Chapter 8

Measuring the Kinetics of Amyloid Fibril Elongation Using Quartz Crystal Microbalances

Alexander K. Buell, Christopher M. Dobson, and Mark E. Welland

Abstract

Kinetic measurements of amyloid growth provide insight into the free energy landscape of this supramolecular process and are crucial in the search for potent inhibitors of the main disorders with which it is associated, including Alzheimer's and Parkinson's diseases and Type II diabetes. In recent years, a new class of surface-bound biosensor assays, e.g., those based on surface plasmon resonance (SPR) and the quartz crystal microbalance (QCM) have been established as extremely valuable tools for kinetic measurements of amyloid formation. Here we describe detailed protocols of how QCM techniques can be used to monitor the elongation of amyloid fibrils in real time and to study the influence of external factors on the kinetics of amyloid growth with unprecedented accuracy.

Key words: Amyloid disorders, Alzheimer's disease, Parkinson's disease, Quartz crystal microbalance, Biosensor, Kinetics, AFM, Surface attachment

1. Introduction

The study of the kinetics of amyloid formation and growth has attracted much attention in recent years, as a detailed understanding of the effect of external factors, such as temperature, ionic strength, or small molecules on the growth rates of amyloid fibrils provides fundamental insight into the free energy surfaces governing this process (1). In addition, in the light of the medical relevance of the amyloid form of proteins, the quest for potent inhibitors, the efficiency of which is primarily evaluated through their influence on the kinetics of amyloid formation, is a highly active field of research (see, e.g., ref. 2). Amyloid formation and growth kinetics from soluble precursor proteins have traditionally been studied in solution using either amyloidophilic dyes, such as Thioflavin T or spectroscopic techniques, such as FTIR or CD

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spectroscopy (3). However, in recent years, a new set of techniques has been developed by adapting pre-existing biosensing approaches for the measurement of amyloid growth, especially those based on surface plasmon resonance (SPR) (4, 5) and the quartz crystal microbalance (QCM) (1, 6, 7). In this chapter, we focus on the latter and describe how to apply this technique to obtain precise and reproducible kinetic data.

The principle of a QCM is that a piezoelectric quartz disc, coated with gold to form electrodes, is stimulated to vibrate at its resonant frequency via the application of a rapidly oscillating AC field, using the inverse piezoelectric effect. The resonant frequency is determined by, amongst other things, the total mass of the quartz crystal. When an analyte molecule attaches to the surface of the crystal, its mass increases and its resonant frequency decreases accordingly, as described by the Sauerbrey (8) equation:

$$\Delta f = -a\Delta m,$$

where Δf and Δm are the changes in resonant frequency and mass, respectively, and a is a proportionality factor that can be calculated from first principles for simple situations such as a thin rigid attached layer in contact with air or a vacuum (8). QCM can, however, also be applied in a liquid environment, and here an additional shift in resonant frequency arises from the coupling of the sensor surface to the liquid (9); in the case of a hydrophilic surface with sticky boundary conditions, some water is moved along with the oscillating crystal and increases the observed drop in frequency compared to the situation where the crystal is in contact with a vacuum. In addition, the friction that the crystal experiences in solution leads to a rapid decay of the vibrational motion. In the case where the material that is attached to the quartz crystal surface has viscoelastic properties, it is also possible to predict the induced shift in resonant frequency, if the viscosity and elasticity of the layer are known and if the layer is smooth and homogeneous (10, 11). In cases where the added material forms a rough layer, the induced frequency shift often has to be empirically calibrated, as we show, for example, in Subheading 3.6. Figure 1 illustrates the general principles of a QCM operating in liquid, and the effect of the attachment of additional material that increases the total mass of the crystal and the coupling to the liquid, leading to both a decrease in resonant frequency and a faster decay of the excited vibration.

Amyloid formation can be decomposed into at least three different sub-processes, namely the formation of seeds (nucleation), the growth of those seeds to form fibrils (elongation), and finally secondary nucleation processes (such as fibril fragmentation) that increase the number of fibrils and that depend on the number of existing fibrils (12). Each of these steps has a different mechanism and, therefore, represents the dynamics of the system in a different region of the global free energy surface. Traditional solution based

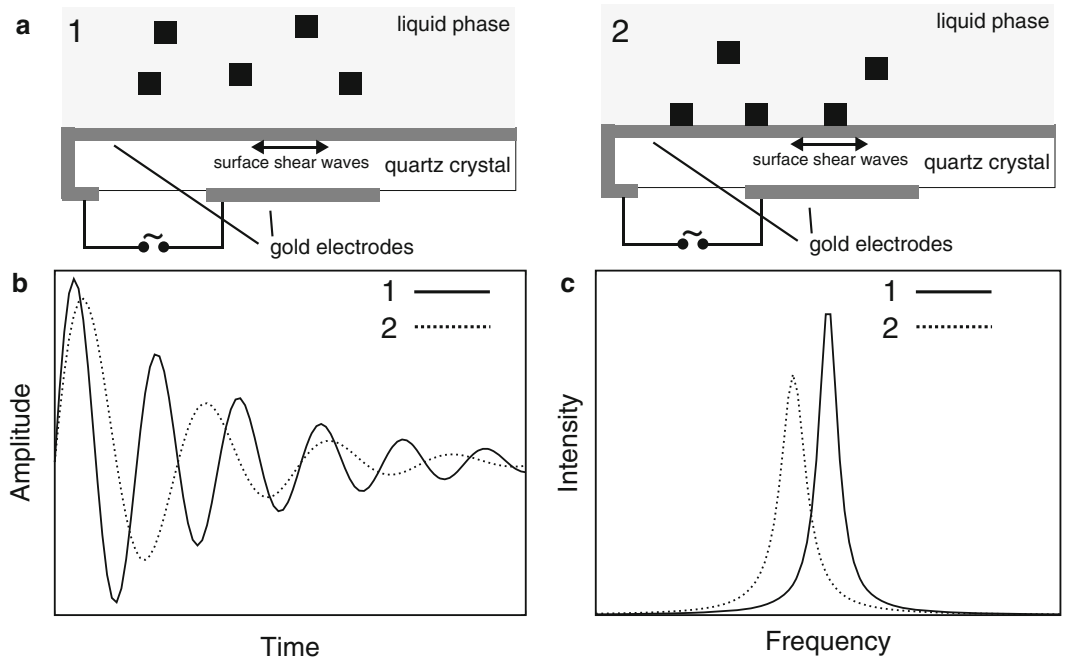

Fig. 1. Principle of a QCM operating in liquid. (**a**) A rapidly oscillating AC voltage is applied across a piezoelectric quartz disc and excites shear waves in the quartz crystal. The attachment of molecules from the solution phase is detected as an increase in mass. (**b**) The vibration of a quartz crystal immersed in liquid is characterized by a frequency and an exponential decay constant. The decay is due to the strong coupling between the liquid and the sensor surface. Additional mass attachment will lead to a decrease in resonant frequency and, if the attachment increases the coupling of the surface to the liquid, will lead to a more rapid exponential decay. (**c**) Schematic frequency spectra of the time domain record of (**b**). The decrease in resonant frequency and the increase in the full width at half maximum are recorded and represent the data that can be analysed to characterize the amount and structure of the attached material.

assays of amyloid kinetics are sensitive to all of the above-mentioned processes, especially in unseeded experiments. A unique strength of the QCM technique is that it is able to isolate the elongation step and allows its separate study. We will limit ourselves in this chapter to this particular application of QCM; it has been shown, however, that QCM can also be used to study the entire process of amyloid formation from the soluble precursor to the mature amyloid fibril (6, 7).

The study of the single step of fibril elongation is achieved via covalent, irreversible attachment of pre-grown amyloid seed fibrils to the surface of the QCM sensor, followed by passivation of the remaining exposed surface of the sensor (13). The growth of the fibrils, when brought into contact with a solution containing soluble amyloidogenic precursor molecules is then monitored via the change in resonant frequency of the QCM (1). This change in frequency upon elongation of the surface-bound seed fibrils has several origins. First, the increase in surface-bound mass (protein molecules and their associated water molecules). Second a change

in the coupling to the liquid; the elongation of the seeds increases the roughness of the surface and results in water being trapped between the fibrils, which leads to additional frequency shifts and therefore an overestimation of the attached mass (14). Third, the viscoelastic properties of the surface-bound fibrils (their finite rigidity) lead to an underestimation of the attached mass (11), as they will not be able to follow the oscillation of the QCM crystal perfectly. We will discuss the consequences and net result of these effects in more detail in Subheading 3.6.

The unique strength of these surface-bound kinetic techniques, of which QCM is a prominent representative, is the fact that the growth of a constant ensemble of seed fibrils can be probed repeatedly. Therefore, the growth rates upon incubation of the same seeds with different monomer solutions or under different conditions can be directly compared. Furthermore, the QCM sensor can be imaged with an atomic force microscope before and after the experiments. This procedure allows an estimation to be made of the number of seed fibrils that have contributed to the growth signal, as well as acting as a control for the absence of secondary pathways such as amorphous aggregation on the surface.

2. Materials

2.1. QCM Instrumentation: Basic Equipment and Accessories

We carried out our experiments with the QCM models D-300 and E4 from Q-Sense (Västra Frölunda, Sweden). These instruments allow the simultaneous monitoring of the resonant frequency and the dissipation of the vibrating crystal (QCM-D technology). The dissipation measurements are, however, not crucial for any of the applications presented in this chapter; therefore, any QCM with nanogram sensitivity is suitable. When we quote numerical values of frequency shifts in any of our examples, they are from experiments with the E4 microbalance and may vary for other systems.

The introduction of the buffer and protein solutions into the liquid cell in our experiments was accomplished either via a simple gravity flow system (D300) or via a peristaltic pump (E4). The QCM sensors we use are almost always gold-coated QSX 301 crystals (Q-Sense, Västra Frölunda, Sweden), with a fundamental shear mode resonant frequency of 5 MHz and a Sauerbrey mass sensitivity of 17.7 ng/Hz. The gold coating not only acts as an electrode but also allows irreversible covalent attachment via a gold sulphur bond to be achieved in a relatively straightforward manner. In addition, this type of sensor can be cleaned and reused multiple times.

2.2. Proteins

In our experience, the QCM is suitable for measuring amyloid elongation rates which span more than four orders of magnitude. This breadth of measurable elongation rates between the fastest

growing proteins we studied (Aβ 1–42 (15) and Ure 2p yeast prion) and the slowest (HEW lysozyme (16), bovine β-lactoglobulin) is possible because of the ability to vary the concentration of the protein solutions and of the extremely stable baseline of the QCM that can detect rates of frequency change as small as 3 Hz/h (for a 5 MHz crystal) or less, therefore enabling the most interesting amyloid systems to be studied with high accuracy. The more crucial factor in the general applicability of the QCM technique is the ability to produce in a reproducible manner well-defined seed fibril suspensions and their reliable attachment to a sensor surface (see Subheading 3 and (13)). In cases where the peptide or protein is readily available commercially (e.g., Aβ 1–42, hen lysozyme, bovine insulin), we used the lyophilized powder without further purification. However, in some cases (e.g., commercially available recombinantly produced human lysozyme), we found that dialysing the protein extensively prior to the kinetic experiments decreased the growth rate considerably (under otherwise identical conditions); this effect can be attributed to residual salts in the lyophilized commercial protein. Therefore, in all cases where the absolute aggregation rate needs to be accurately defined, dialysis or chromatographic separation of the precursor protein from any possible contaminant to achieve high levels of purity should be carried out. Recombinantly expressed protein should also be as highly purified as possible and salt free. In some cases, e.g., in the case of the commercially available Aβ (1–42) peptide (available as a trifluoroacetic acid (TFA) salt in most cases), specific procedures must be adopted (see Subheading 3) to ensure the absence of large oligomers and fibril seeds from the precursor solutions.

2.3. Chemicals

Apart from standard analytical solutions such as phosphate buffer and hydrochloric acid, which are used to provide conditions for reproducible amyloid formation rates other chemical treatment is needed in cases where the amyloid seed fibrils do not contain cysteine residues to allow covalent attachment to the gold surface. Indeed, most amyloidogenic peptides and proteins do not contain cysteine residues. For the well-established procedures for covalent attachment of fibrils to an activated self-assembled monolayer (SAM) (see, e.g., ref. 4), the required chemicals are mercaptoundecanoic acid (MUA), or a similar carboxy-terminated SAM forming molecule, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), and ethanolamine.

We recently reported a novel attachment strategy (13) that does not involve the attachment of the amyloid seeds to an activated SAM, but rather attaches a small sulphur-containing molecule to the seed fibrils, that in turn enables their covalent attachment to the gold. The chemical species required for this method are 2-imminothiolane ("Traut's reagent"), 2,2′-dithiobis(ethylamine) ("cystamine"), and methoxy-terminated PEG thiol (m-PEG thiol,

Polypure, Oslo, Norway). For Aβ (1–42) experiments, in particular, we recommend a chemical treatment of the commercially available peptide to disrupt any pre-existing aggregates. For this protocol (17), TFA of high purity and hexafluoroisopropanol (HFIP) are required. In addition, 7 M NaOH solution is used for the cleaning of the QCM sensors prior to reuse.

2.4. Further Requirements

Direct imaging of fibril functionalized surfaces is critical, especially in the initial stages of a study that involves the use of QCM methods to probe amyloid growth. AFM measurements should be used to confirm the successful production of seed fibrils as well as their attachment to the gold surface of the sensor. In this way, the starting point of the experiment can be easily verified, i.e., the number of seeds present on the sensor can be estimated. In addition, at the end of all experiments, the QCM sensor surface should be imaged again and the increase in length of the fibrils estimated, as well as ensuring the absence of amorphous aggregates or other off-pathway products.

Furthermore, a sensitive (to 0.1 mg) balance, a tabletop centrifuge, a heat plate with magnetic stirrer and a sonication probe (e.g., 500 W ultrasonic homogenizer, Cole Parmer, Hanwell, UK) are required. If reuse of the QCM sensors is desired, an ozone plasma cleaner (e.g., 172 nm Excimer Radiation System SUS-128, Ushio, Livingston, UK) is ideal; alternatively, the SAM can also be removed electrochemically. Here, only an electrical power supply that can deliver 2-V DC voltage is required. For the Aβ (1–42) preparation, a SpeedVac concentrator is also needed.

3. Methods

3.1. Seed Fibril Formation

In most cases where the kinetics of amyloid formation is the object of the study in question, conditions will be well established that enable the formation of appropriate seed fibrils. Therefore, we will not enter here into comprehensive details of the optimal conditions for fibril formation; instead we shall give examples of suitable conditions for three systems from our own work, involving bovine insulin, human α-synuclein, and human Aβ (1–42). The first example represents a globular protein that is widely used as a model system for studies of protein misfolding and aggregation. The second example is the natively unfolded 140-residue protein whose aggregation is associated with the formation of intracellular Lewy bodies in Parkinson's disease, and the third, the 42-residue fragment of the amyloid precursor protein whose aggregation results in extracellular amyloid plaques in Alzheimer's disease.

3.1.1. Insulin

Incubate a solution of 5 mg/ml (ca. 1 mM) of bovine insulin in HCl pH 2 (final pH not adjusted) at 60°C for 4 days, under con-

tinuous magnetic stirring. The solution will turn into a gel while the fibrils form (13).

3.1.2. α-Synuclein

Incubate a solution of 1.5 mg/ml (ca. 100 μM) human α-synuclein in PBS buffer at 37°C for 4 days under magnetic stirring (16). The stirring in this case is absolutely essential; no fibrils will form from highly purified protein solutions without stirring or shaking or other mechanical agitation, with stirring being the most efficient and preferable way.

3.1.3. Aβ (1–42)

Dissolve 0.5 mg commercially available Aβ (1–42) (TFA salt, e.g. from Bachem, Basel, Switzerland) without additional purification steps in 100 μl 10 mM NaOH solution. Divide into ten aliquots of 10 μl each, and store at −20°C. When a QCM sensor is to be prepared for a measurement, add 400 μl of 100 mM phosphate buffer pH 7.4 to each aliquot to be used. Mix well and incubate for 5 min. Then proceed without delay to "Sensor preparation". This protocol relies on the presence of oligomers and fibril seeds in the commercially available peptide and is, therefore, susceptible to a certain variability in terms of the mass and length distribution of fibrils obtained. Recently, a protocol has been established that enables the preparation of Aβ monomer and fibrils in a highly reliable and reproducible way (18) and which has potential to improve the control of the length distribution of seed fibrils attached to the QCM surface.

In most cases, the final fibril suspensions, or the resulting gels, can be stored at room temperature for months, especially the samples at acidic pH. In the case of α-synuclein and Aβ (1–42), however, the fibrils are stable only for a few days and, in addition, the proteins are prone to bacterial degradation, unless sodium azide is added. Fresh fibrils should, therefore, be grown every time a new set of experiments is performed.

The amyloid fibrils in these gels are up to several micrometres in length and the length distribution has to be greatly decreased to optimize the number of fibril ends on the QCM sensor to which the soluble protein molecules attach, and therefore to maximize the measured signal (1, 13). The required decrease in size distribution is achieved via ultrasonication. With the instrument we have been using, a minimum volume of 300 μl is required, as the sample will otherwise increase in temperature as a result of the energy that is dissipated in the sample. We routinely use cycles of 3 s sonication at 100 W and 3 s of recovery time for amyloid fibrils made from all proteins. The total number of sonication cycles necessary to obtain an average length of only a few hundred nanometres varies, however, quite strongly between the amyloid fibrils formed from different proteins, ranging from a single cycle for bovine β-lactoglobulin to 100 or more for bovine insulin. This variability is due to the differences in mechanical properties between fibrils consisting of different proteins. See Note 1 for details on the total sonication times recommended for amyloid fibrils from commonly used peptides and proteins.

3.2. Amyloid Fibril Activation

Two distinct strategies to attach the amyloid seed fibrils to the QCM sensor surface have been established. In the first, amyloid fibrils that are stable at neutral pH can be attached via standard amide coupling to an activated SAM (4, 5, 15). In the second, the fibrils can be directly attached to the gold surface, either via intrinsic sulphhydryl groups/disulphides (from cysteine residues in the protein) or via the attachment of small sulphur-containing molecules (cystamine and 2-imminothiolane) to side chains, a method that can be easily adapted to the desired pH value. We have described the latter approach in detail in ref. (13), as well as the difference between such a covalent attachment to gold and a purely non-covalent absorption of the fibrils onto the surface. Here we describe the procedures by means of two proteins as examples, α-synuclein and bovine PI3-SH3, for which amyloid fibrils are readily formed at neutral and acidic pH, respectively.

3.2.1. Attachment of Cystamine to PI3-SH3 at pH 2

For this protocol, non-sonicated fibrils must be used to facilitate the separation of the activated fibrils from excess reagent via centrifugation. The procedure is as follows:

1. Add 1 mg of EDC and 100 µl of a solution of 1 M cystamine to 300 µl of 1 mM PI3K-SH3 amyloid fibrils in HCl pH 2.
2. Mix thoroughly and make up to 2 ml with HCl pH 2.
3. Centrifuge for 1 h at 8,000 × *g* or more.
4. Remove the supernatant, make up to 2 ml with HCl pH 2 again and resuspend pellet.
5. Repeat centrifugation and resuspension a total of three times. Finally resuspend in 300 µl.
6. Sonicate for a total of 30 s under the conditions described above.

3.2.2. Attachment of 2-Imminothiolane to Human α-Synuclein at pH 7.4

In this case, the fibrils must be sonicated immediately prior to activation, as 2-imminothiolane (Traut's reagent) is only stable for a limited amount of time in its active form (13), and therefore the time delay between fibril activation and attachment to the surface needs to be minimized. The procedure is, therefore, to sonicate 300 µl of a 100 µM suspension of α-synuclein fibrils (in PBS) for a total of 5 min. Add 50 µl of a 1 mg/ml solution of Traut's reagent in water to the sonicated fibril solution. Incubate for 5 min, then proceed to the step described below as Subheading 3.3 without further delay.

3.3. Sensor Preparation

The principal aim of this step is to prepare a homogeneous seed fibril density on the sensor surface. The surface concentration of fibrils needs to be high enough that the growth of these fibrils generates a sufficiently strong, measurable change in resonant frequency of the QCM chip. This objective is obtained by incubating the surface with a concentrated suspension of sonicated fibrils.

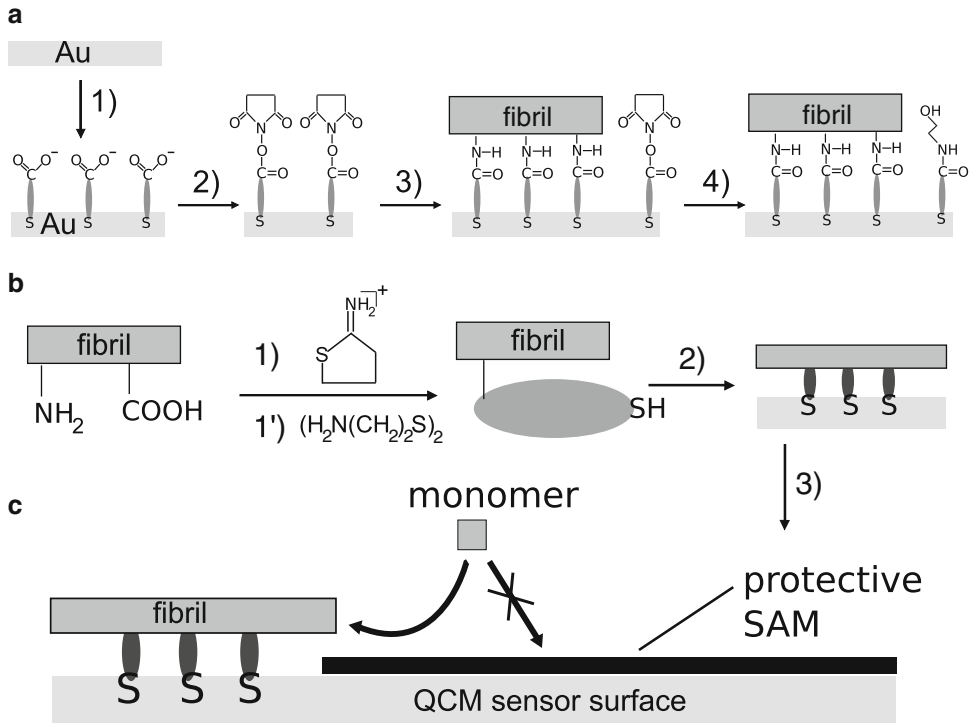

Fig. 2. Schematic illustration of the seed fibril attachment to a gold-coated QCM sensor. (**a**) Attachment of seed fibrils to a chemically activated SAM: (1) formation of SAM of mercaptoundecanoic acid (MUA), (2) activation of SAM with EDC and NHS to form reactive NHS esters, (3) coupling of the seed fibrils to the activated SAM, (4) passivation of the remaining activated MUA with ethanolamine; (**b**) attachment of chemically modified amyloid fibrils to a gold surface: coupling of Traut's reagent (1) or cystamine (1′) to the seed fibrils, (2) attachment of modified seeds to the gold surface, (3) passivation of the remaining exposed gold surface with an SAM; (**c**) QCM measurement where the surface-bound seeds are exposed to a solution of soluble amyloidogenic protein. The non-specific attachment of protein onto the surface is prevented by the SAM, and the attachment is uniquely directed onto the seeds.

The remaining sensor then has to be passivated in the cases where the fibrils are directly attached to the gold rather than to an SAM (13). We note here that it is important to incubate the QCM sensor with the fibril suspension outside the QCM cell. Not only does this procedure minimize the volume of fibril suspension needed for the surface preparation, but it also avoids the adsorption of amyloid fibrils in the flow system of the QCM.

As mentioned above, we can distinguish two attachment strategies, namely the reaction of seed fibrils with an activated SAM or the incubation of a bare gold surface with fibrils that have exposed sulphhydryl or disulphide groups. Examples of both types of attachment procedures will be given here and an illustrative summary is shown in Fig. 2.

In all cases where QCM experiments are performed with a protein not previously studied, or where the solution conditions are drastically changed, control experiments must be performed in

which no seed fibrils are attached to the surface of the QCM sensor, but the gold surface is only passivated by a SAM; when such a control sensor is brought into contact with a protein solution, no frequency shift should be observed. This control experiment is necessary to be able to attribute the observed frequency shift in experiments with surface-bound seed fibrils upon exposure to soluble protein entirely to the elongation of these fibrils, rather than at least in part to non-specific absorption of protein molecules to the surface.

3.3.1. Attachment of Aβ (1–42) Amyloid Fibrils to an SAM on a Gold Surface

This protocol follows a description in ref. 15 and is as follows. Incubate a gold-coated QCM sensor overnight in a solution of 1 mM MUA in absolute ethanol. Rinse the sensor with ethanol and dry with nitrogen and then incubate it for 20 min in a solution of 0.3 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 0.5 mg/ml NHS. Rinse with water and dry with nitrogen, and then add 100 µl of the fibril suspension onto the gold surface and leave for 5–10 min. Rinse surface with water, add 100 µl of a ca. 0.5 M solution of ethanolamine (pH adjusted with HCl to < 9) and incubate for 15 min. Rinse the surface again with water and insert into the microbalance (see Note 2).

3.3.2. Attachment of Insulin Fibrils to the Gold Surface of the QCM Sensor

Dilute the 5 mg/ml stock solution 1:20 into HCl at pH 2, sonicate for 10 min (under the conditions described above), and incubate the surface of a QCM sensor with 100 µl of the fibril suspension for 1 h. Rinse extensively with HCl at pH 2, then incubate the surface for 30 min with a solution of 1 vol.% of m-PEG thiol in HCl at pH 2. Then the surface is washed again with HCl at pH 2 and the sensor is inserted into the microbalance (see Note 2).

3.3.3. Attachment of Activated PI3K-SH3 and α-Synuclein Amyloid Fibrils to a Gold Surface

Add 100 µl of the activated and sonicated fibril suspension (see Subheading 3.2) onto a clean gold-coated QCM sensor. Incubate the sensor surface for 1 h in an atmosphere of 100% humidity (e.g., in a closed Petri dish with a few ml of water added). Rinse with HCl at pH 2 in the case of PI3K-SH3 and with PBS in the case of α-synuclein. In the case of the cystamine-activated fibrils, no further steps are required, as the remaining cystamine in the solution plays the role of an SAM forming molecule, and the sensor can be directly inserted into the microbalance (see Note 2). In the case of the α-synuclein activated with Traut's reagent, however, the formation of an SAM, as in the case of insulin described above, is required. It is carried out in an analogous manner, except that the mPEG thiol is dissolved in PBS at pH 7.4 instead of HCl at pH 2.

In general, it is useful to take AFM images of the QCM sensors after their initial preparation, to be able to define the length distribution and surface density of the attached seed fibrils. The images acquired at this point then provide the reference for images acquired after the experiment is complete. It is not necessary to carry out this procedure routinely, but for a new set of conditions it is indispensable.

It is possible to dry the sensor for the imaging process without interfering with the seeding capabilities of the surface-bound protein, although if the sensor surface is left dry for an extended period of time (hours to days), the seeding capability can eventually be lost.

3.4. Purification/ Treatment of Commercially Available Proteins and Peptides

When the amyloid formation reaction of commercially available protein (e.g., lysozyme, α-lactalbumin, and β-lactoglobulin) is studied and when absolute elongation rates are of interest, the soluble protein, even if reported to be of very high purity, might require dialysis against a large volume of water prior to the experiments, because of the possible presence of residual salts. The protein can be lyophilized again for convenient storage. If the growth rates (see Subheading 3.5) are indistinguishable between dialyzed and non-dialyzed protein, the dialysis step can be omitted.

To be able to use some highly aggregation prone systems, e.g., Aβ (1–42), for kinetic measurements, careful pre-treatment is needed to remove pre-existing aggregates and seeds that can induce rapid fibril formation when the peptide is dissolved in a buffer. To achieve this aim, various methods have been proposed in the literature, such as chemical treatment or size exclusion chromatography. In most cases, we used for Aβ (1–42) and its analogues the treatment with TFA and HFIP (17), as it yields good results on control QCM sensors, while being easy to carry out:

1. Dissolve 0.5 mg Aβ (1–42) in 500 μl of TFA, and sonicate for 30 s on ice with a probe sonicator (see above).
2. Flash freeze in liquid nitrogen and lyophilize over night.
3. Redissolve the peptide in 1 ml HFIP and incubate on ice for 15 min, as the dissolution can take some time.
4. Make aliquots of 50 μl and evaporate the HFIP in a speed vac. Store the aliquots (ca. 10 nmol) in a freezer.
5. Determine the exact amount of peptide per aliquot (amino acid analysis).

3.5. Measurements

Once the sensor is inserted into the liquid cell, the resonant frequency can be monitored and the establishment of a stable baseline can be followed. Changes in the temperature of the cell can lead to a large shift in the resonant frequency, despite the quartz crystal being specifically (AT) cut to minimize that dependency, because of the different thermal expansion coefficients of the quartz and the gold electrodes. The temperature at which the experiments are to be carried out should, therefore, be already established during the equilibration period.

To perform a measurement of the elongation rate of the surface-bound fibrils, they have to be brought into contact with a solution of soluble protein under conditions where amyloid growth

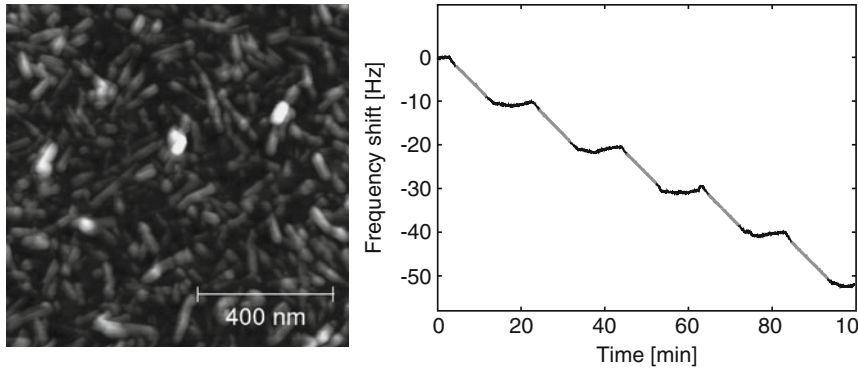

Fig. 3. The AFM image on the *left hand side* shows seed fibrils of the protein insulin attached to a QCM sensor surface, before the series of experiments shown on the *right hand side* of the figure were carried out. The surface number density is estimated to be $500 \pm 200\ \mu m^{-2}$. On the *right hand side*, a typical QCM experiment, in this case a test of the intrinsic reproducibility is shown. The surface-bound seed fibrils are repeatedly exposed to a solution of 0.2 mg/ml insulin in HCl pH 2 at 40°C. The *grey lines* indicate the sections of the frequency trace (here the frequency overtone with $N = 5$, see main text for details) that were fitted with a linear function to extract the rates of frequency change. The average rate of the five repeats shown here is 0.96 ± 0.01 Hz/min.

is favourable; as an example, we describe a measurement of the elongation of insulin fibrils. As the surface-bound seed fibrils are brought into contact with a solution of 0.2 mg/ml insulin in HCl at pH 2 and 40°C, the resonant frequency will start to decrease linearly (see Fig. 3 and Note 3). The rate of change of frequency in this case is ca. 1 Hz/min, but in general can range from <0.05 to >20 Hz/min, therefore spanning several orders of magnitude, depending on the temperature, solution conditions, and the nature of the protein under study. The experiment is performed in the absence of flow, i.e. the pump or gravity flow is stopped once the liquid cell has been completely filled with protein solution. The change in frequency will cease when the insulin solution in the flow cell is replaced by HCl at pH 2. It is important to use the same buffer or other solution to dissolve the protein and to wash the liquid cell after each measurement (see Note 4). When very concentrated protein solutions are used, a shift in resonant frequency can be observed upon contact of the QCM sensor with the protein solution, due to the increased viscosity and density of the latter when compared with pure buffer. This shift is, however, reversible when the liquid cell is filled again with buffer.

The linearity in time of the decrease in resonant frequency greatly facilitates the analysis of the data (see below). To observe this linearity, significant depletion effects within the liquid cell, due to the addition of the soluble protein on the surface-bound seed fibrils, must not occur. This requirement arises because the fibril elongation rate is linearly dependent on the concentration of soluble protein over a wide concentration range, and, as this concentration decreases, so does the rate. It is, therefore, useful to carry out these QCM measurements under conditions where only a very

small fraction of the protein in the liquid cell is consumed, such that the concentration is effectively constant. In practical terms, this situation results for all except the most aggregation prone peptides and proteins where concentrations as low as 1 μM are used to obtain rates that are sufficiently slow to measure accurately (see Note 5).

A first experiment should always be a test of the reproducibility of the rate of frequency shift and therefore of the fibril elongation rate. This can be achieved by filling the liquid cell repeatedly with an identical protein solution (Fig. 3). The slopes of the change in resonant frequency can be expected to be identical to within less than 10%, due to the fact that the growth of the same, constant ensemble of fibril seeds is examined. In the example in Fig. 3, the observed rates of change in resonant frequency in five consecutive incubations are 0.96 ± 0.01 Hz/min. This exceptionally high reproducibility is a main strength of the biosensing approach and enables very small changes in aggregation rate to be detected (16).

If the total shift in frequency, and therefore the total increase in fibril mass reaches a certain threshold value, however, the sensor will not be sensitive to further addition of protein (see Note 5). This threshold value depends on the surface density of fibrils and on the specific protein contained within the fibrils and defines the "life time" of the sensor. Only within this "frequency-shift window", reliable and reproducible measurements can be carried out. The manifestation of this finite usability of a given sensor is the slow decrease in rate of frequency shift over time. The approximate time of onset of this decrease should be determined before a systematic study of the effects of changes in conditions is carried out.

Once this preliminary characterization has been carried out, a freshly prepared QCM sensor can be successively exposed to solutions of soluble protein and conditions such as temperature, ionic strength, the presence of small molecule compounds, and so on can be varied. This ability to carry out a series of experiments with the same fibrils enables a highly accurate study of the influence of such conditions on the fibril elongation rate, and therefore on the barriers that separate the soluble state of the protein on its energy landscape from the aggregated state. This characteristic enables in-depth studies of the mechanism of aggregation, as well as enabling a systematic and high throughput (due to the short duration of a single measurement) search for inhibitors of this process (15, 16).

At the end of one or a series of measurements, especially at the initial stages of a study, the sensor surface should be routinely imaged with an atomic force microscope (Fig. 3). Not only do these images allow an estimate of the average length increase of the surface-bound fibrils to be made (see Note 6) in cases where absolute elongation rates are required, but they can also be used, as mentioned above, to detect the presence of other aggregation products such as amorphous species, which can be induced by certain extrinsic factors, such as high ionic strength, and which can also give rise to a shift in resonant frequency if the aggregates attach to the surface.

3.6. Data Analysis

Two types of kinetic experiments designed to study amyloid fibril elongation are readily carried out using the QCM technique. In the first type, only relative changes of aggregation rate upon some change in conditions are of interest, e.g. a comparison of the fibril elongation rate in the presence and absence of an inhibitor. In the second type, the absolute rate of fibril elongation is needed, i.e. the number of protein molecules that add on to a single fibril end per time unit. For the first type of measurement, no knowledge of the total number of growing fibril ends on the QCM sensor surface is required (as long as it remains constant during the measurement), and the exact proportionality factor between the mass of added protein and the frequency shift do not have to be known. As long as it can be established that the decrease in resonant frequency is, within some range of the total frequency shift, proportional to the mass of added protein, and therefore to the total length increase of the surfac-bound seed fibrils, the rates of frequency change under different conditions can be directly compared.

QCM devices often allow for the fundamental resonant frequency, as well as several of its higher-order harmonics, to be monitored simultaneously. The fundamental frequency is most sensitive to perturbations, such as pressure and temperature changes, and is often not analysed. For surface-attached material of high rigidity, all the (normalized) frequency overtones ideally show the same shift. However, in the case of a rough, viscoelastic surface layer, there can be substantial deviations between the harmonics, as a result of, e.g., frequency-dependent viscoelastic properties of the attached material and different penetration depths of the different overtones of the shear wave into the attached layer. In such cases, the growth rate analysis should be carried out using either one particular frequency or an average of several overtones. The latter possibility can increase signal-to-noise ratio, and is possible because despite the fact that the different overtones show different mass sensitivity coefficients, the relative changes in rate upon changes in condition are in most cases extremely similar.

As mentioned above, experiments to measure growth rates are ideally carried out under conditions where no significant depletion of protein molecules in the liquid cell occurs during a measurement. In this case, the rate of change in frequency is very well approximated as a linear function of time and can be easily fitted to such a function. If a certain change in conditions leads to a faster rate of change in frequency, then it can be concluded that the rate of protein addition to the seed fibrils is enhanced and therefore that the given change in conditions promotes aggregation. The converse holds for inhibition giving rise to a decrease in the rate of frequency change.

3.6.1. Determination of Absolute Reaction Rates

However, the determination of absolute reaction rates in terms of microscopic rate constants requires knowledge about the number density of seed fibrils on the sensor surface and, in addition, the proportionality factor between the attached mass and the observed frequency

shift must be defined. We will describe below how these quantities can be estimated. Knowledge of these two quantities allows calculation of the absolute mass, and therefore of the total sum of protein molecules that have become attached to the fibril ends. At the same time, one has to bear in mind that this quantity will be an ensemble average, not least because amyloid fibrils may elongate via "stop and go" kinetics in which there may be pauses in between periods of rapid growth of individual fibrils (19). If these ensemble averages of absolute aggregation rates are analysed in the framework of an appropriate kinetic model, however, it is possible to determine key quantities such as the free energy barrier that separates the soluble protein from its fibrillar state (1, 20) and characteristic time scales involved in the attachment of a soluble protein molecule on a fibril end (20). Such information is key in the elucidation of the mechanism of amyloid formation.

3.6.2. Determination of the Seed Fibril Number Density on the Surface

This objective can be achieved by imaging of the surface with an AFM before or after the experiment (Fig. 3) and manual counting. The surface can be dried and simply imaged in air. In the case of high surface densities, it can, however, be challenging to obtain an accurate estimate of the number of density of seed fibrils. The fibril density should then be determined on several independent regions of the same sensor and the average value should be used.

3.6.3. Determination of the Proportionality Factor Between Attached Mass and Observed Frequency Shift

As mentioned in Subheading 1, due to the complex hydrodynamics of a network of surface-bound fibrils, it is not possible to interpret the frequency shift induced by the elongation of the fibrils in terms of a simple Sauerbrey (8) or Kelvin–Voigt (11) model. It is, therefore, necessary to determine empirically the proportionality coefficient between fibril length increase and frequency shift. Once this mass sensitivity coefficient has been determined, it can be assumed to be identical for similar experiments, involving amyloid fibrils made from the same protein.

Two methods for the calibration can be distinguished. The first is based on the determination of the length increase of amyloid fibrils by AFM imaging. The average length of amyloid fibrils is measured before and after a QCM experiment and the increase in length is correlated with the observed decrease in resonant frequency of the sensor during the experiment. In addition, the number of fibrils (see above) as well as their diameter and density needs to be known. The diameter can also be determined from AFM measurements, and the density can be assumed to be similar to that of a globular protein. From these data, the total mass of added fibrillar material can be estimated and compared to the frequency shift. Such calculation is illustrated in the following section. The second method, which we mention here only briefly, is based on the finding mentioned above that when low concentrations of soluble proteins are used in the case of highly aggregation prone peptides and proteins, depletion effects can be observed

provided that the volume of the liquid cell is sufficiently small. In these cases, effectively all the available protein in the liquid cell can be incorporated into the fibrils and the total observed frequency shift due to the addition of this protein on to the seed fibrils can be measured. The known amounts of protein (due to known concentrations and volumes) in contact with the seeds can then be correlated with the observed frequency shift.

3.6.4. Example Estimation of Mass Sensitivity Coefficient for E4 QCM Used to Probe Insulin Amyloid Growth

As an illustration of the principles detailed above, we carry through a calculation that allows us to estimate the microscopic rate constant of the insulin amyloid fibrils shown in Fig. 3. The average length increase per fibril is estimated from Fig. 4 to be 200 ± 100 nm. The number density of seed fibrils (Fig. 3) is estimated to be $500 \pm 200\ \mu m^{-2}$. The diameter of a fibril is 5 nm, and the density is assumed to be 1.2 g/cm^3, as for a globular protein. The active area of a QSX-301 sensor is 1 cm^2. Therefore, the observed frequency shift of ca. 50 Hz corresponds to between 70 and 500 ng of added fibrillar protein, yielding a mass sensitivity coefficient of between 1.4 and 10 ng/Hz. If this is compared to the Sauerbrey mass sensitivity of the sensor of 17.7 ng/Hz, it can be seen that an additional contribution to the frequency shift, not stemming from dry mass occurs. Using this range of mass sensitivity coefficients, we obtain a molecular rate constant for insulin fibril elongation of 1350–9630 $M^{-1}s^{-1}$ assuming growth occurs from one end only, or half these values if growth is symmetric. Such data can be used to calculate the free energy barriers that separate the soluble from the aggregated protein (20).

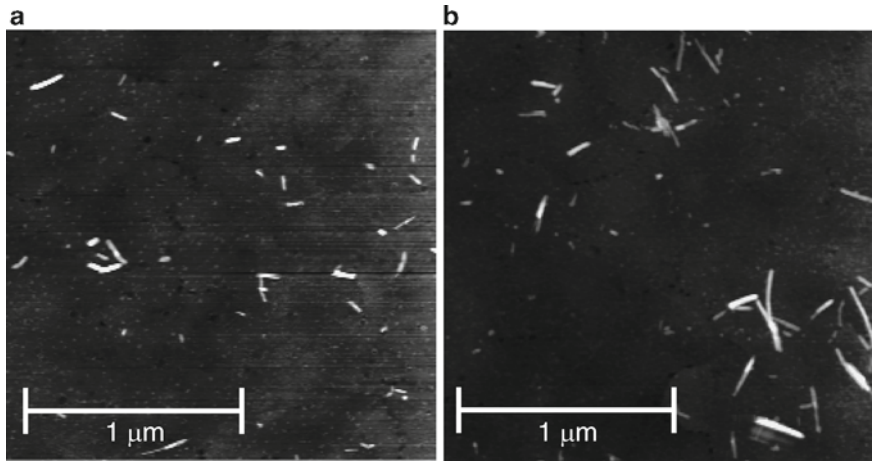

Fig. 4. These two AFM images were used to estimate the length increase that the surface-bound insulin seeds had undergone in the experiment shown in Fig. 3. Gold substrates had been prepared in a similar way to the QCM sensors, albeit with a much lower surface density, which greatly facilitates estimation of the length increase. For an actual QCM measurement, the elongation of surface-bound fibrils at such a low surface density would not give rise to a measurable signal. (**a**) At the beginning of the experiment. (**b**) After incubation for 1 h at 40°C with an insulin solution of 0.2 mg/ml in HCl pH 2. The average length increase is estimated to be 200 ± 100 nm.

3.7. Cleaning of QCM Sensors

In most cases, the gold-coated QCM sensors can be cleaned and reused (13). However, to remove the attached fibrils, relatively harsh conditions have to be applied. We found that heating the sensors to 80°C in 7 M NaOH solution removed >95% of the protein aggregates, as judged from AFM images taken after the cleaning process. When the sensor is reused for the same type of experiments, this level of cleanliness is sufficient. In cases where an SAM has been applied, it can be removed electrochemically by applying a voltage of −1 to −2 V against a gold counter electrode at neutral pH using any inert electrolyte, such as phosphate buffer. Even better results for the removal of the SAM (as judged from contact angle measurements) and residual protein material were obtained via UV/ozone cleaning with exposure times of 10 min, after which the sensors are ready for reuse.

4. Notes

1. The total sonication times used to achieve an appropriate length distribution under the conditions described above (see Subheading 3.1) are as follows: insulin: 10 min, PI3K-SH3: 30s, lysozyme: 15–30 s, insulin B-chain: 2 min, β-lactoglobulin: 3 s, α-lactalbumins: 15 s, α-synuclein: 5 min.
2. When the QCM sensor is inserted into the microbalance, the reverse of the chip (where no fibrils are attached and which is not in contact with the liquid during the experiment) needs to be dry. Otherwise, the baseline will not readily stabilize as a result of evaporation of the residual solution.
3. Often, the frequency shift is non-linear for the first few minutes of incubation of a freshly prepared sensor in contact with a protein solution. This instability is presumably due to a certain amount of non-specific absorption of soluble protein to the surface, despite the passivation of the surface with an SAM, as well as to lateral attachment of protein molecules to the seed fibrils. The extent to which this additional protein adsorption occurs depends strongly on the specific protein. The first contact of a new sensor with a protein solution should therefore always be repeated to check for the completion of this adsorption process that interferes with the signal associated with fibril elongation.
4. The resonant frequency of a QCM sensor in contact with liquid is also dependent on the density, viscosity and, in some cases, ionic strength of the solution involved. If any of these parameters is changed, a shift in resonant frequency will be observed, which is normally reversible when the solution is replaced by the previous solution. This effect can be important in cases, for

example, when the binding of small molecules to amyloid fibrils is being studied (15). The binding can lead to a shift in resonant frequency, but if different solvent conditions are needed to solubilise the small molecules, the shift induced by the change in solvent can be much larger than the shift induced by the binding and the latter can, therefore, be masked.

5. We have observed depletion effects in the case of the Yeast prion protein Ure 2 and for the peptide Aβ (1–42). In these cases, the concentration of soluble polypeptide was as low as 1 μM. Such low concentrations were required to slow down the growth rates sufficiently to prevent the seed fibrils on the sensor surface elongating at too high a rate. When the fibrils become too long, the sensor does not respond any more to the contact with soluble protein. This "saturation" is presumably due first to crowding of fibrils on the surface and second to the effect of their finite rigidity, leading to a loss of vibrational energy within the fibrillar layer.
6. In many cases, the surface density of fibrils on a QCM sensor will be too high to allow an easy estimation of the length increase via AFM imaging of the sensor surface. Such a high surface density might, however, be required to achieve a sufficiently high signal-to-noise ratio. In such cases, a gold substrate can be prepared with a much lower surface density of seeds than on the original QCM sensor and incubated with a monomer solution under identical conditions for the same amount of time. It is then much easier to estimate the length increase and correlate it with the observed frequency shift (Fig. 4).

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