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Development and Application of Methodology for Rapid Screening of Potential Amyloid Probes

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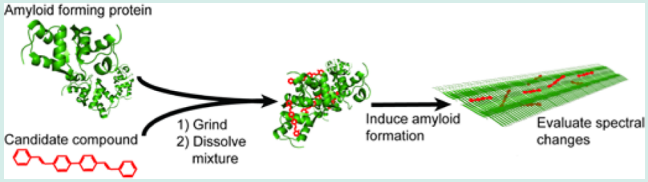
Supporting Information

ABSTRACT: Herein, we demonstrate that it is possible to rapidly screen hydrophobic fluorescent aromatic molecules with regards to their properties as amyloid probes. By grinding the hydrophobic molecule with the amyloidogenic protein insulin, we obtained a water-soluble composite material. When this material is dissolved and exposed to conditions promoting amyloid formation, the protein aggregates into amyloid fibrils incorporating the hydrophobic molecule. As a result, changes in the fluorescence spectra of the hydrophobic molecule can be correlated to the formation of amyloid fibrils, and the suitability of the hydrophobic molecular skeleton as an amyloid probe can thus be assessed. As a result, we discovered two new amyloid probes, of which one is the well-known laser dye DCM. The grinding method can also be used for rapid preparation of novel composite materials between dyes and proteins, which can be used in materials science applications such as organic electronics and photonics.



KEYWORDS: *amyloid probes, rapid screening, fluorescent, aromatic molecules, laser dye DCM*

INTRODUCTION

A large number of proteins are known to aggregate into amyloid plaques or amyloid fibrils in vivo.[1,2] Such amyloid materials are associated with a number of pathological states such as Alzheimers's, Huntinton's, and Parkinson's disease.[1,2] The detection of amyloid materials is thus of significant interest and one of the most important analytical methods is based on the use of optical probes, such as Congo Red or Thioflavin T (ThT).[3−5] The processes by which different proteins aggregate into amyloid materials can also be studied under more controlled conditions in vitro. Under appropriate conditions amyloidogenic proteins will aggregate into amyloid-like fibrils with typical diameters in the nanometer range and lengths in the micrometre range.[1] The structural similarity between amyloid fibrils formed in vivo and amyloid-like fibrils formed in vitro is well established.[1] Both types of structures are built up from protofilaments that in turn are built up from β-strands oriented perpendicularly to the long fibril axis.[1] This leads to a high extent of pleated β-sheet structure that, whether the structures were formed in vivo or in vitro, will lead to specific interactions with dyes such as Congo Red or ThT. These two dyes are at present the benchmark for investigation of the presence of amyloid structures by optical probes.[3−5] Nevertheless, the design, synthesis, and evaluation of dyes with amyloid probing function is an intensively pursued research topic.[6−24] Probes for amyloid fibrils typically consist of a large fraction of aromatic rings that are intrinsically hydrophobic. However, for dyes to function in biological applications they need to be sufficiently water-soluble to enable staining of the amyloid material in an aqueous environment. The two dyes mentioned above, Congo Red and ThT, contains charged groups rendering the molecules hydrophilic (see Figure 1 for molecular structures). In the case of ThT, the positively charged group is part of the chromophore in the form of a methylated nitrogen. Alternatively, a more flexible approach is to use dyes substituted with polar side chains. This is the case for Congo Red where polar sulfonate groups are attached to the aromatic skeleton. Apart from Congo Red and ThT, a variety of optical probes have been synthesized and evaluated to find probes with alternative photophysical, biological, and chemical properties.[6−24] However, the fact that amyloid probes need polar substituents to render them water-soluble introduces complications from an organic synthetic perspective. At present organic synthetic methodology works best in organic solvents and water is commonly regarded as a nuisance preferably minimized by using carefully distilled solvents and inert reaction conditions.[25] Most organic synthetic methodology have thus been developed under water-free conditions and as a result a wide range of commonly employed reagents are highly sensitive to water. A wide variety of interesting chromophores are available from the organic electronics/photonics field where insolubility in aqueous media is the norm. A methodology enabling a quick test of the suitability of a hydrophobic aromatic skeleton as an amyloid probe would thus be of high value as this could significantly speed up the screening process. It should be noted that a common method, enabling the use of slightly hydrophobic compounds, is to use an organic cosolvent miscible with water, such as MeOH, THF, or DMSO. For

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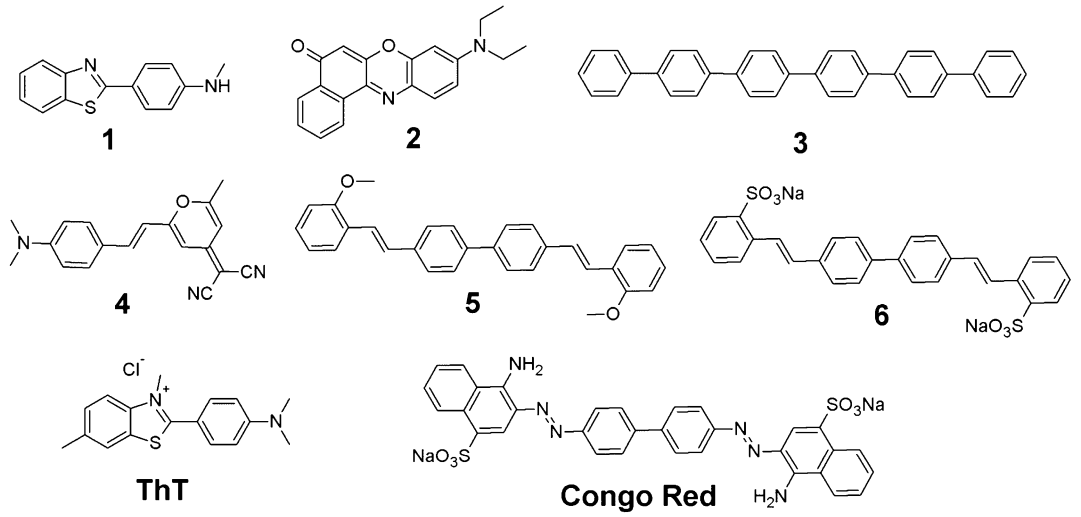

Figure 1. Chemical structures of studied compounds: BTA-1 (**1**), Nile red (**2**), *para*-sexiphenyl (**3**), DCM (**4**), 4,4′-bis(2-methoxystyryl)-biphenyl (**5**), and disodium 4,4′-bis(2-sulfonatostyryl)biphenyl (**6**). For reference, the structures of ThT and Congo Red are shown.

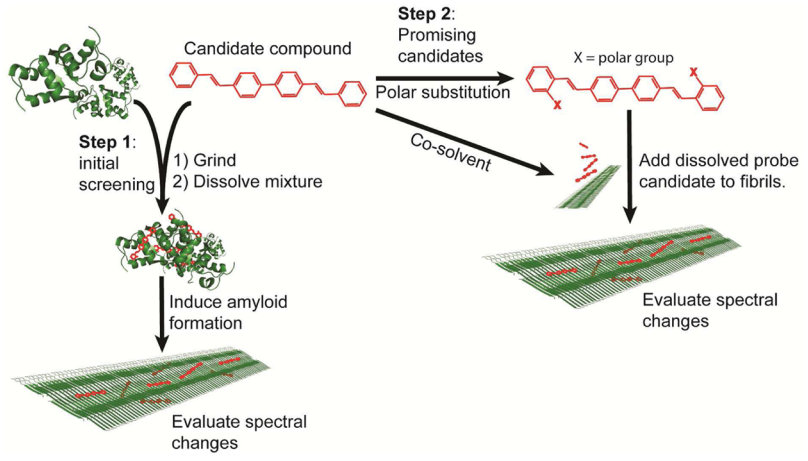

Figure 2. Schematic illustration of the methodology for rapid amyloid probe screening. In step 1, a hydrophobic compound is ground with bovine insulin and after solvation and amyloid formation spectral changes are evaluated. For promising candidates, the corresponding hydrophobic compound substituted with polar groups is tested in step 2, by addition of the potential probe to preformed amyloid fibrils. Alternatively, if the probe is sufficiently hydrophilic a cosolvent miscible with water can be employed.

borderline cases involving slightly hydrophobic compounds this may enable solubility in the organic−aqueous solvent mixture. However, with more strongly hydrophobic compounds this method will lead to dye precipitation or an extremely low concentration of the dye. We recently developed a novel methodology for forming composite materials consisting of hydrophobic molecules and amyloid-like fibrils.[26,27] Bovine insulin, which is a protein capable of forming amyloid-like structures, is mixed with the hydrophobic substance by grinding in the solid state. This results in a composite material soluble in acidic water. Upon appropriate stimuli (i.e., acidic conditions and heating), the protein aggregates into amyloid-like structures incorporating the hydrophobic molecule. Because of the insolubility of the hydrophobic substance it will stay associated with the protein throughout the aggregation process. Therefore, using our methodology we can effectively probe for interactions between the protein and a hydrophobic molecule of our choice. Herein we report our results regarding preparation of composite materials between bovine insulin and a variety of hydrophobic compounds as well as the associated spectral changes upon amyloid formation. The chemical structures of the investigated compounds **1**−**6**, as well as the two well-known amyloid probes Congo Red and ThT are shown in Figure 1. Compounds **1** and **2** are known hydrophobic amyloid probes. Compounds **3**−**5** are typical structures employed in organic electronics and photonics applications and **6** is a hydrophilic analogue of **5**. Compounds **1**−**5** all are relatively flat linear hydrocarbons, which is a common geometry among reported amyloid probes.[3−24] At the same time, compounds **1**−**5** present a large structural variety allowing us to assess the robustness of the grinding methodology toward presence of heteroatoms and changes in the detailed chemical structure. Moreover, as the fibrillation reaction is carried out under acidic conditions it is important that the investigated dye is stable toward acid. A criterion that is fulfilled for all compounds investigated in the present study.

RESULTS AND DISCUSSION

To evaluate the effectiveness of the grinding methodology for finding amyloid probes we tested several hydrophobic compounds with regards to their amyloid detection properties.

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The methodology for amyloid probe screening is summarized in graphical form in Figure 2.

Screening for Probes with the Grinding Method. In a typical procedure samples were prepared by grinding Bovine insulin and 2 wt % of the hydrophobic compound with a mortar and pestle.[26,27] The resulting composite material was dissolved in 25 mM HCl. The sample was then filtered through a 0.2 μm PVDF-filter and the resulting clear solution was heated at 65 °C for up to 24 h in order to induce formation of amyloid fibrils. In order to minimize formation of larger protein aggregates the reactions were stirred for the initial 30 min. Formation of amyloid fibrils was confirmed by AFM and ATR-FTIR measurements performed on aliquots removed after 24 h of heating. A typical result is shown in Figure 3, for the case of

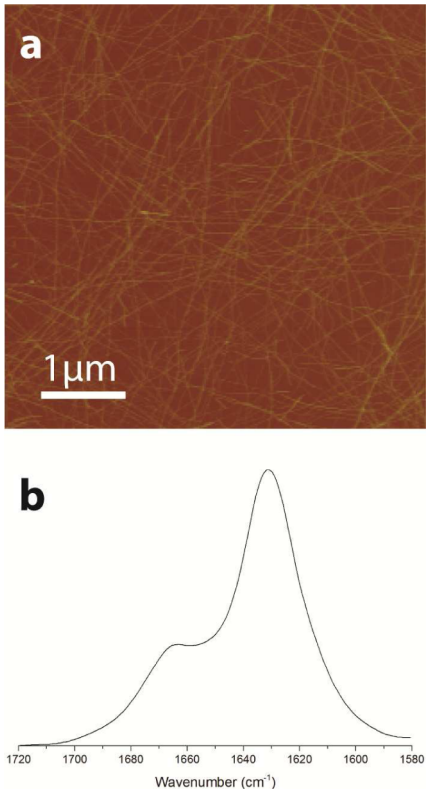

Figure 3. Data for insulin ground with DCM after fibrillation: AFM image (a) and ATR-FTIR spectrum (b).

insulin ground with DCM. The AFM image clearly showed the formation of fibrillar objects (Figure 3a). To confirm the presence of the amyloid β-sheet structure the ATR-FTIR spectrum was analyzed in the amide-I region. The maximum peak position is located at1631 cm^{-1} with an additional local maximum at 1662 cm^{-1}, demonstrating the presence of β-sheet[28,29] (Figure 3b). The corresponding spectra and images for other combinations of insulin and dyes **1**−**5** are shown in Supporting Information Figure S1−S2. In all cases the AFM images (Supporting Information Figure S1) and ATR-FTIR spectra (Supporting Information Figure S2) demonstrate the formation of amyloid-like fibrils. For reference an AFM-image and ATR-FTIR spectrum of fibrillated inulin-only (without any dye) is shown in Supporting Information Figure S1f and Figure S2f, respectively.

Once we have confirmed that the presence of the hydrophobic dye do not inhibit fibril formation, it is possible to evaluate the dyes suitability as amyloid probe by comparing the absorption and fluorescence spectra before and after fibrillation. The results from these tests are shown in Figure 4, where fluorescence spectra are shown for acidic aqueous solutions of composite materials formed by grinding five different hydrophobic substances **1**−**5** with bovine insulin (the corresponding absorption spectra are given in the Supporting Information Figure S3. We first discuss the results for the combination of insulin with BTA-1 (**1**) and Nile Red (**2**) as these two molecules already are well accepted amyloid probes. Because of the hydrophobic nature of BTA-1 and Nile Red, which are insoluble in pure aqueous solvent, previously published studies[16−18] utilized the cosolvent approach.

BTA-1 has the same molecular skeleton as ThT. However, BTA-1 is uncharged making it a hydrophobic analogue of ThT. This makes BTA-1 a perfect candidate for incorporation into amyloid fibrils by the grinding method. We investigated aliquots taken before (0 h) and after fibril formation (24 h) by UV−vis and fluorescence spectroscopy. In the 0 h sample the absorption maximum is located at 410 nm (see Supporting Information, Figure S3a). After heat treatment for 24 h there is a blue-shift with an absorption maximum at 366 nm (Supporting Information Figure S3a). At the same time the fluorescence spectra display dramatic shifts in both intensity and position of the emission maximum (Figure 4a) when excited at 386 nm. In the 0 h sample the emission is relatively weak with a maximum at 432 nm. In the sample heated for 24 h there is a 30 fold intensity increase of the emission maximum, now located at 418 nm. These results thus indicate the suitability of the grinding methodology for rapid amyloid probe screening. Another compound previously shown to be an effective amyloid probe using ethanol as a cosolvent is Nile Red.[17,18] The poor aqueous solubility of Nile Red enabled us to test this compound by the grinding method. When comparing the unfibrillated (0 h) and fibrillated (24 h) samples, the 0 h sample has an absorption maximum at 520 nm, whereas there is a red-shift for the fibrillated sample with a maximum at 554 nm (see Supporting Information, Figure S3b). When excited at 554 nm, Nile Red fluorescence exhibited a blue shift for the fibrillated sample, from 653 nm (sample heated for 0 h) to 629 nm (sample heated for 24 h) with a concomitant 45 fold intensity increase (Figure 4b). These changes are similar to what has been previously reported for Nile Red in combination with amyloid fibrils,[17,18] again verifying the suitability of the grinding method for amyloid probe screening.

Having confirmed the effectiveness of the grinding methodology on known amyloid probes, we then screened three more hydrophobic compounds with unknown properties as amyloid probes. Para-sexiphenyl (**3**)[30] exhibited only slight changes in both absorbance (Supporting Information Figure S3c) or fluorescence spectra (Figure 4c) upon fibrillation. In fact, when excited at 310 nm, the fibrillated sample (24 h) exhibited a slight decrease of fluorescence intensity compared to the 0 h sample, likely as a result of formation of insoluble protein aggregates. These results are discouraging from the point of view of using para-phenylenes as amyloid probes.

4,4′-Bis(2-methoxystyryl)-biphenyl (**5**),[31] a fluorescent whitening agent also known as Fluorescent brightener 378, is an interesting compound as its central part (a biphenyl moiety) has structural similarity to Congo Red. A large variety of stilbene/styryl compounds have been investigated as amyloid probes, but to the best of our knowledge these studies does not involve any molecules with a central biphenyl moiety.[22−24]

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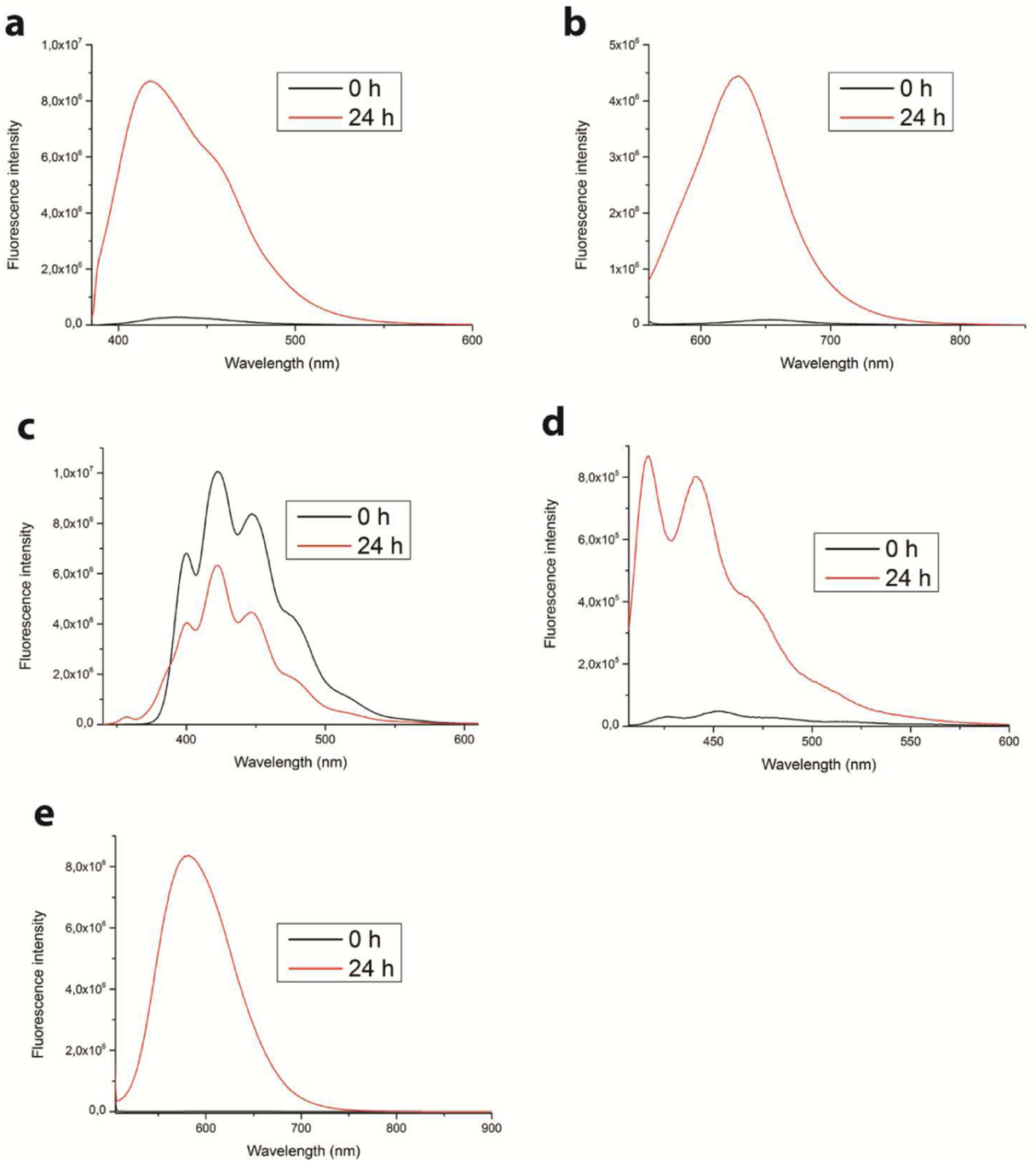

Figure 4. Fluorescence spectra at pH 1.6 (25 mM HCl) for samples of bovine insulin ground with BTA-1 (a), Nile red (b), para-sexiphenyl (c), 4,4′-bis(2-methoxystyryl)-biphenyl (d), and DCM (e) before (0 h) and after (24 h) fibril formation.

However, employing the grinding method the test for amyloid probe properties can be rapidly performed. Investigation by absorbance spectroscopy of aliquots taken before (0 h) and after fibril formation (24 h) showed a slight red shift of absorption maximum (Supporting Information Figure S3d) with concomitant appearance of vibronic fine structure in the fibrillated sample. Investigation by fluorescence spectroscopy of aliquots taken before (0 h) and after fibril formation (24 h) showed a 17-fold intensity increase and a slight blue shift in emission maximum upon fibrillation (Figure 4d). Due to the interesting results we performed further tests using a commercially available water-soluble version of the employed stilbene molecule (see below). Of the compounds tested by the grinding method we obtained the most dramatic differences between the native protein and the amyloid form when employing the laser dye DCM.[32] DCM does not dissolve in 25 mM HCl and is thus suitable for amyloid probe testing by the grinding methodology. We investigated aliquots taken before (0 h) and after fibril formation (24 h) by UV−vis and fluorescence spectroscopy. After heat treatment for 24 h there is a red shift compared to the unfibrillated sample with a local absorption maximum appearing at 500 nm (see Supporting Information, Figure S3e).When comparing DCM fluorescence in the presence of unfibrillated (0 h) and fibrillated protein (24 h), the 24 h sample shows a fluorescence maximum at 582 nm (blue-shifted by 20 nm from the 0 h sample) with a

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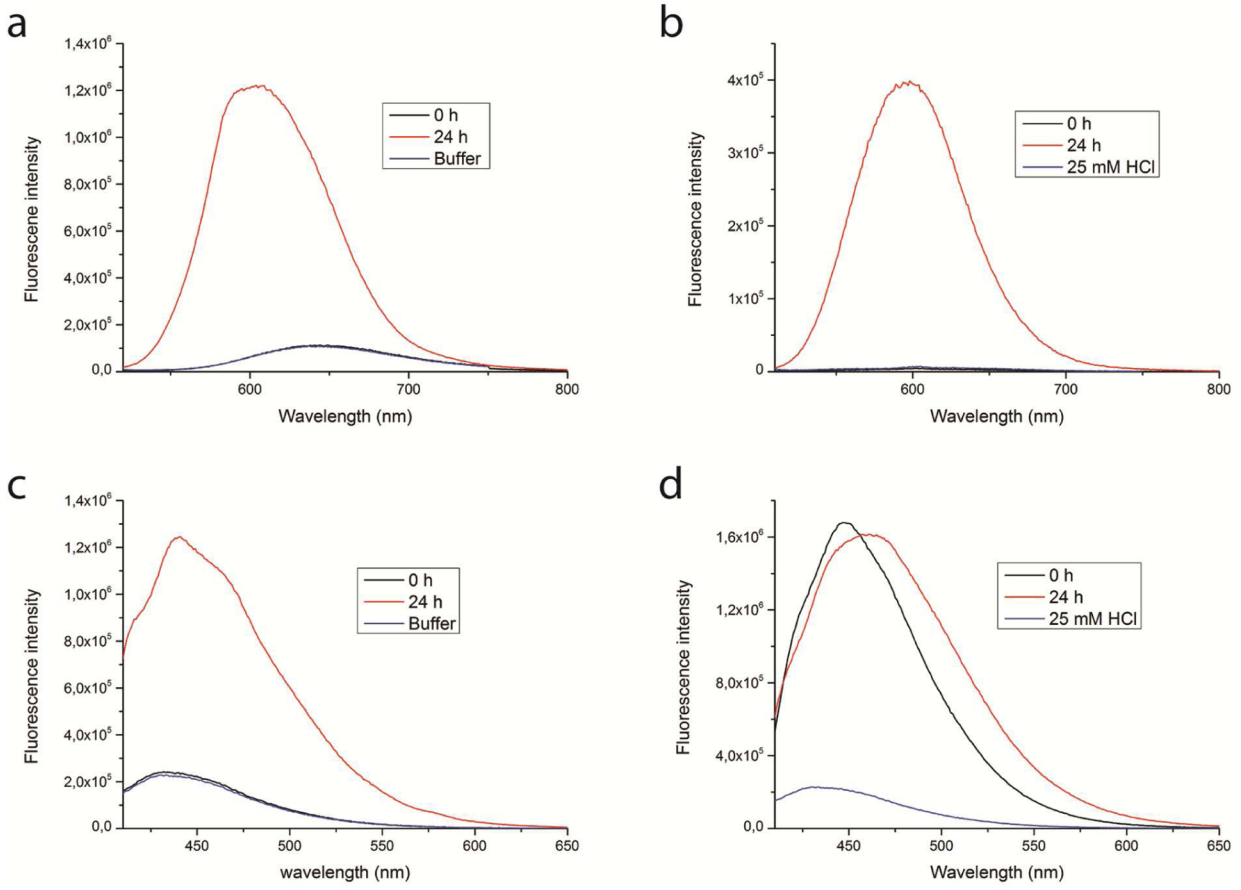

Figure 5. Fluorescence spectroscopy data for samples of bovine insulin before (0 h) and after (24 h) fibrillation. (a) DCM added to protein using MeOH as cosolvent at pH 7.4. Also shown in blue is DCM-MeOH added to pure buffer solution. (b) DCM added to protein using MeOH as cosolvent at pH 1.6. Also shown in blue is DCM-MeOH added to pure 25 mM HCl. (c) Stilbene (**6**) added to protein at pH 7.4. Also shown in blue is stilbene **6** dissolved in pure buffer solution. (d) Stilbene **6** added to protein at pH 1.6. Also shown in blue is stilbene **6** dissolved in 25 mM HCl.

concomitant 1000 fold intensity increase (Figure 4e). DCM has been used for sensing applications related to proteins such as BSA and HSA,[33−35] and for various other sensing applications.[36−38] Moreover, DCM derivatives have been used for protein sensing.[39] However, to the best of our knowledge DCM has never been reported as an optical probe for amyloid formation.

From Preliminary Test to Verification: Tests in Aqueous Environment by Substitution with Polar Groups or Cosolvent Method. With the results from the initial screening in hand we performed further studies on the two promising types of candidates (stilbene **5** and DCM (**4**)). Of the two candidates identified as described above, the stilbene **5** is extremely hydrophobic and the approach employing substitution with polar groups is suitable. On the other hand DCM is a hydrophobic compound containing many polar heteroatoms, and accordingly, although completely insoluble in pure water, DCM can be dissolved in water by use of polar organic cosolvents.

Test of DCM as Amyloid Probe. DCM is sufficiently polar to enable use of the cosolvent method for staining of amyloid fibrils. Accordingly, we dissolved DCM in methanol and then added the resulting solution to samples containing either insulin or fibrillated insulin. The tests were performed in both 25 mM HCl (corresponding to pH 1.6) and pH 7.4 buffer (Figure 5a and b). Samples were excited at 500 nm. Amyloid detection is possible in both 25 mM HCl and pH 7.4 buffer. Just as for the sample prepared by grinding DCM with insulin there is a dramatic shift in fluorescence intensity with a concomitant blue shift for the fibrillated sample compared to the unfibrillated one. However, the difference in intensity between the unfibrillated protein and the fibrillated protein is largest in 25 mM HCl (c.f., Figure 5a and 5b). When comparing the intensity of DCM emission for fibrillated samples in 25 mM HCl and pH 7.4 buffer, the absolute intensity of DCM emission is higher for the pH 7.4 buffer. However, at pH 7.4 there is also stronger emission from the mixture of native protein and DCM (as well as from DCM in only buffer). These effects likely originate from a larger shift in absorption maximum for DCM upon binding to fibrils (relative to the absorption of DCM in the presence of native protein) at low pH (c.f., Supporting Information Figure S3e and Figure S4a). At low pH a larger fraction of the DCM ensemble will absorb at 500 nm. Note that the changes in absorbance that DCM undergoes are readily observable by the naked eye. (see Supporting Information Figure S5, where photographs are shown for DCM added to fibrillated and unfibrillated bovine insulin.). The spectral changes of DCM emission upon binding to fibrils may be explained by binding of DCM to hydrophobic elements of amyloid fibrils. DCM consists of an electron donating moiety (the dimethylamino part) and an electron accepting moiety (the dicyanomethylene part) joined by an unsaturated bridge, and is thus a typical example of a molecule displaying intramolecular charge transfer (ICT). ICT molecules

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will generally exhibit an increase in dipole moment upon excitation and are therefore very sensitive to changes in their external environment.[40] In a polar environment, the excited state will thus be stabilized relative to the ground state, leading to a red-shift (relative to a hypothetical vacuum environment) of fluorescence in polar solvents. Conversely, in a hydrophobic surrounding of low polarity a blue shift of emission is expected.[33] In the case of DCM, a blue shift occurs for the fluorescence in the presence of fibrils indicating that DCM may fit into hydrophobic pockets within the β-sheet framework of the amyloid structure.

Test of Disodium 4,4′-Bis(2-sulfonatostyryl)biphenyl as Amyloid Probe. Disodium 4,4′-bis(2-sulfonatostyryl)-biphenyl (**6**), hereafter designated as stilbene **6** is a well-known fluorescent brightener and laser dye (also known by names such as fluorescent brightener 351, Tinopal cbs, and stilbene 420), and many studies have been published on staining of cellulose and cotton by this dye.[41−43] The test of the combination of amyloid fibrils and stilbene **6** was performed in both 25 mM HCl (corresponding to pH 1.6) and pH 7.4 buffer (Figure 5c and d). The red shift of stilbene **6** upon binding to fibrils can be exploited in order to increase the difference in emission intensity by exciting the sample at the relatively high wavelength 400 nm. In both pH 7.4 buffer and 25 mM HCl stilbene **6** displays an emission spectrum with a maximum located at about 430 nm. Unlike DCM, which can readily discriminate between unfibrillated and fibrillated protein both at pH 7.4 and at pH 1.6, stilbene **6** only shows a small red shift at pH 1.6 when comparing fluorescence from samples with unfibrillated (0 h) and fibrillated protein (24 h). At low pH electrostatic interactions between negatively charged sulfonate groups on stilbene **6** and the positively charged protein (the isoelectric point of bovine insulin is 5.4.) may lead to unspecific interactions between proteins and stilbene **6** leading to an increase in emission intensity for the unfibrillated sample. In contrast, when stilbene **6** is added to a solution of unfibrillated insulin (0 h) at pH 7.4, no significant spectral changes are observed compared to when stilbene **6** is added to only buffer solution. However, upon addition of fibrillated insulin (24 h) dramatic spectral changes are observed. There is a slight red shift of absorption (see Supporting Information Figure S4b). Moreover, in the presence of fibrillated protein there is an increase of emission intensity as well as sharpening of the vibronic structure, when the sample is excited at 400 nm. At pH 7.4, insulin will have a net negative charge, and there will thus be no attractive electrostatic interactions between negatively charged stilbene **6** and insulin, and hence specific interactions between stilbene **6** and the amyloid β-sheet structure gain in importance. The sharpening of the vibronic fine structure that can be observed upon addition of fibrils thus indicates a conformational stabilization of the dye structure upon interaction with amyloid fibrils. This result is in agreement with other studies on the interaction of stilbene **6** with various biomolecular materials such as cellulose, cotton, and silk.[41−44]

For comparison, we studied the fibrillation kinetics of bovine insulin by means of the optical probes ThT, DCM, and stilbene **6**. A methanol solution of DCM or an aqueous solution of stilbene **6** was added to aliquots taken at regular time intervals from an insulin reaction mixture heated to 65 °C and the resulting fluorescence intensity was plotted against the corresponding data obtained from following the same reaction with ThT (Figure 6). The graphs for all probes show typical sigmoidal kinetics, again verifying the suitability of DCM and stilbene **6** to act as amyloid probes.

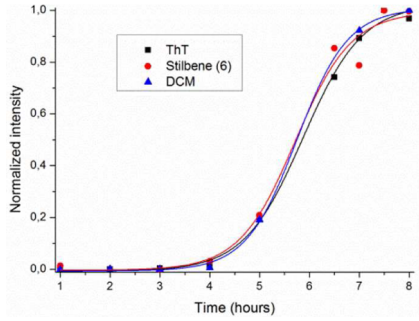

Figure 6. Insulin amyloid fibril formation kinetics followed by ThT (solid black squares), stilbene **6** (solid red circles), and DCM (solid blue triangles) fluorescence measured at pH 7.4. Fluorescence intensities were normalized on a scale of 0−1.

Because of its biological importance the amyloid structure has been intensively studied.[1,2] Such studies have established that the extended β-sheet structure leads to the formation of hydrophobic channels where small molecules may bind.[45] It has been demonstrated that both Congo Red and ThT interacts with amyloids by binding to such channels. We speculate that both stilbene **6** and DCM **4** binds to fibrils in a similar fashion as ThT and Congo Red. Structural elements, such as extended β-sheets are rare in native proteins thus explaning the high selectivity of linear hydrophobic probes for amyloid structures. This mode of binding would readily explain the trends in fluorescence that we have observed for DCM and stilbene **6** upon addition to amyloid fibrils. The blue shift and intensity increase of DCM fluorescence is thus a result of binding into a hydrophobic structural element present (i.e., hydrophobic channels resulting from the extended β-sheet structure) in fibrils but not in the unfibrillated protein.

The screening method where hydrophobic chromophores are ground with a protein capable of self-assembly into amyloid fibrils presents an opportunity to rapidly screen combinations of materials from the organic electronics/photonics field (which generally are extremely hydrophobic) and amyloidogenic proteins. Such work may lead to development of novel amyloid probes, as outlined within the present study, or may lead to development of novel hybrid materials for applications in electronics or photonics. Amyloid materials have for example been incorporated into OLEDs and photovoltaic cells,[46−48] and silk materials have been used for various photonics applications.[44,49] A large number of biological systems utilizes protein scaffolds functionalized with chromophores. Herein, we have demonstrated that amyloid fibrils can be noncovalently functionalized with a variety of extrinsic hydrophobic chromophores. As outlined above this enables rapid screening of a hydrophobic chromophores potential as a probe for amyloid formation. In addition the same methodology is a rapid scalable way of preparing functionalized protein nanofibrils with novel properties for materials science applications.

CONCLUSIONS

We have demonstrated that it is possible to rapidly screen hydrophobic fluorescent aromatic molecules with regards to their properties as amyloid probes. By grinding the hydrophobic molecule with the amyloidogenic protein insulin a

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water-soluble composite material is obtained. When this material is dissolved and exposed to conditions promoting amyloid formation, the protein aggregates into amyloid fibrils. As a result, changes in the fluorescence spectra of the hydrophobic molecule can be correlated to the formation of amyloid fibrils, and the suitability of the hydrophobic molecular skeleton as an amyloid probe can thus be assessed. In order for the probe to function in biological settings the amyloid probe must be water-soluble. For a hydrophobic molecule this can be achieved either by employing cosolvents or by substitution of the hydrophobic molecule by polar groups. In our screening of amyloid probes we found representatives of both approaches; thus, the hydrophobic laser dye DCM can be employed as an amyloid probe through the use of water-miscible cosolvents such as MeOH. On the other hand, extremely hydrophobic molecules can be modified by attachment by polar groups. As an example we identified a promising hydrophobic stilbene skeleton through the grinding method, and verified its use as amyloid probe by performing tests with the corresponding water-soluble sulfonated stilbene. We note that various DCM and stilbene derivatives can be systematically modified by organic synthesis, and the results reported herein may thus inspire future development of novel amyloid probes. Moreover, the grinding approach enables rapid preparation of composites between hydrophobic materials and self-assembling proteins. This will in turn allowing rapid screening of materials in search for novel properties suitable for organic electronics and photonics applications.

EXPERIMENTAL PROCEDURES

Materials and Methods. Bovine insulin was purchased from Sigma-Aldrich and used as received. ThT, Nile Red, and BTA-1 were obtained from Sigma-Aldrich and used as received. Para-sexiphenyl, 4,4′-bis (2-methoxystyryl)-biphenyl, and (disodium 4,4′-bis(2-sulfonatostyryl)biphenyl) were obtained from TCI and used as received. DCM (4-(Dicyanomethylene)-2-methyl-6-(4-dimethylaminostyryl)-4*H*-pyran) was obtained from Exciton and used as received. In a typical procedure, 25 mg of bovine insulin was ground with 0.5 mg of a hydrophobic compound. The grinding was done with a mortar and pestle for 10 min. The resulting composite material was dissolved in 5 mL of 25 mM hydrochloric acid and filtered through a 0.2 μm PVDF filter. The filtered samples were then diluted 2 times with 25 mM hydrochloric acid and subsequently heated at 65 °C for up to 24 h yielding a final insulin concentration of 2.5 g/L. Solutions were stirred with magnetic stirring for the first 30 min of the reaction.

Absorption Measurements. Absorption data was obtained using a PerkinElmer Lambda 950 UV/vis spectrometer. Prior to measuring samples were diluted 8 times from the reaction solution concentration.

Fluorescence Spectroscopy Measurements. The fluorescence data were collected using a Horiba Jobin Yvon Fluoromax-4 spectrofluorometer. Prior to measuring, samples were diluted 20 times from the reaction solution concentration.

ATR-FTIR Spectroscopy. Two microliter aliquots were taken from reaction mixtures after 24 h of heating and drop casted onto an ATR ZnSe crystal surface. The sample chamber was continuously purged with dried air throughout measuring. Measurements were performed with a resolution of 2 cm^{-1} at room temperature on a Bruker Vertex 70 FTIR spectrometer. Spectra were normalized with respect to intensity in the region between 1720 and 1580 cm^{-1}.

AFM. Atomic force microscopy measurements were performed in tapping mode using a Digital instruments Dimension 3100 atomic force microscope. To obtain the AFM images, samples were diluted 10 times from the reaction solution prior to applying to silicon substrates and left to dry for 1 min. Excess fluid was removed by applying a nitrogen gas flow.

Insulin Amyloid Fibril Formation Kinetics. Insulin (2.5 g/L) in 25 mM HCl was kept at 65 °C without agitation. Aliquots were withdrawn at regular intervals, mixed with either ThT, stilbene 6 or DCM directly in the fluorescence cuvette (clear faced 10 mm path length UV range PMMA cuvettes) and diluted 100 times in Robinson-Britton buffer made from 0.04 M H_3BO_4, 0.04 M H_3PO_4, and 0.04 M CH_3COOH and adjusted to pH 7.4 by addition of NaOH, before subsequent study with a fluorospectrometer. One aliquot were taken from three separate reaction vials for each time point measured and the resulting mean value was plotted in Figure 5.The nonlinear curve fitting for the data in Figure 6 was done using the Origin software with a Boltzmann model using the equation $y = (A_2 + ((A_1 - A_2)/(1 + \exp(x - x_0)/dx)))$, with A_1 being the initial value, A_2 the final value, and x_0 the center value.

ASSOCIATED CONTENT

Supporting Information

Absorbance spectra, additional ATR-FTIR spectra and AFM images, and photographs of insulin solutions with DCM added before (0 h) and after (24 h) fibril formation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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