Emicizumab improves the stability and structure of fibrin clot derived from factor VIII-deficient plasma, similar to the addition of factor VIII

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Running title; Emicizumab-induced fibrin clot

Types of article; Original Article

Support; This work was partly supported by a Grant-in-Aid for Scientific Research (KAKENHI) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) to KN (18K07885).

Statement of prior presentation; An account of this work was presented at the XXVII Congress of the International Society on Thrombosis and Haemostasis**,** 2019, Melbourne, Australia.

Word counts; Abstract: 236, text; 2,991: Reference: 31, Figure: 7

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Abstract

Introduction. Emicizumab is an anti-factor (F)IXa/FX bispecific antibody, mimicking FVIIIa cofactor function. Emi prophylaxis effectively reduces bleeding events in patients with hemophilia A. The physical properties of emicizumab-induced fibrin clots remain to be investigated, however. ***Aim.*** We have investigated the stability and structure of emicizumab-induced fibrin clots. ***Methods.*** Coagulation was initiated by activated partial thromboplastin time (aPTT)-trigger and prothrombin time (PT)/aPTT-mixed trigger in FVIII-deficient plasma with various concentrations of emicizumab or recombinant FVIII. The turbidity and stability of fibrin clots were assessed by clot waveform and clot-fibrinolysis waveform analyses, respectively. The resulting fibrin was analyzed by scanning electron microscopy (SEM). ***Results.*** Using an aPTT-trigger, the turbidity was decreased and the fibrinolysis times were prolonged in the presence of emicizumab dose-dependently. Scanning electron microscopy imaging demonstrated that emicizumab improved the structure of fibrin network with thinner fibers than in its absence. Although emicizumab shortened the aPTT dramatically, the nature of emicizumab-induced fibrin clots did not reflect the hypercoagulable state. Similarly, using an PT/aPTT-mixed trigger that could evaluate potential emicizumab activity, emicizumab improved the stability and structure of fibrin clot in a series of experiments. In this circumstance, fibrin clot properties with emicizumab at 50 and 100 μg/ml appeared to be comparable to those with FVIII at ~12 and ~24-32 IU/dl, respectively. ***Conclusion.*** Emicizumab effectively improved fibrin clot stability and structure in FVIII-deficient plasma and the physical properties of emicizumab-induced fibrin clots were similar to those with FVIII.

Key words; Emicizumab, Scanning Electron Microscopy, fibrin clot structure, hemophilia A, fibrinolysis

Introduction

Hemophilia (H)A results from a deficiency or defect of factor (F)VIII procoagulant protein, and is the most common of the severe inherited bleeding disorders. The development of regular prophylactic therapy using FVIII products has dramatically improved quality of life in these patients [1]. The need for frequent intravenous infusions of FVIII, however, presents a mental and physical burden especially for pediatric patients [2]. Moreover, the development of anti-FVIII inhibitor antibodies renders standard FVIII supplementation ineffective and makes hemostatic treatment difficult to predict in these patients [3].

In this context, emicizumab, a recombinant, bispecific monoclonal antibody that binds to FIX/FIXa and FX/FXa and mimics the cofactor activity of FVIIIa in the tenase complex was developed [4,5]. This antibody mediates an appropriate conformational structure on FIXa-catalyzed activation of FX. In phase 1, 1/2 and 3 clinical trials, once-weekly, bi-weekly or tetra-weekly subcutaneous administration of emicizumab substantially reduced bleeding episodes in patients with HA (PwHA), regardless of the presence of inhibitors [6-12]. The antibody has already received regulatory approval for routine prophylaxis in PwHA with and without inhibitors in US, EU, and Japan.

Unlike FVIII, emicizumab does not require activation by thrombin, and the shortening effect of the antibody on the activated partial thromboplastin time (aPTT) is more pronounced than that of FVIII [4,6,13]. The results obtained in aPTT-based clotting assays, therefore, do not directly reflect potential emicizumab activity. Alternatively, comprehensive coagulation assays including clot waveform analysis (CWA) [14], thrombin generation assay [15], and rotational thromboelastometry [16] have been reported as useful techniques for assessing hemostasis in these circumstances. Since these comprehensive assays are dependent on a variety of trigger reagents, however, the results may not represent precise physiological aspects hemostasis. It could be informative, therefore, to examine the relationship between emicizumab activity and the physical properties of fibrin clots derived from PwHA.

Fibrin is formed by thrombin-mediated conversion of fibrinogen, and the specific concentrations of thrombin and fibrinogen are the most important variables influencing fibrin clot structure [17]. Several groups have analyzed the nature of fibrin by scanning electron microscopy (SEM) in the presence of depressed thrombin generation in blood from severe PwHA [18-20]. For example,

Antovic *et al*. [19] demonstrated that fibrin clot structure in plasma from severe PwHA was porous and composed of thick and short fibers, prone to fibrinolysis. The administration of FVIII to these patients resulted in a compact fibrin clot structure comprising thinner fibers, and relatively resistant to fibrinolysis. Emicizumab is known to mimic FVIIIa cofactor function, but the physical properties of emicizumab-induced fibrin clots remain to be examined. Here, we investigated the stability and structure of emicizumab-induced fibrin clots in plasma from PwHA using comprehensive functional assays and SEM.

Materials and Methods

Reagents **-** Recombinant FVIII (Advate®; Takeda Japan, Tokyo, Japan), FVIII-deficient plasma from PwHA (George-King, Overland Park, KS), and recombinant tissue-type plasminogen activator (tPA; American Diagnostica Inc., Stamford, CT) were purchased from the indicated vendors. aPTT reagent (Thrombocheck®APTT-SLA; Sysmex Corp., Kobe, Japan) and PT reagent (Revohem®PT; Sysmex) were used as sources of ellagic acid and tissue factor, respectively. We confirmed that FVIII activity was <1% in three different batches of FVIII-deficient plasma using a one-stage clotting assay (data not shown).

Turbidity of fibrin clot utilizing CWA **-** FVIII-deficient plasma was mixed with emicizumab (0-100 μg/ml) or FVIII (0-100 IU/dl), and clot waveforms were examined using the CS-2400™ analyzer (Sysmex) with aPTT- or PT/aPTT-mixed trigger reagents (PT/aPTT/buffer, 1:15:135 ratio; v:v:v) [14]. Transmitted light intensity was recorded every 0.1 sec at a 660 nm wavelength to monitor the process of fibrin formation [21]. The clot waveforms were computer-processed using the commercial kinetic algorithm (Figure 1A). The horizontal axis shows the running time (sec), and the vertical axis shows the transmittance (%) defined as transmitted light intensity from the pre-coagulation to post-coagulation phase. The clot time, reflecting the pre-coagulation phase, was defined as the time from the addition of the trigger reagent to the point where the transmittance reduced to a predefined level. The coagulation phase was defined as the time from the beginning of fibrin formation to the point of minimum transmittance. The post-coagulation phase was defined as the period of stable transmittance after the end of fibrin formation. The difference in transmittance from the initial level to the post-coagulation phase was regarded as 'Turbidity' of the fibrin clot.

Clot stability utilizing clot-fibrinolysis waveform analysis (CFWA) **-** We have recently described

a novel CFWA to simultaneously assess fibrin formation and clot lysis [22]. FVIII-deficient plasma mixed with emicizumab (0-100 μg/ml) or FVIII (0-100 IU/dl) (50 μl) was mixed with aPTT- or PT/aPTT-trigger reagent (50 μl) prior to incubation with a solution of $CaCl_2$ (50 μl) containing r-tPA (final concentration; 0.63 μg/ml). The resulting waveforms were computer-processed using the commercial kinetic algorithm, and the pre-coagulation and coagulation phases were defined as for the standard CWA method. The post-coagulation phase was followed by an increase in transmittance, however, reflecting fibrinolysis induced by the added r-tPA. In the present study, the stability of the fibrin clot during this fibrinolysis phase was determined using a previously established parameter 'Lys50 (sec)' [23], represented by the time from minimum %transmittance to that at 50% of the maximum (*see* Figure 2B).

Preparation of fibrin clots - Emicizumab (100 μg/ml) or FVIII (0, 30, and 100 IU/dl) was added to FVIII-deficient plasma (50 μl) and preincubated for 3 min in the analytical cuvette with either aPTT- or PT/aPTT-mixed trigger reagent (50 μl) to ensure adequate activation of the contact clotting factors. Coagulation was initiated by the addition of 0.02 M $CaCl_2$ (50 μl), and fibrin formation was examined 60 sec after the post-coagulation phase (*see* the CWA). After clot formation was confirmed, an equal amount of 2% glutaraldehyde in phosphate-buffered saline (PBS) was added at 4°C for fixation overnight.

Scanning electron microscopy (SEM) - Fibrin clots were transferred into glass vials containing a poly-*L*-lysine-coated glass slide. If necessary, the clots were dissected into several pieces beforehand. The fibrin was adhered to the glass by centrifugation at 700×*g* for 5 min. Samples were washed twice with PBS for 5 min, and post-fixed with 1% osmium tetroxide in PBS at 4°C for 45 min in the dark. The fixed samples were washed twice with PBS for 10 min, dehydrated serially in 50, 70, and 90% ethanol and three times with 100% ethanol, and then incubated with 50% *t*-butyl alcohol at 30°C for 5 min and with 100% *t*-butyl alcohol at 30°C for 30 min. The samples were frozen at 4°C for 10 min, and freeze-dried. The samples were mounted with Aquadiag®E Collodai Graphite (TED PELLA. Redding, CA) and coated with osmium. Five images of each sample were captured at ×30,000 magnification using the HITACHI-SU8020 (Hitachi High-Technology, Tokyo, Japan).

Data analysis - The data are presented as the average and standard deviation of three separate experiments. Measurements of fibrin diameters were obtained using Image-Pro Premier (ver. 9.0,

Media Cybernetics, Rockville, MD). One-way ANOVA was performed on experiments with n ≥3 using GraphPad Prism software (GraphPad Software, La Jolla, CA). *P*-values of <0.05 were considered to be statistically significant.

Results

Turbidity of aPTT-triggered fibrin clots in FVIII-deficient plasmas with emicizumab - To investigate the physical property of emicizumab-triggered fibrin, fibrin polymerization was initially examined as an indirect assessment of fibrin clot by monitoring the changes in turbidity of fibrin clot during aPTT measurements. Previous studies have indicated that the size and density of fibrin fibers can be determined from turbidity measurements [24]. In our studies, we used CWA to define the change of turbidity as the difference in transmittance (%) from initial to post-coagulation phases in clot waveforms. Hence, increased turbidity in the presence of thicker fibers is reflected in reduced transmittance. Representative aPTT-based CWA curve and turbidity calculation in FVIII-deficient plasma with FVIII (0-100 IU/dl) or emicizumab (0-100 μg/ml) are shown in Figures 1A-C, respectively. The presence of FVIII significantly decreased the change of turbidity compared to its absence (p<0.001), consistent with the earlier report [25]. The effects of FVIII were dose-dependent, supporting the presence of formation of thinner fibrin fibers. The addition of emicizumab also resulted in a significant reduction in turbidity change relative to the FVIII-deficient state (p<0.001), and the effect of the antibody was dose-dependent.

Stability of aPTT-triggered fibrin in the presence of emicizumab - The physical properties of fibrin were also evaluated as 'stability' by monitoring changes in transmittance mediated by fibrinolysis in the presence of exogenous r-tPA (CFWA). Lys50 was defined as a parameter of stability, and was recorded as the period from maximum change of %transmittance (completion of fibrin clot formation) to the recovery to 50% of the maximum change [23] (Figure 2C). A representative fibrinolytic curve in aPTT-triggered CFWA in FVIII-deficient plasma in the presence of various amounts of FVIII and emicizumab are shown in Figures 2A and 2B, respectively, and the Lys50 value calculated in each sample is shown in Figure 2D. Even the addition of low level of FVIII (10 IU/dl) significantly increased Lys50 relative to the FVIII-deficient state (p<0.001), in keeping with the generation of more stable fibrin in the presence of FVIII [19,22]. The presence of emicizumab also resulted in a significant increase in Lys50 relative to the FVIII-deficient state (p<0.001).

SEM images of aPTT-triggered fibrin in FVIII-deficient plasma with emicizumab - To further directly visualize the fibrin fibers within fibrin clot, SEM images of aPTT-triggered fibrin clots derived from FVIII-deficient plasma samples incubated with emicizumab were compared to those with various amounts of FVIII. In this experiment, we utilized higher concentrations of emicizumab (100 μg/ml) than clinically therapeutic concentrations (~50 μg/ml) to ensure maximum activity for fibrin formation *ex vivo*. The SEM images of fibrin fibers in native FVIII-deficient plasma under these conditions looked rough, short and thick. In the presence of exogenous FVIII, however, the fibrin fibers were relatively thin and the matrices appeared to be denser dose-dependently (Figure 3A), in keeping with a previous report [19]. Similarly, emicizumab-induced fibrin clots were thinner and denser compared to those in FVIII-deficient plasma. To quantify the properties of fibrin fibers, images were randomly selected and examined by four colleagues not involved in the study (25 each per person; total numbers of 100), and the average diameters of fibers in each clot-sample were calculated (Figure 3B). Average fibrin-diameters in the presence of emicizumab were significantly smaller (125±45 nm) than in the absence of FVIII (196±48 nm, $p<0.001$) or in the presence of 30 IU/dl FVIII (147±40 nm, $p<0.001$), and were similar to those at 100 IU/dl FVIII (125±28 nm). The data suggested that the marked shortening of the aPTT mediated by emicizumab was associated with enhanced fibrin formation and improved fibrin clot stability.

Turbidity and stability of PT/aPTT-triggered fibrin clots with emicizumab - In original aPTT-triggered experiments, the potential of emicizumab-induced fibrin formation appeared to be greater than the equivalent intrinsic coagulation activity of FVIII (0.2-0.4 IU/dl per μg/ml) [26]. In this context, we previously demonstrated that a combined, extrinsic-intrinsic trigger reagent could provide a useful means to assess sufficiently the global coagulation potential of emicizumab activity [14]. To assess the natural potential of emicizumab activity, therefore, the above experiments were repeated using a PT/aPTT-mixed activator.

The turbidity of fibrin clots induced by the combined trigger reagent was analyzed using CWA. Representative PT/aPTT-based CWA curves and turbidity calculations in FVIII-deficient plasma with FVIII or emicizumab are shown in Figures 4A-C, respectively. The addition of FVIII appeared to decrease the change of turbidity relative to that in FVIII-deficient plasma dose-dependently although the differences were not statistically significant. The addition of emicizumab also resulted in a slight decrease in change of turbidity dose-dependently. It was

speculated that the turbidity at the concentration of emicizumab (50 μg/ml) appeared to be close to that in the presence of FVIII at above 10 IU/dl.

Furthermore, clot stability measurements, represented by Lys50, in these PT/aPTT-based CFWA experiments are illustrated in Figures 5A-C. The addition of FVIII increased Lys50 relative to that in FVIII-deficient plasma, and the effects were dose-dependent. Similarly, the addition of emicizumab resulted in an increase in Lys50 relative to the FVIII-deficient state in a dose-dependent manner. In particular, the Lys50 at the concentration of emicizumab (50 μg/ml) was comparable to that in the presence of FVIII at above 10 IU/dl.

SEM images of PT/aPTT-triggered fibrin in FVIII-deficient plasmas with emicizumab - The PT/aPTT-induced fibrin clots derived from FVIII-deficient plasmas in the presence of added emicizumab or FVIII were analyzed by using the SEM. Representative images are shown in Figure 6A. The fibrin fibers derived from FVIII-deficient plasma appeared to be slightly thicker than those in the presence of FVIII or emicizumab (100 μg/ml), but the differences were difficult to distinguish visually. The diameters of randomly selected fibrin fibers (n=100) were quantitatively compared as before (Figure 6B). Average diameters in the presence of emicizumab (130±24 nm) or 30 and 100 IU/dl of FVIII (131±29 and 126±29 nm, respectively) were less than that in the absence of FVIII (138±23 nm, $p<0.01$). The mean diameter in the presence of emicizumab (100 μg/ml) seemed to be comparable to that of ~30 IU/dl of FVIII.

The results of these analyses of stability and structure of PT/aPTT-triggered fibrin clots are summarized in Figure 7. Overall, from the view of stability and structure, our data suggested that emicizumab activity at 50 and 100 μg/ml was comparable to FVIII activity of ~12 and ~24-32 IU/dl, likely similar to previous reports [14, 26].

Discussion

The structure of natural hemostatic clots is highly dependent on the concentrations of fibrinogen and thrombin, ionic strength, divalent metal ions, negatively charged substances, etc [17]. In addition, von Willebrand factor also has been shown to modulate fibrin structure and fibrinolysis [27]. Also, high doses of rFVIIa improved clot structure and stability in a HB model [28]. These studies demonstrated, therefore, that SEM and clot-lysis analyses help to clarify the role of coagulation-related factors in physiologic fibrin formation and dissolution.

Emicizumab provides a novel approach for HA therapy, but the impact of this product on the nature of hemostatic clots remains to be determined. We for the first time demonstrated the structure of fibrin clots formed in the presence of emicizumab using the CWA and SEM analysis. Emicizumab-induced fibrin clots were composed of the thinner fibers, as compared to those from severe HA plasma. These findings were consistent with an earlier report demonstrating that FVIII therapy in PwHA constructively improved the generation of fibrin fibers [19]. Moreover, our observations confirmed that changes in fibrin matrices were governed by FVIII concentration [20], and provided a strong indication that the effect of emicizumab was comparable to that of FVIII. Furthermore, our results were compatible with a previous study on the turbidity of fibrin clots [24], and highlighted a correlation between the diameter of fibrin fibers and clot turbidity in the presence of emicizumab as well as FVIII. The evidence suggested, therefore, that measurements of turbidity complement SEM analyses for the assessment of clot structure.

The therapeutic benefits of emicizumab in HA are independent of FVIII activation by thrombin, and conventional aPTT-based assays of coagulation activity are unreliable in these instances [4,6,13]. In our experiments, the physical property of aPTT-triggered fibrin clots formed in the presence of 50 μg/ml emicizumab were similar to those in the presence of ~50 IU/dl FVIII, and although emicizumab markedly shortened the aPTT (<20 sec), the physical properties of the fibrin clots did not indicate the presence of a hypercoagulable state. Similarly, the structure of fibrin clots induced by the PT/aPTT-trigger reagent in the presence of emicizumab (50 μg/ml) was comparable to that of ~12 IU/dl FVIII. These data were consistent with our earlier report on the use of this combined reagent in CWA for the assessment of comprehensive coagulation function [14]. Moreover, our potential activity of emicizumab appeared to be similar to that predicted in pre-clinical studies (0.2-0.4 IU/dl per μg/ml) [26]. Hence, emicizumab at a clinically therapeutic concentration of 50 μg/ml would be equivalent to 10-20 IU/dl FVIII. Our results of PT/aPTT-triggered fibrin clots predicted that 100 μg/ml emicizumab was equivalent to 20-30 IU/dl FVIII.

There are some limitations to our study, however. First, the trigger and tPA concentrations may have an influence on results, although the fact that emicizumab has a comparable effect on clot structure and stability is valuable information. Emicizumab may have a comparable effect on thrombin generation than FVIII, since the kinetics of thrombin generation has a major effect of clot

structure [19, 28]. Secondly, we focused on the impact of emicizumab on fibrin fibers in plasma samples. Leong *et al*. [25] demonstrated, however, that the presence of platelets and red blood cells also affected the formation of fibrin clots. Further investigations using whole blood are required to clarify emicizumab-induced clot formation under physiological blood flow conditions.

Effective hemostasis is governed not only by fibrin clot structure but also by clot stability [29]. In this context, fibrinolytic potential has been commonly assessed by adding tPA or plasmin to fibrin clots *ex-vivo*. More recently, our group has demonstrated by CFWA that the stability of fibrin clots in plasma samples from hemophilia patients was weak and fragile compared to normal control [22]. In the present study, clot stability was evaluated with a time-associated parameter, Lys50, and the results in the presence of emicizumab were similar to those in the presence of FVIII. Clot lysis of thick fibrin fibers is known to be more rapid than that of thin fibers [30], and our data were in keeping with those characteristics. CFWA has been established mainly for rapid determination of fibrinolysis in clinical practice, however, and Nogami *et al*. [31] reported that FVIII is rapidly activated and/or inactivated by plasmin. Further studies are required to examine whether the stability of emicizumab-induced clots reflect physiological responses in the absence of FVIII.

In conclusion, the current findings have shown that emicizumab significantly increased clot stability as assessed by fibrinolytic resistance and improved clot structure as indicated by the morphology of fibrin fibers. These effects of the antibody were similar to those mediated by FVIII.

Acknowledgements

We would like to greatly thank Ms. Shiori Matsuhiroya and Ms. Nozomi Kojima for special technique supports on Scanning electron microscopy.

Authorship

Contributions; NS; performed experiments, analyzed the data, prepared figures and wrote the manuscript, KN; designed all experiments, interpreted the data, prepared figures, wrote and edited the manuscript, KO, TM, FN; performed experiments, TS; interpreted the data and supervised this study, MH; prepared emicizumab, NA, MS; supervised this study.

Conflict-of-interests; NS, KN, KO, TM and MS receive research support from Chugai Pharmaceutical Co., Ltd. (Chugai), and are engaged in clinical studies sponsored by Chugai and F. Hoffmann-La Roche. KN and MS receive (consulting) honoraria from these companies, and are inventors of the patents relating to emicizumab. MH and TS are employees of Chugai Corp, and TS is an inventor of the patents to emicizumab. FN and NA are employees of Sysmex Corp.

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Figure Legends

Figure 1. Fibrin clot turbidity measured by aPTT-triggered CWA in FVIII-deficient plasmas with FVIII or emicizumab added *ex vivo* - FVIII-deficient plasma was mixed with various concentrations of FVIII (0, 10, 30, 50, and 100 IU/dl; **A**) or emicizumab (0, 10, 50, and 100 μg/ml; **B**) and were incubated with aPTT reagent prior to the addition of $CaCl_2$. The lines represent the clot waveforms obtained in each of plasma sample. The segments from (a) to (b), (b) to (d), and (d) to (e) show the phases of pre-coagulation, coagulation and post-coagulation (completion of fibrin clot), respectively. The difference in transmittance from the initial level to post-coagulation phase was regarded as 'a change of turbidity (%)'. *Panel* (**C**) shows turbidity (%) in the presence of FVIII or emicizumab from the clot waveforms illustrated in (A). Experiments were performed at least three separate times, and the average values and SD were calculated. Significant differences between FVIII-deficient plasma and the other groups were considered as $p<0.05$. $^{**}p<0.001$

Figure 2. Fibrin clot stability measured by aPTT-triggered CFWA in FVIII-deficient plasma in the presence of FVIII or emicizumab added *ex vivo* - FVIII-deficient plasma was mixed with various concentrations of FVIII (0, 10, 30, 50, and 100 IU/dl; **A**) or emicizumab (0, 10, 50, and 100 μg/ml; **B**) and incubated with aPTT trigger reagent containing tPA (0.63 μg/ml) prior to the addition of $CaCl_2$. The lines represent fibrin clot formation, followed by clot lysis in each sample. Stability of fibrin clot was assessed by the "Lys50 (sec)" parameter as difference between the time at maximum change of %transmittance and that at 50% of the maximum change (**C**). *Panel* (**D**) shows the Lys50 in the presence of FVIII or emicizumab from waveforms obtained in (A). Experiments were performed at least three separate times, and the average values and SD were calculated. Significant differences between FVIII-deficient plasma and the other groups were considered as $p<0.05$. $^{**}p<0.001$

Figure 3. SEM analyses of aPTT-triggered fibrin clots in FVIII-deficient plasmas with FVIII or emicizumab added *ex-vivo* - (A) SEM images; Fibrin clots in FVIII-deficient plasma in the presence of rFVIII (0, 30, and 100 IU/dl) or emicizumab (100 μg/ml) were generated by the addition of aPTT reagent and $CaCl_2$, and analyzed by SEM. The fibrin fibers were visualized at a magnification of 30,000-fold. **(B)** Measurements of fibrin fibers; the diameters of fibrin fibers (total n=100) selected randomly from the images illustrated in (A) were measured. The short horizontal lines in the graphs indicate average values. Average values and SD were calculated.

Significant differences between groups were considered as $p<0.05$. *$p<0.01$, **$p<0.001$

Figure 4. Fibrin clot turbidity measured by PT/aPTT-triggered CWA in FVIII-deficient plasma added FVIII or emicizumab *ex vivo* - FVIII-deficient plasma was mixed with various concentrations of FVIII (0, 10, 30, 50, and 100 IU/dl; **A**) or emicizumab (0, 10, 50, and 100 μg/ml; **B**) and were incubated with PT/aPTT-mixed trigger reagents prior to the addition of $CaCl_2$. The lines represent the clot waveforms obtained in each of plasma sample. (*Inset*) An enlarged view at the post-coagulation phase (within square area) that was shown in (A) and (B). *Panel* (**C**) shows turbidity (%) in the presence of FVIII or emicizumab from the clot waveforms illustrated in (A). Experiments were performed at least three separate times, and the average values and SD were calculated. Significant differences between groups were considered as $p<0.05$.

Figure 5. Fibrin clot stability measured by PT/aPTT-triggered CFWA in FVIII-deficient plasma added FVIII or emicizumab *ex vivo* - FVIII-deficient plasma was mixed with FVIII (0, 10, 30, 50, and 100 IU/dl; **A**) or emicizumab (0, 10, 50, and 100 μg/ml; **B**) and incubated with PT/aPTT-mixed trigger reagents containing tPA (0.63 μg/ml) prior to the addition of $CaCl_2$. The lines represent fibrin clot formation, followed by clot lysis in each sample. Stability of fibrin clot was assessed by the "Lys50" parameter as difference between the time at maximum change of %transmittance and that at 50% of the maximum change. *Panel* (**C**) shows the Lys50 in the presence of FVIII and emicizumab from waveforms obtained in (A and B, respectively). Experiments were performed at least three separate times, and the average values and SD were calculated. Significant differences between groups were considered as $p<0.05$.

Figure 6. SEM analyses of PT/aPTT-triggered fibrin clots in FVIII-deficient plasmas with FVIII or emicizumab added *ex vivo* - (A) SEM images; Fibrin clots in FVIII-deficient plasma mixed with rFVIII (0, 30, and 100 IU/dl) or with emicizumab (100 μg/ml) *ex vivo* were generated by the addition of PT/aPTT reagent and $CaCl_2$, and analyzed by SEM. The fibrin fibers were visualized at a magnification of 30,000-fold. **(B)** Measurements of fibrin fiber; the diameters of fibrin fibers (total n=100) selected randomly in the images illustrated in (A) were measured. The short horizontal lines in the graphs indicate average values. Average values and SD were calculated. Significant differences between groups were considered as $p<0.05$. *$p<0.01$, **$p<0.001$

Figure 7. Equivalent FVIII activity levels of emicizumab, predicted from the stability and structure of PT/aPTT-triggered fibrin clots - The figures illustrate the **(A)** turbidity, **(B)** stability, and **(C)** fibers diameters of fibrin clots obtained from the PT/aPTT-triggered assays shown in Figures 4-6. The dotted lines represent the levels of FVIII activity equivalent to the turbidity and stability in the presence of emicizumab of 50 μg/ml (*gray lines*) and 100 μg/ml (*black lines*), respectively. The FVIII activity levels were ~12 IU/dl at 50 μg/ml and ~24-32 IU/dl at 100 μg/ml.

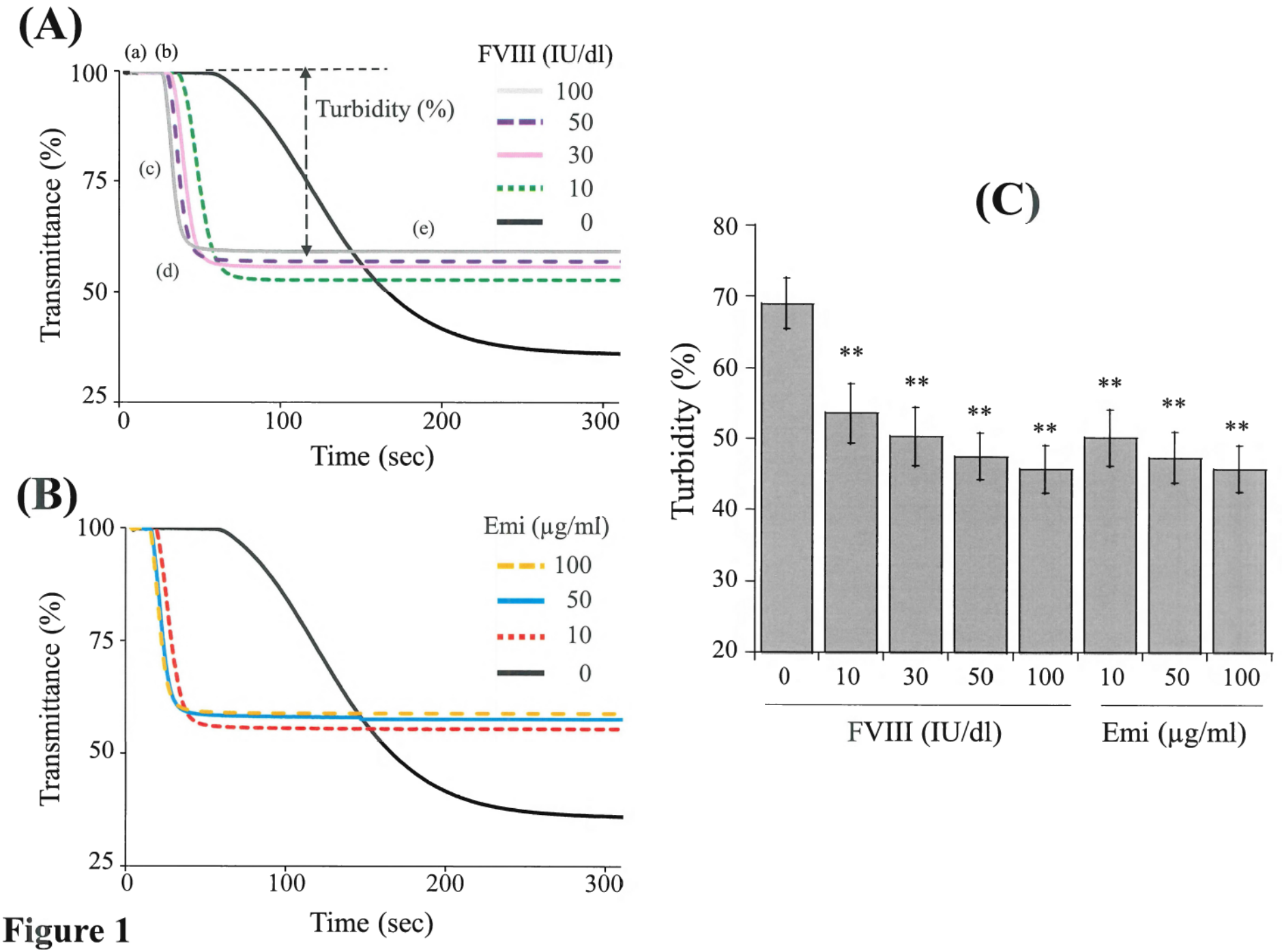
(A)
(a) (b)
FVIII (IU/dl)
100
50
30
10
0
Turbidity (%)
(c)
(d)
(e)
Transmittance (%)
100
75
50
25
0
100
200
300
Time (sec)
(B)
Emi (μg/ml)
100
50
10
0
Transmittance (%)
100
75
50
25
0
100
200
300
Time (sec)
(C)
Turbidity (%)
80
70
60
50
40
30
20
**
0
10
30
50
100
10
50
100
FVIII (IU/dl)
Emi (μg/ml)

Figure 1

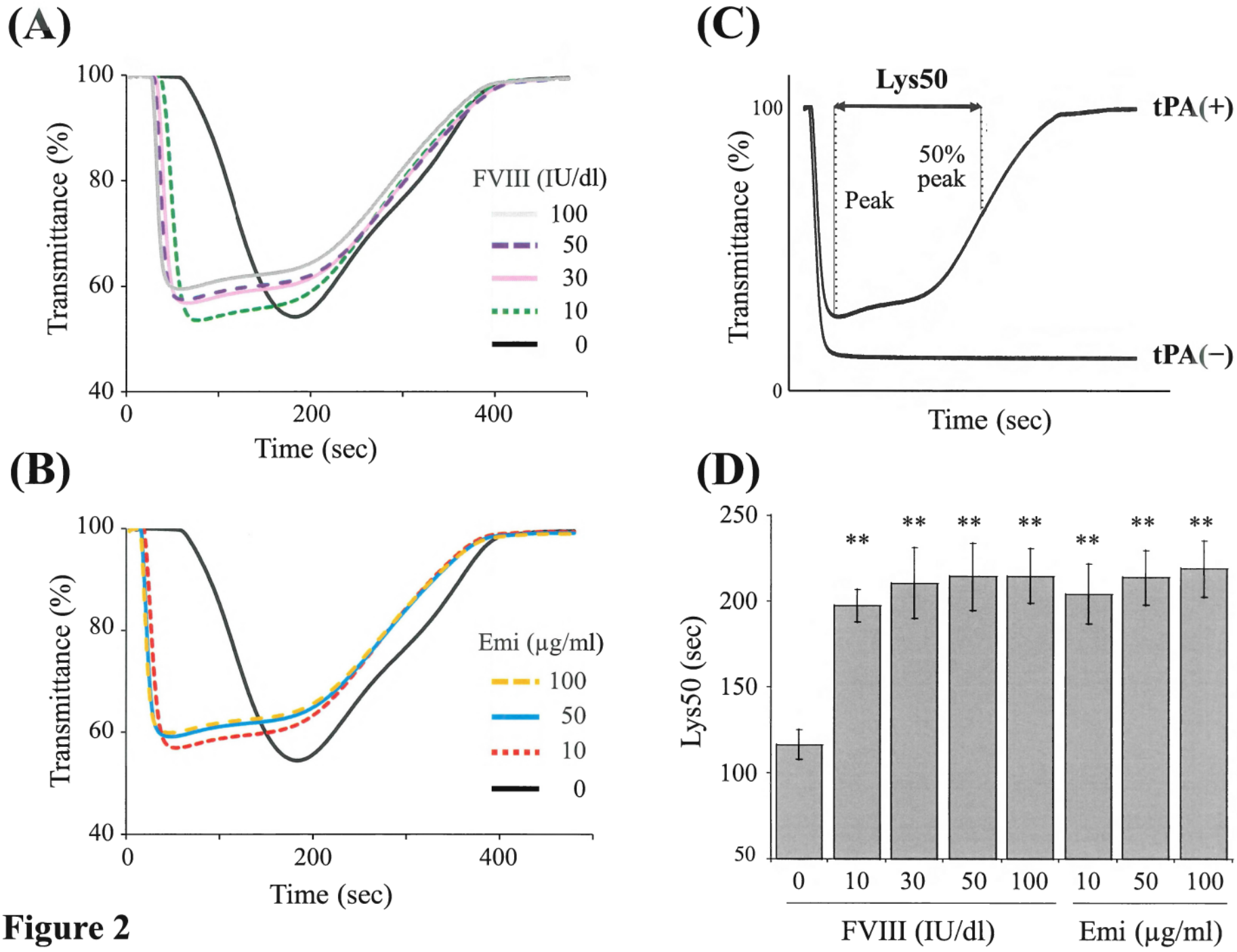
(A)
100
80
60
40
Transmittance (%)
0
200
400
Time (sec)
FVIII (IU/dl)
100
50
30
10
0
(B)
100
80
60
40
Transmittance (%)
0
200
400
Time (sec)
Emi (μg/ml)
100
50
10
0
(C)
Lys50
100
0
Transmittance (%)
Peak
50%
peak
tPA(+)
tPA(−)
Time (sec)
(D)
250
200
150
100
50
Lys50 (sec)
**
0
10
30
50
100
10
50
100
FVIII (IU/dl)
Emi (μg/ml)

Figure 2

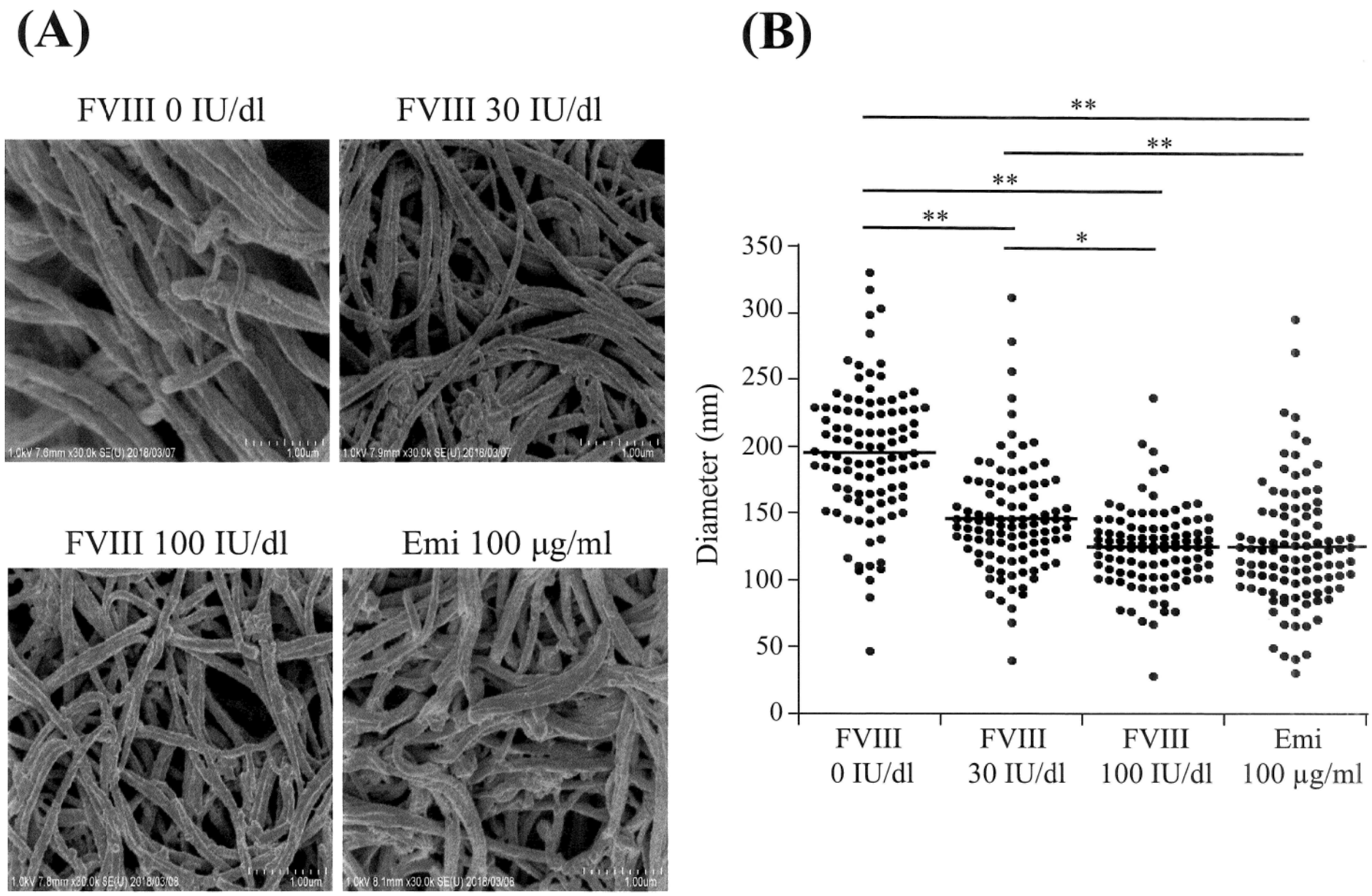
(A)
FVIII 0 IU/dl
FVIII 30 IU/dl
FVIII 100 IU/dl
Emi 100 µg/ml
(B)
**
**
**
**
*
Diameter (nm)
350
300
250
200
150
100
50
0
FVIII 0 IU/dl
FVIII 30 IU/dl
FVIII 100 IU/dl
Emi 100 µg/ml

Figure 3

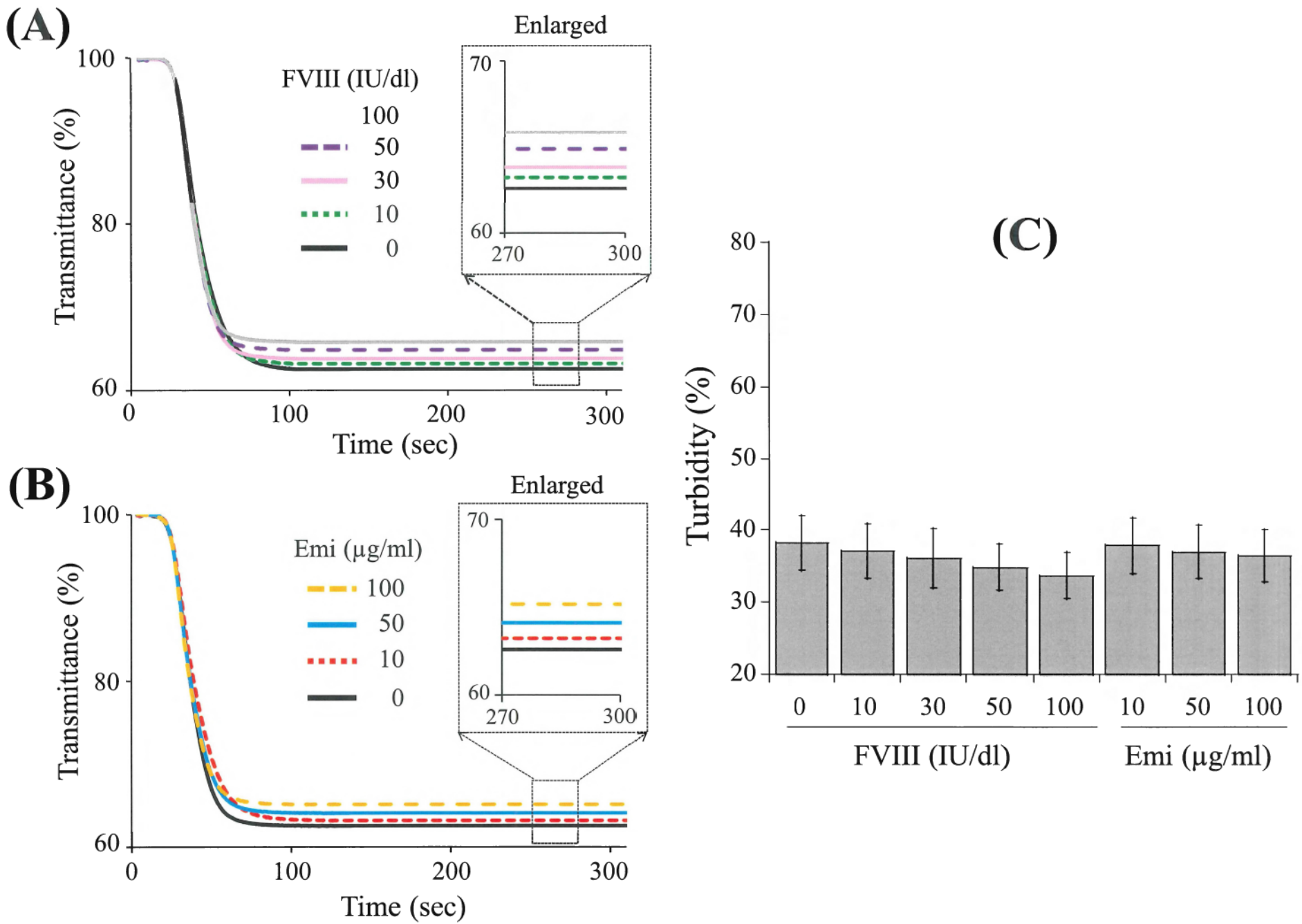
(A)
Enlarged
FVIII (IU/dl)
100
50
30
10
0
Transmittance (%)
Time (sec)
(B)
Enlarged
Emi (μg/ml)
100
50
10
0
Transmittance (%)
Time (sec)
(C)
Turbidity (%)
FVIII (IU/dl)
Emi (μg/ml)

Figure 4

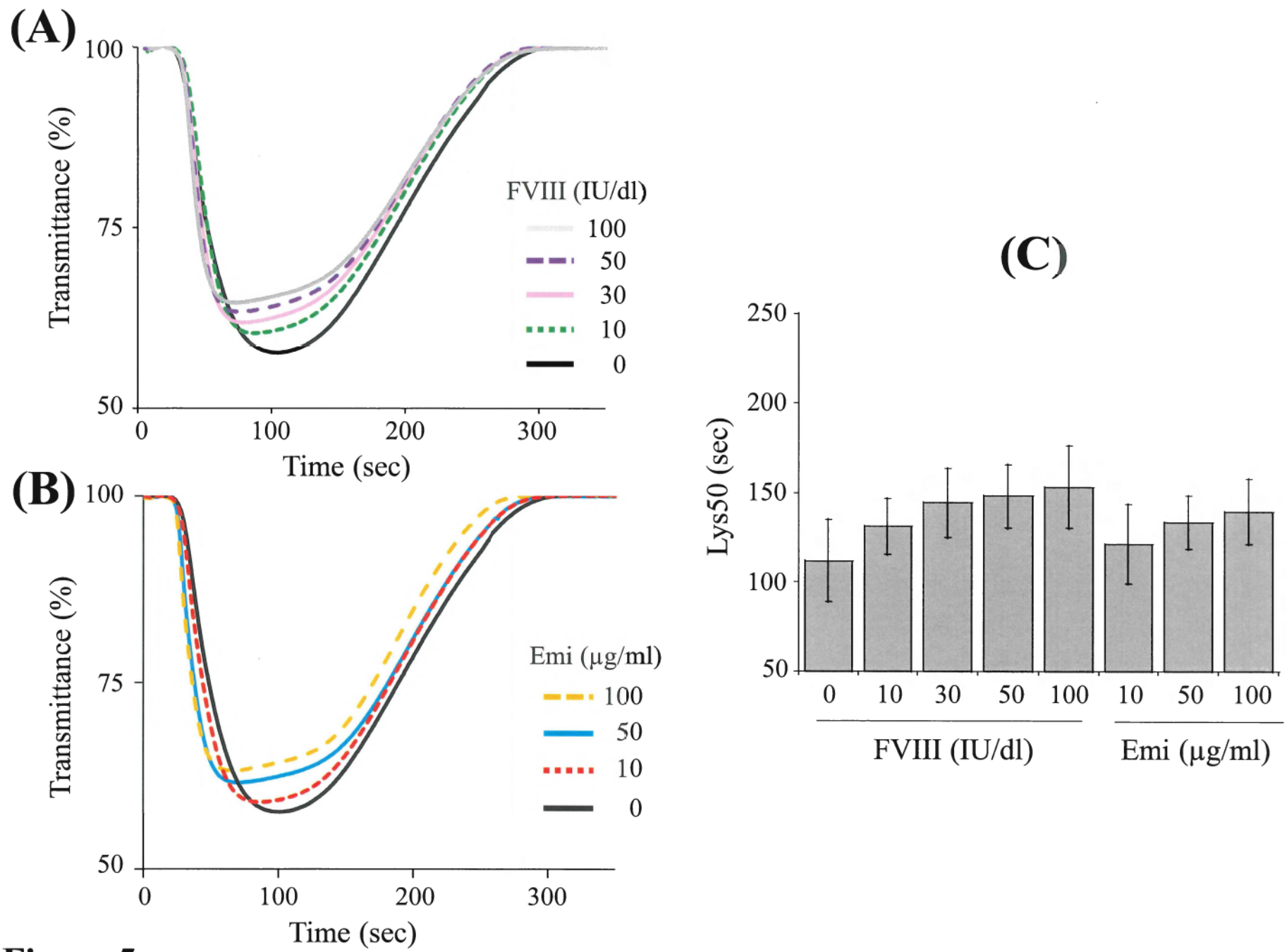
(A)
100
75
50
Transmittance (%)
0
100
200
300
Time (sec)
FVIII (IU/dl)
100
50
30
10
0
(B)
100
75
50
Transmittance (%)
0
100
200
300
Time (sec)
Emi (μg/ml)
100
50
10
0
(C)
250
200
150
100
50
Lys50 (sec)
0
10
30
50
100
10
50
100
FVIII (IU/dl)
Emi (μg/ml)

Figure 5

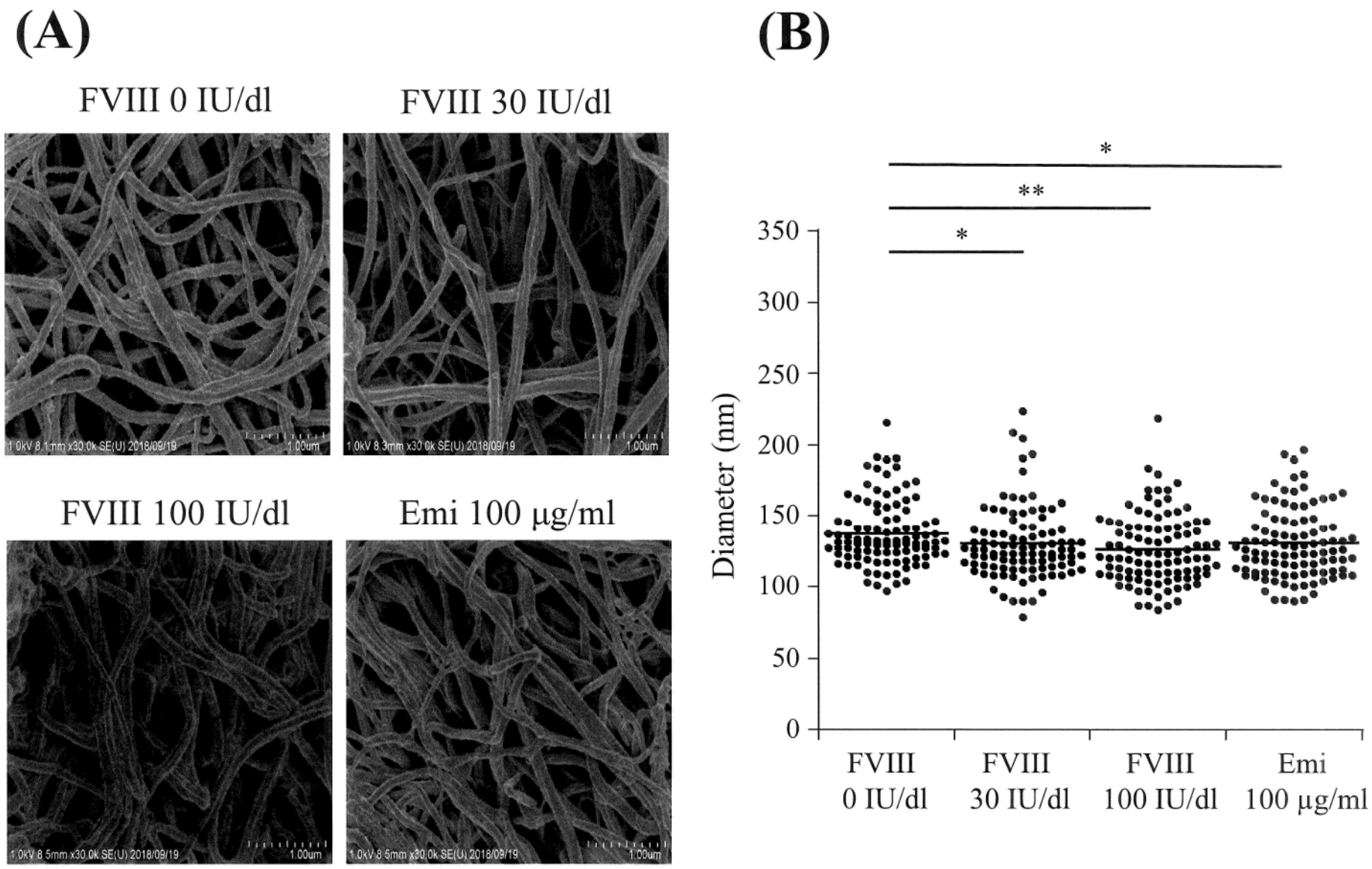
(A)
FVIII 0 IU/dl
FVIII 30 IU/dl
FVIII 100 IU/dl
Emi 100 µg/ml
(B)
Diameter (nm)
350
300
250
200
150
100
50
0
*
**
*
FVIII 0 IU/dl
FVIII 30 IU/dl
FVIII 100 IU/dl
Emi 100 µg/ml

Figure 6

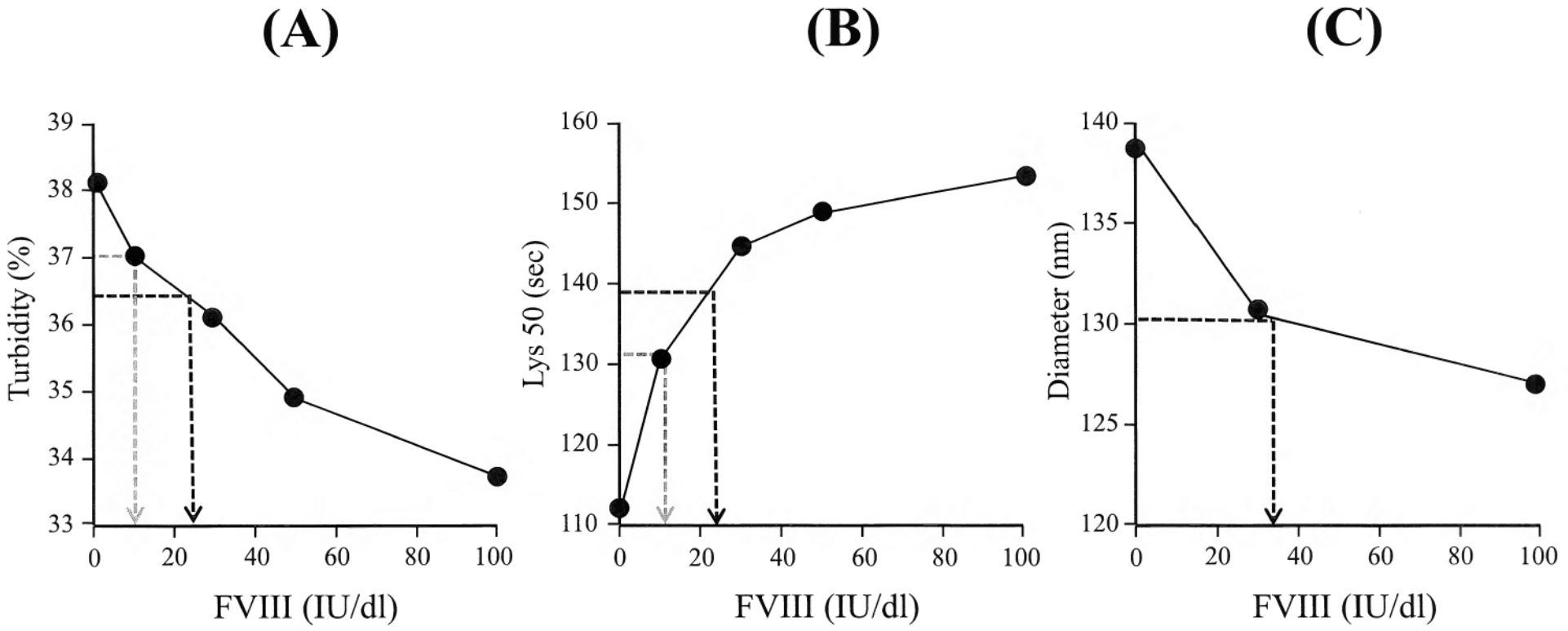
(A)
(B)
(C)
Turbidity (%)
39
38
37
36
35
34
33
0
20
40
60
80
100
FVIII (IU/dl)
Lys 50 (sec)
160
150
140
130
120
110
0
20
40
60
80
100
FVIII (IU/dl)
Diameter (nm)
140
135
130
125
120
0
20
40
60
80
100
FVIII (IU/dl)

Figure 7