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New Human Fibrinogen Multi-Peptide Affinity Ligands Allowing High Temperature Virus Inactivation During Chromatographic Process

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Abstract

According to the 8 algorithms approach it was developed the high affinity multi-peptide chromatographic gel for purification human fibrinogen – one of the basic components of the fibrin surgery glue or sealant. Given the next step of fibrinogen virus inactivation directly in the chromatographic column, the multi-point peptide-affinity gels had to bind this protein at several spatially spaced points in order to limit the protein's degree of freedom for holding it at high buffer flow rates or elevated buffer temperature without denaturation.

Based on previous experiments, an multi-point peptide-affinity chromatographic gel had the following properties: the dynamic binding capacity with enough residence time 20 min was around 54-58 mg×mL^{-1} led to the >92% purity of isolated fibrinogen without virus-inactivation and to the 96,4% after in-column virus-inactivation. The virus inactivation of this protein (coupled by the gel) directly in chromatographic column shown a highly effective virus elimination (log10>9) for both nonenveloped and lipid enveloped viruses.

Using fibrinogen sequence from UniProt_KB and dates from more than 25 literature sources on the protein interaction, affinity peptides were calculated against several sequences or separate amino acid fragments. Then these peptides were modified to reach more affinity enhancement and multi-point affinity-peptide chromatographic gel was synthetized and tested.

By synthetized multi-point affinity-peptide chromatographic gel and one step chromatography the virus inactivated human fibrinogen was purified (96,4% pure) with acceptable quantity (71-76%) and ability to polymerize.

Keywords: Human Fibrinogen, Affinity Peptide, Chromatography Ligand, Virus Inactivation

List of Abbreviation

*h*Fg – human fibrinogen
S/D – solvent/detergent
LEV/LEVs – virus(es) enveloped by lipid membrane
NLEV/NLEVs – non-enveloped virus(es)
Vc – column volume
DBC – dynamic binding capacity
TDC – temperature-depending binding capacity
IE – index of efficiency
GC – gas chromatography
IFA – indirect fluorescent analysis
FID detector – flame ionization detector
UV-detector – ultra violet detector

Introduction

Fibrin sealant is a two-component material consisting of the human fibrinogen (*h*Fg) and thrombin. In the presence of

small amounts of calcium and factor XIII, the human thrombin (*h*Thr) converts *h*Fg into insoluble fibrin, the final stable form of the agent. Fibrin sealant now has over a century of development and use.

The safety of sealant's composed liquid agents is most dependent on the fact that multiple donor plasma pools are used for their preparation. Both contain highly concentrated forms of the *h*Fg (70-85 mg×mL^{-1}) and *h*Thr (500-1000 IU×mL^{-1}), respectively, derived from large pools of plasma. Thus, these forms of human pooled plasma fibrin sealant may be associated with viral disease (Parvovirus B19, hepatitis, HIV) or prion disease (CJD - Creutzfeldt Jakob) transmission [1,2].

Fibrinogen purification has traditionally been obtaibed from side-fraction of the purification of FVIII from cryoprecipitate using heparin affinity chromatography or directly from plasma through a series of ethanol precipitation steps [3]. The more complicated method can include several chromatographic steps on cation exchanger, hydrophobic gel and/or dye gel or mixed mode gel (for example, Capto MMC) [3-5]. Recently, the tetra- or hexapeptide ligands (GPRP FLLVPL identified from a peptide library, was claimed to allow, at experimental scale, isolation of fibrinogen under a highly pure and functional state. Such affinity-purified fibrinogen retained a FXIII crosslinking activity claimed to be required to prepare elastic and high tensile-strength fibrin sealants [6-8].

A simplified procedure was described for the purification of prothrombin from pooled human plasma. The initial steps, which are common to prior purification techniques, include adsorption onto and elution from barium citrate, ammonium sulfate fractionation, and DEAE-Sephadex chromatography [9-15].

Naturally, the isolated protein components of fibrin glue must be processed to remove possible viral contaminants.

Treatment with solvent/detergent (S/D) is a widely used method for ensuring the virus safety of the manufactured proteins from human plasma. It is important to note two significant weaknesses of the S/D treatment. First, it did not inactivate non-enveloped viruses (NLEVs) and it was necessary for further NLEVs reduction to use wet/dry heat treatment at 60-100°C or nanofiltration or UV-/gamma irradiation [16-20]. Second, the exposure at 25-37°C to S/D a relevant labile high-molecular weight protein can cause its denaturation which is summarized with additional losses of the denatured target product caused by high turbulence filtration process using for S/D and NLEVs removing [21,22].

Recently we demonstrated a simple and useful procedure for proteins virus inactivation directly in chromatographic column [23]. This virus elimination was possible to introduce into purification process and obtain the factor of enveloped (LEVs) and non-enveloped (NLEVs) viruses concentration decrease values that was higher than provided by known technologies of virus inactivation in solution [20-24]. These results were based on the two well-known principals: 1) the chromatographic gel with high dynamic binding capacity should retain the target protein by binding a large number of sites to deprive the protein of degrees of freedom and thus save it from denaturing by S/D at elevating temperature of 30-45 °C, that treatment guarantees high degree LEVs elimination; and 2) the S/D treatment during several hours mechanically washes out NLEVs and LEVs traces/fragments from the column.

According to this procedure we received high pure and active virus-inactivated thrombin and fibrinogen [25]. Unfortunately due to fibrinogen ligands lost capture forces under high temperature (>35 °C) or buffer flow (>200 cm×h^{-1}) the spent time and costs of spent reagents left much to be desired.

The development of such multi-point peptide-affinity gel for virus inactivation introduced in the chromatographic purification process of human fibrinogen is presented in this paper.

Materials and Methods

All reagents were purchased from Sigma-Aldrich (Green Chemistry LLC, distributor in Mongolia) unless otherwise indicated. Reagents and equipment from other manufacturers are indicated below. Human (donor's) plasma, obtained by plasmapheresis, was bought from Ulaanbaatar blood collection center (Mongolia, Ulaanbaatar). Human fibrinogen native protein was obtained from Abcam (ab81752) through Abcam Hangzhou (China).

Peptide Array Library, Array Peptide Synthesis, SPOT Peptide Array Experiments

Peptide array library was calculated according to 8 developed principles described before using nucleotide sequence of the human genes and amino acid sequences of the *h*Fg from Protein Knowledgebase - UPKB (UniProtKB/Swiss-Prot) [26].

The synthesis and SPOT peptide array experiments were carried on as described before [27-29].

Total Proteins and Human Fibrinogen Determination

The *h*Fg quantity was measured by the standard method with Human Fibrinogen ELISA Quantitation kit (GenWay Biotech. Inc., San Diego, USA), using analytical equipment Typhoon Trio Imager, Biotrak II visible plate reader and UV/VIS-spectrophotometer Ultrospec 3300*pro* (all from GE Healthcare AB, Sweden, Uppsala).

The total protein in the collected samples was determined by Bradford method with Stoscheck modification [30,31].

Peptide-Affinity and Multi-Point Peptide-Affinity Chromatographic Gels Synthesis and Properties Checking

All developed by early publish method 7-, 8- and 9-mer peptides (cycle/non cycle) were coupled with WorkBeads 40/10000 ACT matrix according to manufacturer's recommendations (Bio-Works Technologies AB, Uppsala, Sweden) [27-29]. In case of peptide affinity gel synthesis for fibrinogen purification the WorkBeads 40/10000 ACT matrix was used.

For checking affinity peptides properties the peptide 1% solution in Na-carbonate-bicarbonate buffer, pH 9.4, was pumped with flow rate 10 Vc (column volume) per hour through ECO^{PLUS} glass columns Ø10×125 mm (YMC Europe GmbH, Germany, Dinslaken), packed with 12 mL WorkBeads 40/10000 ACT matrix (Bio-Works Technologies AB, Uppsala, Sweden) by packing density factor 1,178 during 48 hours at 4 °C. All other manipulations were carried on according to recommendation of activated gel manufacturer. The peptide density on the WorkBeads gel was determined by quantifying the difference between total peptide quantity in start solution and unbound peptide by fluorescence of tryptophan in the spacer measured by Typhoon Trio (GE Healthcare AB, Sweden, Uppsala).

The chromatographic column for *h*Fg purification was preparing on the following way: 6% mixture of six affinity peptides in equal proportions selected according to their properties was pumped through ECO^{PLUS} glass columns Ø50×120 mm (into cooling jacket) packed with 110 mL WorkBeads ACT 40/10000 gel as described above for tests columns.

Finally, the columns were rinsed with deionized water (10 Vc) to remove the blocking agent and before using – with working buffer for equilibration or before storage – with 20% ethanol.

Determination of the Peptide-Affinity Chromatographic Gel Dynamic (DBC) and Temperature-Dependent Binding (TDC) Capacities

The DBC and TDC of the peptide-affinity gels were determined on ECO^{PLUS} glass columns Ø10×125 mm (YMC Europe GmbH, Germany, Dinslaken) packed with 3 mL of peptide-affinity gel, bed Ø:bed height 15×15 mm (packing density factor 1,15-1,20).

DBCs were determined according to standard procedure [32,33]. TDCs were determined by the following way. Target proteins was applied on the peptide-affinity gel. The quantity of protein samples was calculated 10% lower than maximum level of adsorbent DBC at 20°C. After sampling and washing the column/buffer temperature were slowly raised on 5 degrees each step and determined the target protein quantity in eluates. 5 or more independent experiments were performed according to chromatographic conditions stipulated above.

Human Fibrinogen Partially Purification

All chromatographic experiments were performed using ÄKTA*explorer* system (GE Healthcare AB, Uppsala, Sweden) and different size ECO^{PLUS} glass columns (YMC Europe GmbH, Germany, Dinslaken).

The target protein – *h*Fg – was manufactured by early described cascade technology from human blood plasma [34] with our following modifications. The batch of donor's plasma was divided on high (H) and low-middle molecular weight (L/M) protein fractions eluted from Sepharose 6 Fast Flow (GE Healthcare AB, Sweden, Uppsala) according to our developments [35]. On the next steps the H fraction was used for *h*Fg manufacturing.

In-Column S/D Treatment of Human Fibrinogen and its Chromatography Final Purification

*h*Fg was partially purified from blood plasma by H-fraction separation [34-35]. H-fraction, buffer changed on application buffer citric acid-Na_2HPO_4, pH 6.0, and concentrated to protein content 10 mg×mL^{-1}, was applied on ECO^{PLUS} column Ø50×120 mm (into cooling jacket), packed with 110 mL multi-point affinity-peptide-WorkBeads 40/10000 gel, with flow rate 2 Vc×h^{-1}. 2 Vc of the application buffer was used for column washing with same flow rate. After these manipulations the column with captured *h*Fg was ready for virus inactivation/elimination process.

In the same works, the scheme of the process itself is also presented in detail and the stages of manipulations with the temperatures of the column and buffers in the process of virus inactivation/elimination are described. It should be noted that the removal of model viruses [24,28,29] from *h*Fg was carried out according to the scheme used for IgG with max T°=45 °C] due to dates of TDC that the critical zone for proteins retentions by multi-peptide affinity gel was beginning after 49-52 °C [28].

The S/D treatment was beginning after target protein capturing on the affinity column. The experimental scheme and stages of the virus-inactivation/elimination process, developed and presented earlier, did not change when studying the behavior of chromatographically bounded target proteins under the temperature-dependent treatment. The exceptions were temperature levels, buffers volume and the using one S/D blend only: TnBP/Triton X-100.

The Common Conditions of S/D Treatment Were the Following

- **First Washing:** 20 Vc of equilibration buffer (0,5 Vc×min^{-1}) to complete absence of virus material in eluate.

• **The Raising of S/D Inactivation Buffer Gradient:** 15 Vc inactivation buffer with flow rate 0.5 Vc×min^{-1} was automatically created by programming linear increasing concentration from 0 to 100% and applied on the column. The final inactivation buffer included 1.0%/2.5% TnBP/Triton X-100.

• **The Raising Inactivation Temperature Gradient:** on the beginning of inactivation process for inactivation buffer and column the termostabilized gradient was started from 20 to 45°C during 30 min, therefore a temperature gradient grew in parallel to S/D gradient in all cases.

(Stages 2 and 3 combined in one process named in the finale table Gr.)

• **The Second washing After Raising Gradients – Actual Treatment Process:** column was washed by 45 Vc under 45°C with same flow rate 0.5 Vc×min^{-1}; the column temperature was kept at corresponding buffer temperature (Gs).

• **The Falling Down S/D Inactivation Buffer and Temperature Gradients:** after the washing the reverse S/D and T° gradients were used to reach the initial conditions for 36 minutes - 15 Vc with same flow rate 0.5 Vc×min^{-1} (*Gf*).

• **The Third Column Washing:** when the start T° 20°C was reached simultaneously with falling down gradient finishing, the column was washed by 40 Vc equilibration buffer to complete removing S/D materials, flow rate 0.5 Vc×min^{-1}.

The elution of target protein was carried out with 2 Vc of 20 mM citric acid-Na_2HPO_4 buffer, pH 2.6, 0.04 Vc×min^{-1}. The eluate was automatically titrated by 0.05 M NaOH to pH 6.2-6.0.

The all eluates were collected for concentration/yield and purity of target protein determination and their impurities and ligands determination by ELISA, and electrophoresis, and HPLC analysis.

Column regeneration and sanitization were performed with 2 Vc of 0.1 M glycine-HCl, pH 2.5, and 2 Vc of 0.05 M NaOH with flow rate 0.5 Vc×min^{-1}.

Virus Titer and Nuclear Acid Content

Virus titers were calculated using Kaerber and Spearman method and expressed as log 50% tissue culture infectious dose ($TCID_{50}$) [36,37,24,28,29].

For extraction and purification viral RNA/DNA from the protein samples "QIAamp Viral RNA/DNA Mini Kit" from QIAGEN China Co., Shanghai, China was used. Quantitative Real-time PCR was performed on the Rotor-Gene Q 2plex HRM System instrument (QIAGEN China Co., Ltd., China, Shanghai). The data was expressed as log10 of ratio between virus DNA/RNA detected in infected protein sample before and after purification [24,28,29].

S/D Determination in the Target Proteins After Virus Inactivation

The solvent level in the final protein concentrate after S/D treatment was measured by GC method with FID the detergent level - by HPLC with UV-detector (both instruments from E-Chrom Tech, Taiwan) [38,39].

Statistical Analysis

The statistical processing of results was carried out by the standard methods [40]. A value of $p<0.05$ was considered statistically significant. Data was presented as a mean ± standard errors (SEM) of at least 5 independent experiments unless otherwise indicated.

Proteins Sequences Numeration

Proteins and peptides sequences numeration was done according to the whole molecule represented in UniProtKB (Swiss-Prot): human Fibrinogen alpha chain - P02671, beta chain - P02675, gamma chain - P02679.

Results and Discussion

Early our publication described the development of high affinity ligand for fibrinogen purification with following amino acid sequences FFFFRIF [26]. This peptide ligand coped quite satisfactorily with the specific adsorption of target protein. However, the efficiency of protein retention by affinity-peptide chromatographic gel when the temperature of the virus-inactivating buffer was increased to 40 °C was found to be definitely low. The DBC and temperature depended dynamic capacity (TDC) of the peptide-affinity gel, as expected, dropped sharply with increasing buffer speed (>200 cm×h^{-1}) or temperature (>35 °C). Thus, virus inactivation directly in the chromatographic column was carried out at 35 °C and a buffer speed of 0.5 Vc×min-1, which naturally increased the operating time, the consumption of buffers and, ultimately, the process cost.

During the development of affinity ligands for IgG_{1-4} we showed that the chromatographic gel binding of the target protein at several sites separated from each other provides both reliable retention of the protein on the gel at high speed

(>200 cm×h^{-1}) and temperature (>48 °C) of the buffer, as well as effective prevention of protein denaturation [29].

Using our eighth algorithms approach of calculating affinity peptides for target proteins, developed in previous investigations we decided to identified several potentially affinity peptides for Fg at different parts of protein molecules for designing multi-point peptide-affinity gel [27-29].

High Affinity Peptides Calculation for the Human Fg Specific Coupling

First, based on the previous investigations it was decided to use the affinity peptide FFFFRIF, which as a ligand of the FFFFRIF-WorkBeads 40/10000 chromatographic gel allowed the maximum binding of fibrinogen at 25 min of residence time (92% of total Fg) with purity around 83% from high (H) molecular weight protein fraction of the total human plasma [26]. It should also be noted that this ligand provided fairly good conditions for the virus inactivation of fibrinogen directly in the column: buffer flow rate 200 cm×min^{-1} and temperature 40 °C. The affinity FFFFRIF peptide was developed against fibrinogen sequences αC 196-209, γC 432-445/439-452.

Laudano and Doolittel and Hsieh studying the polymerization of fibrin monomer showed that peptides Aα 17-23[1] (36-45) and Bβ 15-25[1] (45-52) of fibrinogen chains are available for interaction and include highly affinity polymerization sites [41-43]. In particular, they are also capable to associating with thrombin, which causes exposure it catalytic site and re-exposure of exosite-1. Maurer *et al* [44]. by new flexible docking approach applied to determine the structure of a fibrinogen αC peptide (^{7}DFLAEGGGVRGPRV^{20}) bound to an active site mutant of thrombin shown that the N-terminal portion of this peptide (^{7}D-R^{16}) involves a chain reversal, whereas the C-terminal portion (^{17}GPRV^{20}) adopts a fold that exists in several different orientations. However, Calvete et al [45]. determined that GPIIb/IIIa as an inducible receptor for fibrinogen has a polypeptide 656-667 which binds to soluble fibrinogen during its polymerization, but is not a RGD-domain.

According to our eighth algorithms approach the calculation of the 10-mer affinity antisense peptides for fibrin monomer peptides Aα 17-23 (^{36}GPRVVERHQSACK^{48}) and Bβ 15-25 (45-52) are presented in Figure [27-29]. 1 and Table.1. Aα 17-23 and Bβ 15-25 peptides sequences numeration was done by authors according to the whole fibrin molecule, our numeration at the round brackets – according to the whole fibrinogen molecule.

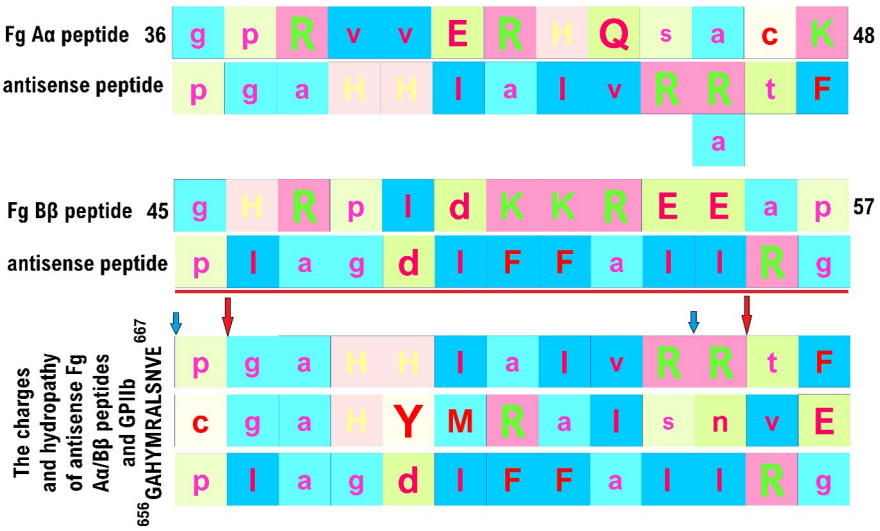

Figure 1: TIF The Calculation of Anti-Sense Sequences of the Aα and Bβ Fg Peptides which are Responsible for the Interaction with GPIIb Site 656-667 According to [41-43]

The following comparison Aα and Bβ Fg sequences by charges and hydropathy of amino acids with possible by sequence of the interaction site 656-667 on GPIIB [45]. Positive charges of amino acid shown by green and lite green letter colors: from +10.8 (R) through +9.7 (K) to +7.6 (H). Negative charges shown by burgundy-red-rose letter colors: from -2.8 (D) through -5.4 (N) to -6.5 (P). Hydropathy index shown by cell colors: crimson – strong (R, K), yellow – weak (D, Q, N, E, H, S, T) hydrophilic; ivory – in fact neutral (P, Y, C); blue – weak (G, A, M) and dark blue – strong (L, V, F) hydrophobic. Molecular weights shown by letters sizes: high MW>80 Da (F, R, Y, W) – by big capital letters; medium MW 71-80 Da – by small capital (H, M, E, Q, K) letters; low MW 30-58 Da – by non-capital (G, D, N, L, T, V, P, C) letters and very low MW – by low non-capital (A) letter. Sequences selected for study are indicated by small blue and long red arrows

As can be seen from Figure. 1, the availability of Aα, Bβ and peptide ^{656}G-E^{667} of GPIIb to interactions shown by Laudano and Doolittel Hsieh and Calvete *et al* [41-43,45]. is actually confirmed by the physicochemical properties of the amino acids these sequences. Thus, the antisense peptide Aα had only one mismatch with peptides ^{656}G-E^{667} or ^{655}C-E^{667}. Moreover, the antisense amino acid PGAHH is almost identical to the sequence CGAHY except for two pairs: P↔C and H↔Y. First: the complementarity of the sense PRV sequence (Aα peptide) to the GAH sequence (peptide ^{656}G-E^{667}) is unconditional [46]. Second: for the neutral pair P↔C, it should be noted that these amino acids have almost identical

properties: 1) MW_P=40 Da, MW_C=46 Da, 2) pI_P=6.5, pI_C=5.1, 3) hydropathy (HP) HP_P=-0.3 , HP_C=0.2 (both are neutral in fact). In addition, according to Biro amino acid glycine (sense peptide Aα) is able to form a complementary pair with cysteine (peptide ^{655}C-E^{667}) [46]. Third: the same applies to the negative-neutral amino acids pair H↔Y. They are almost neutral by charge: pI_H=7.6, pI_Y=5.7; according to hydropathy histidine is weak hydrophilic HP_H=-1.7, and tyrosine is neutral in fact HP_Y=0.1. The sense amino acid valine (Aα peptide) is also able to form a complementary pair with the tyrosine (peptides ^{656}G-E^{667} or ^{655}C-E^{667}) [46]. That is, the sequences PGAHH (Aα Fg) and ^{655}C-E^{667} (GPIIb) determined by a number of researchers are complementary [41-43, 45]. The following differences between the antisense peptide Aα and the peptide ^{655}C-E^{667} are minor (the pairs L↔M, L↔A, V↔L, R↔S, R↔N are formed). The completely opposite physicochemical properties of the last three pairs of amino acids A↔R, T↔V, F↔E indicate that sense acids of the Aα peptide will oppose as follows R↔R, C↔V, K↔E and not contribute to the interaction, but will push back and separate the discussed sequences (Figure. 1). Excluding amino acids ^{666}V and ^{667}E from the sequence ^{655}CGAHYMRALSNVE^{667,} will choose a complementary replacement, first, for ^{661}R, and more complementary substitutions for ^{659}Y, ^{660}M, ^{662}A-N^{665}, a calculated high-affinity peptide for the Aα of Fg can be generated.

From the given data, it becomes clear the lowest affinity of the Bβ peptide to the ^{656}G-E^{667} GPIIb sequence, which had about 30% of the Aα peptide affinity, namely the presence of non-complementary pairs of amino acids: ^{48}P↔H^{658,} ^{51}K↔R^{661}, ^{54}E↔S^{664}, ^{55}E↔N^{665}, ^{56}A↔V^{666}, ^{57}P↔E^{667} and the low complementary pair ^{49}L↔Y^{659} (Figure. 1). Thus, we become sure that for Fg Aα and Bβ peptides it will be possible to calculate and construct high-affinity 7-10-mer peptides using appropriate amino acid substitutions at the sequence ^{655}CGAHYMRALSN^{665}.

Based on the above dates and "summarized" antisense peptide obtained after recognition of the most stable/matching structure (shown by black arrows on the Figure. 1) an initial library of potential affinity peptides was created (Table 1). These peptides were tested on the Aα (No. 1, 5) and Bβ (No. 9, 13) *h*Fg binding in 0.05 M Na_3-citrate buffer, pH 7.2, – a future coupling buffer in the chromatographic process of *h*Fg purification.

All tested potentially affinity peptides showed approximately equal and high affinity to Aα and Bβ *h*Fg (Table 1, no statistically significant affinity changes between peptides No. 1 and 5, 9 and 13 - p>0.1) enough for strong coupling protein and for its desorption at non-denaturation level pH=2,5÷3,2. Adequate mutations R/A, A/S and combine mutation in ^{1}PGA(S)HHLALVR(A)^{10} or ^{2}GA(S)HHLALVR(A)R^{11} did not strengthened affinity to Aα *h*Fg same as mutation A/S in position 3 and 9 in ^{1}PGA(S)GHLFFA(S)L^{10} or ^{2}GA(S)GHLFFA(S)LL^{11} resulted a visible decrease of affinity to Bβ *h*Fg. Moreover, the decrease in activity in some cases (especially with double mutation) reached statistically significant values (for peptides No. 2, 4, 8, 11, 12, 16 – p<0.05).

The core of both peptides, which have the highest affinity for Aα and Bβ *h*Fg, is almost identical in physicochemical properties excepting histidine↔glycine at position 4. We suggested that the replacement of ^{4}H in ^{2}GAHHLALV^{9} and ^{4}G in ^{2}GAGHLFFA^{9} by amino acid forming a complementary pair with both valine in peptide Aα and proline in peptide Bβ, will open the possibility to create a summarized high-affinity sequence for both *h*Fg peptides.

Affinity peptide No												
	G	P	R	V	V	E	R	H	Q	S	A	Aα peptide *h*Fg
	C	G	A	H	Y	M	R	A	L	S	N	^{655}C-N^{665} GPIIb sequence
	"Summarized" antisense peptide (amino acid position number at the peptide)											Affinity to Aα *h*Fg by $pH_{-50\%}$ (M±SEM, n=5)
	1	2	3	4	5	6	7	8	9	10	11	
1	P	G	A	H	H	L	A	L	V	R	-	3.42±0.08
2	P	G	A	H	H	L	A	L	V	A	-	3.69±0.09
3	P	G	S	H	H	L	A	L	V	R	-	3.51±0.06
4	P	G	S	H	H	L	A	L	V	A	-	3.73±0.09
5	-	G	A	H	H	L	A	L	V	R	R	3.34±0.07
6	-	G	A	H	H	L	A	L	V	A	R	3.57±0.12
7	-	G	S	H	H	L	A	L	V	R	R	3.48±0.05
8	-	G	S	H	H	L	A	L	V	A	R	3.65±0.07
Affinity peptide No												
	G	H	R	P	L	D	K	K	R	E	E	Bβ peptide *h*Fg
	C	G	A	H	Y	M	R	A	L	S	N	^{655}C-N^{665} GPIIb sequence
	"Summarized" antisense peptide (amino acid position number at the peptide)											Affinity to Bβ *h*Fg by $pH_{-50\%}$ (M±SEM, n=5)
	1	2	3	4	5	6	7	8	9	10	11	
9	P	G	A	G	H	L	F	F	A	L	-	3.46±0.07
10	P	G	A	G	H	L	F	F	S	L	-	3.55±0.10
11	P	G	S	G	H	L	F	F	A	L	-	3.61±0.08
12	P	G	S	G	H	L	F	F	S	L	-	3.72±0.11
13	-	G	A	G	H	L	F	F	A	L	L	3.36±0.08
14	-	G	A	G	H	L	F	F	S	L	L	3.44±0.10
15	-	G	S	G	H	L	F	F	A	L	L	3.53±0.14
16	-	G	S	G	H	L	F	F	S	L	L	3.68±0.09

Table 1: The Peptide Array Library for the Development High Affinity Peptides Separately to Aα and Bβ Peptides *h*Fg Interacted with ^{655}CGAHYMRALSN^{665} GPIIb Sequence

Note 1: rose → light rose → yellow → light green → green colors are shown the calculated peptide affinity increasing to the Aα and Bβ Fg peptides

Note 2: blue color cells are representing the mutations A/S and R/A were caused by the presence of the amino acid serine in the sense or antisense peptides, which can express amphoteric properties in the hydropathy

Table 2 Presents the Library of Summarized Sequences and the Results of Determining their Affinity for Both *h*Fg Peptides.

All developed sequences shown the high combine affinity to Aα and to Bβ peptides *h*Fg (no significant differences, in all cases $p>0.05$), although three sequences 17, 19, 21 (GARHLALV, GARHLAFV and GARHLFFV) showed a tendency for greater complementarity for both Fg peptides. Further researches were continued with them.

Affinity peptide No	G	P	R	V	V	E	R	H	Q	S	A	Aα peptide *h*Fg	
		G	A	H	H	L	A	L	V	R		antisense (affinity) sequence	
	G	H	R	P	L	D	K	K	R	E	E	Bβ peptide *h*Fg	
		G	A	G	H	L	F	F	A	L		antisense (affinity) sequence	
	"Summarized" antisense peptide (amino acid position number at the peptide)											Affinity by $pH_{-50\%}$ (M±SEM, n=5) to	
	1	2	3	4	5	6	7	8	9	10	11	Aα *h*Fg	Bβ *h*Fg
17	-	G	A	R	H	L	A	L	V	-	-	3.35±0.11	3.37±0.09
18	-	G	A	R	H	L	F	F	A	-	-	3.39±0.13	3.40±0.07
19	-	G	A	R	H	L	A	F	V	-	-	3.34±0.08	3.36±0.10
20	-	G	A	R	H	L	A	L	A	-	-	3.46±0.14	3.49±0.11
21	-	G	A	R	H	L	F	F	V	-	-	3.32±0.13	3.38±0.12
22	-	G	A	A	H	L	F	F	V	-	-	3.44±0.11	3.47±0.09
23	-	G	A	L	H	L	F	F	V	-	-	3.52±0.10	3.49±0.07
24	-	G	A	Y	H	L	F	F	V	-	-	3.50±0.09	3.54±0.13

Table 2: The Secondary Peptide Array Library for the Development the Combine Affinity Peptides to Aα and Bβ Peptides *h*Fg

Note 1: light rose → light green → green colors are shown the calculated peptide affinity increasing to the Aα and Bβ Fg peptides

Note 2: blue color cells are representing the different mutations for reaching the high affinity of the sequences

As have been shown fibrinogen αC residues 242-424 had a major regulatory role in the factor XIII-A2B2 (FXIII-A2B2) activation [47]. Smith *et al.* have characterized the binding of recombinant (r)FXIII-A subunit and FXIII-A2B2 with fibrin(ogen) and fibrin αC residues 233-425. Using recombinant truncations of the fibrin αC region 233-425 and surface plasmon resonance, they found that activated rFXIII-A bound αC 233-425 (K_d=2.35±0.09 μM) which was further localized to αC 389-403. Site-directed mutagenesis of this region highlighted Glu^{396} as a key residue for binding of activated rFXIII-A [48].

8-mer antisense peptides to the fibrinogen αC sequence ^{408}PDWGTFEEVSGNVSPG^{423} (389-404 – number according fibrin molecule) synthetized that the antisense amino acid leucine to key glutamine residue remains in the peptide are presented in Table 3.

Synthesized peptides No. 25-29 have a high affinity to αC fibrinogen (no significant differences, in all cases, $p>0.05$). A tendency to increase affinity was observed in the case of the central location of leucine in the synthetized sequences (peptides 27 and 28 showed the maximum affinity). This confirmed the conclusion Smith *et al* [48]. that complementary glutamine is an essential amino acid for the interaction of the αC fibrinogen peptide. We should also note that lysine was very important for increasing affinity trends, the loss of which in peptide 29 significantly reduces the affinity to αC fibrinogen. The absence of leucine and lysine in peptide 30 practically leads to a statistically significant loss of affinity ($p<0.05$).

Peptide No.	PDWGTFE**E**VSGNVSPG	Peptide 389-404 interacted with FXIII by Smith *et al.* [48]	
	GLTPWKL**L**HAPFHAGP	Antisense sequence developed according our method [27-29]	
25	GLTPWKL**L**	3.46±0.10	Affinity by $pH_{-50\%}$ (M±SEM, n=5) to
26	TPWKL**L**HA	3.43±0.07	
27	WKL**L**HAPF	3.37±0.08	
28	CKL**L**HAPF	3.39±0.09	
29	L**L**HAPFHA	3.50±0.07	
30	HAPFHAGP	3.77±0.13	

Table 3: Six Affinity Peptides Developed to Fibrinogen α-Chain Amino Acid Sequence Interacted with FXIII

Note 1: $^{(396)415}$glutamine as a key residue shown as bold E, complementary leucine shownz as bold L Synthesis and verification of the sequence with the W/C mutation (peptide No. 28), were done due to the fact that threonine has two equally complementary amino acids, tryptophan and cysteine. The appearance of cysteine did not change the affinity of the CKLLHAPF peptide, which in the future will allow the synthesis of a cyclo-peptide that will have greater stability and lifetime as an affinity ligand.

In the case of sequences No. 27 and 28, improved affinity by selecting amino acids with slightly different physicochemical properties was not required, since the affinity shown was already significant and practically limiting for protein desorption from a similar ligand by lowering buffer pH to 2.5-3.0.

By the same way the results of number authors investigations were transformed for development of affinity peptides. Thus Brehm *et al* [49]. studying the mechanisms of inositol phosphate (InsP6) influencing on platelet aggregation and then Vottero *et al.* testing the invermectin effects on hypercoagulability caused by the interaction of coronavirus (SARS-CoV-2) spike protein (SP) with fibrinogen independently identified the binding site on βC *h*Fg for InsP6 and SP namely ^{156}QKRQKQVK^{163} and ^{158}RQKQVKDN^{165}, respectively [50]. Synthesized antisense peptides VFAVFVHF, VFSVFVH, AVFVHFLF and SVFVHFLF for both sequences showed a high affinity interaction with fibrinogen measurement as $pH_{-50\%}$: 3.38±0.12, 3.46±0.10, 3.32±0.14 and 3.40±0.09, respectively [41-43]. The A/S mutation was tested due to the fact that arginine has complementarity with alanine and reverse complementarity with serine [46]. Subsequently, VFAVFVHF and SVFVHFLF were chosen for testing as chromatographic ligands, since the VFSVFVH and VFSVFVH peptides were rejected, because first required too low pH <2.5 buffer for the elution of fibrinogen, second did not have enough fibrinogen retention forces at high speed and temperature of the chromatographic buffer.

Also Vottero *et al.* at the same investigation at fibrinogen γC discovered essential for the interaction amino acids $^{254(228)}$LxxEKxxL$^{261(235)}$ belonged to the fibrinogen site LGNEKIHL of leukocyte receptor $\alpha_M\beta_2$ (Mac-1), previously identified by Yakovlev et al. [50,51]. The high affinity of the synthesized antisense peptides (DPFLFYLD, EPFLFYLE and NPFLFYLN) to fibrinogen confirmed that this sequence is accessible for interaction and may be the leukocyte receptor αMβ2 (Mac-1) site on fibrinogen. Mutations D/E and D/N were used due to the fact that leucine has complementarity with aspartic acid, asparagine and glutamic acid [46]. In addition, a simple analysis of the amino acid sequence of the leukocyte receptor $\alpha_M\beta_2$ (Mac-1) revealed the presence of several sequences very similar in physicochemical properties to the synthesized antisense peptide of the site on fibrinogen, shown in Figure. 2 as examples and speculation.

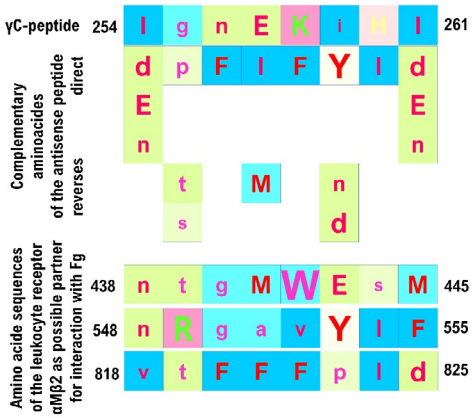

Figure 2: TIF Examples of Amino Acid Sequences that Possible (According to Physicochemical Properties) to be an Interaction Sites with Fibrinogen on Leukocyte Receptor $\alpha_M\beta_2$ (Mac-1). Color Amino Acid Letters and Frames Designation Shown in FigureL: 1

Important for creation an affinity ligand for the N-end γC of fibrinogen was Gonzalez-Durruthy et al. investigation, where was studied the fibrinogen and the beta-blockers acebutolol and propranolol interaction [52]. Using the molecular docking method and Density Functional Theory, the authors identified several sites fibrinogen-drugs interaction, in in particular, γC site ^{41}FxxxCPxxxG^{50}. Later, independently from González-Durruthy Votero et al. by the method of molecular docking simulation determined the core of this site ^{43}SICPTT^{48} [50]. Thus we began investigations with 12-mer sequence ^{39}ERFGSICPTTCG^{50} (keeping in the mind one idea which will be explained lower), synthetized against them several antisense sequences and checked their affinity to fibrinogen. The results are presented in the Table 4.

Excluding sequence No. 32 with ^{5}A/R mutation, all other sequences were highly affine to fibrinogen. As shown above, the $^{9/10}$C/W mutations practically did not affect the affinity of the sequence (statistically insignificant difference, $p>0.5$) same as a shortening of the sequence from the C-end on 3 amino acids. In addition, ^{2}A/C substitution did not statistically significantly affect affinity ($p>0.5$), due to close physicochemical properties both amino acids and which was very significant for the synthesis of cyclic peptide with the aim of increasing its lifetime as an affinity ligand. For the purpose of the peptide cyclization two additional amino acids E and R were used from the N-terminus of the sequence. It is important that the antisense pair for arginine is alanine with very similar properties as cysteine (HP_A=1.0, HP_C=0.2; pI_A=6.0, pI_C=5.1), which allows their adequate replacement. ^{1}Lysine - antisense pair for glutamic acid - served as the point of attachment to the gel matrix from the peptide N-terminus, and ^{10}W - from the C-terminus.

Affinity peptide No	E	R	F	G	S	Y	C	P	T	T	C	G	γC peptide *h*Fg [50, 52]
	Antisense peptide (amino acid position number at the peptide)												Affinity to *h*Fg by $pH_{-50\%}$ (M±SEM, n=5)
	1	2	3	4	5	6	7	8	9	10	11	12	
31	L	A	K	P	A	M	T	G	W	W	T	P	3.37±0.12
32	L	A	K	P	R	M	T	G	W	W	T	P	3.71±0.14
33	-	A	K	P	A	M	T	G	C	W	T	P	3.39±0.09
34	-	A	K	P	A	M	T	G	C	C	T	P	3.41±0.10
35	L	C	K	P	A	M	T	G	W	W	T	P	3.39±0.07
36	-	C	K	P	A	M	T	G	C	W	T	P	3.44±0.12
37	-	C	K	P	A	M	T	G	C	-	-	-	3.38±0.08
38	-	C	K	P	A	M	T	G	C	-	-	-	3.40±0.10
39	-	C	K	P	A	M	T	G	C	W	-	-	3.41±0.10
40	L	C	K	P	A	M	T	G	C	-	-	-	3.36±0.15

Table 4: Ten Affinity Peptides Developing to Fibrinogen γ-Chain Amino Acid Sequence Acebutolol and Propranolol

Cyclization of two cysteines did not lead to a significant loss of affinity (statistically insignificant difference between non-cycle/cycle CKPAMTGC or LCKPAMTGC/CKPAMTGCW, in all cases $p>0.5$) as well as additional ^{1}L or ^{10}W from the N- or C-terminus. For further investigation were chose peptides No. 37-40.

Finally we calculated, synthetized and checked affinity of 15 peptides designed for different parts αC, βC and γC *h*Fg, one of them 7-mer, twelve 8-mer with one cycled and two cycled 9-mer.

Properties of Peptide-Affinity Chromatographic gels and Peptides Selection for Multi-Point Peptide-Affinity Chromatographic Gel Synthesis

After peptides "sealing" according to activated gel manufacturer recommendations the 15 chromatographic gels were obtained into 3 mL ECO^{PLUS} glass columns (Table 5). Due to early expressed stipulations for coupling affinity peptides

to the gel matrics we used tested before short spacer with 2-3 tryptophan including as for easy amount coupled peptide calculation as for the ligand leaching from the chromatographic matrics [26]. In addition, the introduction of acidic residues, like D or E, into the spacer will make it possible to easily dissolve a highly hydrophobic peptide in Na-carbonate-bicarbonate buffer, pH 9.4. All other properties of the synthetized gels are shown in the Table 5.

Peptide No.	Peptide ligands of the gels	Chromatographic affinity peptide gels basic properties				
		Peptide density on the gel	DBC at flow rate 30 cm×h^{-1}	Residence time *h*Fg max coupling	*h*Fg purity (*h*Fg$_{pur}$)	IE
		mg/μmol×ml^{-1}	mg *h*Fg	min	%	
17	**GARHLALV**	22,4/26,8	52,4	19	88,7	173,4
19	**GARHLAFV**	22,2/25,5	50,8	17	90,2	181,8
21	**GARHLFFV**	23,1/24,4	56,5	18	94,6	219,1
27	**WKLLHAPF**	23,0/22,7	54,9	20	92,1	222,7
28	**CKLLHAPF**	22,8/24,6	53,4	22	91,8	199,3
37	**CKPAMTGC**	21,5/26,5	51,5	20	93,2	181,1
38	**CKPAMTGC**	21,9/27,7	50,1	20	91,5	165,5
39	**CKPAMTGCW**	21,4/21,5	52,9	19	93,2	229,3
40	**LCKPAMTGC**	21,2/23,0	45,8	21	92,7	200,3
41	**VFAVFVHF**	22,4/23,2	53.7	19	94,4	218,5
42	**SVFVHFLF**	22,7/22,8	39,4	17	90,1	155,7
43	**DPFLFYLD**	22,3/21,7	45,2	15	92,3	192,3
44	**EPFLFYLE**	22,9/21,7	48,7	14	93,8	210,5
45	**NPFLFYLN**	22,6/22,0	43,9	14	89,4	178,4
46	**FFFFRIF**	23,7/23,2	56,2	18	95,7	231,8

Table 5: Basic Characteristics of Synthetized Peptide-Affinity Chromatographic Gels

All calculated affinity peptides were high or medium hydrophobic with MW from 790 to 1057 Da. The peptide coupling to the chromatographic matrics occurred almost uniformly without statistically significant deviations and ranged from 20,5 to 23,7 mg×ml^{-1} or 21,6-27,7 μmol×ml^{-1} matrix. There was also no statistically significant difference in the residence time for maximum saturation of affinity gels with fibrinogen (17-22 min) at the buffer speeds at which the preliminary volumes of DBC were determined. Therefore, for a more objective selection of the most effective affinity peptide from groups that were determined for different areas of fibrinogen, we allowed ourselves to introduce an IE efficiency index, which was calculated from the ability to bind human fibrinogen with a certain purity shown on Figure. 3.

$$IE = \frac{DBC_{(mg\ hFg)} \times hFg_{(purity,\%)}}{Peptide\ density_{(\mu mol \times ml^{-1})}}$$

Figure 3: TIF Formula for Calculating the Affinity Peptide Efficiency Index

Thus 6 peptides No. 21 (GARHLFFV designed for Aα and Bβ hFg peptides), No. 27 (WKLLHAPF designed for 408-423 αC), No. 46 (FFFFRIF designed for 196-209 αC), No. 41 (VFAVFVHF designed for 156-165 βC), No. 39 (S-S-cycled CKPAMTGCW designed for 40-50 γC) and No. 44 (EPFLFYLF designed for 254-261 γC) were chose for synthesis of multi-point affinity-peptide chromatographic gel under the name Fg-MAP-Workbeads 40/10000 gel.

The peptides density on the Fg-MAP-Workbeads 40/10000 gel was 23,1 mg×mL^{-1} or 23,6 μmol×mL^{-1} according to averaged MW calculated from the amount of each peptide bound to the matrix. The dynamic binding capacity (DBC) with enough residence time 18-20 min for *h*Fg was between 54 and 58 mg×mL^{-1} with buffer flow rate 30 cm×h^{-1}. After crude *h*Fg fraction uploading the level of DBC was not fall down to 450 ml×h^{-1}. According to this DBC level the temperature limit of TDC was reached stability before 51 °C and thus we can carry on virus inactivation process directly in chromatographic column under 45 °C. The *h*Fg purity after H fraction processing [35] on the Fg-MAP-Workbeads 40/10000 gel was 92,7% that was quite satisfactory for affinity chromatography.

Direct in-Column Virus Inactivation/Elimination by S/D Treatment During Human Fibrinogen Purification by Multi-Peptide Affinity Chromatography

*h*Fg was partially purified from blood plasma by H-fraction separation and finally purification on ECO^{PLUS} column Ø50×120

mm (with cooling jacket) packed with 110 mL Fg-MAP-Workbeads 40/10000 gel with packing density factor 1,15-1,20 [35].

Of cause, just for model investigations of virus inactivation/elimination the *h*Fg of the H-fraction was infected by viruses and then applied on the multi-point peptide-affinity column.

Solvent/detergent (S/D) treatment of *h*Fg was performed according to scheme shown earlier and described in the "Materials and Methods" [24,28,29]. Our published dates and previous studies on model virus inactivation of streptokinase peptide (SK^{1-61}), lysozyme, fibrino(geno)lytic enzyme complex FVIII/vWF and ceruloplasmin and immunoglobulins dates have shown that proper virus removal during chromatographic purification depends on the nature of the S/D and their concentration, time and temperature of action on the protein adsorbed by the chromatographic gel [24,28,29].

After early experiments we noted that too much buffer (30 Vc) was used to completely wash out viruses that did not "infect" the protein. In the last 10 Vc we collected such small viruses quantity that bordered on the method sensitivity and in no way could affect the indicators of the virus inactivation process. Experimentally we came to the conclusion that for the first washing it was enough to apply the buffer volume equal to 20 Vc. On the other hand, there were cases of partial contamination of protein preparations with detergent, so it was necessary to increase the volume of the third wash to 40 Vc. In addition, in recent experiments with IgG it was observed that faster viruses inactivation/elimination took place at Gs phase temperature 45 °C, which requires less inactivation buffer consumption and, accordingly, process time. In this case, the time and the buffer amount on phases G*r* (increasing T°-gradient and S/D concentration), G*f* (fall down specified gradients) and G*s* (T°=45 °C, max S/D concentrations) were reduced by 2 times. Thus it was possible to significantly, almost by 2 times, reduce the time and reagents, which, accordingly, led to a significant reduction the virus inactivation process cost [29].

So, the most effective virus inactivation/elimination conditions were selected for the presented work, namely: TnBP/Triton X100 concentrations 1.0% and 2.5%, respectively; temperature 45°C and exposure to high T° for 2,5 hours (linear buffer gradient, which T° increases from 20 to 45°C during 30 min; exposure to constant buffer T° 45°C during 90 min; linear buffer gradient, which T° decreases from 45 to 20°C during 30 min).

Results of the *h*Fg S/D virus inactivation in the chromatographic column depended from process temperature are presented in Table 6.

S/D treatment stage	Virus determination by:	Decreasing the factor of virus concentration (FVD) as a $\log_{10}$ (M±SEM, n=5)					
		$BVDV^{LEV}$	$DHBV^{LEV}$	$MuLV^{LEV}$	PRV^{LEV}	CPV^{NLEV}	BEV^{NLEV}
Gr	titer	1,07±0,29	0,04±0,01	0,24±0,09	0,23±0,11	2,19±0,40	1,94±0,28
	NA	1,63±0,43	2,02±0,28	1,25±0,33	1,72±0,23	2,10±0,27	1,96±0,34
Gs	titer	3,94±0,55	0,12±0,03	0,05±0,02	0,04±0,01	3,82±0,49	3,82±0,51
	NA	7,86±0,82	7,65±0,44	8,32±0,76	7,96±0,83	7,51±0,68	7.79±0,70
Gf	titer	bsl	bsl	bsl	bsl	bsl	bsl
	NA	bsl	bsl	bsl	bsl	bsl	bsl
Target *h*Fg elution	titer	0	0	0	0	0	0
	NA	0	0	bsl	bsl	0	bsl
Whole process summed FVD	titer	5,01±0,62	0,16±0,03	1,49±0,09	0,27±0,11	6,01±0,63	5,76±0,58
	NA	9,49±0,93	9,67±0,52	9,57±0,72	9,68±0,86	9,61±0,89	9,75±0,78

Table 6: Direct Virus Inactivation/Elimination By S/D Treatment During Hfg Chromatographic Purification Process: Virus Titer, Nucleic Acid Content And Protein Quantity Functional Assessment

Note:1: Gr, Gs, Gf – process stages, more explanation at the section "Materials and Methods – In column S/D treatment…"
Note 2: NA – nucleic acid
Note 3: bsl – value was below the method's sensitivity level

For further comparison we continue to evaluate the inactivation by three or two mentioned methods in the present study. In fact, we received full repetition of previously detected pattern: the method of the virus titer determining gave an adequate result according to target protein infectivity in the case of LEV and NLEV models: 1) the LEV titer determination didn't leave the hope to calculate process kinetics since the loss in virus infectivity due to the virus particles destruction and protein denaturation and was more acceptable for NLEV infectivity mass balance determination; 2) determination of viral proteins by IFA approximately simulated the virus titer and nucleic acids measurement but could in no way to confirm the presence or absence of infectivity; 3) quantification of viral nucleic acids by RT-PCR in target proteins and buffer fractions obtained during inactivation allowed to calculate the process mass balance; 4) the present investigation demonstrated again that virus inactivation by S/D treatment of peptide/protein preparation directly in the chromatographic column cooperates two processes, namely: a) destruction of a virus particles by solvent and/or detergent (mainly for LEVs with minor effect for NLEVs) and b) dissociation and washing away of virus material which was associated with protein where the outcome that was successful both for LEVs and NLEVs [24,28,29].

In addition, the present study ambiguously showed that increasing the virus-inactivation processing temperature, leaving the protein tightly bound on the adsorbent intact (here fibrinogen retained its polymerization properties, anti-Rubella IgG retained its antiviral activity led to rapid removal/destruction model viruses [29]. Reducing processing time significantly, almost twice, reduced time and materials, reducing the cost of works.

Target Protein Yield and Activity After in-Column Treatment

Our results suggested that protein "nailed" to chromatographic gel didn't lose any activity and can be eluted from column with satisfied yield 71-76%. Small (lower than 5%) fluctuations in the target protein yield we explained by ordinary deviations in chromatographic process. Solvents and detergents were not detected in target protein preparations.

Another significant fact was the elution from the gel after high temperature virus inactivation of more purified fibrinogen. Without virus inactivation we obtained Fg with a purity of 92,7%, then after virus inactivation - with a purity of 96,4%. We assume that the buffer at a temperature of 45 °C washed out additional amounts of impurities firmly adsorbed on the gel or fibrinogen itself.

Conclusion

The liquid chromatography of proteins using specific synthetic peptides as affinity ligands seemed quite exotic until recently. All thanks to the complex multi-step search for the peptide itself, which would be sufficiently affinity to the target protein, which significantly increased the cost of the affinity gel. In the pharmaceutical industry, this "exoticism" made it possible to use this approach only for the purification of either absolutely necessary or highly valuable proteins as the basis of future medicines. But the attractive efficiency of peptide-affinity chromatography prompted the development of simpler, and therefore cheaper, approaches to the development of affinity ligands. As a result of 20 years researches, the cost of peptide affinity chromatography has become more or less acceptable with some caveats.

Jan C. Biro investigations allowed us to make the development of a peptide ligand cheaper, as it seems, to a minimum. In one of the last works it was shown that 35 high affinity peptides were developed during 12 hours by one person. Another 72 hours were spent for peptide libraries synthesis and experimental determinations of the affinity level of the calculated peptides. We really lost more time and effort for discussing results and writing that manuscript [27]. The presented work on the development of affinity peptides for human fibrinogen once again confirms that the cost of affinity gels will soon be comparable to ion exchange gels.

In addition, thanks to modern chromatographic matrices, the dynamic capacity of chromatographic gels has increased significantly, which allows temperature-dependent virus inactivation/virus elimination to be carried out directly during the chromatographic process in the chromatographic column. The protein "crucified" on the adsorbent loses the ability to denature at temperatures of 45 °C [24,28,29]. Presented method allowed to obtain for fibrinogen the factor of virus concentration decrease values that were higher than those provided by known technologies and was sufficient to fully inactivate viruses. The virus inactivation/elimination of these proteins (coupled by the gels) directly in chromatographic column shown a highly effective virus elimination (log_{10}>9) for both nonenveloped and lipid enveloped viruses.

And yet, the most important thing in the interaction between the protein and peptide ligand, as well as the development of the ligand itself, is the understanding that this interaction occurs mainly between linear amino acid sequences, and the interaction due to the conformational convergence of partner amino acids, which takes place, is most likely an additional factor. This emphasizes once again the non-randomness and the logical genetic program (sense and antisense sequences on each of the interaction partner proteins) of recognition, such as receptor recognition by the effector.

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Statements and Declaration

Ethical Approval

Not applicable.
This article does not contain any studies with human participation or animal performed by any of the authors.

Consent to Participate

Not applicable.
This article does not contain any studies with human participation.

Consent to Publish

Not applicable.

CRediT Authorship Contribution Statement

Serhiy P. Havryliuk – written the first draft of the manuscript, methodology.

Heorhii L. Volkov – written first draft and original of the manuscript.

Olena S. Havryliuk – written revue and editing.

All authors contributed to the study conception and design, material preparation, dates collection and analysis, commented on previous versions of the manuscript
All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest. The study was performed at a time when authors were employees of Neutromics Ukraine TOV and working together at the biotechnology Pilot plant at Raining (Boroo) Valley, Mongolia. The authors have no relevant financial or non-financial interests to disclose.

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The Neutromics Ukraine TOV has allowed publication in the open press with the transfer of rights on the article to the appropriate publisher.

The using of research and production facilities of the Pilot plant at the Raining (Boroo) Valley belongings to Shijir International Co. Ltd, Sukhbaatar sq., Bodi Tower building, Ulaanbaatar, Mongolia, by Neutromics Ukraine TOV is grounding on the non-finance cooperation between both parties. This agreement was signed due to the fact that as the project of Pilot plant same as all started technologies and plant staff education were designed and implemented by Neutromics Ukraine TOV according to the order by Shijir Int. Co. Ltd. The active cooperation of two companies will continue on the base that new methods and/or technologies developed by Neutromics Ukraine TOV firstly are offering for Shijir Int. Co. for buying and start-upping on the own decision.

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Declaration of Generative AI and AI-Assisted Technologies in the Writing Process.

During the preparation of this work the authors did not use ani AI and AI-assisted technologies in order tin the writing process.

Availability of Data and Materials

Not applicable.

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Tables

Affinity peptide No												
	G	P	R	V	V	E	R	H	Q	S	A	Aα peptide *h*Fg
	C	G	A	H	Y	M	R	A	L	S	N	^{655}C-N^{665} GPIIb sequence
	"Summarized" antisense peptide (amino acid position number at the peptide)											Affinity to Aα *h*Fg by pH$_{-50\%}$ (M±SEM, n=5)
	1	2	3	4	5	6	7	8	9	10	11	
1	P	G	A	H	H	L	A	L	V	R	-	3.42±0.08
2	P	G	A	H	H	L	A	L	V	A	-	3.69±0.09
3	P	G	S	H	H	L	A	L	V	R	-	3.51±0.06
4	P	G	S	H	H	L	A	L	V	A	-	3.73±0.09
5	-	G	A	H	H	L	A	L	V	R	R	3.34±0.07
6	-	G	A	H	H	L	A	L	V	A	R	3.57±0.12
7	-	G	S	H	H	L	A	L	V	R	R	3.48±0.05
8	-	G	S	H	H	L	A	L	V	A	R	3.65±0.07
Affinity peptide No												
	G	H	R	P	L	D	K	K	R	E	E	Bβ peptide *h*Fg
	C	G	A	H	Y	M	R	A	L	S	N	^{655}C-N^{665} GPIIb sequence
	"Summarized" antisense peptide (amino acid position number at the peptide)											Affinity to Bβ *h*Fg by pH$_{-50\%}$ (M±SEM, n=5)
	1	2	3	4	5	6	7	8	9	10	11	
9	P	G	A	G	H	L	F	F	A	L	-	3.46±0.07
10	P	G	A	G	H	L	F	F	S	L	-	3.55±0.10
11	P	G	S	G	H	L	F	F	A	L	-	3.61±0.08
12	P	G	S	G	H	L	F	F	S	L	-	3.72±0.11
13	-	G	A	G	H	L	F	F	A	L	L	3.36±0.08
14	-	G	A	G	H	L	F	F	S	L	L	3.44±0.10
15	-	G	S	G	H	L	F	F	A	L	L	3.53±0.14
16	-	G	S	G	H	L	F	F	S	L	L	3.68±0.09

Table 1: The Peptide Array Library for the Development High Affinity Peptides Separately to aα and bβ Peptides hfg Interacted with 655Cgahymralsn665 gpiib Sequence

Note 1: rose → light rose → yellow → light green → green colors are shown the calculated peptide affinity increasing to the Aα and Bβ Fg peptides

Note 2: blue color cells are representing the mutations A/S and R/A were caused by the presence of the amino acid serine in the sense or antisense peptides, which can express amphoteric properties in the hydropathy

p e	G	P	R	V	V	E	R	H	Q	S	A	Aα peptide *h*Fg	
		G	A	H	H	L	A	L	V	R		antisense (affinity) sequence	
	G	H	R	P	L	D	K	K	R	E	E	Bβ peptide *h*Fg	
		G	A	G	H	L	F	F	A	L		antisense (affinity) sequence	
	"Summarized" antisense peptide (amino acid position number at the peptide)											Affinity by $pH_{-50\%}$ (M±SEM, n=5) to	
	1	2	3	4	5	6	7	8	9	10	11	Aα *h*Fg	Bβ *h*Fg
17	-	G	A	R	H	L	A	L	V	-	-	3.35±0.11	3.37±0.09
18	-	G	A	R	H	L	F	F	A	-	-	3.39±0.13	3.40±0.07
19	-	G	A	R	H	L	A	F	V	-	-	3.34±0.08	3.36±0.10
20	-	G	A	R	H	L	A	L	A	-	-	3.46±0.14	3.49±0.11
21	-	G	A	R	H	L	F	F	V	-	-	3.32±0.13	3.38±0.12
22	-	G	A	A	H	L	F	F	V	-	-	3.44±0.11	3.47±0.09
23	-	G	A	L	H	L	F	F	V	-	-	3.52±0.10	3.49±0.07
24	-	G	A	Y	H	L	F	F	V	-	-	3.50±0.09	3.54±0.13

Table 2: the Secondary Peptide Array Library for the Development the Combin Affinity Peptides to aα and bβ Peptides hfg

Note 1: light rose → light green → green colors are shown the calculated peptide affinity increasing to the Aα and Bβ Fg peptides

Note 2: blue color cells are representing the different mutations for reaching the high affinity of the sequences

Peptide No.	PDWGTFE**E**VSGNVSPG	Peptide 389-404 interacted with FXIII by Smith *et al.* [48]	
	GLTPWKL**L**HAPFHAGP	Antisense sequence developed according our method [27-29]	
25	GLTPWKL**L**	3.46±0.10	Affinity by $pH_{-50\%}$ (M±SEM, n=5) to
26	TPWKL**L**HA	3.43±0.07	
27	WKL**L**HAPF	3.37±0.08	
28	CKL**L**HAPF	3.39±0.09	
29	L**L**HAPFHA	3.50±0.07	
30	HAPFHAGP	3.77±0.13	

Table 3: Six Affinity Peptides Developed to Fibrinogen α-Chain Amino Acid Sequence Interacted with FXIII

Note 1: $^{(396)415}$glutamine as a key residue shown as bold E, complementary leucine shown as bold L

Affinity peptide	E	R	F	G	S	Y	C	P	T	T	C	G	γC peptide *h*Fg [50, 52]
	Antisense peptide (amino acid position number at the peptide)												Affinity to *h*Fg by $pH_{-50\%}$ (M±SEM, n=5)
	1	2	3	4	5	6	7	8	9	10	11	12	
31	L	A	K	P	A	M	T	G	W	W	T	P	3.37±0.12
32	L	A	K	P	R	M	T	G	W	W	T	P	3.71±0.14
33	-	A	K	P	A	M	T	G	C	W	T	P	3.39±0.09
34	-	A	K	P	A	M	T	G	C	C	T	P	3.41±0.10
35	L	C	K	P	A	M	T	G	W	W	T	P	3.39±0.07
36	-	C	K	P	A	M	T	G	C	W	T	P	3.44±0.12
37	-	C	K	P	A	M	T	G	C	-	-	-	3.38±0.08
38	-	C	K	P	A	M	T	G	C	-	-	-	3.40±0.10
39	-	C	K	P	A	M	T	G	C	W	-	-	3.41±0.10
40	L	C	K	P	A	M	T	G	C	-	-	-	3.36±0.15

Table 4: Ten Affinity Peptides Developing to Fibrinogen γ-Chain Amino Acid Sequence Acebutolol and Propranolol

Note 1: rose color is shown the very low peptide affinity to Fg
Note 2: blue color cells are representing the different mutations for reaching the high affinity of the sequences

Peptide	Peptide	Chromatographic affinity peptide gels basic properties				
		Peptide density on the gel	DBC at flow rate 30 $cm \times h^{-1}$	Residence time *h*Fg max coupling	*h*Fg purity (hFg_{pur})	IE
		$mg/\mu mol \times ml^{-1}$	mg *h*Fg	min	%	
17	GARHLALV	22,4/26,8	52,4	19	88,7	173,4
19	GARHLAFV	22,2/25,5	50,8	17	90,2	181,8
21	GARHLFFV	23,1/24,4	56,5	18	94,6	219,1
27	WKLLHAPF	23,0/22,7	54,9	20	92,1	222,7
28	CKLLHAPF	22,8/24,6	53,4	22	91,8	199,3
37	CKPAMTGC	21,5/26,5	51,5	20	93,2	181,1
38	CKPAMTGC	21,9/27,7	50,1	20	91,5	165,5
39	CKPAMTGCW	21,4/21,5	52,9	19	93,2	229,3
40	LCKPAMTGC	21,2/23,0	45,8	21	92,7	200,3
41	VFAVFVHF	22,4/23,2	53.7	19	94,4	218,5
42	SVFVHFLF	22,7/22,8	39,4	17	90,1	155,7
43	DPFLFYLD	22,3/21,7	45,2	15	92,3	192,3
44	EPFLFYLE	22,9/21,7	48,7	14	93,8	210,5
45	NPFLFYLN	22,6/22,0	43,9	14	89,4	178,4
46	FFFFRIF	23,7/23,2	56,2	18	95,7	231,8

Table 5: Basic Characteristics Of Synthetized Peptide-Affinity Chromatographic Gels

S/D treatment stage	Virus determi-nation by:	Decreasing the factor of virus concentration (FVD) as a log_{10} (M±SEM, n=5)					
		$BVDV^{LEV}$	$DHBV^{LEV}$	$MuLV^{LEV}$	PRV^{LEV}	CPV^{NLEV}	BEV^{NLEV}
Gr	titer	1,07±0,29	0,04±0,01	0,24±0,09	0,23±0,11	2,19±0,40	1,94±0,28
	NA	1,63±0,43	2,02±0,28	1,25±0,33	1,72±0,23	2,10±0,27	1,96±0,34
Gs	titer	3,94±0,55	0,12±0,03	0,05±0,02	0,04±0,01	3,82±0,49	3,82±0,51
	NA	7,86±0,82	7,65±0,44	8,32±0,76	7,96±0,83	7,51±0,68	7.79±0,70
Gf	titer	bsl	bsl	bsl	bsl	bsl	bsl
	NA	bsl	bsl	bsl	bsl	bsl	bsl
Target *h*Fg elution	titer	0	0	0	0	0	0
	NA	0	0	bsl	bsl	0	bsl
Whole process summed FVD	titer	5,01±0,62	0,16±0,03	1,49±0,09	0,27±0,11	6,01±0,63	5,76±0,58
	NA	9,49±0,93	9,67±0,52	9,57±0,72	9,68±0,86	9,61±0,89	9,75±0,78

Table 6: Direct Virus Inactivation/Elimination By S/D Treatment During *h*fg Chromatographic Purification Process: Virus Titer, Nucleic Acid Content and Protein Quantity Functional Assessment

Note:1: Gr, Gs, Gf – process stages, more explanation at the section "Materials and Methods – In column S/D treatment…"
Note 2: NA – nucleic acid
Note 3: bsl – value was below the method's sensitivity level