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Enantiomeric study using a QCM-D with nanoparticles functionalised on the sensor surface

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Title (English)

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Title (Swedish)

Abstract

Interactions between purified enantiomers of chiral drugs and human serum proteins were used as models to investigate the use of the QCM-D technique for enantiomeric studies. In order to increase the sensitivity of the QCM-D, the sensor crystal was functionalised with nanoparticles and reached the monolayer coverage of 80 %. There is evidence that the increased resolution was able to resolve differences in enantiomeric affinity of the S-form of propranolol compared to the R-form binding to α_1-acid glycoprotein.

Keywords

QCM-D, enantiomer, nanoparticles, biosensor, drug-protein interaction, chiral, DMSO

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Trikien Quach

Sammanfattning

För att en kemisk reaktion ska kunna ske så måste molekylerna komma tillräckligt nära varandra och stereokemin har därför en avgörande betydelse. Vid tillverkning av ett läkemedel får man förutom den aktiva formen med den önskade effekten ibland även en annan molekyl som är en spegelbild av produkten och i vissa fall har andra egenskaper som dessutom kan vara skadliga för kroppen. Detta kan ske om molekylen innehåller en eller flera asymmetriska centra. Det typiska exemplet gällande spegelisomerers förödande effekt är neurosedynskandalen som inträffade i slutet på 1950-talet då den inaktiva formen av läkemedlet Neurosedyn orsakade fosterskador, vilket ledde till att många barn föddes med missbildningar. På grund av spegelisomerers likartade kemiska och fysikaliska egenskaper blir separationen av den ena formen från den andra komplicerad. Den grundläggande förståelsen om hur spegelbildsformer av molekyler växelverkar med målproteiner är därför mycket viktig. Att utveckla bättre metoder för analys av dessa är därför syftet med detta projekt, vilket också är mycket attraktivt för industrin.

Kvartsmikrovågen är en sensor som detekterar massförändringen på en kristall som uppstår under en molekylinteraktion. Teknikens tillämpning för interaktionsstudier av spegelisomerer kommer att undersökas med hjälp av en sensoryta som är modifierad med nanopartiklar så att fler ligander kan bindas upp. Detta ger i sin tur en flerfaldig ökning av känsligheten som resultat.

Examensarbete 20 p
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1. Introduction

1.1. Project background

Many pharmaceuticals are chiral molecules. A chiral molecule[1] consists of one or more chiral centres, in which atoms of the molecule are arranged in spatially distinct forms, with one form a mirror image of the other. Just like the left and right hand, both contain the same components but differ in arrangement, therefore, they have a difference in conformation and function. In nature, chirality is a common occurrence and occurs when one carbon atom binds to four different functional groups. Any molecule that is different from its reflection is said to be chiral. The two different forms of a chiral molecule are called enantiomers labelled with the prefix R (rectus) or S (sinister). Enantiomers of drugs have identical physical and chemical properties (e.g. energy, melting point, boiling point, solubility etc.), but when interacting with other enantiomers e.g. in a biological system, they may behave very differently physiologically and in pharmacokinetics. Conformations of the drugs have a significant effect on the binding affinity to different sites on proteins and thereby also an effect on biological processes. A typical example of the problems associated with enantiomeric drugs is the thalidomide catastrophe[2] in the late 1950's. The drug which was marketed as a sedative was sold as a racemate, a mixture of both enantiomers, where the active enantiomer was a sedative, whereas the nonactive enantiomer was later found to cause foetal abnormalities.

The thalidomide catastrophe highlighted the potentially dramatic different physiological response to enantiomers and the acute need for new and better techniques for asymmetric syntheses. Separation techniques of chiral compounds have been developed encouraged of the guideline by FDA[3] (U S Food and Drug Agency) and EMEA[4] (European Medicine Agency) and the trend is toward single enantiomeric production. Yet, many chiral pharmaceuticals on the market are still sold as a racemate. The reason is that it is still very difficult to separate enantiomers with high efficiency and for low costs. Improved drug development requires a fundamental understanding about how the drug is being transported in our bodies and how molecules interact with each other. Interaction studies under physiological conditions are essential. To achieve this, very high resolution analysis methods are required and great improvements have been made in the area of chiral High Performance Liquid Chromatography (chiral HPLC), Surface Plasmon Resonance (SPR), Stopped Flow, Capillary Electrophoresis etc. In a recent study, SPR and HPLC-PM (perturbation method), were compared for detailed characterization of chiral drug-protein interactions. The conclusion was that HPLC-PM is best suited for analysis of weaker interactions, whereas SPR is the better alternative for stronger interactions[5]. However, the combination of different analytical

techniques often provides a much better understanding of a complete molecular process than a single measurement.

Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) measures the change in resonance frequency of a quartz crystal to detect adsorbed mass. Importantly QCM-D can also obtain viscoelasticity information which is directly related to the structural conformation of the adsorbed molecules. This technique shares the same advantages as SPR e.g. label free detection in real time of adsorption or desorption events, but is more economical. Most pharmaceuticals are rather small molecules compared to a transport protein which makes it difficult to detect them. It is of great interest to the industry to investigate if the QCM-D technique is sensitive enough to determine differences in adsorbed mass due to enantiomeric selectivity and it is also the purpose of this project.

1.2. Aims of the project

The aims of this project are to investigate the use of QCM-D as an analytical tool for enantiomeric studies and to quantify the detection limit of this technique. In addition, possibilities to improve the sensitivity of the technique using nanoparticles will be investigated.

The drug-protein combinations used in this study are Warfarin binding to human serum albumin (HSA) and Propranolol binding to α_1-acid glycoprotein (AGP). Both albumin and AGP are transport proteins with a fairly high affinity and enantioselectivity to their respective drug and the detections of affinity differences of the enantiomers are of interest. Enantiopurified substances will be used. Also the effect of the common organic solvent Dimethyl sulfoxide (DMSO) on drug-protein binding will be investigated.

Proteins will be immobilised onto surfaces of gold and polystyrene using common methods such as physical adsorption (PA) and the formation of self-assembled monolayers (SAM). Furthermore, active surface modification based on a decoration with nanoparticles is of particular interest. The challenge is to improve the sensitivity for detecting very low molecule weight or low concentration of analytes. Nanoparticles will increase the active surface area where the selective interactions are taking place and consequently provide a larger signal.

Atomic Force Microscopy (AFM)[6] will also be used as a complimentary technique to visualize the active surface.

2. Theoretical Background

2.1. The theory of QCM-D

The Curie brothers discovered in 1880 that anisotropic crystals, if mechanically stressed, will provide an electric signal and equally an applied electric signal will cause mechanical deformation. This phenomenon is known as the Piezo-electric effect. Quartz is the most commonly known piezo-electric material but other piezo-electric materials include tourmaline, barium titante and polyvinylidene fluoride. In 1959, Sauerbrey showed that a small change of the mass of the quartz crystal is directly proportional to its shift in the resonance frequency. This fact was later developed into the QCM technique[7] as a very sensitive microbalance device with an incredible sensitivity of detecting mass change at the nanogram level. The QCM sensors are made of quartz crystal which is forced to oscillate at its resonance frequency (5-10 MHz) by an oscillating electric potential applied between gold electrodes coated on both sides of the quartz. As the mass is increased or decreased during the analyte-ligand interaction, it will cause a shift in resonance frequency. If the adsorbed film is thin and rigid, the adsorbed mass (ΔM) is directly related to the frequency shift via the Sauerbrey relationship[8]

$$\Delta M = \frac{-C\Delta F}{n}$$

where ΔF, is the frequency shift , n = is the overtone number and C is constant related to the material properties of the crystal. In this case C = 17.7 ng / (Hz cm^{-2}) for a 5 MHz quartz crystal.

For biological investigations, the QCM measures the mass of the molecules but also the mass of the water associated with the layer. Due to this hydrodynamic effect the calculated mass of the adsorbed biomolecules is usually greatly overestimated. In most cases the adsorbed film cannot be considered rigid and the simple Sauerbrey relationship becomes invalid. In this case it is important to understand the effects of the viscoelasticity of the system. However, the mass frequency relationship works well as a guideline.

The traditional QCM technique was improved in 1999 by Rodahl and Höök. They developed a method to measure the change of dissipation factor ΔD in combination with change of resonance frequency ΔF. QCM-D became a much more powerful analysis tool, very suitable in protein adsorption studies. Generally the viscoelasticity (ΔD) of an adsorbed layer increases as more loosely bound matter or big molecules are attached to the sensor surface because they do not fully follow the oscillation. Energy is

dissipated due to the frictional losses and as a consequence the oscillation will be damped. The dissipation is defined as:

$$D = \frac{E_{Dissipated}}{2\pi E_{Stored}}$$

where $E_{Dissipated}$ is the energy loss (dissipated) during one oscillation cycle and E_{stored} is the total energy stored in the oscillator.

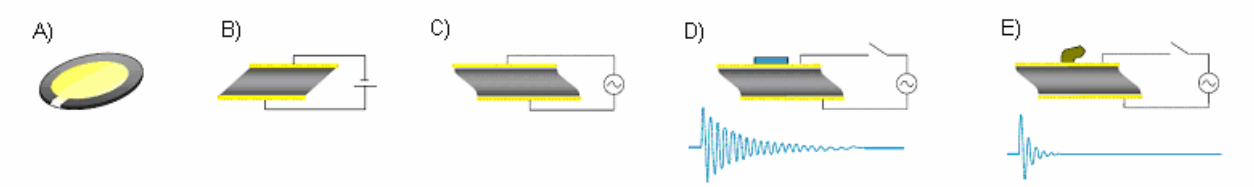


FIG 1. (A) Schematic presentation of a crystal. (B) Deformation of the crystal caused by an electric potential. (C) Oscillation of the crystal caused by AC. (D) Dissipation by a rigid molecule. (E) Dissipation of a viscoelastic molecule.

2.2. The Q-Sense instrument

In this study, the QCM-D Q-Sense D300 instrument[9] was used and the data was analyzed using the Q-Tools software (Q-Sense Inc., Sweden). A complete QCM-D instrument consists of an electronic unit, a measurement chamber with sensor crystal and acquisition software. The electronic unit is the core of the instrument conducting the measurement chamber and containing the temperature control system for the thermoelectric module. An external pump could be connected as an accessory for flow injection feed measurements. The crystal is the sensing element excited to oscillation by the drive generator. After every 5 ms the drive generator interrupts itself and the oscillation decays exponentially. The frequency shift and dissipation energy, ΔF and ΔD value, during an interaction process are plotted on the computer screen in real time. This process repeats itself automatically. QCM-D300 has a mass sensitivity better than 5 ng /cm^2.

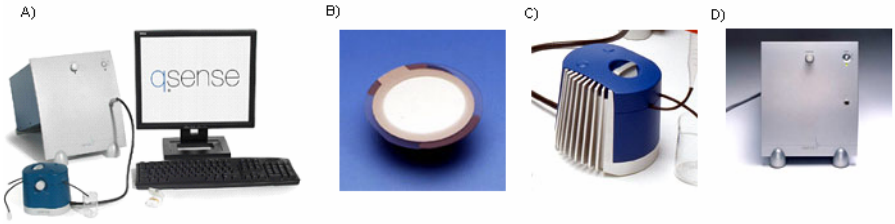


FIG 2. (A) The QCM-D300 instrument. (B) The sensor crystal. (C) The chamber. (D) The electronic control unit.

2.3. Surface of nanoparticles

Since most proteins have high affinity to surfaces of hydrophobic materials e.g. polystyrene (PS), the most convenient way to adsorb proteins is by simple physisorption forming a thin film by hydrophobic interactions. However, proteins unfold when forced to adsorb onto a surface and change their conformation becoming denatured. For this reason physical adsorption may not always be suitable for affinity studies. One way to preserve the specific binding properties is to modify the surface with a neutral self-assembled monolayer[10] which will allow the adsorbed proteins to maintain their structural conformation.

In some cases, attachment of the ligands on nanoparticles is preferred because of the advantages of a much lager surface area compared to a flat surface in a defined area. Also there are positive effects on mass transport kinetics as the analytes can bind with reduced steric barriers. Previous studies have shown a 10-fold increase in the binding constant of the streptavidin–biotin complex with a corresponding increase in the particle surface curvature[11].

All biosensors consist mainly of two principal components; transducer and chemical interface. The function of the transducer is to convert a chemical signal on the interface and present it into a readable electrical signal. In this study, with ambition to improve the sensitivity of QCM-D measurement using nanoparticles there are some important issues. To maximise the surface area, small particles should be used. The smaller the particles, the lager the surface area and the lower the background noise as the interaction is taking place closer to the sensor surface. Two types of particles were used: 160 nm PS particles and 120 nm silica particles pretreated with streptavidin. Several different strategies were investigated as methods to deposit the particles to the sensor surfaces. The PS particles were adsorbed to the surface by simple physisorption processes and also by functionalising the PS with albumin and thiolating this complex using SPDP reagent. The silica particles were bound to the surface utilising the strong coupling between biotin and streptavidin, either coupling directly to biotin bound to the surface or incorporating biotin functionalised albumin. Multilayer build up of the particle layer was not desirable because the signal has a tendency to be noisy.

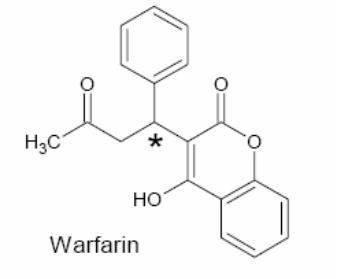
3. Experimental: Materials

3.1.1. The proteins

Albumin is the most common protein in blood serum and albumin-like proteins can be found in all mammals. 50-60% of the plasma proteins in our blood vessels are albumin. Its consistence of three globular subunits and many hydrophobic packets are making it an excellent carrier of fatty acids and hydrophobic drugs. This transport protein with a molecular weight of 66 kDa, is one of the best characterised proteins and widely used as a model in drug-protein studies[12].

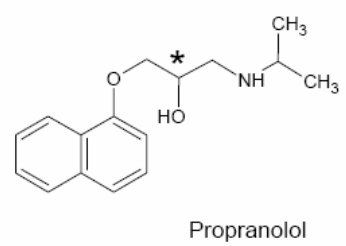
α_1-acid glycoprotein (AGP) is an acute-phase plasma protein, meaning that in an acute-phase reaction the concentration of it increases several fold. This protein has a molecule weight of 40 kDa and about 45 % of its weight is composed by five polysaccharides internally cross-linked by two disulfide bridges. The polypeptide chains give the protein its acidic character (pI.2.7). AGP is synthesised mainly in the liver and is one of the most important plasma proteins for the distribution of basic drugs[13].

3.1.2. The substrates



Warfarin

Warfarin (MW 308.3 Da) is used as an anticoagulant and works by inhibiting vitamin K-dependent coagulation factors, which prevents blood clots from forming or growing larger. It is mainly used after a heart attack or heart valve replacement surgery. Warfarin can also be used with other drugs in the treatment of some lung cancers and is well characterised. In this study we investigate the main binding site on HSA, called the "warfarin-site".



Propranolol

Propranolol (MW 295.81 Da) is a β-blocker and works as a competitive antagonist to β-receptor sites. The drug is used to reduce high blood pressure and to treat irregularities in hart beat. Propranolol is administered as a racemate but its therapeutic activity is tied to the S-form. The S-form of both drugs has been reported to have stronger binding to their target proteins than the R-form[14].

FIG 3. Chemical structure of the substrates. *Asymmetrical centre.

3.1.3. The chemicals

The buffer used in all studies in this project was 10 mM PBS at pH 7.4 unless otherwise indicated. PBS was made from PBS powder (Sigma) diluted in high purity mQ water.

Glutaraldehyde (GA), HSA (99%, fatty acid free), AGP, Glycine, Hydrogen peroxide, 4-aminothiophenol (ATPh) 97%, Propranolol ([(*R*)-1-(isopropylamino)-3-(1-naphthyloxy)-2-propanol hydrochloride] and [(*R*)-1-(isopropylamino)-3-(1-naphthyloxy)-2-propanol hydrochloride)]), Periodic acid 98% and Cysteamine hydrochloride were purchased from Sigma. Warfarin (Chemoswede AB) was kindly provided by AztraZeneca, Mölndal Sweden.

PS latex particles (160 nm, 10% solids) and silica particles (120 nm, CP01N) pretreated with streptavidin were purchased from Bangs Laboratories Inc. (Fishers, IN, USA). Sulfo NHS LC biotinylation kit was purchased from PIERCE.

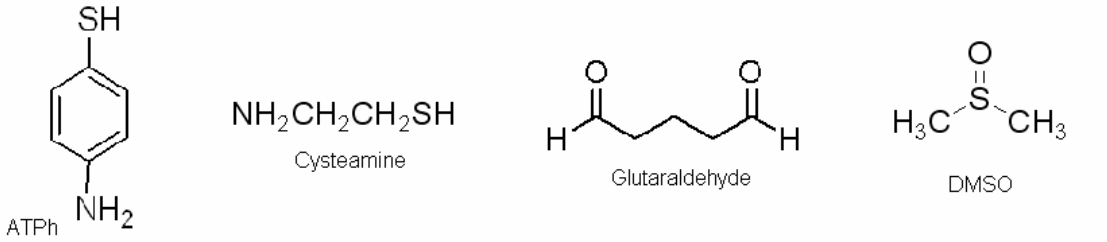


FIG 4. Chemical structure of substances used in study.

3.1.4. The sensor crystals

The commercially available crystals come prepared with gold substrates or with coatings such as PS and SiO_2. If necessary, the substrate can be further modified by a thin film of biomaterials (proteins, vitamin, antibody, DNA, polymers, particles, cells etc.[15]) with the ability to selectively interact with the target analyte. All sensor crystals were purchased by Q-sense. The chemical cleaning and regeneration procedures are described in section 3.2.1.

3. Experimental: Methods

3.2.1. Cleaning the QCM-D instrument

The tubing

For an effective surface modification the surface has to be free from contaminations. Proteins usually stick very hard to surfaces and there are no gentle ways to remove them all. All tubing in the QCM-D instrument were cleaned with 2 % SDS or 1 % Hellmanex and then rinsed thoroughly with mQ water. The solutions were pumped through the tubing using an external pump (see fig. 7).

The gold crystals

For removal of lipids and proteins, the sensor crystals were immersed in 1:3 (v/v) H_2O_2 / H_2SO_4 (piranha solution) for 5 min at room temperature and then rinsed with ethanol and blow dried with pure nitrogen gas. Alternative, a solution of mQ water, ammoniac (25%) and H_2O_2 (30%) (5:1:1) was heated up in a water bath to 70-75 ºC and the sensor crystals were immersed in the solution for 5 minutes. Then the crystals were rinsed in mQ water and blow dried with nitrogen gas.

The PS crystals

The sensor crystals were immersed in different solutions in the following order and each was sonicated for 2 minutes; a solution of 1% sodium dodecyl sulphate (SDS), a mixture of ethanol and mQ water (1:1) and ethanol. After this the sensor crystals were blow dried in pure nitrogen. For removal of the PS, the sensor crystals were treated with the same procedure as above except for with the two first solutions which were exchanged for a mixture of hexane and mQ water (1:1) and a mixture of toulen and mQ water (1:1).

3.2.2. Sample preparations

Sample preparation

A series of different concentrations of Warfarin (R and S) and Propranolol (R+ and S-) were made in PBS. Pure Warfarin has an extremely poor solubility in water and the PBS was modified with 1-5 % DMSO to increase the solubility and make the analysis possible to perform. Caution is needed because DMSO is an organic solvent and has an ability to penetrate physiological barriers and is toxic at high doses. DMSO is commonly used for dissolving substances e.g. carbohydrates, peptides, polymers, gasses and inorganic salts. For that reason always use gloves for protection!

3.2.3. Protein preparations

All proteins dissolved in buffer should always be vortexed and not shaken which causes protein denaturation. “Protein solution, stirred not shaken!” All proteins in this study are human materials. For that reason always use gloves for protection!

Preparation of nanoparticles

The 160 nm PS nanoparticles in water solution were accumulated by centrifugation at 14 000 rpm for 45 minutes and the pellet was dispersed in new mQ water and vortexed. This washing procedure was repeated three times. Finally the pellet was dispersed in glycine buffer (10 mM, pH 7.4) or mQ water for storage. During the adsorption step, a 0.5% (w/v) suspension of PS nanoparticles was incubated with 5 mg / ml albumin for 1 h at room temperature and vortexed gently for a few seconds (2.5 mg protein is needed per m^2 PS surface). After PS was totally saturated with albumin, free and loosely bound albumins were removed by centrifugation and the pellet was once more dispersed in glycine buffer (10 mM, pH 9.2). The newly exchanged buffer with pH around 9-10 would make the chemistry coupling of SPDP more effective. 30 µl SPDP (30 mM) was needed per 2 mg protein and the particles were left to react for 30 minutes. The newly introduced 2-pyridyldisulfide was then cleaved with DTT to provide the thiolated albumin. For 1 µl SPDP 100 µl DTT was added and the reaction took place for 5-20 minutes. This mixture was then centrifuged for 30 seconds, at 8000 rpm, through a filter for removal of by-products (only a small part of the solution was removed). The supernatant was further dispersed in buffer to get the desired concentration and was then immersed on a gold surface immediately. 120 nm silica nanoparticles were already pre-adsorbed with streptavidin further dispersed in buffer.

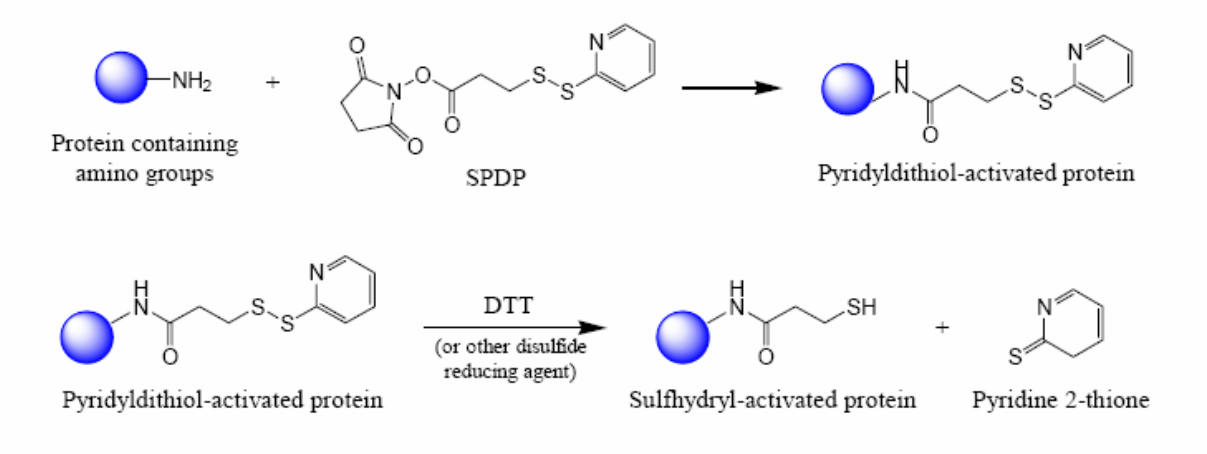


FIG 5. Reaction of SPDP reagents with primary amine.

Biotinylation of albumin

Biotin, a vitamin (244 Da), binds with extremely high affinity to both avidin and streptavidin (10^{15} M^{-1}). Streptavidin is a much bigger tetrameric protein and contains four subunits that can each bind one molecule of biotin. This strong noncovalent interaction in the avidin-biotin complex is widely used as a versatile mediator particularly for binding antibody–antigen interactions e.g. separation of bio molecules in affinity chromatography, localisation of bio molecules in affinity cytochemistry, diagnostics for immunoassays etc.[16]. Biotins have a simple chemistry coupling on primary amines existing in most proteins. The biological activity of the protein is usually not affected much because of the relatively small size of biotin. Avidin binds to the other end and acts like a secondary reagent conjugating with biotin-protein.

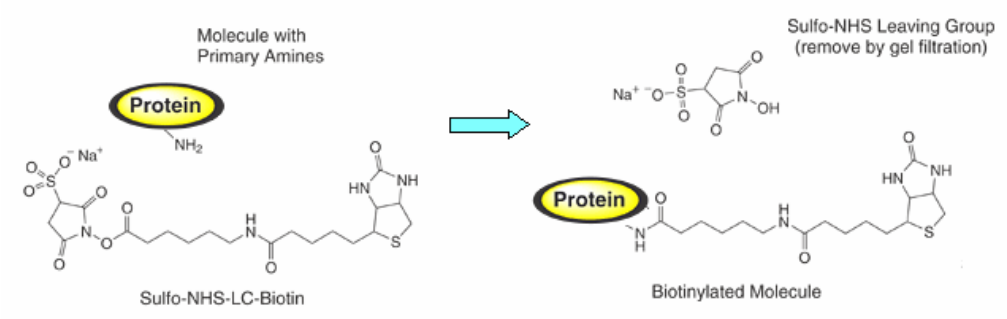


FIG 6. Reaction of Sulfo-NHS-LC-Biotin with primary amine.

3.2.4. Surface modifications

Chemical coupling of ligands onto a surface are achieved by three basic steps; surface activation, functional modification and ligand immobilisation. Physical adsorption, oxidized dextran spacer methods, thioamine thiolation methods, thiol-gold chemisorption of self-assembled monolayer (SAM) etc. could be applied depending on the type of ligands being immobilised. Contaminations of surfaces are inevitable since the air is full of bio molecules and dust. For a successful chemical immobilisation the surface has to be cleaned by rinsing it thoroughly with EtOH and then blow drying it with pure nitrogen gas just before modification.

Physical immobilisation of proteins

Most proteins have a high affinity to surfaces of hydrophobic material and to gold, which makes physical immobilisation very easy to perform. The sensor crystal is simply immersed in a protein solution (1-2 mg/ml) for 1 h and then rinsed with PBS for removal of loosely bound proteins. If the solution is a mixture of different proteins they will compete with each other for available binding sites. The ones with the highest arrival rate will cover the surface first but they are gradually displaced by proteins with higher affinity to the surface[17].

Self assembled monolayers of Aminothiophenol and Cysteamine

Gold coated crystals were immersed in 20 mM ATPh[18] dissolved in DMSO or alternatively 10 mM Cysteamine dissolved in mQ water for 5 h. Due to the thiol-gold chemisorption the amino groups in ATPh or Cysteamine were left sticking up on the surface forming a neutral thin film. After adsorption, loosely bound ATPh or Cysteamine were removed by rinsing with DMSO or mQ water respectively. The amine groups were further activated by immersing the crystal in 2.5 % glutaraldehyde dissolved in 50 mM phosphate buffer for 1 h. Then the crystal was rinsed again with PBS before the immobilisation of albumin (1-2mg/ml) for 1 h. The ε-amino groups on the albumin were covalently attached to glutaraldehyde. Finally, one last rinse was done with PBS for removal of all loosely bound albumins. The functionalisation of the crystals with ATPh or Cysteamine kept the functionality of the albumin intact.

3.2.5. The measurement procedure

The sensor crystal was installed into the QCM chamber and the resonance frequency and dissipation were checked in air and in water. The measurement is very sensitive to air bubbles inside the reaction chamber and they were removed by injecting water back over the sensor using a syringe. The water was then exchanged for buffer and left to stabilize for typically 1 h. Experiments were performed with either single static injection or continuous flow using a pump. The QCM apparatus is extremely temperature sensitive. The samples were passed into a temperature stabilising loop for 1 min to stabilise the temperature before they are injected into the sensor cell. All experiments were carried out at 22 ºC and each sample (3 ml) was measured for 15-60 minutes or until equilibrium was reached.

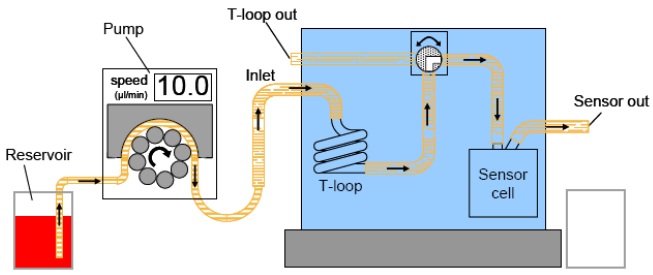


FIG 7. Schematic of flow injections approach using an external pump.

4. Results and Discussion

During the adsorption process, the general trends in the signals were a decrease in frequency and an increase in dissipation. In all charts, blue colour represents the 3rd overtone, red the 5th overtone, green the 7th overtone and black the 3rd dissipation in QCM measurements.

4.1. Influence of DMSO on the measured signal

The solvent is considered to be one of the crucial parts in all interaction studies because it keeps the solutes in good shape during the interaction process. DMSO is a very common solvent and it is used extensively in fine organic synthesis. The QCM-D measurement is highly sensitive to density differences even between different solvents. In a recent study it was confirmed that DMSO has a substantial competition between certain drugs for protein binding, weakening the binding of Propranolol and Cel7a to each other, by a factor of three[4]. Warfarin is not soluble in pure water so the PBS has to be modified with DMSO, regardless of it affecting the binding in a negative way or not. It was therefore important to quantify the changes in QCM signal due solely to the DMSO before the effects of adding drugs to the proteins could be measured.

Figure 8 shows the change in signal due to introducing PBS containing DMSO in different amounts. Although, there was no attachment onto the sensor surface a significant signal was detected. The signal contribution is around 2 Hz per increasing 0.5% DMSO which is a relatively big frequency shift in model systems like ours. The change in signal was directly proportional to the amount of DMSO, and as the Δf vs. ΔD plot in figure 8B shows there is a clear linear relationship between changes in viscoelasticity with the amount of DMSO in PBS. This confirmed that the difference was due to different viscosities of the buffers. Importantly the linear relationship meant that the change in signal due to DMSO could be quantified and substracted from the frequency changes due to protein and drug adsorption. The analytes being used which have a combination of low molecule weight (approx. 300 Da) and low concentration level (below 1 mM), should give signal responses expected to be around 0.5 Hz, which is several times lower compared to the solvent changes of 0.5 % DMSO. In single molecule interaction studies only one parameter should be varied at a time and the rest should be kept constant, otherwise it is impossible to distinguish the signal which is coming from the interaction process or from the background. QCM-D is a blind technique in the sense that it detects total mass change but can not distinguish the solvent from the adsorbates.

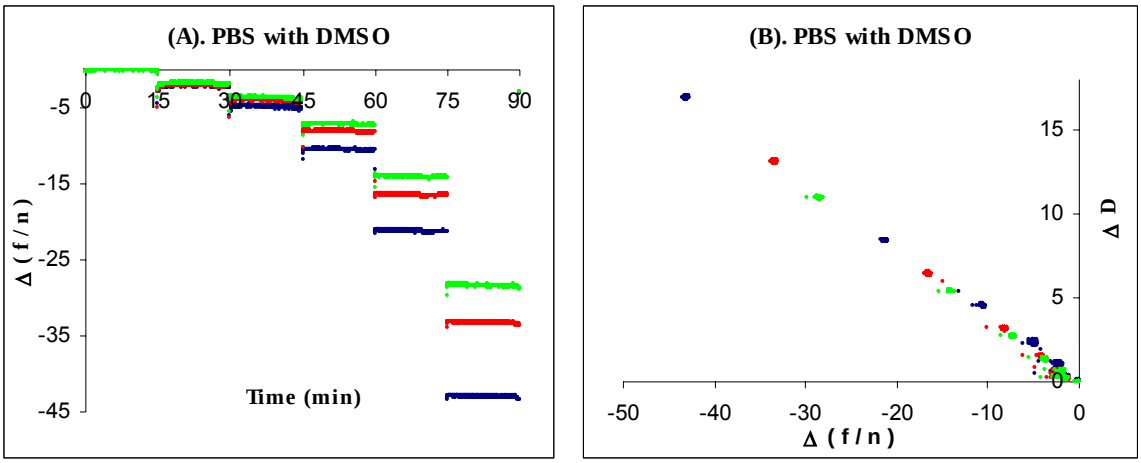


FIG 8. Steady states QCM measurement on surface of gold. (A) The baseline was of PBS and after every 15 minutes new injections of PBS containing 0.5 %, 1.0 %, 2.0 %, 4.0% and 8.0 % DMSO were made. (B) Change of resonance frequency (Δ f / n) plotted against change of dissipation (Δ D * 10 $^{-6}$).

Adsorption of Warfarin onto HSA

Figures 9 and 10 show the measured changes in frequency and dissipation during Warfarin adsorption to HSA modified surfaces. The DMSO concentration was 1% and 5% respectively. Note that in Figure 9 the HSA was physisorbed directly onto gold in situ whereas in Figure 10 the HSA was attached to the gold via covalent bonding to an adsorbed thiolated monolayer. In figure 9, additions of Warfarin on a surface of albumin resulted in small negative frequency shifts and small positive changes of dissipation, which implies low mass adsorption and a change of viscoelasticity. There is a systematic decrease in frequency with increasing Warfarin concentration showing that the adsorbed amounts are well below saturation coverage. The linear gradient of the ΔD/ΔF slope indicates that the Warfarin adsorbs to the HSA in the manner of increasing concentration. Experimental data shows that PBS modified with 5 % DMSO (figure 10) gave a higher signal response and a better signal separation than with PBS modified with 1 % DMSO (figure 9). The previous experiment has already confirmed that different viscosities of the buffer have a great influence on the resonance frequency change. However, the relative frequency shifts between two samples were expected to be the same because the only difference was the Warfarin concentration, the DMSO concentration was constant. One reasonable explanation for the difference in relative shifts is that in the case of 1 % DMSO the Warfarin did not dissolve completely in the buffer and neither did it maintain the correct conformation and thus affected the binding to albumin on the surface. In contrast, in the case with 5 % DMSO, large fractions of it could have attached to the Warfarin which had increased the total mass and therefore increased the signal response. In other words the signal was not representing the Warfarin only! The ΔD/ΔF slopes in both experiments were quite similar. Another important consideration is the adsorption of the protein to the crystal. In the case of figure 10, the albumin was covalently attached to the surface via a thiol spacer and is expected to be in a much more natural state compared to the protein that was physisorbed onto gold.

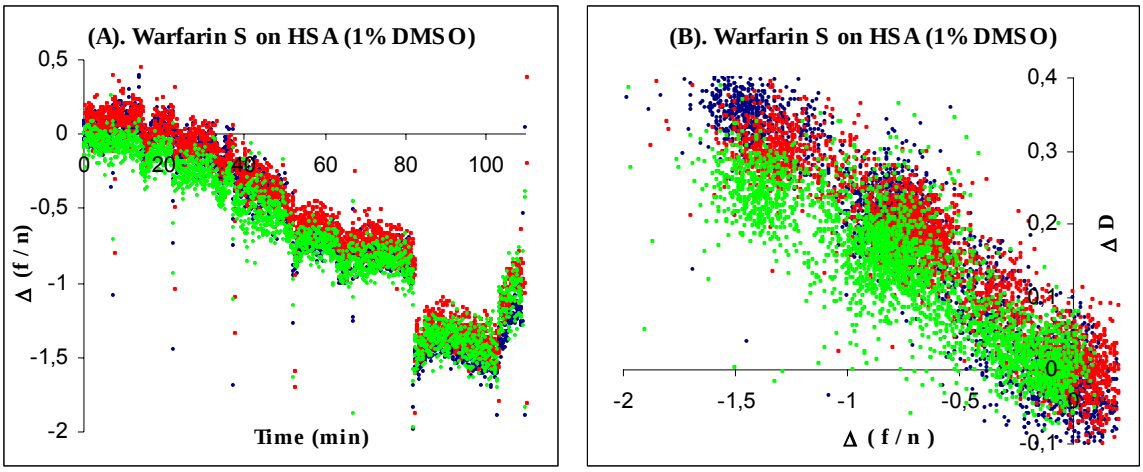


FIG 9. Static adsorption QCM measurement on surface of albumin physical adsorbed on gold. (A) Baseline was of PBS (1% DMSO), followed by new injections of Warfarin solutions; 12.5 µM, 25.0 µM, 50.0 µM, 100 µM, 200 µM and 1000 µM. (B) Change of resonance frequency (Δ f / n) plotted against change of dissipation (Δ D * 10 $^{-6}$).

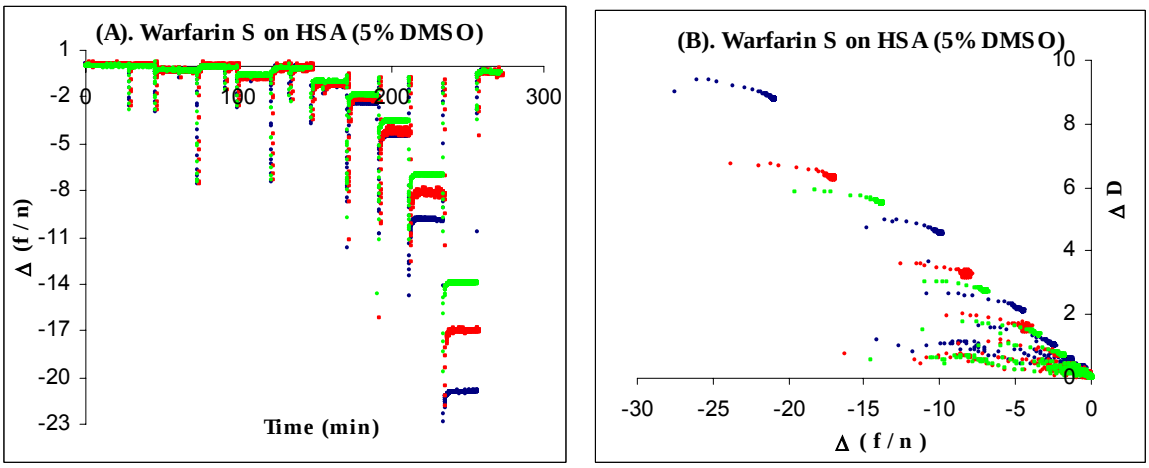


FIG 10. Static adsorption QCM measurement on surface of albumin attached by SAM (Au/ATPh/GA/HSA). (A) Baseline was of buffer (PBS, 5% DMSO), followed by new injections of solutions of; buffer, 15 µM, buffer, 30 µM, buffer, 62.5 µM, 125 µM, 250 µM, 500 µM and 1000 µM Warfarin. (B) Change of resonance frequency (Δ f / n) plotted against change of dissipation (Δ D * 10 $^{-6}$).

Adsorption of Propranolol to AGP

Figures 11 and 12 present measurements of propranolol adsorption onto AGP. The AGP was adsorbed directly onto polystyrene surface in situ prior to drug addition. Figure 11 shows that there is a direct and systematic decrease in frequency with increasing concentration of propranolol. The frequency shifts are similar for the R and S form although the frequency decrease is larger for the S form which is the enantiomer with higher affinity for AGP. The Δf/ΔD plots shown in Figure 12 indicate that the S form of the propranolol binds in with a less diffuse conformation to AGP which is consistent with tighter binding and higher affinity. So there is good evidence that the QCM can discriminate the enantiomeric affinity. However, even the lowest concentration detectable is 0.125 mM which is well above the levels detectable with for example SPR and also above levels useful for industry. Clearly the sensitivity of this technique needs to be increased if it is to be used for enantiomeric selectivity.

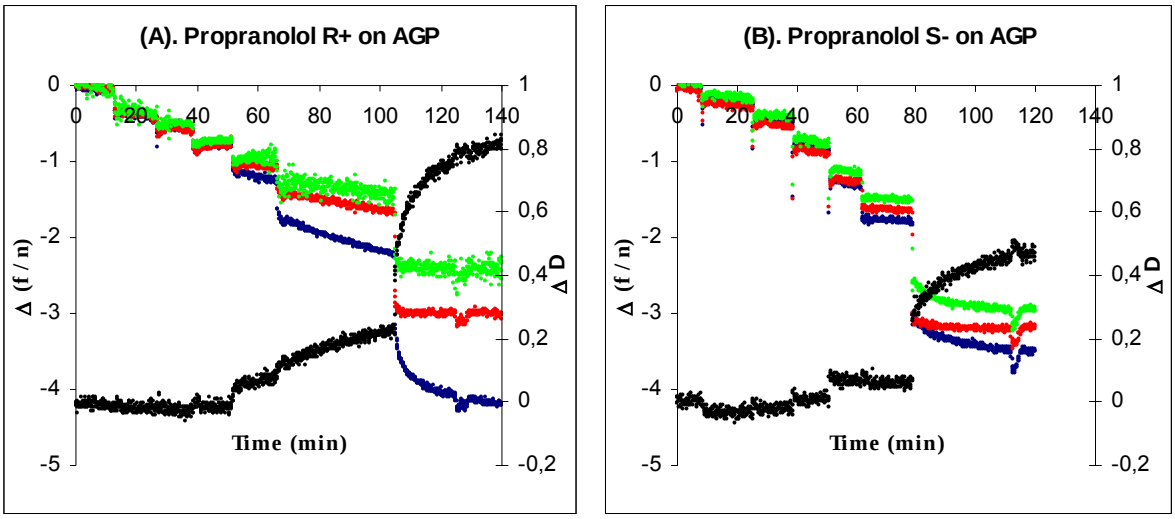


FIG 11. Static adsorption QCM measurement on surface of AGP physical adsorbed on PS. (A) Propranolol R+ and (B) Propranolol S- baseline of PBS, followed by new injections of 0.125 mM, 0.250 mM, 0.500 mM, 1.0 mM, 2.0 mM and 3.0 mM.

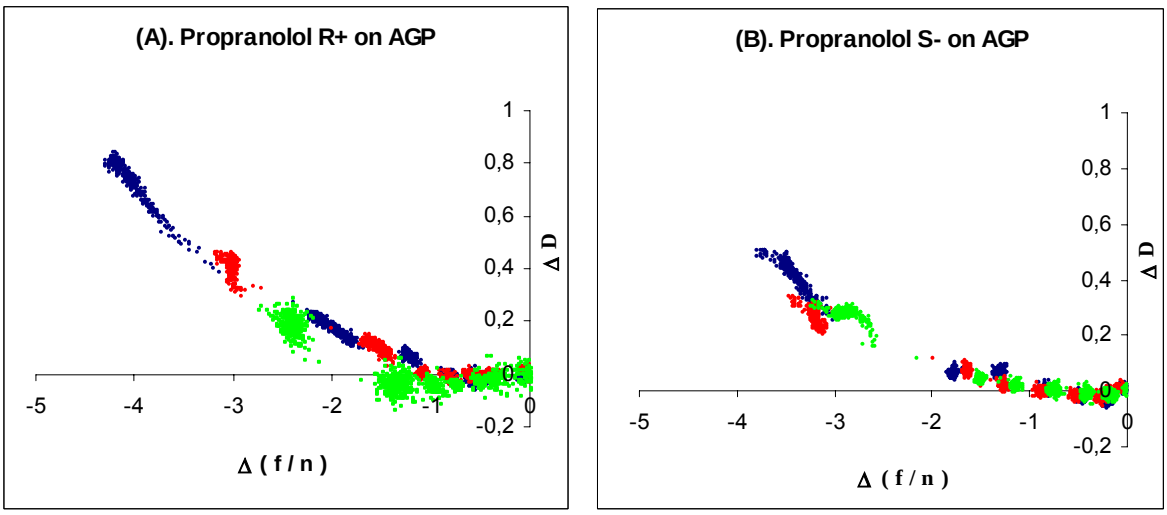


FIG 12. Change of resonance frequency (Δ f / n) plotted against change of dissipation (Δ D * 10 $^{-6}$). (A) Propranolol R+ and (B) Propranolol S-.

4.2. Sensitivity of the QCM: detection limit

Warfarin (308.3 Da) is about 200 times smaller in mass compared to albumin (66 kDa). Adsorption of albumin gave a frequency shift of 22 and 25 Hz depending on whether it was physisorbed or covalently attached respectively. Consider one to one binding of Warfarin to albumin, then the signal response should be about 0.1 Hz which is on the edge of the detection limit of the QCM-D technique. However, the lowest concentration detected was 10 μM Warfarin S on albumin and a noisy signal response of 0.1 Hz (figure 9) could have been produced by any disturbance e.g. small DMSO deviations. A believable signal should be around 0.5 Hz representing a Warfarin concentration of 100 μM, but the warfarin selective sites on albumin were already saturated by then and no differences in affinity could be found between Warfarin R+ and S- (data not shown). QCM-D provided a very good signal above 100 μM in all experiments but to obtain binding constants of enantiomers, the measurements have to be performed below 10 μM.

Protein adsorption strategies

Experiments (data not shown) indicated that longer time was required for equilibrium the more DMSO was used in the buffer. In theory, the ATPh should have formed a compact thin monolayer but experiments indicated that there always was a small mass change over a long time period if the buffer contained DMSO. Because ATPh "likes" DMSO (hydrophobic interactions) a "two equilibrium system" was suspected; Warfarin to albumin and DMSO to monolayer. When working on a model, which is a much more simplified version of reality, the proteins immobilised on surface may not behave exactly as they would in free solution and all of them are not properly functional. Physical adsorption seems to be the most convenient method, easy and time saving. Streptavidin adsorbed very fast onto a surface of gold and formed a very compact film (almost no change of ΔD, data not shown). However, some proteins may unfold on the surface and result in loss of biological properties. In two separate experiments (data not shown) AGPs and Thrombins were denaturized when attached on a surface of gold. AGP absorbed on a surface of PS on the other hand was successful when letting PS stabilize in buffer and leaving it to swell overnight before immobilisation. An inert monolayer is achieved by using spacers like ATPh and Cysteamine. Also, bare surface areas should be covered by blocking agents, e.g. Ceasine, in order to avoid unspecific binding of the drugs. All immobilisations (table 1) on a flat surface gave a maximum frequency shift of 33 Hz which was not enough to provide a significant signal response for low concentration measurements. To increase the sensitivity, extra weights could be added to the analytes or one could measure the capture of high mass target proteins onto a surface imprinted with the analytes. In this work, we simply increased the access of proteins by increasing the sensor surface using nanoparticles. All data points will be transformed to a higher concentration range for affinity comparisons.

4.3. Nanoparticle modification

An aim of this project is to test whether the QCM sensitivity can be increased by modifying the surface with nanoparticles. The rationales behind using nanoparticles are firstly dramatically increasing the surface of the measuring area and secondly to produce a surface with multiple points of high curvature which is favourable for protein adsorption compared to a flat surface.

Nanoparticle adsorption onto gold

Figure 13 shows the QCM measurement of silica nanoparticles (120nm, 0.05mg/ml) adsorption onto a gold surface under continuous flow of 4 ml/min for 1 h. There is a rapid initial adsorption rate which quickly plateaus. The large change in frequency indicates a large adsorbed amount of particles and the AFM image in 13B, taken after the adsorption study, clearly shows significant particle coverage. The surface coverage is estimated to be close to 50% which is consistent with the 54.7% predicted as the jamming limit for particles adsorbed under Random sequential adsorption (RSA)[19]. Note that there is significant aggregation which may have happened during drying after taking the crystal out of the QCM or the PBS buffer.

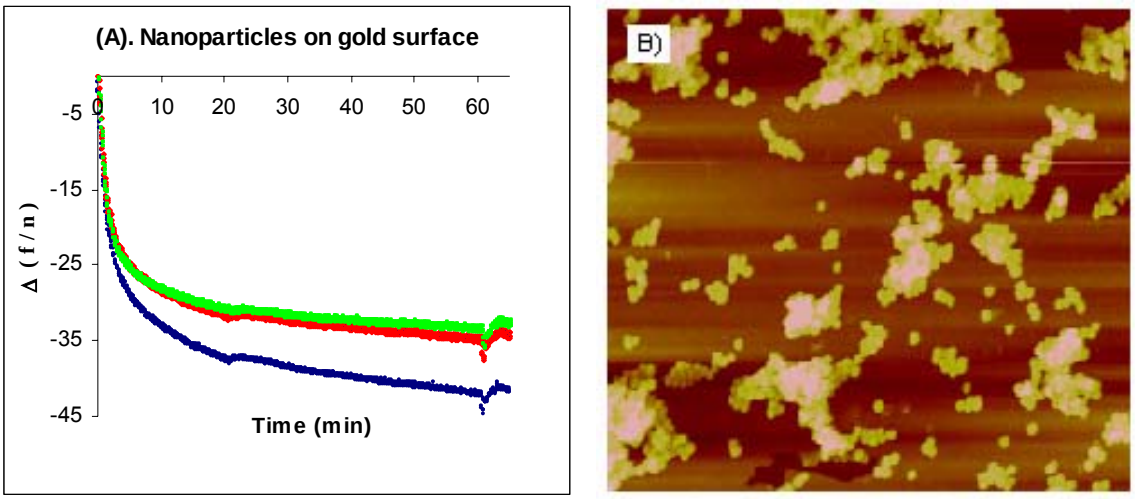


FIG 13. (A) Flow injection of 120 nm Silica nanoparticles on gold surface. The particles were pretreated with streptavidin dispersed in PBS. (B) AFM images of the surface.

Nanoparticle adsorption onto biotintylated surface

An alternative strategy to nanoparticle physisorption onto gold is using the strong biotin streptavidin binding. At this end, the QCM crystal was functionalised by adsorbing biotin prior to introducing the silica particles precoated with streptavidin. Figure 14 A shows the QCM measurement of particle adsorption onto a biotin coated surface. The same particle concentration and flow rate were used as in figure 13. In contrast to adsorption onto gold the nanoparticle adsorption rate was much slower and there was a much lower adsorbed amount. The signal to noise level also increased. All these trends reflect that the particle adsorption is now dominated by the protein binding interaction rather than the non specific physisorption onto gold. The AFM image 14 B reveals much lower particle coverage but the particles are generally less aggregated.

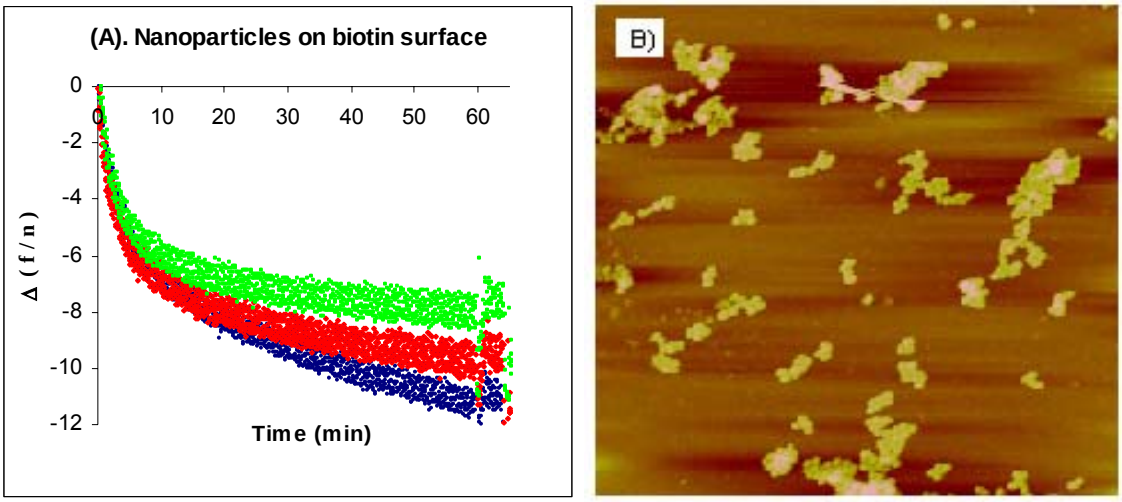


FIG 14. (A) Flow injection of 120 nm Silica nanoparticles on biotin surface. The particles were pretreated with streptavidin dispersed in PBS. (B) AFM images of the surface.

Biotintylated BSA nanoparticle multilayers

Following on from the biotin-streptavidin strategy, a new method was devised incorporating biotintylated BSA. BSA was shown to have high affinity for gold and is used routinely as a blocking agent for non specific binding in protein studies. Furthermore absorbing biotin labeled BSA will render less biotin adsorbed to the surface and leave more available for binding with streptavidin. Figure 15 shows the sequential adsorption profile of biotin labeled BSA (0.6 mg/ml) followed by streptavidin coated silica nanoparticles (0.05 mg/ml). There is rapid adsorption of the BSA onto gold which quickly reaches plateau coverage. The particle adsorption is much slower than the protein mainly due to diffusion rate limited by the increased size of the particles. It is most interesting to see that a second addition of BSA rapidly adsorbs onto the particle coated layer. This is to be expected since the particles contain multiple streptavidin molecules and so can bind more than one biotin per particle. A second addition of particles resulted in a large but slow uptake of particles showing that it is possible to build up multilayers of protein and particles in a sandwich layer.

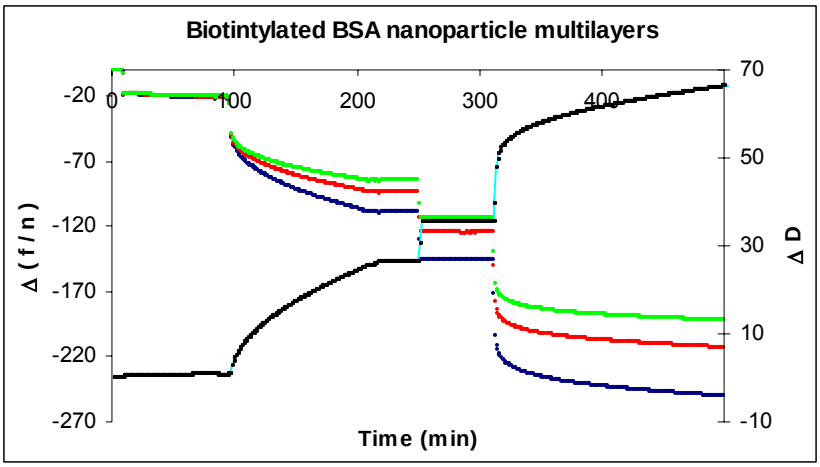


FIG 15. Static adsorption QCM measurement on surface of gold. Increasing of surface area using nanoparticles. Injection of Biotin-labelled Bovine albumins at 10 min, nanoparticles pretreated with streptavidin at 100 min, more albumins were added at 250 min and nanoparticles at 310 min.

Surface modification in situ has the advantage that the adsorption process can be monitored in real time and the reaction can be terminated when the satisfied condition is reached. The drawback was that a large amount of material was needed. For in vitro surface modification, only a drop of sample is needed, but in the case of using nanoparticles the properties of the sensor crystal were changing so much that it became a problem to find its resonance frequency. The removal of the nanoparticles from the sensor surface was found to be difficult, except for the oscillating part sandwiched by the two electrodes. This observation suggested that an oscillating surface weakened the binding of material. In other words, for an effective attachment of material onto a sensor surface, the QCM instrument should be switched off.

Methods	**Active surface**	**Proteins ΔF(Hz)**	**Detection limit (uM)**	**Analyte**	**ΔF (Hz)**
PA	PS/AGP(1mg/ml)	33	125	Pro R +	0.3
	Au/HSA (1mg/ml)	22	50	War S	0.2
SAM	Au/ATPh/GA/HSA (2mg/ml)	25	100	War R	0.1
	Au/Cy/GA/HSA(0.4mg/ml)	*	100	Pro R+	0.1
	Avidin/Biotin-HSA(1mg/ml)	6	-		-
Nano	SiNano/Biotin-AGP(2mg/ml)	12	12.5	Pro R+	0.2
	SiNano/Biotin-BSA(0.6mg/ml)	>> 40	-		-

Table 1. Comparisions of immobilisation methods on flat and nanoparticle surfaces on a QCM crystal. PA = physical absorption; SAM = self-assembled monolayer; SiNano = silica nanoparticles pre-treated with streptavidin; PSNano = polystyrene nanoparticles pre-treated with HSA. * in vitro immob.

Propranolol adsorption onto nanoparticle functionalised AGP

Following the successful application of nanoparticle functionalisation using biotin streptavidin binding, the adsorption of Propranolol to AGP was investigated with nanoparticle modified surfaces. AGP was biotintylated according to the method described in the experimental section. This protein was then adsorbed onto a PS surface which had been pretreated by adsorption of the streptavidin coated silica nanoparticles. Propranolol was introduced to the protein treated crystal. Figure 16 shows the QCM measured frequency change upon introducing propranolol in the R and S form. These results should be compared to the data shown earlier in Figure 11. It is clear that in Figure 16 B, a significant detection due to drug binding can be detected at 25 μM which is a five fold increase in sensitivity compared to AGP adsorbed directly to the flat polystyrene surface. It is evident that the nanoparticle functionalisation has greatly improved the sensitivity of this technique by providing a greatly increased surface area and a more favourable adsorption conformation of the AGP. Note that there is also much better resolution of the data for the S form of propranolol which indicates that this measurement is capable of resolving enantiomeric selectivity.

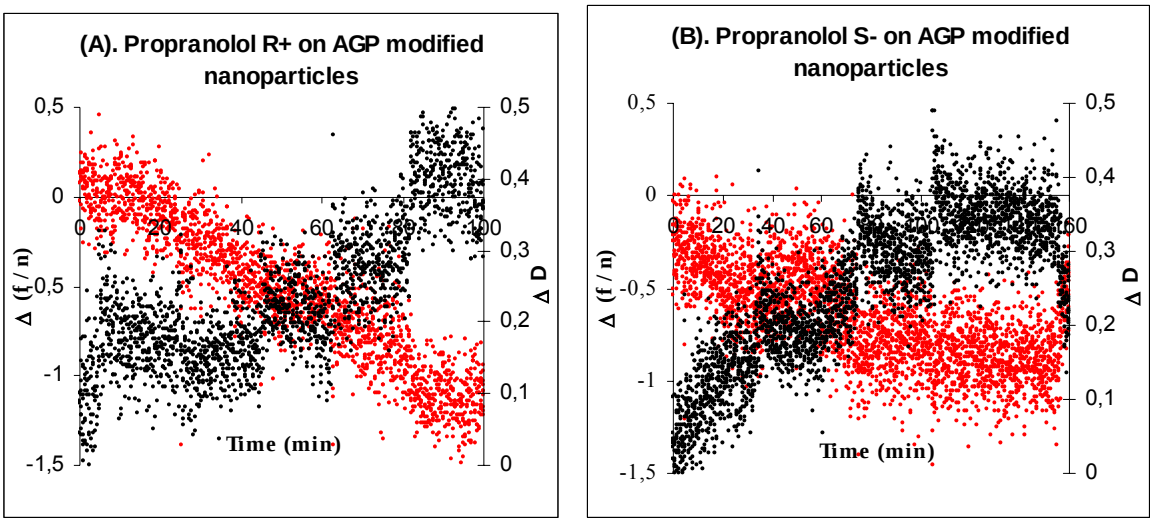


FIG 16. Static QCM measurement on 120 nm AGP modified silica nanoparticles attached on surface of PS. The particles were pre-treated with streptavidin in which biotinylated AGP were attached to. The baselines were of PBS followed by new injections of (A) 12.5 μM, 25.0 μM, 50.0 μM, 100 μM and 200 μM Propranolol R+ and (B) 25.0 μM, 50.0 μM, 100 μM and 200 μM Propranolol S-.

Further strategies of nanoparticle functionalisation

Figures 17, 18 and 19 show AFM images of QCM crystals functionalised with nanoparticles following different adsorption protocols. Figure 17 shows the particle coverage achieved when a drop containing particles dispersed in PBS was left to dry on a gold crystal. Clearly, large particle coverage is attained and as the image 17 A shows a multilayer of particles is formed. Rinsing with pure water removed most of the multilayer particles.

In figure 18 and 19, the particles were covalently bound to a surface of gold via SPDP which acted as a linker molecule. The degrees of coverage could be adapted by varying the amount of free thiol and the incubation time. Coverage of 80% was reached when SPDP was cleaved by DTT for 7 minutes and glycine buffer prevented the particles from clustering together. Caution is needed when using this immobilisation method since DTT cleaves all disulfide bridges including the ones in proteins.

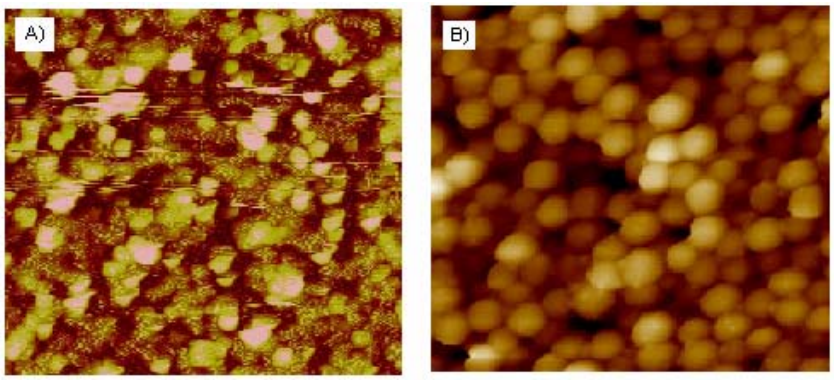


FIG 17. AFM images. 120 nm nanoparticles of silica as above, immobilised in vitro on gold. The particles were dissolved in PBS (0.1% w/w) and incubated (A) for 3 h followed by rinsing with mQ water and (B) without rinsing and let dry in air. Triple (A) and multilayer (B) layer of particles was formed.

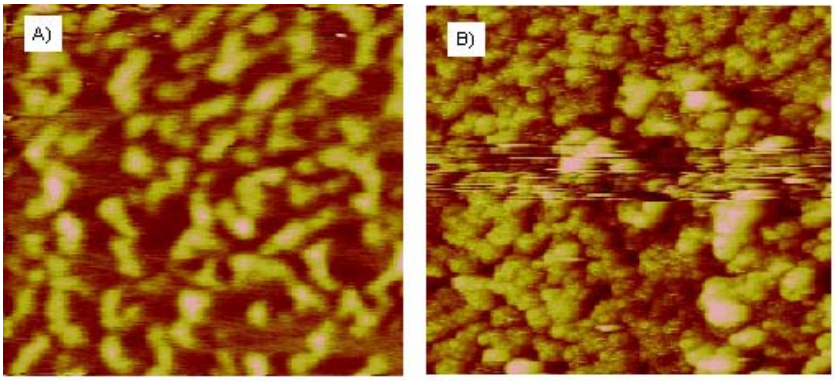


FIG 18. AFM images. 160 nm nanoparticles of PS, immobilised in vitro on gold. The particles were pre-treated with albumin and further modified with SPDP. After cleavage by DTT, the particles were incubated on gold (A) for 1 h and (B) 16 h, followed by rinsing with mQ water. The running buffer was glycine.

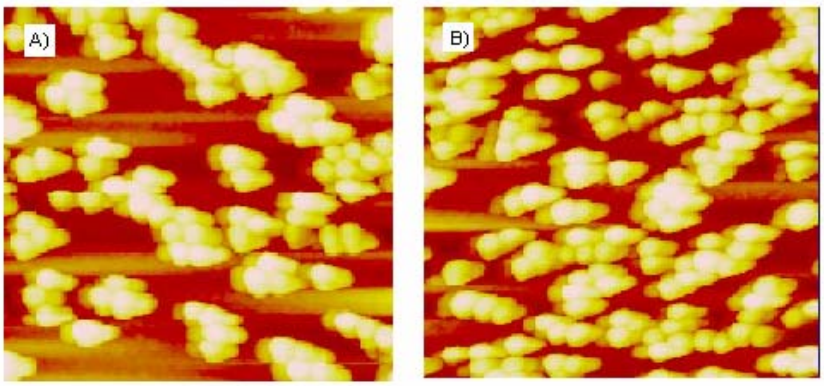


FIG 19. AFM images; height, 5 μm^2. PS nanoparticles modified with albumin and SPDP on gold. SPDS were cleaved by DTT (A) for 5 minutes and (B) for 7 minutes and immersed on gold for 1 h.

5. Conclusions

5.1. Conclusions and future work

QCM-D has been used to measure the binding of chiral drugs to model proteins.

At fairly high concentrations, QCM-D gave strong and clear quantitative measurement of drug-protein adsorption. However, the limit of sensitivity was higher than the need to detect enantiomeric selectivity.

Successful strategies were employed to improve the sensitivity of QCM-D technique by functionalising the QCM crystal with nanoparticles. This had the dual benefit of significantly increasing the surface area of the crystal and also providing a surface more amenable to protein adsorption. There is evidence that the increased resolution was able to resolve differences in enantiomeric affinity of the S form of propranolol compared to the R form binding to AGP.

Further improvements are using streptavidin biotin complex to immobilise protein in order to preserve the activity. At the final stage of this project, a flat surface was successfully being modified with PS nanoparticles via SPDP and reached monolayer coverage of 80 %. The sensitivity is estimated to be at least ten fold.

To obtain the binding constants of both enantiomeric forms of Warfarin and Propranolol to HSA and AGP respectively, the measurement should be performed below 10 μM. For maximising active surface area and for reducing background noise, smaller nanoparticles stabilized by cross-linking should be used and bare surface areas should be covered by blocking agents.

DMSO is often used in organic synthesis and is relevant for drug-protein binding studies. In this study DMSO was needed to dissolve the Warfarin but its presence was also found to influence the interaction with albumin. The QCM data suggested that DMSO incorporated within and changed the active surface, and thereby increased the background signal over and above the change due to drug binding.

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