Measurement of the Osmotic Properties of Thin Polymer Films and Biological Tissue Samples

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A new type of micro-osmometer is described in which water absorption of small tissue samples is measured by a quartz crystal microbalance (QCM). The swelling of the sample deposited on the surface of a quartz crystal is determined by monitoring the change in resonance frequency of the quartz sensor as a function of the vapor pressure in the surrounding environment. The measurement principle is verified by studying the water uptake of poly(vinyl alcohol) films. Reasonable agreement is found between the results obtained by the QCM-based osmometer and previous osmotic pressure measurements made on a similar poly(vinyl alcohol) sample. The feasibility of the new method is demonstrated by measuring the osmotic response of tissue-engineered cartilage samples. It is found that the osmotic pressure of cartilage substantially increases with culture time. The present result is consistent with cartilage models, suggesting that the proteoglycan content governs the compressive resistance of the tissue.

Introduction

Precise measurement of water uptake from the environment is of great significance in a variety of contexts. The physical properties of both synthetic and biopolymers are strongly influenced by their water content. Understanding the relationship between hydration and function is critical in biology and biophysics, as well as in many biotechnological applications, such as the stabilization of protein preparations for pharmaceutical use or food preservation.[1−4]

Biological soft tissues swell or shrink in response to changes in osmotic pressure or composition of their environment. For example, in articular cartilage, swelling governs both tissue mechanics and function.[5−8] Changes in the osmotic swelling pressure of cartilage may also serve as an indicator of degenerative joint disease. In the initial stage of osteoarthrosis, cartilage swelling may be the first detectable macroscopic event, occurring before cartilage erosion and loss.[9,10]

Studying the osmotic properties of tissue samples poses several challenges. Most commercial osmometers have been developed to investigate homogeneous polymer solutions. In conventional osmotic pressure measurements the minimum sample size requirement is at least a few hundred microliters. In general, however, biological specimens are inhomogeneous and exhibit gel-like properties, in addition to often being limited in quantity.

To address these issues, we have developed a new micro-osmometer that can measure minute amounts of water absorbed by thin polymer films or small tissue samples as a function of the water activity in the surrounding vapor phase. The measurement principle is based on the high sensitivity of the resonance frequency of the quartz crystal to small changes in the amount of material deposited on its surface (quartz crystal microbalance, QCM, technique). The polymer- (or tissue-) coated quartz sensor is placed in an environment where the relative humidity is precisely controlled. The change in resonant frequency due to water uptake of the specimen is measured and related to its water content. High sensitivity mass change measurements allow us to determine the osmotic properties of very small samples (<1 μg). In this way osmotic pressure and tissue hydration can be measured simultaneously.

QCM is a well-established mass sensing technique for investigating polymer systems at the solution−surface interface, that is, when the electrode is immersed in a solution.[11−14] Until now, much less attention has been paid to the use of this method in the vapor phase.[15−17] In this paper we demonstrate that QCM is a reliable technique for determining the water uptake of both thin polymer films and tissue samples. The swelling of poly(vinyl alcohol) (PVA) films is determined as a function of the vapor pressure of water in the surrounding environment. Because the osmotic properties of the PVA/water system are well-documented in the literature, these measurements are used to validate the QCM-based osmotic technique.[18−20] The effect of the thickness of the polymer layer on the kinetics of water uptake and the reversibility of the swelling/shrinking process are also investigated.

To demonstrate the applicability of the QCM technique to biological tissues we report osmotic measurements made on tissue-engineered cartilage samples. Knowledge of the concentration dependence of the osmotic swelling pressure allows us to estimate the compressive resistance of the tissue. The latter quantity is particularly important because it quantifies the load-bearing properties of cartilage. Change

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in the osmotic behavior may reflect age and disease related changes in the tissue structure and function.[6−10]

First we describe briefly the measurement technique and the basic features of the apparatus. Then water sorption measurements made on PVA films are presented. The equilibrium swelling data are analyzed in terms of the Flory−Huggins theory, which is the standard framework for describing the osmotic pressure of polymer solutions.[21] The QCM results are compared with osmotic data obtained by independent measurements performed on similar PVA samples. Finally, we report osmotic swelling pressure measurements made on tissue-engineered cartilage samples after different culture times.

Experimental Section

QCM. In the osmometer the mass sensor is a QCM (QCM-9000, ELCHEMA, Potsdam, NY).[22] In the apparatus a precision piece of AT cut quartz crystal is sandwiched between two gold electrodes. The vibration modes excited by an alternating electric potential result in a transverse acoustic standing shear wave pattern in the crystal. A rigid thin film deposited on the surface couples to the oscillations of the crystal and alters its resonant frequency. The resonant frequency of the quartz crystal is inversely proportional to the thickness. If this thickness is increased by the deposition of material, the frequency decreases. Sauerbrey[23] showed that the decrease in the resonant frequency, Δf, is proportional to the mass, Δm, deposited per unit area

$$\Delta f = f_{dry} - f = (2f_0^2/\rho v)\Delta m \quad (1)$$

where f_0 is the resonant frequency of the quartz crystal and ρ and v are the density and shear velocity of sound waves in the quartz crystal, respectively.

Measuring the electrical response of the crystal monitors changes in the resonant frequency. The electronics used in the present instrument allow us to measure frequency changes within 1 Hz. The 10-MHz quartz crystal is suitable for detecting a mass change $\approx$ 10 ng/cm^2, which corresponds to a thickness of approximately a 0.1-nm water layer over the area of the electrode. This makes the QCM an extremely sensitive technique for measuring very small changes in the amounts of material deposited on the surface of the crystal.

The vapor sorption measurements are carried out in a temperature-controlled sample chamber containing NaCl solution of a known water activity. The sample chamber is placed in a Faraday cage. In the prototype apparatus, the volume of the sample chamber is approximately 200 cm^3, suitable to accommodate six quartz crystal electrodes. First the resonant frequency of the uncoated quartz crystal is determined. After the material (polymer or tissue) is deposited onto the crystal the new resonant frequency is measured. The resonant frequency decreases when the polymer absorbs water (swells) and increases when water molecules leave the film (deswells). Each experiment begins with a 2-h conditioning period in air. This conditioning is necessary to minimize the effect of the prior history of the sample (residual volatile components, etc.) on the mass changes measured in the actual run. The 2-h period was chosen arbitrarily and may be decreased for certain samples. [In the case of PVA films, no appreciable differences were observed between the results obtained after 1, 2, and 12 h (overnight) conditioning.] Changing the concentration of the NaCl solution in the sample chamber induces stepwise changes in the water vapor pressure. The response of the quartz crystal is monitored until no further change in the resonant frequency is detected. The dry weight of the sample is measured at the end of each experiment by removing the water from the sample with dry air.

Table 1. Biochemical Analysis of Tissue-Engineered Cartilage Constructs

culture time (days)	collagen (% wet weight)	glycosaminoglycan (% wet weight)	DNA (μg/mg wet weight)
10	0.18 ± 0.04	1.1 ± 0.2	0.46 ± 0.06
20	0.33 ± 0.07	1.4 ± 0.3	0.49 ± 0.06
30	0.56 ± 0.15	1.8 ± 0.3	0.67 ± 0.07

Sample Preparation. *PVA Gel Film.* PVA (molecular weight = 90 000; degree of hydrolysis: 99%) was purchased from Sigma-Aldrich. The polymer was dissolved in deionized water at 99 °C.

Polymers from aqueous solutions can be relatively easily deposited on hydrophilic surfaces using standard coating techniques (e.g., drop coating), because they spontaneously wet the surface. Prior to the coating process, the surface of the gold electrode was cleaned with Piranha solution (one part 30% H_2O_2 in three parts 98% H_2SO_4). The quartz crystal was immersed in a Piranha solution for 20 min and then was rinsed with deionized water. After drying with nitrogen the gold surface was drop coated with a 0.1% (w/w) PVA solution.

Cartilage Sample. Cartilage was aseptically harvested from a chick embryo sternum (16 days old). Chondrocytes were isolated by digestion with collagenase and resuspended in culture medium (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, antibiotics, and 50 μg/mL ascorbate). PVA hydrogel disks (diameter, 25 mm; thickness, 2 mm) were swollen with the medium and seeded uniformly with chondrocytes (125 million cells per disk). The cell-laden hydrogel scaffolds were cultured under static conditions at 37 °C in a humid environment with 5% CO_2 for 5 weeks. The medium was replaced every 1−2 days.

After each culture period, the resulting cartilagenuous tissue was gently removed from the surface of the PVA scaffold and suspended in 0.05% (w/w) PVA solution. The gold electrode was uniformly coated with the suspension. The role of dissolved PVA was to improve adhesion between the tissue and the gold surface.

For biochemical assays cartilage samples taken after different culture times were digested by papain and analyzed to determine the total DNA content,[24] total sulfated glycosaminoglycan content,[25] and total collagen content[26] (based on hydroxyproline). Typical results of the biochemical analysis[27] are shown in Table 1.

Results and Discussion

When the surface of the crystal is coated with polymer the resonant frequency is decreased. The Sauerbrey equation

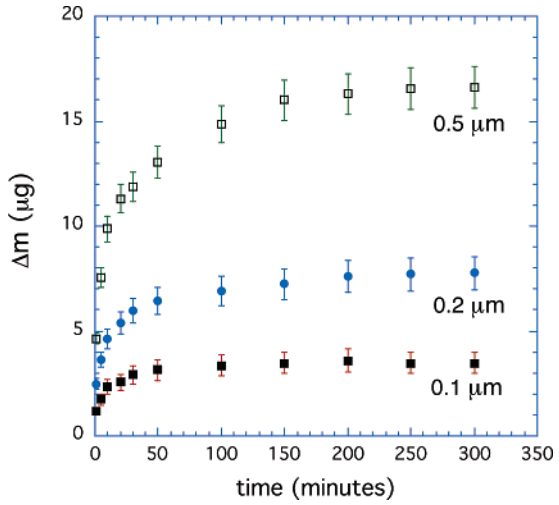

Figure 1. Water uptake, Δm, as a function of time for PVA films of different thicknesses. The water vapor pressure was set by a 2% (w/w) NaCl solution.

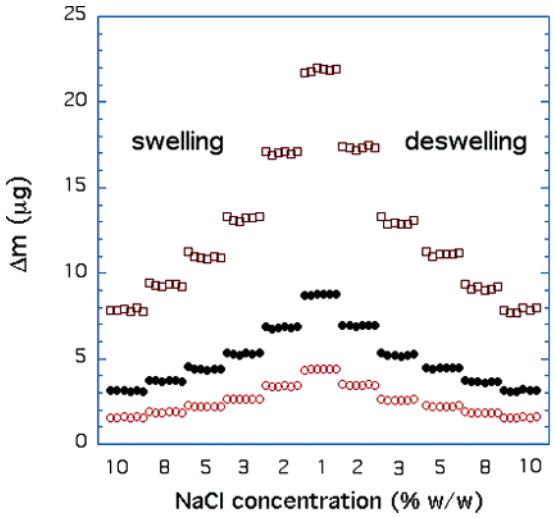

Figure 2. Equilibrium amount of absorbed water, Δm, as a function of NaCl concentration in the equilibrium bath at 25 °C for the PVA samples shown in Figure 1.

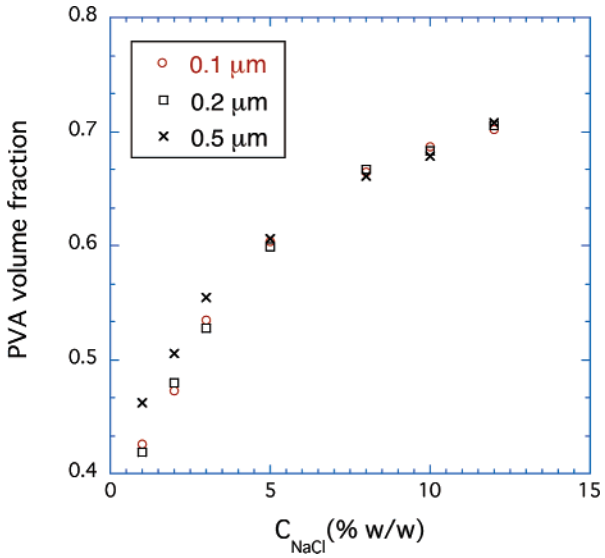

Figure 3. PVA volume fraction as a function of NaCl concentration in the equilibrium bath at 25 °C.

predicts that when the film is rigidly attached to the surface, the change in resonant frequency is linearly proportional to the mass of the material deposited on the electrode. In a nonideal system, however, many factors (e.g., softening, viscoelastic energy dissipation, nonuniform film thickness, poor adhesion, interfacial slippage) may contribute to the deviation from the linear response.

Vapor Sorption Measurements on PVA Films. Figure 1 shows the kinetics of water uptake for three PVA films of varying thickness. The thicknesses of these films, estimated from the weight and density of the dry PVA ($\rho = 1.269$ g cm^{-3}) are approximately 0.1, 0.2, and 0.5 μm, respectively. In the vapor sorption measurements the PVA films were equilibrated with 2% (w/w) NaCl solution. Equilibrium is attained when the water vapor is absorbed and desorbed at the same rate. The data indicate that both the initial rate of water uptake and the equilibration time increase with the thickness of the polymer film. The initial rapid swelling can be attributed to the larger chemical potential difference across the water vapor/polymer interface. It is likely that in the region in contact with the vapor phase the water concentration rapidly reaches its equilibrium value. This rapid initial swelling creates osmotic stresses in the film, which are dissipated by viscous flow. In thick films the relaxation of the high osmotic stress takes longer, that is, the equilibration time increases.

In Figure 2 the equilibrium amount of water, Δm, for the three PVA films shown in Figure 1, is plotted as a function of increasing and decreasing NaCl concentration in the equilibrium bath at 25 °C. The results indicate that the swelling/deswelling cycle is reversible; that is, the amount of water being absorbed into the film is the same as that being desorbed for the same change in NaCl concentration. At a constant NaCl concentration the water content, Δm, increases with the thickness of the film.

It is important to note that the rate of water vapor uptake by the polymer layer deposited on the quartz crystal is significantly slower than that of a free PVA film immersed in pure water. The diffusion coefficient for PVA is in the range 2×10^{-11} m^2 s^{-1}. Using this value, the equilibration time of a 1-μm film should be of the order of seconds. The experimental results indicate that the actual equilibration time is longer than 1−2 h. Presumably, this time can be reduced somewhat by using a smaller sample chamber, that is, by reducing the distance (diffusion path) between the sample and the NaCl solution. The large deviation between the calculated and measured swelling equilibration times, however, suggests that the kinetics of swelling of the PVA layer attached to the crystal surface is not a simple diffusion-controlled process. Other effects, such as viscoelastic relaxation of the polymer, water diffusion in the nonuniformly swollen matrix, mechanical constraints imposed by the solid surface, and so forth, may also be important.

Although the Δm versus c_{NaCl} plots differ for PVA films of various thicknesses the equilibrium polymer concentration in the swollen films should be the function of the NaCl concentration only. In Figure 3 the same data are displayed as in Figure 2 but in a different representation. It can be seen that at high polymer concentration (volume fraction > 0.6) all data collapse on the same curve. Deviation from this

behavior can only be observed in the case of the thickest PVA film at high water content. In thick films the acoustic wave may decay before it reaches the outer boundary of the film, and the frequency shift is no longer proportional to the mass change. Other factors such as viscoelastic effects or poor adhesion may also cause deviation from the simple Sauerbrey expression. We observed that at higher swelling ratios (>2.5) the QCM did not oscillate. When the water activity in the surrounding vapor phase was reduced, however, oscillation was regained. Measurements reported here were made in the concentration range in which the change in the frequency shift with the water activity was reproducible.

At thermodynamic equilibrium the chemical potential of the solvent (in the present case water) is the same in the coexisting phases, that is,

$$(\Delta\mu_1)_{sol} = (\Delta\mu_1)_{gel} = (\Delta\mu_1)_{vapor} = RT \ln a_1 \qquad (2)$$

where the subscripts sol, gel, and vapor refer to the solution, gel, and vapor phases, respectively, R is the gas constant, T is the absolute temperature, and a_1 is the activity of the water. In polymer solutions, the chemical potential of the solvent is directly related to the osmotic pressure $\Delta\mu_1 = -\Pi V_1$, where V_1 is the partial molar volume of the solvent.

In binary polymer mixtures Π is given by the Flory−Huggins equation[21]

$$\Pi = -(RT/V_1)[\ln(1-\varphi) + \varphi + \chi_0\varphi^2 + \chi_1\varphi^3] \qquad (3)$$

where φ is the volume fraction of the polymer and χ_0 and χ_1 are constants that depend on the interactions between the polymer and the solvent molecules.

Because the QCM response may reflect uncontrollable changes in the physical (e.g., viscoelastic) properties of the polymer film, it is important to validate the results by independent techniques. Several methods have been developed to determine the swelling properties of polymer films and gels.[28−32] Here we compare the QCM results with that obtained by osmotic deswelling.[19,32] The latter is a simple and powerful technique that has been applied successfully for a variety of synthetic and biopolymer systems. In the osmotic deswelling experiment the sample of unknown osmotic pressure is enclosed in a semipermeable membrane (dialysis bag) surrounded by a polymer solution of known osmotic pressure. At equilibrium, the osmotic pressure of the sample in the dialysis bag is equal to the osmotic pressure exerted by the polymer solution outside. The role of the semipermeable membrane is to prevent the penetration of the polymer molecules from the solution into the gel. This technique requires much larger samples (>0.1 g) than the QCM (<0.1 mg), and the equilibration time is significantly longer (>3−4 days).

Figure 4 shows the dependence of the osmotic pressure on the PVA volume fraction obtained by these two independent experimental techniques. The dashed curve is the least-squares fit of eq 3 to the osmotic deswelling data (triangles).[19] This fit yields for the interaction parameter $\chi_0 = 0.477$ and $\chi_1 = 0.414$. The QCM data lie close to the extrapolated curve. (Note that the QCM experiment probes the polymer at a higher polymer concentration.) The deviation from the calculated curve at high PVA concentration ($\varphi > 0.65$) may be attributed to higher-order interactions and/or structural changes occurring in the PVA film. Increasing the PVA concentration favors association (due to hydrogen bonding) and crystallization.[33] Molecular associations and crystallites act like physical cross-links. Equation 3 is only applicable to un-cross-linked systems at moderate polymer concentrations.

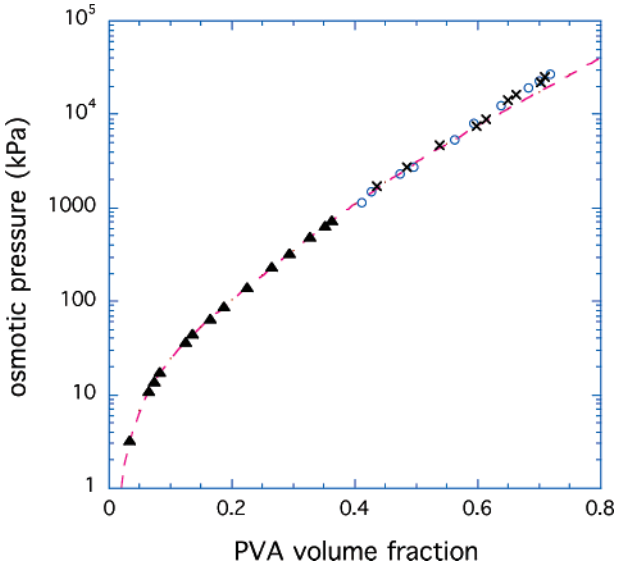

Figure 4. Dependence of the osmotic pressure on the volume fraction of the PVA. Open symbols, data measured by the QCM-based osmotic technique; filled triangles, data measured by osmotic deswelling. The dashed curve is the least-squares fit of eq 3 to the data obtained by osmotic deswelling.

Vapor Sorption Measurements on Tissue-Engineered Cartilage. Swelling is known to be critically important in the biomechanical function of cartilage, supporting compressive loads and maintaining tissue hydration.[5−10] In light of the success of the QCM measurements on PVA films, we made vapor sorption measurements on tissue-engineered cartilage samples. Our goal was to demonstrate the applicability of the QCM-based method for determining the osmotic properties of biological tissues. It is important to note that if structural change occurs in the tissue sample, it may affect the resonance frequency of the quartz crystal.[34] In this case the frequency shift is no longer proportional to the mass change; that is, the Sauerbrey equation cannot be used.

In general, biological tissues are not homogeneous. Cartilage consists of two major phases: a fluid phase (60−80% of the tissue wet weight) and a solid matrix, with only 4−5% of the total tissue volume occupied by chondrocytes (cartilage cells).[10] The fluid phase is mainly a physiological electrolyte solution. The solid phase consists of proteins (mainly collagen) and proteoglycan molecules. The latter are immobilized within the collagen network. It is generally assumed that the amount of proteoglycans governs the extent of cartilage swelling.[9,35,36]

The QCM technique probes the interactions of the tissue with the electrode surface. To improve the adhesion between the tissue sample and the quartz crystal, we first suspended the tissue in a dilute PVA solution and then coated the

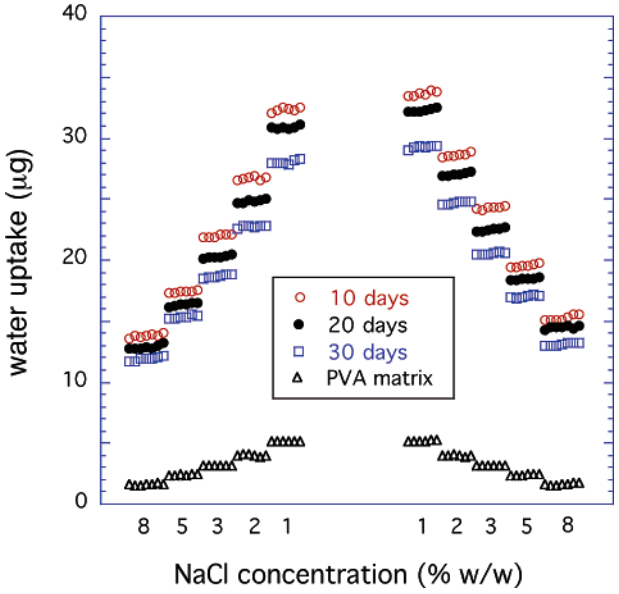

Figure 5. Water uptake of tissue-engineered cartilage samples after different culture times as a function of the NaCl concentration in the bath at 25 °C. The amount of water absorbed by the PVA matrix is also shown in the figure (open triangles).

electrode with the suspension. When the coated quartz crystal is exposed to water vapor both the tissue and the PVA absorb water. Thus, the equilibrium swelling data should be corrected for the amount of water absorbed by the PVA.

Quartz crystal electrodes coated by known amounts of cartilage tissue were equilibrated with water vapor at 25 °C. The amount of absorbed water was determined as a function of the NaCl concentration (Figure 5). This figure also shows the water uptake of the unloaded (cartilage-free) PVA film. The equilibration time at each NaCl concentration was 2 h. The complete measurement consisted of five sorption/desorption steps. After the fifth step the water vapor pressure was kept constant for 12 h and then was reduced in the next steps. It can be seen that even after 12 h of equilibration time the amount of absorbed water slightly increases, indicating that the system is not in equilibrium. This is also reflected when comparing the absorption and desorption data. When the water vapor pressure is decreased the amount of water leaving the tissue should be the same as that absorbed at the same water activity when the vapor pressure is increased. It appears that desorption data lie slightly above the absorption data. This hysteresis may be caused by slow structural or chemical changes (e.g., degradation of high molecular weight proteoglycans) occurring during the sorption/desorption cycle.

The swelling degree, m/m_0, of the cartilage was obtained from the measured swollen and dry weights and the known amount of PVA added to the tissue

$$m/m_0 = (m_{\text{total}}^{\text{swollen}} - m_{\text{PVA}}^{\text{dry}} - m_{\text{PVA}}^{\text{water}})/(m_{\text{total}}^{\text{dry}} - m_{\text{PVA}}^{\text{dry}}) \quad (4)$$

where $m_{\text{total}}^{\text{swollen}}$ and $m_{\text{total}}^{\text{dry}}$ are the weights of the swollen and dry samples, respectively, $m_{\text{PVA}}^{\text{dry}}$ is the weight of the dry PVA, and $m_{\text{PVA}}^{\text{water}}$ is the amount of water absorbed by the PVA.

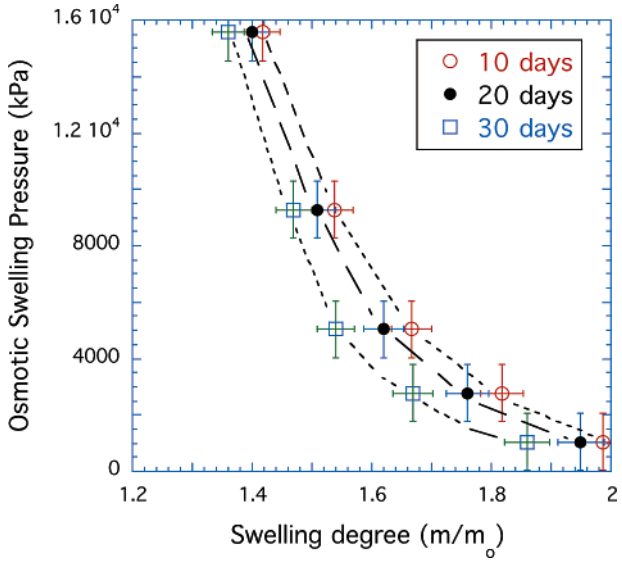

Figure 6. Dependence of the osmotic swelling pressure on the swelling degree in tissue-engineered cartilage samples. The curves through the data points are guides to the eye.

The swelling curves determined for cartilage samples after different culture times (10, 20, and 30 days) are shown in Figure 6. The osmotic pressure of the cartilage rapidly decreases with increasing water content. The rate of decrease, $[\partial\Pi/\partial(m/m_0)]$, is proportional to the compressive resistance of the sample. The results show that the compressive resistance of tissue-engineered cartilage increases with culture time. Cartilage models predict that strong repulsive interaction between negatively charged proteoglycan molecules enables cartilage to resist compression under load. The present results are consistent with this prediction.

Conclusions

In this work, the suitability of the QCM is demonstrated for studying the osmotic properties of polymers and tissue samples. The osmotic pressure of thin PVA layers deposited on the surface of a quartz crystal is determined. Reasonable agreement is found between the results obtained by the QCM and those found by macroscopic osmotic pressure observations.

QCM provides a powerful means to measure the osmotic response of biological tissue samples. For cartilage, whose primary function is to absorb shock and protect the underlying bone, knowing the biomechanical properties is essential. Preliminary results on tissue-engineered cartilage samples indicate that the compressive resistance of the neo-cartilage significantly increases with the culture time.

The QCM-based osmotic technique requires considerably shorter equilibration time than conventional osmometry. Moreover, the small sample requirement of the QCM (<1 mg) allows probing the local osmotic properties of inhomogeneous tissue samples. For example, cartilage matrix composition and collagen fiber orientation vary with the distance from the articular surface. Existing osmotic techniques could not provide information on the osmotic properties at such a high spatial resolution.

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