Interactions between Amyloidophilic Dyes and Their Relevance to Studies of Amyloid Inhibitors

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ABSTRACT Amyloid fibrils are filamentous aggregates of peptides and proteins implicated in a range of neurodegenerative disorders such as Alzheimer's and Parkinson's diseases. It has been known almost since their discovery that these β-sheet-rich proteinacious assemblies bind a range of specific dyes that, combined with other biophysical techniques, are convenient probes of the process of amyloid fibril formation. Two prominent examples of such dyes are Congo red (CR) and Thioflavin T (ThT). It has been reported that in addition to having a diagnostic role, CR is an inhibitor of the formation of amyloid structures, and these two properties have both been explained in terms of the same specific noncovalent interactions between the fibrils and the dye molecules. In this article, we show by means of quartz-crystal microbalance measurements that the binding of both ThT and CR to amyloid fibrils formed by the peptide whose aggregation is associated with Alzheimer's disease, Aβ(1–42), can be directly observed, and that the presence of CR interferes with the binding of ThT. Light scattering and fluorescence measurements confirm that an interaction exists between these dyes that can interfere with their ability to reflect accurately the quantity of amyloid material present in a given sample. Furthermore, we show that CR does not inhibit the process of amyloid fibril elongation, and therefore demonstrate the ability of the quartz-crystal microbalance method not only to detect and study the binding of small molecules to amyloid fibrils, but also to elucidate the mode of action of potential inhibitors.

INTRODUCTION

The use of fluorescent probes to measure chemical and physical processes in biological systems is well established (1). The fluorescent entities can be intrinsic to the molecule under study, for example, tryptophan in proteins (2); covalently attached to it, as in fluorescence resonance energy transfer experiments (3); or able to interact noncovalently with the structures of interest, as in Thioflavin T (ThT) fluorescence assays of amyloid fibril formation and growth (4). This latter example is of increasing practical and fundamental interest, due to the implication of amyloid fibrils, highly ordered protein aggregates, in a variety of disorders including Alzheimer's and Parkinson's diseases (5).

To gain insight into any process under study using fluorescence probes, knowledge is required of the fundamental steps that link the observed fluorescence signal with the molecular events that are taking place. The study of amyloid fibril formation is a particular case where fluorescent dyes such as ThT have been used as probes for many decades, although despite extensive experimental (6–8) and computational (9–11)) studies, there is no definitive agreement as to the exact mechanism, stoichiometry, and mode of binding to these supramolecular proteinacious fibrils (12). Several distinct ThT binding sites on amyloid fibrils have been identified that can differ in their affinities and binding modes. For example, it has been reported that ThT binds in the form of large micelles (6), but also that it binds in monomeric form (7). Molecular dynamics simulations (10,11) seem to suggest that the binding occurs in monomeric form. However, it is difficult to simulate high concentrations of dye molecules; this difficulty might lead to a bias toward monomeric forms of the dye in simulations.

Whereas the shift in the Congo red (CR) absorption spectrum and its induced birefringence upon binding are commonly used as a qualitative test for the presence of amyloid structures (13), ThT fluorescence assays have been established as a more quantitative method for kinetic studies in particular. When ThT binds to β-sheet-rich amyloid fibrils, its absorption maximum and fluorescence spectrum undergo a red shift and the fluorescence emission at 480 nm upon excitation at 440 nm is within certain limits commonly assumed to be proportional to the quantity of amyloid fibrils present. This property can then be used to follow time courses of amyloid growth, and such kinetic studies give valuable insight into the molecular mechanism of amyloid formation. Moreover, reduction in ThT fluorescence is often taken to be an indication of the inhibition of this macromolecular self-assembly process. The quest for potent inhibitors is a very active research direction (14) due to the close connection between amyloid formation and neurodegenerative and other diseases (5). CR is an example of a compound the inhibitory properties of which have been studied, but published results on its effects have given rise to very different conclusions (15–20). Moreover, in the context of studies of potential inhibitors of amyloid formation, it has been shown recently that inhibitor candidates that are themselves fluorescent can interfere with the

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ability of dye fluorescence to reflect accurately the quantity of amyloid present (21).

To explore avenues that avoid such interference effects, label-free approaches focusing on the use of biosensors, including surface plasmon resonance and quartz-crystal microbalances (QCM), have been developed and applied to probe the growth of amyloid fibrils (22–27). Surface plasmon resonance has also been used to study the binding of amyloidophilic dyes to surface-bound protein aggregates (28,29).

Equilibrium binding constants describing the interaction between amyloidophilic dyes and protein aggregates, as well as their kinetics of binding, have been widely studied (6,8,12,30,31)). In this article, we describe the use of a QCM method to probe the dye binding to surface-attached amyloid fibrils and use the label-free nature of the experimental technique to probe the binding process. The results of these experiments, in conjunction with complementary solution-state light scattering and fluorescence experiments, reveal that an interaction occurs between the dyes CR and ThT that interferes with the ability of ThT to reflect accurately the quantity of amyloid fibrils present. In addition, we demonstrate that CR does not influence the kinetics of amyloid fibril elongation and that therefore, the observed inhibitory effects are likely to involve interference at earlier stages of the process of fibril formation.

MATERIALS AND METHODS

Amyloid fibril surface attachment and QCM experiments

The covalent surface attachment of Aβ(1–42) amyloid fibrils (Fig. 1, *a* and *b*) closely follows previously published protocols (32). All chemicals were from Sigma Aldrich (Gillingham, United Kingdom) unless stated otherwise. Briefly, gold-coated QSX 301 QCM crystals (Q-Sense, Västra Frölunda, Sweden) were immersed in a solution of 0.5 mg/ml mercaptoundecanoic acid in ethanol for 24 h. The crystals were rinsed with ethanol and dried with nitrogen gas. They were then immersed in an aqueous solution of 0.3 mg/ml N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride and 0.5 mg/ml N-hydroxy succinimide for 20 min, rinsed with water, and dried with nitrogen gas.

Aliquots of 50 μg Aβ(1–42) (Bachem, Basel, Switzerland) in 10 μl 0.01 M NaOH were diluted into 400 μl of 100 mM PB, pH 7.4, and 100 μl of the resulting solution were put onto each biosensor for ~15 min in an atmosphere of 100% humidity. This preparation of an Aβ fibril suspension is reliant on the presence of preformed seeds in the commercial dry peptide, and its kinetics therefore subject to a certain variability. The crystals were then rinsed with water and incubated with 100 μl of a 0.7 M ethanolamine solution (pH 9 with HCl) for 20 min. Finally, the crystals were rinsed with water and put into the microbalance flow cell (Q-Sense E4). As a control experiment, for some sensors no fibrils were attached; rather, the self-assembled monolayer was directly passivated with ethanolamine. The sensors were left to equilibrate overnight in 100 μM PB, pH 7.4.

When a stable baseline was attained, the QCM measurements were carried out. To maximize the coverage of the QCM crystal surface with Aβ(1- 42) amyloid fibrils, the surface-bound seed fibrils were brought into contact with a solution of the Aβ(1–42) peptide for ~30 min (Fig. 1 *c*) to allow the seed fibrils to elongate. Fig. 1 shows the record of this growth period followed via the decrease in resonant frequency. The crystals with the Aβ(1–42) fibrils attached were then used for the study of the dye-amyloid interactions. The dye solutions (50 μM Congo red and 100 μM ThT) were flowed through the liquid cell using a peristaltic pump at a flow rate of 150 μl/min. For the analysis, the frequency overtone at 15 MHz was used (N = 3).

The peptide used for the preparation of the surface-bound initial seed fibrils was used as received from the supplier. However, the peptide that was to be used for the subsequent growth was treated with trifluoroacetic acid and hexafluoroisopropanol, as described previously (20), to minimize the number of oligomers and seeds present in the initial solution. This procedure is necessary to direct the growth entirely to the surface-bound seeds.

The kinetic experiments probing the inhibitory effect of CR on elongation of the surface-bound fibrils (see Fig. 4) were carried out in an analogous manner, except for a difference in duration of the initial fibril attachment. We incubated the sensors for only 5–10 min to avoid the fibrils becoming too long before the kinetic experiments began. For the inhibition study, the fibrils were first exposed to an ~2-μM solution of soluble Aβ(1–42) in 100 mM PB at pH 7.4, then twice to a solution of 50 μM CR in the same buffer, and then to a mixture of 2 μM Aβ(1–42) and 50 μM CR. The rate of elongation of the seed fibrils on the sensor surface was constant during the time course of the experiment, as judged from the constant rate of change of resonant frequency. On this timescale, there is no appreciable depletion of the peptide within the liquid cell. We fitted the frequency shifts with linear functions and compared the slopes, which are proportional to the elongation rates, in the absence and presence of CR.

Dynamic-light-scattering experiments

Solutions of 50 μM CR in 100 mM PB, pH 7.4, and 30 μM ThT in the same buffer were freshly prepared and the size distribution was measured using dynamic light scattering (DLS) with a Malvern Zetasizer (Malvern Instruments, Malvern, United Kingdom). The ThT solution was weakly scattering and could not readily be distinguished from pure buffer. A 1:1 mixture of these solutions was then prepared, i.e., a solution containing 25 μM CR and 15 μM ThT, and the size distribution was again measured. The presence of larger aggregates in the more highly concentrated ThT solution made it necessary to decrease the initial ThT concentration for the light-scattering experiments to 30 μM to facilitate detection of the formation of ThT-CR clusters.

ThT fluorescence experiments

The fluorescence spectra of solutions of 1.2 μM ThT were measured with a UV/VIS fluorimeter (constant excitation wavelength 440 nm, emission scanned from 450 to 600 nm). Then, the fluorescence of a solution of 2 μM Aβ(1–42) fibrils and 1.2 μM ThT was measured, the initial ThT fluorescence background was subtracted from these data, and the resulting measurements were plotted (see Fig. 3 *b*). Then, 1.2 μM of Congo red were added, the solution was mixed thoroughly, and the fluorescence spectrum was immediately measured again. The resulting spectrum, again after subtracting the ThT background fluorescence, is also plotted (see Fig. 3 *b*).

Atomic force microscope imaging

The atomic force microscope (AFM) images shown here were acquired with tapping mode in air using a PicoPlus (Molecular Imaging, Tempe, AZ) AFM. The QCM sensors were imaged after rinsing with water and drying.

RESULTS AND DISCUSSION

To study the binding of small molecules to amyloid fibrils, we attached short, freshly grown Aβ(1–42) amyloid fibrils to the gold surface of a QCM crystal, following established

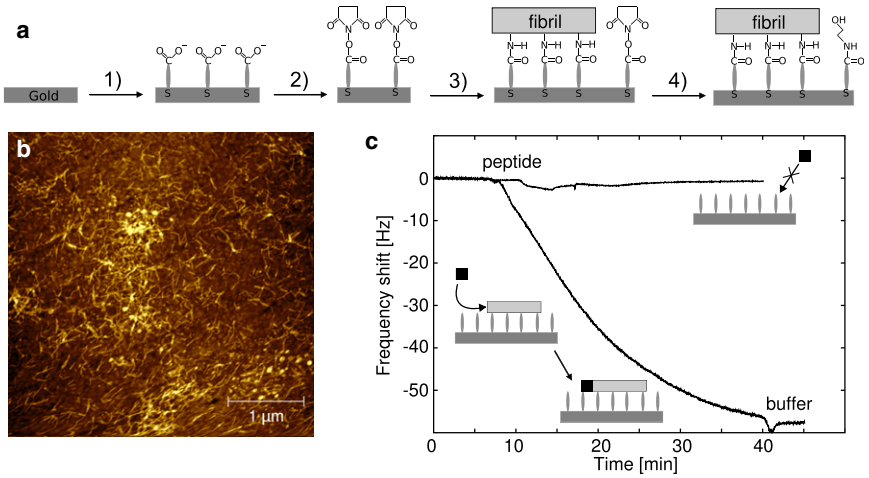

FIGURE 1 (*a*) Covalent attachment of Aβ(1–42) amyloid fibrils to the gold surface of a QCM crystal (see Methods section for details), showing formation of a self-assembled monolayer (SAM) of mercaptoundecanoic acid (*1*); activation of the SAM with a mixture of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride and NHS (*2*); coupling of Aβ(1–42) amyloid fibrils to the activated SAM (*3*); and passivation of the remaining surface with ethanolamine (*4*). (*b*) AFM image of the QCM crystal after attachment of the amyloid fibrils. (*c*) QCM experiment monitoring the growth of the surface-bound Aβ(1–42) amyloid fibrils. After ~10 min, a 2-μM solution of the Aβ peptide in 100 mM PB, pH 7.4, was introduced and the elongation of the surface-bound fibrils was monitored via the decrease in resonant frequency of the overtone at 15 MHz (n = 3). After 40 min, the liquid cell was flushed with buffer, which stopped the elongation. For comparison, a typical frequency trace is shown from an experiment (*horizontal line*) where soluble Aβ(1–42) is brought into contact with a QCM chip with no attached fibrils, only covered by a passivated SAM.

protocols (32) (see Methods section for details). Fig. 1 *a* shows the steps of the covalent attachment process, and the AFM image in Fig. 1 *b* demonstrates the resulting successful attachment. The AFM image suggests that the surface-bound protein is mainly fibrillar in nature; however, we cannot exclude that in this step, small oligomers could also attach to the QCM sensor surface. However, to maximize the signal resulting from the binding of the dye molecules specifically to fibrillar material, we incubated the surface with a solution of soluble Aβ(1–42) to increase the length of the attached fibrils and therefore the mass of fibrillar material on the sensor. The elongation of the seed fibrils was monitored through the shift in resonant frequency of the QCM (Fig. 1 *c*). During this step, it is highly likely that the peptide predominantly adds on to the preexisting seed fibrils as opposed to creating new aggregates; indeed, incubation of a sensor surface that has no attached fibrils shows <5% of the frequency shift of a sensor with attached seeds (Fig. 1 *c*). We therefore conclude that we do not introduce new fibrils or oligomers onto the surface to an appreciable extent but rather elongate the previously attached fibrils.

Fig. 2 *a* shows the result of a QCM measurement of the binding of CR to amyloid fibrils formed from Aβ(1–42), the peptide implicated in Alzheimer's disease. Contact between the biosenors and a dilute solution of the dye (50 μM in 100 mM PB, pH 7.4) leads to a frequency shift of several Hz. In a control experiment where no fibrils were attached to the surface (Fig. 2 *a*, *upper AFM image*), no change in resonant frequency was observed upon contact with the dye solution.

The observed frequency shift in the presence of fibrils therefore stems from the binding of dye molecules to the amyloid fibrils. With the Sauerbrey QCM-crystal mass sensitivity of 17.7 ng Hz^{-1} cm^{-2}, this shift would formally correspond to a surface density of ~1 dye molecule/nm^2. Since according to the AFM images only part of the surface is coated with amyloid fibrils, this result indicates either that the dye binds in the form of large clusters or that a significant part of the frequency shift arises from a change in the hydrodynamic mass of the crystal (i.e., from coupling to the liquid (33) rather than simply in the inertial mass of the bound dye molecules). The binding of CR is saturable, as the change in resonant frequency is complete within 2 min, and the frequency remains constant even when the injection of dye solution is continued. The finding that the binding of CR to surface-bound amyloid fibrils can be monitored in real time led us to design the sequence of experiments shown in Fig. 1 *b*. These experiments make it possible to 1), probe the binding of ThT to surface-attached amyloid fibrils; and 2), detect any competition between the two dyes for binding sites on the protein fibrils. Previous studies have reported that ThT and CR have very different stoichiometries of binding to fibrils (34) and occupy different binding sites (35). It can be seen that binding of ThT (100 μM solutions in 100 mM PB, pH 7.4) to the fibrils can also be detected and gives rise to a smaller frequency shift than that observed for CR binding. The corresponding control experiment, where a sensor without fibrils was exposed to a solution containing ThT, was carried out (data not shown) and confirmed that the presence of fibrils is necessary to observe a significant change in the resonant frequency. The observed difference in the magnitude of the frequency shifts between CR and ThT binding can be rationalized based on several factors. The signal is dependent on the surface density of fibrils as well as the stoichiometry of binding. Also, the exact mode of binding is likely to influence the frequency response of the QCM: if the dye binds in the form of large micelles, the overall roughness of the surface increases more strongly compared to the situation where the dye binds in monomeric form. The dye concentrations in these QCM experiments for both CR (36) and ThT

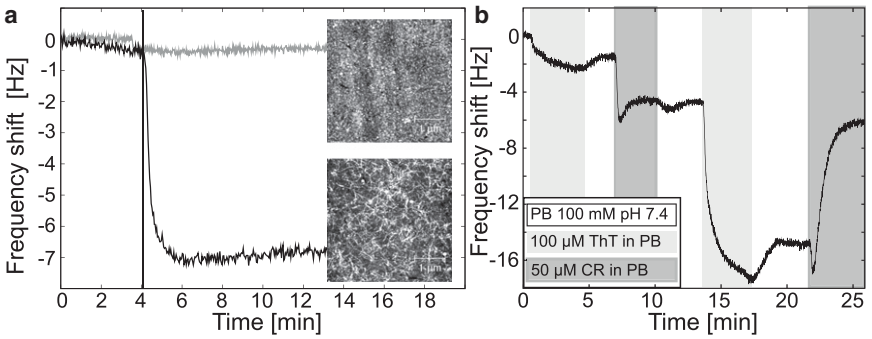

FIGURE 2 (*a*) QCM measurements probing the binding of the amyloidophilic dye Congo red to surface-bound Aβ(1–42) fibrils, with the dye injection site marked (*vertical line*). The binding gives rise to a frequency shift of several Hz (shown is the frequency response of the overtone N = 3). In the absence of amyloid fibrils (*upper AFM image*), no frequency response is detected (*gray line*). (*b*) Binding of ThT to the surface-bound fibrils can be detected in an analogous way. The presence of bound ThT does not prevent the binding of CR. Renewed contact with a ThT solution leads to a drastically increased drop in resonant frequency, compared to the response to the initial contact with ThT; this decrease in frequency can be reversed by subsequent contact with CR solution. These observations strongly suggest that ThT and CR do not bind independently to the fibrils.

(6,7) were above the critical concentration for the formation of supramolecular structures; however, the size of the dye molecule clusters can differ, leading to a different increase in roughness for an equivalent amount of binding and therefore to a differential QCM response (33). The raw frequency shifts can therefore not readily be converted to accurate stoichiometries.

The binding of ThT can be seen from the present data to be partly reversible, as revealed by the increase in frequency upon flushing the surface with fresh buffer. It is interesting to note that our experimental setup allows the creation of a nonequilibrium situation within the QCM cell through continuous flushing with buffer for several minutes and therefore of favorable conditions for measuring the dissociation of high-affinity binders such as ThT (10).

A subsequent incubation with 50 μM CR solution also shows binding, similar to the mechanism shown in Fig. 1 *a*. We were surprised to find, however, that when the ThT solution is injected again into the liquid cell, a large drop in resonant frequency is observed. Even more remarkable, renewed contact with the CR solution leads to an apparent loss in mass that is approximately equal to the original mass associated with the addition of ThT. These observations, which have been confirmed in multiple repeats of these experiments, lead us to the conclusion that the binding of the two dyes to the amyloid fibrils is not independent; rather, the presence of CR has a strong effect on the binding of ThT. This conclusion can be drawn even under the assumption that only part of the frequency shift directly stems from rigidly attached inertial mass of dye molecules; under the assumption of independent binding sites, the frequency shifts induced by the binding of the dyes should be additive regardless of whether or not a change in rigidly attached mass or hydrodynamic mass gives rise to the change in frequency.

To obtain independent experimental insight into the potential interaction between the two dyes revealed by the QCM experiments, we performed DLS experiments (Fig. 3 *a*) with solutions of the individual dyes and with a mixture of the two molecules (see Methods section for details). The DLS measurements indicate that in the latter situation, the structures formed are of considerably larger size than the monomers or small oligomers observed in the corresponding pure dye solutions. Such an intermolecular interaction can be attributed at least in part to the ionic nature of the two dyes: CR is negatively charged at

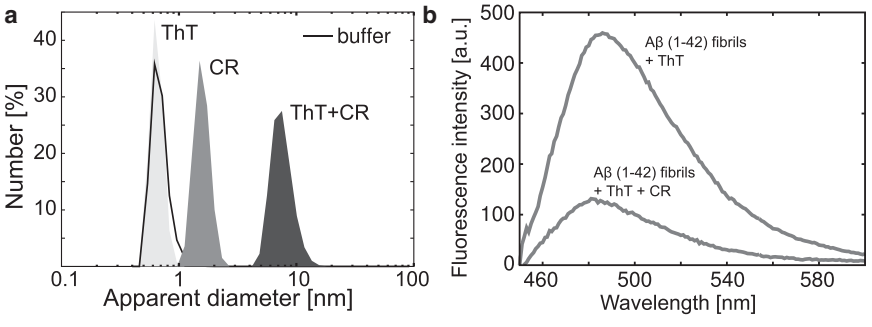

FIGURE 3 (*a*) DLS measurements probing the interactions of CR and ThT. The results of four independent particle-size distribution measurements are shown: 100 mM PB, pH 7.4 (*dark line*), 30 μM ThT solution in PB, 50 μM CR solution, and a 1:1 (vol/vol) mixture of the ThT and CR solutions. The data for the ThT solution are indistinguishable from those for buffer, indicating that the scattering intensity from monomeric/oligomeric ThT is too weak at this concentration to be detected. A mixture of CR and ThT shows the presence of considerably larger-sized structures than observed for the two components separately. (*b*) The fluorescence of a solution of ~2 μM Aβ(1–42) and 1.2 μM ThT was measured (excitation wavelength 440 nm, with ThT background signal subtracted). CR was then added to the sample to obtain a concentration of ~1.2 μM and the fluorescence was again immediately measured. As a result, the observed fluorescence drastically decreases, an effect attributable to the quenching of ThT fluorescence or the displacement of ThT by CR (see text).

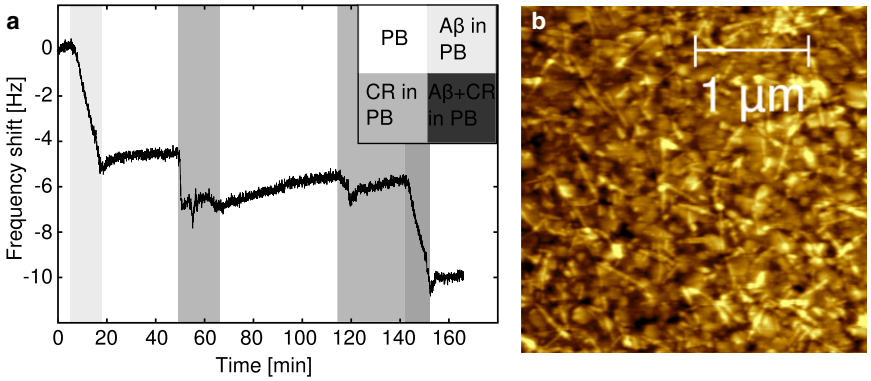

FIGURE 4 (*a*) Probing the influence of CR on the elongation of Aβ(1–42) amyloid fibrils. The surface-bound fibrils were exposed to a 2-μM solution of peptide, and then to a 50-μM solution of CR. Subsequently, they were brought into contact with a solution containing both CR and Aβ(1–42), and the slopes of the resulting frequency shifts, proportional to the elongation rates, were compared. Within experimental error, the fibrils grow with the same rate in the presence or absence of CR. (*b*) AFM image of the QCM crystal after the experiment, showing the absence of nonfibrillar aggregates, confirming that the fibrils grow in the presence of CR.

physiological pH, because of its sulfonic acid groups and the reduced basicity of its amines due to partial delocalization of the nitrogen lone pairs, whereas ThT, since it is a quaternary ammonium cation, is positively charged. The large frequency shift during the QCM experiment described above is consistent with the formation of such CR-ThT clusters on the surface of Aβ(1–42) amyloid fibrils. Indeed, the binding of CR is likely to increase the negative surface charge density of the Aβ(1–42) fibrils (the net charge/peptide monomer at pH 7.4 is -3), as every dye molecule carries a net negative charge, therefore facilitating the binding of the positively charged ThT molecules.

To explore further the significance of this result, we performed ThT fluorescence experiments with Aβ(1–42) amyloid fibrils in the presence and absence of CR (Fig. 3 *b*). The fluorescence was first measured in the absence of CR; then, CR was added and the fluorescence was immediately measured again. The fluorescence spectra obtained in these experiments indicate that the presence of CR leads in this case to a substantial suppression of ThT fluorescence. A similar phenomenon, observed previously (18,34), was interpreted as either fluorescence quenching or displacement of the ThT from the fibrils. It is interesting to note that this effect already occurs at concentrations of ThT and CR that are below their respective critical concentrations for the formation of supramolecular aggregates.

Because our experimental system is not reliant on optical detection, we can study the effects of the presence of CR on the rate of elongation of the Aβ(1–42) fibrils (see Fig. 4 and Methods section for details) without the interference of CR on the signal used to evaluate the kinetics. First, the fibrils were exposed to the soluble Aβ(1–42) peptide in the absence of CR. Then, they were brought into contact first with a solution of CR and then with a mixture of peptide and CR. We then analyzed the slopes of the linear frequency shift induced by the addition of soluble peptide to the surface-bound fibrils, which report on the fibril elongation kinetics (23). We found that the elongation rates in the presence and absence of CR was identical within error (0.46 Hz/min and 0.49 Hz/min, respectively), and we therefore conclude that the presence of CR does not interfere with the process of elongation of the fibrils. Recent work of Lendel et al. shows, however, that soluble Aβ(1–42) peptide can interact with CR micelles (20). It appears, however, that these interactions are not strong enough to prevent the peptide from incorporating into the fibrils. Fibril formation therefore competes successfully with the weaker interactions between peptide and dye, an observation in agreement with the data of Lendel et al. (20).

Taken together, therefore, these results show that the use of ThT to detect amyloid growth requires careful controls if it is to be used in quantitative studies. In particular, care has to be taken to exclude interactions with ThT that can interfere with its potential to reflect accurately the quantity of amyloid present at a given time point. Monitoring a quantity more directly related to the incorporation of soluble peptide into fibrils, namely, the shift of resonant frequency of a biosensor in a QCM, is therefore a powerful and independent method for the study of amyloid growth. In addition, the same procedure can be used to probe the interaction between small molecules and amyloid fibrils. This biosensor approach complements established bulk solution measurements, which probe the effects of small molecules on protein aggregation, as it monitors highly selectively one of the many processes that contribute to the overall aggregation pathway, namely, the elongation of amyloid fibrils. It therefore enables a detailed study of the mechanism of small molecule/amyloid interactions and has the potential to contribute significantly to an effective search for inhibitors of amyloid formation.

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