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Acoustic determination of early stages of intravascular blood coagulation

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The blood coagulation system (BCS) is a complex biological system playing a principal role in the maintenance of haemostasis. Insufficient activity of the BCS may lead to bleeding and blood loss (e.g. in the case of haemophilia). On the other hand, excessive activity may cause intravascular blood coagulation, thromboses and embolization. Most of the methods currently used for BCS monitoring suffer from the major disadvantage of being invasive. The purpose of the present work is to demonstrate the feasibility of using ultrasonic methods for non-invasive registration of the early stages of blood coagulation processes in intensive flows. With this purpose, a special experimental set-up was designed, facilitating the simultaneous detection of optical and acoustic signals during the clotting process. It was shown that (i) as microemboli appear in the flow during the early stage of blood coagulation, the intensity of the Doppler signal increases twofold, and (ii) microemboli formation in the early stages of blood clotting always reveals itself through an acoustic contrast. Both of these effects are well defined, so we hope that they may be used for non-invasive BCS monitoring in clinical practice.

Keywords: acoustically detected microemboli; blood coagulation; clot formation

1. Introduction

(*a*) *Biochemical blood coagulation system*

Changes in the aggregate state of human blood are regulated by the activation of platelets and/or by a special biochemical blood coagulation system (BCS; Esmon 2000; Davie 2003). The so-called 'intrinsic' and 'extrinsic' biochemical pathways of blood coagulation are schematically presented in figure 1 (Davie 1995; Schenone *et al.* 2004). Here, Roman numerals are used for inactivated coagulation factors that pre-exist in the blood. The figure illustrates how these inactive factors are converted into active forms through the influence of activation factors (denoted in the scheme by Roman numerals with a suffix 'a').

Factor IIa, known as thrombin, is generated from prothrombin. Thrombin in turn catalyses the conversion of fibrinogen molecules to fibrin. The polymerization of fibrin in vessels gives rise to thromb formation. Symbol A (figure 1)

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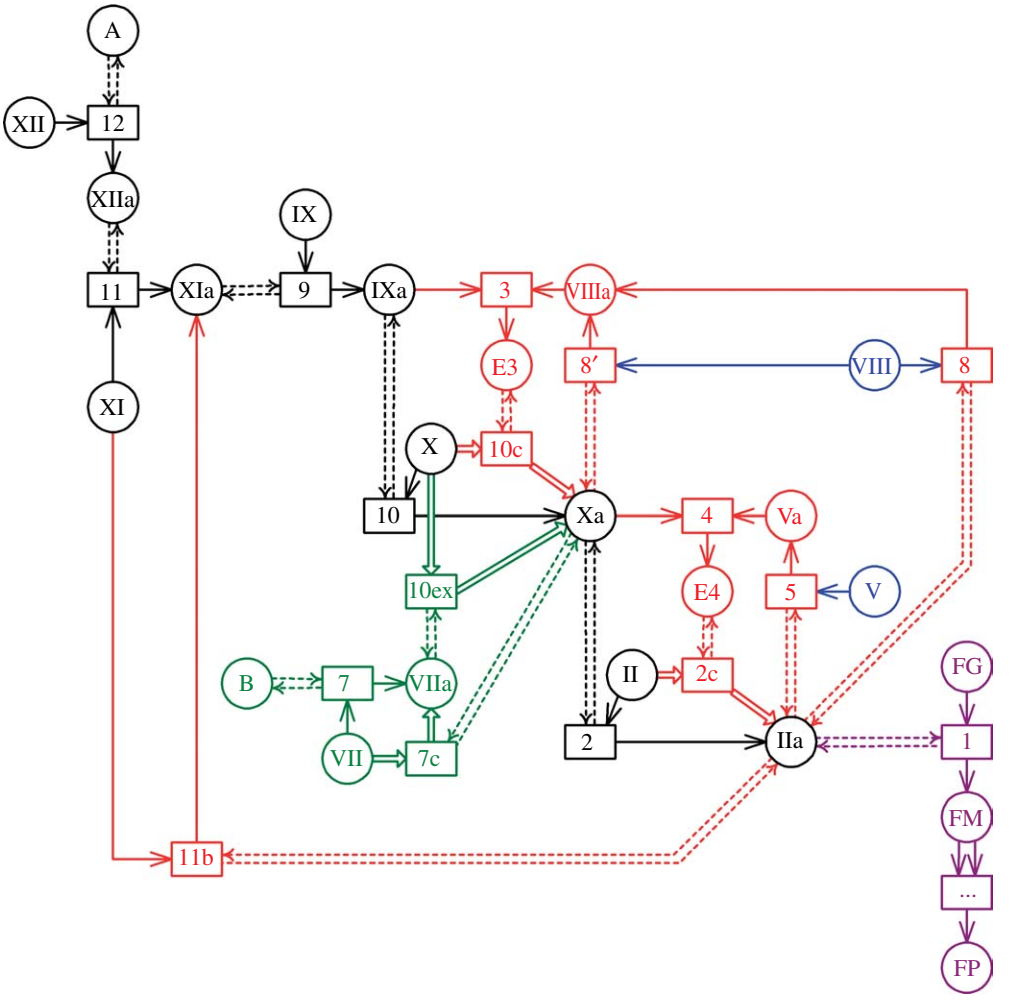

Figure 1. Schematic of the blood coagulation cascade. The intrinsic and extrinsic pathways of coagulation are shown in black and green, respectively. The violet colour represents the polymerization process and red represents positive feedbacks. The circles represent coagulation factors and the rectangles represent biochemical reactions. Non-active factors are denoted by Roman numerals and their active forms by Roman numerals with suffix 'a'. Reactions are denoted by Arabic numbers, which refer to the active factors produced in them. A solid arrow from factors to reaction represents participation in the reaction, and an arrow from reaction to factor means that the factor is a product of the reaction. Dotted arrows represent a catalysing action of the substance. The meaning of wide arrows is the same as that of the solid ones, but they indicate that the process proceeds intensively. 'A' and 'B' represent primary initiation of intrinsic and extrinsic pathways, respectively. E3 represents the complex of active forms of factors IXa and VIIIa, and E4 the complex of factors Xa and Va. Other terms used are: II, prothrombin; IIa, thrombin; FG, fibrinogen (also known as factor I); FM, fibrin monomer; FP, fibrin polymer.

denotes negatively charged surfaces exposed naturally or artificially in human blood. On these surfaces, the so-called contact activation of the primary reaction of factor XIIa generation takes place (Vogler *et al.* 1995). It is generally accepted that the generation of factor XIIa initiates a chain reaction cascade

known as the intrinsic coagulation pathway. The extrinsic pathway is activated by a tissue factor (denoted on the scheme as B) via reaction with factor VII (Mann *et al.* 1998). It should also be considered that all active coagulation factors in human blood are gradually inactivated by antithrombin III (ATIII) and by some less important inhibitors. The intensity of the inhibition of blood coagulation by ATIII is increased many times by heparin, an endogenous cofactor of ATIII (Zwaal & Hemker 1986). In clinical practice, fraxiparin (low-molecular-weight heparin) as well as unfractioned heparin is used for the same purposes.

Simple analysis demonstrates that the higher the intensity of inhibition of the main coagulation factors, the higher must be the perturbation to initiate the cascade of autocatalytic reactions drawn in figure 1. The range of stability of the liquid state of blood depends on the level of inhibition as well as on the concentration of coagulation factors that pre-exist in the blood (Khanin & Semenov 1989).

It is well known that insufficient factor VIII is responsible for haemophilia A (Collins *et al.* 2007). Deficit of factor IX directly decreases blood coagulation intensity in the case of haemophilia B (Zwaal & Hemker 1986; Esmon 2000). In contrast to both bleeding cases, mutations in factor V (known as a Leiden mutation) give rise to a significant increase in the production of factor Va, resulting in the acceleration of fibrin clot formation. In this case, patients suffer from intravascular thrombosis (Horne & McCloskey 2006).

(*b*) *BCS monitoring in medicine*

The blood coagulation processes can develop quite rapidly (Hemker & Beguin 2000). In some cases, the formation of a macroscopic thrombus takes only a few minutes (Levi 2007). Under normal conditions, blood coagulation stops the bleeding. In cases of BCS dysfunctions, its activation may lead to intravascular blood coagulation, thromboses and embolization. For instance, such well-known pathologies as cardiac infarction, thrombotic pulmonary embolism and disseminated intravascular coagulation result from dysfunctions of the BCS (Furie & Furie 2007; Levi 2007).

According to recent statistical reports, up to 29 per cent of lethal cases worldwide are directly caused by BCS dysfunctions (World Health Organization 2004). Thus, the development of methods for the early diagnosis of BCS dysfunctions is of vital importance. The complexity of the BCS (see figure 1) and the diversity of types of essential malfunctions are so large that it is practically impossible to monitor the activity of all blood coagulation factors in real time. In practice, measurements of indirect kinetic parameters, reflecting a series of relevant times, measured in off-line or online experiments, are used for the estimation of BCS state (Colman *et al.* 2006). The most widely used methods for BCS control include such coagulation tests as the following: activated clotting time (ACT); activated partial thromboplastin time (aPTT); prothrombin time (PT), etc. (Barrowcliffe *et al.* 2006; Colman *et al.* 2006). The majority of coagulation test procedures are based on measuring the temporal characteristics of blood or plasma coagulation under the influence of a particular activator. Either intrinsic (ACT, aPTT) or extrinsic (PT) pathways can be used for activation.

Hence one may point out that present-day coagulation tests are focused on the estimation of the coagulation *potential* of BCS, i.e. its principal ability to ensure thrombus formation. Depending on whether the clotting time is long or short, one may derive conclusions about how high this potential actually is. If the clotting exceeds some statistical standard, one may speak about a hypo- (in the case of a delayed coagulation) or hyperactivation (in the case of rapid coagulation) of BCS.

However, knowledge of the general processes of blood coagulation does not allow prediction of the actual starting point of the process. In many clinical situations, a patient's life directly depends on the ability to perform coagulation tests quickly. For instance, for cardiac surgery and intensive care units, real-time assessment of haemostasis properties is often required.

A significant increase in the efficiency of coagulation tests has been achieved through the development of automated haemostasis testing systems (so-called point-of-care testing devices). This kind of device could be applied directly at the bedside of a patient, hence eliminating turnaround times, which are inevitable in the case of laboratory testing. Several types of point-of-care device are used clinically (Prisco & Paniccia 2003). The time required for test completion varies from 2 to 10 min depending on the device and test type, as well as on the method of BCS activation. Usage of modern point-of-care devices allows relatively quick assessment of haemostasis alterations, but all the practically used testing methods are invasive. Moreover, it is worth mentioning that, owing to the indirect character of test indicators, the results of measurements in many cases could not be unambiguously interpreted. Particularly, based on indirect kinetic data, it is impossible to conclude that the process of fibrin polymerization has already started and that regional or general haemodynamics abnormalities will follow inevitably. Therefore, creating non-invasive and direct methods for BCS monitoring is a matter of great interest.

Significant achievements in the elaboration of non-invasive methods of blood monitoring were reached by the development of capillaroscopy and its introduction into clinical practice (Gurfinkel *et al.* 1998). This method is based on the microscopic observation of capillary blood flow under the surface of the nail. However, capillaroscopy cannot be applied for BCS monitoring in deep-seated blood vessels, including the majority of large veins and arteries, whereas thrombus formation in these very vessels is of the greatest risk.

We suppose that ultrasonic detection of microemboli appearing in the early stages of blood clotting may be used for non-invasive BCS monitoring in large veins and arteries.

It is well known that the scattering of an acoustic wave is determined by the inner microheterogeneity of the medium in which the wave propagates (Strutt 1896). In blood coagulation under intensive flow conditions, the process of fibrin polymerization and blood cell aggregation leads to the formation of microemboli, increasing gradually in size (Zwaal & Hemker 1986; Turitto & Hall 1998). As soon as, in the early stages of blood coagulation, the size of microemboli becomes comparable to the wavelength of an acoustic wave, the scattering of sound on microemboli should significantly increase.

There has been wide-scale discussion regarding the mechanisms of the so-called spontaneous acoustic echogenicity in blood vessels. A smoke-like signal is sometimes observed during ultrasonic examination of ventricular cavities and other

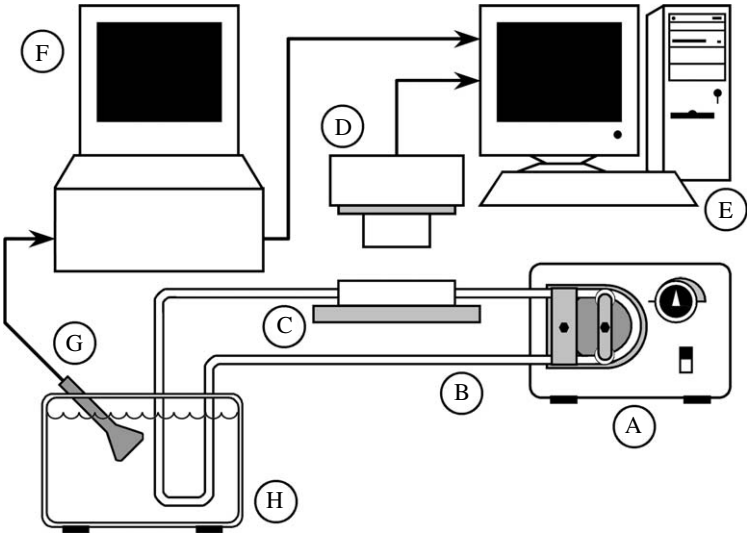

Figure 2. Layout of the experimental set-up: A denotes a peristaltic pump; B a system of transparent flexible silicone tubes; C a screw clamp; D a camcorder; E a personal computer; F the ultrasonic scanner; G the ultrasonic sensor; and H a water bath.

sections of the circulatory system (Iliceto *et al.* 1985; Ercan *et al.* 2003). There is currently no general agreement concerning the mechanisms of spontaneous echo-contrast generation. Among the mechanisms suggested to explain the origin of spontaneous acoustic echo contrast, the following seem to be the most probable: mechanisms dealing with biochemical blood coagulation processes (Ercan *et al.* 2003), with the aggregation of thrombocytes and leucocytes (Zotz *et al.* 2001), or with rouleaux formation (Wang *et al.* 1992).

In the present paper, we demonstrate that the formation of microemboli in the flow, which precedes macroscopic thrombus formation, always causes the appearance of an acoustic echo contrast.

2. Material and methods

The experiments were carried out with platelet poor plasma and whole blood. The blood was taken from healthy donors and preserved in 'Baxter' plastic bags containing citrate phosphate dextrose anticoagulant (CPDA) solution. Plasma was obtained from donor blood by centrifugation at $2350g$ for 15 min.

The experimental set-up for the registration of optical and acoustic signals during the clotting process under intensive flow conditions is shown in figure 2.

In our experiments, we used a closed-circuit system of transparent flexible silicone tubes (B in figure 2) of 4 mm diameter and with total volume of 15 ml, used in Baxter haemodialysis devices. The tube system was filled with blood plasma or whole blood depending on the type of experiment. The flow velocity was set by power regulation of the peristaltic pump and kept constant through the experiment (A in figure 2). The average flow speed value in different experiments varied in the range from 10 to 30 cm s^{-1}. The usage of a screw clamp (C in figure 2) facilitates the regulation of the cross-clamping value. Recalcification was

performed at the beginning of each experiment by the injection of 300–500 μl of 10 per cent $CaCl_2$ solution into the tube. All experiments were performed at room temperature, $t=23\pm1°C$.

The optical image was registered in the reflected light of a laser diode, with a wavelength of 640 nm, by means of a digital camcorder (D in figure 2). An ultrasonic scanner HP Sonos 4500 (F in figure 2) with an ultrasonic sensor (G in figure 2) working within the frequency range of 3.9–7.1 MHz was used. During every experiment, the ultrasonic scanner made it possible to register the Doppler signal reflected by the flow or to observe a two-dimensional image of the flow in the tube (in B-mode).

In the experiments with platelet poor plasma, the optical and acoustic signals were registered simultaneously. In the experiments with whole blood (mean haematocrit = 50%), owing to its optical opacity, only the acoustic signal was registered. Data were recorded on a personal computer (Intel Pentium 4 CPU, 2.40 GHz, 512 Mb RAM) with two video capture boards (AverTV WDM Video Capture) installed. Video data acquisition rate was 25 frames per second.

3. Results

In the experiments with blood plasma, it was discovered that the coagulation processes in an intensive flow take place in several successive stages. Fragments of the record of a typical clotting process are reproduced in figure 3. A Doppler spectrum time sweep obtained by means of an ultrasonic scanner is given at the bottom of every frame. A graph of the intensity of the Doppler signal reflected by clotting plasma is presented in figure 4, where the letters (A, B, C, …) denote the time moments corresponding to the frames presented in figure 3*a–f*.

Over 20 min after recalcification, the intensity of the Doppler signal remains at the initial level (area '0' in figure 4), while the plasma in the tube remains permanently transparent (figure 3*a*). Within the first stage of the clot formation process (area '1' in figure 4), primary microemboli appear in the flow (figure 3*b*). The concentration of microemboli in the flow grows rapidly with time. The avalanche-like formation of microemboli is accompanied by a drastic growth of the Doppler signal intensity (the interval '1' in figure 4). In 17 experiments performed with blood plasma, the intensity increases on average 6.2 ± 1.5 times compared with its initial level. This abrupt intensity increase took place over a period of approximately 28 ± 6 s.

In the second stage of the coagulation process (the interval '2' in figure 4), the mutual aggregation of microemboli takes place. The latter leads to the formation of macroscopic emboli (figure 3*c*). Every embolus passing through the cross-clamping area is accompanied by an outburst in Doppler signal intensity (figure 3*d*,*e*).

Further development of clotting processes (the interval '3' in figure 4) leads to the formation of large, up to 10 cm long, clots in the flow (figure 3*e*). Sometimes similar clots may block the stream, leading to the complete cessation of flow (figure 3*f*). The corresponding Doppler signal intensity then abruptly turns to zero (F in figure 4).

A drastic change in Doppler signal intensity in the clotting process was also detected in the series of six experiments with whole blood. A typical graph of the time dependence of Doppler signal intensity during the blood coagulation process

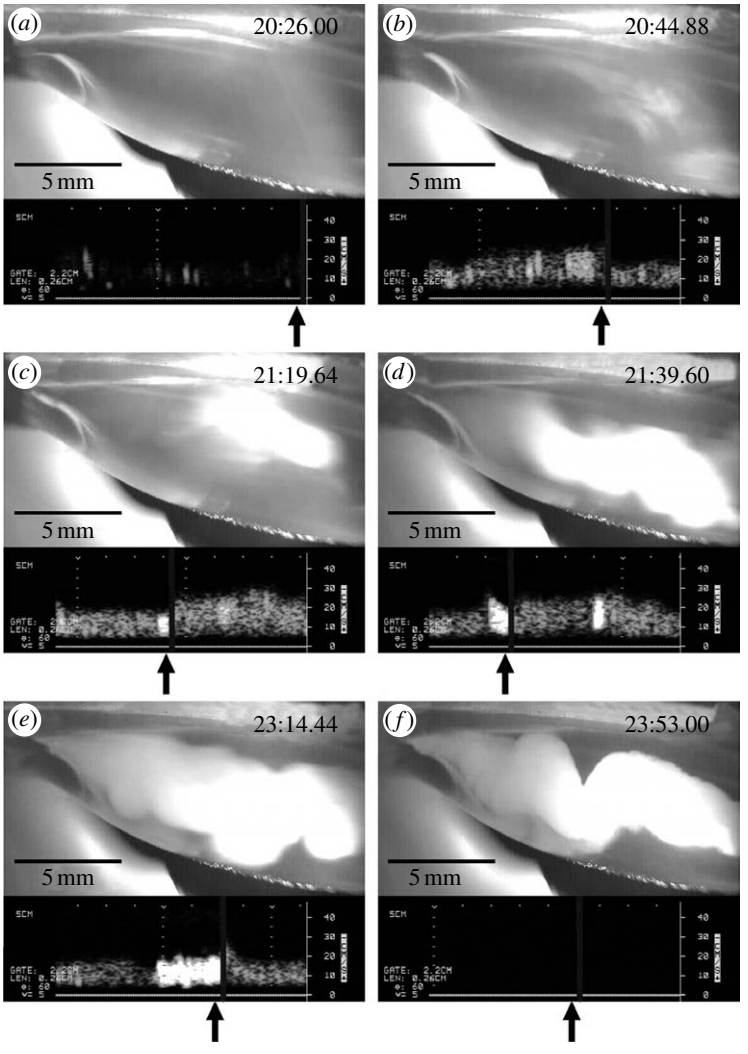

Figure 3. (*a–f*) Fragments of a typical clotting process record. An optical image is presented on top of every frame. A Doppler spectrum time sweep is given below the frames. The arrows point to the moments during the sweep that are relevant to the optical images. The time (min : s) is given in the right upper corner.

is given in figure 5. A similar increase in the Doppler signal intensity was observed in all experiments with whole blood. In the experiments, blood coagulation was accompanied by at least 1.85 times increase of the Doppler signal intensity. The intensity increase took 50 ± 5 s.

As illustrated in figure 6, two-dimensional ultrasonic imaging of the blood flow revealed that the beginning of the clotting process was always accompanied by the appearance of an acoustic echo contrast. The acoustic echo contrast inside the tube could easily be seen after the clotting process had begun (figure 6*b*), while before the coagulation starts there is no echo contrast to observe (figure 6*a*). These data obtained in our experiments seem to be important for the interpretation of echo-contrast phenomena detected in clinical practice.

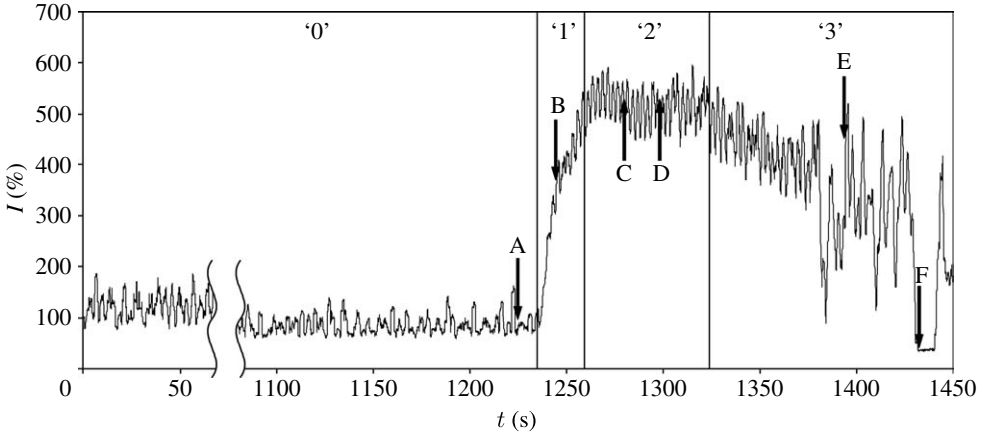

Figure 4. Variation of the Doppler signal intensity during a clotting process in blood plasma. The stages of the clotting process (see details in the text) are marked with numbers. The letters (A, B, C, ...) denote the time moments corresponding to the frames presented in figure 3*a–f*.

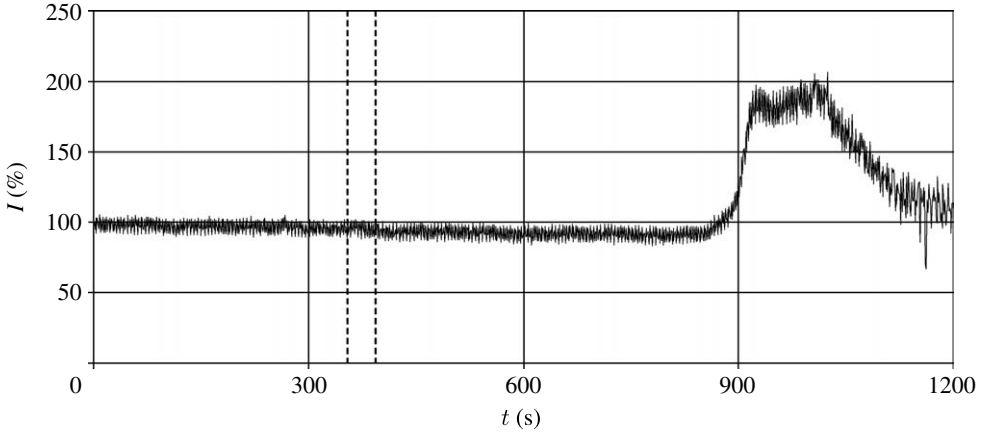

Figure 5. Time dependence of the intensity of the Doppler signal obtained from the blood during coagulation. Vertical dashed lines mark the recalcification period.

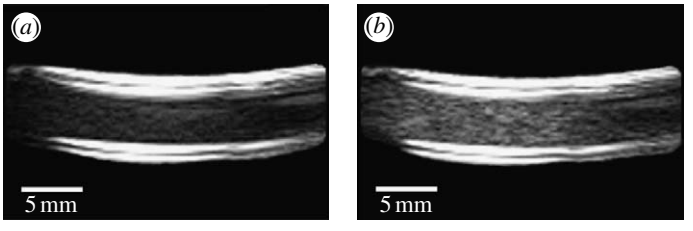

Figure 6. Ultrasonic images of the blood flow in the tube (*a*) before and (*b*) after the formation of microemboli.

4. Discussion

The data presented in this work unambiguously point to the possibility of acoustic determination of the early stages of intravascular blood coagulation. In all our experiments, the beginning of clotting processes in the flow caused a practically twofold increase in the Doppler signal intensity. The formation of microemboli in blood flow always entails the appearance of an acoustic echo contrast in the ultrasonic signal. The large amplitude of the discovered effects suggests that the obtained results could be used for the development of a novel non-invasive technique for early-stage intravascular coagulation diagnostics.

Changes of the parameters of the acoustic signal from coagulating blood have been studied in a number of experimental works (Shung *et al.* 1984; Voleisis *et al.* 2002; Ossant *et al.* 2004; Calor-Filho & Machado 2006). In all these works, coagulation was noted to bring on significant changes in such acoustic signal characteristics as sound speed, backscatter amplitude and/or attenuation of permeated signal. Measurements of ultrasound backscattering, ultrasound velocity and attenuation in the papers cited above have been used to detect the process of blood coagulation under static conditions. The coagulation processes in moving blood were studied with acoustic methods in recent works (Huang & Wang 2007; Uzlova *et al.* 2007). In contrast to the works listed above, we performed simultaneous optical and acoustic registration of the clotting processes. Taking advantage of parallel optical and acoustic methods in the experiments, we reveal a direct correlation between the changes in amplitude of acoustic backscattering and the formation of fibrin clots.

The early stages of fibrin clot formation have been described theoretically. We are aware of a few mathematical models that can qualitatively describe certain stages of the blood coagulation cascade (Khanin & Semenov 1989; Willems *et al.* 1991) as well as threshold phenomena in BCS (Jesty *et al.* 2005). More extended models have been suggested for the description of the aPTT test usually used in clinical practice (Kogan *et al.* 2001; Kramoroff & Nigretto 2001). Unfortunately, an explicit mathematical expression for the partial thromboplastin time as a function of the kinetic parameters of the coagulation cascade has not been established. Moreover, some problems in the interpretation of continuous thrombin generation assays have recently been revealed (Butenas & Mann 2007).

It has recently been established that, owing to the internal complexity of the kinetic cascade, there are important limitations to the simulation of the clotting system (Wagenvoord *et al.* 2006). It was found that the large majority of biochemical reaction mechanisms suitable for successful simulation of a thrombin generation curve do not contain more information than can be expressed by four parameters. A rather limited kinetic mechanism of only six reactions (comprising proteolytic activation of factor X and factor II, feedback activation of factor V and thrombin inactivation by antithrombin) can simulate practically any thrombin generation curve in a number of different ways. Thus, those authors came to the conclusion that 'successful simulation of experimental data therefore does not validate the underlying assumptions. *A fortiori*, simulation that is not checked against experimental data lacks any probative force. The information content of a thrombin generation curve is smaller than the

information required to describe a physiologically realistic reaction scheme. Consequently, much of the input information is irrelevant for the output.'

These arguments explain in part why purely phenomenological mathematical models are widely applied for the modelling of blood coagulation in reaction–diffusion systems without convection (Ataullakhanov *et al.* 1998) and in a weak flow environment (Chulichkov *et al.* 2000; Guy *et al.* 2007).

For clot formation under high-flow conditions, any theoretical approach is restricted by hydrodynamic limitations associated with the as yet unsolved 'turbulence problem' (general instability of hydrodynamic flow under high Reynolds numbers; Reynolds 1895; Falkovich & Sreenivasan 2006). Thus, any rigorous theoretical description of blood coagulation under the intensive flow conditions relevant to large human vessels seems to be improbable in the near future. For this reason, the development of an efficient experimental approach for the determination of the primary stages of intravascular blood coagulation is of significant importance.

Primary microemboli generation as a result of intravascular blood coagulation has recently been investigated theoretically (Zlobina & Guria 2006). The activation of BCS, regardless of its direct cause, initiates a cascade of autocatalytic reactions that lead to avalanche-like formation of fibrin monomers. The polymerization of fibrin takes place together with the increase in its concentration. At first, fibrin molecules form dimers, trimers and oligomers. Then fibrin microemboli appear, and their further mutual aggregation leads to the formation of fibrin fibres and nets of macroscopic scale. The final stage of the fibrin polymerization process is the formation of solid clots. The accumulation of fibrin monomer after BCS activation happens quite swiftly. In the case of oversaturated solution, the fibrin polymerization process develops in a blow-up manner (de Gennes 1979; Gennisson *et al.* 2006; Guria *et al.* in press). Therefore, the time interval between the development of primary detectable microemboli in the flow and the formation of emboli of considerable size in our experiments does not exceed 60 s. As the aggregates reach a size comparable with the acoustic wavelength, the scattering of ultrasonic waves increases significantly. The two-dimensional visualization (B-mode) of the signal from the blood flow at that moment demonstrates the appearance of an acoustic echo contrast.

The reasons for the appearance of spontaneous acoustic echo contrast in blood flow have been widely discussed (Wang *et al.* 1992; Fatkin *et al.* 1997; Zotz *et al.* 2001; Ercan *et al.* 2003). The underlying mechanisms have been studied both *in vitro* (Fatkin *et al.* 1997) and *in vivo* (Wang *et al.* 1992), and discussion still continues (Tanaka & Saijo 2007). The nature of the processes causing acoustic echo-contrast generation is not always confined to blood coagulation processes alone (Wang *et al.* 1992; Fatkin *et al.* 1997). However, the results we have obtained unambiguously point to the fact that the formation of microemboli in the early stages of clotting in intensive flows is always accompanied by the appearance of acoustic echo contrast.

The development of the acoustic method for the diagnosis of intravascular coagulation processes in their early stage is of great interest owing to the non-invasiveness of the procedure. Moreover, all practically used coagulation tests only allow the evaluation of BCS state in the whole organism, providing no data about local changes of BCS state. At the same time, thrombotic events can occur as a consequence of locally developing processes. For instance, the development

of some pathological process in tissue may be followed by the infiltration of BCS activating substances into the blood flow. Another example is local BCS activation due to hydrodynamic perturbations. The usage of ultrasonic methods would facilitate the registration of local changes in the aggregative state of the blood in potentially the most dangerous parts of the circulatory system. The latter may be of practical interest, for instance, in post-operational monitoring of the patient's coagulation status in cardiovascular surgery. It should be noted that an important advantage of acoustic methods is the possibility to use them for permanent real-time observation of the BCS state.

The described ultrasonic method for detection of the early stages of blood coagulation is based on the registration of microemboli. The latter are formed in blood only when the BCS has already been locally supercritically activated. Furthermore, our experiments showed that the registration of blood coagulation processes by acoustic methods is possible only after the appearance of sufficiently large aggregates in the flow. So far the acoustic methods we have used cannot provide any information about the BCS state before the beginning of microemboli formation. Therefore, the approach described here could be used particularly for detection of the early stages of the intravascular coagulation process but not as a diagnostic tool to determine the proximity of the BCS to its activation threshold.

We hope that new non-invasive ultrasonic methods for the detection of the early stages of blood coagulation processes will find clinical applicability soon.

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