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The thioflavin T fluorescence assay for amyloid fibril detection can be biased by the presence of exogenous compounds

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Abstract

Thioflavin T (ThT) dye fluorescence is used regularly to quantify the formation and inhibition of amyloid fibrils in the presence of anti-amyloidogenic compounds such as polyphenols. However, in this study, it was shown, using three polyphenolics (curcumin, quercetin and resveratrol), that ThT fluorescence should be used with caution in the presence of such exogenous compounds. The strong absorptive and fluorescent properties of quercetin and curcumin were found to significantly bias the ThT fluorescence readings in both in situ real-time ThT assays and single time-point dilution ThT-type assays. The presence of curcumin at concentrations as low as 0.01 and 1 μM was sufficient to interfere with the ThT fluorescence associated with fibrillar amyloid-b(1-42) (0.5 μM) and fibrillar reduced and carboxymethylated kappa-casein (50 μM), respectively. The ThT fluorescence associated with fibrillar amyloid-b(1-42) was also biased using higher concentrations of resveratrol, a polyphenol that is not spectroscopically active at the wavelengths of ThT fluorescence, implying that there can be direct interactions between ThT and the exogenous compound and/or competitive binding with ThT for the fibrils. Thus, in all cases where ThT is used in the presence of an exogenous compound, biases for amyloid-associated ThT fluorescence should be tested, regardless of whether the additive is spectroscopically active. Simple methods to conduct these tests were described. The Congo red spectral shift assay is demonstrated as a more viable spectrophotometric alternative to ThT, but allied methods, such as transmission electron microscopy, should also be used to assess fibril formation independently of dye-based assays.

Keywords

fluorescence, assay, t, exogenous, thioflavin, presence, biased, be, can, detection, fibril, amyloid, compounds, CMMB

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

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The thioflavin T fluorescence assay for amyloid fibril detection can be biased by the presence of exogenous compounds

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Keywords

amyloid fibril; thioflavin T; congo red; polyphenol; κ -casein

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Thioflavin T (ThT) dye fluorescence is used regularly to quantify the formation and inhibition of amyloid fibrils in the presence of anti-amyloidogenic compounds such as polyphenols. However, in this study, it was shown, using three polyphenolics (curcumin, quercetin and resveratrol), that ThT fluorescence should be used with caution in the presence of such exogenous compounds. The strong absorptive and fluorescent properties of quercetin and curcumin were found to significantly bias the ThT fluorescence readings in both *in situ* real-time ThT assays and single time-point dilution ThT-type assays. The presence of curcumin at concentrations as low as 0.01 and 1 μM was sufficient to interfere with the ThT fluorescence associated with fibrillar amyloid- β (1-42) (0.5 μM) and fibrillar reduced and carboxymethylated κ -casein (50 μM), respectively. The ThT fluorescence associated with fibrillar amyloid- β (1-42) was also biased using higher concentrations of resveratrol, a polyphenol that is not spectroscopically active at the wavelengths of ThT fluorescence, implying that there can be direct interactions between ThT and the exogenous compound and/or competitive binding with ThT for the fibrils. Thus, in all cases where ThT is used in the presence of an exogenous compound, biases for amyloid-associated ThT fluorescence should be tested, regardless of whether the additive is spectroscopically active. Simple methods to conduct these tests were described. The Congo red spectral shift assay is demonstrated as a more viable spectrophotometric alternative to ThT, but allied methods, such as transmission electron microscopy, should also be used to assess fibril formation independently of dye-based assays.

Structured digital abstract

- MINT-7259867: *RCM κ -CN* (uniprotkb:P02668) and *RCM κ -CN* (uniprotkb:P02668) bind (MI:0407) by *electron microscopy* (MI:0040)
- MINT-7258930: *RCM κ -CN* (uniprotkb:P02668) and *RCM κ -CN* (uniprotkb:P02668) bind (MI:0407) by *fluorescence technologies* (MI:0051)
- MINT-7259878: *Amyloid beta* (uniprotkb:P05067) and *Amyloid beta* (uniprotkb:P05067) bind (MI:0407) by *fluorescence technologies* (MI:0051)

Abbreviations

CR, congo red; RCM κ -CN, reduced and carboxymethylated κ -casein; TEM, transmission electron microscopy; ThT, thioflavin T.

Introduction

Over 40 debilitating and incurable human diseases are associated with the failure of a specific peptide or protein to adopt, or remain in, its native functional state [1]. These conditions are referred to as protein misfolding diseases and include pathologies as diverse as Alzheimer's disease, Parkinson's disease, cataracts and type-II diabetes [1,2]. The majority of misfolding diseases are associated with amyloidosis, a pathological state involving the self-aggregation of specific proteins (or protein fragments) into cytotoxic filamentous deposits known as amyloid fibrils, which adopt a characteristic cross- β -sheet structure [1–3].

In this field, extrinsic fluorescence of the benzothiazole dye, thioflavin T (ThT), is widely used for the identification and quantification of amyloid fibrils *in vitro*, and has become the premier technique used to monitor fibrillation kinetics in real-time [4,5]. When ThT is added to samples containing β -sheet-rich deposits, such as the cross- β -sheet quaternary structure of amyloid fibrils, it fluoresces strongly with excitation and emission maxima at approximately 440 and 490 nm, respectively [6,7]. Free ThT in an aqueous environment, however, shows only weak fluorescence, with lower (blue-shifted) excitation and emission maxima at 350 and 440 nm, respectively [6]. Although the mechanism of interaction between ThT and amyloid fibrils remains poorly understood, the most widely accepted theory involves the intercalation of ThT molecules within grooves between solvent-exposed side chains of the amyloid fibril that run parallel to the fibril axis [5,8,9]. Binding within the channels is thought to provide rigidity and to prevent the formation of a less-radiative twisted internal charge-transfer state of the ThT molecule in the excited singlet state, which is normally accompanied by growth of the torsion angle between the benzothiazole and benzene ring from 37° to 90° [10]. As there is a stoichiometric and saturable interaction between ThT and amyloid fibrils, fluorescence from the amyloid–ThT complex provides accurate quantification of amyloid fibril formation as a function of amyloid fibril length and number [4]. In the so-called ThT fluorescence assay, ThT is simply added to samples containing fibril structures at a concentration in excess of the number of potential ThT–fibril binding sites, and steady-state ThT fluorescence emission is monitored at \sim 490 nm (excitation at \sim 440 nm). There are two common variations to this assay: the *in situ* real-time ThT assay, whereby fibril formation is carried out in the presence of ThT, thus providing a means to monitor fibrillation kinetics in real-time; and a single time-point dilution ThT assay,

in which samples of a fibril-forming polypeptide are diluted into buffered solutions of ThT, providing simpler single time-point readings.

Over the past 5 years, a wide range of compounds, known as anti-amyloids (or anti-aggregates), have been reported to prevent amyloid fibril formation, reverse fibrillation and to protect against amyloid fibril-induced cytotoxicity [11–15]. One class of compounds that has received considerable attention is the natural polyphenols, which comprise over 8000 plant-derived molecules found in high concentrations in wine, tea, spices and berries [16,17]. For instance, recent studies of the most abundant polyphenolic extract from green tea, (-)-epigallocatechin-3-gallate, show that it efficiently suppresses fibril formation by α -synuclein and amyloid- β , the amyloidogenic proteins involved in Parkinson's and Alzheimer's diseases respectively, and redirects their aggregation down an off-pathway yielding nontoxic unstructured oligomers [18]. We have recently reported that (-)-epigallocatechin-3-gallate also inhibits fibril formation by the milk protein, reduced and carboxymethylated κ -casein (RCM κ -CN), through a distinct mechanism whereby the protein is constrained in its native-like state [19]. The phenolic aromatic rings of polyphenols are often connected via conjugated systems, resulting in strong $\pi \rightarrow \pi^*$ electronic transitions and making them chromophoric and even intrinsically fluorescent [20–22]. This spectroscopic activity commonly occurs at the principal wavelengths of ThT fluorescence, which in theory makes the ThT fluorescence assay unsuitable for studies where the fibril-forming polypeptide is mixed with such compounds. However, when reviewing the literature on polyphenols, it is found that despite the caution of such practices by LeVine [23], the ThT fluorescence assay is employed widely in the presence of such polyphenols (i.e. as a means to assess their anti-amyloid activity) [24–34]. In fact, it is of concern that in many studies the ThT technique is used as the sole measure of the *in vitro* anti-aggregation efficacy of these compounds [24,27,30,32–34].

In this study, we illustrated the potential unsuitability of ThT as a probe for amyloid fibril formation in the presence of exogenous additives by examining the effect of three polyphenols (curcumin, quercetin and resveratrol) on the fluorescence emission of ThT in both *in situ* real-time ThT assays and single time-point dilution ThT-type assays. We utilized the generic model amyloid fibril-forming protein with which we have considerable experience, namely RCM κ -CN, which is easily prepared and is highly amyloidogenic

under conditions of physiological temperature and pH, and does not require denaturants [35,36]. As theorized, it is found that the presence of the polyphenols significantly biases the RCM κ -CN fibril-associated ThT fluorescence, and these findings are corroborated using fibrillar amyloid- β (1-42) peptide [37]. The use of the absorptive dye, Congo red (CR), is demonstrated as a viable alternative to ThT, but it is also stressed that other methods, such as transmission electron microscopy (TEM) should be used to assess fibril formation independently of dyes.

Results

The coupled effect of polyphenols on amyloid fibril formation and on the *in situ* real-time ThT assay

In order to explore the coupled effects of the selected polyphenolics, curcumin, quercetin and resveratrol (Fig. 1A), on amyloid fibril formation and on *in situ* real-time ThT fluorescence, a solution of native RCM κ -CN (50 μ M) containing ThT (10 μ M) was incubated at 37 $^{\circ}$ C in the presence and absence of the polyphenols (1–100 μ M) and the change in ThT

fluorescence emission was monitored (Fig. 1). As previously reported, the initial decrease in *in situ* ThT fluorescence emission during the first 30 min of incubation is attributable to the samples warming to the incubation temperature [36]. This was confirmed by monitoring the temperature-dependence of ThT fluorescence induced with fibrillar RCM κ -CN (Fig. S1). The ThT fluorescence of RCM κ -CN incubated alone increased rapidly after the equilibration period and reached a plateau after 800–1000 min, which is indicative of RCM κ -CN amyloid fibril formation (0, Fig. 1B–D) [35,36]. The addition of quercetin led to a concentration-dependent decrease in the apparent ThT fluorescence (Fig. 1B), which on its own suggests the efficient concentration-dependent inhibition of fibril formation. Resveratrol, at concentrations up to 100 μ M, had little effect on the ThT fluorescence response up to 400 min, and only a small concentration-dependent decrease in fluorescence was observed at later time-points (Fig. 1C). In stark contrast to the effects of quercetin and resveratrol, the apparent ThT fluorescence was found to vary markedly with the concentration of curcumin (Fig. 1D). At a concentration of 1–10 μ M, curcumin increased the apparent ThT fluorescence, whereas at 50 and 100 μ M, the fluorescence was

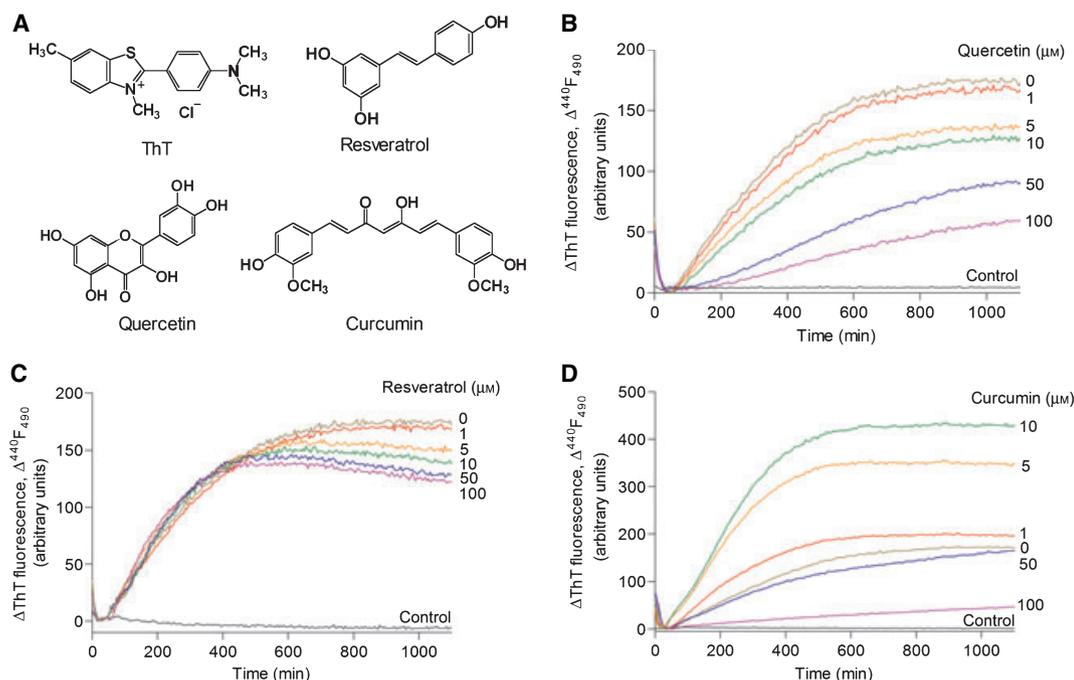


Fig. 1. Effect of the polyphenols, quercetin, resveratrol and curcumin, on RCM κ -CN amyloid fibril formation and the associated increase in *in situ* ThT fluorescence. (A) The structure of ThT and the polyphenols used in this study. (B–D) *In situ* real-time ThT fluorescence assays of solutions of fresh native RCM κ -CN (50 μ M) incubated at 37 $^{\circ}$ C in the presence of quercetin (B), resveratrol (C) and curcumin (D). For each assay, polyphenol (100 μ M) without RCM κ -CN was also monitored (control). Values shown are the mean readings from three individual experiments, and the standard error for each data point is within 4% (not visible).

decreased, implying an inhibition of amyloidogenesis. When the polyphenols were incubated with ThT in the absence of RCM κ -CN, there was no significant change in ThT fluorescence over the course of the incubation period, indicating that they do not form amyloid fibrils themselves, nor are they likely to interact directly with free, solvated ThT (control, Fig. 1B–D).

Curcumin, quercetin and resveratrol affect RCM κ -CN fibril morphology with similar efficacy

The morphological effects of the polyphenols on RCM κ -CN aggregation were assessed simultaneously by TEM (Fig. 2). In agreement with our previous studies [35,36], TEM micrographs of RCM κ -CN alone, taken before fibril formation, indicated that RCM κ -CN exists initially as globular micelle-like species with diameters of ~ 30 nm (Fig. 2A). At plateaus of the *in situ* ThT fluorescence curves (i.e. post-incubation), most prefibrillar entities have been incorporated into mature amyloid fibrils (Fig. 2B). In contrast to the

previous *in situ* real-time ThT fluorescence experiments, all polyphenols (at the same concentration) appeared to affect RCM κ -CN aggregation similarly, giving a combination of stunted fibrillar and prefibrillar assemblies when RCM κ -CN was incubated in their presence (Fig. 2C–E). To investigate this discrepancy between the TEM and real-time ThT assays, in relation to the possibility that the polyphenols bias the apparent ThT fluorescence, the spectroscopic properties of ThT and the polyphenols in the presence of fibrils were examined more closely.

The spectroscopic activity of quercetin and curcumin bias ThT fluorescence

To explore the spectroscopic relationships between ThT and the polyphenols, UV-visible absorption spectra (320–650 nm) of curcumin, quercetin and resveratrol solutions (100 μ M) were recorded (Fig. 3A). The absorption spectrum of RCM κ -CN (50 μ M) + ThT (10 μ M), pre-incubated for 20 h to induce the forma-

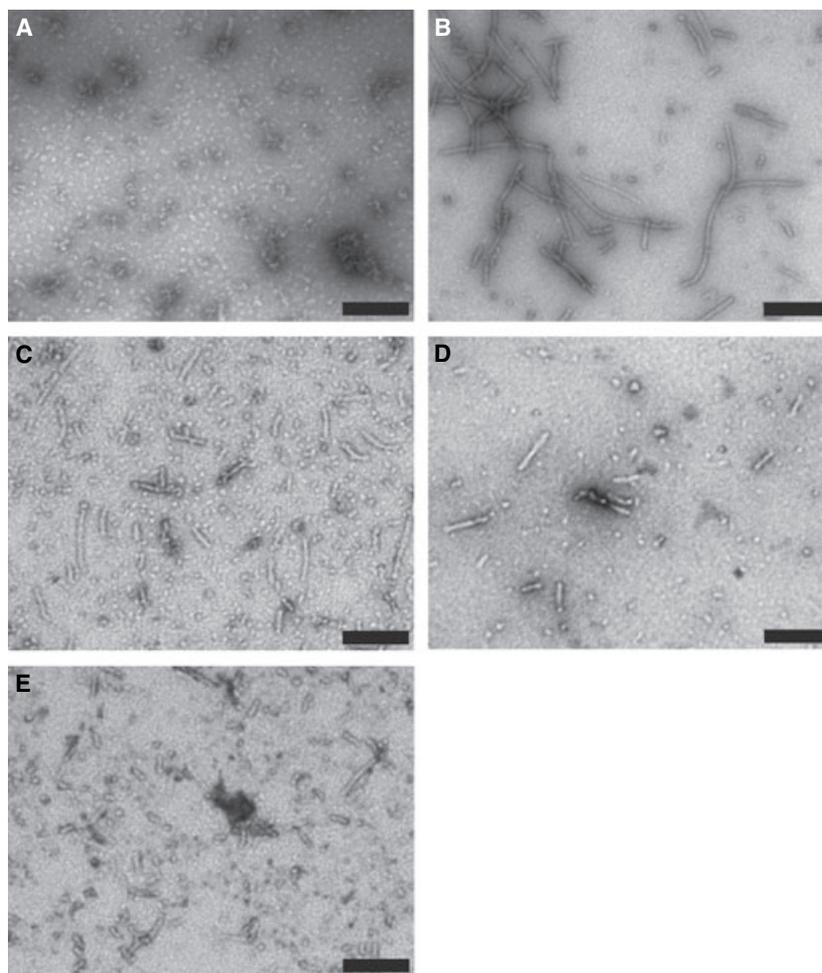


Fig. 2. TEM micrographs of samples from the *in situ* ThT fluorescence assays (see Fig. 1), taken before or after the 1100 min incubation period to investigate the morphological effects of quercetin, resveratrol and curcumin on RCM κ -CN aggregation. (A) Fresh native RCM κ -CN (50 μ M) before incubation, (B) RCM κ -CN alone (50 μ M) following incubation and (C–E) RCM κ -CN (50 μ M) in the presence of polyphenols (100 μ M) after incubation: quercetin (C), resveratrol (D) and curcumin (E). Bars represent 200 nm.

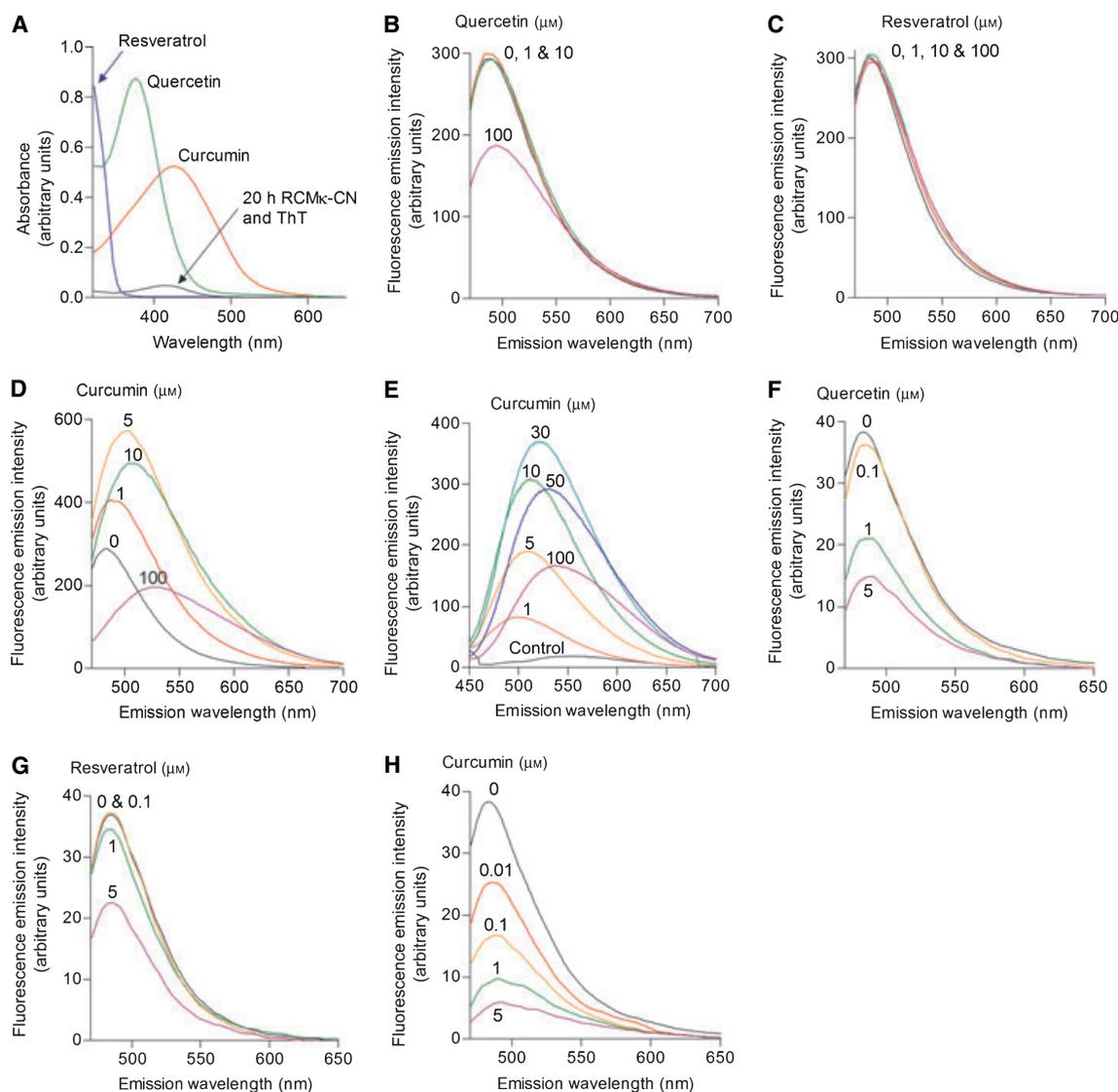


Fig. 3. UV-visible absorption, ThT interaction and steady-state fluorescence emission characteristics of curcumin, quercetin and resveratrol. (A) The absorption spectra (320–650 nm) of curcumin, quercetin and resveratrol solutions (100 μM) are shown. Also shown is the absorption spectrum of pre-incubated fibrillar RCM κ -CN (50 μM) and ThT (10 μM). (B–D) Steady-state ThT fluorescence emission spectra (λ_{ex} 440 nm, λ_{em} 470–700 nm) of pre-incubated fibrillar RCM κ -CN (50 μM) and ThT (10 μM) in the absence of polyphenols (0 μM). Quercetin (B), resveratrol (C) or curcumin (D) were added at various concentrations (1–100 μM) and the fluorescence spectra were re-acquired immediately. (E) Steady-state curcumin fluorescence emission spectra (λ_{ex} 426 nm, λ_{em} 450–700 nm) of fresh native RCM κ -CN (50 μM) with curcumin (1–100 μM). The spectrum of curcumin (100 μM) without RCM κ -CN is also shown (control). (F–H) Steady-state ThT fluorescence emission spectra (λ_{ex} 440 nm, λ_{em} 470–650 nm) of fibrillar amyloid- β (1–42) (0.5 μM) and ThT (5 μM) in the absence of polyphenols (0 μM). Quercetin (F), resveratrol (G) or curcumin (H) were added at various concentrations (0.01–5 μM) and the fluorescence spectra were re-acquired immediately.

tion of fibrils, was also acquired (20 h RCM κ -CN and ThT; Fig. 3A). At these concentrations, the broad absorption bands of the highly conjugated chromophores of quercetin and curcumin (absorption maxima at 374 and 426 nm, respectively) completely dominated the ThT absorption (maxima at approximately 440 nm; i.e. the excitation wavelength for the ThT fluorescence assay). This implies that when quercetin

and curcumin are present in the real-time ThT assay, they will cause significant inner filter effects leading to effective quenching of the ThT fluorescence (i.e. by absorbing both the light intended to excite ThT and the ThT fluorescence radiation emitted). By contrast, resveratrol showed no absorption above 350 nm and is thus unlikely to influence ThT fluorescence on a spectroscopic basis (Fig. 3A).

Next, the direct effect of the polyphenols on the ThT fluorescence emission spectrum (λ_{ex} 440 nm, λ_{em} 470–700 nm) was examined by adding curcumin, quercetin or resveratrol (1–100 μM) to pre-incubated fibrillar RCM κ -CN (50 μM) and ThT (10 μM) (Fig. 3B–D). The fluorescence spectra were acquired immediately after the addition of the polyphenol. As hypothesized, the addition of quercetin at high concentrations (100 μM) significantly decreased the ThT fluorescence yield (37% at 490 nm) (Fig. 3B) and the presence of resveratrol did not alter the ThT fluorescence spectra from fibrillar RCM κ -CN alone (Fig. 3C). Based on these data, the real-time ThT fluorescence assay for RCM κ -CN treated with resveratrol (Fig. 1C) appeared not to be influenced by resveratrol, therefore indicating that resveratrol has little anti-aggregation activity against RCM κ -CN. The concentration-dependent inhibition effect observed for quercetin (Fig. 1B) is, however, clearly affected by quenching of the ThT fluorescence and cannot be attributed solely to an inhibitory effect on fibril formation (compare Fig. 1B with Fig. 2C). Curcumin added at low concentrations (1, 5 and 10 μM) enhanced the apparent emission of ThT fluorescence, but at higher concentrations (100 μM) the fluorescence yield was substantially decreased (Fig. 3D). Furthermore, the spectrum shape was altered, with the maximum emission wavelength increasing (red-shifted) at higher concentrations of curcumin.

These results for curcumin may be explained by recent studies showing that although curcumin is weakly fluorescent in water, it becomes highly fluorescent in aqueous solutions containing detergents and as a result of binding to hydrophobic regions on proteins such as human serum albumin or bovine serum albumin [22,38,39]. This implies that curcumin will become fluorescent in the presence of amyloid fibril-forming proteins, such as RCM κ -CN, which inherently have exposed hydrophobic regions that mediate the process of aggregation [2,36]. Hence, intrinsic curcumin fluorescence may contribute to the apparent real-time ThT fluorescence (Fig. 1D) and this may help to explain the different ThT-interfering effects observed for curcumin and quercetin (compare Fig. 3B with Fig. 3D). To verify this, the steady-state curcumin fluorescence emission spectra (λ_{ex} 426 nm, λ_{em} 450–700 nm) of freshly prepared native RCM κ -CN (50 μM) with curcumin (1–100 μM) were acquired (Fig. 3E). The control curcumin solution (100 μM) showed weak fluorescence. As postulated, for all concentrations of curcumin in the presence of RCM κ -CN (50 μM), the fluorescence yield of curcumin was substantially enhanced. The emission intensity was maximal up to a saturation

concentration of curcumin of between 10 and 50 μM . At higher concentrations, the additional solvated and nonfluorescent curcumin is likely to re-absorb the fluorescence radiation, leading to self-quenching [40]. The curcumin fluorescence spectra are also red-shifted with increasing concentrations, which suggests increased solvent relaxation [40]. Significantly, the wavelength range of curcumin fluorescence emission overlaps significantly with the emission spectrum of ThT fluorescence (compare Fig. 3B–D with Fig. 3E). Hence, the apparent ThT fluorescence spectrum in the presence of curcumin would be influenced by a combination of both the absorptive (subtractive) and fluorescent (additive) properties of free curcumin and solvent-shielded curcumin, respectively (depending on the concentration of curcumin) (compare Fig. 3A with Fig. 3E). These spectral properties provide an explanation for the complex profiles of fluorescence observed for the *in situ* real-time ThT assay for RCM κ -CN aggregation conducted in the presence of curcumin (Fig. 1D).

Dilution ThT assay techniques do not overcome the spectroscopic biases of polyphenols and reveal the occurrence of nonspectral interference effects

In the previous sections it is demonstrated that *in situ* real-time ThT fluorescence assays are heavily biased by the presence of the polyphenols, quercetin and curcumin. These real-time ThT fluorescence assays are performed typically with relatively high concentrations of fibril-forming polypeptide to necessitate amyloidogenesis. However, the effect of any exogenous compound on ThT fluorescence is, of course, relative to its concentration and the magnitude of ThT fluorescence emission (a function of ThT concentration, concentration and type of amyloidogenic protein under study, temperature and the buffer system employed) [23,41]. Exploiting this fact, some studies suggest that the ThT-interfering effects of additives to the ThT fluorescence assay (particularly polyphenols), can be overcome by performing single time-point dilution ThT assays where protein samples are diluted into a ThT solution (i.e. thereby diluting out the effects of the added chromophores while in theory maintaining a sufficiently high intensity of fibril-induced ThT fluorescence for detection) [25,26,28,29].

To investigate these claims and to substantiate the previous findings made using RCM κ -CN, dilution ThT assays were performed using the standard dilute concentration of pre-incubated fibrillar amyloid- β (1–42) (0.5 μM). Curcumin, quercetin or resveratrol was then added at concentration ranges that are typical of those

used in previous studies [24–34] (Fig. 3FH). As seen with RCM κ -CN, the immediate addition of quercetin to fibrillar amyloid- β (1-42) led to a dramatic concentration-dependent decrease in ThT fluorescence (i.e. a 42 and 60 % decrease in fluorescence intensity was observed at 490 nm upon the addition of 1 and 5 μ M quercetin, respectively) (Fig. 3F, compare with Fig. 3B).

The addition of resveratrol, by contrast, had a markedly different effect on the ThT fluorescence induced with amyloid- β (1-42) in comparison to RCM κ -CN. The ThT fluorescence yield was reduced significantly at the higher concentrations of resveratrol, despite resveratrol being spectroscopically inactive in the ThT fluorescence range (Fig. 3A). For example, there was a 39 % decrease in ThT fluorescence intensity at 490 nm in the presence of 5 μ M resveratrol (Fig. 3G, compare with Fig. 3C). These findings clearly indicate that nonspectroscopic-based interferences primarily account for this bias in the ThT fluorescence. Moreover, because there is insufficient time for resveratrol to disrupt the highly stable fibril complexes after its initial addition, the effect is most likely to involve direct interactions between resveratrol and ThT, or competitive binding between resveratrol and ThT for sites along the fibril structure. The ThT fluorescence spectra of the samples with resveratrol were also monitored continuously over a period of 48 h, but there was no significant decrease in intensity after the large initial decrease (data not shown). It therefore appears that an equilibrium involving these nonspectral interactions is rapidly established. This nonspectroscopic interference may not be observed in the same assay with identical molar ratios of resveratrol to fibrillar RCM κ -CN (Fig. 3C) as a result of the inherent variations in the make-up of the amyloid- β (1-42) and RCM κ -CN fibrils, or simply because of the presence of a greater concentration of fibrillar RCM κ -CN for ThT. Alternatively, assuming that a competitive amyloid-resveratrol interaction is involved in both cases, this may suggest that resveratrol has a greater affinity for amyloid- β (1-42) and displaces amyloid-bound ThT.

Curcumin exhibits a clear concentration-dependent inhibitory effect on the ThT fluorescence yield in the presence of preformed amyloid- β (1-42) fibrils, with no significant change observed in the shape of the emission spectrum (Fig. 3H). This effect was also markedly different to that observed with RCM κ -CN (Fig. 3D), implying that, like quercetin, the spectral effects of curcumin are primarily absorptive, such that, at the diluted concentrations, curcumin is not adequately solvent-shielded by the hydrophobic peptide to become fluorescent. Curcumin modulates the ThT fluorescence to the greatest extent of all the studied polyphenols,

giving dramatic interfering effects at a concentration as low as 0.01 μ M. Therefore, it is evident that even after diluting fibril samples, the low concentrations of exogenous additive can still adversely bias the ThT fluorescence response. Moreover, when comparing spectroscopic versus possible nonspectroscopic interference in the cases of quercetin and curcumin, it is clear that spectroscopic effects are the prominent contributor to the interference of ThT fluorescence by these two polyphenols.

CR dye binding as an alternative to ThT

The CR dye is a less commonly used spectrophotometric alternative to ThT for easily quantifying amyloid fibril formation [5,42]. CR is structurally similar to ThT and is believed to bind to β -sheet-rich structures in a similar manner [8,43]. Binding induces a characteristic increase in absorption and red shift in the CR absorption band from 490 to 540 nm [43,44]. Although CR is known to partially inhibit fibril formation and is thus unsuitable for an *in situ*-type assay, its use in single time-point experiments (the CR spectral shift assay) has been well established (i.e. where CR is added to aliquots of fibril-forming polypeptide and the CR absorption band is recorded immediately) [42,45,46]. Because the CR spectral shift assay relies on obtaining absorption spectra at longer wavelengths than used for the ThT fluorescence assay, it potentially enables the quantification of amyloid fibrils in the presence of coloured compounds that interfere with ThT fluorescence (or possibly even other types of ThT-interfering compounds) [7,42].

To investigate this possibility, the CR absorption spectra (450–600 nm) of solutions of pre-incubated fibrillar RCM κ -CN (50 μ M) and CR (20 μ M), with and without added curcumin, quercetin or resveratrol (100 μ M), were recorded (Fig. 4A). The absorption spectra were acquired immediately after the addition of the polyphenol. The spectra of CR (20 μ M) alone was also recorded (CR only, Fig. 4A). For all mixtures, the absorption shoulder at 540 nm from the CR control is indicative of CR binding to amyloid fibrils [43,44]. Importantly, it appears that the polyphenols only affect the CR spectral shift curve as a result of their spectroscopic properties, which can be easily accounted for via subtraction of the absorption spectrum of a control solution. For instance, the CR spectra of fibrillar RCM κ -CN in the presence and absence of quercetin or resveratrol are identical, as both quercetin and resveratrol do not absorb significantly over the range of 450–600 nm (Fig. 3A). However, the chromophore of curcumin does absorb considerably

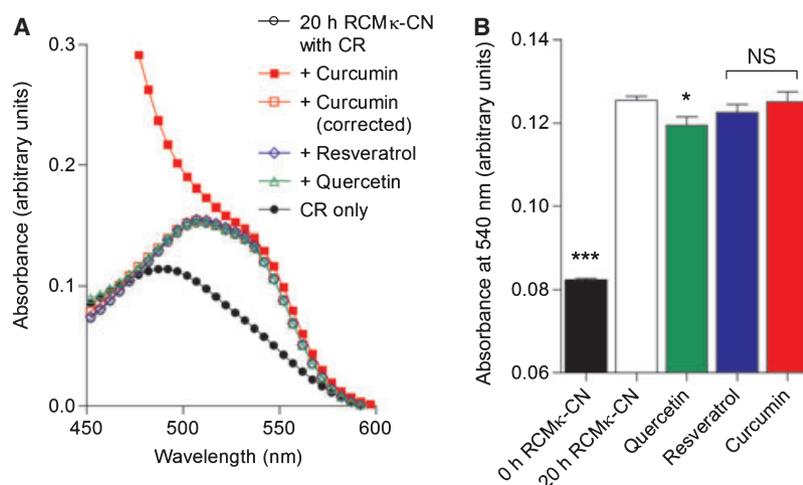


Fig. 4. The Congo red (CR) spectral shift assay as an alternative to ThT fluorescence for quantifying amyloid fibril formation in the presence of curcumin, quercetin and resveratrol. (A) CR absorption spectra (450–600 nm) of pre-incubated fibrillar RCMκ-CN (50 μM) and CR (20 μM) in the absence of polyphenols (20 h RCMκ-CN with CR). Curcumin, quercetin or resveratrol (100 μM) were added and the absorption spectra were re-acquired immediately. The spectrum of CR alone (20 μM) is also shown. The strong spectral contribution from curcumin is clearly visible below 520 nm. This has been accounted for by subtracting the absorption spectrum of a solution containing curcumin alone (100 μM) (see Fig. 3A). (B) CR absorbance, at 540 nm, of fresh native RCMκ-CN (50 μM) or RCMκ-CN (50 μM), which was incubated at 37 °C for 20 h in the presence and absence of quercetin, resveratrol or curcumin (100 μM). All samples have CR (20 μM) added immediately before reading the absorbance. For samples with curcumin, the absorbance readings were corrected for the contribution from the curcumin chromophore by subtracting the absorbance at 540 nm of a solution of curcumin alone (100 μM) (see Fig. 3A). Values represent mean ± SEM ($n = 6$); NS, not significantly different; * $P < 0.05$ and *** $P < 0.001$ versus RCMκ-CN pre-incubated at 37 °C for 20 h alone (one-way ANOVA, Dunnett's post hoc test).

over these wavelengths, but subtraction of the absorption spectrum of the control solution of curcumin alone (Fig. 3A) resulted in a spectrum identical to that of fibrillar RCMκ-CN alone [compare 20 h RCMκ-CN with CR and curcumin (corrected), Fig. 4A]. In a similar assay, performed using native RCMκ-CN (50 μM) instead of fibrillar RCMκ-CN (because CR, like ThT, partially binds native RCMκ-CN), no difference in the absorption spectra were observed in the presence and absence of the polyphenols after the spectral properties of curcumin alone were taken into account (data not shown). Therefore, because the studied polyphenols do not significantly affect CR-binding or its subsequent absorptive properties, it appears that the CR assay may permit accurate quantification of fibril formation in the presence of ThT-interfering compounds, provided that any inherent absorptive properties of the additives can be accounted for.

With that in mind, an experiment similar to the ThT fluorescence assay shown in Figure 1 was performed, using CR spectral shift (i.e. measurement of the absorbance at 540 nm upon the addition of 20 μM CR) to quantify fibril formation before and after incubation, and therefore to determine the anti-fibril effect of the polyphenols. The CR absorbance at 540 nm was recorded for samples of fresh native RCMκ-CN

(50 μM) and RCMκ-CN (50 μM) incubated at 37 °C for 20 h in the presence and absence of curcumin, quercetin or resveratrol at the highest concentration used previously (100 μM) (Fig. 4B). The absorbance of RCMκ-CN alone increased significantly following incubation, which is indicative of CR binding to RCMκ-CN fibrils. All three polyphenols appeared to have similarly poor efficacy to prevent the formation of β-sheet structures capable of binding to CR. Treatment with quercetin showed the greatest reduction in CR absorbance at 540 nm compared with RCMκ-CN alone (14 ± 4%). These findings were consistent with both the TEM data, which showed that all the polyphenols studied had a similar effect on RCMκ-CN aggregation (Fig. 2), and the seemingly unbiased real-time ThT assay for RCMκ-CN with resveratrol, which showed a weak anti-aggregation action (Fig. 1C). Hence, it appears that although curcumin, quercetin and resveratrol significantly modulate the morphology of RCMκ-CN fibril formation, their presence yields little inhibition of the formation of β-sheet-rich structures.

Discussion

In this study, it is shown that ThT fluorescence (excitation and emission maxima at approximately 440 and

490 nm, respectively) should be used with caution when quantifying amyloid fibril formation in the presence of exogenous additives such as polyphenols. By employing both single time-point dilution ThT assays and *in situ* real-time ThT assays in the presence of standard assay concentrations of curcumin, quercetin and resveratrol, we demonstrated that such compounds can significantly bias fibril-associated ThT fluorescence.

Quercetin was strongly absorptive in the spectral region of ThT fluorescence (Fig. 3A) and at higher concentrations (relative to the concentration of fibrils) led to severe inner filter effects and concentration-dependent quenching of the ThT fluorescence intensity (Fig. 1B, 3B and 3F). Free solvated curcumin was also highly absorptive in the spectral range of ThT (Fig. 3A). In addition, whilst curcumin is only weakly fluorescent in water, it becomes highly fluorescent in hydrophobic environments, such as when it interacts with an amyloidogenic protein (Fig. 3E) [22,39]. Based on studies of curcumin and other hydrophobic proteins, including human serum albumin and bovine serum albumin, it is believed that such hydrophobic species act as vehicles in which curcumin is carried and protected from exposure to solvent [22,39]. These fluorescent properties of curcumin also overlapped the wavelengths of ThT fluorescence (Fig. 3E). Hence, the *in situ* real-time ThT fluorescence assay was affected by both the strong absorptive and fluorescent properties of free curcumin and solvent-shielded curcumin, respectively (depending on its concentration, compare Fig. 1D with Fig. 3D). However, in the absence of sufficient amyloid fibrils to elicit curcumin fluorescence, as was the case in the single-point dilution ThT assay, the absorptive properties of curcumin dominate and a concentration-dependent quenching effect was observed (Fig. 3H). Importantly, in the presence of fibrillar amyloid- β (1-42) (0.5 μM) and fibrillar reduced and carboxymethylated κ -casein (RCM κ -CN) (50 μM) the biasing effects of curcumin were found to extend to curcumin concentrations as low as 0.01 and 1 μM , respectively (Fig. 3D,H).

Resveratrol had no significant absorption within the spectral ranges of ThT fluorescence (Fig. 3A). The real-time ThT assay for RCM κ -CN treated with resveratrol thus appears to be able to quantify fibril formation, indicating that resveratrol does not prevent RCM κ -CN aggregation efficiently (Fig. 1C and 3C). However, in the dilution ThT assay, resveratrol at higher concentrations (5 μM) significantly decreased the ThT fluorescence induced by fibrillar amyloid- β (1-42) (Fig. 3G), suggesting that nonspectroscopic effects of polyphenols can also play a role in biasing

ThT fluorescence. As this interfering effect was observed immediately after the addition of resveratrol to the ThT-fibrillar amyloid- β (1-42) complex, and its magnitude did not change over time, it is unlikely to be attributable to fibril dissolution. Rather, the results suggest that either resveratrol interacts directly with ThT (in the amyloid-bound or unbound state), decreasing its fluorescence intensity, or resveratrol competitively binds with ThT for β -sheet-rich sites along the length of fibrils and is able to displace ThT from these sites. This finding is perhaps not unexpected in light of the similar aromatic structures of ThT and polyphenols (Fig. 1A).

The nonspectral biases identified using resveratrol are an important finding of this work because they indicate that an exogenous compound added in ThT fluorescence assays does not necessarily have to be spectroscopically active to bias the ThT fluorescence response. For example, one concern is that because ThT exists in micelles at the concentrations used to monitor amyloid fibrils (~ 5 –20 μM) [41,47], and the added compounds that modulate fibril formation are often hydrophobic (a property which helps direct them towards the inherently hydrophobic amyloid core), they are likely to incorporate themselves into the ThT micelles, which may affect the solution-state spectroscopic properties of the ThT [47]. Hence, the results presented herein clearly highlight the need to investigate for interference to ThT fluorescence when any extrinsic compound is to be used in combination with the ThT chromophore. This is, of course, particularly relevant for conjugated aromatic molecules such as polyphenols, which are typically spectroscopically active in the ThT fluorescence range. Assessment should first involve establishing any UV-visible absorbance and fluorescence properties of the exogenous compound in the presence and absence of preformed fibrils (at the intended ThT assay concentrations), to ensure that there is no spectral activity in the regions of 440 and 490 nm. Additionally, nonspectroscopic-based interference of ThT fluorescence should then be investigated by adding the exogenous compound to samples of preformed fibrils and ThT, and monitoring for any changes in the ThT fluorescence emission spectra over short time frames.

Herein, we also explored the use of the CR dye spectral shift assay (absorbance at 540 nm) as a potential alternative to the ThT fluorescence assays. It was found that the absorptive contributions of the three studied polyphenols could be accounted for via subtraction in this assay and therefore allowed the quantification of fibril formation (Fig. 4A). In using this technique to assess the anti-amyloid activity of the

polyphenols (Fig. 4B), it was shown that the polyphenols are all poorly efficacious at preventing RCM κ -CN from forming β -sheet structures to which CR is capable of binding. Complementary TEM analysis indicated that despite their weak ability to inhibit β -sheet formation, the polyphenols dramatically alter the morphology of RCM κ -CN aggregation (Fig. 2). Their presence yields a combination of stunted fibrillar and prefibrillar RCM κ -CN assemblies, indicating that they may interfere with the monomer/oligomer stacking events that occur during fibril elongation [1,2]. It is worth noting that the CR spectral shift approach assumes that even if the added exogenous compound affects fibril formation, its contribution to the CR spectrum will remain unchanged. Furthermore, given that the CR dye is also similarly aromatic in structure to ThT [5], the potential for nonspectral interference remains (even though we found no evidence of this in our system). Therefore, in all circumstances where a fibril-forming polypeptide is in the presence of an added exogenous compound, complementary methods should be used to assess fibril formation independently of ThT fluorescence and CR spectrophotometric assays (e.g. electron microscopy or atomic force microscopy to visualize fibril morphology, X-ray fibre diffraction to confirm cross- β -sheet structure, or even other optical techniques, such as using the mid-UV absorbance of the peptide bond or intrinsic fluorescence of the added compound) [7,48–50].

Experimental Procedures

Materials

Unless specified otherwise, all materials and reagents used were of analytical grade and were obtained from the Sigma Chemical Company (St Louis, MO, USA). Black μ Clear 96-microwell plates were supplied by Greiner Bio-One (Stonehouse, UK) and Thin Seal self-adhesive plate covers were purchased from Excel Scientific (Chicago, IL, USA). Carbon-coated 400-mesh nickel TEM grids were purchased from SPI Supplies (West Chester, PA, USA) and uranyl acetate was bought from Agar Scientific (Stansted, UK). κ -Casein was reduced and carboxymethylated as described by Shechter *et al.* [51], with minor modifications as per Farrell *et al.* [52]. Lyophilized RCM κ -CN was stored at -20 °C and all solutions of native RCM κ -CN were prepared in sodium phosphate buffer (50 mM, pH 7.4) at 4 °C immediately before use. The desired RCM κ -CN concentration of 50 μ M was achieved by standard spectrophotometric methodology using a CARY 5000 UV-Vis-NIR spectrophotometer (Varian, CA, USA), a 1-cm path-length cuvette and a molar extinction coefficient of 0.95 mL \cdot mg $^{-1}$ \cdot cm $^{-1}$ at

280 nm [53]. Fibrillar RCM κ -CN solutions (50 μ M) were prepared by pre-incubating native RCM κ -CN solutions (50 μ M) at 37 °C for 20 h to permit the formation of mature amyloid fibrils [35,36]. Amyloid- β (1-42) was purchased from AnaSpec Inc. (San Jose, CA, USA) and a 1-mg sample was reconstituted in 60 μ L of ammonium hydroxide buffer (1%), as directed by the manufacturer. The reconstituted peptide was then diluted in sodium phosphate buffer (50 mM, pH 7.4) to 25 μ M and incubated at 37 °C for 4 h with vigorous shaking to induce the formation of mature amyloid- β (1-42) fibrils. After fibrillation, the fibril solution was diluted to the desired concentration of 0.5 μ M in glycine-NaOH buffer (50 mM, pH 8.5) to enhance the ThT fluorescence induced by the dilute amyloid- β (1-42) fibrils [23]. MilliQ water and all buffers were filtered using a 0.2 μ m syringe filter before use.

Preparation of samples for assay

The polyphenols, curcumin, quercetin and resveratrol, were first solubilized in dimethylsulfoxide to stock concentrations of 0.001, 0.01, 0.1, 0.5, 1, 3, 5 and 10 mM. To achieve the desired molar ratio of polyphenol to native or fibrillar protein (as indicated in each figure), the polyphenol stocks were added to protein solutions (prepared as described above) such that the final concentration of dimethylsulfoxide was 1% (v/v) and the protein solution was not significantly diluted. The residual concentration of dimethylsulfoxide was confirmed to have no significant effect on fibrillation or ThT/CR binding (data not shown). Both quercetin and curcumin are not soluble above 100 μ M without using higher concentrations of dimethylsulfoxide. Samples of polyphenol alone were prepared by substituting the protein solution for the same volume of sodium phosphate buffer. Where appropriate, ThT (5 or 10 μ M) or CR (20 μ M) was added to the samples immediately before the assay from stock solutions of ThT and CR (200 μ M) prepared in the appropriate assay buffer. It was established that a ratio of 20 μ M CR to 50 μ M native and fibrillar samples of RCM κ -CN resulted in the most practicable and detectable change in CR absorbance at 540 nm as a result of the presence of fibrils (data not shown) and these concentrations were therefore used in these experiments.

In situ ThT fluorescence assay

Samples (200 μ L) with ThT were incubated at 37 °C in black μ Clear 96-microwell plates that were sealed to prevent evaporation. The ThT fluorescence intensity of each sample was recorded every 5 min using a FLUOstar OPTIMA plate reader (BMG Labtechnologies, Melbourne, VIC, Australia) with 440/490-nm excitation/emission filters set. As native RCM κ -CN binds some ThT as a result of its β -sheet content [52], the ThT data were normalized by

plotting the change in ThT fluorescence (in arbitrary units, a.u.), ΔF , as determined by the equation: $\Delta F = F - F_{30}$, where F is the mean ThT fluorescence reading (in arbitrary units, a.u.) from triplicate experiments and F_{30} is the mean ThT fluorescence reading (in arbitrary units, a.u.) after 30 min of incubation once all samples had evenly equilibrated to 37 °C. The presence of ThT at the concentration used did not affect RCM κ -CN fibril formation [35].

TEM

Aliquots (2 μ L) were taken from samples of the *in situ* ThT fluorescence assays, before or after the 1100 min incubation period (as indicated in each figure), and were transferred onto the surface of carbon-coated 400-mesh nickel TEM grids. The grid surface was then washed with MilliQ water passed through a 0.2- μ m syringe filter (3 \times 10 μ L) and negatively stained with 10 μ L of uranyl acetate solution [2% (w/v), in MilliQ water]. Between each wash, and after staining, excess solution was removed by blotting with filter paper. The grid was left to air dry and was then viewed under 25 000–64 000 \times magnification using a Philips CM100 Transmission Electron Microscope (Philips, Eindhoven, the Netherlands) with an 80 kV excitation voltage.

Absorption and fluorescence emission spectra

UV-visible solution-state absorption spectra, or single-wavelength absorbance of samples with and without ThT or CR (as indicated in each figure), were recorded using a CARY 5000 UV-Vis-NIR spectrophotometer and 0.2-cm path-length cuvettes. Steady-state fluorescence emission spectra of samples were acquired using a Cary Eclipse fluorescence spectrophotometer (Varian, CA, USA) and a 0.2-cm path-length cuvette with the excitation and emission slit widths set at 10 and 20 nm, respectively. All absorption and fluorescence spectra were recorded at room temperature to prevent significant amyloid fibril formation by RCM κ -CN over the period of spectral acquisition. The absorption spectrum of buffer was subtracted from all sample absorption spectra. Fluorescence emission spectra were corrected for minor contributions from scattered excitation radiation by subtracting the spectrum of buffer alone.

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References

- 1 Chiti F & Dobson CM (2006) Protein misfolding, functional amyloid, and human disease. *Annu Rev Biochem* **75**, 333–366.
- 2 Ecroyd H & Carver JA (2008) Unraveling the mysteries of protein folding and misfolding. *IUBMB Life* **60**, 769–774.
- 3 Westermark P, Benson MD, Buxbaum JN, Cohen AS, Frangione B, Ikeda S, Masters CL, Merlini G, Saraiva MJ & Sipe JD (2005) Amyloid: toward terminology clarification. Report from the Nomenclature Committee of the International Society of Amyloidosis. *Amyloid* **12**, 1–4.
- 4 Bolder SG, Sagis LM, Venema P & van der Linden E (2007) Thioflavin T and birefringence assays to determine the conversion of proteins into fibrils. *Langmuir* **23**, 4144–4147.
- 5 Hawe A, Sutter M & Jiskoot W (2008) Extrinsic fluorescent dyes as tools for protein characterization. *Pharm Res* **25**, 1487–1499.
- 6 LeVine H III (1993) Thioflavine T interaction with synthetic Alzheimer's disease beta-amyloid peptides: detection of amyloid aggregation in solution. *Protein Sci* **2**, 404–410.
- 7 Nilsson MR (2004) Techniques to study amyloid fibril formation in vitro. *Methods* **34**, 151–160.
- 8 Krebs MR, Bromley EH & Donald AM (2005) The binding of thioflavin-T to amyloid fibrils: localisation and implications. *J Struct Biol* **149**, 30–37.
- 9 Biancalana M, Makabe K, Koide A & Koide S (2008) Molecular mechanism of thioflavin-T binding to the surface of beta-rich peptide self-assemblies. *J Mol Biol* **385**, 1052–1063.
- 10 Stsiapura VI, Maskevich AA, Kuzmitsky VA, Turovov KK & Kuznetsova IM (2007) Computational study of thioflavin T torsional relaxation in the excited state. *J Phys Chem A* **111**, 4829–4835.
- 11 Findeis MA (2000) Approaches to discovery and characterization of inhibitors of amyloid beta-peptide polymerization. *Biochim Biophys Acta* **1502**, 76–84.
- 12 Sacchettini JC & Kelly JW (2002) Therapeutic strategies for human amyloid diseases. *Nat Rev Drug Discov* **1**, 267–275.
- 13 Gazit E (2005) Mechanisms of amyloid fibril self-assembly and inhibition. Model short peptides as a key research tool. *FEBS J* **272**, 5971–5978.
- 14 Rochet JC (2007) Novel therapeutic strategies for the treatment of protein-misfolding diseases. *Expert Rev Mol Med* **9**, 1–34.

- 15 Barten DM & Albright CF (2008) Therapeutic strategies for Alzheimer's disease. *Mol Neurobiol* **37**, 171–186.
- 16 Porat Y, Abramowitz A & Gazit E (2006) Inhibition of amyloid fibril formation by polyphenols: structural similarity and aromatic interactions as a common inhibition mechanism. *Chem Biol Drug Des* **67**, 27–37.
- 17 Feng BY, Toyama BH, Wille H, Colby DW, Collins SR, May BC, Prusiner SB, Weissman J & Shoichet BK (2008) Small-molecule aggregates inhibit amyloid polymerization. *Nat Chem Biol* **4**, 197–199.
- 18 Ehrnhoefer DE, Bieschke J, Boeddrich A, Herbst M, Masino L, Lurz R, Engemann S, Pastore A & Wanker EE (2008) EGCG redirects amyloidogenic polypeptides into unstructured, off-pathway oligomers. *Nat Struct Mol Biol* **15**, 558–566.
- 19 Hudson SA, Ecroyd H, Dehle FC, Musgrave IF & Carver JA (2009) (-)-Epigallocatechin-3-gallate (EGCG) maintains κ -casein in its pre-fibrillar state without redirecting its aggregation pathway. *J Mol Biol* **392**, 689–700.
- 20 Banwell CN & McCash EM (1994) *Fundamentals of Molecular Spectroscopy*, 4th edn. McGraw-Hill, London.
- 21 Falkovskaia E, Sengupta PK & Kasha M (1998) Photo-physical induction of dual fluorescence of quercetin and related hydroxyflavones upon intermolecular H-bonding to solvent matrix. *Chem Phys Lett* **297**, 109–114.
- 22 Kunwar A, Barik A, Pandey R & Priyadarsini KI (2006) Transport of liposomal and albumin loaded curcumin to living cells: an absorption and fluorescence spectroscopic study. *Biochim Biophys Acta* **1760**, 1513–1520.
- 23 LeVine H III (1999) Quantification of beta-sheet amyloid fibril structures with thioflavin T. *Methods Enzymol* **309**, 274–284.
- 24 Conway KA, Rochet JC, Bieganski RM & Lansbury PT Jr (2001) Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct. *Science* **294**, 1346–1349.
- 25 Ono K, Yoshiike Y, Takashima A, Hasegawa K, Naiki H & Yamada M (2003) Potent anti-amyloidogenic and fibril-destabilizing effects of polyphenols in vitro: implications for the prevention and therapeutics of Alzheimer's disease. *J Neurochem* **87**, 172–181.
- 26 Ono K, Hasegawa K, Naiki H & Yamada M (2004) Curcumin has potent anti-amyloidogenic effects for Alzheimer's beta-amyloid fibrils in vitro. *J Neurosci Res* **75**, 742–750.
- 27 Kim H, Park BS, Lee KG, Choi CY, Jang SS, Kim YH & Lee SE (2005) Effects of naturally occurring compounds on fibril formation and oxidative stress of beta-amyloid. *J Agric Food Chem* **53**, 8537–8541.
- 28 Ono K, Hirohata M & Yamada M (2005) Ferulic acid destabilizes preformed beta-amyloid fibrils in vitro. *Biochem Biophys Res Commun* **336**, 444–449.
- 29 Ono K & Yamada M (2006) Antioxidant compounds have potent anti-fibrillogenic and fibril-destabilizing effects for alpha-synuclein fibrils in vitro. *J Neurochem* **97**, 105–115.
- 30 Byeon SR, Lee JH, Sohn JH, Kim DC, Shin KJ, Yoo KH, Mook-Jung I, Lee WK & Kim DJ (2007) Bis-styrylpyridine and bis-styrylbenzene derivatives as inhibitors for Abeta fibril formation. *Bioorg Med Chem Lett* **17**, 1466–1470.
- 31 Matsuzaki K, Noguch T, Wakabayashi M, Ikeda K, Okada T, Ohashi Y, Hoshino M & Naiki H (2007) Inhibitors of amyloid beta-protein aggregation mediated by GM1-containing raft-like membranes. *Biochim Biophys Acta* **1768**, 122–130.
- 32 Reinke AA & Gestwicki JE (2007) Structure-activity relationships of amyloid beta-aggregation inhibitors based on curcumin: influence of linker length and flexibility. *Chem Biol Drug Des* **70**, 206–215.
- 33 Akaishi T, Morimoto T, Shibao M, Watanabe S, Sakai-Kato K, Utsunomiya-Tate N & Abe K (2008) Structural requirements for the flavonoid fisetin in inhibiting fibril formation of amyloid beta protein. *Neurosci Lett* **444**, 280–285.
- 34 Byun JH, Kim H, Kim Y, Mook-Jung I, Kim DJ, Lee WK & Yoo KH (2008) Aminostyrylbenzofuran derivatives as potent inhibitors for Abeta fibril formation. *Bioorg Med Chem Lett* **18**, 5591–5593.
- 35 Thorn DC, Meehan S, Sunde M, Rekas A, Gras SL, MacPhee CE, Dobson CM, Wilson MR & Carver JA (2005) Amyloid fibril formation by bovine milk kappa-casein and its inhibition by the molecular chaperones alphaS- and beta-casein. *Biochemistry* **44**, 17027–17036.
- 36 Ecroyd H, Koudelka T, Thorn DC, Williams DM, Devlin G, Hoffmann P & Carver JA (2008) Dissociation from the oligomeric state is the rate-limiting step in fibril formation by kappa-casein. *J Biol Chem* **283**, 9012–9022.
- 37 LaFerla FM, Green KN & Oddo S (2007) Intracellular amyloid-beta in Alzheimer's disease. *Nat Rev Neurosci* **8**, 499–509.
- 38 Adhikary R, Mukherjee P, Kee TW & Petrich JW (2009) Excited-state intramolecular hydrogen atom transfer and solvation dynamics in the medicinal pigment curcumin. *J Phys Chem B* **113**, 5255–5261.
- 39 Leung MH & Kee TW (2009) Effective stabilization of curcumin by association to plasma proteins: human serum albumin and fibrinogen. *Langmuir* **25**, 5773–5777.
- 40 Lakowicz JR (1984) *Principles of Fluorescence Spectroscopy*. Plenum Press, New York.
- 41 Eisert R, Felau L & Brown LR (2006) Methods for enhancing the accuracy and reproducibility of Congo red and thioflavin T assays. *Anal Biochem* **353**, 144–146.

- 42 Klunk WE, Jacob RF & Mason RP (1999) Quantifying amyloid by congo red spectral shift assay. *Methods Enzymol* **309**, 285–305.
- 43 Frid P, Anisimov SV & Popovic N (2007) Congo red and protein aggregation in neurodegenerative diseases. *Brain Res Rev* **53**, 135–160.
- 44 Meehan S, Berry Y, Luisi B, Dobson CM, Carver JA & MacPhee CE (2004) Amyloid fibril formation by lens crystallin proteins and its implications for cataract formation. *J Biol Chem* **279**, 3413–3419.
- 45 Lorenzo A & Yankner BA (1994) Beta-amyloid neurotoxicity requires fibril formation and is inhibited by congo red. *Proc Natl Acad Sci USA* **91**, 12243–12247.
- 46 Klunk WE, Jacob RF & Mason RP (1999) Quantifying amyloid beta-peptide (A β) aggregation using the Congo red-A β (CR-A β) spectrophotometric assay. *Anal Biochem* **266**, 66–76.
- 47 Khurana R, Coleman C, Ionescu-Zanetti C, Carter SA, Krishna V, Grover RK, Roy R & Singh S (2005) Mechanism of thioflavin T binding to amyloid fibrils. *J Struct Biol* **151**, 229–238.
- 48 Ahn JS, Lee JH, Kim JH & Paik SR (2007) Novel method for quantitative determination of amyloid fibrils of alpha-synuclein and amyloid beta/A4 protein by using resveratrol. *Anal Biochem* **367**, 259–265.
- 49 Hamley IW (2007) Peptide fibrillization. *Angew Chem Int Ed Engl* **46**, 8128–8147.
- 50 Riviere C, Richard T, Quentin L, Krisa S, Merillon JM & Monti JP (2007) Inhibitory activity of stilbenes on Alzheimer's beta-amyloid fibrils in vitro. *Bioorg Med Chem* **15**, 1160–1167.
- 51 Shechter Y, Patchornik A & Burstein Y (1973) Selective reduction of cystine I-VIII in alpha-lactalbumin of bovine milk. *Biochemistry* **12**, 3407–3413.
- 52 Farrell HM Jr, Cooke PH, Wickham ED, Piotrowski EG & Hoagland PD (2003) Environmental influences on bovine kappa-casein: reduction and conversion to fibrillar (amyloid) structures. *J Protein Chem* **22**, 259–273.
- 53 Fox PF & McSweeney PLH (1998) *Dairy Chemistry and Biochemistry*. Blackie Academic and Professional, London.

Supporting information

The following supplementary material is available:

Fig. S1. Supporting data shows the temperature-dependence of RCM κ -CN fibril-induced ThT fluorescence.

This supplementary material can be found in the online version of this article.

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