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## Characterization of thin biological layer by scanning acoustic microscope (SAM)

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In this paper, the importance of study of adhesion level of biofilms is discussed at the beginning which the main driving force of this project. Then, the simple case of a various plates immersed in water is discussed and the results are presented. An extreme case of a 150  $\mu\text{m}$  thick glass plate is illustrated in order to show the mixed signals resulting a central frequency of transducer that is not high enough. Therefore, temporal and frequential studies have been explored in order to understand the possibilities that might exist for the special case that the wavelength of transducer beam is larger than the thickness of sample to be studied. Finally results of measurements done for these methods have been presented.

## 1 Introduction

Regarding France health organization, from every 20 people, one person is died because of nosocomial diseases, which is about 3500 to 9000 people per year in France, from 2008 to 2017. This is without counting the people died from bacterial resistance against antibiotics. Most of these nosocomial diseases have been caused by Staphylococcus Aureus, Escherichia Coli and Pseudomonas Aeruginosa [1] Nosocomial diseases are usually due to formation of biofilm on various surfaces in touch with patients like catheters, implants, beds, sinks, toilettes etc. Biofilms adhesion on surfaces needs an intermediate thin layer of protein who serves as the nourishing medium to the biofilm. There are various types of proteins who promote or weaken the adhesion of biofilm to various substrates. In the project resulting in current paper, first of all the intermediate layer of protein is characterized by means of scanning acoustic microscope. In the second step, the adhesion level of various proteins with various environmental conditionings is studied which is the subject of our other papers.

In this paper, first of all, a method of characterizing various thin substrates by SAM has been introduced, then considering the non-practicability of this method at wavelengths shorter than thickness of studied layer, it has been tried to characterize one of the most commonly used types of proteins, Bovine Serum Albumin (BSA) using other temporal and frequential methods.

## 2 Methodology

A solid plate immersed in water (plate is in contact with water at both sides) with the thickness of  $d$  is considered to be placed at distance  $L$  to the transducer. The first ultrasound echo reflected spectrum from the top of the plate is called  $\underline{S}_1(f)$  and the second ultrasound echo reflected spectrum from the bottom of plate is called  $\underline{S}_2(f)$ . These formula have been developed during current project but they are also found to be to some extent similar to the formulas developed by Strohm et Kolios [2]. It could be written as following:

$$\begin{cases} \underline{S}_1(f) = \underline{S}_0(f) \cdot \underline{R}_{wg} e^{-j \cdot 2 \cdot k_w \cdot L} \\ \underline{S}_2(f) = \underline{S}_0(f) \cdot \underline{T}_{wg} \underline{T}_{gw} \underline{R}_{gw} e^{-j \cdot 2 \cdot k_w \cdot L} e^{-j \cdot 2 \cdot k_g \cdot d} \end{cases} \quad (1)$$

where  $\underline{S}_0(f)$  is the reference spectrum,  $\{\underline{R}_{ij}, \underline{T}_{ij}\}$  are the reflection and transmission coefficients, respectively, between the layers indexed  $\{i, j\} = \{w, g\}$  for water and glass, respectively.

The transfer function is defined from eq. (1) as follows:

$$\underline{T}_{21} = \frac{\underline{S}_2}{\underline{S}_1} = \frac{-4 \cdot \underline{Z}_w \underline{Z}_g}{(\underline{Z}_w + \underline{Z}_g)^2} e^{-j \cdot 2 \cdot k_g \cdot d} \quad (2)$$

Finally, the speed of ultrasound  $C_g$  and the attenuation  $\alpha_g$  in plate (glass in the present case) are defined as a function of  $\underline{X}_{wg}$  expressed from  $\underline{T}_{21}$  in eq. (2):

$$\underline{X}_{wg} = -\underline{T}_{21} \cdot \frac{(\underline{Z}_w + \underline{Z}_g)^2}{4 \cdot \underline{Z}_w \underline{Z}_g} \quad (3)$$

Then the wanted expression can be deduced from eq. (3):

$$\begin{cases} C_g = \frac{2 \cdot \omega d}{\arg\{\underline{X}_{wg}\}} \\ \alpha_g = \frac{-1}{2d} \log(|\underline{X}_{wg}|) \end{cases} \quad (4)$$

## 3 Plate immersed in water

In order to apply the previously discussed method, 3 samples have been measured using SAM. A glass pate of 1 mm thick, a glass plate of 150  $\mu\text{m}$  thick and a polyethylene (PE) film of 300  $\mu\text{m}$  thick. The time signals measured by a plane transducer at 50 MHz central frequency. Nevertheless, the central frequency has been rechecked for each sample by calculating the fast Fourier transform (FFT) of the specular echo for each time signal. The aforementioned specular echo corresponds to the first surface echo, clearly separated from the second roundtrip echo. The results are presented in following sections.

Figure 1 shows the first and second echoes of the ultrasound reflection on the 1 mm thick glass plate. The time time-of-flight difference between the first and second echo corresponds to the longitudinal wave velocity in this glass plate, evaluated at 5676 m/s. This value is very close to that found in literature [3], for glass at 5660 m/s.

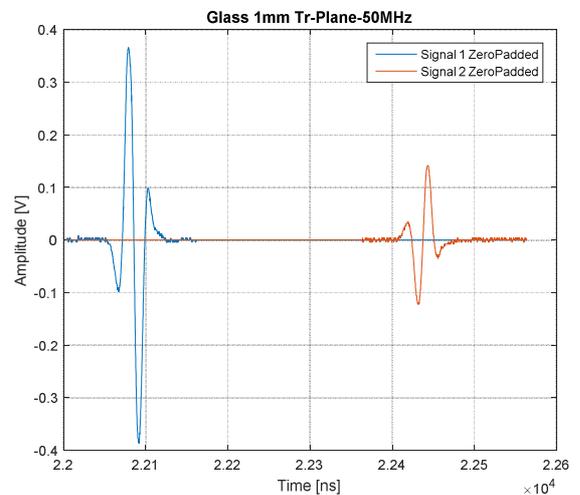


FIGURE 1 – Echoes from a 1 mm thick glass plate.

Figure 2 shows the longitudinal wave velocity in 1 mm thick glass plate as a function of frequency. One could observe an increasing trend in this chart except in the 10 to 15 MHz frequency range, which is quite far from the central frequency of transducer. In this lower frequency range, the signal-to-noise ratio is not sufficient. It should be noted that the nominal frequency of transducer is 50 MHz but it is around 35 MHz when considering the FFT of the first echo.

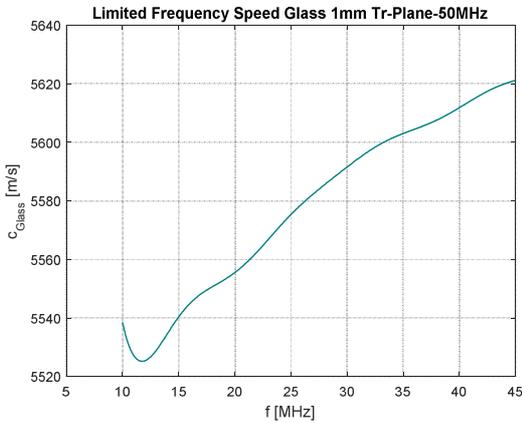


FIGURE 2 – Longitudinal wave velocity as a function of frequency for a 1 mm thick glass plate.

Figure 3 presents the attenuation of ultrasound in 1 mm thick glass plate. One could see many irregularities but in general a low average attenuation is observed, especially in vicinity of 35 MHz which is the measured central frequency of transducer, where the average attenuation is less than 10 Neper/m. The quite high attenuation at lower frequencies, between 10 and 15 MHz should not be considered regarding the assumed non-linearity of transducer response far from its central frequency.

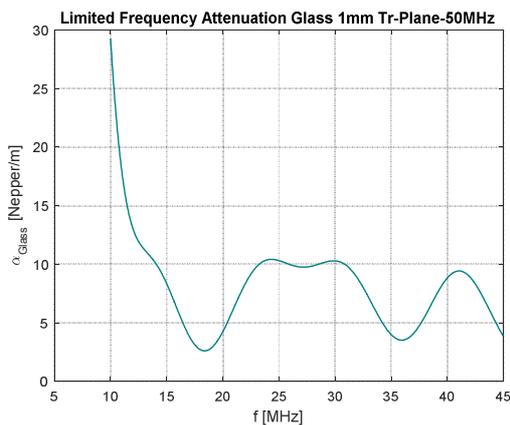


FIGURE 3 – Ultrasound attenuation in a 1 mm thick glass plate.

Figure 4 demonstrates the multiple reflection in a 150 μm thick glass plate. These echoes, include the first, second and subsequent reflections in the thin the glass plate that are near the extreme case of being mixed together, as we are near the axial resolution of our transducer. This case has been chosen to demonstrate the situation in which the wavelength of the transducer becomes close to, but still slightly shorter than, the thickness of the studied sample.

More precisely, the wavelength is equal to 113 μm considering the calculated longitudinal wave velocity in the glass plate and the central frequency of the transducer, which is very close but slightly shorter than the thickness of plate resulting in this extreme case of resolving the time signal.

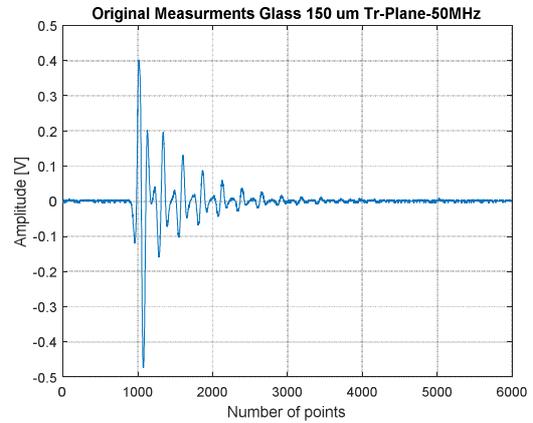


FIGURE 4 – Multiple reflections in a 150 μm thick glass plate.

Figure 5 shows the first and second echoes precisely separated from each other. By calculating the intercorrelation of Hilbert transform of these 2 signals, one could find the time-of-flight difference between first echo and second echo. Hence, that the longitudinal wave velocity in the glass plate, is evaluated at 5639 m/s. It is noted that this value is close to that found for the 1 mm thick glass plate, which is made of glass but not necessarily the same type of glass.

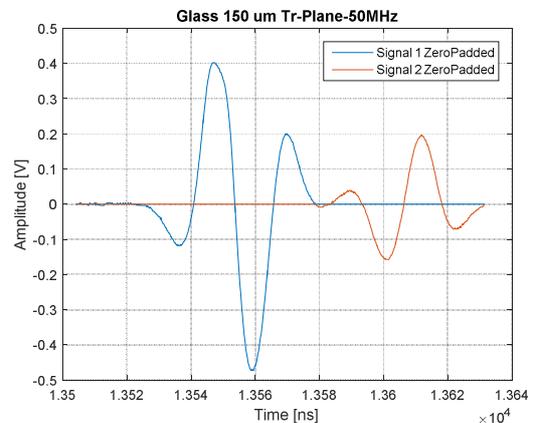


FIGURE 5 – First and second echoes separated for a 150 μm thick glass plate.

Figure 6 shows the longitudinal wave velocity in a 150 μm glass plate as a function of frequency, in the frequency range of the transducer. One could observe a smooth decrease of the velocity in this frequency range. One could note that the average velocity in this frequency range in this glass plate is close to the nominal value calculated before, inferred from the time-of-flight difference between the first and second echoes. It should be noted that Figure 6 shows a quite zoomed presentation of the speed curve, while one could see that the total variation of the longitudinal wave velocity in this glass plate, is only about 0.3% of the nominal calculated value.

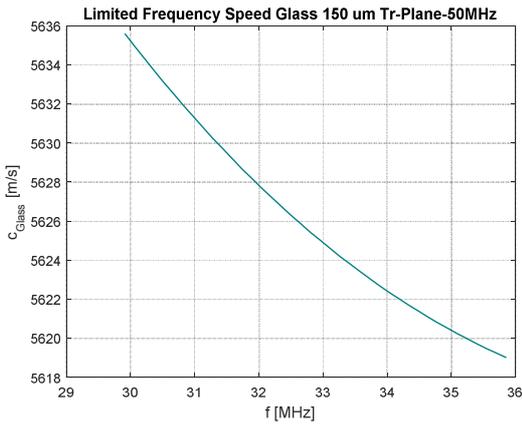


FIGURE 6 – Wave velocity in a 150  $\mu\text{m}$  glass plate.

Figure 7 shows the ultrasound attenuation in the glass plate as a function of frequency. One could see a quite great variation of attenuation as a function of frequency which is not expected. This could be because of instabilities in computations or not being centered on central frequency of transducer, etc. Exploring this curve in other frequency ranges presents negative values of attenuation that are unacceptable.

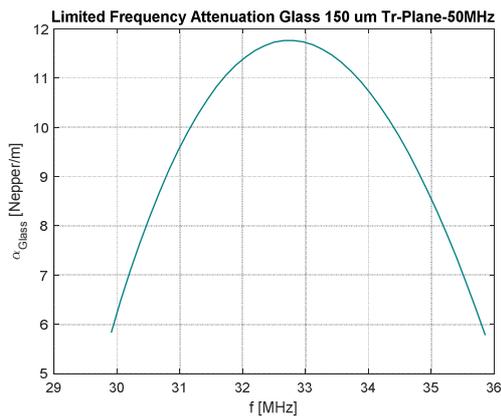


FIGURE 7 – Attenuation in a 150  $\mu\text{m}$  thick glass plate.

Figure 8 shows the first and second echoes, respectively from the top and bottom of a 300  $\mu\text{m}$  polyethylene film. By calculating the correlation between the Hilbert transform of these two echoes, the time-of-flight between the two echoes leads to an evaluated longitudinal wave velocity inside the polyethylene plate, of 1953 m/s. This value is in accordance with reported value in literature [3], that is between 1950 m/s and 2430 m/s, depending on the polyethylene density.

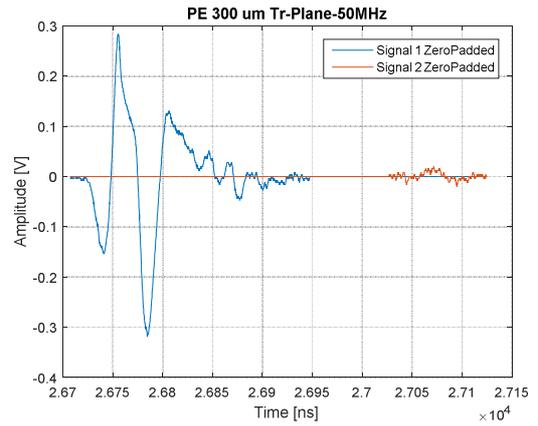


FIGURE 8 – First and second echoes in a 300  $\mu\text{m}$  thick polyethylene.

Figure 9 presents the longitudinal wave velocity as a function of frequency in a 300  $\mu\text{m}$  thick film of polyethylene. The evaluated value is relatively stable in the frequency range from 10 up to 70 MHz, with values between 1960 m/s and 2140 m/s. In the very low frequency range, i.e. where the frequency is less than 10 MHz, the signal-to-noise ratio is not sufficient. These values are in good accordance with previously calculated wave velocities based on time-of-flight measurements, as well as measures from the literature.

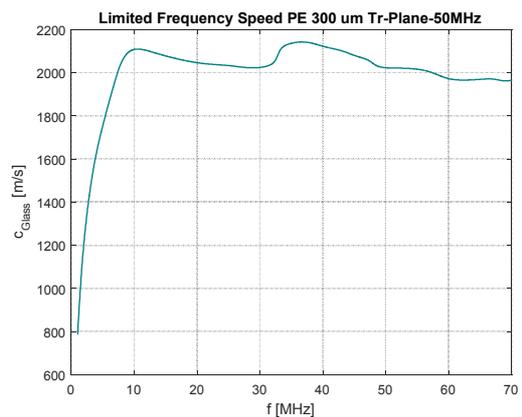


FIGURE 9 – Velocity in a 300  $\mu\text{m}$  thick polyethylene film.

Figure 10 shows the attenuation of ultrasound as a function of frequency in a 300  $\mu\text{m}$  thick film of polyethylene. One could see high so much irregularities in this curve that could be because of irregularities in the initial time signal measurements or computation instabilities.

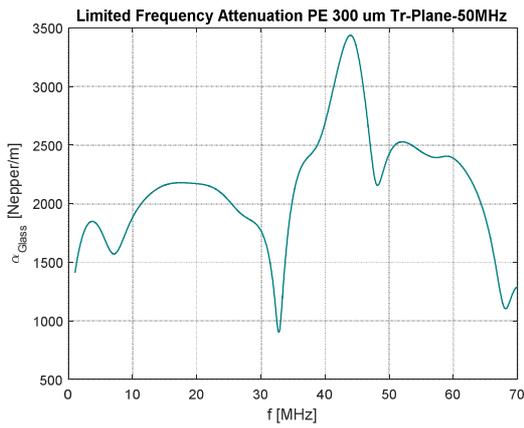


FIGURE 10 – Ultrasound attenuation in a 300  $\mu\text{m}$  thick film of polyethylene.

## 4 Exploring characterization methods of a thin layer considering the mixed signals

As mentioned in the case of a 150  $\mu\text{m}$  thick glass plate, when the frequency of transducer is less than the required limit, in other words the ultrasound beam wavelength is longer than the thickness of the layer to be characterized, the first and second echoes become closer together and beyond specific wavelength, they become mixed with each other, thus making their resolving very difficult. Actually, this is the case with the frequency of our actual transducers. As a result, other temporal and frequential methods have been explored.

### 4.1 Temporal method

In this experiment, a BSA protein layer of approximately 5  $\mu\text{m}$  thick has been deposited on a glass plate of 1mm. Then 2 echoes to be analyzed, have been chosen, namely top echo on the BSA and top echo on the glass plate without BSA. It should be noted that the signal obtained from top of the BSA, is the same signal obtained from top of glass plate, but it has been time-shifted toward left part of the time axis (it arrives sooner than the echo of glass plate) and additionally it has been slightly attenuated by BSA layer. Figure 11 presents these two echoes. The time shift between these two echoes gives a good estimation of thickness of the BSA layer. In this case, the time shift is 4.2 ns. it should be noted that this beam has been flown in the water, so one could calculate the thickness of the BSA layer using this time-of-flight and speed of ultrasound in water. This gives the thickness of 3.11  $\mu\text{m}$ .

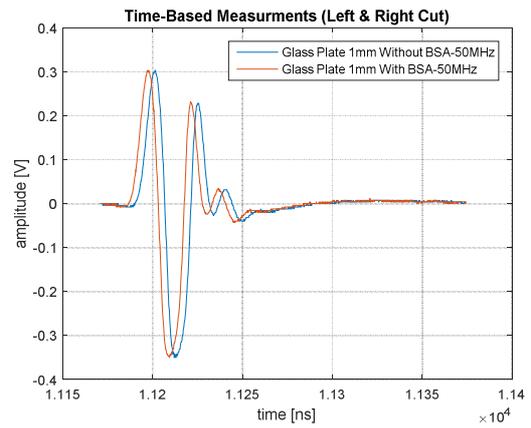


FIGURE 11 – Top echoes of a 5  $\mu\text{m}$  thick BSA layer vs. 1 mm thick glass plate.

In order to make these two echoes comparable, we superpose the peak of echo of top of BSA to the echo of glass plate and we math their amplitudes. This is presented in Figure 12.

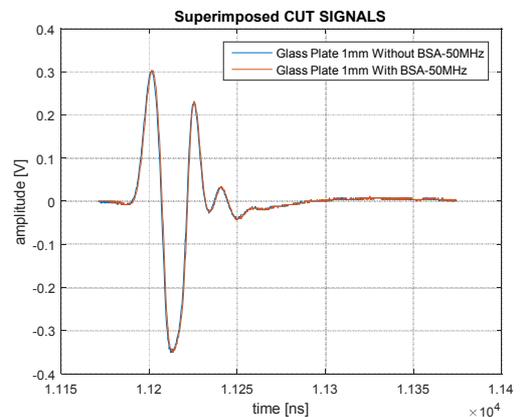


FIGURE 12 – Superposed echoes of top of BSA layer and glass plate.

Once the two echoes are comparable, the echo of BSA top is subtracted from the echo of glass plate, which has been taken as the reference signal. The resulting signal, could be interpreted as the signal that has been flown through the thin layer of BSA. This is presented in Figure 13.

As a result, the difference between the extremum point (maximum or minimum) of the subtracted signal and the peak of reference signal has been calculated. This time-of-flight, gives us the speed of ultrasound in BSA layer or the thickness of layer, based on the information that we have in hand. For example, referring to the literature, [4], the speed of ultrasound in BSA layer is 1920 m/s. Thus, one could estimate the thickness of BSA layer using new time-of-flight that is 5.8 ns (difference between  $t_3$  and  $t_1$  considering Figure 13). Thus, one could estimate the thickness of BSA layer to be 5.57  $\mu\text{m}$ .

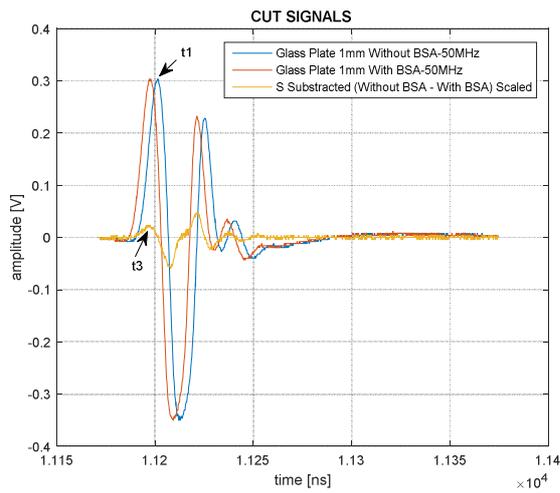


FIGURE 13 – Signals resulting from subtraction of top echoes of BSA layer and glass plate.

### 4.2 Frequency domain analysis

For this method, the following experiment has been set up. The first echo or top echo on the BSA layer has been measured in addition to the surface echo on the glass plate. These two echoes are compared together in Figure 14. It could be seen that the two echoes are separated from each other by a time shift corresponding to different time-of-flight over BSA layer and glass plate, as well as an attenuation. If one calculates the difference of time-of-flight in water, which is 13.59ns, over BSA layer and glass plate, the thickness of BSA would be estimated to be 10.19  $\mu\text{m}$  at measurement point X1, which is nearly double of the value about 5  $\mu\text{m}$  previously estimated!

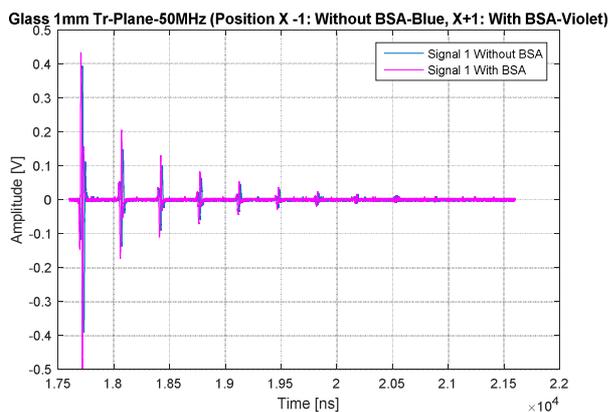


FIGURE 14 – The time-domain reflected echoes from the surface of the glass plate (blue) and from the surface of the BSA layer (magenta).

At the next step, the first echo has been suppressed and then the FFT of the remaining signal including the second echo together with subsequent echoes, has been computed. This process is done once for the signal on the BSA layer and once for the glass plate without BSA layer. The result is presented in Figure 15.

The sharp peaks could be observed in both FFTs that represent the resonance frequencies of examined sample by the perpendicular incidence of the sample surface by the incident wave front. One could observe that the peaks are more or less superimposed but not exactly. This variation of resonance frequencies between echoes on glass sample and on BSA layer, is the source of inspiration in this part of study.

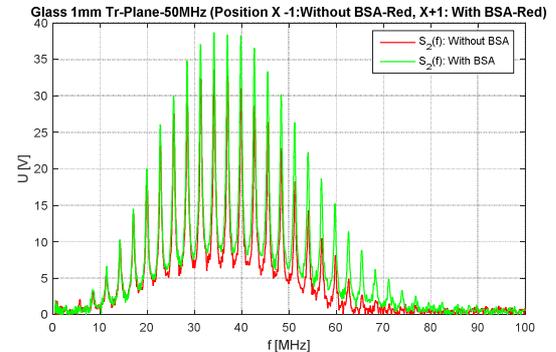


FIGURE 15 – The frequency-domain result from the FFT of reflected echoes from the surface of the glass plate (red) and from the surface of the BSA layer (green).

The peak frequencies over the higher band of frequency range, f1-f6, have been chosen because the difference of frequency variation tends to be higher at these ranges. For each frequency, the exact value for measurement points X1-X5 has been taken, then their maximum variation has been calculated for each peak f1-f6, and then their basic statistics like count, average and standard deviation (StD) have been calculated. This is done to assure that the frequency variation among different measurement points for each of the two main measurements situations (without and with BSA layer) is relatively small. This process has been repeated for the two situation (without and with BSA layer) and the results are summarized in Table 1. These results show that the maximum variation for f1-f6, varies between 50 kHz and 110 kHz for the case without BSA and from 20 kHz to 70 kHz for the case with BSA layer. Therefore, the minimum frequency variation for both cases and all frequency peaks and measurement points, is 20 kHz and the maximum value is 110 kHz.

TABLE 1 – Max variation between points X1-X5 for each frequency f1-f6 [kHz]

High frequency range	f1	f2	f3	f4	f5	f6	Average	StD
Without BSA	50	60	80	60	110	100	76,6	24,2
With BSA		40	20	50	70	60	43,3	20,7

On the other hand, in order to infer from these data, whether the variations of frequencies are remarkable between two cases (without and with BSA layer) or not, the maximum variation of all peak frequencies, f1-f6, for various measurements points, X1-X5, have been recapitulated in Table 2. It could be seen that the minimum value for these variations is 110 kHz and the maximum value is 190 kHz.

Thus, comparing two cases without and with BSA layer, it could be inferred that the maximum peak frequency variations, varies at least 1.7 times and at most 5.5 times the individual variations of frequency for each case, which is quite significant. Therefore, it could be deduced that this method could be a promising method for distinguishing the case with adhered thin layer from the substrate alone, when our transducer frequency is not high enough.

TABLE 2 – Max variation between  $f_{BSA^-}$  and  $f_{BSA^+}$  cases, for the frequencies f1-f6 for each point X1-X5 [kHz]

Measurement points	X1	X2	X3	X4	X5	Average	StD
$f_{BSA^-} - f_{BSA^+}$ (for f1-f6)	110	160	140	190	190	158	34,2

## 5 Conclusion

This paper has been prepared as part of a PhD project for studying the adhesion level of adhering layer of biofilms (proteins) to various substrates. At first, the case of various plates immersed in water has been studied. Then an extreme case of transducer low wavelength comparing to thickness of plate has been studied where the time signals are hardly resolved. At the second step, the temporal and frequential methods have been explored to estimate at least the longitudinal wave velocity and thickness of layer, in the case where the time signals are completely mixed up. It has been experimentally shown that these methods might be promising for the cases where the frequency of transducer is lower than necessary.

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