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Fluorescence detection of cationic amyloid fibrils in human semen ☆

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ABSTRACT

Cationic amyloid fibrils, including the Semen Enhancer of Virus Infection (SEVI), have recently been described in human semen. Simple methods for quantitating these fibrils are needed to improve our understanding of their biological function. We performed high-throughput screening to identify molecules that bind SEVI, and identified a small molecule (8E2), that fluoresced brightly in the presence of SEVI and other cationic fibrils. 8E2 bound SEVI with almost 40-fold greater affinity than thioflavin-T, and could efficiently detect high molecular weight fibrils in human seminal fluid.

Amyloid fibrils have been identified in various disease states,[1] as well as in normal tissues, including seminal fluid.[2–4] Seminal amyloid fibrils are formed by self-assembling cationic peptides, and strongly enhance human immunodeficiency virus type 1 (HIV-1) infection.[2–4] Furthermore, the magnitude of HIV-1 infection enhancement by individual semen samples is correlated with the relative levels of these fibrillogenic peptides.[3,5–7] As a result, there is interest in developing methods to rapidly detect and quantitate amyloid fibrils in semen and other samples—including SEVI.[3,5,8,9] However, the quantitative detection of SEVI is challenging because of its cationic charge, but also because it exists in a highly disordered structure, when compared to other amyloid fibrils.[10–12]

The reagent most widely used to detect amyloids is thioflavin-T (ThT).[13] However, ThT may be suboptimal for binding to cationic fibrils,[14,15] and is also ill-suited for studies of the disruption of amyloid fibrils by EGCG (epigallocatechin-3-gallate) and related compounds,[12] since EGCG and ThT exhibit competitive binding for SEVI and other amyloid fibrils.[16–19]

We therefore set out to identify novel small molecules capable of binding to the cationic seminal amyloid fibril, SEVI. To do this, we designed and optimized a high throughput assay to identify small molecules that could bind to SEVI (see Supplementary data; Fig. S1). We then used this assay to screen a targeted library of polyaromatic compounds (Fig. S2). Several SEVI-binding small molecules were identified (Fig. S3, Table S1), including a benzo[4,5]-imidazo[1,2-*a*]pyridine derivative that fluoresced brightly in the presence of SEVI; designated 8E2 (Fig. 1).

8E2 exhibited a broad absorbance spectrum (from ~400 to 450 nm) and a very strong fluorescence emission maximum in

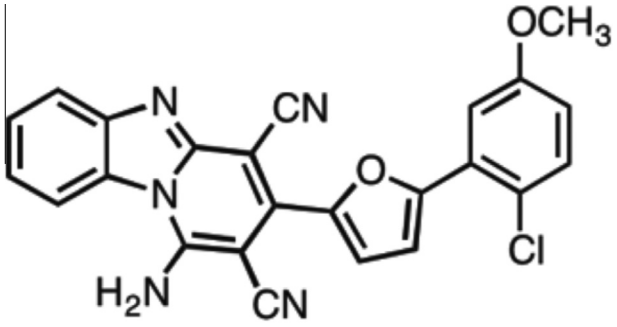

Figure 1. Structure of 8E2.

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the presence of SEVI (Fig. 2). 8E2 fluoresced brightly only in the presence of SEVI fibrils, and not in presence of the monomeric $PAP_{248-286}$ peptide (Fig. 3). The molar extinction coefficient (ε) of 8E2 was roughly twice that of ThT when compared at their respective excitation absorbances, indicating its ability to absorb light more efficiently than ThT. The efficiency of fluorescence process was measured by the quantum yield (Φ) calculations. 8E2 exhibited considerably higher quantum yield compared to ThT (see Tables S2 and S3).

8E2's fluorescence properties were also evaluated in the presence of other amyloid fibrils, including matched pairs of fibrils that were designed to differ in their overall charge, but only minimally in their amino acid composition. For this purpose, we used SEVI (formed from the cationic $PAP_{248-286}$ peptide) and SEVI-Ala (formed from a non-cationic derivative of this peptide[7]). We also used fibrils formed from Aβ42 and from a cationic derivative of this peptide (Aβ42+; see Supplementary data). We then compared the peak fluorescence for 8E2 and ThT in the presence of these fibrils. 8E2 fluoresced much more strongly than ThT in the presence of the cationic amyloids (SEVI and Aβ42+; Fig. 4). Consistent with this, the disassociation constant (K_d) for binding of 8E2 to SEVI was approximately 40-fold lower than the K_d for ThT (Table 1; Figs. S4 and S5). In contrast, the K_d of 8E2 for SEVI-Ala was only six-fold higher than for ThT (Table 1; Figs. S4 and S5). We tentatively attribute this to charge–charge repulsion between the quaternary heterocyclic nitrogen on ThT and the positively charged surface cationic fibrils.[14,15] However, it is possible that other factors may also play a role, since 8E2 and ThT exhibited similar K_d's for Aβ42 fibrils and their cationic Aβ42+ counterparts (Table 1).

Since ThT and 8E2 have quite divergent K_d's for binding to various amyloid fibrils, we wondered whether the two molecules might bind fibrils via different mechanisms. To test this, we performed a binding competition experiment. To do this, we incubated SEVI (5 μM) with 8E2 (0.25 μM) and ThT (80 μM), and measured the peak of fluorescence intensity for each molecule under these conditions (Fig. 5). The experimentally observed peak of fluorescence intensity was almost exactly equal to the sum of the peaks of fluorescence intensity for 8E2 and ThT alone (Fig. 5).

This shows that 8E2 binding to SEVI was not competed away, even in the presence of a 320-fold molar excess of ThT, and suggests that the two molecules bind SEVI in a distinct (and non-competitive) manner.

We next wished to test if 8E2 could detect amyloid fibrils in seminal fluid (SF). As a first step, we examined the effect of viscosity on 8E2 fluorescence. This is important because (i) seminal fluid is viscous, and (ii) 'molecular rotor' dyes such as ThT fluoresce strongly in viscous solutions that impede their rotation.[20]

ThT and 8E2 fluorescence was sharply increased in the presence of high concentrations of glycerol, but not in solutions containing ⩽25% glycerol (Fig. S6). Thus, we diluted SF samples in Dulbecco's phosphate-buffered saline (DPBS), to a final concentration of 10%, and then examined 8E2 (and ThT) fluorescence. A pooled SF stock

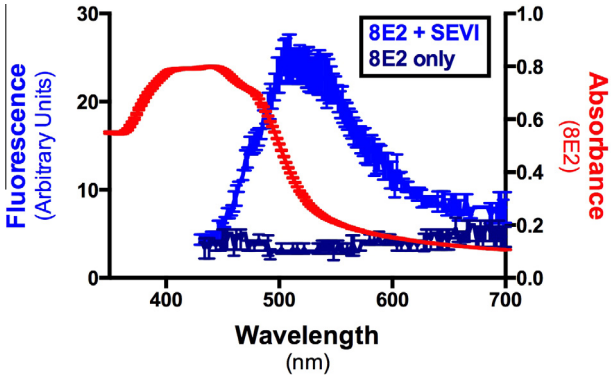

Figure 2. The spectral properties of 8E2. Wavelength scanning analysis for 8E2 absorbance (red). 8E2 (1 μM) was also incubated in the presence (light blue) or absence (dark blue) of SEVI (5 μM), and fluorescent intensities were measured at excitation 418 nm. Error bars represent the standard error of the mean (SEM) from one experiment performed in triplicate.

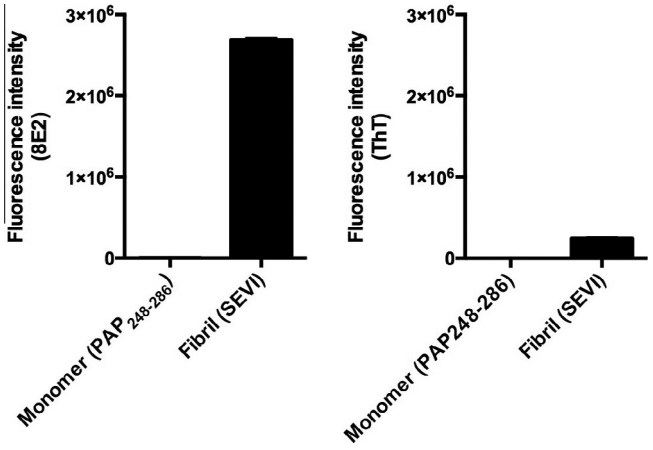

Figure 3. 8E2 fluorescence is fibril specific. ThT or 8E2 (50 μM) were incubated with monomeric $PAP_{248-286}$ peptide or SEVI fibrils (25 μM). Fluorescence intensity was measured at excitation 465 nm, emission 535 nm. Background fluorescence intensity values were subtracted from experimental data. Data represent results from three independent experiments performed in triplicate; error bars denote the SEM.

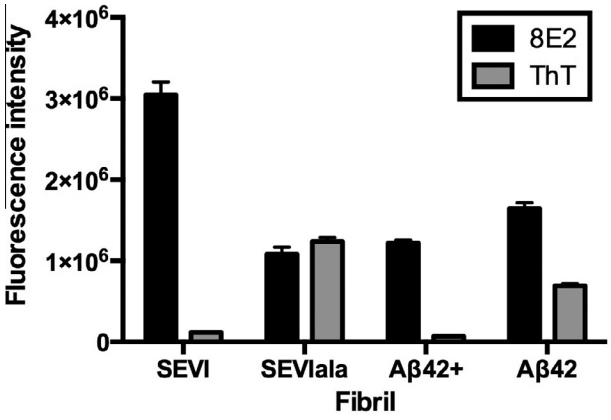

Figure 4. ThT exhibits reduced fluoresence in the presence of cationic amyloid fibrils, while 8E2 does not. ThT or 8E2 (50 μM) were combined with 25 μM of SEVI, SEVI-Ala, Aβ42, or Aβ42+ fibrils. Fluorescence intensities were measured at excitation 465 nm, emission 535 nm. Background fluorescence intensity values were subtracted from experimental data. Data represent results from three independent experiments performed in triplicate; error bars denote the standard error of the mean.

Table 1
Dissociation constants (K_d) for binding of 8E2 and ThT to SEVI, SEVI-Ala, Aβ42+, Aβ42 (5 μM) fibrils

	SEVI (μM)	SEVI-Ala (μM)	Aβ42+ (μM)	Aβ42 (μM)
8E2	1.10 ± 0.09	1.61 ± 0.282	4.52 ± 0.753	2.60 ± 0.034
ThT	42.5 ± 4.2	0.246 ± 0.011	6.49 ± 1.78	1.83 ± 0.091

K_d data were determined by plotting the reciprocal of the fluorescence maximum (F_{max}) versus the reciprocal of the concentration of the probe. The x-intercepts were extrapolated from the linear regression and the negative reciprocal was taken yielding the apparent K_d. Data were reported as an average of a triplicate, and error was the standard error of the mean.