, Piscataway, NJ).

Primary antibody					
	Host Animal	Manufacturer	Cat #	Vendor	Dilution
anti-His	Mouse	Calbiochem	13/45/31/2	EMD bioscience	(1:100)
anti-EPCR	Rabbit	Zymed	40-2900	Invitrogen	(1:1000)
anti-TM	Mouse	abcam	6980	abcam	(1:1000)
anti-myc	Mouse	Invitrogen	R950-25	Invitrogen	(1:5000)
anti-flag	Mouse	Sigma	F1804	Sigma	(1:1000)

Table 4. List of primary antibodies used for detection of proteins expressed in Pichia strain of X-33 cell.

Secondary antibody					
		Manufacturer	Cat #	Vendor	Dilution
anti-His	anti-Mouse	KPL	074-1806	KPL	(1:2000)
anti-EPCR	anti-rabbit	KPL	074-1506	KPL	(1:1000)
anti-TM	anti-Mouse	KPL	074-1806	KPL	(1:1000)
anti-myc	anti-Mouse	KPL	074-1806	KPL	(1:1000)
anti-flag	anti-Mouse	KPL	074-1806	KPL	(1:5000)

Table 5. List of secondary antibodies used for detection of proteins expressed in Pichia strain of X-33 cell.

Protein	MW (kDa)	Cat #	Manufacturer
Human Protein C	62	HPC1001	Enzyme Research lab
human α thrombin	37	HT1002a	Enzyme Research lab

Table 6. List of proteins used in binding ability of proteins expressed in *Pichia* strain of X-33 cell with human protein C and human α thrombin.

Antibody	Cat #	Manufacturer	Dilution
HRP-conjugated anti-human protein C	SHPC-180	Enzyme Research lab	(1:1000)
HRP-conjugated anti-human α thrombin	SHAT-140	Enzyme Research lab	(1:1000)

Table 7. List of HRP conjugated antibodies used in binding ability of proteins expressed in *Pichia* strain of X-33 cell with human protein C and human α thrombin.

SDS-PAGE

Proteins that were partially purified from supernatant and pellet lysate were analyzed by SDS-PAGE gel electrophoresis on a 4-12% Bis-Tris NuPAGE gels (Invitrogen, Carlsbad, CA) under reducing condition. For partially purified protein from pellet lysate, 10 µl of LDS buffer (Invitrogen, Carlsbad, CA) and 4 µl of reducing agent (Invitrogen, Carlsbad, CA) were added to chimera I (7ul), TM2-His (10ul), and chimera II (10ul). The rest of sample volume was filled up with water to make a total volume of 40 µl. For partially purified protein from supernatant, 10ul of LDS buffer (Invitrogen, Carlsbad, CA) and 4ul of reducing agent (Invitrogen, Carlsbad, CA) were added to TM2-His (1.5ul), chimera I (2ul), and EPCR-His (2ul). The rest of sample volume was filled up with water to make a total volume of 40 µl. These samples were heated at 100˚C for 10 minutes, cooled down on the ice, centrifuged for 1 minute at 13,000 rpm, and loaded on 4-12% Bis-Tris NuPAGE gels (Invitrogen, Carlsbad, CA). The gel was run at 200 V for 50 minutes. Proteins were visualized by staining with Silver Quest™ Silver Staining Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After gel electrophoresis, the gel was fixed in fixative solution for overnight, followed by incubation in staining solution for 15 minutes and in developing solution until the bands

were visualized in the gel. Once the desired band intensity was achieved 10 ml of stopper solution was added to the gel.

Solution Phase Protein C Activation Assay

The ability of partially pure chimera I and TM2-His to catalyze the activation of protein C was assayed in the solution phase. Protein solutions ofTM2-His, chimera I, human α thrombin (TR, Enzyme Research Labs, South Bend, IN), human Protein C (hPC, Enzyme Research Labs, South Bend, IN), and human activated Protein C (aPC, Enzyme Research Labs, South Bend, IN), were prepared in reaction buffer (0.1 M NaCl, 0.003 M $CaCl_2$, 0.0006 M MgCl2, 0.02 M Tris-HCl, 0.1 mg/ml Bovine Serum Albumin, pH 7.5). For chimera I, 25 µl of reaction buffer, 25 µl of a 0.05 mg/ml hPC solution, and a various concentration of partially pure chimera I from supernatant and pellet lysate were incubated for 15 minutes at room temperature in a sterile 96 well cell culture plate (Fisher Scientific, Pittsburgh, PA). At the same time, 25µl of reaction buffer and 25µl of a 0.05 mg/ml hPC solution were incubated for 15 minutes at room temperature in the same cell culture plate, followed by addition of 25 µl of various concentration of partially pure TM2-His from supernatant and pellet lysate. Finally, 25 µl 0.03 µg/ml TR was added to the reaction mixture of chimera I and TMs-His, which was incubated at 37˚C for 30 minutes. For the standard curve of activated Protein C, 100 µl of various concentration of human activated Protein C was added to the same 96 well cell culture plate. To measure the amount of activated PC from each well, 25 µl of spectrozyme aPC (American Diagnostica Inc., Stamford, CT) was added to a final concentration of 0.0004M. The reaction was incubated at 37˚C and the absorbance was taken after 0, 30

and 60 minutes at 405 nm using a Beckman Coulter AD 340 UV/Vis microplate reader. The concentration of generated APC was calculated using an APC in reaction buffer calibration curve on the same cell culture plate.

Results

Vector construction and expression of chimeric genes in a *Pichia pastoris*

Upon the completion of subcloning chimera I and chimera II into TOPO vector, ten clones each from experiment were selected and were further sequenced to ascertain the DNA sequence of the desired insert comparing the reference sequence of insert. The clones that are determined to possess the correct sequence (sequences are listed in the Appendix) listed in Table 8.

After sequence verification, clone #1 from chimera I and clone #2 from chimera II were selected to further insertion into pPICZ α A vector. The PCR product of TM2-His was directly inserted into a pPICZ α A vector. Table 9 shows that five clones from chimera I, eight clones from chimera II, and six clones from TM2-His are in frame with pPICZ α A vector. Error free Clone #1-6 from chimera I, #2-4 from chimera II, and #1 from TM2-His were selected for expression in *Pichia* host X-33 strain.

Optimization of Induction Time

Clones were first expressed in small scale cultures (~15 ml) and Western blotting was performed to aid in the selection of viable clones for further growth and optimization in a large scale expression (~80 ml). Typically, all clones were grown up to 96 hours and samples were taken at 24, 48 and 96 hours. Figure 10 shows the result of Western blot analysis on the samples obtained at 24 hour induction time from small scale culture. Panel-A shows the supernatants obtained from transformed *Pichia* cells carrying the gene for chimera-I. Based on observed band intensities, clones R2, R6 and R7 were chosen for further western blot analysis. Panel-B shows the supernatants obtained from transformed *Pichia* cells carrying the gene for chimera-II. Based on observed band intensities, clones

O3 and R2 were chosen for further western blot analysis. Panel-C shows the supernatants obtained from transformed *Pichia* cells carrying the gene for TM2-His. Based on observed band intensities, clones O1, O4, and R2 were chosen for further western blot analysis.

In the next step, samples obtained at 24, 48, and 96 hour from selected clones were also analyzed by western blot analysis (Figure 11/12/13). With increasing induction time, all selected clones show higher levels of expression. Clone z100 R2 from chimera I, z100 R2 from chimera II, and z100 O1 from TM2-His show stronger signal, implying higher expression. Based on the band intensity, *Pichia* cells carrying the gene for chimera I was grown up to 48 hours, *Pichia* cells carrying the gene for chimera II was grown up to 96 hours , and *Pichia* cells carrying the gene for TM2-His was grown up to 96 hours in a large scale culture.

Large Scale Expression of Recombinant Proteins

The samples obtained at 24, 48, and 96 hours of supernatant and pellet lysate from a large scale culture were analyzed by Western blotting analysis to confirm the expression levels of selected clones. Figure 14 shows clone #1-6 z100 R2 carrying genetic elements encoding chimera I in *Pichia* strain of X-33 cell. Figure 15 shows clone #2-4 z100 R2 carrying genetic elements encoding Chimera II in *Pichia* strain of X-33 cell. Figure 16 shows clone #1 z100 O1 carrying genetic elements encoding TM2-His in *Pichia* strain of X-33 cell. With increasing induction time, all selected clones show higher levels of expression. In the next step, supernatant and pellet lysate of chimera I at 48 hour, chimera II at 96 hour, and TM2-His at 96 hour were subjected to further

Western blot analysis to confirm whether each chimeric protein preserves all genetic elements.

Detection of expressed chimera I, chimera II, and TM2-His protein

Proteins obtained from supernatant and pellet lysate of chimera I, chimera II, and TM2-His were detected by western blot analysis with various antibodies. First, reduced Western blots probed with a mouse monoclonal antibody against histidine are shown in Figure 17. Panel-A shows the proteins obtained from the supernatant of TM2-His (lane 1), EPCR-His (lane 2), chimera I (lane 3), and chimera II (lane 4). The expected molecular weight of TM2-His was 42 kDa and the protein was detected at 42 kDa. The expected molecular weight of EPCR-His was 38 kDa and the protein was detected at 38 kDa. The expected molecular weight of chimera I was 78 kDa and the protein was detected at 78 kDa with a light protein band around 40 kDa. The expected molecular weight of chimera II was 78 kDa and the protein was detected at 78 kDa with a light protein band around 40 kDa. Panel-B shows the proteins obtained from the pellet lysate of TM2-His (lane 1), chimera I (lane 2), and chimera II (lane 3). All proteins from the pellet lysate show multiple bands with a band at their expected molecular weight.

Second, reduced Western blots probed with a rabbit polyclonal antibody against EPCR are shown in Figure 18. Panel-I shows the proteins obtained from the supernatant of EPCR-His (lane 1), chimera I (lane 2), and chimera II (lane 3). The expected molecular weight of EPCR-His was 38 kDa and the protein was detected as expected. The expected molecular weight of chimera I was 78 kDa and the protein was detected at 78 kDa with a light protein band around 40 kDa. The expected molecular weight of chimera II was 78 kDa and the protein was detected at 78 kDa with a light protein band

around 40 kDa. Panel-II shows the proteins obtained from the pellet lysate of TM2-His (lane 1), chimera I (lane 2), and chimera II (lane 3). All proteins from the pellet lysate show multiple bands, and chimera II has a strong signal at 78 kDa.

Third, reduced Western blots probed with a mouse monoclonal antibody against thrombomodulin are shown in Figure 19. Panel-I shows the proteins obtained from the supernatant of TM2-His (lane 1), EPCR-His (lane 2), chimera I (lane 3), and chimera II (lane 4). The expected molecular weight of TM2-His was 42 kDa and the protein was detected as expected. EPCR-His was not detected. The expected molecular weight of chimera I was 78 kDa and the protein was detected at 78 kDa with a light protein band around 40 kDa. The expected molecular weight of chimera II was 78 kDa and the protein was detected at 78 kDa with a light protein band around 40 kDa. Panel-II shows the proteins obtained from pellet lysate of TM2-His (lane 1), chimera I (lane 2), and chimera II (lane 3). All proteins from the pellet lysate show multiple bands with a band at the expected molecular weight.

Fourth, reduced Western blots probed with a mouse monoclonal antibody against myc are shown in Figure 20. Panel-I shows the proteins obtained from the supernatant of TM2-His (lane 1), EPCR-His (lane 2), and chimera I (lane 3). TM2-His and EPCR-His was not detected. The expected molecular weight of chimera I was 78 kDa and the protein was detected at 78 kDa with a light protein band around 40 kDa. Panel-II shows the proteins obtained from the pellet lysate of TM2-His (lane 1), chimera I (lane 2), and chimera II (lane 3). TM2-His and chimera II were not detected on the blot. The expected molecular weight of chimera I was 78 kDa and the protein was detected at 78 kDa with other multiple bands.

Fifth, reduced Western blots probed with a mouse monoclonal antibody against flag are shown in Figure 21. Panel-I shows the proteins obtained from the supernatant of TM2-His (lane 1), EPCR-His (lane 2), chimera I (lane 3), and chimera II (lane 4). The expected molecular weight of chimera II was 78 kDa and the protein was detected at 78 kDa with a strong protein band around 40 kDa and other bands. TM2-His, EPCR-His, and chimera I were not detected on the blot. Panel-II shows the proteins obtained from the pellet lysate of TM2-His (lane 1), chimera I (lane 2), and chimera II (lane 3). All proteins from the pellet lysate show multiple bands. The results of Western blot analysis to detect the functional elements and the linkers of chimeric proteins are summarized in Table 10.

Western blot analysis of proteins obtained from His-resin purification

Proteins of chimera I, chimera II, TM2-His and EPCR-His obtained from His-resin purification were analyzed to check the purity of these proteins by Western blot with an antibody against histidine. The expected molecular weight of chimera I, chimera II, TM2-His, EPCR-His was 78, 78, 42, and 38 kDa, respectively. Figure 22 shows proteins from the pellet lysate of TM2-His (lane 1), chimera II (lane 2), and chimera I (lane 3) and proteins from the supernatant of EPCR-His (lane 4), TM2-His (lane 5), chimera II (lane 6), chimera I (lane 7), and EPCR-His (lane 8). Most of proteins were detected at their corresponding molecular weight along with multiple bands. Protein from the supernatant of chimera II (lane 6) was not detected at all and protein from the supernatant of EPCR-His (lane 8) was barely detected with a faint signal.

Silver staining analysis of proteins obtained from His-resin purification

Proteins of chimer I, chimera II, TM2-His and EPCR-His obtained from His-resin purification were analyzed to check the purity of these proteins were analyzed by SDS-PAGE and silver staining (Figure 23). The expected molecular weight of chimera I, chimera II, TM2-His, EPCR-His was 78, 78, 42, 38 kDa, respectively. Proteins from the pellet lysate of chimera I (lane 1), chimera II (lane 2), and TM2-His (lane 3) have multiple bands. All of purified proteins have a band at 62 kDa. Proteins from the supernatant of TM2-His (lane 5), chimera I (lane 7), and EPCR-His (lane 9), and have multiple bands. TM2-His (lane 5) has one band at 42 kDa with other multiple bands. Chimera I (lane 7) has one band at 45 kDa and the other band at 62 kDa. EPCR-His (lane 4), chimera I (lane 6), and EPCR-His (lane 8) have only one band at 62 kDa.

Binding ability of partially pure proteins with human Protein C and human α thrombin

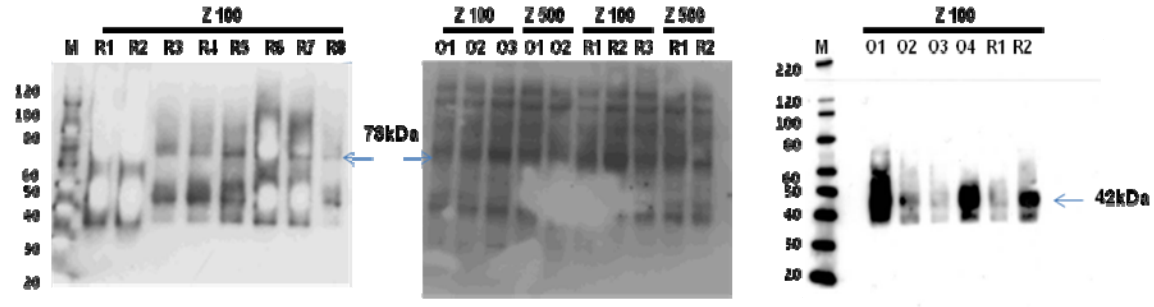
Figure 24 shows that partially pure proteins of chimera I, chimera II, TM2-His, and EPCR-His, were electroblotted, incubated with human protein C, and detected with a HRP-conjugated anti-human protein C. All of proteins are bound to human protein C, except for protein from the supernatant of chimera II (lane 6). Figure 25 shows that partially pure proteins of chimera I, chimera II, TM2-His, and EPCR-His, were electroblotted, incubated with human α thrombin, and detected with a HRP-conjugated anti-human α thrombin. All of proteins are bound to human α thrombin, except for protein from the supernatant of chimera II (lane 6) and EPCR-His (lane 8).

Solution Phase Protein C Activation Assay

The ability of partially pure chimera I and TM2-His to catalyze the activation of Protein C was assayed in the solution phase. We used the APC values obtained from 4762, 2381, 595, and 297 nM of TM2-His (supernatant) in the experiment to extrapolate the APC value of 961 nM of TM2-His (supernatant). To extrapolate the APC values of 320 nM and 160 nM of TM2-His (pellet), we used the APC values obtained from 595 and 297 nM of TM2-His (pellet) in the experiment. The final APC values presented in this study were averaged from 30 minute and 60 minute reading at 405 nm after the activation of Protein C.

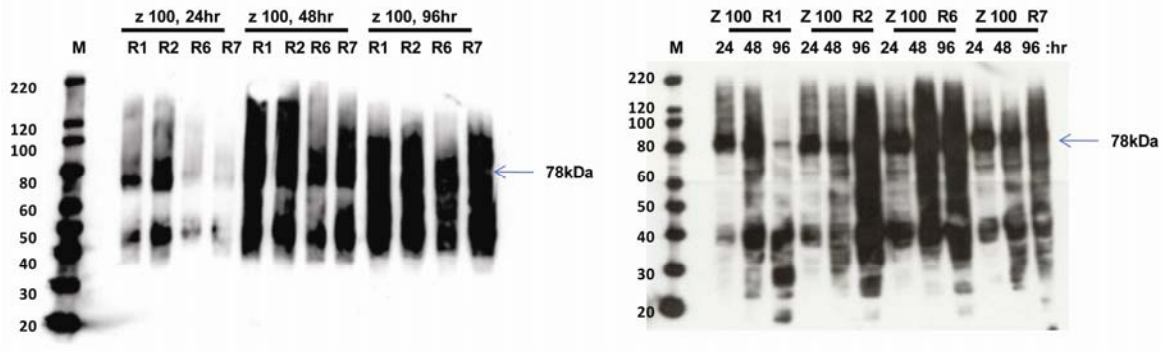
Figure 26 shows the amount of APC generated from partially pure proteins of chimera I and TM2-His. Chimera I (supernatant) at 961 nM produced 0.979 nM of APC, chimera I (pellet) at 320 nM produced 3.768 nM of APC, and chimera I (pellet) at 160 nM produced 1.370 nM of APC. TM2-His (supernatant) at 961 nM produced 0.540 nM of APC, TM2-His (pellet) at 320 nM produced 2.048 nM of APC, and TM2-His (pellet) at 160 nM produced 0.676 nM of APC. Human Protein C alone, thrombin alone, or human Protein C and thrombomodulin together did not produced activated Protein C. The control sample produced 9.1 nM of APC with commercial proteins of hPC, rabbit Lung TM, and thrombin in the absence of EPCR.

Overall the activity level of chimera I is twice higher than that of TM2-His at the same concentration of proteins from both supernatant and pellet lysate, and the activity level of protein from the pellet lysate is higher than that of proteins from the supernatant.



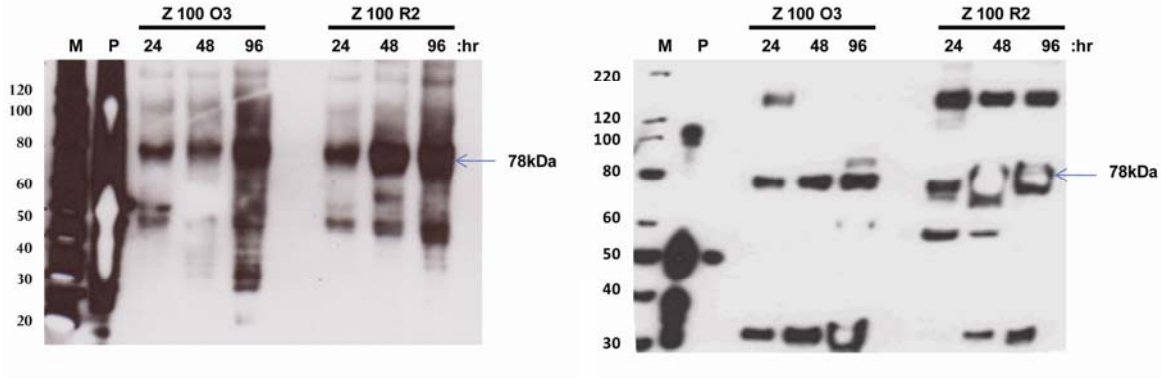
(A) Chimera I, Supernatant,24hr (B) Chimera II, Supernatant,24hr (C) TM2-His, Supernatant,24 hr

Figure 10. Western blotting analysis of small scale induction experiments probed with anti-His. Selected clones of *Pichia* cells carrying the gene for chimera I, chimera II, and TM2-His were first grown in BMGY, followed by BMMY and fractions were analyzed at 24 hours induction in cell culture. Supernatants were separated from via centrifugation and pellets were lysed. Supernatants were analyzed by western blotting and probed with a mouse monoclonal antibody against. (Z100: Zeocin 100 µg/ml, z 500: Zeocin 500 µg/ml, O: original plate, R: restreaked plate)



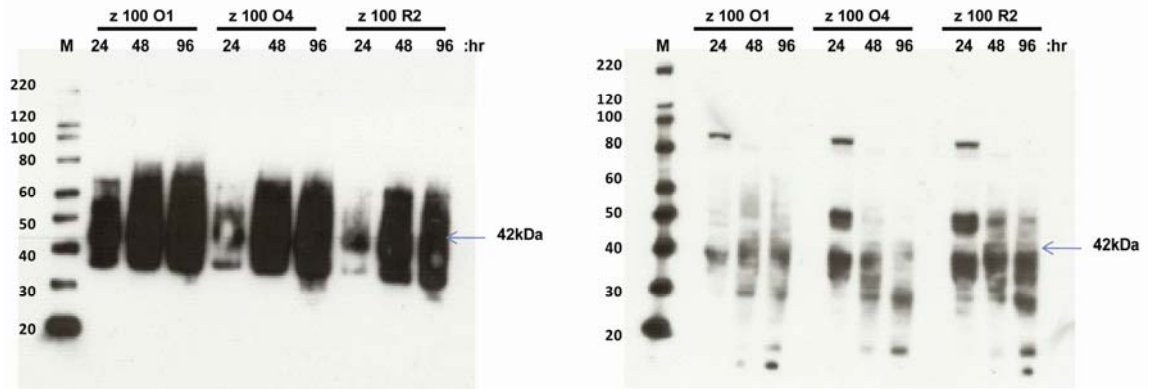
(A) Chimera I, Supernatant **(B) Chimera I, Pellet lysate**

Figure 11. Western blotting analysis of small scale induction experiment probed with anti-His. *Pichia* cells carrying the gene for chimera I (clone #1-6) were first grown in BMGY, followed by BMMY and fractions were analyzed at 24, 48, and 96 hours upon induction in cell culture. Supernatants were separated from via centrifugation and pellets were lysed. 10 µl of supernatant and 5 µl of soluble lysate from pellet were analyzed by Western blotting and probed with a mouse monoclonal antibody against histidine. (Z100: Zeocin 100 µg/ml, R: restreaked plate)



(A) Chimera II, Supernatant **(B) Chimera II, Pellet lysate**

Figure 12. Western blotting analysis of small scale induction experiment probed with anti-His. *Pichia* cells carrying the gene for chimera II (clone #2-4) were first grown in BMGY, followed by BMMY and fractions were analyzed at 24, 48, and 96 hours upon induction in cell culture. Supernatants were separated from via centrifugation and pellets were lysed. 30 µl of supernatant and 5 µl of soluble lysate from pellet were analyzed by Western blotting and probed with a mouse monoclonal antibody against histidine. (Z100: Zeocin 100 µg/ml, O: original plate, R: restreaked plate).



(A) TM2-His, Supernatant **(B) TM2-His, Pellet lysate**

Figure 13. Western blotting analysis of small scale induction experiment probed with anti-His. *Pichia* cells carrying the gene for TM2-His (clone #1) were first grown in BMGY, followed by BMMY and fractions were analyzed at 24, 48, and 96 hours upon induction in cell culture. Supernatants were separated from via centrifugation and pellets were lysed. 10 µl of supernatant and 5µl of soluble lysate from pellet were analyzed by Western blotting and probed with a mouse monoclonal antibody against histidine. (Z100: Zeocin 100 µg/ml, O: original plate, R: restreaked plate).

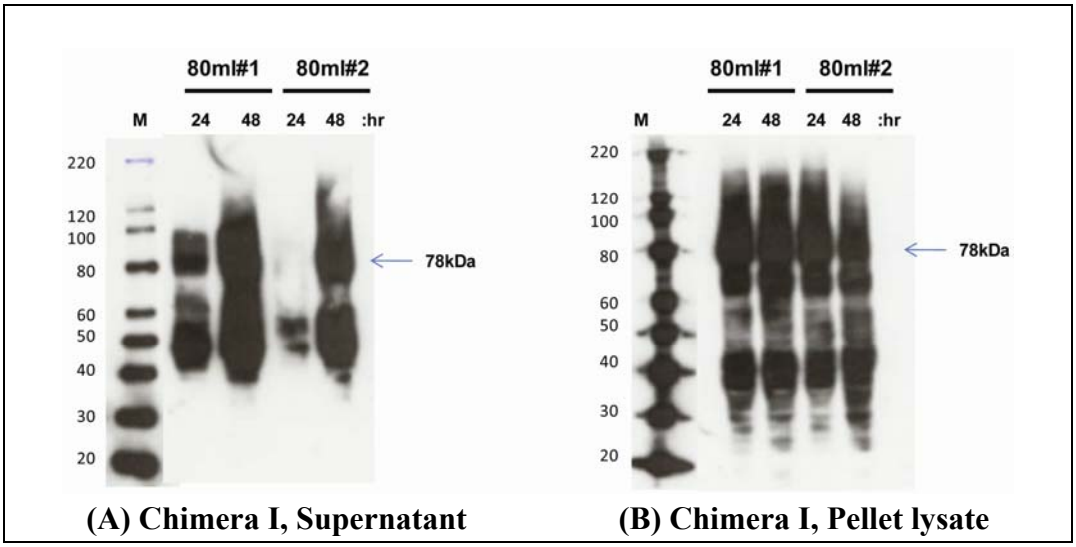


Figure 14. Western blotting analysis of large scale induction experiment probed with anti-His. *Pichia* cells carrying the gene for chimera I (clone #1-6z100 R2) were first grown in BMGY, followed by BMMY and fractions were analyzed at 24 and 48 hours upon induction in cell culture. Supernatants were separated from via centrifugation and pellets were lysed. 10 µl of supernatant and 5 µl of soluble lysate from pellet were analyzed by Western blotting and probed with a mouse monoclonal antibody against histidine.

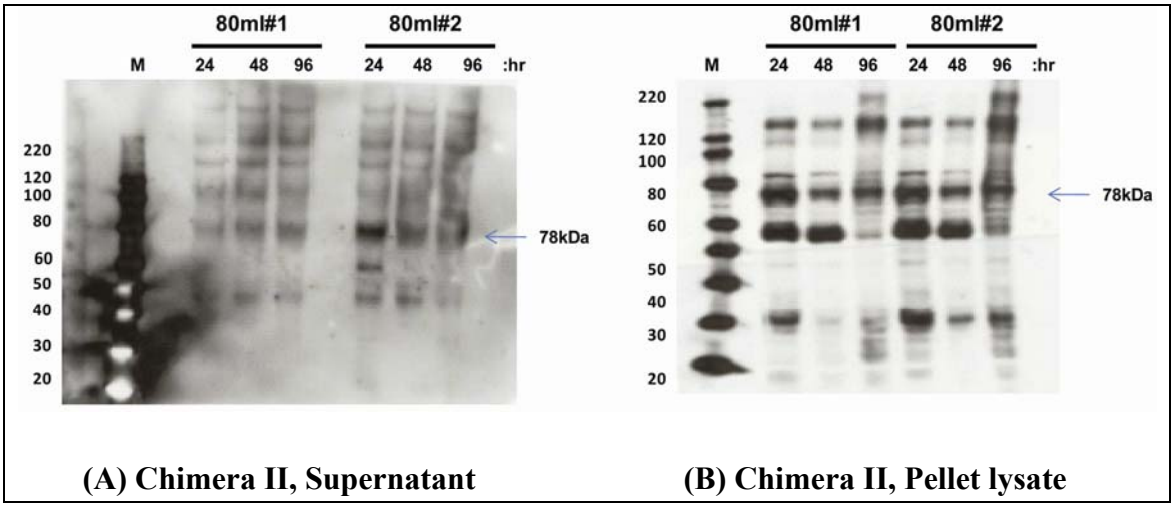
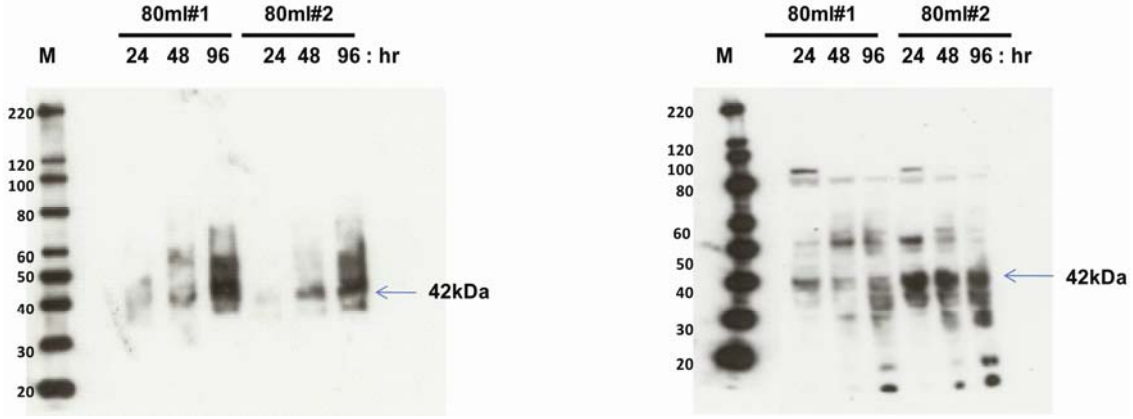


Figure 15. Western blotting analysis of large scale induction experiment probed with anti-His. *Pichia* cells carrying the gene for chimera II (clone #2-4 z100 R2) were first grown in BMGY, followed by BMMY and fractions were analyzed at 24 and 48 hours upon induction in cell culture. Supernatants were separated from via centrifugation and pellets were lysed. 30 µl of supernatant and 5 µl of soluble lysate from pellet were analyzed by Western blotting and probed with a mouse monoclonal antibody against histidine.



(A) TM2-His, Supernatant **(B) TM2-His, Pellet lysate**

Figure 16. Western blotting analysis of large scale induction experiment probed with anti-His. *Pichia* cells carrying the gene for TM2-His (clone #1 z100 O1) were first grown in BMGY, followed by BMMY and fractions were analyzed at 24, 48, and 96 hours upon induction in cell culture. Supernatants were separated from via centrifugation and pellets were lysed. 10 µl of supernatant and 5ul of soluble lysate from pellet were analyzed by Western blotting and probed with a mouse monoclonal antibody against histidine.

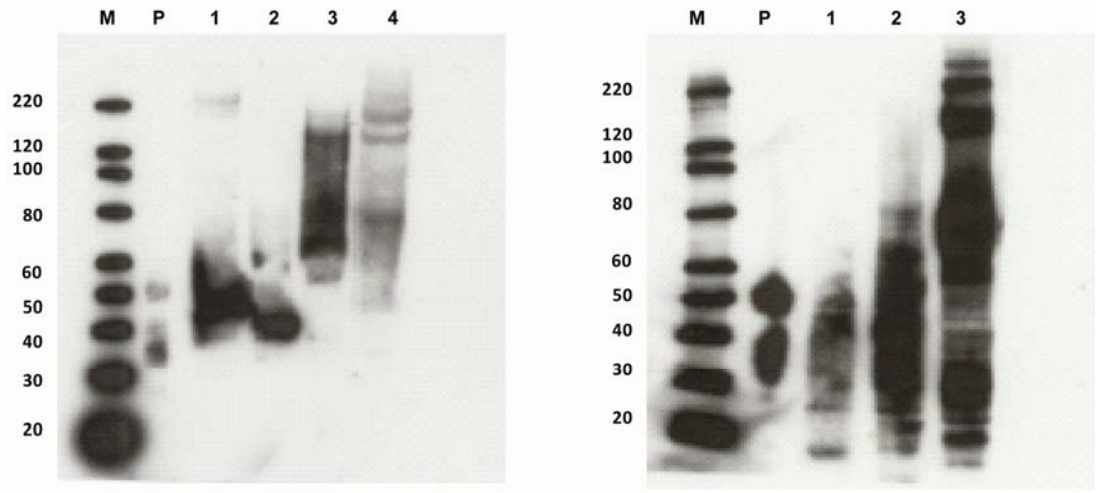
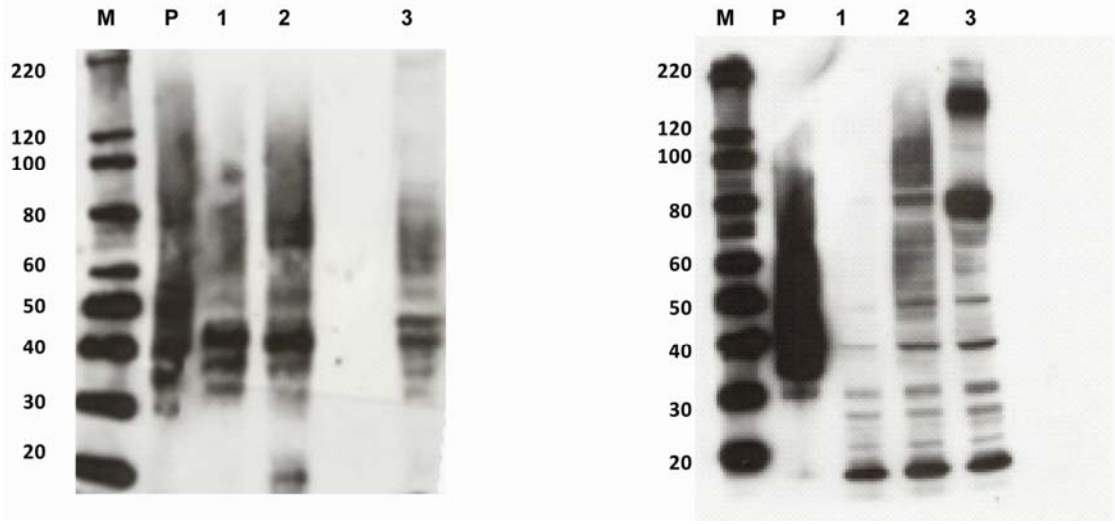


Figure 17. Western blotting analysis of proteins expressed in *Pichia Pastoris* probed with anti-His. Transformed *Pichia* strains were first grown in BMGY, followed by BMMY. Supernatants were separated from via centrifugation and pellets were lysed. Supernatant and soluble lysate from pellet were analyzed by western blotting and probed with a mouse monoclonal antibody against histidine. (I) P: positive control (Positope), lane 1: TM2-His (5X concentrated), lane 2: EPCR-His (5X concentrated), lane 3: Chimera I (5X concentrated), lane 4: Chimera II (20X concentrated) **(**II) P: positive control (Positope), lane 1: TM2-His, lane 2: Chimera I, lane 3: Chimera II



(A) Supernatant before purification **(B) Pellet lysate before purification**

Figure 18. Western blotting analysis of chimeric proteins expressed in *Pichia Pastoris* probed with anti-EPCR. Transformed *Pichia* strains were first grown in BMGY, followed by BMMY. Supernatants were separated from via centrifugation and pellets were lysed. Supernatant and soluble lysate from pellet were analyzed by western blotting and probed with a rabbit polyclonal antibody against EPCR. (I) P: positive control (143.5ng of EPCR-LL), lane 1: EPCR-His (5X concentrated), lane 2: Chimera I (5X concentrated), lane 3: Chimera II (20X concentrated) **(**II) P: positive control (143.5ng of EPCR-LL), lane 1: TM2-His, lane 2: Chimera I, lane 3: Chimera II

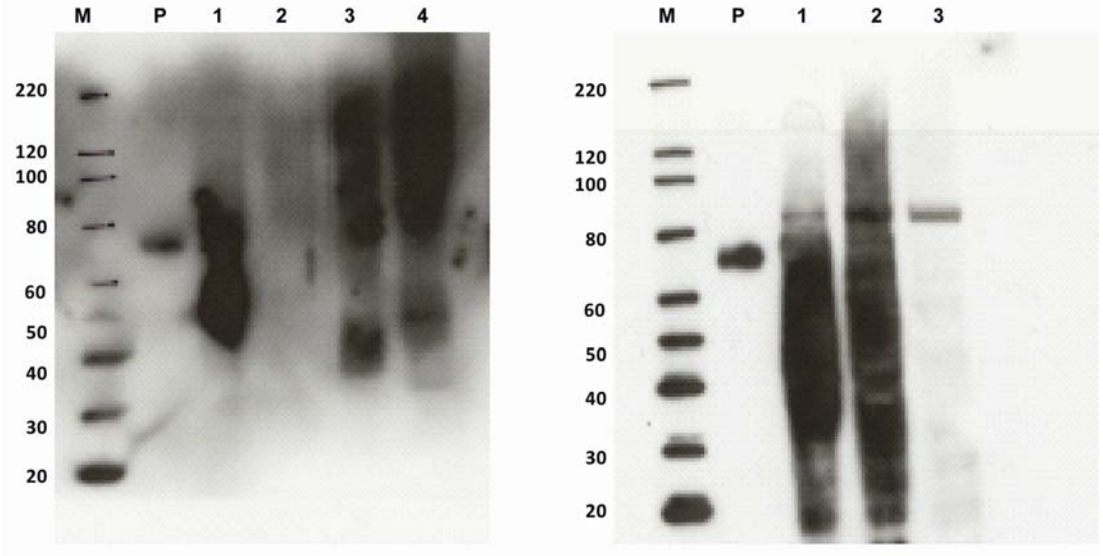
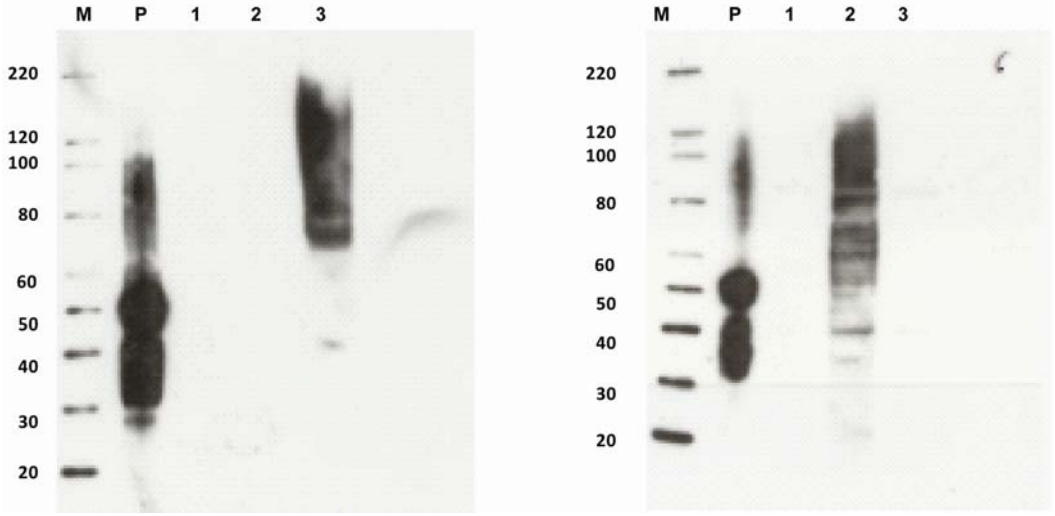


Figure 19. Western blotting analysis of chimeric proteins expressed in *Pichia Pastoris* probed with anti-TM. Transformed *Pichia* strains were first grown in BMGY, followed by BMMY. Supernatants were separated from via centrifugation and pellets were lysed. Supernatant and soluble lysate from pellet were analyzed by western blotting and probed with a mouse monoclonal antibody against thrombomodulin. (I) P: positive control (50ng of human Thrombomodulin), lane 1: TM2-His (5X concentrated), lane 2: EPCR-His (5X concentrated), lane 3: Chimera I (5X concentrated), lane 4: Chimera II_20X concentrated **(**II) P: positive control (100ng of human Thrombomodulin), lane 1: TM2-His, lane 2: Chimera I, lane 3: Chimera II



(A) Supernatant before purification **(B) Pellet lysate before purification**

Figure 20. Western blotting analysis of chimeric proteins expressed in *Pichia Pastoris* probed with anti-myc. Transformed *Pichia* strains were first grown in BMGY, followed by BMMY. Supernatants were separated from via centrifugation and pellets were lysed. Supernatant and soluble lysate from pellet were analyzed by western blotting and probed with a mouse monoclonal antibody against myc. (I) P: positive control (250ng of Positope), lane 1: TM2-His (5X concentrated), lane 2: Chimera I (5X concentrated), lane 3: Chimera II (20X concentrated) **(**II) P: positive control (250ng of Positope), lane 1: TM2-His, lane 2: Chimera I, lane 3: Chimera II

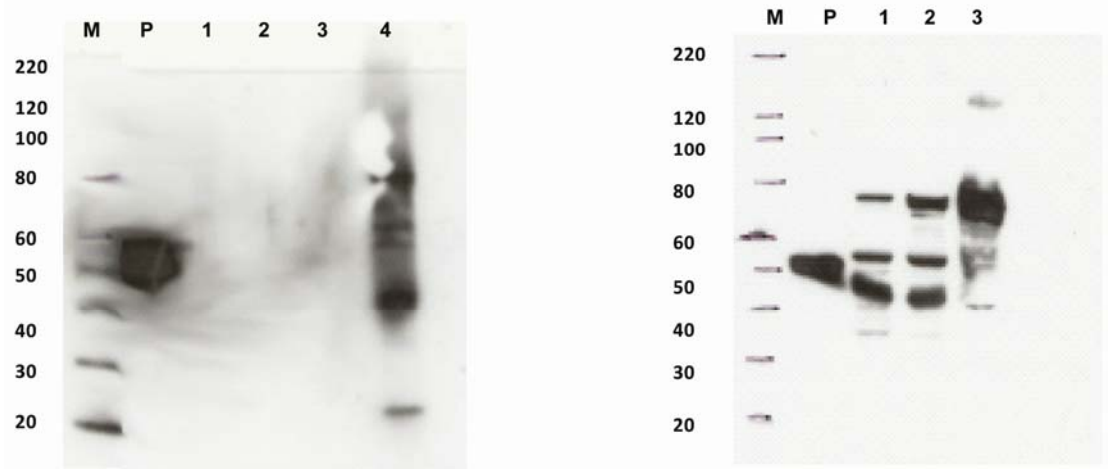


Figure 21. Western blotting analysis of chimeric proteins expressed in *Pichia Pastoris* probed with anti-flag. Transformed *Pichia* strains were first grown in BMGY, followed by BMMY. Supernatants were separated from via centrifugation and pellets were lysed. Supernatant and soluble lysate from pellet were analyzed by western blotting and probed with a mouse monoclonal antibody against flag. (I) P: positive control (50ng of flag fusion protein), lane 1: TM2-His (5X concentrated), lane 2: EPCR-His (5X concentrated), lane 3: Chimera I (5X concentrated), lane 4: Chimera II (20X concentrated) (II) P: positive control (50ng of flag fusion protein), lane 1: TM2-His, lane 2: Chimera I, lane 3: Chimera I

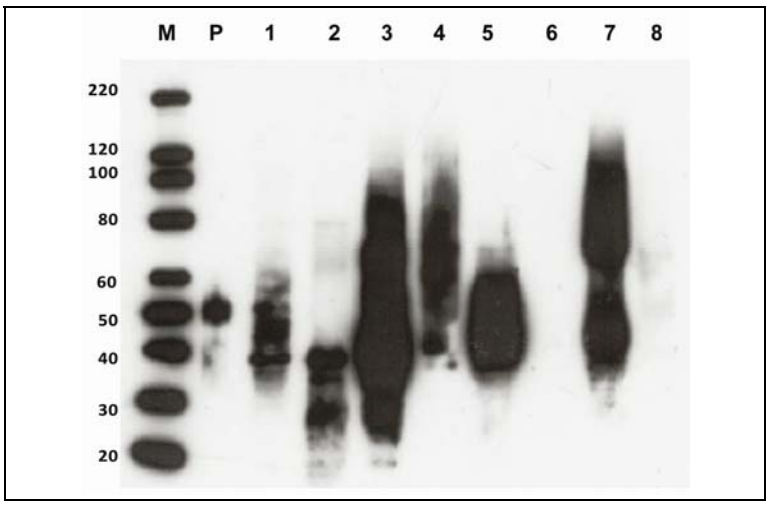


Figure 22 Western blotting analysis of His-resin purified chimeric proteins expressed in *Pichia Pastoris* probed with anti-His. Transformed *Pichia* strains were first grown in BMGY, followed by BMMY. Supernatants were separated from via centrifugation and pellets were lysed. Supernatant and soluble lysate from pellet were purified by His-resin and analyzed by western blotting and probed with a mouse monoclonal antibody against histidine. (I) P: positive control (Positope), lane 1: TM2-His from pellet, lane 2: Chimera II from pellet, lane 3: Chimera I from pellet, lane 4: EPCR-His from supernatant, lane 5: TM2-His from supernatant, lane 6: Chimera II from supernatant, lane7: Chimera I from supernatant, lane 4: EPCR-His from supernatant.

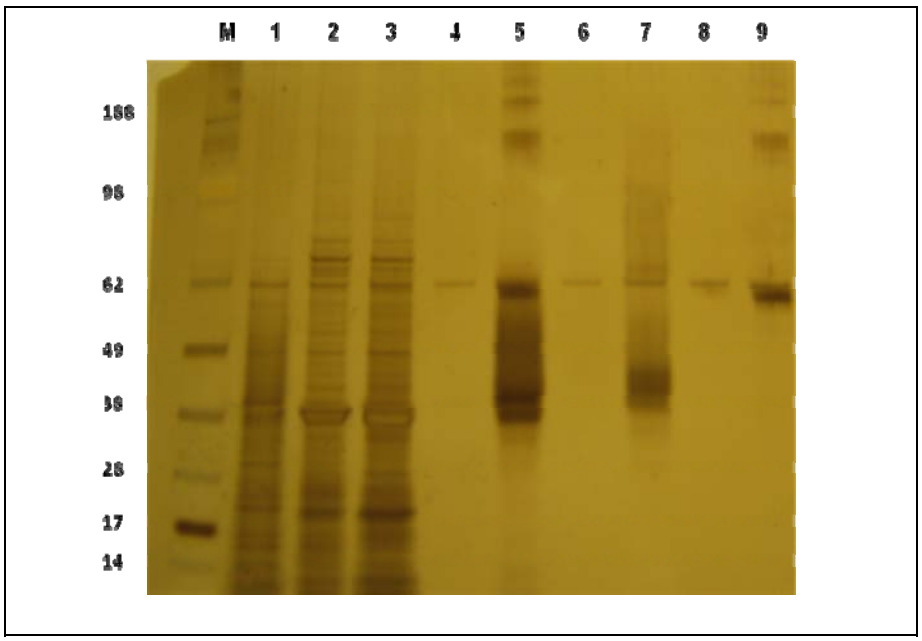


Figure 23. SDS-PAGE analysis of His-resin purified chimeric proteins expressed in *Pichia Pastoris* with silver staining. Transformed *Pichia* strains were first grown in BMGY, followed by BMMY. Supernatants were separated from via centrifugation and pellets were lysed. Supernatant and soluble lysate from pellet were purified by His-resin and analyzed by SDS-PAGE with silver staining. Lane1: TM2-His from pellet, lane 2: Chimera II from pellet, lane 3: Chimera I from pellet, lane 4: EPCR-His from desalted supernatant, lane 5: TM2-His from supernatant, lane 6: Chimera II from supernatant, lane 7: Chimera I from supernatant, lane 8: EPCR-His from desalted/lyophilized supernatant, lane 9: EPCR-His from desalted supernatant.

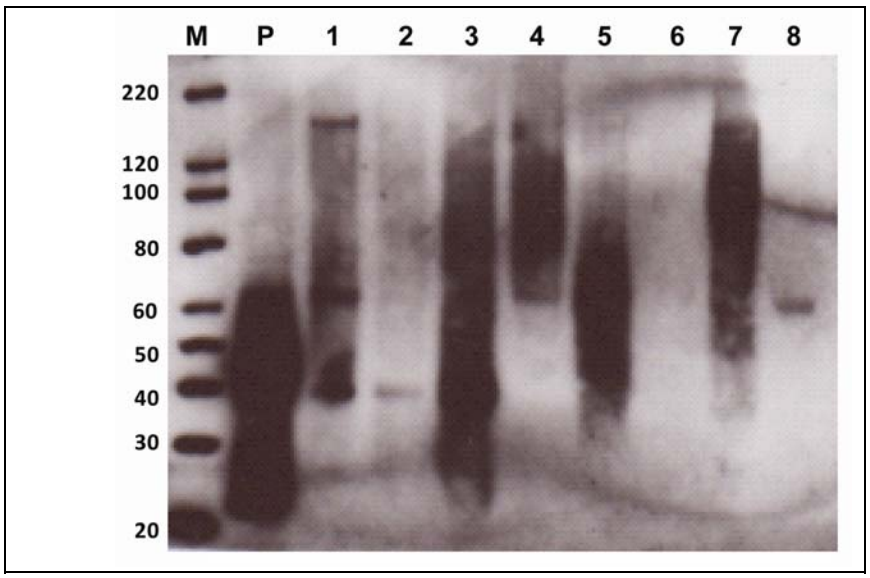


Figure 24. Western blotting analysis of His-resin purified chimeric proteins expressed in *Pichia pastoris* to check the binding ability with human protein C. Transformed *Pichia* strains were first grown in BMGY, followed by BMMY. Supernatants were separated from via centrifugation and pellets were lysed. Supernatant and soluble lysate from pellet were purified by His-resin and analyzed by western blotting and incubated with 2ug/ml human protein C overnight at 4C. The signal was detected with a HRP-conjugated sheep anti-human protein C. (I) P: positive control (500ng human protein C), lane 1: TM2-His from pellet, lane 2: Chimera II from pellet, lane 3: Chimera I from pellet, lane 4: EPCR-His from supernatant, lane 5: TM2-His from supernatant, lane 6: Chimera I from supernatant, lane7: Chimera II from supernatant, lane 4: EPCR-His from supernatant.

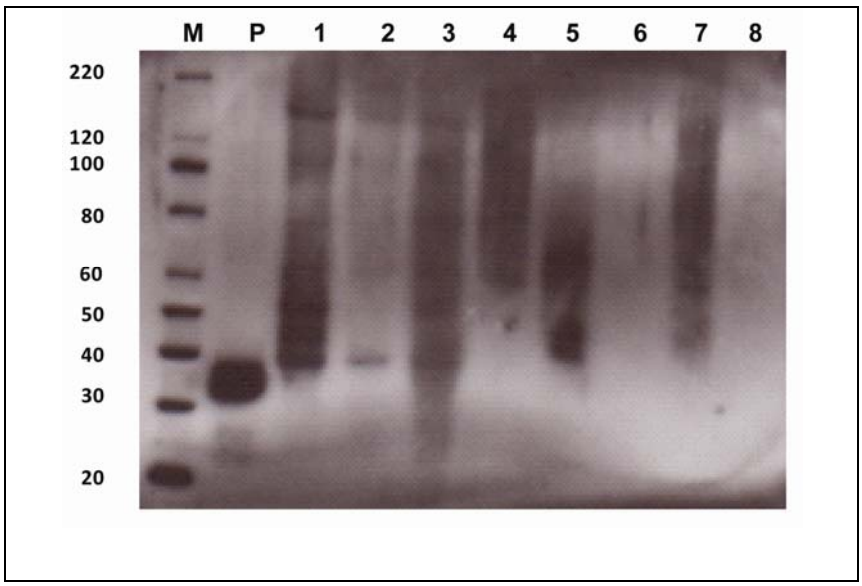


Figure 25. Western blotting analysis of His-resin purified chimeric proteins expressed in *Pichia pastoris* to check the binding ability with human α thrombin. Transformed *Pichia* strains were first grown in BMGY, followed by BMMY. Supernatants were separated from via centrifugation and pellets were lysed. Supernatant and soluble lysate from pellet were purified by His-resin and analyzed by western blotting and incubated with 2ug/ml human α thrombin overnight at 4C. The signal was detected with a HRP-conjugated sheep anti- human α thrombin. (I) P: positive control (500ng human protein C), lane 1: TM2-His from pellet, lane 2: Chimera II from pellet, lane 3: Chimera I from pellet, lane 4: EPCR-His from supernatant, lane 5: TM2-His from supernatant, lane 6: Chimera I from supernatant, lane7: Chimera II from supernatant, lane 4: EPCR-His from supernatant.

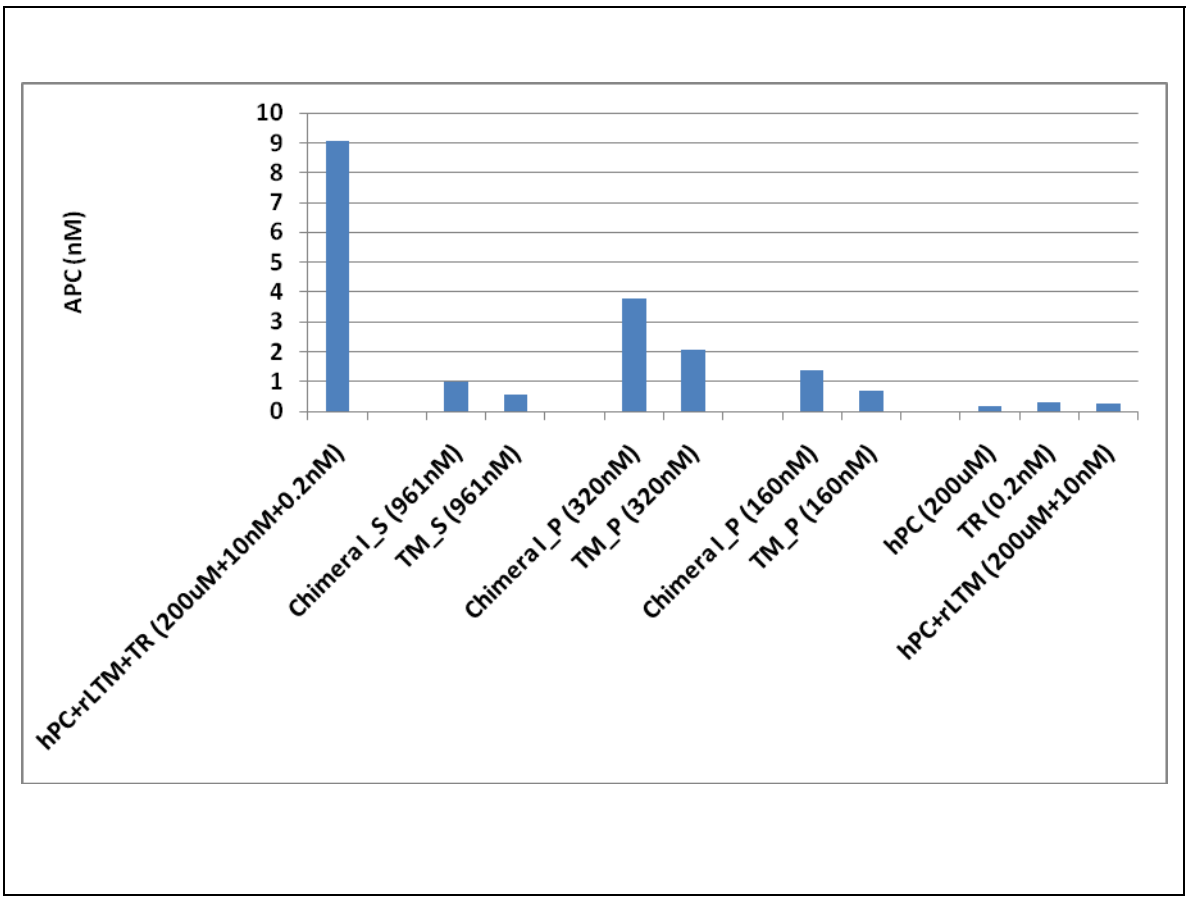


Figure 26. Solution phase Protein C activation assay of purified chimera I and TM2-His from supernatant. Protein solutions ofTM2-His, chimera I, human α thrombin, human Protein C, human activated Protein C, were prepared in reaction buffer. For chimera I, 25 µl of reaction buffer, 25 µl of a 0.05 mg/ml hPC solution, and a various concentration of partially pure chimera I from supernatant and pellet lysate were incubated for 15 minutes at room temperature in a sterile 96 well cell culture plate. At the same time, 25µl of reaction buffer and 25µl of a 0.05 mg/ml hPC solution were incubated for 15 minutes at room temperature in the same cell culture plate, followed by addition of 25 µl of various concentration of partially pure TM2-His from supernatant and pellet lysate. Finally, 25 µl 0.03 µg/ml TR was added to the reaction mixture of chimera I and TMs-His, which was incubated at 37˚C for 30 minutes. For the standard curve of activated Protein C, 100 µl of various concentration of human activated Protein C was added to the same 96 well cell culture plate. To measure the amount of activated PC from each well, 25 µl of Spectrozyme aPC was added to a final concentration of 0.0004M. The reaction was incubated at 37˚C and the absorbance was taken after 0, 30 and 60 minute at 405 nm using a Beckman Coulter AD 340 UV/Vis microplate reader. The concentration of generated APC was calculated using an APC in reaction buffer calibration curve on the same cell culture plate.

Name of chimera	Composition & Size	Positive clones
chimera I	EPCR-myc-TM2-His_6 (1395 bp)	#1, #4, #8
chimera II	EPCR-flag-2MT-His_6 (1416 bp)	#2

Table 8. Sequence result of subcloned chimera I and chimera II with TOPO vector. After amplification, gel-purified PCR products of chimera I and chimera II were subcloned into TOPO vector. The DNA plasmids were transformed into chemically competent cells of *E. coli* strain TOP-10. 10-50 µl from each transformation was spread on a pre-warmed LB plates containing 50 µg/ml kanamycin and incubated overnight at 37°C. Ten positive clones of transformants from each chimera were selected and transferred into 5ml of LB medium containing 50 µg/ml kanamycin to be cultured overnight at 37°C. Then, plasmid DNA from each chimera was isolated and was sequenced at University of Nebraska-Medical Center sequencing facility with gene specific primers. Sequence analysis was performed using ChromasPro v1.5 software.

Name	Composition	Positive clones
chimera I	EPCR-myc-TM2-His_6	#1-2, #1-4, #1-6, #1-7, #1-9
chimera II	EPCR-flag-2MT-His_6	#2-1, #2-2, #2-3, #2-4, #2-6, #2-7, #2-8, #2-9
TM2-His	2MT-His_6	#1, #2, #6, #10, #11, #13

Table 9. Cloning results of chimera I, chimera II, and TM2-His with pPICZ α A vector. Clones from chimera I, chimera II, and TM2-His were digested by Xho I and Not I for 3hours at 37˚C and then directionally ligated with pPICZ α A vector that was digested with the same enzymes. The ligated plasmid was transformed into chemically competent cells of *E. coli* strain TOP-10 according to manufacturer's protocol. 50-100 µl of each transformants were spread on a prewarmed LB plates containing 25 µg/ml Zeocin and incubated overnight at 37°C. Ten positive clones from each transformant were selected and transferred into 5ml of LB medium containing 25 µg/ml Zeocin to be cultured overnight at 37°C. Then, plasmid DNA from each chimera was isolated and sequenced at University of Nebraska-Medical Center sequencing facility with gene specific primers. Sequence analysis was performed using ChromasPro v1.5 software.

	Pellet lysate			Supernatant			
	TM2-His	Chimera I	Chimera II	TM2-His (5X)	Chimera I (5X)	Chimera II (20X)	EPCR-LL-His (5X)
anti-His	+	+	+	+	+	+	+
anti-EPCR	-	+	+	-	+	+	+
anti-TM	+	+	+	+	+	+	-
anti-myc	-	+	-	-	+	-	-
anti-flag	-	-	+	-	-	+	-

Table 10. Summary of Western blotting analysis with various antibodies to detect the functional elements and the linkers of chimeric proteins

Discussion

The goal of this study is to express genes encoding the functional elements of EPCR and TM in an attempt to generate APC in-situ. The synthetic genes encoding chimera I, chimera II, and TM2-His were successfully expressed in yeast *Pichia* host X-33 strain. However, the expression and purification of these chimeric proteins need improvement to enhance secretion of mature protein and obtain pure chimeric proteins of chimera I, chimera II and TM2-His. When *Pichia* cells carrying the gene for chimera I was grown up to 96 hours, the overexpression was observed on Western blotting analysis. To resolve this issue, chimera I was grown up to 48 hours (Figure 11). Overall, the expression level of all chimeric proteins from the pellet lysate was as high as that of chimeric proteins from the supernatant, which indicated that the secretion of mature protein into the medium was not sufficient. Secretion of mature protein is important since it allows easy and efficient purification from the extracellular medium [84]. The purpose of using pPICZ α A vector in this study is to secrete mature protein into the medium with α-factor leader sequence. The partial secretion of chimeric proteins in this study might be due to the ineffective signal cleavage at Kex2 cleavage site. To provide a hydrophilic environment at Kex2 cleavage site, the addition of $(Glu\text{-}Ala)_2$ is recommended, which will be further removed by Ste 13 dipeptidyl aminopeptidase [85, 86]. Brake et al. suggested that the repeating residue of (Glu-Ala) was not necessary for a proper processing of fusion protein hEGF, but the presence of repeating sequence of (Glu-Ala) might prevent the accumulation of intracellular active α-factor and be essential for the correct folding of fusion between α-factor leader and foreign proteins.

Along with the partial secretion of chimeric proteins, proteolytic degradation of secreted chimeric proteins has been observed in Figure 17/18/19/20/21. All crude chimeric proteins from the supernatant have multiple small fragments with a strong band at their expected molecular weight. These small fragments are caused by the inactivation of protease inhibitors in an aqueous reaction environment over a 48 hour period [87] even though 2 mM of PMSF (Phenylmethanesulphonyfluoride, protease inhibitor) in 100% methanol has been added to the cell culture at the first 12 hour induction. A large amount of oxygen consumption for methanol induction and additional methanol feeding every 12 hours contribute to the accumulation of hydrogen peroxide as a by-product inside the cell, which can cause oxidative stress on the cell to bring out more protease activity. Sinha et al. reported that proteolytic activity was observed when the carbon source switched from glycerol to methanol and reached a maximum between 60 to 72 hours induction time [88]. In this sense, the lower molecular weight fragments of chimera I protein and chimera II proteins are suspected to be a truncated form of chimeric proteins caused by the proteolytic activity. Both chimera I protein and chimera II protein show a strong band at the expected molecular weight of 78 kDa with another strong band at 40 kDa. Li et al. have observed the similar phenomena in the attempt of producing 58 and 75 kDa fusion proteins, MBP-FXa-cargo-mcy-His6 [89]. All these proteins had a band at 45 kDa corresponding molecular weight of MBP, and Li et al. suggested that the proteolysis occurred at or close to FXa or in the C-terminal MBP region. For chimera I protein and chimera II protein, the proteolysis occurred in the C-terminal EPCR region since both these proteins were detected with anti-His, anti-EPCR, ant-His, and anti-myc/anti-flag.

To reduce the photolytic degradation of secreted proteins, it is important to explore the choice of protease inhibitor with reduced induction times on methanol.

Another interesting fact is that the expression level of *Pichia* cells carrying the genes for chimera I, and chimera II, and TM2-His was different from each other (Figure 14/15/16). The expression of *Pichia* cells carrying the gene for TM2-His was more successful than others while the expression of *Pichia* cells carrying the gene for chimera II was poor. This might be due to the composition of each chimeric protein. A chimeric protein TM2-His contains a partial gene sequence (EGF 4/5/6) of the whole thrombomodulin molecule, so the expression is much easier than the expression of bifunctional proteins such as chimera I and chimera II. Protein chimera I and protein chimera II contain both EPCR (binding of Protein C and activated Protein C) and TM (binding of thrombin). The expression of *Pichia* cells carrying the gene for chimera II is even worse than that of chimera I. This might be explained by the reversed amino acid sequence of thrombomodulin in chimera II since other elements of both genes are identical except for the linker protein and the order of amino acid sequence for TM. The reversed amino acid sequence of TM is not natural and it might affect the stability of the protein construction. Another important domain in chimeric protein is an appropriate linker peptide for the multi-functional protein to work independently by keeping the spatial separation of the multi-functional domains [65]. Aral et al. introduced α-helical linker, $(EAAAK)_n$ to separate two green fluorescent protein (GFP) variants and demonstrated a good separation of the functional domains and kept them independent, without disturbing domains. The linker peptide used for chimera I and chimera II is a myc epitope and a flag epitope, respectively. These linker peptides are flexible but they

do not provide much space between EPCR and TM, so it might affect the biological function of the chimeric proteins. All these factors can contribute to the expression of chimeric proteins.

Prior to testing the biological activity of the chimeric proteins to activate Protein C, His-resin affinity purification is used for a single step purification of protein containing His-tag sequence by metal chelation chromatography. His-tag sequence binds to Ni^{2+} ion immobilized on His-bind resin in a batch mode and the results are shown in Figure 21 and Figure 22. The final products of the chimeric proteins are partially pure since every sample with silver staining has a mysterious band at 62 kDa. Especially, the result of EPCR-His protein purification is not agreeable with the literature reported previously [90]. Mamedov et al. purified the same EPCR-His protein with His-resin affinity column purification and the final product showed only one protein band at 38 kDa. These results suggest that the efficiency of column purification is better than batch mode purification since the batch mode has a limitation in complete removal of residues from each step, especially after binding and washing steps. This is probably why the final protein products after purification have a protein band at 62 kDa. In addition, purified protein from the supernatant of chimera II was not detected on both Western blot and silver staining. This could be the result of inappropriately handling protein samples along with a low expression level. After the purification, the protein sample prepared for Western blot analysis showed a sign of pH problem by turn into yellow instead of purplish blue. The sample was too acidic to run on the gel with the running buffer at 7.0, which led to an aggregation of protein sample in the gel well. This could be fixed by a series of dialysis immediately after purification to remove a residual imidazole from

elution buffer. Proteins purified from the pellet lysates show numerous protein bands on silver staining, which is occurred by a combination of a strong proteolytic activity inside the cell and degradation of protein due to inadequate protease inhibitor use. The cell lysis buffer used in this study contains protease inhibitors, 1 mM of EDTA and 2 mM of PMSF, but the combination of EDTA and PMSF is not sufficient to prevent the degradation of protein. More options for protease inhibitors need to be explored and shorten induction times to reduce the amount of vacuolar proteases and hydrogen peroxide accumulation inside the cell. In spite of all the issues, the biological activity of the final protein products was tested.

Solution phase assay was conducted to demonstrate that chimera I protein and TM2-His protein were biologically active to catalyze activation of Protein C (Figure 26). Chimera I protein and TM2-His protein activated the Protein C; however, the activity of these two proteins is much less than that of control with commercial proteins of hPC, rabbit lung TM, and thrombin in the absence of EPCR. The activity of control is ~80-fold higher than chimera I protein from pellet lysate and ~910-fold higher than chimera I protein from supernatant. The activity of control is ~180-fold higher than TM2-His protein from pellet lysate and ~1600-fold higher than TM2-His protein from supernatant. In addition, the activity of proteins from pellet lysate generated more APC than proteins from supernatant. It is possible that the protein from pellet lysate still remain the α-factor signal sequence so it might alter the protein folding process inside the cell, which might affect the function of protein. Nonetheless, the overall activity of chimera I protein is almost 2-fold higher than that of TM2-His protein. The activity ratio of chimera I and TM2-His is 1.8 at 961 nM (supernatant), 1.84 at 320 nM (pellet), and 2.0 at 160 nM

(pellet). This can be explained by the unique molecular composition of chimera I (EPCR-flag-2MT-His_6). While thrombomodulin in TM2-His protein activates Protein C only by forming a complex with a thrombin, EPCR in chimera I protein stimulates Protein C activation mediated by the thrombin/thrombomodulin complex [37]. The Gla-domain of PC/APC interacts with EPCR [33], and the Gla-domain of PC also interacts with TM and/or TR[49]. The result of PC activation assay proves our hypothesis that incorporating EPCR with TM in the chimeric protein can stimulate the activation of Protein C better than TM alone.

Conclusion

The synthetic genes encoding chimera I, chimera II, and TM2-His have been successfully expressed in the yeast, *Pichia pastoris* X-33 strain. Overall, expression level of chimera I, chimera II, and TM2-His is low, and mature proteins are partially secreted into the medium, and the final products of purified proteins were partially pure. In spite of all these issues, this study demonstrates that chimera I protein and TM2-His protein remain the biological activity to activate Protein C from the solution phase experiment. Further, this study has shown that the activity of chimera I is almost twice higher than that of TM2-His by co-expressing EPCR and TM in the chimera I protein.

Future Work

This study demonstrates the expression of genes encoding functional elements of EPCR and TM in an attempt to generate APC in-situ and the biological activity of chimera I protein to activate Protein C. However, several issues need to be resolved to improve the production of chimeric proteins. To optimize the secretion of target protein, the addition of repeated sequence of (Glu-Ala) after Kex2 cleavage site needs to be considered in the design of DNA sequence. To improve the function of multi-functional protein, the use of a helical linker between EPCR and TM might be a better choice by keeping the spatial separation of the multi-functional domains. To reduce or prevent the degradation of protein, the use of protease inhibitor cocktail is recommended for the induction and process of protein at any stage, which will affect the quality of final protein product. Once chimera I and chimera II are successfully expressed and purified, these proteins can be coated onto the surface of the biomaterial surfaces to delay the blood clot.

References

1. Peppas, N.A. and R. Langer, *New challenges in biomaterials.* Science, 1994. **263**(5154): p. 1715-20.
2. Ratner, B.D., *Blood compatibility--a perspective.* J Biomater Sci Polym Ed, 2000. **11**(11): p. 1107-19.
3. Wu B, G.B., Grinnell BW, Meyerhoff ME, *Polymeric coatings that mimic the endothelium: combining nitric oxide release with surface-bound active thrombomodulinand* 28, 2007: p. 4047-4055.
4. Xue, L. and H.P. Greisler, *Biomaterials in the development and future of vascular grafts.* J Vasc Surg, 2003. **37**(2): p. 472-80.
5. Abbott, W.M., et al., *Prosthetic above-knee femoropopliteal bypass grafting: results of a multicenter randomized prospective trial. Above-Knee Femoropopliteal Study Group.* J Vasc Surg, 1997. **25**(1): p. 19-28.
6. Kannan, R.Y., et al., *Current status of prosthetic bypass grafts: a review.* J Biomed Mater Res B Appl Biomater, 2005. **74**(1): p. 570-81.
7. Tiwari, A., et al., *Improving endothelial cell retention for single stage seeding of prosthetic grafts: use of polymer sequences of arginine-glycine-aspartate.* Eur J Vasc Endovasc Surg, 2003. **25**(4): p. 325-9.
8. Hermens, R.A., et al., *Influence of luminal pore size on the patency rate and endothelialization of polymeric microvenous prostheses.* Microsurgery, 1995. **16**(7): p. 482-7.
9. Ravi, S., Z. Qu, and E.L. Chaikof, *Polymeric materials for tissue engineering of arterial substitutes.* Vascular, 2009. **17 Suppl 1**: p. S45-54.
10. Ottani, V., M. Raspanti, and A. Ruggeri, *Collagen structure and functional implications.* Micron, 2001. **32**(3): p. 251-60.
11. Cummings, C.L., et al., *Properties of engineered vascular constructs made from collagen, fibrin, and collagen-fibrin mixtures.* Biomaterials, 2004. **25**(17): p. 3699-706.
12. Ye, Q., et al., *Fibrin gel as a three dimensional matrix in cardiovascular tissue engineering.* Eur J Cardiothorac Surg, 2000. **17**(5): p. 587-91.
13. Swartz, D.D., J.A. Russell, and S.T. Andreadis, *Engineering of fibrin-based functional and implantable small-diameter blood vessels.* Am J Physiol Heart Circ Physiol, 2005. **288**(3): p. H1451-60.
14. Brash, J.L., *Exploiting the current paradigm of blood-material interactions for the rational design of blood-compatible materials.* J Biomater Sci Polym Ed, 2000. **11**(11): p. 1135-46.
15. Ishihara, K., et al., *Inhibition of fibroblast cell adhesion on substrate by coating with 2-methacryloyloxyethyl phosphorylcholine polymers.* J Biomater Sci Polym Ed, 1999. **10**(10): p. 1047-61.
16. Pasche, B., et al., *Binding of antithrombin to immobilized heparin under varying flow conditions.* Artif Organs, 1991. **15**(6): p. 481-91.

17. Gorbet, M.B. and M.V. Sefton, *Biomaterial-associated thrombosis: roles of coagulation factors, complement, platelets and leukocytes.* Biomaterials, 2004. **25**(26): p. 5681-703.
18. Hanson, S., *Device thrombosis and thromboembolism.* Cardiovasc Pathology, 1993. **2**: p. 157S-165S.
19. Mackman, N., R.E. Tilley, and N.S. Key, *Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis.* Arterioscler Thromb Vasc Biol, 2007. **27**(8): p. 1687-93.
20. Gailani, D. and T. Renne, *Intrinsic pathway of coagulation and arterial thrombosis.* Arterioscler Thromb Vasc Biol, 2007. **27**(12): p. 2507-13.
21. Ishihara, K. and Y. Iwasaki, *Reduced protein adsorption on novel phospholipid polymers.* J Biomater Appl, 1998. **13**(2): p. 111-27.
22. Tseng, P.Y., et al., *Membrane-mimetic films containing thrombomodulin and heparin inhibit tissue factor-induced thrombin generation in a flow model.* Biomaterials, 2006. **27**(12): p. 2637-50.
23. Wu, B., et al., *Polymeric coatings that mimic the endothelium: combining nitric oxide release with surface-bound active thrombomodulin and heparin.* Biomaterials, 2007. **28**(28): p. 4047-55.
24. Ebert, C.D., E.S. Lee, and S.W. Kim, *The antiplatelet activity of immobilized prostacyclin.* J Biomed Mater Res, 1982. **16**(5): p. 629-38.
25. Taite, L.J., et al., *Nitric oxide-releasing polyurethane-PEG copolymer containing the YIGSR peptide promotes endothelialization with decreased platelet adhesion.* J Biomed Mater Res B Appl Biomater, 2008. **84**(1): p. 108-16.
26. Gutowska, A., et al., *Heparin release from thermosensitive polymer coatings: in vivo studies.* J Biomed Mater Res, 1995. **29**(7): p. 811-21.
27. Di Cera, E., *Thrombin.* Mol Aspects Med, 2008. **29**(4): p. 203-54.
28. Li, J.M., et al., *Immobilization of human thrombomodulin to expanded polytetrafluoroethylene.* J Surg Res, 2002. **105**(2): p. 200-8.
29. Esmon, C.T., *The roles of protein C and thrombomodulin in the regulation of blood coagulation.* J Biol Chem, 1989. **264**(9): p. 4743-6.
30. Tseng, P.Y., et al., *Catalytic efficiency of a thrombomodulin-functionalized membrane-mimetic film in a flow model.* Biomaterials, 2006. **27**(13): p. 2768-75.
31. Robert W, C., Victor J. Marder, Alexander W. Clowes, James N. George, Samuel Z. Goldhaber, *Hemostasis and Thrombosis: Basin Principles and Clinical Practice.* 2006, Lippincott Williams & Wilkins
32. Dahlback, B. and B.O. Villoutreix, *Regulation of blood coagulation by the protein C anticoagulant pathway: novel insights into structure-function relationships and molecular recognition.* Arterioscler Thromb Vasc Biol, 2005. **25**(7): p. 1311-20.
33. Esmon, C.T., et al., *The protein C pathway: new insights.* Thromb Haemost, 1997. **78**(1): p. 70-4.
34. Esmon, C.T., J. Stenflo, and J.W. Suttie, *A new vitamin K-dependent protein. A phospholipid-binding zymogen of a serine esterase.* J Biol Chem, 1976. **251**(10): p. 3052-6.
35. Kisiel and . *Human Plasma Protein C: isolation, characterization and activation by human thrombin.* J Clin Invest, 1979. **64**: p. 761

36. Stenflo, J., *A new vitamin K-dependent protein. Purification from bovine plasma and preliminary characterization.* J Biol Chem, 1976. **251**(2): p. 355-63.
37. Fukudome, K., et al., *Activation mechanism of anticoagulant protein C in large blood vessels involving the endothelial cell protein C receptor.* J Exp Med, 1998. **187**(7): p. 1029-35.
38. Marconi, W., A. Piozzi, and D. Romoli, *Preparation and evaluation of polyurethane surfaces containing immobilized plasminogen.* J Biomater Sci Polym Ed, 1996. **8**(4): p. 237-49.
39. Esmon, C.T. and W.G. Owen, *The discovery of thrombomodulin.* J Thromb Haemost, 2004. **2**(2): p. 209-13.
40. Weisel, J.W., et al., *The shape of thrombomodulin and interactions with thrombin as determined by electron microscopy.* J Biol Chem, 1996. **271**(49): p. 31485-90.
41. Kurosawa, S., et al., *A 10-kDa cyanogen bromide fragment from the epidermal growth factor homology domain of rabbit thrombomodulin contains the primary thrombin binding site.* J Biol Chem, 1988. **263**(13): p. 5993-6.
42. Owen, W.G. and C.T. Esmon, *Functional properties of an endothelial cell cofactor for thrombin-catalyzed activation of protein C.* J Biol Chem, 1981. **256**(11): p. 5532-5.
43. Fukudome, K. and C.T. Esmon, *Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor.* J Biol Chem, 1994. **269**(42): p. 26486-91.
44. Fukudome, K. and C.T. Esmon, *Molecular-Cloning and Expression of Murine and Bovine Endothelial-Cell Protein C/Activated Protein-C Receptor (Epcr) - the Structural and Functional Conservation in Human, Bovine, and Murine Epcr.* Journal of Biological Chemistry, 1995. **270**(10): p. 5571-5577.
45. Kurosawa, *Identification of functional endothelial cell protein C receptor in hman plasma.* J Clin Invest, 1997. **100**: p. 411-418.
46. Simmonds, R.E. and D.A. Lane, *Structural and functional implications of the intron/exon organization of the human endothelial cell protein C/activated protein C receptor (EPCR) gene: comparison with the structure of CD1/major histocompatibility complex alpha1 and alpha2 domains.* Blood, 1999. **94**(2): p. 632-41.
47. Regan, L.M., et al., *The interaction between the endothelial cell protein C receptor and protein C is dictated by the gamma-carboxyglutamic acid domain of protein C.* J Biol Chem, 1997. **272**(42): p. 26279-84.
48. Oganesyan, V., et al., *The crystal structure of the endothelial protein C receptor and a bound phospholipid.* J Biol Chem, 2002. **277**(28): p. 24851-4.
49. Olsen, P.H., et al., *Ca2+ dependence of the interactions between protein C, thrombin, and the elastase fragment of thrombomodulin. Analysis by ultracentrifugation.* Biochemistry, 1992. **31**(3): p. 746-54.
50. Shin, Y.H., et al., *Polymer-coated tips for patterning of viruses by dip-pen nanolithography.* Angew Chem Int Ed Engl, 2010. **49**(50): p. 9689-92.
51. Nyamjav, D., S. Rozhok, and R.C. Holz, *Immobilization of motile bacterial cells via dip-pen nanolithography.* Nanotechnology, 2010. **21**(23): p. 235105.

52. Piner, R.D., et al., *"Dip-Pen" nanolithography.* Science, 1999. **283**(5402): p. 661-3.
53. Salaita, K., Y. Wang, and C.A. Mirkin, *Applications of dip-pen nanolithography.* Nat Nanotechnol, 2007. **2**(3): p. 145-55.
54. Stern, E., D.J. Mooney, and T.M. Fahmy, *A biomimetic approach for the creation of two-dimensional microscale surface patterns: creation of isolated immunological synapses.* Int J Biomater, 2009. **2009**: p. 821308.
55. You, C., et al., *Affinity capturing for targeting proteins into micro and nanostructures.* Anal Bioanal Chem, 2009. **393**(6-7): p. 1563-70.
56. Lu, R.L., et al., *The active site of the thrombin-thrombomodulin complex. A fluorescence energy transfer measurement of its distance above the membrane surface.* J Biol Chem, 1989. **264**(22): p. 12956-62.
57. Adams, T.E., et al., *The crystal structure of activated protein C-inactivated bovine factor Va: Implications for cofactor function.* Proc Natl Acad Sci U S A, 2004. **101**(24): p. 8918-23.
58. Yegneswaran, S., et al., *Protein S alters the active site location of activated protein C above the membrane surface. A fluorescence resonance energy transfer study of topography.* J Biol Chem, 1997. **272**(40): p. 25013-21.
59. Kador, K.E., et al., *Sequential Co-immobilization of Thrombomodulin and Endothelial Protein C Receptor on Polyurethane: Activation of Protein C.* Acta Biomater, 2011.
60. Kador, K.E. and A. Subramanian, *Surface Modification of Biomedical Grade Polyurethane to Enable the Ordered Co-immobilization of Two Proteins.* J Biomater Sci Polym Ed, 2010.
61. Perez-Casal, M., et al., *Activated protein C induces the release of microparticle-associated endothelial protein C receptor.* Blood, 2005. **105**(4): p. 1515-22.
62. Goeddel, D.V., et al., *Expression in Escherichia coli of chemically synthesized genes for human insulin.* Proc Natl Acad Sci U S A, 1979. **76**(1): p. 106-10.
63. Wriggers, W., S. Chakravarty, and P.A. Jennings, *Control of protein functional dynamics by peptide linkers.* Biopolymers, 2005. **80**(6): p. 736-46.
64. Hayward, S., *Structural principles governing domain motions in proteins.* Proteins, 1999. **36**(4): p. 425-35.
65. Arai, R., et al., *Conformations of variably linked chimeric proteins evaluated by synchrotron X-ray small-angle scattering.* Proteins, 2004. **57**(4): p. 829-38.
66. Fantinato Sonia, P.L., Pilone S. Mirella, *Engineering, expression and purification of a His-tagged chimeric D-amino acid oxidase from Rhodotorula gracilis.* Enzyme and Microbial Technology, 2001. **29**: p. 407-412.
67. Hu, Y.C., *Generation of chimeric baculovirus with histidine-tag displayed on the envelope and its purification using immobilized metal affinity chromatography.* Enzyme and Microbial Technology, 2003. **33**: p. 445-452.
68. Andersen, D.C. and L. Krummen, *Recombinant protein expression for therapeutic applications.* Curr Opin Biotechnol, 2002. **13**(2): p. 117-23.
69. Gerngross, T.U., *Advances in the production of human therapeutic proteins in yeasts and filamentous fungi.* Nat Biotechnol, 2004. **22**(11): p. 1409-14.

70. Chu, L. and D.K. Robinson, *Industrial choices for protein production by large-scale cell culture.* Curr Opin Biotechnol, 2001. **12**(2): p. 180-7.
71. Daly, R. and M.T. Hearn, *Expression of heterologous proteins in Pichia pastoris: a useful experimental tool in protein engineering and production.* J Mol Recognit, 2005. **18**(2): p. 119-38.
72. Makrides, S.C., *Strategies for achieving high-level expression of genes in Escherichia coli.* Microbiol Rev, 1996. **60**(3): p. 512-38.
73. Coloma, M.J., et al., *The role of carbohydrate in the assembly and function of polymeric IgG.* Mol Immunol, 2000. **37**(18): p. 1081-90.
74. Humphreys, B., *Cholesterol drugs dominate.* MedAdNews, 2003. **22**: p. 42-57.
75. Grabenhorst, E., et al., *Genetic engineering of recombinant glycoproteins and the glycosylation pathway in mammalian host cells.* Glycoconj J, 1999. **16**(2): p. 81-97.
76. Grabowski, G.A., et al., *Enzyme therapy in type 1 Gaucher disease: comparative efficacy of mannose-terminated glucocerebrosidase from natural and recombinant sources.* Ann Intern Med, 1995. **122**(1): p. 33-9.
77. Pandhal, J. and P.C. Wright, *N-Linked glycoengineering for human therapeutic proteins in bacteria.* Biotechnol Lett, 2010. **32**(9): p. 1189-98.
78. Inan, M. and M.M. Meagher, *Non-repressing carbon sources for alcohol oxidase (AOX1) promoter of Pichia pastoris.* J Biosci Bioeng, 2001. **92**(6): p. 585-9.
79. Lijnen, H.R., et al., *Functional properties of a recombinant chimeric protein with combined thrombin inhibitory and plasminogen-activating potential.* Eur J Biochem, 1995. **234**(1): p. 350-7.
80. Davami, F., et al., *Expression of a novel chimeric truncated t-PA in CHO cells based on in silico experiments.* J Biomed Biotechnol, 2010. **2010**: p. 108159.
81. Weaver, W.D., *The role of thrombolytic drugs in the management of myocardial infarction. Comparative clinical trials.* Eur Heart J, 1996. **17 Suppl F**: p. 9-15.
82. Lopez, M., A. Koehler, and G. Nowak, *High-level secretion of dipetarudin, a chimeric thrombin inhibitor, by Pichia pastoris.* Protein Expr Purif, 2007. **52**(1): p. 8-13.
83. Lopez, M., et al., *Cloning, purification and biochemical characterization of dipetarudin, a new chimeric thrombin inhibitor.* J Chromatogr B Analyt Technol Biomed Life Sci, 2003. **786**(1-2): p. 73-80.
84. Tan, N.S., B. Ho, and J.L. Ding, *Engineering a novel secretion signal for cross-host recombinant protein expression.* Protein Eng, 2002. **15**(4): p. 337-45.
85. Brake, A.J., et al., *Alpha-factor-directed synthesis and secretion of mature foreign proteins in Saccharomyces cerevisiae.* Proc Natl Acad Sci U S A, 1984. **81**(15): p. 4642-6.
86. Kim, T.R., et al., *High-level expression of bovine beta-lactoglobulin in Pichia pastoris and characterization of its physical properties.* Protein Eng, 1997. **10**(11): p. 1339-45.
87. Sinha, J., et al., *Causes of proteolytic degradation of secreted recombinant proteins produced in methylotrophic yeast Pichia pastoris: case study with recombinant ovine interferon-tau.* Biotechnol Bioeng, 2005. **89**(1): p. 102-12.

88. Sinha, J., et al., *Improved production of recombinant ovine interferon-tau by mut(+) strain of Pichia pastoris using an optimized methanol feed profile.* Biotechnol Prog, 2003. **19**(3): p. 794-802.
89. Li, Z., et al., *Secretion and proteolysis of heterologous proteins fused to the Escherichia coli maltose binding protein in Pichia pastoris.* Protein Expr Purif, 2010. **72**(1): p. 113-24.
90. Mamedov TG, S.M., Pattanayak A, Subramanian A, *Human Protein C binding affinities of Endothelial Protein C Receptor (EPCR) and its Variants upon Surface Immobilization.* Applied Biochemistry and Biotechnology 2010. **Submitted**.

Appendices

1. Chimera I Design

a t c t c g a g a a a a g a F C S Q D A S D G L Q R L H M L Q I S Y F R D P Y H V W Y Q G N A S L G G H L T H V L E G P D T N T T I I Q L Q P L Q E P E S W A R T Q S G L Q S Y L L Q F H G L V R L V H Q E R T L A F P L T I R C F L G C E L P P E G S R A H V F F E V A V N G S S F V S F R P E R A L W Q A D T Q V T S G V V T F T L Q Q L N A Y N R T R Y E L R E F L E D T C V Q Y V Q K H I S A E N T K G S Q T S R S Y T S E Q K L I S E E D L N S A V D C S V E N G G C E H A C N A I P G A P R C Q C P A G A A L Q A D G R S C T A S A T Q S C N D L C E H F C V P N P D Q P G S Y S C **Met** C E T G Y R L A A D Q H R C E D V D D C I L E P S P C P Q R C V N T Q G G F E C H C Y P N Y D L V D G E C V E P V D P C F R A N C E Y Q C Q P L N Q T S Y L C V C A E G F A P I P H E P H R C Q **Met** F C N Q T A C P A D C D P N T Q A S C E C P E G Y I L D D G F I C T D I D E C E N G G F C S G V C H N L P G T F E C I C G P D S A L V R H I G T D C D S G K H H H H H H Stop g c g g c c g c t a

---

Lower-case: nucleotides	Upper Case: Amino Acids
Black- added sequence	Red- EPCR mature peptide
Green- Flag epitope	Blue- TM-2 (Asp^{226} to Lys^{466}) normal
Orange- His 6 tag	Underline-Xho1 and Not1 sites

2. Chimera I Starting original nucleotide sequence

ATCTCGAGAAAAGATTTTGTAGTCAAGATGCTTCTGATGGTCTACAAAGATTG
CATATGTTGCAAATCTCTTACTTTCGTGATCCTTATCATGTTTGGTACCAAGGT
AATGCTTCTTTGGGTGGACATTTGACTCATGTTTTGGAAGGTCCAGATACTAA
TACTACTATCATTCAATTGCAACCTTTGCAAGAACCTGAAAGTTGGGCTCGTA
CTCAAAGTGGTTTGCAATCTTACTTGTTACAATTTCATGGTCTAGTTCGTTTGG
TTCATCAAGAACGTACTTTGGCTTTTCCTTTGACTATCCGTTGTTTTTTGGGTT
GTGAATTGCCTCCTGAAGGTTCTAGAGCTCATGTTTTTTTTGAAGTTGCTGTTA
ATGGTAGTTCTTTTGTTAGTTTTCGTCCAGAAAGAGCTTTGTGGCAAGCTGAT
ACTCAAGTCACCTCTGGAGTTGTCACCTTTACCTTGCAACAATTGAATGCTTA
CAATCGTACTCGTTATGAATTACGTGAATTTCTTGAAGATACTTGTGTTCAAT
ATGTTCAAAAACATATTTCTGCTGAAAATACTAAAGGTAGTCAAACTAGTCG
TTCTTACACTTCTGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCC
GTCGACTGTTCTGTTGAAAATGGTGGTTGTGAACATGCTTGTAATGCTATTCC
AGGTGCTCCAAGATGTCAATGTCCAGCTGGTGCTGCTTTGCAAGCTGATGGTA
GATCTTGTACTGCTTCTGCTACTCAATCTTGTAATGATTTGTGTGAACATTTCT
GTGTTCCAAATCCAGATCAACCAGGTTCTTATTCTTGTATGTGTGAAACTGGT
TATAGATTGGCTGCTGATCAACATAGATGTGAAGATGTTGATGATTGTATTTT
GGAACCATCTCCATGTCCACAAAGATGTGTTAATACTCAAGGTGGTTTCGAAT
GTCATTGTTATCCAAATTATGATTTGGTTGATGGTGAATGTGTTGAACCAGTT
GATCCATGTTTCAGAGCTAATTGTGAATATCAATGTCAACCATTGAATCAAAC
TTCTTATTTGTGTGTTTGTGCTGAAGGTTTCGCTCCAATTCCACATGAACCACA
TAGATGTCAAATGTTCTGTAATCAAACTGCTTGTCCAGCTGATTGTGATCCAA
ATACTCAAGCTTCTTGTGAATGTCCAGAAGGTTATATTTTGGATGATGGTTTC
ATTTGTACTGATATTGATGAATGTGAAAATGGTGGTTTCTGTTCTGGTGTTTG
TCATAATTTGCCAGGTACTTTCGAATGTATTTGTGGTCCAGATTCTGCTTTGGT
TAGACATATTGGTACTGATTGTGATTCTGGTAAGCATCATCACCATCACCACT
AAGCGGCCGCTA

3. Chimera II Design

a t c t c g a g a a a a a g a F C S Q D A S D G L Q R L H **M** L Q I S Y F R D P Y
H V W Y Q G N A S L G G H L T H V L E G P D T N T T I I Q L Q P L Q E P
E S W A R T Q S G L Q S Y L L Q F H G L V R L V H Q E R T L A F P L T I
R C F L G C E L P P E G S R A H V F F E V A V N G S S F V S F R P E R A L
W Q A D T Q V T S G V V T F T L Q Q L N A Y N R T R Y E L R E F L E D
T C V Q Y V Q K H I S A E N T K G S Q T S R S Y T S K K R K K D Y K D
D D D K K R K R K C D T G I H R V L A S D P G C I C E F T G P L N H C V
G S C F G G N E C E D I D T C I F G D D L I Y G E P C E C S A Q T N P D C
D A P C A T Q N C F M Q C R H P E H P I P A F G E A C V C L Y S T Q N L
P Q C Q Y E C N A R F C P D V P E V C E G D V L D Y N P Y C H C E F G
G Q T N V C R Q P C P S P E L I C D D V D E C R H Q D A A L R Y G T E C
M C S Y S G P Q D P N P V C F H E C L D N C S Q T A S A T C S R G D A Q
L A A G A P C Q C R P A G P I A N C A H E C G G N E V S C D W A R K K
K R H H H H H H Stop g c g g c c g c t a

Lower-case: nucleotides	Upper Case: Amino Acids
Black- added sequence	Red- EPCR mature peptide
Green- Flag epitope	Blue- TM-2 (Ala^{224} to Cys^{462}) flipped
Orange- His 6 tag	Underline-Xho1 and Not1 sites

4. Chimera II Starting original nucleotide sequence

ATCTCGAGAAAAGATTCTGCTCTCAAGATGCTTCTGATGGATTGCAAAGATTG
CATATGTTGCAAATTTCTTACTTCAGAGATCCTTACCACGTTTGGTACCAGGG
TAACGCTTCTTTGGGTGGACATTTGACTCACGTTTTGGAGGGTCCTGATACTA
ACACTACTATCATTCAATTGCAACCTTTGCAGGAACCTGAATCTTGGGCCAGA
ACCCAATCTGGATTGCAGTCTTACTTGTTGCAGTTTCATGGTTTGGTTAGATT
GGTTCACCAGGAGAGAACTTTGGCCTTTCCTTTGACCATTAGATGTTTCTTGG
GATGTGAATTGCCTCCAGAAGGTTCTAGAGCTCATGTTTTTTTCGAAGTTGCT
GTTAACGGTTCTTCTTTCGTTTCTTTCAGACCTGAGAGAGCCTTGTGGCAGGC
TGATACTCAGGTTACCTCTGGAGTTGTTACTTTCACCTTGCAACAGTTGAACG
CTTATAACAGAACCAGATATGAATTGAGAGAATTCTTGGAAGATACTTGTGT
TCAGTATGTTCAAAAGCACATTTCTGCCGAAAACACCAAGGGTTCTCAAACTT
CTAGATCTTACACCTCTAAGAAAAGAAAGAAGGATTACAAAGACGACGACG
ATAAGAAGAGAAAGAGAAAATGTGACACTGGTATCCACAGAGTTTTGGCTTC
TGACCCTGGATGCATTTGTGAGTTTACTGGACCTTTGAACCACTGTGTTGGTT
CTTGTTTTGGTGGTAACGAGTGTGAGGACATTGACACTTGCATTTTTGGAGAC
GATTTGATTTATGGTGAGCCATGCGAATGTTCTGCCCAAACTAACCCTGATTG
CGACGCCCCATGTGCCACTCAAAACTGTTTCATGCAGTGTAGACACCCTGAA
CATCCTATTCCAGCTTTCGGTGAAGCTTGCGTTTGTTTGTATTCTACTCAGAAC
TTGCCACAGTGCCAGTACGAGTGTAACGCTAGATTTTGCCCTGACGTTCCTGA
AGTTTGTGAGGGTGATGTTTTGGACTACAACCCATACTGTCATTGCGAATTCG
GTGGACAGACTAACGTTTGCAGACAACCTTGCCCTTCTCCAGAATTGATCTGT
GATGACGTTGATGAATGCAGACACCAGGATGCCGCCTTGAGATATGGAACCG
AATGTATGTGTTCTTATTCTGGTCCTCAAGATCCTAACCCAGTTTGTTTCCATG
AGTGCTTGGACAATTGTTCTCAGACCGCTTCTGCCACCTGTTCTAGAGGAGAT
GCCCAGTTGGCTGCTGGTGCTCCTTGCCAATGTAGACCTGCTGGTCCAATTGC
TAACTGTGCTCACGAATGTGGAGGTAATGAGGTTTCTTGTGACTGGGCTAGA
AAGAAGAAGAGACATCATCACCATCACCACTAAGCGGCCGCTA

5. **TM2-His Design**

ATCTCGAGAAAAGA A W D C S V E N G G C E H A C N A I P G A P R C Q C P A
G A A L Q A D G R S C T A S A T Q S C N D L C E H F C V P N P D Q P G S Y S C
M C E T G Y R L A A D Q H R C E D V D D C I L E P S P C P Q R C V N T Q G G F
E C H C Y P N Y D L V D G E C V E P V D P C F R A N C E Y Q C Q P L N Q T S Y
L C V C A E G F A P I P H E P H R C Q **M** F C N Q T A C P A D C D P N T Q A S C
E C P E G Y I L D D G F I C T D I D E C E N G G F C S G V C H N L P G T F E C I C
G P D S A L V R H I G T D C CATCATCACCATCACCACTAAGCGGCCGCTA

Blue-TM-2 (Ala224 to Cys462)

Underline-Xho I and Not I sites

Orange-His 6 tag DNA sequence

6. **Chimera II Starting original nucleotide sequence**

ATCTC-GAG, AAA, AGA

GCTTGGGATTGCTCTGTCGAAAATGGAGGTTGTGAACATGCTTGTAATGCCAT
CCCAGGTGCCCCAAGATGCCAGTGTCCAGCAGGAGCTGCCTTGCAAGCTGAT
GGTAGATCTTGTACCGCATCTGCAACTCAGTCCTGTAATGATTTGTGCGAGCA
CTTCTGTGTTCCTAACCCAGATCAACCTGGATCCTACTCTTGTATGTGTGAGA
CAGGTTACAGATTGGCTGCTGATCAACATAGATGTGAAGATGTTGACGACTG
TATTTTGGAACCTTCTCCATGTCCACAAAGATGTGTCAACACTCAAGGTGGTT
TTGAGTGTCATTGCTATCCAAATTACGATTTGGTTGATGGAGAGTGCGTCGAA
CCAGTTGACCCATGTTTCAGAGCTAATTGTGAGTACCAGTGCCAGCCATTGAA
TCAAACTTCCTATTTGTGTGTCTGCGCCGAAGGTTTTGCCCCAATCCCTCATG
AACCTCACAGATGTCAGATGTTTTGTAATCAGACTGCCTGTCCAGCCGACTGC
GACCCAAATACTCAGGCCTCCTGTGAATGTCCAGAAGGATACATTTTGGATG
ATGGTTTTATTTGTACAGACATTGATGAGTGTGAAAACGGTGGATTTTGTTCC
GGTGTCTGTCACAACTTGCCTGGTACTTTCGAATGCATTTGCGGACCTGATTC
CGCTTTGGTCAGACACATCGGAACTGACTGT

CAT, CAT, CAC, CAT, CAC, CAC, TAA, GCG-GCCGCTA