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Direct interaction of fibrinogen with lipid microparticles modulates clotting kinetics and clot structure

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Abstract

Extensive studies revealed the role of blood lipid microparticles (liposomes, microvesicles) in activation of coagulation cascade. The direct interaction of fibrinogen/fibrin with lipid surfaces and its consequence for hemostasis received much less attention. We observed pronounced changes in both clot morphology and kinetics of fibrin clotting in the presence of artificial liposomes. The evidence was obtained that lipid microparticles per se present a diffusion barrier to the three-dimensional fibril assembling and pose spatial restrictions for fiber elongation. On the other hand, fibrinogen adsorption results in its high local concentration on liposome surface that accelerates fibrin polymerization. Adsorption induces Fg secondary structure alterations which may contribute to the abnormal clot morphology. In dependence on lipid composition and size of microparticles, the interplay of all the outlined mechanisms determines functionally important changes of clot morphology. The obtained results contribute to the knowledge of clotting mechanisms in the presence of artificial and natural lipid microparticles.

Key words: Liposomes; Fibrinogen; Adsorption; Fibrin clotting

Fibrinogen (Fg) is a 340 kDa homodimeric water-soluble protein present in human blood plasma. Fibrinogen is a key participant of hemostasis and thrombosis, forming insoluble gelling clots based on proteolytic transformation to fibrin.[1] To maintain the coagulation equilibrium both the Fg concentration in blood and its processivity are important. Significant effects on blood coagulation come from the presence of lipids.[2,3] Coagulation cascade is greatly accelerated upon the binding of coagulation factors to negatively charged lipids of membranes. Being bound to the membrane surface, coagulation proteases become thousands of times more active due to the formation of spatially arranged multi-enzyme complexes facilitating the surface-directed transfer of the activated coagulation factors between the various complexes.[2]

Apart from cell membranes, the lipids can present in the bloodstream in the form of high and low density lipoproteins and lipid microvesicles (MVs), detached from the outer cell membrane during activation and apoptosis of blood and endothelial cells. MVs are the microparticles, surrounded by a phospholipid membrane, with the size from approximately 30-100 nm (exosomes) to 1 μm (cellular MVs) or more (apoptotic bodies).[4] The main population of circulating lipid microparticles in blood is formed by the platelet MVs, released into the bloodstream under the platelets activation. It has been established that MVs in blood can influence fibrin formation, clot structure and resistance to lysis due to their participation in the generation of active thrombin and plasmin.[5–9]

Besides, there are the evidences that effect of lipid MVs on the structure and properties of fibrin clot is not limited by thrombin and plasmin generation, but also can be defined by a

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direct binding of MVs to fibrin in the process of clot formation.[7,10–16]

In addition to naturally circulating blood MVs, a similar effect on clotting can be associated with the artificial lipid particles[17] used as drug carriers[18] or platelet substitutes.[19] For instance, the evidence has been provided that synthetic lipids promote the fibrin deposition on damaged vessels.[11] The authors argued that DPPC liposomes possess significantly higher procoagulant activity than liposomes from DPPE, DPPS and their mixture with DPPC.

Despite a huge amount of research works devoted to the complex action of lipid component of blood on hemostasis and thrombosis, the studies devoted to direct interaction of fibrinogen/fibrin with lipids and lipidic surface are relatively scarce. One can anticipate that the interaction of Fg with a surface of lipid bilayer will modify the process of fibrin clotting, since the Fg secondary and tertiary structures as well as the orientation of protein molecules relative to the surface are known to affect the Fg polymerization properties.[20–23] It seems especially reasonable due to the known propensity of Fg molecules to reversible self-assembling upon concentration in solution[24] or near the surfaces.[25,26] In turn, the alteration of the fibrin polymerization kinetics evokes changes in clot porosity, fiber thickness and branching,[27] which determine mechanical stability, permeability and rate of clot lysis.[28,29]

Herein we report the structural studies of fibrin gels obtained by thrombin action on Fg in suspensions of liposomes of different composition. The nanostructure of fibrin clots is compared with zeta potential of liposomes and kinetics of fibrin polymerization and hydrolysis. The obtained results provide insight into molecular interactions of Fg with lipid particles of various origin that could affect the morphology and properties of fibrin clots.

Methods

Chemicals

Fibrinogen (Fg) from bovine plasma was from Calbiochem, thrombin from bovine plasma (Tb) and trypsin from porcine pancreas were purchased from Sigma. Rabbit brain cephalin (CPH, M.w. 768.6) (Technology standard, Russia), Egg phosphatidylcholine (EPC, M.w. 770), soy phosphatidylcholine (SPC, M.w. 758.06), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, M.w 733.6), and 1,2-dipalmitoyl-rac-glycero-3-phosphoethanolamine (DPPE, M.w. 691.96) were purchased from Sigma (USA). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] sodium salt (POPG, M.w. 770.5), 1-palmitoyl-2-oleoyl-*sn*-glycero-3 phosphocholine (POPC, M.w. 759.6), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS, M.w. 783.99) were purchased from Avanti Polar Lipids, Inc. (USA). Other chemicals were of analytical grade. Details of lipid nomenclature and structure can be found in Supplementary Material.

Preparation of solutions

Fg stock solutions were prepared in buffer 1 (20 mM Tris–HCl with 150 mM NaCl, pH 7.4) or in buffer 2 (5 mM Tris–HCl with 30 mM NaCl, pH 7.4). Tb and trypsin were dissolved in buffer 1 only. Details of solution preparation can be found in the Supplementary Material.

Liposome preparation

To test the influence of particle size on fibrin formation two kinds of liposomes, large multilamellar vesicles (MLV) and small unilamellar vesicles (SUV), were prepared according to proper protocol.[30] Details of preparation can be found in the Supplementary Material.

Turbidimetry

The kinetics of fibrin polymerization and lysis was studied by turbidimetry[31] using a Lambda 25 spectrophotometer (Perkin-Elmer). Apparent absorbance of Fg in solution or in liposome suspensions prepared on buffer 1 was monitored at 350 nm after Tb addition. Kinetics was registered at 37 °C during 1 h. Fibrin lysis was initiated after complete clot formation spreading 10 μL of trypsin stock solution on the top of clot surface.

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to investigate the structure of clots formed in the presence or in the absence of MLV particles. Clots were generated by adding Tb to Fg solution or lipid MLV in buffer 1. Final concentrations were: Fg 1 mg/ml, lipid 1 mg/ml, Tb 0.26 NIH. All details of the procedure are described in the Supplementary Material.

Zeta potential and mean hydrodynamic diameter of lipid microparticles

To examine the interaction of Fg with lipid particles we measured zeta-potential and hydrodynamic size of SUV upon titrating with Fg solution. Lipid suspensions and Fg solutions were prepared in buffer 2, centrifuged 10 min at 10,000 ×*g* and measured on a Zetasaizer Nano (Malvern Panalytical Ltd.) spectrometer at 37 °C following the published recommendations.[32,33] Particle polydispersity index (PDI) retains the constant value of 0.08 ± 0.02 over the entire set of data. Detailed description of measurement procedure can be found in the Supplementary Material.

FTIR spectroscopy

Fourier-transform infrared (FTIR) spectra were recorded using IR Affinity-I FTIR spectrometer (Shimadzu) equipped with attenuated total reflectance (ATR) accessory with ZnSe single bounce optical element and flow cell. MLV and Fg solutions prepared on buffer 2 were flowed sequentially above the ZnSe element at 37 °C. Spectra of SUV adsorbed on ZnSe, and Fg adsorbed on the liposomes were sequentially measured and subtracted to obtain pure spectra of Fg adsorbed on liposomes. Non-adsorbed MLV and Fg were washed out. Spectra of free Fg dissolved in buffer 2 were obtained in transmission mode using two CaF_2 plates and 6 μm thick ring-shaped spacer.

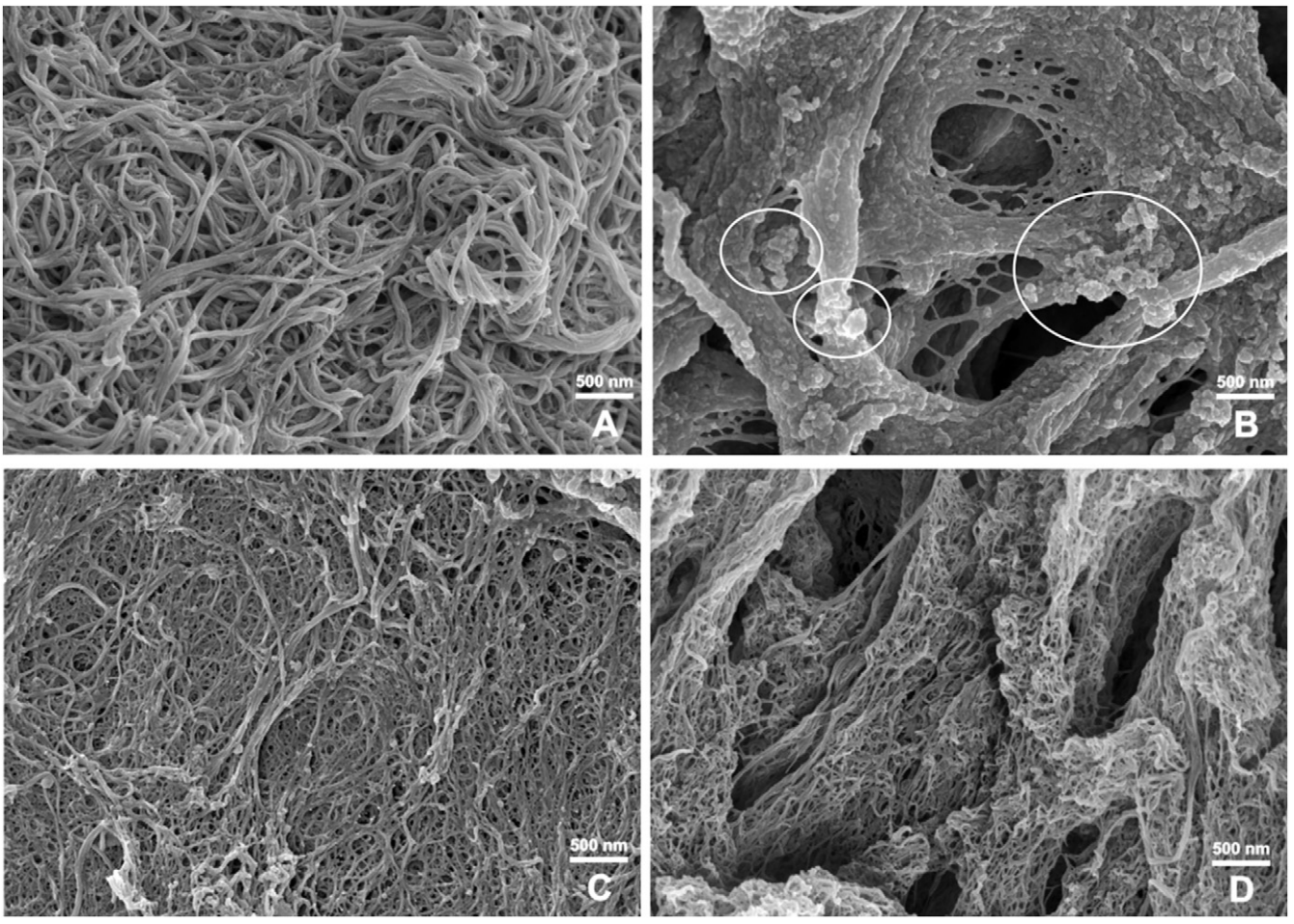

Figure 1. **SEM images of fibrin clotted in the presence of MLV**: (**A**) control, (**B**) DPPC, (**C**) CPH, (**D**) DPPC/10%POPG. DPPC MLV aggregates, imbedded into clot structure, are outlined by circles.

Statistical analysis

Descriptive statistics are given as mean ± standard error of mean. Statistical analysis was performed using Origin 8 (OriginLab, MA, USA).

Results

Lipid MVs influence fibrin clot structure

SEM images reveal that the structure of lipids in the studied model systems has a significant impact on the morphology of fibrin gels. In the absence of lipids (control), fine-porous gels are formed, composed of long homogeneously packed fibers of medium thickness (Figure 1, *A*). In the presence of MLV made of EPC or CPH, the structure of the gels looks somewhat denser and more branched, but in general does not differ much from the control one (Figure 1, *C*). The lipid particles are not retained in the gel structure and are easily removed during washing. In contrast, in the DPPC MLV suspension the heterogeneous large-pore gels are formed with thin branched fibers and lipid particles incorporated into the gel structure (Figure 1, *B*). The images reveal many free fiber terminations. Fibers appeared to be highly adhesive forming dense mats and tight bundles. However, it is sufficient to add only 10% of negatively charged lipid POPG to the DPPC liposomes to obtain a radically changed structure of the lace-like network of the thin non-adhesive branched fibers (Figure 1, *D*). The fibers are combined in loose bundles free of lipid particles with large pores between them. From these results, we conclude that lipid particles indeed could have an impressive influence on fibrin architecture.

Lipid MVs influence kinetics of fibrin polymerization

The kinetics of fibrin polymerization displays the diversity of effects in the presence of lipid particles depending on their size and structure (Figure 2). In pure Fg solution the turbidity curve has the shape typical for fibrin formation.[27] Tb addition initiates Fg cleavage and formation of fibrin-monomers prone to spontaneous association. A distinct lag period is observed which corresponds to the time required for diffusion of fibrin monomers and formation of double-stranded protofibrils which account for initially weak turbidity growth. This lag-time is followed by a rapid rise in turbidity resulted from a lateral aggregation of protofibrils that have reached a certain minimum length. Finally, the turbidity is stabilized at the plateau level and

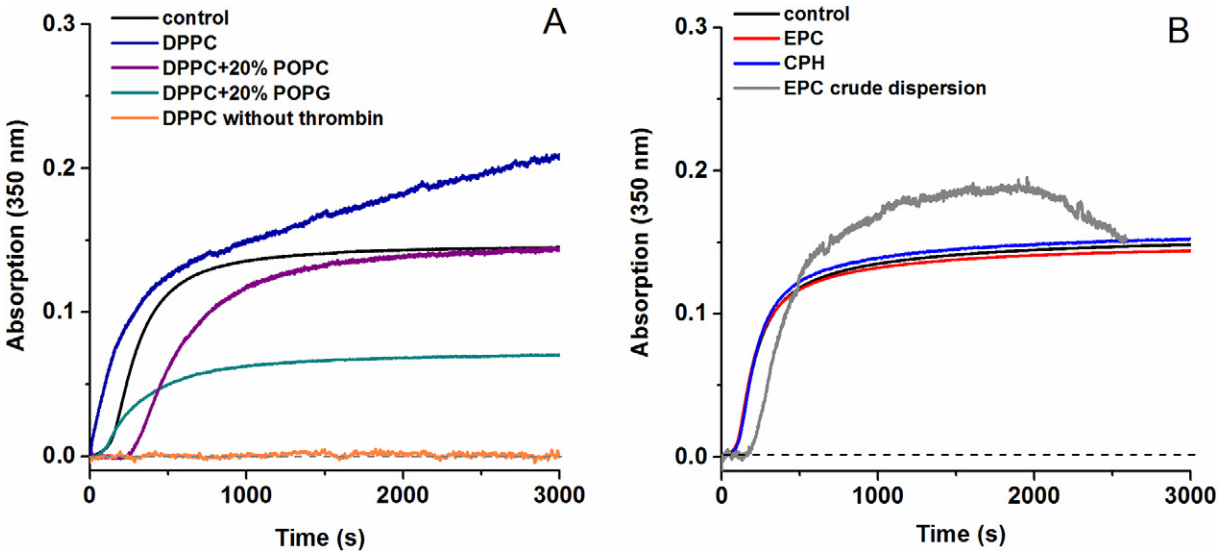

Figure 2. **Time course of optical density at 350 nm in fibrinogen / lipid MLV suspensions after thrombin addition**. (**A**) MLV made from synthetic lipids (pure DPPC and DPPC with 20% POPC or 20% POPG added). (**B**) MLV made of natural lipids (EPC or CPH) and crude dispersion of EPC. Control: without lipids. Concentration of components: [L] = 1.3 mM, [Fg] = 1.5 mkM, [Tb] = 0.26 NIH.

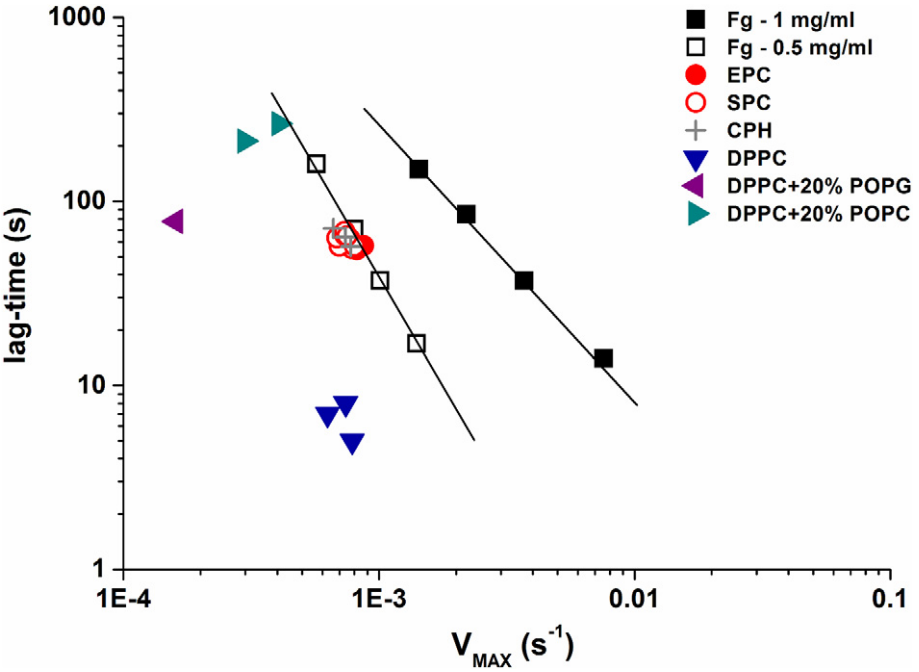

Figure 3. Correlations between the duration of lag period and the maximum rate of turbidity growth in fibrinogen solutions (open and closed squares, points refer to Tb activity 0.13, 0.26, 052 and 1.33 NIH from top to bottom) and in the lipid MLV suspensions (colored symbols). Concentration of components in lipid suspension: [L] = 1.3 mM, [Fg] = 1.5 mkM, [Tb] = 0.26 NIH.

its value is related directly to the thickness of fibers.[27] Among the tested lipids the most pronounced effect has been shown by DPPC. In comparison with control clotting in the absence of lipids, the lag-time in the DPPC MLV suspension is reduced to nearly zero and the stage of steep growth of turbidity is followed by its linear increase, instead of leveling-off (Figure 2, *A*). The addition of 20% of anionic POPG to DPPC returns the lag-time close to the control value, but lowers the maximal rate of turbidity growth and the plateau level. The addition of a 20% of zwitterionic POPC to DPPC extends the lag-time well above the control value with a significant lowering of the fibrin formation rate. In contrast to MLV, the suspension of DPPC SUV does not reveal any noticeable effects on the kinetics of turbidity growth (data not shown) which points to the significance of particle size.

The influence of EPC and CPH MLV on the time-course of turbidity is minimal and consists, if ever, in small variations in the lag

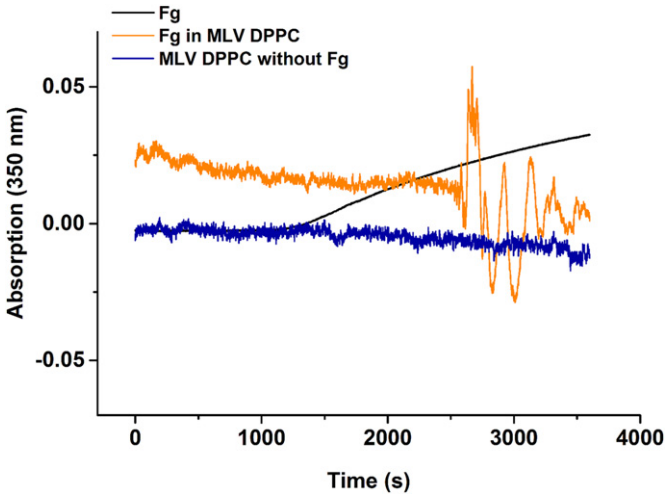

Figure 4. **Turbidity changes during fibrin polymerization at low concentrations of Fg in solution and DPPC MLV suspensions**. Concentration of components: [L] = 1.3 mM, [Fg] = 0.15 mkM, [Tb] = 0.26 NIH.

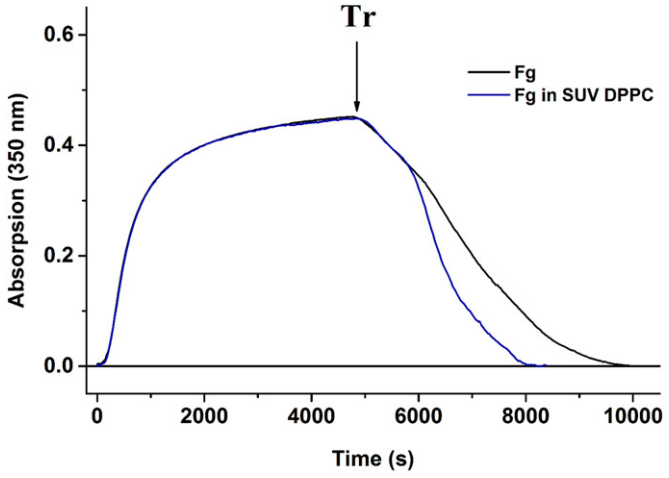

Figure 5. **Turbidity changes in the course of fibrin formation and subsequent dissolution**. Fibrin clotted by Tb with or without DPPC SUV and lysed by trypsin. Arrow points to the moment of trypsin (Tr) addition. Concentration of components: [L] = 1.3 mM, [Fb] = 1.5 mkM, [Tb] = 0.26 NIH.

time duration, the growth rate and the plateau level (Figure 2, *B*). No significant difference was found in the fibrin polymerization kinetics between MLV and SUV made of these lipids. However, in a crude EPC suspension (not exposed to ultrasound), a significant increase in the lag-time and slow fluctuations of scattering at a plateau were observed (Figure 2, *B*).

The formation of fibrin gel is a complex process. The most significant factors affecting the polymerization kinetics in solution are Tb and Fg activities which generally may be changed in the presence of lipid particles. To test this assumption, we compared the ratios between the lag-time and the turbidity growth rate for Fg in solution and in suspensions of liposomes (Figure 3). In the Fg solution both parameters are strongly correlated forming the linear dependence on the Tb concentration — the rate is higher and the lag period is shorter in solutions with higher Tb activity. The decrease of Fg concentration shifts the entire dependence to the left, i.e. to the region of lower rates. In the presence of MLV from EPC, SPC and CPH the data points are grouped close to the corresponding control line. That means that the MLV from natural unsaturated lipids do not have noticeable effect on the kinetics of fibrin formation. In the presence of MLV from DPPC or its mixtures with POPC and POPG the corresponding data shift to the left from the control dependence, indicating the decrease of fibril growth rate due to the formal diminution of Fg concentration in solution. Besides, in the MLV suspension from DPPC a significant reduction of lag period is observed, which formally can be attributed to an increase in the Tb activity.

The kinetics of fibrin clotting at very low Fg concentrations provided the additional insight to the role of lipids in this process. At the 10 times lower Fg concentration in the lipid-free solution the delayed coagulation is observed — the lag period increases and the coagulation rate decreases to an order of magnitude due to the increase in diffusion time of reaction components[27] (Figure 4). Nevertheless, a stable spatial network of very thin

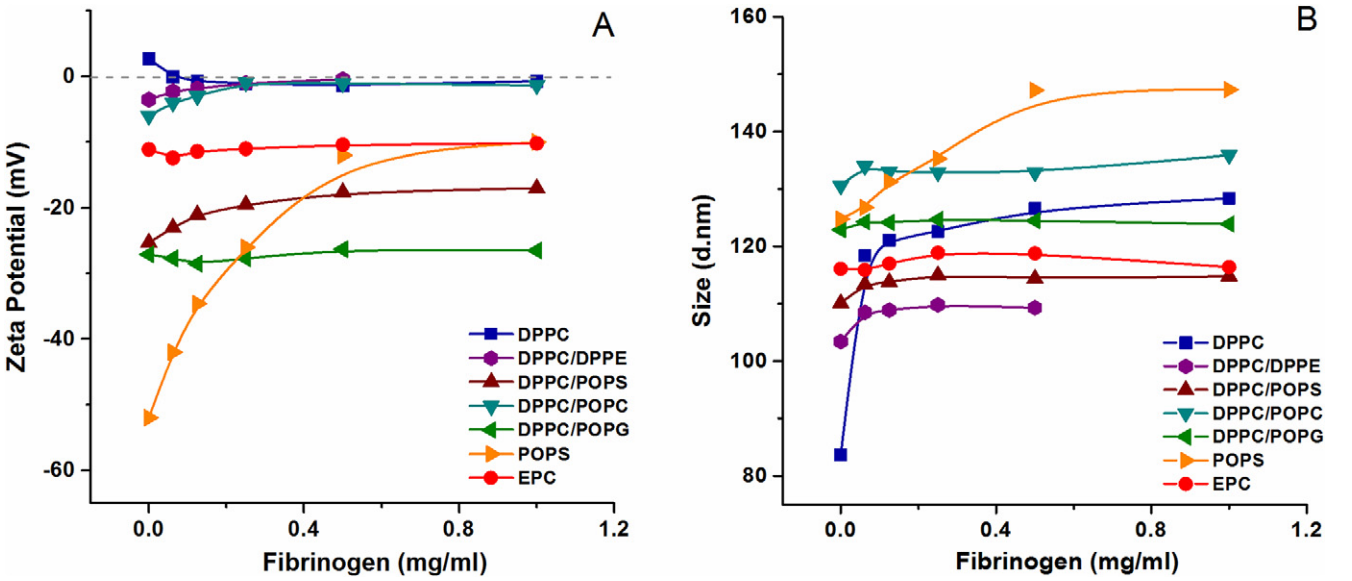

Figure 6. **Dependence of zeta potential (A) and size (B) of lipid SUV on Fg concentration in suspension**. Concentration of components: [L] = 1.3 mM.

fibrin fibers is formed that is evidenced from the low turbidity level attended at a plateau. In the DPPC MLV suspension the lag-time is approximately doubled and instead of steep turbidity growth the scattering fluctuations arise. The lag time elongation indicates a further decrease of Fg concentration in solution, probably due to the binding to liposomes. Intense scattering fluctuations point to adhesion of lipid particles on the fibers of growing fibrin network. We believe that fluctuations arise when the forming weak fibrin fibrils is disturbed by thermal movement of bound lipid particles. This effect looks especially probable bearing in mind the adhesive nature of DPPC particles outlined in the SEM results.

Thus, among the factors mediating the influence of lipid particles on fibrin formation, we suggest the significance of at least two nanoscaled mechanisms: the Fg adsorption on lipid surface and the adhesion of lipid particles on fibrin fibers.

Decreased fibrinolytic resistance of fibrin clots in the presence of lipid MVs

The presence of lipid particles also affects the gel lysis. Figure 5 shows an examination of the turbidity kinetics upon the fibrin clotting and lysis in solution and in the DPPC SUV suspension. Despite the absence of visible difference in the clotting kinetics, there is a clear evidence of impact of the DPPC SUV on the clot lysis by trypsin. The specificity of trypsin is similar to that of plasmin,[34] so the lysis kinetics characterizes the overall availability of Fg tryptic sites to the proteinase. After the spreading of trypsin over the clot surface, the decrease of optical density begins almost immediately, but proceeds non-uniformly. Initially, the kinetics falls down slowly, but after 20-30 min the process is accelerated such that the distinct bend appears on the curve. With the small variations in the duration and steepness, this shape was reproduced in all lysis experiments with the control clots. In the presence of the DPPC SUV the lysis also proceeds in two stages. However, while during the initial lysis stage the clotting behavior is close to the control one, the second stage develops distinctly faster. It seems plausible that reduced resistance of fibrin to lysis is also related to Fg adsorption on the surface of SUV.

Adsorption of fibrinogen on lipid MVs

The interaction of Fg with liposomes in solution was studied by DLS using the SUV made from pure and mixed lipids. The zeta potential data show that liposomes of different composition carry different effective electric charge (Figure 6, *A*). For most of them zeta potential has a negative sign. The only exception is pure DPPC SUV, which have a small positive zeta potential despite the zwitterionic nature of DPPC head group. The addition of 20% of anionic POPG to DPPC shifts the zeta potential to −30 mV because of excess of negative charges introduced. It is noteworthy that the addition of zwitterionic POPC to DPPC also shifts zeta potential to negative values though to the less extent. The last two lipids have identical head groups but differ in saturation degree of fatty acid tails. These data illustrate that the effective charge of the bilayer formed by neutral zwitterionic lipids is generally non-zero due to surface adsorption of counterions and depends on the ratio of saturated and unsaturated fatty acid tails[35] and conformation of head groups.[36,37] Upon the adsorption of any ligand the counterions will be displaced out of the surface that is accompanied by zeta potential variations.[38] Indeed, the titration of Fg into lipid suspension alters zeta potential revealing the adsorption of Fg on SUV. At pH 7.4 Fg carries a net negative charge,[39] however, as follows from the data, the charge itself does not have a decisive influence on the interaction of Fg with lipid bilayer. Fg is adsorbed on the negatively charged POPS SUV even more effectively than on

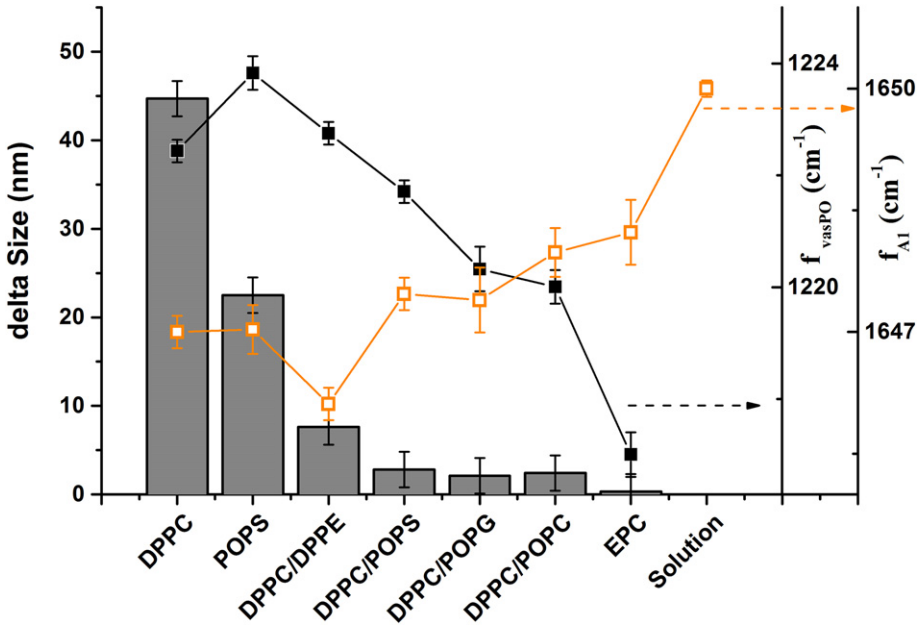

Figure 7. **Correlation between the adsorption capacity of LUV, their surface hydration and alterations of Fg secondary structure for different lipid compositions**. Difference between the effective particle diameters in suspension without Fg and at the maximum protein concentration for various lipid SUV (bars). Frequency position of lipid v_{as}PO band (black squares). Frequency position of Fg amide I band (orange squares).

the less charged SUV from zwitterionic EPC or mixed DPPC/DPPE and DPPC/POPC. At the same time, data reveal only minor Fg adsorption on the negatively charged DPPC/POPG SUV. Thus, the specific structure of bilayer surface, formed by polar lipid head groups, is important as well as the orientation of Fg molecule relative to lipid surface due to heterogeneously distributed charge patches on Fg surface.[39]

Figure 6, *B* shows the dependence of effective hydrodynamic diameter of lipid particles on Fg concentration in solution. In most cases, there is an increase of particle size concomitant with the shift of their zeta potential to less negative values. In turn, for liposomes exhibiting no dependence of size on the Fg concentration, the constancy of zeta potential is observed. The DPPC again behaves notably demonstrating the fast increase in hydrodynamic diameter of lipid particles along with a switching of zeta potential from positive to negative values at very low Fg concentration. It is worth to note that the effective particle size increased mantaining low value of PDI (See Methods), which proves the homogeneity of suspension. The uniform increase of particle size with Fg concentration implies the Fg adsorption on the particle surface rather than particles aggregation. The difference between the effective particle size in a suspension without Fg and at its saturating concentration allows us to assess the degree of Fg adsorption on liposomes (Figure 7). The data exhibit very different Fg affinities to lipid surfaces of varying composition. The highest affinity is observed for the pure DPPC SUV, which is greatly diminished upon mixing with other lipids.

To check the possibility that high speed centrifugation employed may distort the actual size distribution, we performed the same measurements on SUV suspensions not subjected to centrifugation step. As it follows from Figure S1, the overall tendency is preserved except more scattered data in suspensions of DPPC and DPPC/DPPE, more prone to aggregation.

Secondary structure of adsorbed Fg

Seeking for possible reasons of the lipid structure influence on the fibrin clotting properties we examined the secondary structure of Fg upon adsorption on SUV. The FTIR technique has long been established as a powerful analytical approach to investigate protein secondary structure.[40,41] The overall shape and position of amide bands depend on the protein secondary structure content.[42] Indeed, FTIR spectra of Fg in the range of peptide absorbance demonstrate subtle but distinct differences in amide I (centered at ca. 1650 cm^{-1}) as well as in amide II (at ca. 1550 cm^{-1}) band shape and position wavenumber (Figure S2). We used the amide I band position as an overall characteristic of the Fg secondary structure alteration upon its adsorption on liposomes (Figure 7). It follows that the Fg in solution has the amide I band position at 1650 cm^{-1} due to the large helical content. Upon adsorption on the lipid surface the band shifts to lower wavenumbers with the concomitant increase of bandwidth as a result of the growth of the non-helical structures content. The peptide spectral changes look as specific for different lipids, so we tried to correlate them with the spectral features relevant to lipid properties. The most convincing correlation was revealed between adsorption efficiency, amide I and lipid phosphate v_{as}PO band positions. It was shown[43] that the phosphate band shifts to lower wavenumbers in more polar environments. Because the polarity is mainly determined by the hydration of lipid headgroups,[44] we may conclude that the alteration of Fg secondary structure is more pronounced upon adsorption on the less hydrated lipid surface.

Discussion

It has been long recognized that besides the blood cells also the lipid microparticles (liposomes, microvesicles), normally circulating in blood, participate in maintaining the hemostasis, but the true mechanisms are far from being completely elucidated. In this study, we focused on the direct interaction of Fg with artificial liposomes of different composition and evaluated clot structure, kinetics of clotting and the lysis of fibrin in the presence of liposomes. We used purified Fg and Tb for clotting and trypsin for clot lysis to bypass the complicating effects of active Tb and plasmin generation.

Scanning electron micrographs show the discernible effect of lipid MLV on fibrin structure. Both individual fibrils thickness and branching, and overall fibrin density and porosity varied widely upon lipid composition. The particular morphological features as well as the general clot appearance display close similarity with fibrin networks reported for various dysfibrinogenemia,[16,27,28,45–47] pointing to the modified Fg reactivity.

Because of established close relationship between the fibrin morphology and clotting kinetics[27] we examined the kinetics of turbidity evolution upon fibrin clotting in the lipid suspensions. The data reveal the diversity of effects imposed by the lipid particles including either the drastic shortening or enormous extension of lag period, the slowing down of maximal growth rate, the continuously rising or unstable plateau level. The analysis of correlations between lag time and growth rate points to the apparent diminution of Fg concentration in lipid suspensions, suggesting that some of Fg molecules become catalytically inert, obviously due to adsorption on the particles. In extremely low Fg concentrations in suspension of DPPC MLV, the partial fall-out of Fg molecules from polymerization process became clearly evident leading to enormous elongation of lag period and formation of unstable fibrin network associated with lipid particles.

On the other hand, in the DPPC MLV suspensions with normal Fg concentration, despite somewhat lowering of maximal growth rate, the overall duration of fibrin formation shortens due to the collapse of lag time. The reduction of lag time may equally result from the increase of Tb activity or Fg concentration. Both of the possibilities find simultaneous explanation if we suggest that the apparent hyperactivity of Tb in DPPC suspensions stems from the increased local concentration of Fg on the surface of DPPC MLV.

Indeed, the zeta-potential and size measurements in the SUVs/Fg suspensions reveal that the Fg adsorbs on lipid particles with the adsorbed amount is strictly dependent on the lipid structure (See Scheme S1). The highest adsorption is observed for the pure DPPC particles. The mixing of DPPC with either lipid among the tested ones diminishes the adsorption dramatically. The FTIR measurements reveal that the Fg adsorption correlates negatively with the extent of lipid surface hydration which in turn depends on the degree of unsaturation of fatty acid chains. The DPPC, which possessed the fully saturated tails, forms the bilayer surface with least hydration and highest affinity of Fg. The appearance of one double bond in POPS increases the bilayer hydration and reduces the Fg adsorption. EPC contains the fatty acid residues with the great variety of length and unsaturation and forms the most hydrated bilayer with least affinity to Fg molecules. Comparing data in the Table S1 and Figure 7 we may deduce that the hydration of pure lipid bilayers correlates with bilayer fluidity, because bilayers with lower transition temperature are more fluid at a given temperature.[44]

It may be further deduced that the admixing of lipids with unsaturated fatty acid chains to DPPC results in more hydrated and, hence, more fluid surface[48] which adsorbs Fg poorly.

It should be concerned about the influence of DPPE, which is also the lipid with fully saturated tails. Due to the ability of its polar head groups to form strong hydrogen bonds between the adjacent DPPE molecules, the surface of pure DPPE bilayer is poorly hydrated.[49] Nevertheless, being admixed to DPPC bilayers the DPPE increases overall bilayer hydration because of bringing additional water binding sites which accounts for the diminished Fg adsorption on the mixed DPPC/DPPE liposomes.

Previously it was evidenced that the Fg adsorption could modulate its reactivity.[50–52] At low extent of surface coverage, the Fg molecules are adsorbed in parallel to lipid surface (side-on orientation), which results in limited availability of Fg restriction sites for the Tb action. These Fg molecules do not participate in fibrin formation. At the high degree of surface coverage the Fg molecules are oriented mostly perpendicularly to the surface (end-on), maintaining high availability for thrombin.

Fg adsorption creates a new entity — high local concentration of Fg on lipid surface that manifests itself through initial turbidity burst due to fast fibrillation of surface bound Fg. The conformational alterations accompanying the adsorption process may result as well in the altered clotting properties. In the case of sufficiently large particles or high particle concentration the effects became noticeable. Among the tested lipids, the DPPC MLV represents an extreme case, where the sufficiently large dimension of particles, high Fg affinity to the DPPC surface and concomitant conformational changes have been brought together resulting in the abnormal clot morphology. The less adsorbing DPPC/POPC particles simply pose a barrier, which retards the fibril aggregation. Although our data have shown that the negatively charged DPPC/POPG MLV repel Fg, the more complicated interactions may develop after Tb action. The cleavage of fibrinopeptides A and B decreases the overall Fg negative charge dramatically. The resulting redistribution of positive and negative charges between Fg domains may have even more important consequences, raising the possibility of negatively charged particles interfering with clot formation.[53]

The dimensions of lipid particles represent another factor, having noticeable impact on the fibrin clot structure. Influence of size of non-adsorbing EPC particles on clotting kinetics differs in case of large particles in crude suspension and after homogenization by ultrasound down to small sized MLVs (Figure 2, *B*). Small-sized EPC MLVs do not influence kinetics at all. Large-sized particles in crude suspension modify kinetics simply posing spatial barriers to diffusion and fibril propagation. This results in elongation of lag time, in modification of the fibrin fibers thickness and length and in the increasing of branching points.

Thus, the composition and the size of lipid MVs could have an impact on the fibrin polymerization. Apart from enzymatically mediated coagulation and lysis, the direct nonspecific fibrin–lipid interactions contribute significantly to the MVs hemostatic potential, which should be taken into consideration upon the engineering of artificial lipid particles or interpreting the clotting deceases.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2019.102098.

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