



Minireview

Biosensor-based label-free assays of amyloid growth

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ABSTRACT

Uncontrolled fibrous protein aggregation is implicated in a range of aberrant biological phenomena. Much effort has consequently been directed towards establishing quantitative *in vitro* assays of this process with the aim of probing amyloid growth in molecular detail as well as elucidating the effect of additional species on this reaction. In this paper, we discuss some recent approaches based on label-free technologies focussed on achieving these objectives. Several biosensor techniques have been developed to monitor biomolecular assembly without the requirement for fluorophore marker molecules; in particular quartz crystal microbalance and surface plasmon resonance measurements provide advantageous alternatives to traditional spectroscopic methods and are currently receiving increasing attention in the context of amyloid growth assays.

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1. Introduction

Biological functionality provided by protein molecules in living systems is in general contingent on their reliable folding into the native structures encoded in their polypeptide sequences. It is increasingly appearing, however, that polypeptide chains also possess an inherent tendency to access an alternative ordered phase where non-native contacts are formed between adjacent molecules condensed into supra-molecular assemblies [1–3]. Such elongated nanostructures, commonly known as amyloid or amyloid-like fibrils, are implicated in a wide range of pathological conditions including many neurodegenerative diseases as well as localised and systemic amyloidoses [1,4–6]. In addition, it has recently been established that in select cases amyloid-like structures can also possess beneficial biological functionality, for instance as functional bacterial coatings [7–9], as epigenetic switches [10–14], as controlling factors in fungal cell fusion [15,16] and as catalytic scaffolds in melanin synthesis [17]. This natural use of amyloid assembly for functional purposes in biological systems has provided inspiration for the investigation of tailored amyloid-like structures as bionanomaterials for technological applications [18–25].

Soluble forms of many different proteins are capable of transforming into insoluble amyloid structures under a variety of physiological and non-physiological conditions [2,26]. In order to

develop a fundamental understanding of the factors which promote, or on the contrary inhibit, this transition which lies at the heart of biological function and malfunction, it is essential to be able to probe the thermodynamics and kinetics of this process in a reliable way. Traditional experimental methods have focussed on the use of amyloidophilic dyes such as Congo red or thioflavin T which, upon binding to amyloid fibrils, exhibit a change in their spectral properties which can be measured using optical spectroscopy in a conventional manner. Such approaches, however, suffer from their dependence on the knowledge of stoichiometry, sensitivity and mechanism of dye binding [27–30]; aspects which currently remain challenging to quantify. Many of these problems can be avoided when protein monomers are covalently labeled with fluorophores [31], but in such cases care is required in order to ensure that the label does not affect fibril morphology or aggregation kinetics.

Direct, label-free measurements represent an alternative and complementary approach to traditional protein aggregation assays. These direct approaches circumvent the complexities associated with the design, synthesis and detailed understanding of the mechanism of action of fluorophores and the influences they may have on the aggregation reaction. Of particular interest are several label-free surface techniques based on biosensors that have been developed recently and applied to protein aggregation. The general strategy behind such assays is to exploit coupling between the amyloid growth reaction on a sensor surface and the mechanical or optical response of the sensor. We discuss here two such approaches which rely on surface plasmon resonance and mechanical transduction.

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2. Measurement of amyloid growth rates using mechanical transducers

We first focus on the monitoring of amyloid growth through direct measurements, using quartz crystal microbalances, of the mass increase as new protein molecules add on to amyloid fibrils. The operation of a quartz crystal microbalance (QCM) relies on the high sensitivity of the resonant frequency of a mechanical oscillator to the mass which is in movement. In practice the inverse piezo-electric effect is commonly used to transduce oscillating electric fields into acoustic shear waves in thin quartz crystals, resulting typically in resonant frequencies in the megahertz range for the fundamental frequency. The surface of the sensor is on an anti-node of the shear wave, and therefore any changes in surface mass loading consequently are reflected accurately in the resonant frequency of the system. First applications of this principle [32] for practical mass sensing focussed on gas phase adsorption as this provided an environment free from mechanical perturbation or temperature fluctuations that can affect the frequency of the crystal. Significant advances in the 1980s demonstrated the use of QCM in liquid environments [33,34] and as biosensors [35,36] and in recent years there has been increasing interest in applying mechanical resonators as the basis for bio- and immuno-sensing [36–40].

In order to use QCM methodology as the basis for an amyloid growth assay, the protein aggregation reaction has to be directed on to the sensor surface where the changes in mass can be detected effectively. One possibility in this respect is to create specific growth sites on a functionalised surface. This type of functionalisation can be achieved by covalently attaching small pre-formed aggregates [41–43] to the sensor surface; when exposed to soluble protein under aggregation promoting conditions, the monomers present in solution can add on to such seed aggregates, resulting in amyloid growth (Fig. 1b). The controlled preparation of such surfaces is essential for reproducible measurements to be achieved;

the fixation of seed fibrils on to gold surfaces can either exploit naturally occurring surface exposed sulphhydryl groups within cysteine residues of the polypeptide chains composing the aggregates, or when these are absent, specific linker molecules can be used to guarantee effective binding of seed fibrils to the sensor surface [42,43]. The remaining exposed area of the surface can then be passivated using an inert thiol-terminated self-assembling monolayer which binds to the free exposed gold surface and prevents non-specific protein adsorption. It has been demonstrated that this approach enables reliable assays of protein aggregation to be developed for different proteins such as insulin [41], a range of mutants of the SH3 domain (Fig. 2a and b) [42] and the A β peptide [43].

Due to the high sensitivity of QCMs to mass increase at the solid–liquid interface, amyloid growth rates for a given set of conditions can be measured in many cases within a matter of minutes (Fig. 1a); this situation is in contrast to traditional aggregation assays, where typically hours or days are required to measure the time for the conversion of soluble protein into fibrils. In addition, lower detection limits can be achieved with QCM sensors compared to fluorescent assays; for instance for the case of the A β peptide, aggregation rates could be measured for concentrations of soluble peptide as low as 500 nM, a value approximately one order of magnitude lower than the limits reported for traditional fluorescent assays [43]. A further advantage of biosensor measurements is that the growth of the same ensemble of fibrils can be probed repeatedly under different conditions simply by changing the growth solution in contact with the fibrils bound to the sensor; a possibility which is absent if both the monomers and the fibrils are in solution.

An alternative approach to the growth of pre-formed seed fibrils on a sensor is to initiate the reaction with monomeric protein in solution and let polymerisation take place *de novo* starting from the nucleation step at the solid–liquid interface [44–46]. In partic-

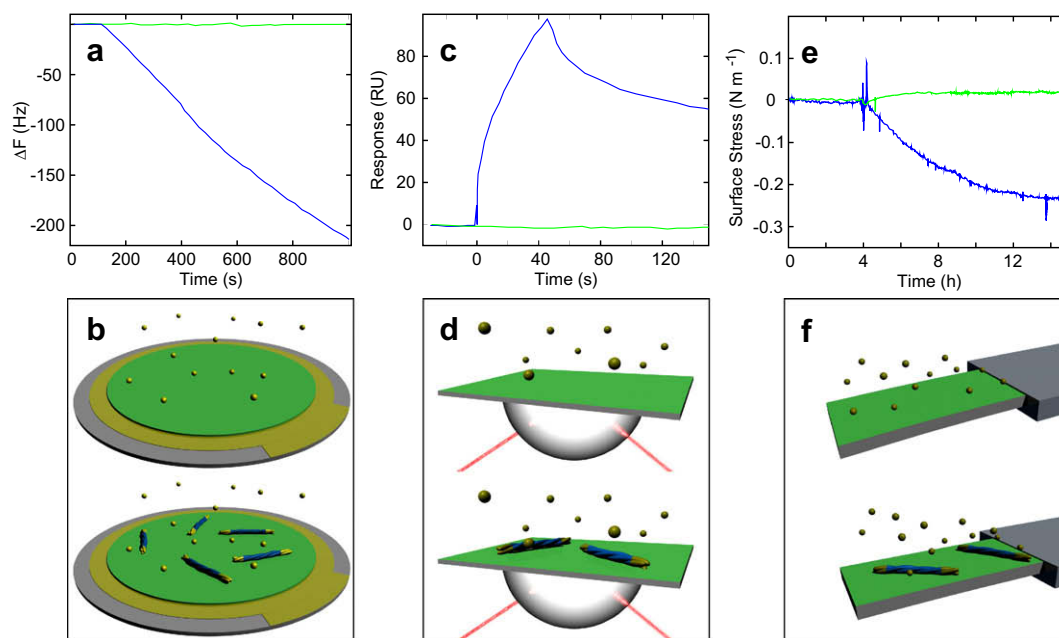


Fig. 1. Label-free measurements of amyloid fibril elongation. In the schematic diagrams (b,d,f) passivated surfaces are shown in green, monomeric protein is represented by yellow spheres, immobilised seed fibrils are shown in blue and the yellow fibril ends represent regions of growth. Three detection principles are illustrated: (a–b): QCM, (c–d): SPR, (e–f) cantilever sensors. (a) A negative shift in resonant frequency is observed upon insulin fibril growth (a, blue line and b, lower schema); control measurements under the same conditions exhibit a very low response (a, green line and b, upper schema). (c) Surface plasmon resonance measurements report a response upon addition of monomeric protein to A β (1–40) seed fibrils, subsequent dissociation is also observed upon rinsing with buffer (blue line); control measurements show little fluctuation (green line). In (e–f) the differential surface stress generated by growing insulin fibrils on a microcantilever (e) relative to a cantilever devoid of seeds exposed protein solutions is used as a probe for amyloid growth. Data from [41,60,81].

ular the complete aggregation profile of glucagon has been followed using QCM and time-lapse atomic force microscopy (AFM) after a tantalum surface was exposed to monomeric protein over a period of 25 h [44].

An important aspect of QCM measurements is the question of the physical origin of the resonant frequency changes. Contributions to the signal result both from an increase in mass bound to the sensor as well as from viscous damping; this latter contribution in turn is dependent on the surface roughness [47–51] and the visco-elastic behaviour of the surrounding fluid as well as of the surface bound species [44,52]. It is in general not possible to predict from first principles the frequency shift resulting from a complex process such as fibril growth on a sensor surface, and one approach is to calibrate the sensor response with independent AFM measurements of the corresponding increase in fibril length [41,42]. Alternatively, under certain conditions, the changes in surface roughness can be neglected, and it is then possible to gain valuable insight into the visco-elasticity of the fibril layer using material models such as the Kelvin–Voight model [53] in conjunction with simultaneous measurements of the changes in the resonant frequency and in the quality factor of the resonator [54,55]. An elegant demonstration of this approach is given in Ref. [44], where a rigid protein monolayer was observed to have formed during the lag-phase of aggregation followed by a dramatic increase in the viscoelasticity of the adsorbed layer attributed to subsequent fibril growth.

An alternative path for transducing chemical reactions into a mechanical response is through the measurement of the surface stress resulting from biomolecular assembly using microcantilever sensors [40,56–59]. This principle has been successfully imple-

mented [60] as a label-free assay of amyloid growth (Fig. 1e and f). Cantilever sensors offer many advantages over QCMs in terms of parallelisation and integration with microfluidic systems; currently, however, the molecular origins of the differential surface stresses associated with given chemical processes have been challenging to identify [61], and therefore at present QCMs remain more straightforward to operate as quantitative amyloid growth assays.

3. Surface plasmon resonance

An alternative strategy for following surface bound protein aggregation is through the use of surface plasmon resonance (Fig. 1d). Collective electron resonances localised at the interface between a metal such as gold or silver and a dielectric can be excited by an incident light beam; for energy to be transferred from the incident photons to the surface plasmons, the surface component of the wave-vector of the light must match the wave-vector of the plasmons in order to satisfy momentum conservation. The characteristic angle satisfying this condition is highly sensitive to the refractive index of the medium close to the metal surface and can consequently be used as a probe for the adsorption of molecules.

Surface plasmon resonance is arguably the most well-established surface-based biosensing technique and has been extensively discussed elsewhere [62–64]. The only prerequisite for adopting SPR to study bio-interactions is, as for QCM, that one species under investigation must be immobilised on the sensor surface. This has led to a diverse range of relevant applications, from the binding of small ligands to amyloid structures [65–69] to inter-

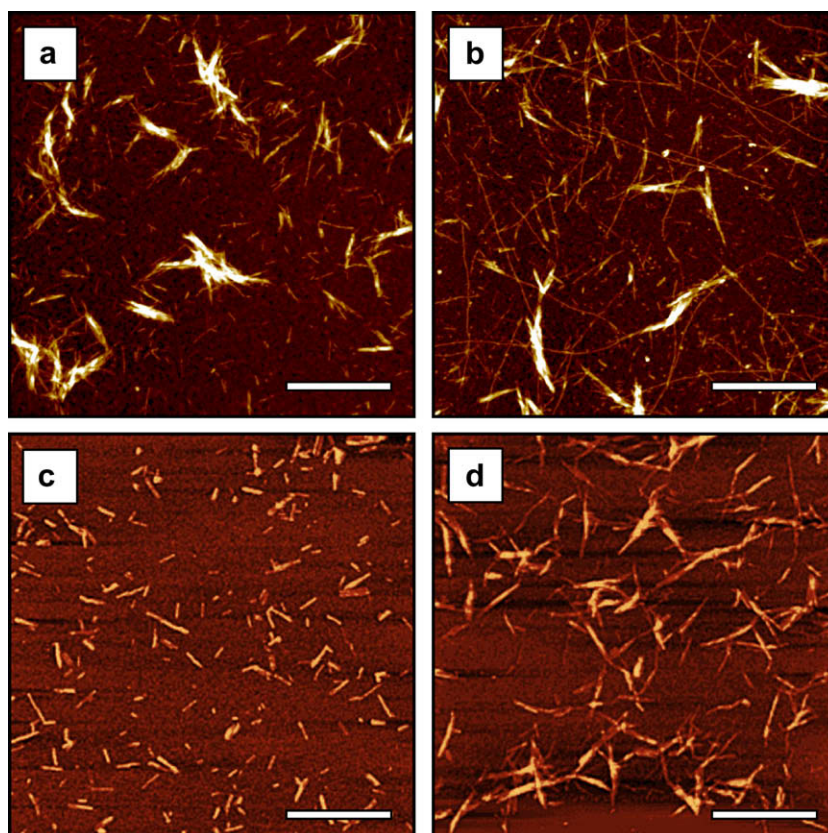


Fig. 2. Tapping mode atomic force microscopy images of amyloid fibrils immobilised on gold biosensor surfaces. (a) Sonicated PI3K-SH3 seed fibrils covalently bound to a gold surface. (b) Fibrils elongate following monomer addition to the seeds. (c) Sonicated A β (1–40) seed fibrils immobilised on a C1 SPR gold sensor. (d) Elongated fibrils are also observed post monomer addition. Images reproduced with permission from [42,80]. Scale bars are 1 μ m.

actions of amyloid with lipids [70–75] and the binding of small heat shock proteins to amyloid-related species [76–78]. Here we focus on reports that are based on surface plasmon resonance as an approach for monitoring the growth of amyloid fibrils.

The strategies adopted for SPR assays to monitor amyloid fibril growth are closely related to those described above for QCM despite differences in the physical principles underlying the detection method. One approach involves immobilisation and extension of pre-formed seed fibrils [79–81], while an alternative method follows the complete aggregation profile from monomeric protein to mature fibrils [82]. While many covalent attachment [80,82] methods are available, the majority of SPR amyloid assays are based on the immobilisation of the protein species on to a sensor surface via amine coupling to a surface bound carboxylated dextran matrix. Surface imaging by AFM confirms that the observed density increase upon exposure of monomeric protein molecules to the functionalised surface is a direct measure of fibril extension rather than non-specific binding or amorphous aggregation (Fig. 2c and d) [80,82].

SPR measurements are highly sensitive to the addition of material to the sensor surface, for example A β concentrations as low as 1 μ M have been observed to extend immobilised amyloid fibrils [80]. The continuous flow capability of biosensor methods ensures that such low concentrations remain constant throughout experimental measurements, allowing complex contributions from monomer depletion to be omitted. Biosensor techniques also benefit from real-time measurements of fibril growth with time intervals on the order of seconds that have allowed irreversible and reversible monomer addition to be identified [79]. Accurate temperature control is an additional advantage that facilitates thermodynamic measurements and has allowed determination of activation barriers for monomer addition to amyloid fibrils [80].

Several important experimental considerations must be made when designing surface assays to allow confidence in quantitative data analysis [83]. Undesirable experimental artifacts such as non-specific binding can be controlled either by duplicating conditions on suitably prepared reference sensors or by optimising the surface chemistry, for instance through the use of inert monolayers to minimise adsorption. An intrinsic feature of all surface-based techniques is the entropic penalty associated with immobilisation of a reactive species; attempts have been made to alleviate these effects by immobilising the reactive species on to a flexible dextran matrix. It can, however, also be argued that surface measurements reproduce more accurately the entropic limitations that cellular organelles, membranes and macromolecules would exert upon protein aggregation in vivo [84].

4. Biosensing of amyloid binding compounds

The previous sections have outlined the potential of the QCM and SPR techniques for monitoring directly the aggregation of amyloid proteins. Accurate kinetic measurements of fibril growth, however, also allow the influence of disparate conditions and additional molecular species on this reaction to be studied in a quantitative manner. Inhibitors of amyloid growth are of particular biological interest, both for the understanding of the mechanisms in place in living systems that prevent uncontrolled protein aggregation and for the development of therapeutic strategies for intervention in the case of protein deposition disorders.

Mechanistically, a molecular species can inhibit fibril extension through direct association with the fibril and preventing fibril growth through steric inhibition or by targeting the soluble monomer and blocking addition. Surface plasmon resonance and the quartz crystal microbalance have been demonstrated as powerful ways to monitor such inhibitory effects.

Surface plasmon resonance measurements have successfully enabled the binding affinities of several ligands to monomeric A β to be related to inhibition of neuronal toxicity by measuring dissociation constants of the ligands from the immobilised monomeric peptide [68]. Molecular chaperones are thought to assist proteins in achieving their native fold rather than forming a partially folded or misfolded state that could prove toxic. In this context the binding of a family of chaperones known as small heat shock proteins (sHsps) to monomeric amyloid proteins has been demonstrated using SPR binding assays [76–78].

As opposed to monitoring binding of chaperones to monomeric proteins, the quartz crystal microbalance has been used as a tool to probe the effect of chemical and molecular chaperones on amyloid fibril extension [41]. Differential effects of a chemical chaperone (Trimethylamine N-oxide, TMAO) and a molecular chaperone (the sHsp α B-crystallin) were demonstrated (Fig. 3) by measuring fibril extension first from a monomer solution, then from a monomer solution also containing the chaperone molecules, and finally from a monomer solution without chaperone once again on the same surface. This systematic approach was able to demonstrate that while both chaperones can significantly inhibit fibril growth, α B-crystallin remained bound to the fibrils (Fig. 3B) and inhibited further growth on that sensor, in contrast to TMAO (Fig. 3A). Binding of a molecular chaperone to A β fibrils and a consequent inhibi-

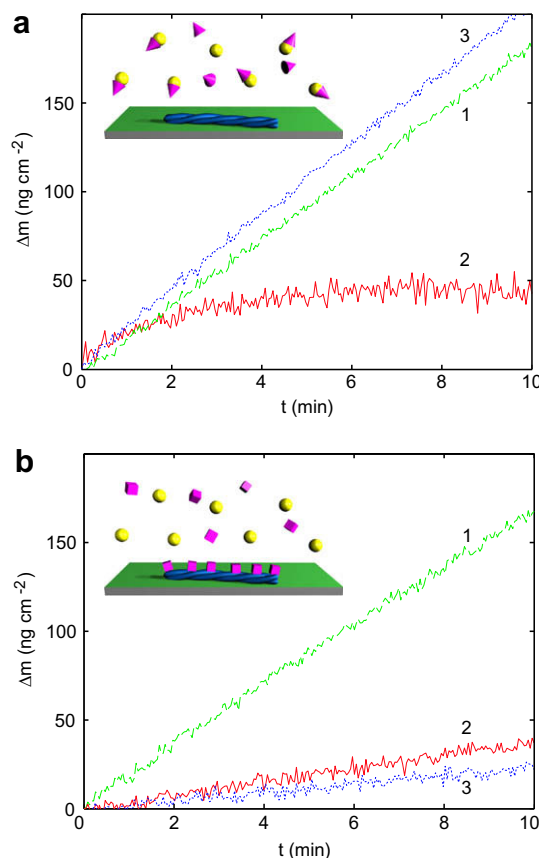


Fig. 3. Mechanisms of amyloid growth inhibition monitored by a QCM assay. (a): Growth inhibition by a chemical chaperone (TMAO). Insulin fibrils are first grown in the presence of soluble protein (1), then in the presence of both protein and chaperone (2) and finally in the presence of protein alone (3). In the absence of chaperone, the growth resumes to the initial level, indicating no permanent interactions between the fibrils and the chaperone. In (b), the growth inhibition assay is repeated for α B-crystallin. The growth rate (3) after exposure to chaperone (2) is significantly lower than that measured for pristine fibrils (1), revealing a strong interaction between the fibrils and α B-crystallin as the basis of the growth inhibition. Data from [60].

tion of aggregation has also been demonstrated using SPR in combination with ThT binding assays [85].

5. Concluding remarks

Label-free kinetic measurements of biophysical processes are becoming a promising alternative approach to traditional spectroscopic methods. In particular, surface plasmon resonance and the quartz crystal microbalance provide the basis for powerful assays for monitoring the kinetic and thermodynamic parameters governing the elongation phase of the growth of amyloid fibrils. Accurate kinetic measurements have allowed the influence of extrinsic species such as molecular chaperones to be determined and provide promise for further elucidation of mechanistic details. Practical benefits such as high sensitivity, high sample throughput, short measurement times with low volumes and facile incorporation into existing fluidic systems make surface-based biosensors an exciting platform for developing a detailed understanding of amyloid growth processes using quantitative assays.

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