MEASUREMENT OF THE INHIBITION OF CELL PROCESSES ON FIVE POLYMERIC FILMS BY THE QUARTZ CRYSTAL RESONATOR : COMPARISON TO THE CELL COUNTING TECHNIQUE

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Abstract

The Thickness Shear Mode (TSM) quartz crystal resonator has been extensively used as sensitive sensor in various electrochemical and biological applications. This technique based on the transverse propagation of an acoustic shear wave, generated by a sinusoidal electric field through a piezoelectric quartz resonator, provides a non destructive and powerful mean for probing changes at solid-solid or solid-liquid interfaces. In this work, we propose to apply these systems to characterize the inhibition properties developed by bioactive polymers towards McCoy fibroblast cells adhesion processes. Thin films of various functionalized poly(methylmethacrylate) (PMMA) based copolymers exhibiting either carboxylate and/or sulfonate functional groups and non functionalized PMMA as control were deposited on quartz samples. The effects of induced inhibition of McCoy fibroblasts cell adhesion onto thin functionalized polymeric films of various chemical compositions were analyzed. In addition, shear acoustical results were further compared to those obtained by the cell counting technique.

Introduction

There are many reports detailing techniques related to the measurement of cell adhesion and the evaluation of the number of attached cells to surfaces. An interesting alternative method based on quartz crystal microbalance (QCM) technique has been proposed to follow the cell attachment and proliferation processes. In 1992, Matsuda et al. [1] showed a correlation between the change in the quartz crystal resonance frequency and the number of deposited cells on quartz. With a quite similar system, Gryte et al. [2] followed the cell attachment and the subsequent detachment induced by a viral infection. However, studies involving the quartz crystal resonator to characterize cell adhesion were relatively few. By using new devices [3-4] allowing the determination of the resonant frequency, it is possible to measure any change in quartz impedance induced by cells, polymers or medium. Moreover, these data provide direct access to the viscoelasticity of the adhered cells layer or deposited polymer film. Therefore, because of its high sensitivity, QCM technique appears to be of high interest in order to study interactions between living systems such as proteins or cells and polymer or metallic surfaces of biomedical devices. The constant improvement and the sophistication of the new synthetic materials devoted to the realization of biomedical device widen the field of its applications and in the same time increase the need of high sensitive methods of evaluation. Thus, the behaviour of cell in contact to new synthetic implant surfaces (cornea, joint prostheses, vascular implants) could be *in vitro* evaluated through QCM technique and results compared to those obtained with more classical techniques : adherent cells counting, cell proliferation [5-7]. The new polymers we propose to investigate are new biomimetic polymers exhibiting defined chemical functions and able to inhibit McCoy fibroblasts cell adhesion and proliferation.

Materials and methods

The thickness shear mode resonator (Fig. 1) is constituted by a AT-cut quartz disk (Mattel, Créteil, France) on both sides of which gold electrodes (5 mm in diameter - 2500 Å thickness) were deposited by evaporation on a thin chromium adhesion layer. The quartz crystal diameter d equals 14 mm and the fundamental resonance frequency f_0 = 9 MHz. The upper side was coated by a thin film of bioactive polymer and exposed to a McCoy fibroblast suspension (250 000 cells /ml). The electrode in contact with the solution is limited by one of the O-ring joints. On the opposite side -in contact with air- a silica gel maintain a constant degree of humidity. The device is placed at 37°C under controlled atmosphere (5 % CO_2) in a cell culture incubator.

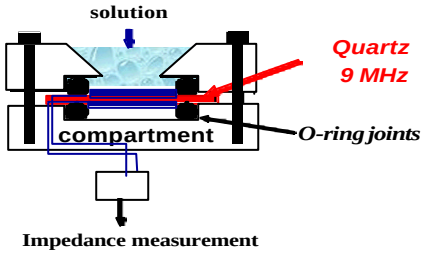
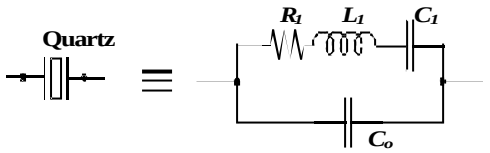


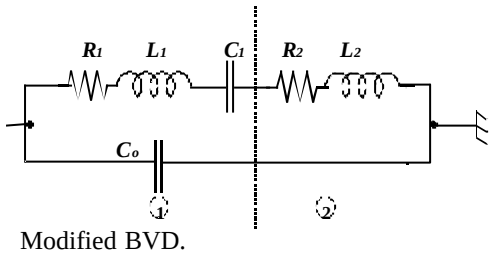
Figure 1 : Scheme of the experimental device.

The electro acoustical response of the sensor device was first investigated for an unperturbed resonator (i.e. without cells).

Data analysis was performed using the lumped element Butterworth-Van Dyke (BVD) equivalent circuit. This circuit consists in a capacitor C_0 in parallel with an inductance L_1, a resistor R_1 and a capacitor C_1 in series (see Fig. 2). When the quartz crystal resonator is coated with a bioactive polymer film or in contact with the cell suspension, this new element can be represented as a resistance R_2 due to specific damping and an inductance L_2 due to the frequency shift. In this system, the R_2 value will depend on the importance of the McCoy fibroblasts cell adhesion.



Butterworth-Van Dyke (BVD) equivalent circuit.



Modified BVD.

Figure 2: BVD equivalent circuits.

Analysis of the impedance.

When McCoy fibroblasts and/or bioactive polymers films are deposited or present at the quartz crystal resonator surface, variations of the R_2 value were observed. These variations are related to modifications of the viscosity at the quartz/polymer or quartz/cell interface. In a first approach, R_2 resistance was directly calculated from the medium viscosity [8, 9]:

$$R_2 = \omega L_2 = \frac{N\pi}{4K_0^2 \omega_S C_0}\sqrt{\frac{\omega \rho_m \eta_m}{2\rho_q \mu_q}} \approx a\sqrt{\rho_m \eta_m}$$

with ρ_m medium density, η_m medium viscosity.

McCoy fibroblasts culture.

McCoy fibroblastic cells were cultured in Dulbecco's Modified Eagle's Medium (D.M.E.M., In Virogen, France). The culture medium was supplemented with 10% (*v/v*) of fetal calf serum (F(FCS), 4 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Fibroblasts were grown in a humidified incubator with 5% CO_2 atmosphere at 37°C. McCoy monolayer were twice washed with DMEM and then detached by Trypsin solution 0.25% (*w/v*) supplemented with 1mM EDTA for 5 min at 37°C. Trypsin digestion was stopped by the addition of an excess of complete culture medium. Fibroblasts were then centrifuged at 110 g for 10 min and its concentration adjusted to 250 000 cells per ml. McCoy fibroblast cells were seeded on quartz crystal resonator or on biopolymers.

Polymers synthesis.

Bioactive PMMA based terpolymers, referenced EF27, EF31, EF32, EF33a, were synthesized by radical copolymerization of the following monomers: methyl methacrylate (MMA), methacrylae acid (MA) and sodium styrene sulfonate (NaSS). Terpolymerizations of MMA, MA and NaSS were carried out in dimethylsulfoxide (DMSO) at 70°C under nitrogen until complete conversion. Terpolymers were precipitated in water and dried at 70°C under vacuum. Compositions were determined by ^{1}H NMR in deuterated DMSO using a Bruker ACE 200. Poly(methyl methacrylate) PMMA - commercially available polymer - was used as control.

Analysis using the quartz crystal resonator and cell counting techniques.

The different bioactive polymers and PMMA (control) were first coated on quartz crystal resonator and then incubated with culture medium for 2 hours. McCoy fibroblastic cells were then seeded at an initial concentration of 250 000 cells/mL and cultured on each polymer for different times varying from 1 to 8 hours. Adhered cells on polymer coated quartz crystal resonator were detached after each hour by tryspin digestion and counted (in triplicate) using a multisizer II coulter.

Observation of adhered cells by fluorescence microscopy.

McCoy fibroblastic cells adhered after 8 hours of culture on five different polymers were observed by fluorescence microscopy after staining with orange acridine.

Results and discussion.

Bioactive polymers synthesis.

Chemical compositions of the various bioactive polymers were determined by ^{1}H NMR 200 MHz. Results expressed in % of each of the three monomers MMA, MA and NaSS are presented in Table 1. Each polymer was also characterized by R which the molar ratio of MA content (COO^-) to MA + NaSS content ($COO^- + SO_3^-$).

Polymers	Molar composition (%) MMA/MA/NaSS	$R = \frac{COO^-}{COO^- + SO_3^-}$
EF27	87.1/3.7/9.2	0.29
EF31	92/0/8	0
EF32	72.3/17.7/0	1
EF33a	85.5/8.5/6	0.59
PMMA	100/0/0	-----

Table 1: Chemical composition of bioactive polymers and control.

Amongst the various synthesized bioactive polymers, some are terpolymers, i.e. composed of three monomers MMA, MA and NaSS (EF27 and EF33a), and some are copolymers i.e. composed of two monomers MMA/MA (EF32) or MMA/NaSS (EF31) .

Analysis by the quartz crystal resonator technique.

Results expressed in variation of the resistance R against time for each polymer coating on quartz are presented in Fig. 3.

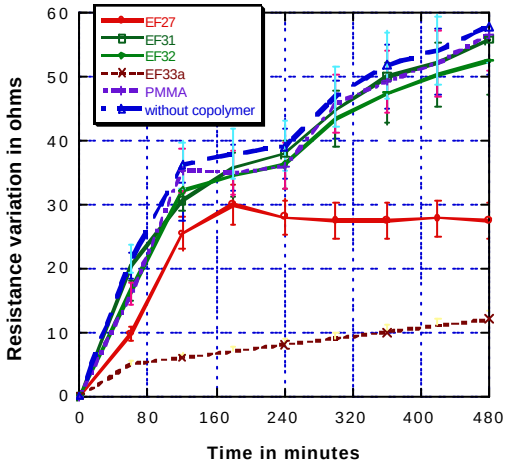


Figure 3: Resistance variation during cell adhesion process.

Results show that the resistance induced by cell adhesion on various surfaces depends on the chemical composition of the polymer used as quartz coating. Observed resistance on quartz (without polymer) was identical to that observed on PMMA (control polymer) or copolymers EF31 and EF32 bearing only one functional carboxylate or sulfonate group whereas it was very much lower on terpolymers EF27 and EF33a bearing both carboxylate or sulfonate groups . These results confirm previous results on cell adhesion and proliferation on the same polymers [10]. Polymers EF27 and EF33a which inhibit cell proliferation and induce low adhesion strength of cells exhibit less resistance with the quartz resonator technique. This suggests that the resistance measured on quartz resonator can be correlated to the intensity of interactions which are developed between cells and surfaces and then to cell adhesion: the lower the R, the lower the cells/surface interactions or cell adhesion.

Analysis by cell counting technique.

Kinetics of cell adhesion were carried out on the different surfaces (quartz coated or not with polymers) and the number of adhered cells on surfaces were determined by cell counting after trypsin detachment. Results are presented in Fig.4.

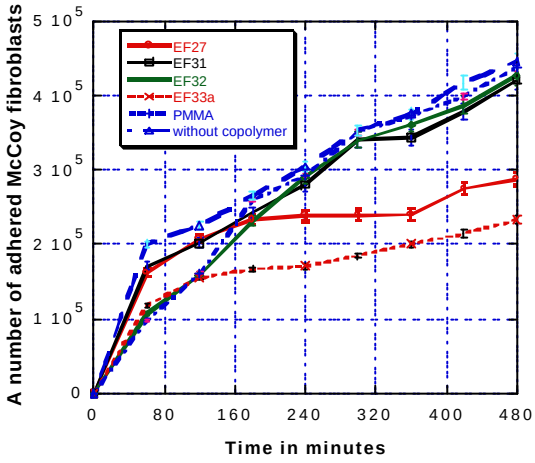


Figure 4: Kinetics of cell adhesion on different surfaces by cell counting technique.

These results are in total agreement with those obtained by the quartz crystal resonator technique. Surfaces inducing high values of R resistance correspond to surfaces on which cells adhered; in contrast surfaces inducing low R values correspond to surfaces on which cells adhered lightly.

Comparison of quartz resonator and cell counting methods.

In order to compare the results obtained with quartz resonator and cell counting techniques, results of each technique were first expressed as inhibition percentage of cell adhesion on polymer coated quartz to cell adhesion on uncoated quartz. Inhibition percentages obtained by both techniques after 8 hours of McCoy fibroblastic cell seeding and culture on different bioactive polymers are presented in Figure 5.

Results show that EF27 and EF33a bioactive polymers present higher inhibiting properties towards cell adhesion thanEF31, EF32 and PMMA: this inhibiting effect is chemical composition dependant and requires both carboxylate and sulfonate functional groups. It is noteworthy that the sensitivity of the quartz

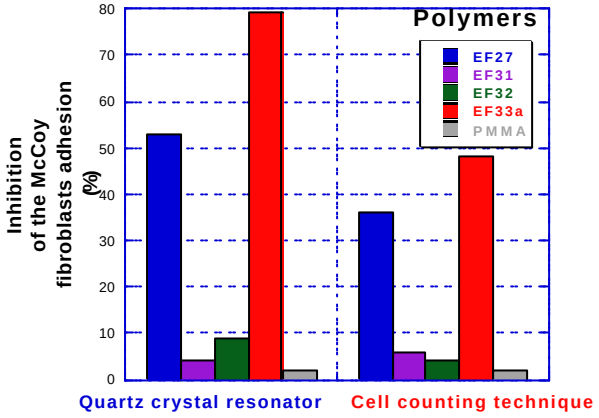


Figure 5: Comparison of cell adhesion by the quartz crystal resonator and cell counting techniques.

resonator technique seems to be better than the cell counting technique.

Observation of cell adhesion by fluorescence microscopy.

The pictures of stained cells cultured for 8 hours on different surfaces are presented in the Figure 6. These images show and confirm the differences in the number of adhered cells when cultured on quartz crystal resonator (a) on PMMA polymer control (b) and on the bioactive functionalized polymers (c to f). Cells adhered strongly and were numerous on quartz resonator, few less cells adhered on PMMA, EF 31 and EF 32 and low amount of cells adhered to EF 27 and 33a inhibiting polymers. EF33a bioactive terpolymer presents the highest cell adhesion inhibiting properties as compared to the other polymers.

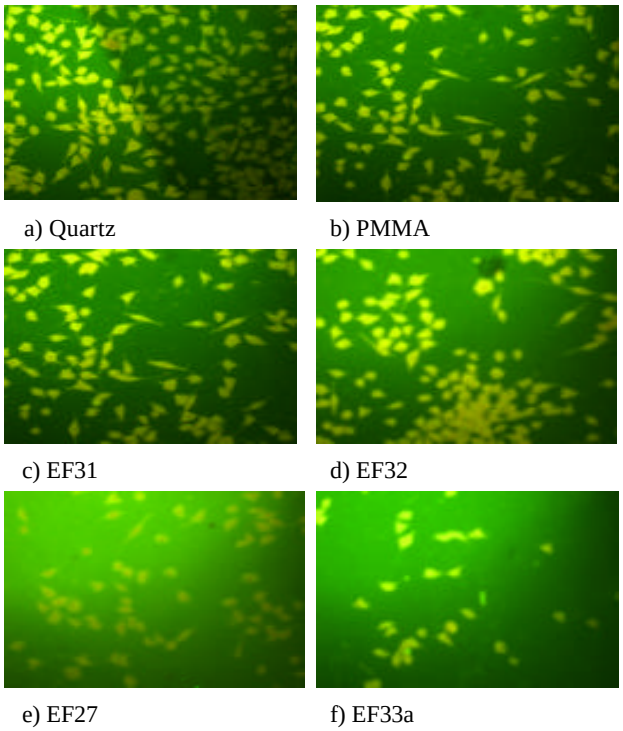


Figure 6: Images of stained cells adhered to different polymers / fluorescence microscopy.

Conclusion

Bioactive PMMA based terpolymers EF27 and EF33a bearing sulfonate and carboxylate groups present inhibiting properties of McCoy fibroblastic cells adhesion when compared to non functionalized polymer PMMA and polymers bearing only one of these functional groups. Both functional groups are required to observe the inhibiting properties. This inhibiting effect was observed and evidenced by three differents techniques : quartz crystal resonator, cell counting and fluorescence microscopy. EF33a polymer is an excellent candidate for biomedical applications where the inhibition of cell adhesion and proliferation is needed : this polymer can be use in the intraocular lens application to prevent the secondary cataract. In addition, it is worthy to note that the quartz crystal resonator technique is very sensitive technique to evaluate cell/surfaces interactions.

References

[1] T. Matsuda, A. Kishida, H. Ebato, Y. Okahata, ‘‘Novel instrumentation monitoring in situ platelet adhesivity with a quartz crystal microbalance’’, ASAIO Journal, pp. 171-173, 1992.

[2] D.M. Gryte, M.D. Ward, W.S. Hu, ‘‘Real time measurement of anchorage-dependent cell adhesion using a quartz crystal microbalance’’, Biotechnol. Prog., vol. 9, pp. 105-108, 1993.

[3] H. Darbeida, C. Gabrielli, M. Gindre, M. Houmady, J.-Y. Le Huéron, H. Perrot, W. Urbach, ‘‘Suivi de l’adhérence de cellules en culture par microrhéologie’’, Les cahiers de rhéologie, vol. 16, pp. 73-75, 1999.

[4] H. Darbeida, M. Gindre, J.-Y. Le Huéron, W. Urbach, ‘‘Instrumentation ultrasonore et adhérence cellulaire’’, Système et microsystèmes pour la caractérisation, pp. 67-73, 2001.

[5] A. J. Garcia, D. Boettiger, ‘‘Integrin-fibronectin interactions at the cell-material interface : initial integrin binding and signaling’’, Biomaterials, vol. 20, pp. 2427-2433, 1999.

[6] K. B. McClary, T. Ugarova, D. W. Grainger, ‘‘RhoA-induced changes in fibroblasts cultured on organic monolayers’’, Biomaterials, vol. 20, pp. 2435-2446, 1999.

[7] Y. Ito, ‘‘Surface micropatterning to regulate cell functions, Biomaterials, vol. 20, pp. 2333-2342, 1999.

[8] S. J. Martin, V. E. Granstaff, G. C. Frye, Characterization of a quartz microbalance with simultaneous mass and liquid loading’’, Anal. Chem., vol. 63, pp. 2272-2281, 1991.

[9] H. L. Bandey, S. J. Martin, R. W. Cernosek, ‘‘Modeling the response of thickness shear mode resonators under various loading conditions’’, Anal. Chem., vol. 71, pp. 2205-2214, 1999.

[10] F. El Kadhali, G. Pavon-Djavid, G. Hélary, V. Migonney, Biomacromolecules, 3, (1), 51-56, 2002.