Engineering of a Novel Inhibitor of Factor XIa with Better Stability and Inhibitory Efficiency

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Abstract Thrombosis is the cause of many cardiovascular diseases, which accounts for 29.2% of deaths in 2018 locally [1]. Current treatment options for it include heparin, warfarin and new oral factor Xa or thrombin inhibitors. However, prevalent limitations have been associated with these drugs, with the most significant being excessive bleeding. This study seeks to target Factor XIa (FXIa), an enzyme in the intrinsic pathway of coagulation. Inhibitors of FXIa have shown to effectively prevent pathological thrombus formation, without increasing bleeding risks in patients [2]. P1395, a putative FXIa inhibiting 77-residue peptide sequence, has been identified from the salivary gland of deer tick [3]. In its wildtype (WT) form, P1395 is able to inhibit FXIa at sub-nanomolar level. Still, its development in therapeutics is severely limited due to two inherent issues. First, the presence of seven cysteine(C) residues results in a free C with a free reactive thiol group after formation of disulfide bonds, reducing the stability of the peptide and increases the possibility of alternative disulfide pairings which are undesirable. Consequently, C was mutated to serine. An enzymatic assay analysis showed that P1395 mutant possesses similar activity to P1395 WT. Therefore, we have successfully eliminated the reactive thiol group to improve the stability of P1395 to be further developed as a drug candidate. Second, P1395 WT folds into a monomeric domain capable of binding to a single FXIa active site, which is a dimer. Given that two P1395 WT molecules are required for complete inhibition of one FXIa molecule, the overall inhibition efficiency of P1395 monomer is halved. This study sought to utilise the free thiol to engineer a dimeric P1395 linked through a peptide linker to improve inhibitory efficiency. However, in our preliminary assessment, the P1395 and peptide linker did not conjugate. Further optimisations of the conjugation are to be conducted.

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H. Guo et al. (eds.), *IRC-SET 2020*,
https://doi.org/10.1007/978-981-15-9472-4_13

Keywords Anticoagulant · Factor XIa · SDS-PAGE · Gene cloning · Protein synthesis · LC-MS · HPLC · Enzymatic assay

1 Introduction

A. *Excessive Blood Coagulation Predicament*

Blood coagulation is an imperative process in averting excessive bleeding upon the rupture of endothelium of blood vessels. Clots typically materialise in veins and arteries, which constitute the circulatory system of mammalian bodies. Given the necessity of blood coagulation systems for survival, the system is highly conserved in most species of Mammalia organisms.

Excessive blood clotting, or thrombophilia, could nevertheless occur, resulting in severely restricted blood flow. Vascular injury due to coagulation is a normal physiological response to imbalance in coagulation, which emanates from pathological clot formation, otherwise known as thrombosis. Thrombophilia has ensued an array of disorders, which comprise of deep vein clotting. The primary distinctive symptoms of deep vein clotting are swelling, throbbing pain and skin decolourisation. When blood flow is obstructed in arteries, tissues such as the heart and brain have a reduced blood supply, leading to a heart attack or stroke. It was reported that, in 2018, 17 individuals died per day from cardiovascular diseases in Singapore alone, accounting for 29.2% of all deaths in that year [1].

B. *Anticoagulants*

Anticoagulants are chemical substances developed to reduce coagulation of blood in order to prolong clotting time. Elongation of clotting time could eminently impede the progression of various diseases, such as myocardial infarction [4] and coronary heart disease [5], consequently saving abundant lives. It is, hence, notable that the capability of anticoagulation mechanisms to suppress clotting factors is indispensable to the survival of humans.

Numerous naturally-occurring anticoagulants are present within blood-sucking animals such as leeches, mosquitoes and ticks. These anticoagulants equip these animals to keep the bite area unclotted for an extended period, therefore allowing the animal to feed continuously. Thrombin, in particular, plays a vital role in blood coagulation by promoting platelet aggregation and by converting fibrinogen to form the fibrin clot in the final step of the coagulation cascade. To date, hirudin is the one and only thrombin inhibitor adopted in clinical agent development. Hirudin was originally isolated from the salivary glands of the medical leech *Hirudo medicinalis* [6].

Given the potency of anticoagulants in resolving multitudes of cardiovascular diseases, there exists an urgent need to develop new anticoagulants, particularly those with high potency and specificity yet minimal bleeding risk.

C. *Factor XIa (FXIa): Prospective Anticoagulant Drug Target*

In this project, we aim to target factor XIa (FXIa), a keenly pursued anticoagulant drug target. It has been shown to effectively prevent pathological thrombus formation, without significantly increasing bleeding risks in patients. P1395, a putative FXIa inhibiting 77-residue peptide sequence, has been identified from the salivary gland of the black-legged tick Ixodes scapularis [3]. *Even though it is able to inhibit FXIa at sub-nanomolar concentrations, several issues hindered the prospect of developing the peptide into a therapeutics. Firstly, the presence of seven cysteine (C) residues resulted in one free cysteine after the formation of three disulfide bridges. The free cysteine results in the presence of a reactive free thiol group, which significantly reduces the stability of the peptide and complicates purification process. It also results in alternative disulfide pairings during folding of the peptide which are undesirable. Hence, a sequence was purchased with the Cys73 mutated into Ser73. Another issue present with P1395 Wild Type (WT) protein is that it folds into a single, monomeric domain capable of binding to a single FXIa active site. However, FXIa is a dimer, thus two P1395 molecules are needed for complete inhibition of every one FXIa molecule, reducing overall efficiency of the inhibition by P1395. Thus, in this study, an attempt was made to evaluate the thiol mutant as well as engineer a dimeric P1395 linked through a peptide linker.*

2 Primary Research Objectives

This study primarily seeks to analytically deduce the best possible efficiency of the bivalent P1395 inhibitor by varying the lengths of protein involved in the formation of this bivalent inhibitor. The Cys73 to Ser73 mutation performed was to increase P1395's stability. This study primarily obtained P1395 from the salivary gland of *Ixodes scapularis*, an inhibitor of factor XIa utilised to prevent blood coagulation. The possibility of P1395 being used as a putative inhibitor with heightened efficiency and fewer harmful side effects was evaluated.

3 Hypotheses

Given the similarity in size between Serine and Cysteine, the hypothesis for Part One is that Serine can replace Cys73 without affecting the overall folding, structure and activity of P1395.

In addition, peptide linkers of different lengths can react with P1395 through the free thiol to form dimers, which can subsequently be assayed to select for the most efficient inhibitor of FXIa.

4 Procedure

A. *Part One: Cloning of P1395*

Cloning of P1395 WT and P1395 mutant (M) were performed by members of our host laboratory.

B. *Part One: Recombinant Expression of P1395 WT and Mutant In Bacterial*

Firstly, the recombinant AVA0421 plasmid containing the P1395 Wild Type(WT) gene was transformed into SHuffle® T7 Competent *E.coli* cells (New England Biolabs) following the manufacturer's instructions and protocol. After heat-shock transformation, 950 μl of Luria broth(LB) was added to the bacteria cell and grown in a 30 °C incubator for 60 min at 200 rpm. 100 μl of the bacterial suspension was plated onto LB plate supplemented with kanamycin. This plating was repeated with 200 μL of bacterial suspension. The plates were incubated overnight at 30 °C.

Next, starter cultures consisting of the transformed SHuffle cells with 50 μg/mL kanamycin and 10 mL of LB were prepared. Thereafter, they were incubated for 18 h at 250 rpm at 30 °C.

Each starter culture was then added into a 2.8 L conical flask containing 1L of LB broth and 50 μg/mL of kanamycin. The cultures were incubated at 30°C at 250 rpm until their optical density(OD) at 600 nm was 0.6–0.8. The cultures were then induced with 0.5 μM of Isopropyl β-d-1-thiogalactopyranoside (IPTG) and further incubated for 16 h and 16 °C at 150 rpm.

The 4 l of recombinant SHuffle T7 cell culture was centrifuged for 30 min at 6000 rpm. The pellet was then resuspended in 25 ml of 1 × Phosphate-buffered saline(PBS) with 8 M urea. The solution was incubated on ice for two hours with rocking before it was sonicated for a total of 22 min, with a pulse setting of 5 s on and 5 s off. Then, the product was centrifuged for 30 min at 4 °C at 10,000 rpm. The supernatant containing the P1395WT protein was kept.

Then, metal affinity chromatography column was prepared by adding 4 ml of Ni-NTA agarose beads into the column. The beads were washed with 8 ml of milli Q water twice before 8 ml of 1XPBS with 8 M urea was added to the column. All the supernatant from the previous step was poured into the column. The column was capped and incubated for 30 min. The column was washed with the following solutions in sequence: 8 ml of 1XPBS with 8 M urea, 8 ml of 1X PBS with 160 μM NaCl and 8 ml of 1XPBS. Thereafter, the Ni-NTA beads were incubated with 1X PBS with 1 mg/mL 3C protease for 16–18 h overnight at room temperature. After that, the protein was eluted out and cleaved by 3C protease. 250 μM of Imidazole was added to the Ni-NTA agarose beads and the eluate was collected immediately. A 10X dilution of the eluent was done using 1X PBS buffer.

The sample was then filtered using a 0.45 μM pore size filter. High Performance Liquid Chromatography(HPLC) and mass spectroscopy(MS) were run. HPLC was ran with an ACE 5 C18-300 250 × 4.6 mm for eluate one and Jupiter 5u C18 300A New Column 250 × 10 mm for eluate two.

C. *Part One: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

10 µl of sample was taken after each extraction or purification step, mixed with 2.5 µl of 6 × Purple Loading Dye from NEB, and pipetted into a 12-well SDS-PAGE gel. 10 µl of Precision Plus Protein™ Standards (Dual Colour) ladder and Buffer tris-glycine Sodium dodecyl sulfate (SDS) were used. Gel electrophoresis was then run for 45 min at 150 V. After confirmation of the positive gel results, we freeze dry the proteins from the pure HPLC fractions.

D. *Part One: Enzymatic Assay*

An enzymatic assay was conducted on P1395 WT and P1395 mutant proteins.

Firstly, a reaction buffer of 50 µM Tris pH 7.4, 0.15 µM NaCl, 0.002 nM $CaCl_2$ was prepared and used to dilute the concentrations of the proteins.

Then, ten wells in a 96-well plate were loaded with different concentrations, in nM, of P1395 proteins, at 300, 90, 30, 9, 3, 0.9, 0.3, 0.09, 0.03and 0.009. Another two wells were loaded with only FXIa and S2366 and served as a control. To each well, 0.75 nM of Factor XIa(FXIa) was added. P1395 and FXIa were then incubated for 45 min. After 45 min, a substrate, S2366, of concentration 3 µM was added. Immediately, OD_{405} of each of the wells were recorded and plotted on a graph of absorbance against time. From there, the Vmax (OD/hr) numerical values and subsequent percentage of inhibition, which corresponds to the effectiveness of the inhibitor, were computed.

E. *Part Two: Synthesis Of Linker Peptides*

Different lengths of linker peptides were synthesized to bind two P1395WT monomers together, forming a dimeric inhibitor. Firstly, 0.10-Resin Swelling(HS) was adopted. Amino acids were subsequently added in the respective sequence using 0.10-Single Coupling(HS), followed by 0.10-Final deprotection(HS). Addition of Cysteine via 0.10-Single 50 °C 10 min Coupling (HS) was then performed. For L25 onwards, the addition of cysteine was performed via 0.10-Double 50 °C 10 min Coupling (HS). For the last 20 amino acids of each sequence, the amino acids were added using 0.10-Double Coupling(HS).

10 ml of Dichloromethane(DCM) was added to the resin transferred into a filter tube. The accent system was then adopted for drainage of the DCM. The process was repeated with 10 ml of methanol.

For each protein, 5 ml of cleavage cocktail was prepared using TFA, milliQ water, TIS and DODT in the ratio of 37:1:1:1. A magnetic stirrer was added to the cocktail and resin and left to spin for three hours. The system was adopted to drain the cocktail into a tube, which facilitated collecting of the desired protein.

After 3 h, 40 ml of cold diethyl ether was added for the peptide to precipitate out. It was centrifuged at 3000 rpm for 3 min, which allowed for the collection of the peptide as a pellet at the bottom. HPLC analysis using ACE C18 5µ 300 Ångstrom 4.6 × 250 µM reverse phase column and MS was then conducted using a similar mechanism as above, keeping the relevant HPLC fractions.

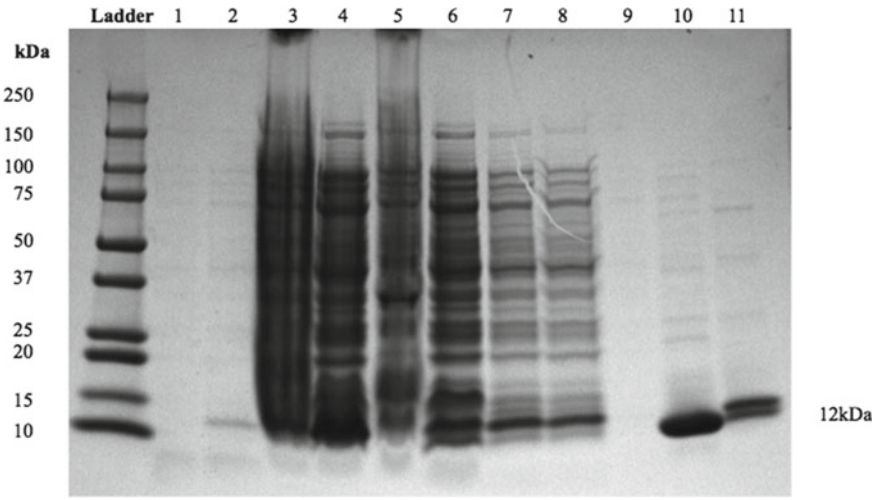

Fig. 1 .

F *Part Two: Conjugation Of Linker Peptides To P1395 WT*

The linker peptides with varied lengths, which were synthesized previously, were mixed. These linker peptides included L19, L13, and L17, with FXIa and P1395 proteins in a buffer solution. For this step, 120 nM of each length of protein was incubated, together with 60 nM of FXIa and 240 nM of P1395WT for 48 h at room temperature. The buffer used was 50 μM Tris pH 7.4, 0.15 μM NaCl, 0.002 nM $CaCl_2$. Finally, an analysis by LC-MS using a reverse-phase C18 Accucore RP column was conducted.

5 Results

A. *P1395 Sequence AVA0421-P1395 (WT)*[1]
B. P1395 Sequence AVA0421-P1395 (Mutant)[b]:
MAHHHHHHMGTLEAQTQ/ [1] GP…**S**…TY
C. *SDS-PAGE*
Figure 1 shows the successful expansion and purification of P1395 WT protein was purified and observed as the 12 kDa bands in the eluate(lane 10) and imidazole(lane 11) fractions. Lane 1 and 2 shows that there is no protein present before and after induction respectively. Lane 3 was taken after sonication, releasing all protein products present in the cell. Lane 4 shows most protein was present in the soluble fraction after centrifugation, and this is supported by lane 5 which shows

[1] sequence of the protein is not disclosed as intellectual property is pending:
MAHHHHHHMGTLEAQTQ/[2]GP…**C**…TY

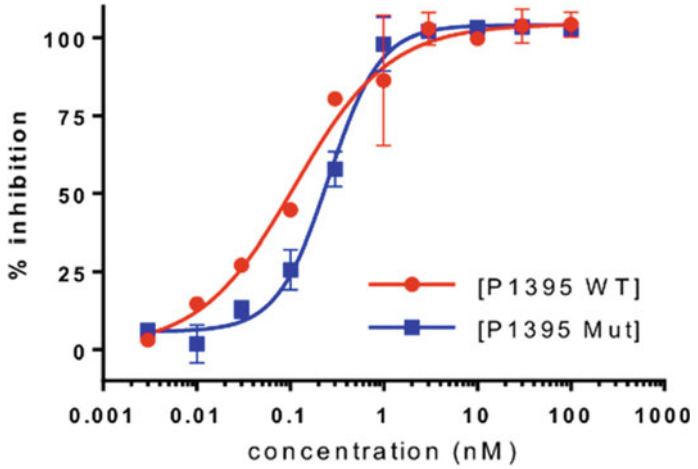

Fig. 2 Dose response curve of the percentage inhibition of FXIa against the concentration of P1395WT and mutant

minimal protein loss in the insoluble fraction. Lane 6 is the flow through during metal affinity chromatography, where non-P1395WT proteins are removed from the column. Lane 7–9 are the washes conducted.

D. Enzymatic Assay
Figure 2 shows the IC_{50} value of P1395 WT is 0.112 and IC_{50} value of P1395 Mutant is 0.257, which is a two-fold drop in activity of P1395. The values of Vmax are the same as the OD_{405} per hour, which is the reaction rate when the enzyme FXIa is fully saturated by substrate S2366, over time (OD_{405}/hour). The percentage inhibition was calculated by using the following formula- $z\frac{V\max(control)-V\max(sample)}{V\max(control)}\times 100\%$. The percentage inhibition against the various concentration of P1395 was plotted using the calculated values to obtain the dose response curve.

E. *PEPTIDE LINKER SYNTHESIS*
Table 1.

6 Discussion

A. *Successful Extraction Of P1395 WT Protein*

SDS-PAGE analysis revealed that P1395 WT was successfully expressed. The successful expression of P1395 was evident in the heightened thickness of the band on the gel at the expected 12 kDa size upon induction by IPTG, which demonstrates overexpression of the P1395 WT protein (Fig. 1). As such, it can be concluded that expression and purification of P1395 WT was successful.

B. *Enzymatic Assay Revealed Two-Fold Drop In FXIa Activity*

Table 1 Success rate of the linker protiens synthesized, ans tested for successful conjugation with the P1395 inhibitor[a]

Name	Molecular weight (g/mol)	Successful synthesis
L9[d]	767.79	No
L13[d]	1026.02	No
L17[d]	1284.84	No
L21[d]	1542.50	No
L25[d]	1800.72	No
L29[d]	2059.00	No
L33[d]	2317.20	No
L9[e]	809.87	Yes
L13[e]	1082.13	Yes
L17[e]	1354.39	Yes
L21[e]	1626.65	Yes (minute)
L25[e]	1898.91	No
L29[e]	2171.18	No
L33[e]	2443.44	No
L25[f]	1989.00	No
L29[f]	2291.28	No
L33[f]	2563.55	No

[a]Fmoc-based solid phase peptide synthesis(gly) was done with 0.17 substitution
[b]Fmoc-based solid phase peptide synthesis(ala) was done with 0.69 substitution
[c]Fmoc-based solid phase peptide synthesis(ala) was done with 0.48 substitution

As evident from the P1395 WT and mutant sequence, the only distinction observed is the last cysteine in the sequence, which was modified into a serine, resulting in merely six cysteines present in the mutant sequence. The presence of six cysteines facilitated the formation of three disulphide bridges, therefore increasing the stability of the inhibitor. In the enzymatic assay, FXIa catalysed the hydrolysis of S2366 into para nitroaniline, which was detected at 405 nm absorbance. From the IC_{50} value, a two fold drop of activity from 0.112 to 0.257 nM from the WT to mutant sequence was observed (Fig. 2). This indicates that the P1395WT is more effective at inhibiting FXIa when compared to P1395 Mutant. However, even though there is a drop in the P1395 Mutant activity, the IC_{50} value of 0.257 nM still suggests a strong inhibiting nature of the mutant. Hence, P1395 Mutant which should be further pursued as a possible anticoagulant drug.

Further analysis on the stability of the P1395 mutant and subsequent contrasting with P1395 WT could be conducted. This would provide firm justification that P1395 mutant is more stable than P1395WT as was inferred from the removal of the thiol group. Further stability analysis via LCMS analysis of both inhibitors over time could

be performed to observe degradation or formation of side products. More extensive enzymatic assays could be conducted to ensure reliable, consistent results.

C. *Unsuccessful Peptide Linker Synthesis*

Initially, it can be seen that the repeating sequence used was "GSGG" across the linker peptides from L9 to L33. However, when HPLC and MS were ran, the purity of the product was extremely low (less than 30% of overall proteins present). It was deduced that this was possibly due to the fact that the repeating sequences caused the probability of the addition of the next amino acid to be significantly lowered. Hence, we introduced alanine into the sequence to increase the sequence variability. We also used a resin with a higher substitution value of 0.69, to increase the probability of a correct sequence being synthesized. This resulted in the successful synthesis of L9–L21. For the larger peptide linkers, the product purity was still low(less than 40% of overall proteins present). Since these peptides are longer, more variability was included in the 3rd run, by alternating "SAGG" and "SASG" sequences until it reached the desired length. The resin was also changed to one with a lower substitution value of 0.48 to prevent inter-peptide interactions. However, the HPLC and MS results still showed low purity levels. In the future, perhaps more unique amino acids are needed for longer sequences or different resins should be used to optimize the conditions.

D. *HPLC Analysis Indicated Unsuccessful Conjugation*

The HPLC analysis observed after mixing revealed negative results. There are two major peaks, which correspond to the mass of a P1395 monomeric inhibitor and dimeric inhibitor without the linker peptide, of 8728 and 17,456 molecular weight respectively, in that order. We were unable to form a dimeric inhibitor with the linker peptide and hence unable to test which peptide length will best to form a dimeric inhibitor for factor XIa. To increase the likelihood of conjugation of the linker peptide and P1395 WT, conditions need to be further optimized. This could possibly be done by altering the pH, temperature or by adding oxidizers and redox shufflers to make the formation of disulphide bonds more kinetically and thermodynamically favourable.

Finally, even though these solutions are mutually exclusive at this stage, the potential for the two approaches to converge through further engineering solutions remains. It is very highly probable that after finding out the optimum peptide linker length, we could clone the P1395WT gene and add residues within the two domains. This allows for the transcripted protein to be a dimer with the peptide linker, so there is no need for conjugation afterwards. Hence, a dimeric, bivalent inhibitor of FXIa that is stable and efficient could be formed.

7 Applications

This study has prominent applications to coagulation disorders.

Quantitative results revealed that in the case of the P1395 mutant inhibitor, stability is marginally improved from the mutation which resulted in removal of the free thiol group. In the case of the P1395 WT, even though P1395 WT was unable to conjugate successfully with the linker peptide currently, with optimized conditions it could be completed to form a dimeric, bivalent inhibitor. This inhibitor could potentially be highly efficient since FXIa is also dimeric.

In both cases, the P1395 inhibitor could be adopted to inhibit FXIa. Hence, when adopted as an anticoagulant drug, minimal bleeding risk would be present, which is critical for drugs used over long time periods. This is an important step forward in cardiology and therapeutics, for the development of the first anticoagulant drug with low bleeding risks.

8 Conclusion

A. *Part One*

As seen from the IC_{50} value, even though there is a two fold drop of activity from the WT to mutant sequence, this drop is very minimal. Thus, this indicates that the mutant is still indeed a strong inhibitor and we could pursue this as a possible anti-coagulant drug provided the compromise between activity and stability of inhibitor is insignificant.

B. *Part Two*

We were unable to test which linker protein will best form a dimeric inhibitor for factor XIa, owing to the fact that the linker protein was not compatible with the P1395 monomers due to difference in size. However, we understand that changing the concentration ratio of P1395 monomers or linker proteins could produce better results. Finally, we believe that if P1395 were to dimerize successfully and effectively inhibit FXIa, it could prove to be more cost-effective as compared to warfarin and heparin, opening up many possibilities in the healthcare industry in the aspects of medical devices and coagulation disorders.

Acknowledgements T. I. and M. Y. thank Professor Koh Cho Yeow and his lab for mentoring us throughout our research journey. We also thank the National University of Singapore, School of Biological Sciences, S2, for providing us with the resources to carry out this project.

References

1. Singapore Statistics. (n.d.). Retrieved from https://www.myheart.org.sg/my-heart/heart-statistics/singapore-statistics/.
2. Al-Horani R. A. and Desai R.U. (2017) Factor XIa inhibitors: A review of patent literature, pg 1–2

3. VectorBase. Ixodes scapularis. (2019, June 25). Retrieved from https://www.vectorbase.org/organisms/ixodes-scapularis.
4. WebMD. Myocardial Infarction (Heart Attack) Causes and Warning Signs. (2019, July 2). Retrieved from https://www.webmd.com/heart-disease/understanding-heart-attack-basics#1.
5. Sig, A. K., Guney, M., Guclu, A. U., & Ozmen, E. (2017). Medicinal leech therapy—an overall perspective. *Integrative Medicine Research*, *6*(4), 337–343.
6. Boston Scientific. What is coronary artery disease and what causes it? (n.d.). Retrieved from https://www.bostonscientific.com/en-US/patients/health-conditions/coronary-artery-disease.html.
7. Weber M., Steinle H., Golombek S., Hann L., Schlensak C., Wendel H. P. and Avci-Adali M. (2018) Blood-Contacting Biomaterials: *In Vitro* Evaluation of the Hemocompatibility, Frontiers in Bioengineering and Biotechnology, Volume 6:99.
8. Rosano, G.L and Ceccarelli, E.A (2014) Recombinant protein expression in *Escherichia coli*: advances and challenges, Frontiers in Microbiology, Volume 5:172.
9. Tanaka-Azevedo A. M., Morais-Zani K.,Torquato R. J. S., and Tanaka A. S. (2010) Thrombin Inhibitors from Different Animals, Journal of Biomedicine and Biotechnology, Volume 2010:641025.
10. Wilson A. C., Neuenschwander P. F. and Chou S. F. (2019) Engineering Approaches to Prevent Blood Clotting from Medical Implants, Archives in Biomedical Engineering & Biotechnology, Volume 1:2.