Combined Electro-Optical Imaging for the Time Evolution of White Thrombus Growth in Artificial Capillaries

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***Abstract*—We present a novel methodology to measure white thrombus volume growth in an artificial microchannel, where whole blood flows. We designed a sensor consisting of a poly-dimethyl-siloxane microchannel and parallel gold electrodes sputtered on the surface of a slide, where induced hemostasis takes place. A novel inversion methodology, based on optical and electrical impedance data simultaneously processed, allows reconstructing the thrombus volume. The advantage of the proposed methodology is to reconstruct the evolution of the thrombus volume as a function of time; this is not possible with the present state of the art optical imaging based on confocal microscopy, which provides the thrombus volume estimation only at the end of the process.**

***Index Terms*—Electrical impedance spectroscopy, whole blood hemostasis.**

I. Introduction

IN THE PERSPECTIVE of a clinical screening, there is an increasing interest in *in vitro* measurement of thrombus formation under flow conditions for the evaluation of the thrombotic process in humans. Current available technologies capture the kinetics of thrombus formation in coated artificial capillary or flow chamber by means of a video imaging technique and image processing, which include a microscopy device either inverted or confocal. In this way, also the anti-thrombotic profiles of therapeutic agents can be also monitored. For the kinematic imaging of white thrombus formation a fluorescent labeling for platelets is used (either Rhodamine or alternatively mepacrine). In the inverted microscope, the quantification of white thrombus formation is performed from a digital bi-dimensional data image of a whole blood sample based on pixel luminance. The 3-D size of the white thrombus is therefore mathematically extrapolated only from the pixel luminance and may not reflect its actual size.

This problem can be overcame by the use of a confocal microscope. However, the size of the instrument and its costs are against its usage in a bedside clinical setting, thus, it is usually confined to an experimental sophisticated laboratory setting. In addition, all other instruments currently on the market (like PFA 100, Ultegra, Impact) do not analyze the kinetics of thrombus formation and/or the thrombus stability.

Here, we present a novel system, which should provide information on the individual predisposition to form thrombi. This methodology will develop a quantitative mean to evaluate the volume of white thrombus during its formation, by monitoring the global electrical impedance of an aggregating blood sample in conjunction with a 2-D optical image of pixel luminance.

Electrical impedance spectroscopy (EIS) applied to blood has received, since early years of last century [1]–[5], a considerable attention because of noninvasive and realtime monitoring of the clotting process. The impedance measurement bench, presented in [6], is formed by an impedance meter and a preliminary low cost sensor based on copper printed circuit board (PCB) technology; measurements are acquired with a control panel on a personal computer. Then in [7], we improved such a preliminary sensor by introducing in the microchannel a pair of gold microwires.

In this letter, in order to characterize blood and thrombus electrical properties, we designed a first device consisting of a microchannel, whose entire bottom and top surfaces are gold sputtered electrodes forming a parallel plates-like geometry. Moreover, to detect thrombus formation, we designed a second sensor consisting of multiple pairs of parallel gold electrodes, sputtered only on the surface of the slide closing the microchannel and transversally with respect to the blood flow direction. Whole blood flows and induced hemostasis takes place and grows only on the surface of the plastic microscope slide. The gold electrodes of the second sensor have been designed in order to reconstruct the thrombus geometry and volume by means of a novel inversion methodology based on optical and impedance data simultaneously processed. With such a sensor, at a time instant and at a specific frequency, the acquired impedance data are post processed and compared with respect to a corresponding computed impedance obtained from an electromagnetic numerical model of the overall system formed by the microchannel, containing the thrombus, and the gold electrodes. The geometry of the thrombus is represented by a solid model deduced from the 2-D optical image of pixel luminance. For each pixel, the height of the thrombus, with

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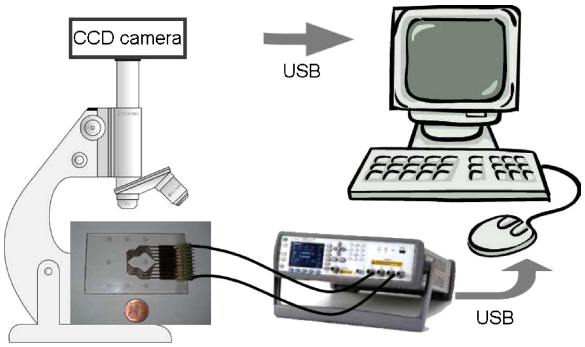

Fig. 1. Block diagram of the measurement setup: the thrombus growth in the microfluidic sensor provides an optical information through a microscope and a CCD camera and an electrical information through an LCR meter. Electrical and optical information are both automatically acquired by a computer.

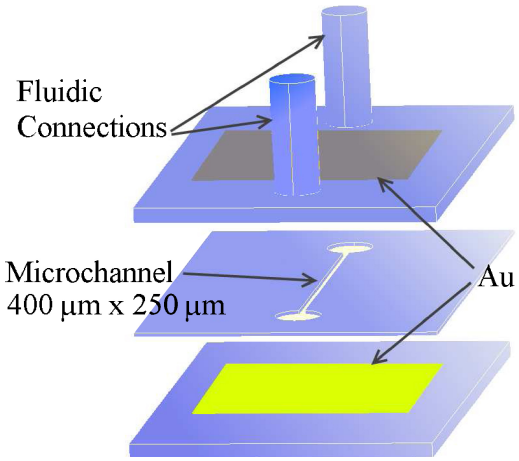

Fig. 2. Assembly of the microchannel used for blood and thrombus characterization.

respect to surface of the slide, is assumed to be proportional to the luminance according to a unique scale factor for all the pixels. In order to determine such a scale factor, a number of 3-D steady-state current conduction analyses of the overall system meshed with tetrahedra are performed by modifying the scale factor in such a way that the computed impedance matches the measured one; the white thrombus volume is then easily evaluated by the thrombus solid model.

The 3-D steady-state current conduction analysis tool is based on the discrete geometric approach developed by the authors [8], [9].

In order to compare the evaluated volumes with the actual volumes of the white thrombus, we considered a typical distribution of the aggregates volumes measured by means of a confocal microscope on the same blood at a reference time instant (5 min); it must be noted that such a reference measurement cannot be performed directly on the flowing blood but, on the contrary, we need to stop the experiment and leave the confocal microscope to focus on different planes with prescribed steps starting from the slide surface for some minutes.

In the experimental results section, we will show how the reconstructed volume is quite in a good agreement with reference thrombus obtained from the same whole blood.

II. System Description

The system description of the measurement procedure is summarized in Fig. 1.

The developed microfluidic chip (described in detail in Section II-B) is electrically connected to an LCR meter and posed under an optical microscope with a charge-coupled device (CCD) camera; both LCR meter and CCD camera communicate with a computer through USB interface and a software control panel simultaneously acquires optical and impedance data.

Blood flows inside the microchannel at a controlled temperature of $37°C$ and at a controlled flow rate in order to simulate the *in vivo* conditions. Collagen is spread on the upper wall of the microchannel in order to induce the aggregation process of platelets, which are marked with quinacrine; in the thrombus, marked platelets when excited with blue (488 nm wavelength) fluorescence, reemit the light in the green zone (522 nm wavelength): increasing the thrombus base, the CCD camera will provide a larger number of green pixels, which delivers a higher light intensity. In particular, we evaluate the light intensity of each pixel by means of the luminance $L = 0.299R+0.587G+0.114B$ [10], being R (Red), G (Green) and B (Blue) the RGB values of the acquired images.

The acquired data are managed by a steady-state current conduction field simulator [8], [9], which meshes the electrodes, the microchannel and the thrombus geometry starting from the optical information provided by the images acquired by the CCD camera; the conductivity of the thrombus with respect to conductivity of the plasma is obtained from an experimental blood electrical characterization.

The luminance of each image provides a map of the thrombus base and the shape factor of the thrombus geometry (i.e., which pixels represent higher thrombi). Finally, the geometry is meshed by multiplying the numeric value of luminance by a height factor which is iteratively changed; with this methodology the thrombi heights are tuned in order to match the overall impedance change at prescribed time instants.

A. Blood Electrical Characterization

In order to validate the impedance measurements during white thrombus formation described in Section III and estimate the electrical parameters to be used in the steady-state current conduction field simulator, a preliminary test chamber chip has been realized to characterize the fluids under investigation.

In Fig. 2, the assembly of the preliminary microfluidic device is shown. It is composed of three layers; the first layer is a Polycarbonate (PC) slide on which a 25 mm $\times$ 25 mm $\times$ 200 nm gold electrode has been sputtered and microfluidic connections are glued. The second layer is a 250 μm thin polydimethyl-siloxane (PDMS) membrane on which a 400 μm wide and 25 mm long microchannel has been carved; the third layer is exactly like the first one, without fluidic connections. The device can be disassembled in order to spread collagen on the upper wall of the microchannel, the assembled three layers are kept together with a clamp and aligned through references

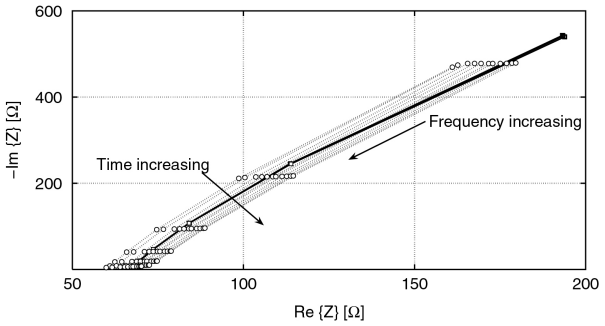

Fig. 3. Impedance spectroscopy with $(\cdot\circ\cdot)$ or without $(-\square-)$ thrombus formation. Test frequencies are 1, 3, 8, 20, 60, 150, and 250 kHz. The time evolution is acquired on a 5 min interval with a resolution of 5 s; for graphical reasons only impedance data acquired every 30 s are plotted.

printed on the layers. The overall behavior of the assembled device is a parallel plates impedance, whose contact surface is 400 μm $\times$ 25 mm with a gap between plates of 250 μm.

The impedance spectroscopy measurements have been performed using the high precision LCR meter Agilent E4980A in the frequency range 1 kHz−250 kHz with seven logarithmic spaced steps, a two-wire configuration and a drive voltage of 100 mV; such drive voltage is by far lower than the standard half cell potential for gold (that is 1.5 V) in order to avoid some artifacts due to redox reactions taking place between electrodes and salts dissolved in plasma. The LCR meter has been connected to the assembled empty device and an open calibration has been performed in order to eliminate the capacitive parasitic effects of the membrane interposed between the electrodes outside the microchannel and the effects of connections. In these conditions, the impedance measurement accuracy is 0.1 % [11]. Blood flows in the microchannel at a flow rate of $Q = 750$ μl/min in order to obtain a target shear rate of $\gamma = 3000$ s^{-1}, according to the relation, valid for rectangular ducts

$$\gamma = \frac{6Q}{wh^2}. \tag{1}$$

Fig. 3 shows the real−imaginary plot of the impedance for blood ($-\square-$ curves) and blood with the collagen spread on the upper wall to induce white thrombus formation ($\cdot\circ\cdot$ curves) over a time interval of five minutes; the time resolution of acquired data is 5 s, but, due to graphical reasons, each plotted curve is shown every 30 s in Fig. 3.

In order to better understand Fig. 3, we have to state that the interaction between a biological fluid and a metal electrode forms a narrow interface, known as double layer, where the current flows as in a capacitive layer but the phase shift of current is less (in magnitude) than $-\pi/2$; for this reason it is useful [12] to define the constant phase element (CPE) and its impedance is given by

$$Z_{CPE}(\omega) = \frac{1}{(j\omega)^{\beta}\, CPE} \tag{2}$$

where β, with $0 \leqslant \beta \leqslant 1$, represents the phase shift and CPE represents a term not depending on frequency.

In Fig. 3, it is remarkable that the double layer plays an important role at low frequencies, while at higher frequencies the measured impedance becomes purely real thus bypassing the double layer CPE. Since before each measurement we performed open and short compensations to remove the parasitic parallel capacitance of cables and electrodes and the parasitic series inductance of cables, we can assume that the equivalent circuit of measured impedance is, in this frequency range, approximated by the series of a CPE and a resistor.

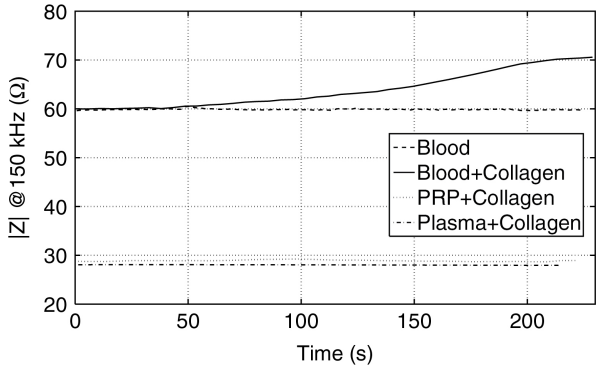

Fig. 4. Impedance magnitude at 150 kHz versus time for plasma, PRP, and blood with and without collagen addition.

TABLE I
PLASMA, PRP, AND BLOOD CONDUCTIVITIES WITH RELEVANT ACCURACIES

Fluid	Conductivity (S/m)	Uncertainty (S/m)
Plasma+Collagen	0.92	0.05
PRP+Collagen	0.89	0.05
Blood	0.42	0.03

Moreover, in [9] we demonstrated that blood cells behave like an electrical insulator if the frequency is lower than 300 kHz, hence there is an optimal frequency interval [100, 300] kHz where the double layer is bypassed and the blood behaves (in this frequency range) like a resistor, whose resistance is increased by the thrombus growth, since the resistive cross section of blood is reduced proportionally to the thrombus height and width.

In Fig. 3, it is also possible to see that during the entire experiment on the same sample of blood without collagen ($-\square-$ curves) there is a very high repeatability while in the sample with blood and collagen there is a drift over time ($\cdot\circ\cdot$ curves). To assure that this drift is strictly related to thrombus formation and not to other artifacts (e. g. changes in collagen electric properties), we compared the impedance drift of plasma with collagen, platelet rich plasma (PRP) with collagen, blood and blood with collagen.

Fig. 4 shows the time plots of impedance magnitudes evaluated at 150 kHz. As it can be seen, the impedance drift takes place only when the aggregation process is involved. From Fig. 4, it is also possible to characterize the electric properties of the fluids known as the device dimensions. Considering an accuracy of 10 μm for the microchannel dimensions and propagating the uncertainty, it is possible to derive the conductivities of plasma with collagen, of PRP with collagen, and of blood as shown in Table I.

Obviously, the derived conductivity data are valid for a specific patient and depend on the specific values of

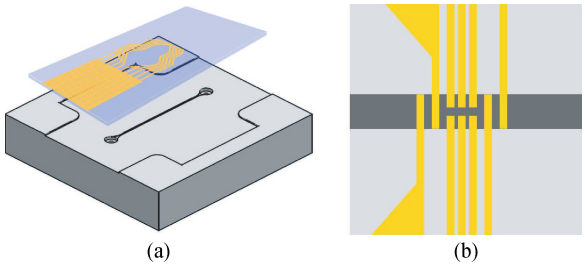

Fig. 5. (a) Assembly of the sensor composed of PDMS microchannel and microelectrodes on PC. (b) Zoom of microelectrodes disposition.

hematocrit, blood cells counts, and dimensions. In particular, in the reported experiment the hematocrit was $H = 44\%$, the red blood cells (RBCs) count was $5.21 \times 10^6/\mu$l and the platelet count was $147 \times 10^3/\mu$l.

The ratio between measured conductivities of blood and plasma $\sigma_{BLOOD}/\sigma_{PLASMA}$ is in good agreement with Maxwell-Fricke theory. In Maxwell-Fricke theory, since the main cellular content in blood is due to RBCs, the conductivity depends mainly on the hematocrit value H and a factor C depending on the RBCs shape ratio, according to

$$\frac{\sigma_{BLOOD}}{\sigma_{PLASMA}} = \frac{1 - H}{1 + (C - 1)H} \tag{3}$$

considering that, at high shear rates, RBCs tend to deform into prolate ellipsoids with their long axis aligned parallel to the flow, and form layers that slide on adjacent plasma layers [13]–[15].

B. Developed Microfluidic Chip

After completing the characterization phase, we developed a new microfluidic chip with gold electrodes specifically designed for thrombus volume estimation. It is composed by a PDMS substrate with fluidic connections and a PC microscope slide with 200 nm thick sputtered gold electrodes, as shown in Fig. 5 (a).

On the PDMS substrate, an open microchannel without the upper wall whose dimensions are 400 μm in width, 250 μm in depth and 25 mm in length is molded; also the fluidic connections have been molded on this substrate. The upper wall of the microchannel is formed by a PC slide with 200 nm thick sputtered gold electrodes, arranged as shown in Fig. 5 (b). Two different electrode arrangements have been investigated during the tests. A first arrangement comprising of a couple of electrodes whose length extends over the entire microchannel width and the electrode width is 100 μm, the electrodes gap is 100 μm; referring to Fig. 5 (b), this arrangement represents the two couples posed on the left and on the right of the sketch. The second electrode arrangement is composed of three couples of facing electrodes whose width is 100 μm, extending 150 μm inside the channel with a transversal gap of 100 μm; each couple of electrodes is separated by 50 μm from adjacent couples. Referring to Fig. 5 (b), this arrangement is posed on the central part of the sketch. In our tests, this second arrangement did not provide satisfying results, since the edge effects due to smaller electrodes masked the phenomenon to be measured; so in Section III all the experimental results refer to the first electrodes arrangement.

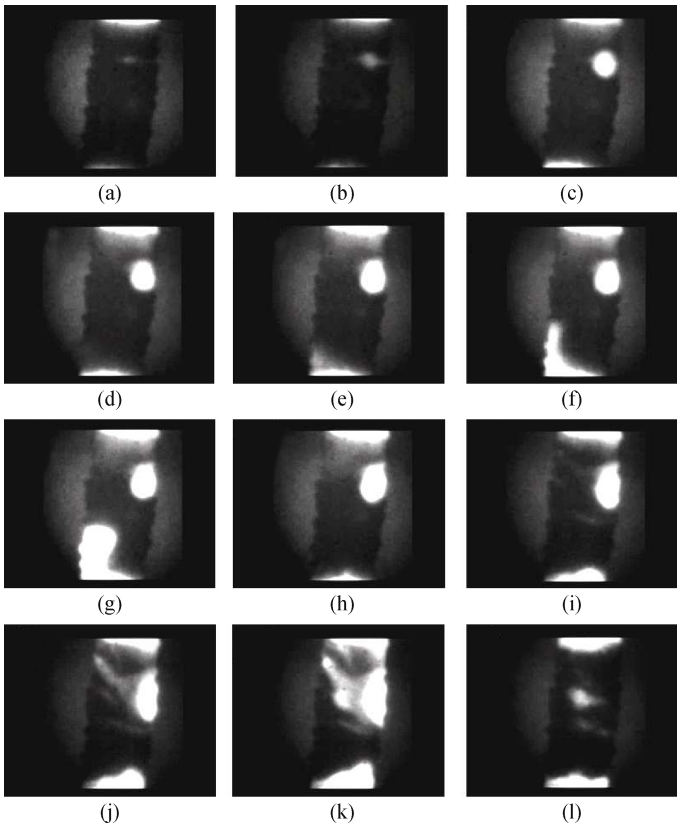

Fig. 6. Frames acquired with microscope during the experiment shown in Fig. 7. (a)–(e): From 0 to 110 s in Fig. 7. (f)–(h): From 110 to 120 s in Fig. 7. (i)–(k): From 120 to 210 s in Fig. 7. (l): 215 s in Fig. 7.

III. Experimental Results and Post Process

The impedance measurements have been performed connecting the slide (representing the top wall of the microchannel) to the E4980A and automatically acquiring the impedance data with a software control panel as shown in Fig. 1.

The control panel has been set up in order to perform a two-wire measurement with the same parameters described in Section II-A in order to avoid redox reactions at the electrodes; before performing measurements on blood it has been measured the impedance of cables and of the empty sensor and then an open-short compensation has been applied in order to achieve maximum accuracy of the instrument.

Blood flow rate has been kept constant in order to obtain a shear rate of 3000 s^{-1} according to (1) in a 37 °C controlled temperature perfusion chamber and experiments have been performed for a standard duration of five minutes.

Fig. 6 shows the most significant frames acquired from the microscope during the experiment shown in Fig. 7; the blood flows from the left to the right of each frame, the electrodes are vertically oriented and they extend to the entire width of the channel. The growing thrombus is represented by the white spots inside the investigation area.

The continuous line plotted in Fig. 7 shows the impedance magnitude at 150 kHz frequency; this plot is highly significant since we can evaluate the thrombus volume also in transient

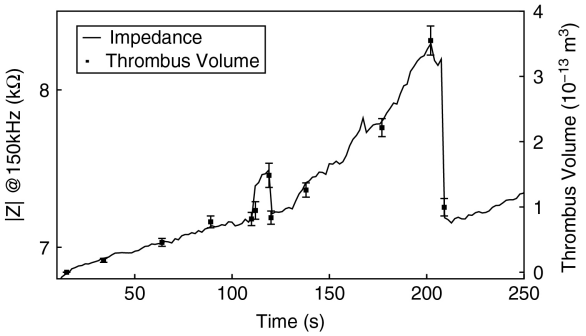

Fig. 7. (–):Behavior of the impedance magnitude versus time at 150 kHz when aggregation is taking place. (■): reconstructed thrombus volume relevant to frames shown in Fig. 6. Error bars represent the uncertainty estimation on volume reconstruction.

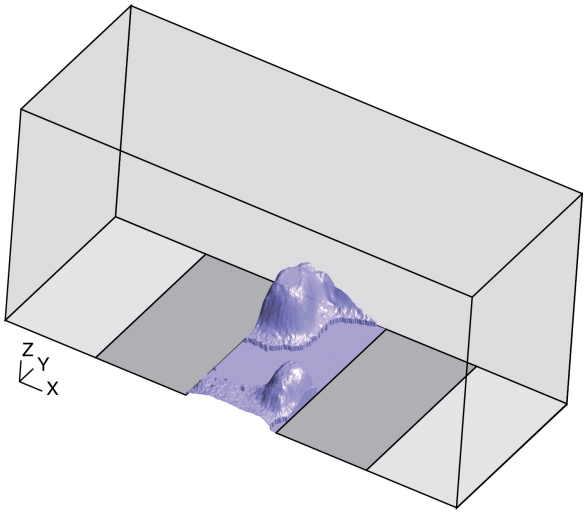

Fig. 8. Thrombus geometry through optical and impedance reconstruction.

changes. Referring also to Fig. 6 (f)–(l), in fact, it is possible to see that in the time intervals [110, 120] s and [130, 210] s a temporary thrombus is brought in the investigation zone by the flow and, when grown too much, is swept away by the blood flow. With standard measurement systems (e.g., confocal microscope), the evaluation and measurement of these phenomena is not always possible, since the transient thrombus formation can be fast enough (few seconds) to impede the operator to stop the flow on time to make the confocal scan.

The markers shown in Fig. 7 represent the thrombus reconstructed volume relevant to the frames shown in Fig. 6.

The uncertainty on the volume reconstruction can be overestimated approximating each reconstructed thrombus with a cylinder; this hypothesis is pejorative since thrombus shape looks like a paraboloid as shown in Fig. 8.

The uncertainty of each thrombus base diameter, D_i, is related to quantization pixel of microscope and can be quantified in $u(D_i) = 1\,\mu$m, while the uncertainty of the height h_i is related to the simulations steps and can be quantified in $u(h_i) = 5\,\mu$m. Since each thrombus volume can be overestimated as

$$V_i = \pi \frac{D_i^2 h_i}{4}. \tag{4}$$

Thus, propagating the uncertainty and considering height and diameter uncorrelated quantities since derived from two different measuring instruments, we obtain

$$u(V_i) = \sqrt{\left(\frac{\pi D_i h_i u(D_i)}{2}\right)^2 + \left(\frac{\pi D_i^2 u(h_i)}{4}\right)^2}. \tag{5}$$

In Fig. 7, the uncertainties relevant to each volume reconstruction are reported as error bars; the maximum relative uncertainty on the volume reconstruction is estimated less than 20%.

IV. Conclusion

We developed a combined methodology coupling light distribution and intensity on a CCD surface from the one hand and impedance measurements from the other. Such a technique allowed us to estimate the volume of thrombus formation as a function of time along a portion of the microchannel under flow conditions. The experimental results demonstrated how the thrombus volume was well correlated with the amplitude of the measured impedance.

V. Acknowledgments

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