

QCM/HCC as a platform for detecting the binding of warfarin to an immobilized film of human serum albumin

G. Zilberman¹, A. L. Smith^{1,2*}

1. Department of Chemistry, Drexel University, Philadelphia, PA 19104
2. Masscal Corporation, Chatham, MA 02633

Abstract

Quartz crystal microbalance/ heat conduction calorimetry (QCM/HCC) is a new measurement technology that has been used to monitor simultaneously the mass and motional resistance of a thin film in conjunction with the heat flow produced by a chemical change in the film initiated by reaction with a gas. In this work we examine the applicability of the QCM/HCC in detecting chemical changes at the solution/thin film interface. Human serum albumin (HSA) was bound to the gold electrode of a 5 MHz AT-cut quartz resonator using three types of linkers and then exposed to buffered solutions of the anticoagulant drug warfarin. Changes in resonator frequency and motional resistance as well as changes in heat flow produced by warfarin binding to HSA were monitored as a function of the warfarin concentration. Differences in frequency and motional resistance changes depend upon the linker and vary both in magnitude and sign, whereas the integrated heat signal is proportional to the concentration of warfarin and independent of the linker chemistry. Quartz crystal microbalance/heat conduction calorimetry can thus be a useful tool for studying protein-ligand interactions at the solution-surface interface, even though the quartz resonator does not behave as a microbalance.

Introduction

QCM/HCC is a new technique for studying chemical processes at surfaces. It has been applied so far to processes occurring at the solid/gas interface¹⁻³. The technique combines two complementary methods – *Quartz Crystal Microbalance* and *Heat Conduction Calorimetry*. Under suitable conditions, QCM measurements can provide the mass change and changes in shear loss modulus of the surface film, whereas heat

conduction calorimetry measures the heat generated/consumed by the surface process. Direct measurement of these properties provides thermodynamic and kinetic information on the chemical process. QCM/HCC can be used both as a technique for characterizing interfacial processes at the gas/solid interface and as a gas sensor.

Over the last decade there has been an increasing need for the analysis and detection of protein-ligand and protein-protein interactions in solutions and at surfaces. Despite the continuing questions surrounding the use of QCM in solution as a mass sensor, the QCM has been shown to be a very sensitive probe of biomolecular adsorption at surfaces, with a sensitivity comparable to surface plasmon resonance⁴⁻⁶.

In this work we show that QCM/HCC is sensitive enough to characterize the binding of a ligand to a layer of protein immobilized on a surface in aqueous solution. Moreover, the technique is more cost-effective than many other techniques. It requires small amount of solutions and does not require labeling of any reagent, so no chemical modification is required. Our aim in this work is to validate the QCM/HCC technology for biological application by demonstrating that it can provide thermodynamic and other information for a model system: the binding of the anticoagulant drug warfarin to human serum albumin (HSA). HSA, the most abundant protein in the plasma, serves as the primary drug transporter in blood. Thermodynamic and kinetic information on drugs binding to HSA is essential in drug discovery. Warfarin is a small drug molecule that binds mainly to HSA Site I of HSA, which is often simply termed the warfarin-binding site. At higher concentration warfarin may be bound also to HSA Site II, which is less preferred energetically. The QCM/HCC is shown here to have the ability to detect the binding of warfarin as well as to obtain important kinetic and thermodynamic information using small amount of reagents, which might be a crucial factor in drug discovery.

The system of HSA/warfarin was also chosen since it is well studied⁷⁻¹² and therefore comparison can be made between the results obtained here and the results of both solution and surface-based techniques. Using the surface chemistry employed by Thompson et al⁹, three thiol linkers were used to attach the HSA covalently to the gold

electrode on the quartz resonator: Cysteamine, 11- Mercaptoundecanoic (11-MUA), and 3,3'-dithio-bis(propionic acid N-hydroxysuccinimide ester (DSP). Pronounced changes in frequency, motional resistance, and heat generation were observed with each linker. The frequency change and motional resistance change signals are found to depend both on the bulk warfarin concentration and on the linker used to immobilize HSA. However, the integrated heat signal is proportional to the warfarin concentration and independent of the linker chemistry.

Experimental

The QCM

QCM is based on a piezoelectric crystal that oscillates in the thickness shear mode. At constant temperature and pressure and in the thin film limit², the response of a coated crystal to the absorption of gas in the coating follows the Sauerbrey equation (1):

$$\Delta f = -C \cdot \Delta m / A \quad (1)$$

where Δf is the change in frequency, $\Delta m/A$ is the change in mass per unit area and C is a proportionality constant that depends only on the properties of quartz. The Sauerbrey equation holds for shear mode piezoelectric sensors when the thickness of the rigid adsorbed film is small compared to the crystal thickness (i.e.~1%), and when the crystal is in contact with a gas. The frequency shift of a QCM with non-rigid layer on its surface and immersed in a liquid and does not obey the Sauerbrey equation.

A non-rigid film on the QCM surface increases the dependency of the frequency shift on the viscoelastic properties of the layer^{13, 14}. Motional resistance is an electrical property of the quartz crystal proportional to its viscoelastic damping by the film and the medium. The technique developed by Kasemo et al¹⁵ and commercialized by Q-Sense AB measures the frequency f and the dissipation D , related to motional resistance R_m by

$$D = R_m / 2\pi f L \quad (2)$$

where L is the inductance of the equivalent RLC circuit of the crystal¹⁵. For the 5 MHz AT-cut crystal, typical inductance values are 0.04 Henry, so $D = 7.92 \times 10^{-7} R_m$.

The Heat Flow Sensor

Heat flow calorimetry measures the thermal power flowing from the sample to a heat sink as a function of the time. The total heat associated with the process is just the time integral of the thermal power. A thermoelectric module measures the thermal power, P , which follows the Tian equation:

$$P = 1/S(U + \tau(dU/dt)) \quad (3)$$

where S is the thermopile sensitivity (V/W), U is the thermopile voltage and τ is the time constant of the calorimeter. At steady state, $U = SP$, and the output voltage is proportional to the thermal power dissipated on its surface. The thermopile sensitivity S was calculated from the heat generated by the QCM³.

The Apparatus

A sketch of a single frequency/heat flow sensor is shown in figure 1. The apparatus used in this work, a prototype of the Masscal™G1, contains two identical sensors – reference and sample. Buffered solutions with or without warfarin were introduced to the system through a flow cell using a dual syringe pump. A further description of the system is given elsewhere¹.

Reagents and Materials

Albumin (HSA) from human serum (fraction V, powder 96-99%w/w protein) was purchased from Sigma-Aldrich and used without further purification. *N*-hydroxysuccinimide (NHS), Dulbecco's phosphate buffer saline (DPBS), glutaraldehyde Grade 2 (GA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 3-(α -acetylbenzyl)-4-hydroxycoumarin (warfarin), 11-Mercaptoundecanoic acid (11-

MUA) and 3,3'-dithio-bis(propionic acid N-hydroxysuccinimide ester (DSP) were all purchased from Sigma-Aldrich.

HSA Immobilization Chemistry

AT-cut, polished 5MHz piezoelectric quartz crystals 2.54 cm in diameter (Maxtek Inc.) with gold electrodes were used. The larger electrode (diameter 1.56 cm) exposed to the liquid flow was cleaned with acetone, ethanol and water then coated according to the following procedures⁹.

A monolayer of cysteamine was formed by immersing the QCM in a 0.1% solution of cysteamine in water overnight prior to being treated with 2.5% GA in PBS buffer for 1 h. A monolayer of 11-MUA was formed by immersing the QCM in 10mM 11-MUA in ethanol solution for at least 24 hours at room temperature. The crystal was then washed with ethanol, dried and then immersed in 1:1 solution of 15 mM NHS (Sigma) and 75 mM EDC(Sigma) for about 1 hour. Attachment of DSP was achieved by immersing the oscillator into a 2 mM solution of DSP in DMSO for 2 hours at room temperature, followed by washing with DMSO followed by a phosphate buffer.

In each case, the device with the linker film was washed with water, dried and then placed into the protein solution of 1mg ml⁻¹ HSA in aqueous DPBS for at least one hour. Warfarin solutions were always prepared in two steps: dissolution of warfarin in DMSO following by dilution to 5% DMSO in DPBS.

Experimental Procedure

The QCM/HCC temperature was stabilized overnight at 25.00°C before an experiment. The placement of the coated crystal involves opening the QCM/HCC for a short time, after which the loaded cell was flushed with a solution of 5% DMSO in DPBS until stable thermal power, frequency shift and motional resistance signals were obtained.

A dual syringe pump feeding a four-way valve permitted the introduction of buffer solution for sample equilibration followed by the introduction of buffered warfarin solution with no interruption in flow. After the signals due to binding had reached steady state (usually 30 minutes), buffer was reintroduced to remove the bound warfarin, again with no interruption in flow. This cycle was repeated twice with warfarin solutions of higher concentration. The flow rate of all reagents was 50 μ l/min.

Results

Detection of warfarin binding to film of HSA immobilized with a cysteamine linker

The results obtained for the binding of warfarin to HSA at three warfarin concentrations are shown in Figures 2 and 3.

There are two sources for the observed thermal power P : P_{bind} , the heat generated by the binding of warfarin to HSA, and P_{cryst} , and the heat generated by the QCM itself. The latter must be subtracted from the thermal power signal in order to obtain the heat generated solely by the reaction: $P_{\text{bind}} = P - P_{\text{cryst}}$. The quantity determining motional resistance logged by the software is the conductance voltage V_{cond} , related to the motional resistance R by Equation 4 below. Equation 5 gives the heat generated by the QCM as a function of the conductance voltage. Eqs. 4 and 5 are provided by Maxtek Inc., the manufacturer of the oscillator driver electronics in the prototype.

$$V_{\text{cond}} = 100 / (R + 20) \quad (4)$$

$$dq/dt_{\text{crystal}} = (V_{\text{soc}})^2 (V_{\text{cond}} / 100) (1 - V_{\text{cond}} / 5) \quad (5)$$

Here $V_{\text{soc}} = 0.125\text{V}$ is the open circuit crystal drive voltage. The thermal power generated by the QCM is shown in Figure 4, and Figure 5 shows P_{bind} , the net thermal power due to the reaction. Figure 6 presents the integrated heat $Q = \int P dt$ for each adsorption/desorption step.

Kinetic Analysis of HSA/warfarin binding

In order to compare the kinetics and thermodynamics of warfarin binding to an immobilized film of HSA with the results obtained from solution studies of this binding, we use the integrated heat signal for Figure 6 as input data for the methodology developed by Willson and Beezer¹⁶⁻¹⁸. As shown by previous work, HSA has two binding sites for warfarin. The equilibrium constant is larger for the first site, but for warfarin concentrations $> 30 \mu\text{M}$ both sites are partially occupied¹⁰. The binding process is described by Eq. 6, and the resulting rate constant expressions are given by Eq. 7.



$$d[AB]/dt = k_1[A][B] - k_{-1}[AB] \quad (7)$$

$$d[A_2B]/dt = k_2[A][AB] - k_{-2}[A_2B]$$

Here A is the warfarin (analyte) concentration, B is the immobilized HSA concentration and AB and A₂B are the concentrations of warfarin bound to the first and second binding site of HSA. k_1 and k_{-1} are the forward and reverse rate constants for the first site occupation on HSA, and k_2 and k_{-2} for the second occupation.

The heat generated by the reaction is:

$$q = n_{AB} \cdot \Delta H_1 + n_{A_2B} \cdot \Delta H_2 \quad (8)$$

Equations were further derived to include the build-up of concentration with time inside the flow cell. Using MATLAB we solved those equations, where the fit parameters were ΔH_1 , ΔH_2 , k_1 and k_{-1} , k_2 and k_{-2} . The initial values for k_1 , k_{-1} , k_2 and k_{-2} were calculated

using the equation in O'Neill et al¹⁸, assuming second order reaction. The initial values for ΔH_1 and ΔH_2 were taken as 50kJ/mol. The obtained results are in agreement with other studies^{7, 8, 10, 12}. It should be noted that there might be a dependency of the results with the initial values given to the fit. Therefore, we tried to estimate the values of the rate constants and to start with reasonable values for the enthalpy.

$$k_1 = 9 \times 10^5 \text{M}^{-1} \text{s}^{-1}$$

$$k_{-1} = 0.6 \text{s}^{-1}$$

$$k_2 = 6 \times 10^3 \text{M}^{-1} \text{s}^{-1}$$

$$k_{-2} = 5 \text{s}^{-1}$$

$$\Delta H_1 = 8.4 \times 10^4 \text{J/mole}$$

$$\Delta H_2 = 2.6 \times 10^4 \text{J/mole}$$

The fitting curve is shown in figure 7. Further description of the methodology and a comparison with prior experiments will be published elsewhere.

Detection of warfarin binding to films of HSA immobilized with two other linkers

In order to determine if the binding signals depended on the immobilization chemistry linking HSA to the resonator surface, we repeated the experiment with two additional linkers: 11-MUA and DSP. The QCM response of HSA/11-MUA linker to warfarin is shown in Figure 8. The net thermal power (Figure 9) and the integrated heat (Figure 10) for this system were derived in similar manner to that of HSA/cysteamine. The raw data for the HSA/DSP system are not given here, but the resulting shifts in frequency, motional resistance, and integrated heat are summarized in Table I for all three linkers. For comparison, also given in Table I are the shifts in frequency and motional resistance measured by Thompson et al⁹ for the same three HSA linkers using a 9 MHz QCM and a 2 mM solution of warfarin.

Discussion

Variation of frequency and motional resistance with linker

The variation both in magnitude and in sign of the changes in frequency shown in Table I cannot be explained by assuming that the shear mode resonator behaves as a microbalance. Usually when the QCM behaves as a microbalance the motional resistance and the frequency shift vary in opposite directions. An increase in the motional resistance, for example, means that the layer adjacent to the crystal surface is more viscous. This behavior is usually accompanied with a decrease in the frequency shift due to the dissipative interaction. This type of behavior is shown for the 11-MUA linker. When the frequency and the motional resistance changes in the same direction, as with the cysteamine linker, effects such as slippage should be considered to explain the resulting signal as discussed below.

If the HSA-coated resonator were a gravimetric sensor, then adsorption of warfarin would always produce a negative frequency shift whose magnitude was independent of the linker. Our results show that for the 11-MUA linker the frequency shift is positive, and that the magnitude of the shift depends upon the linker for a given warfarin concentration. Thompson et al.⁹ observe a positive frequency shift for the DSP linker, in disagreement with our results. The only differences we can see in our experimental procedures are that we used a 5 MHz instead of a 9 MHz resonator, and that the syringe pump directing buffer and warfarin solution through the cell was on continuously.

The QCM model embodied by the Sauerbrey equation is not consistent with the large viscoelastic damping caused by the immobilization of the HSA film. The Sauerbrey equation assumes that the film on the surface is rigid – obviously a poor assumption for an immobilized protein. This is one of the causes of the “missing” mass effect causing departures from the Sauerbrey equation^{13, 14}. When warfarin binds to the HSA, water molecules rearrange and desorb from the protein film, affecting the viscoelastic properties of the film. Many other works report similar phenomena in which trapped water is desorbed from an immobilized surface species due to absorption of a ligand¹⁹⁻²¹.

The changes in motional resistance exhibited in Table I also vary in sign and depend upon the linker. We observe a negative change in motional resistance with the cysteamine linker, in disagreement with the results of Thompson et al⁹.

Importance of slip

The conclusion that the shear mode resonator is not behaving as a microbalance when detecting adsorbed species in solution is not new, since there is ample evidence in the literature of this phenomenon. Recent reviews of the use of quartz crystal microbalances for biomolecular systems at the solution-surface interface have still viewed this sensor as a mass detector^{5, 6}. We disagree, and we believe our experimental results support our conclusion. Models have been developed that describe the response of a shear mode resonator coated with a non-rigid layer immersed in a liquid²²⁻²⁵. All those models take into account the breakdown of the no-slip boundary condition. Slippage may occur between the solid substrate (gold electrode on quartz) and the attached HSA film; this is called inner slip by Ellis and Thompson²⁵. Inner slip is assumed to be largely viscoelastic in nature, related to adhesion and friction and determined by changes in bonding between the linker and the gold. These yield variations in the amount of acoustic energy propagated into the HSA layer from the resonator. For a 5 MHz oscillator the acoustic wavelength will be longer than for a 9 MHz oscillator; thus, inner slip could be more important in our case than for Thompson et al⁹.

Outer slip also may occur between the HSA film and the solution. Outer slip is considered to be a viscous process normally described by a planar discontinuity between the upper layer of HSA and the adjacent solution layer. The adsorption of warfarin by HSA may induce configurational changes in the protein, causing changes in viscous coupling between the film and the solution. Although the linker chemistry affects the interface between the substrate and the HSA film and thus is expected to influence inner slip, it is reasonable to expect both type of slippage in the cases studied in this work, and the influence of each of them cannot be easily separated. It has been shown^{22, 26} that slip

may result in increases or decreases in both motional resistance and frequency large compared with those expected from merely gravimetric response.

An important result of the present study is shown in Figure 11. The changes in integrated heat signal are (a) proportional to the concentration of warfarin in solution and (b) independent of the linker chemistry used to immobilize HSA to the surface. Because the kinetic modeling of the HSA/warfarin binding process at the surface produces binding enthalpies consistent with solution studies, we use the measured integrated heat to determine the number of moles of warfarin bound to HSA and thus the minimum surface coverage of immobilized HSA. We consider that only one site is occupied, which is not the case for the warfarin concentrations higher than 30 μ M; however the second site is not fully occupied and the enthalpy for this site is lower, so for simplicity we ignore its contribution. For 2.6 mM warfarin concentration, from Table I, an integrated heat of 5.5 mJ for this concentration corresponds to $.0055/85000 = 6.5 \times 10^{-8}$ moles of warfarin bound on a 2 cm² area (slightly less if we consider that the second site is partially occupied). The HSA surface coverage must thus be at least 3.4×10^{-8} moles/cm² assuming 1:1 binding. Yet Thompson et al ⁹ estimate the surface coverage of a close-packed HSA monolayer as only 5×10^{-12} moles/cm². Myszka et al ⁷ have used surface plasmon resonance to monitor the build-up of HSA linked by EDC/NHS, and find that after 7 minutes of exposure of the linker surface to an HSA solution three order of magnitude lower in concentration than the one used in our immobilization procedure, the HSA surface coverage is 3.3×10^{-10} moles/cm². In that work they limited the time and the concentration to obtain lower surface coverage. Morton and Myszka ²⁷ discuss thoroughly the many experimental precautions needed to prepare protein surface layers of uniform coverage, and these include capping reactive groups on the protein to prevent coagulation or polymerization and thorough washing to remove noncovalently bound protein. Zini et al ²⁸ show that commercial human serum albumin polymerizes, thus exhibiting a concentration-dependent aggregation in solution. This aggregation may well be occurring on the HSA surfaces used in this work. Since procedures of the type discussed by Morton and Myszka were not used either by Thompson et al or in this work, we are forced to conclude that the HSA surface used here to detect warfarin binding

contained $\sim 10^4$ more mass per unit area than in a monolayer of the protein. Such a large excess of protein would also help to explain the relatively large shifts in both frequency and motional resistance observed in both studies.

Despite this result, we maintain that the calorimetric signal produced by binding is a better analytical measure of warfarin binding to HSA than the signals from the quartz resonator. The integrated heat is proportional to warfarin concentration and independent of the linker chemistry, thus making it more suitable for quantitation.

Conclusions

We have shown that quartz crystal microbalance/heat conduction calorimetry can be a useful tool for studying protein-ligand interactions at the solution-surface interface, even though the quartz resonator does not behave as a microbalance. Three linkers were used to bind HSA to a 5 MHz shear mode resonator surface, and the interactions of the resulting immobilized film with warfarin were determined by following changes in frequency, motional resistance, and integrated heat. Differences in frequency and motional resistance changes depend upon the linker, whereas the integrated heat signal is proportional to the concentration of warfarin and independent of the linker chemistry.

Acknowledgement

The authors wish to acknowledge partial funding for their work by the State of Pennsylvania, Dept. of Community & Economic Development, through the Nanotechnology Institute.

Table I. Summary of changes in frequency, motional resistance, and integrated heat for the interaction of warfarin with HSA immobilized with three linkers.

Max heat (mJ)

Warfarin conc.	0.2mM	0.5mM	1.0mM	1.04mM	1.5mM	2.0mM (Ref. 9)	2.6mM
Cysteamine	0.40				3.0		5.5
11-MUA		0.8	1.8				
DSP	0.65	1.2	2.4				

Frequency shift (Hz)

	0.2mM	0.5mM	1.0mM	1.04mM	1.5mM	2.0mM (Ref. 9)	2.6mM
Cysteamine	-9.6				-28.3	-25/-30	-47.0
11-MUA		32.5		39.8		36/30	
DSP	-3.9	-6.6	-9.0			33/29	

Motional resistance (Ω)

	0.2mM	0.5mM	1.0mM	1.04mM	1.5mM	2.0mM (Ref. 9)	2.6mM
Cysteamine	-7.43				-34.5	7.9/12.2	-49.0
11-MUA		-7		-10		-1.8/-2.5	
DSP	0.9	4.6	6.7			7.8/8.7	

Figure Captions

Figure 1. The QCM/HCC sensor and sample chamber

Figure 2. Changes in frequency and motional resistance due to binding of warfarin to an HSA film/cysteamine linker, at three warfarin concentrations

Figure 3. Changes in thermal power due to binding of warfarin to an HSA film/cysteamine linker

Figure 4. Calculated thermal power P_{cryst} generated by the QCM, HSA film/cysteamine linker

Figure 5. The net thermal power for the binding process, P_{bind} , HSA film/cysteamine linker

Figure 6. The integrated heat for the binding process, P_{bind} , HSA film/cysteamine linker

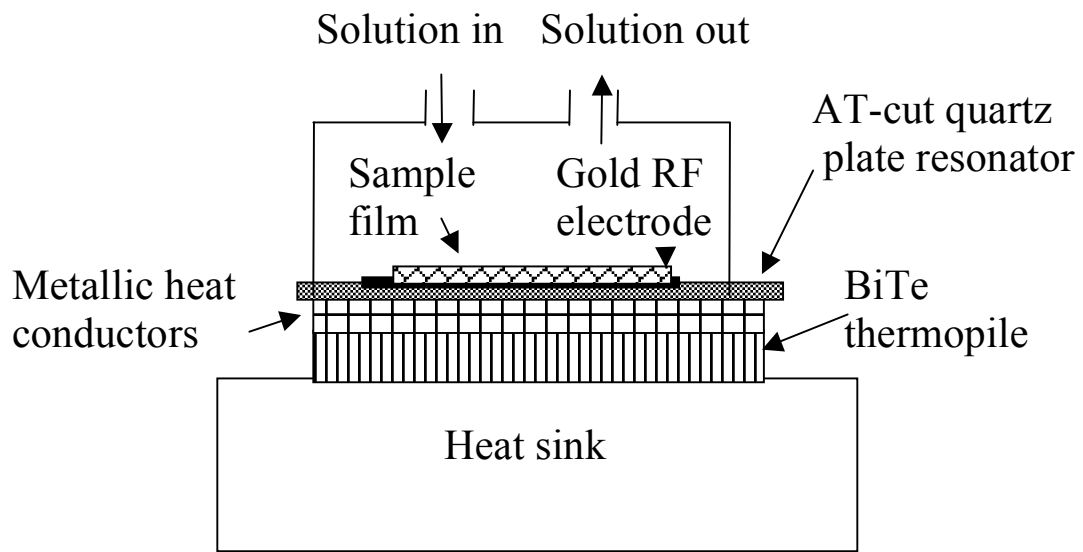
Figure 7. The fitting (open circles) and the experimental (line) signal for the interaction of 1.5mM warfarin with immobilized HSA

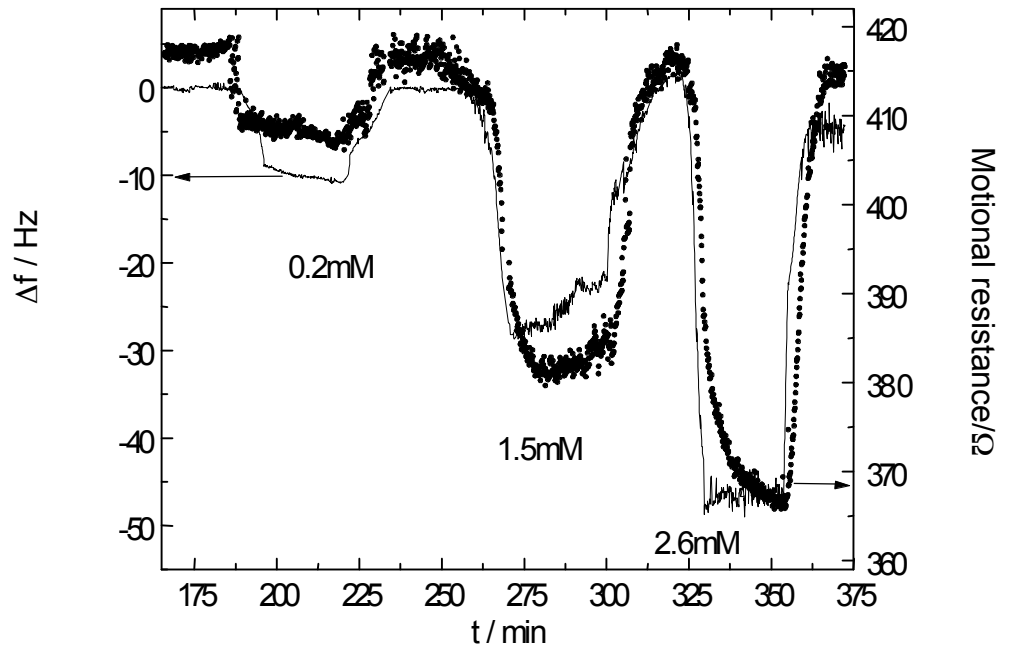
Figure 8. Changes in frequency and motional resistance due to binding of warfarin to an HSA film/11-MUA linker. The first peak is for 0.5mM warfarin, the second for 1.04mM warfarin.

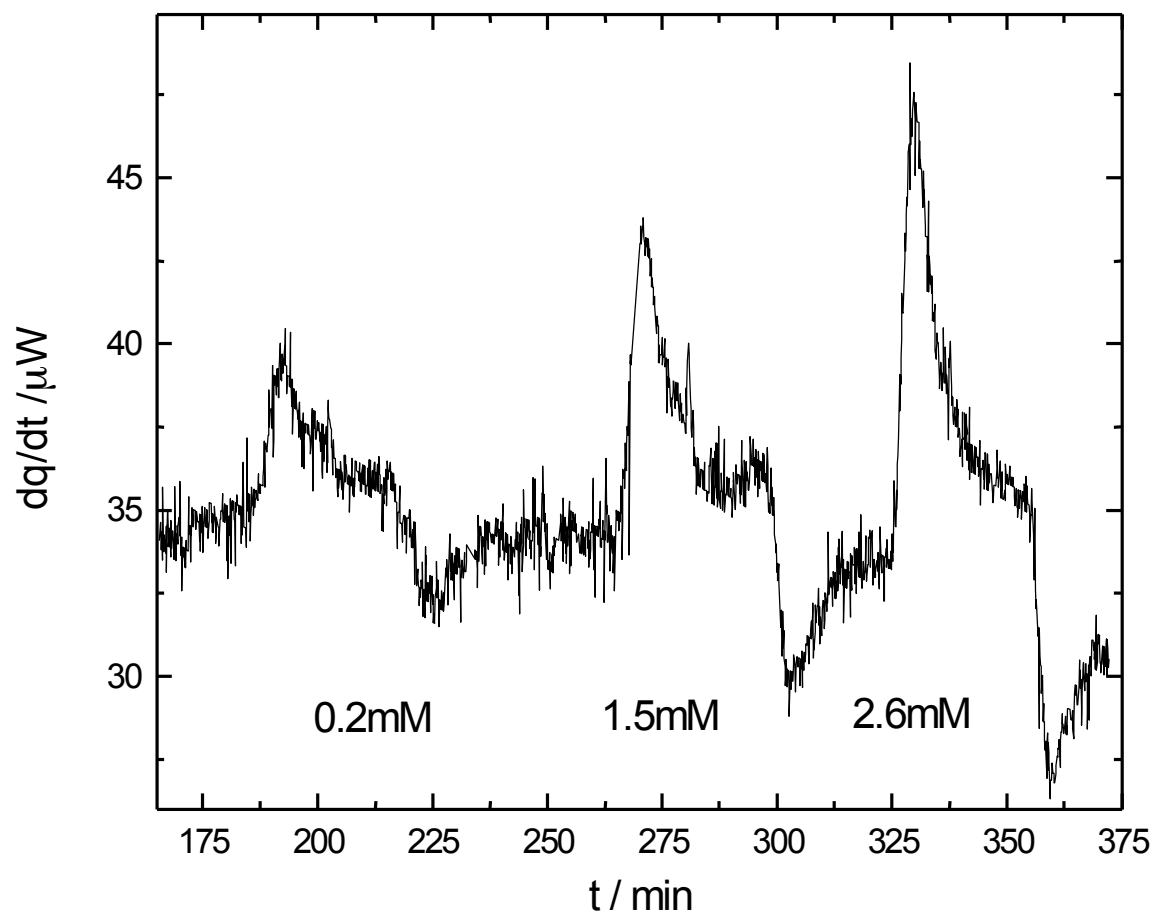
Figure 9. The net thermal power for the binding process, P_{bind} , HSA film/11-MUA linker

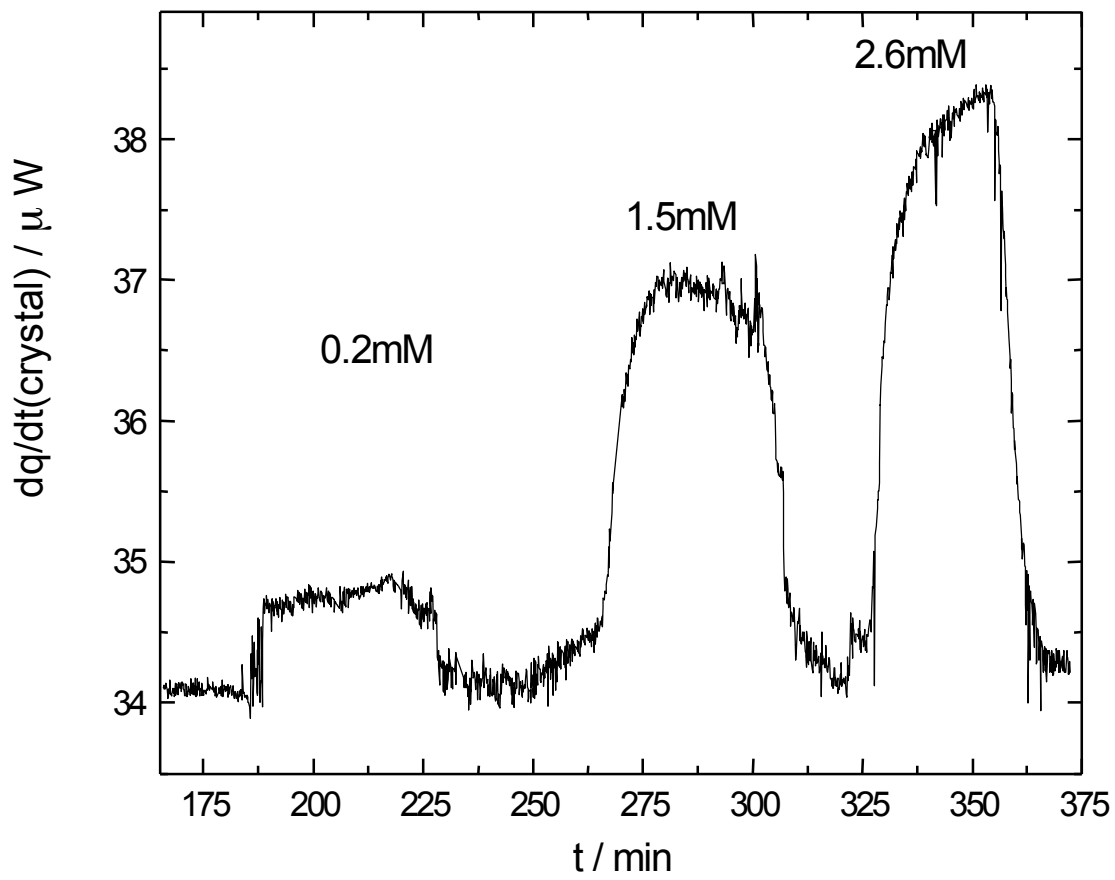
Figure 10. The integrated heat for the binding process, P_{bind} , HSA film/MUA linker

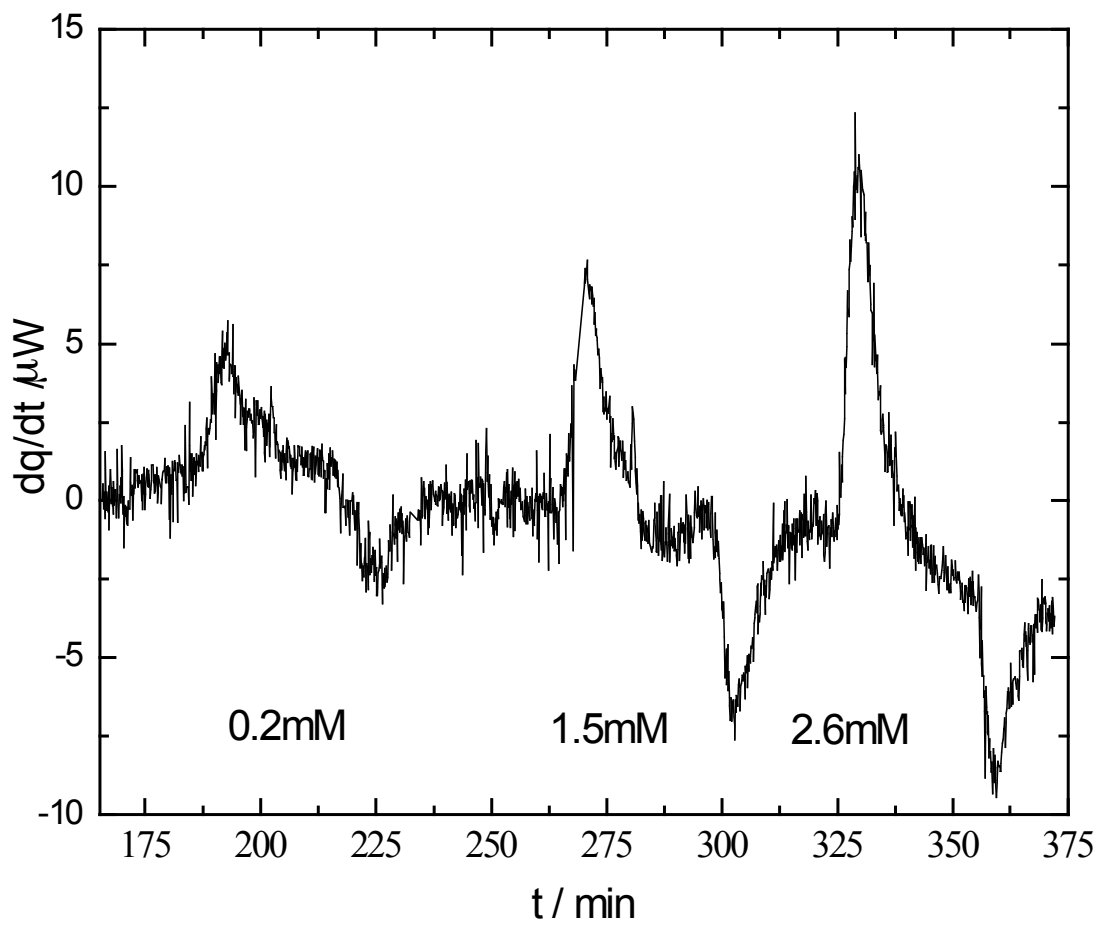
Figure 11. Dependence of integrated heat signal on warfarin concentration for the interaction of warfarin with HSA immobilized by three linkers

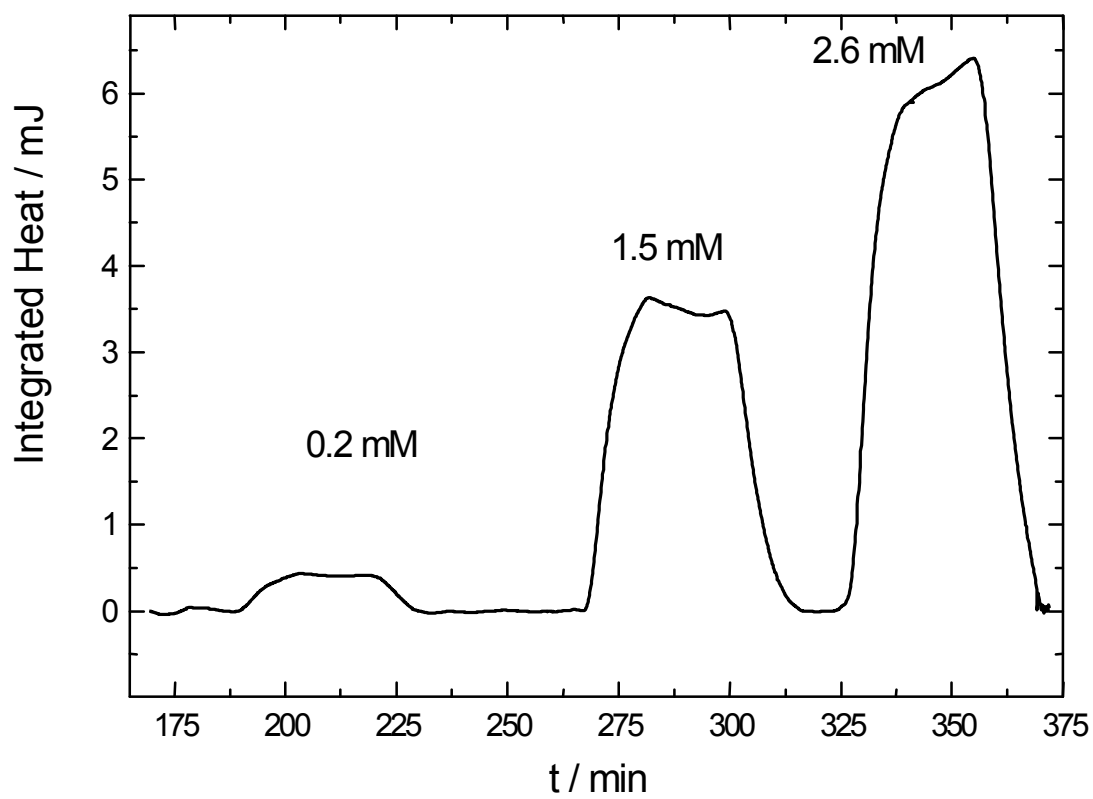


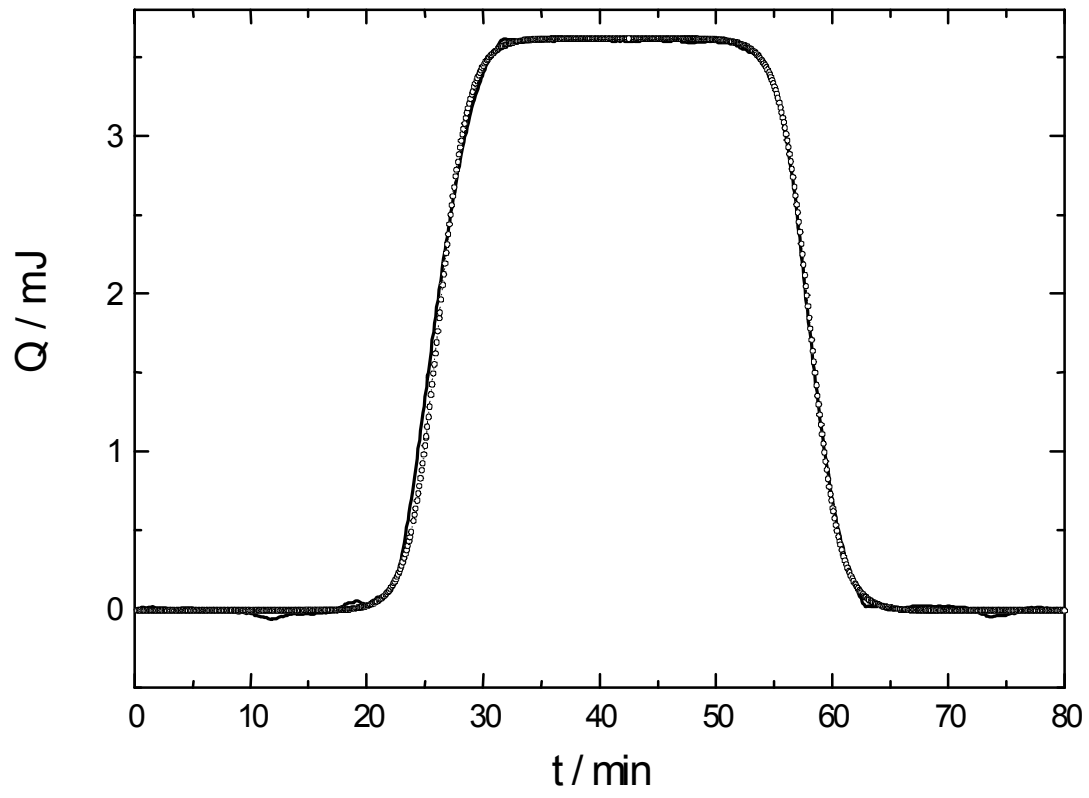


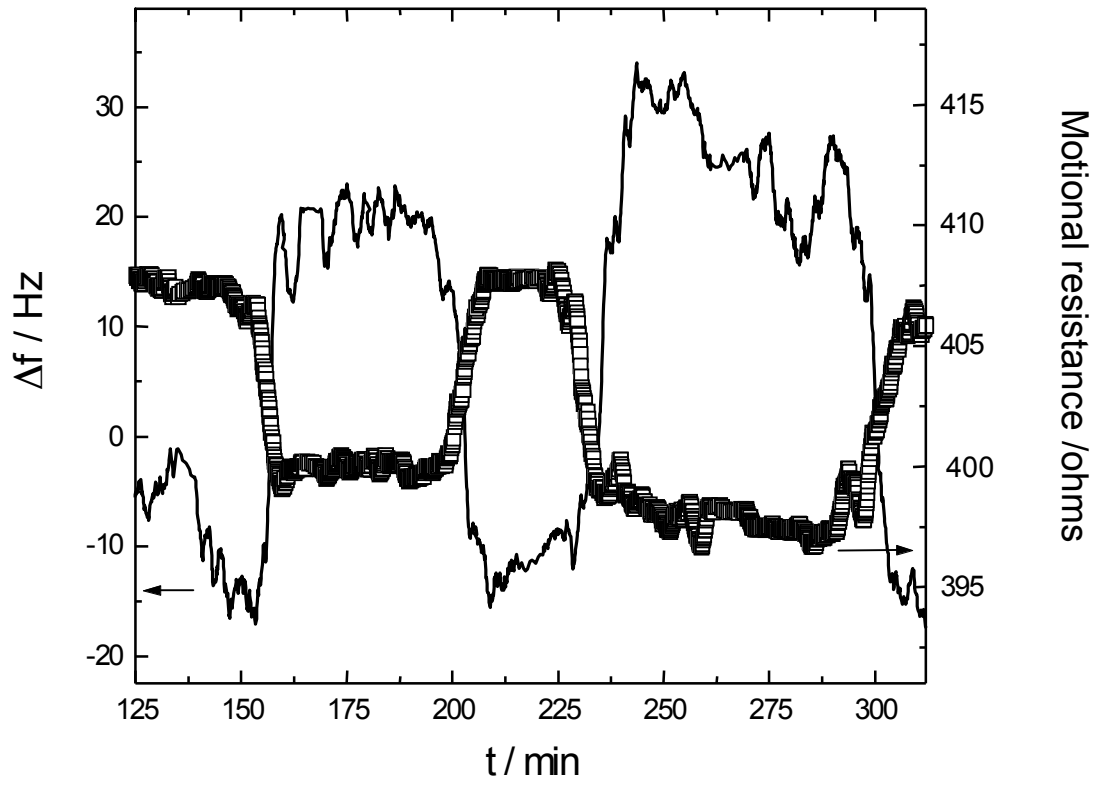


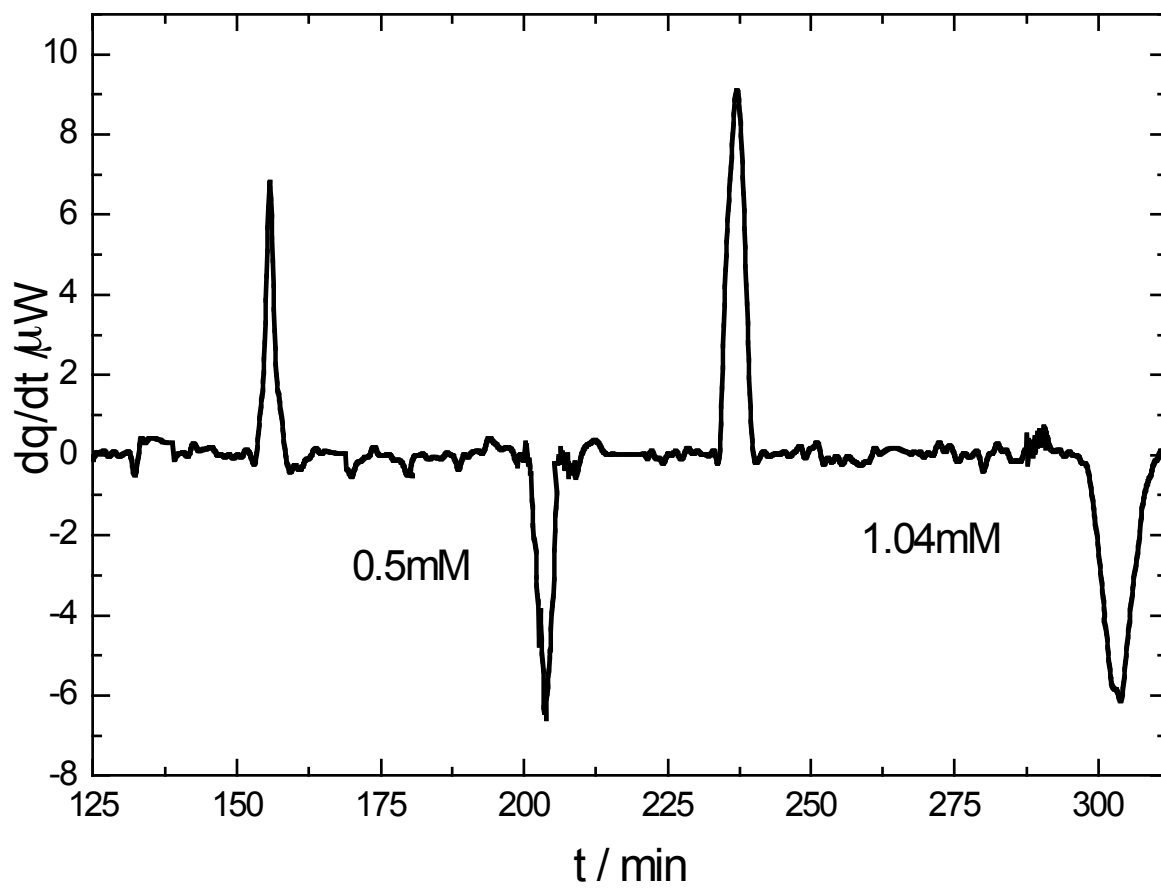


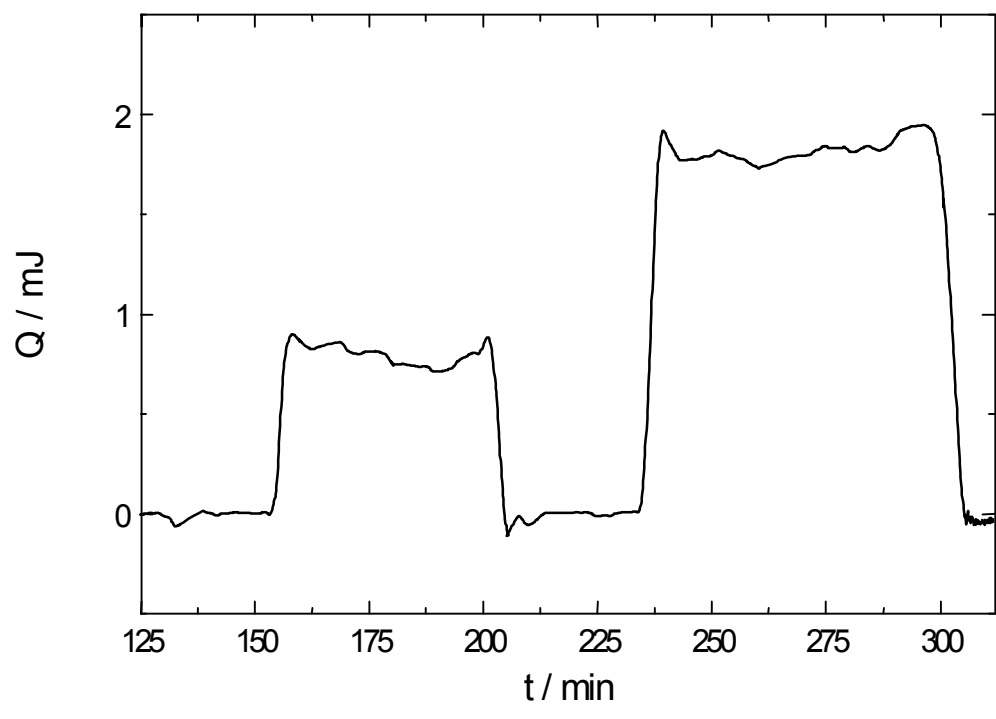


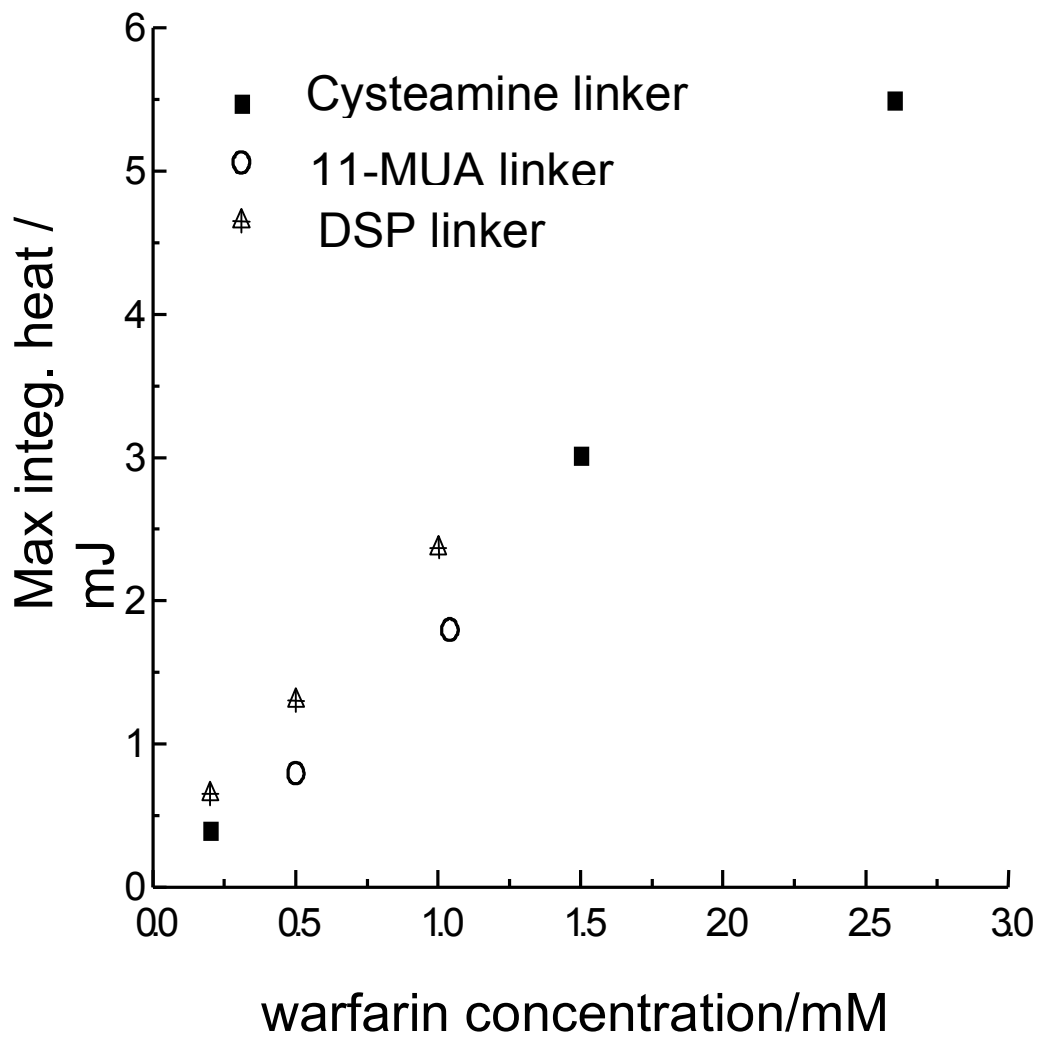












References

- 1 *A. L. Smith and H. M. Shirazi, J. of Thermal Analysis and Calorimetry, 2000, 59, 171-186.*
- 2 *A. L. Smith, S. R. Mulligan, and H. M. Shirazi, J. Polymer Sci. Part B Polymer Physics, 2004, 42, 3893-3906.*
- 3 *A. L. Smith and H. M. Shirazi, Thermochemica Acta, 2005, in press.*
- 4 *E. Reimhult, C. Larsson, B. Kasemo, and F. Höök, Anal. Chem., 2004, 76, 7211-7220.*
- 5 *A. Janshoff, H.-J. Galla, and C. Steinem, Angew. Chem. Int. Ed., 2000, 39, 4004-4032.*
- 6 *K. A. Marx, Biomacromolecules, 2003, 4, 1099-1120.*
- 7 *L. R. Rich, Y. S. N. Day, T. A. Morton, and D. G. Myszka, Anal. Biochem., 2001, 293, 197.*
- 8 *B. Loun and D. S. Hage, Anal. Chem., 1996, 68, 1218.*
- 9 *E.-L. Lyle, G. L. Hayward, and M. Thompson, Analyst, 2002, 127, 1596-1600.*
- 10 *H. Aki and M. Yamamoto, J. Pharm. Sci., 1994, 83, 1712.*
- 11 *D. Pavey, E.-L. Lyle, C. J. Olliff, and F. Paul, Analyst, 2001, 126, 426.*
- 12 *C. Andre, M. Kokkinidis, and D. Tsernoglou, J. Chromatog.B, 2004, 801, 221.*
- 13 *R. Lucklum and P. Hauptmann, Measurement Science & Technology, 2003, 14, 1854-1864.*
- 14 *M. V. Voinova, M. Jonson, and B. Kasemo, Biosensors and Bioelectronics, 2002, 17, 835-841.*
- 15 *M. Rodahl, F. Hook, A. Krozer, P. Brzezinski, and B. Kasemo, Rev. Sci. Instr., 1995, 66, 3924-3930.*
- 16 *R. J. Willson, A. E. Beezer, J. C. Mitchell, and W. Loh, Journal Of Physical Chemistry, 1995, 99, 7108-7113.*
- 17 *A. E. Beezer, A. C. Morris, M. A. A. O'Neill, R. J. Willson, A. K. Hills, J. C. Mitchell, and J. A. Connor, Journal Of Physical Chemistry B, 2001, 105, 1212-1215.*
- 18 *M. O'Neill, A. E. Beezer, G. J. Vine, R. B. Kemp, D. Olomolaiye, P. L. O. Volpe, and D. Oliveira, Thermchim. Acta., 2004, 413, 193-199.*
- 19 *S. Cusack, C. Berthet-Colominas, M. Hartlein, N. Nasser, and R. Leberman, Nature, 1990, 347, 249.*
- 20 *T. G. Oas, L. P. Mcintosh, E. K. O'Shea, F. W. Dahlquist, and P. S. Kim, Biochemistry, 1990, 29, 2891.*
- 21 *R. S. Hodges, Zhou, N.E., C. M. Kay, and P. D. Semchuk, Peptide Research, 1990, 3, 123.*
- 22 *G. McHale, R. Lucklum, M. I. Newton, and J. A. Cowen, J. Appl. Phys., 2000, 88, 7304-7312.*
- 23 *G. L. Hayward and M. Thompson, J. Appl. Phys., 1998, 83, 2194.*
- 24 *J. S. Ellis and M. Thompson, Chemical Communications, 2004, 1310-1311.*
- 25 *J. S. Ellis and M. Thompson, Physical Chemistry Chemical Physics, 2004, 6, 4928-4938.*
- 26 *J. S. Ellis and M. Thompson, Chem. Comm., 2004, 11, 1310.*

²⁷

T. A. Morton and D. G. Myszka, Methods of Enzymology, 1998, 295, 268-294.

²⁸

R. Zini, J. Barre, F. Bree, J. P. Tillement, and B. Sebille, J. Chromatog., 1981, 216, 191-198.