

## LETTER

## Two-photon excited lasing of Coumarin 307 for lysozyme amyloid fibrils detection

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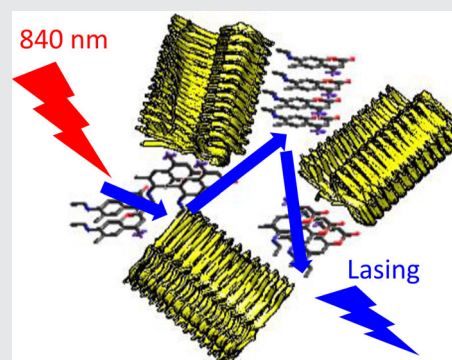
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**Abstract**

Amyloid fibrils are a well-recognized hallmark of neurodegeneration. A common approach to detect amyloid fibrils is staining with organic molecules and monitoring optical properties using fluorescence spectroscopy. However, the structural diversity of amyloids necessitates new sensitive methods and probes that can be reliably used to characterize them. Here, Coumarin 307 is applied for lysozyme fibrils detection by observation of laser action in the process of two-photon excited stimulated emission. It is shown that the lasing threshold and spectrum significantly depend on the adopted structure ( $\alpha$ -helix or  $\beta$ -sheet) of the lysozyme protein, whereas fluorescence spectrum is insensitive to the protein structure. The applications of coherent stimulated emission light that can be emitted deep inside a scattering medium can be particularly promising for imaging and therapeutic purposes in the neurodegeneration field. Two-photon excitation with the near-infrared light, which allows the deepest penetration of tissues, is an important advantage of the method.

**KEYWORDS**

neurodegeneration, protein aggregation, stimulated emission, time-resolved fluorescence, two-photon absorption



## 1 | INTRODUCTION

Coumarin dyes have drawn much attention due to their broad pharmacological activities [1]. Many coumarins exert anti-coagulant, anti-tumor, anti-viral, anti-inflammatory and antioxidant effects, as well as anti-microbial and enzyme inhibition properties [2]. Coumarin derivatives were found to act also as amyloid fibril growth inhibitors [3]. Amyloids are molecules composed of misfolded proteins that create  $\beta$ -sheets ladder-like

fibrillar structures [4]. Those structures are considered to be involved in neurodegenerative diseases such as Alzheimer's [5], Parkinson's [6] or familial renal amyloidosis (FRA) [7].

Some of the coumarin derivatives were tested in clinical trials for inhibiting brain diseases progression [8]. However, coumarins are relatively poorly characterized in terms of their spectroscopic properties in crowded environments. Better understanding of their physical and chemical properties in the aggregated state may help

designing more effective and multifunctional dyes that merge detection and inhibitory attributes [9]. Since coumarins are listed on Food and Drug Administration (FDA) list, the authorization of new derivatives for application in neurodegeneration field should be relatively fast and easy.

Inhibitors with selective optical properties that target the aggregation of amyloidogenic proteins are highly demanded for early stage detection of malfunctioning proteins long before visible disease symptoms occur. Among proteins involved in FRA is human lysozyme protein [10]. Here we use lysozyme analogue from chicken egg [11] that is relevant to the disease-related protein. We study nonlinear optical response—two-photon excited lasing—of Coumarin 307 [12] in the context of lysozyme aggregation. Lasing or stimulated emission (SE) is attracting much attention in fluorescence-based detection methodology because it is considered to be more sensitive than standard fluorescence [13]. In the process of light amplification the observed response can be influenced by changes of photons' path lengths inside the medium as they undergo multiple scattering events [14–16]. In consequence, lasing spectra become sensitive to self-aggregation of emitting molecules or presence of other constituents such as proteins.

We expand our previous research on employing SE for amyloid fibrils detection in thin films [17–19] to dye suspended in solution with native monomer or aggregated lysozyme protein, what is relevant to biological conditions. In the present work we show that lasing in a scattering environment can be induced through a two-photon excitation process using near-infrared light. It is an important advantage that lasing can be induced within the therapeutic window of light spectrum. This makes it possible to develop lasing-based methods for imaging amyloids with low photodamage and deep light penetration [20]. Our findings also suggest that Coumarin 307 is capable of being sensed at high signal-to-noise ratio in relevance to diagnostics of neurodegenerative diseases.

## 2 | MATERIALS AND METHODS

### 2.1 | Coumarin

Coumarin 307 was purchased from Exciton and used as obtained, dissolved in a set of concentrations from 0.5 to 27.7 mM in 99.8% ethanol. For lifetime measurements and studies with protein Coumarin 307 was also dissolved in 70% and 90% (vol.) ethanol/water mixtures.

### 2.2 | Lysozyme protein

Native monomers of lysozyme protein from chicken egg white were purchased from Sigma-Aldrich. The protein was dissolved to 2.1 mM in 70% or 90% (vol.) ethanol/water mixture and filtered through a 0.25  $\mu\text{m}$  filter. The stock solution at 90% was then incubated in 45°C. After 3 days a viscous solution was obtained indicating formation of lysozyme fibrils. Fibrils formation was additionally confirmed by a standard test with thioflavin T (ThT). As expected, ThT showed several times stronger fluorescence in the incubated solution than in the solution of monomeric lysozyme protein prepared in 70% ethanol (Supporting Information Figure S1). Stock solutions of Coumarin 307 (27.7 mM) and lysozyme (2.1 mM) were mixed in 1:1 volume ratio in 70% and 90% (vol.) ethanol/water mixture, stirred and heated at 37°C for 1 h prior to the stimulated emission measurements.

### 2.3 | Fluorescence measurements

Steady-state fluorescence spectra of highly concentrated solutions and fluorescence decay curves of Coumarin 307 under various conditions were recorded in custom-built experimental setups described in detail in Appendix S1.

### 2.4 | Stimulated emission measurements

Scheme of the experimental setup is shown in Figure S2. The sample was illuminated with femtosecond pulses using 5 kHz Legend Elite Duo (Coherent Inc.) Ti:sapphire regenerative amplifier and Opera Solo Optical Parametric Amplifier providing energy of 20–30  $\mu\text{J}$  in femtosecond pulses at the wavelength used in the current study (840 nm). A focusing lens with a focal length of 100 mm was mounted slightly off-center in a continuously rotating mount. Thus, the focal point of the excitation beam, located inside a  $10 \times 10 \text{ mm}^2$  cuvette with Coumarin 307 solution, was moving circularly, which helped to prevent populating the triplet state of dye molecules and their photodegradation. SE was detected along the excitation beam, simultaneously in the forward (total emitted power measurements) and backward (spectral analysis) directions. A 50:50 beamsplitter located between the focusing lens and the cuvette reflected part of the back-emitted SE to an Ocean Optics USB 2000 fiber spectrometer, whose spectral resolution was around 4 nm. Forward-emitted SE was filtered from the excitation beam using a 60° prism made of SF10 glass and a short-pass (edge at 550 nm) or bandpass (central wavelength 500 nm, FWHM 40 nm) filter and directed to the sensor (PD300-3 W-V1) of a power-meter (Ophir Nova II). We took advantage of the effect of the cuvette walls acting as an optical resonator for SE light by aligning the cuvette with

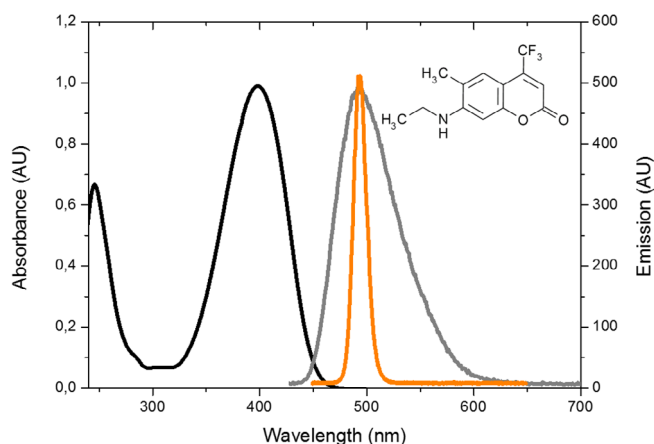
respect to the excitation beam in order to obtain maximum SE intensity [21].

### 3 | RESULTS AND DISCUSSION

In our experimental conditions light amplification in ethanol solution of Coumarin 307 occurs above 10 mM dye concentration for two-photon excitation at 840 nm. The threshold level at this concentration was 1  $\mu$ J pumping energy, whereby the observed emission spectrum becomes significantly narrower (FWHM = 15 nm) than at lower energies (Figure 1). However, such a large dye concentration often causes self-aggregation of the dye molecules. In consequence it can lead to fluorescence quenching and shorter excited-state lifetimes [22].

In order to evaluate fluorescence self-quenching, fluorescence lifetime measurements were performed in Coumarin 307 concentration range from 0.01 mM to 28 mM (Figure 2 and Table 1). Fluorescence decays are monoexponential with decay times independent on the dye concentration up to approximately 3 mM. Above that concentration the decays remain monoexponential but become shorter with increasing dye concentration (Inset in Figure 2). Most plausible explanation of this effect is related to the excitation energy transfer between dye monomers and aggregates formed at elevated concentrations.

To verify applicability of Coumarin 307 for amyloid fibrils detection we first studied fluorescence and lasing for a concentrated (27.7 mM) solution of Coumarin 307 in 90% and 70% (vol.) ethanol/water mixtures (Figure 3). These ethanol/water systems have biological significance because lysozyme protein in 70% ethanol remains in its native monomeric form dominated by  $\alpha$ -helix whereas in 90% fibrils dominated by  $\beta$ -sheet structures are formed [23].



**FIGURE 1** Absorption (black), emission (gray) and two-photon excited lasing (orange) spectra of Coumarin 307 in 99.8% ethanol with chemical structure of Coumarin 307 as inset

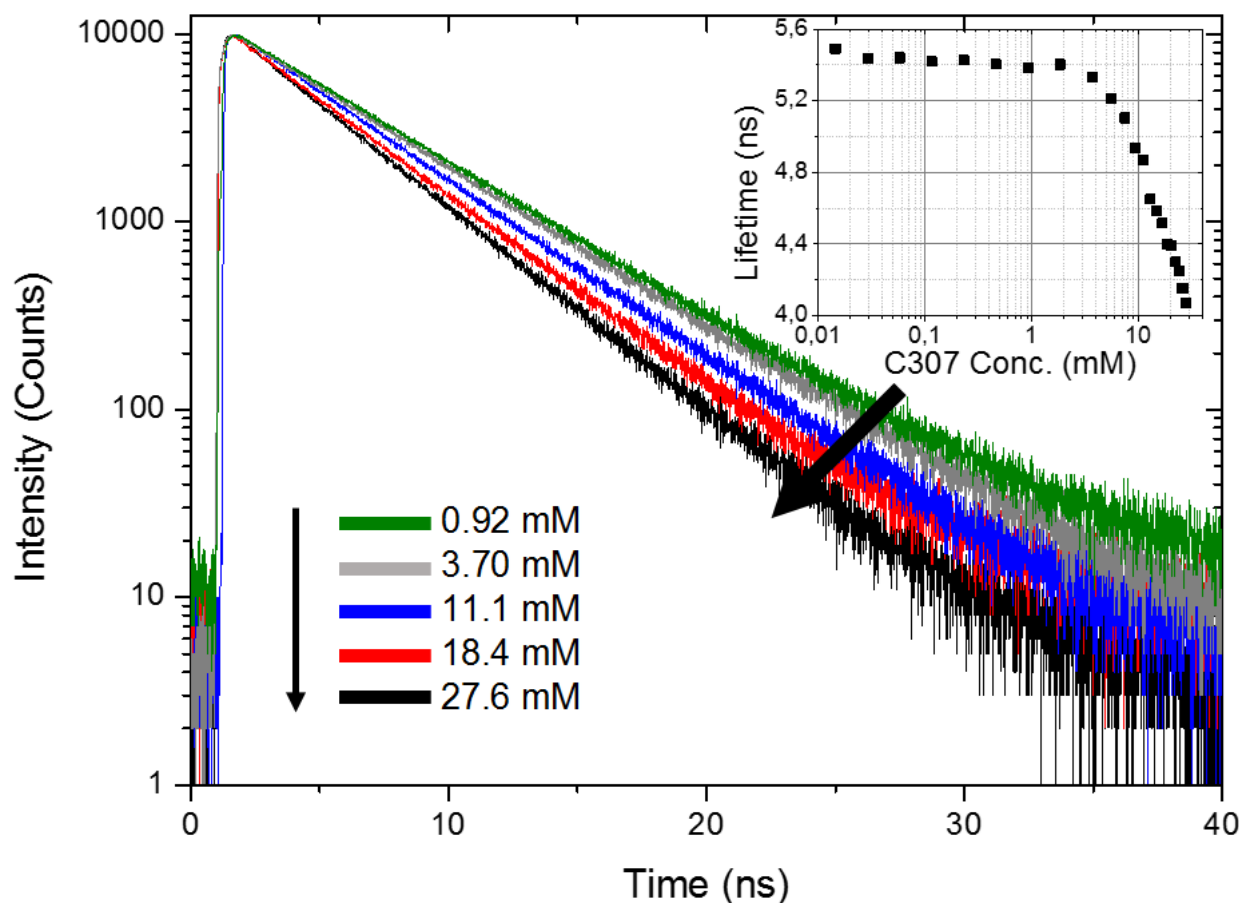
Addition of water to ethanol solution of Coumarin 307 causes spectral red-shift of both spontaneous emission and lasing spectra (Figure S3) accompanied by the increase of the fluorescence lifetime (Figure S4). Both effects can be attributed to the increase of the polarity of the solvent induced by adding more polar water to less polar ethanol. It has been already demonstrated for various coumarins that increasing solvent polarity results in a red-shift of the fluorescence spectrum [24] and increase of the fluorescence lifetime [25]. We note that at 27.7 mM concentration in 99.8% ethanol the dye is already strongly aggregated thus we do not expect a significant increase of the aggregation degree due to the increased water content.

Figure 3(A-C) shows that spontaneous emission and lasing spectra as well as lasing thresholds of Coumarin 307 in 70% ethanol are the same for the pristine dye and dye mixed with native lysozyme. It indicates that addition of monomeric protein has no influence on spectral properties of dye aggregates. In contrast, formation of lysozyme fibrils in 90% ethanol leads to a clear red-shift of the lasing spectrum whereby spontaneous emission remains intact (inset in Figure 3D). Moreover, the lasing threshold increases from 1.8 to 2.9  $\mu$ J upon addition of lysozyme fibrils (Figure 3E-F).

We consider two possible mechanisms that can be responsible for the change of the lasing characteristics. First, upon misfolding, proteins become strongly scattering. A scattering environment introduces energy losses which counteract light amplification. These scattering losses increase with the frequency of the emitted light and thus are lower at the longer wavelengths [26]. Therefore in a strongly scattering environment of the aggregated protein the lasing spectrum is red-shifted. Simultaneously the pump energy required to obtain the laser action increases in order to overcome the energy losses.

Second, fibrillization causes rearrangement of the protein structure, where  $\alpha$ -helices become replaced by the  $\beta$ -sheets. Since the core structure of the fibril, dominated with densely packed  $\beta$ -sheets, is hydrophobic, there is an increase of the repulsion force on dye molecules upon fibrillization. In a highly crowded environment this may lead to dye crystallization that reduces the amount of molecules contributing to light amplification. This, in turn, results in the increase of the lasing threshold.

The sensitivity of Coumarin 307 to detect lysozyme fibrils and their structure by lasing methodology was further studied by recording its lasing spectra upon reduction of ethanol content in the fibrils solution from 90% down to 70%. A test experiment in which fluorescence intensity of thioflavine T was measured showed that fibrils prepared in 90% ethanol remain dominated by  $\beta$ -sheets even in 70% ethanol but structural reorganization occurred (Figure S1).



**FIGURE 2** Fluorescence decays recorded at different Coumarin 307 concentrations. The inset shows dependence of the fluorescence lifetime on the dye concentration

**TABLE 1** Fluorescence lifetimes of Coumarin 307 in ethanol at different dye concentrations<sup>a</sup>

Concentration (mM)	Lifetime (ns)	Concentration (mM)	Lifetime (ns)
0.01	5.49	9.2	4.87
0.03	5.43	11.1	4.65
0.06	5.41	12.9	4.58
0.12	5.42	14.8	4.51
0.23	5.40	16.6	4.39
0.46	5.38	18.4	4.38
0.92	5.40	20.3	4.29
1.84	5.33	22.1	4.25
3.68	5.21	24.0	4.15
5.53	5.10	25.8	4.07
7.37	4.93	27.7	4.04

<sup>a</sup>Uncertainties of lifetimes were estimated at 0.03 ns.

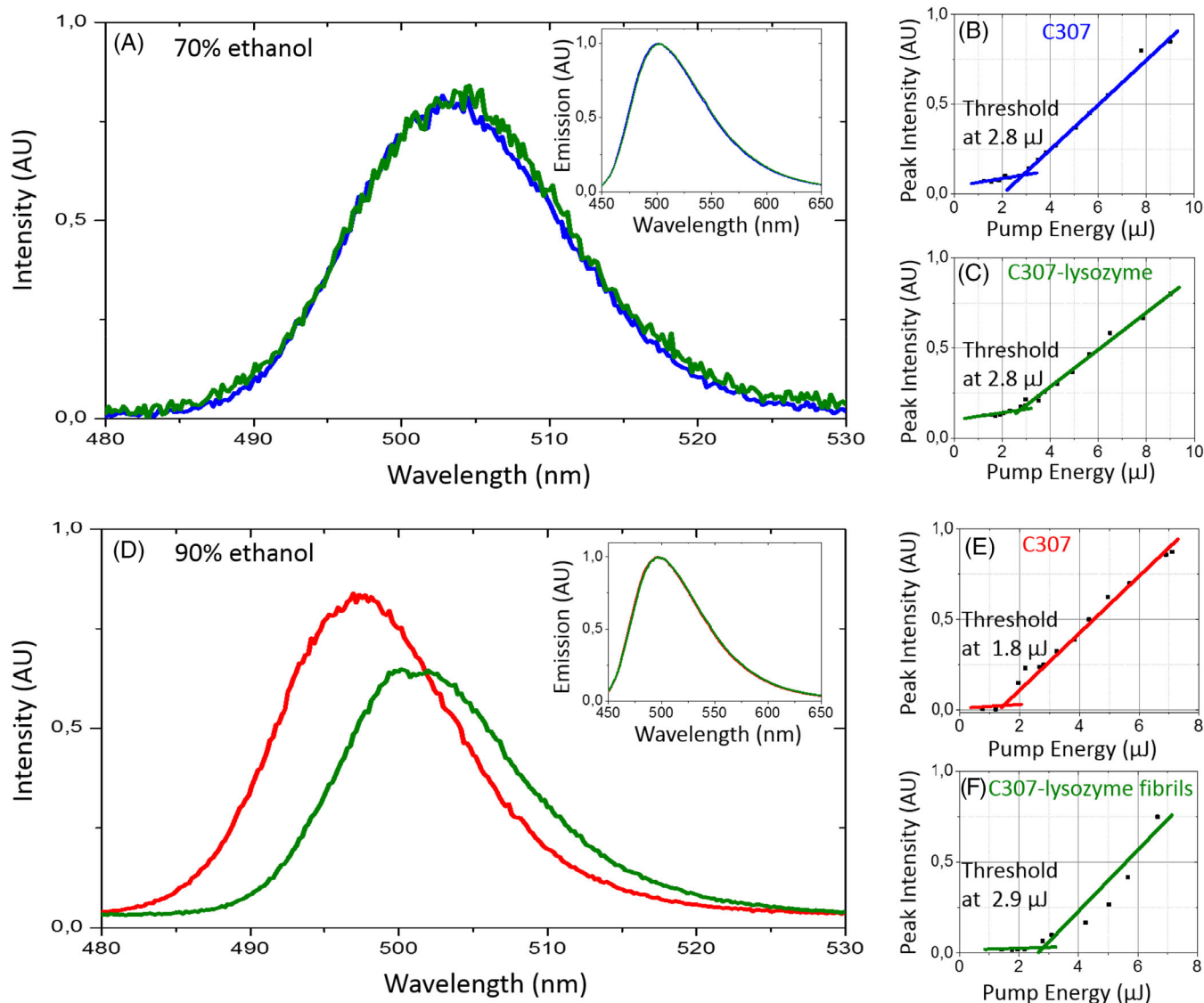
This change of the fibrils structure is also reflected in the lasing characteristics of Coumarin 307. The spectra recorded in the fibrils solution in 70% ethanol are still different from

the monomeric protein dissolved directly in 70% ethanol but they also clearly differ from spectra recorded for mature fibrils prepared in 90% ethanol (Figure 4). This experiment confirms that structure of fibrils affects the characteristic features of lasing in the studied system.

It has been demonstrated that by changing the solvents ratios it is possible to influence the protein structure and even restore monomers functionality [27]. Here we show that by using highly sensitive lasing methodology it is possible to detect transition forms of the fibrils by monitoring lasing spectral signatures and lasing thresholds. In the studied case, when mature fibrils formed in 90% ethanol are transferred to 70% ethanol, they can create a more loosely packed structure. This effect may be responsible for the appearance of a distinct shoulder around 528 nm in the lasing spectra of Coumarin 307, which indicates that dye molecules also reorganize in response to fibrils structure (Figure 4). Such a hypothesis finds the support also in the second parameter, the lasing threshold, which is lower for fibrils in 70% ethanol than in 90% ethanol.

In summary, two possible reasons of the change of the Coumarin 307 lasing characteristics after protein





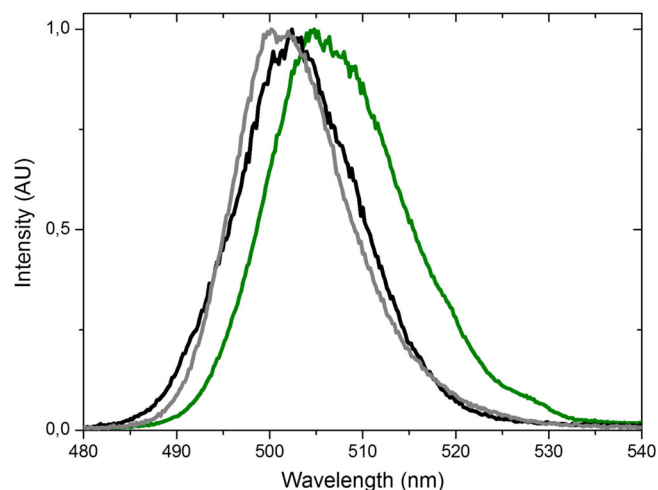
**FIGURE 3** Lasing spectra of pristine Coumarin 307 (blue) and recorded in presence of monomer lysozyme protein (green) in 70% ethanol (A) with respective thresholds (B, C). Lasing spectra of pristine Coumarin 307 (red) and in presence of lysozyme protein fibrils (green) in 90% ethanol (D) with respective thresholds (E, F). Insets in (A) and (D) show fluorescence spectra with and without the protein, which overlap each other to such an extent that the two curves in each graph are indistinguishable

reorganization are proposed: light scattering appearing due to protein aggregation and dye crystallization controlled by the hydrophobic nature of amyloid fibrils. We emphasize also that both effects rather co-exist and their interplay determines the lasing characteristics in the studied systems. The observed effects clearly demonstrate the great advantage of the amyloid detection scheme based on lasing over standard fluorescence techniques based on spontaneous processes.

## 4 | CONCLUSION

We demonstrated that lasing of Coumarin 307 can be induced by a two-photon excitation both in solution and in a

crowded environment of aggregated proteins. Spectral signatures of the resulting laser action are significantly influenced by reorganization of the lysozyme protein, where  $\alpha$ -helices are replaced by  $\beta$ -sheets in the aggregated form of the protein. This is in contrast to the fluorescence spectrum of the dye which is totally insensitive to the structure of the protein. Our experiments confirm that extending classical fluorescence spectroscopy to laser-based nonlinear spectroscopy greatly improves sensitivity towards recognition of specific structural motifs in proteins. Two-photon excitation used to induce the laser action allows application of near-infrared light from the therapeutic region of the spectrum which ensures the deepest tissue penetration depths. Our findings should be extendable to other dyes capable of light



**FIGURE 4** SE spectra of Coumarin 307 in presence of lysozyme fibrils in 90% ethanol (gray), 70% ethanol (green) and fresh native lysozyme in 70% ethanol (black)

amplification upon two-photon excitation. The results indicate that amyloid sensing techniques based on two-photon excited stimulated emission of light in scattering environments may find applications in the field of diagnostics of neurodegenerative disorders.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article. Appendix S1. Supporting Information.

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