Designing of New Optical Immunosensors Based on 2-Amino-4-(anthracen-9-yl)-7-hydroxy-4H-chromene-3-carbonitrile for the Detection of Aeromonas hydrophila in the Organs of Oreochromis mossambicus Fingerlings

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INTRODUCTION

Genus of Aeromonas mainly consists of oxidase-positive, facultative, anaerobic Gram-negative bacteria; among these disease-causing pathogen community, Aeromonas hydrophila (AH) is noted as an important motile Gram-negative bacillus pathogen which is mostly found in water sources, and it is more pathogenic, especially for fish and other cold-blooded species. It is also pathogenic for both humans and animals. On the basis of the physiological and host properties, they are mainly divided into two major groups. The first group which mainly includes the motile aeromonads with AHs, a typical representative, and the other group consists of nonmotile species, which is represented by Aeromonas salmonicida. Aeromonas species are said to be pathogenic because of the production of a wide range of extracellular enzymes and possess all of the requirements of pathogenic bacteria through the production of flagella, pili, and adhesins. The main isolation source for the AH is food materials such as fish, meat products, milk, and vegetables, which ranges from $10^2$ to $10^9$ CFU/g. The amount of Aeromonas percentage in some food products such as meat and poultry (3–70%), dairy products (4%), and vegetables (26–41%) has also been reported. However, the sea food (31–72%) is the major source for the isolation of Aeromonas-positive samples.

The motile aeromonads with AH possess some virulence factors including hemolysins, aerolysins, proteases, adhesins, enterotoxins, phospholipases, and lipases; simultaneously, the infections caused by this could lead to gastroenteritis, septicemia, meningitis, respiratory, and hemolytic uremic. As a consumer, human community has been receiving gastro-enteritis because of the intake of stressed or ill fish, and those who are easily affected with this AH will also undergo hemorrhagic septicemia. In humans, the gastrointestinal tract carries the Aeromonas species in both of their symptomatic and asymptomatic individuals. For nondisease conditions, the rate of fecal carriage ranges from 0 to 4%, whereas for diarrheal illness, it ranges from 0.8 to 7.4%. Because of these adverse effects, people undergo so many clinical diagnosis procedures to detect the pathogenicity of Aeromonas species, but it requires both sensitivity and specificity.

Culture-based detection methods are generally used to grow Aeromonas species in differential isolation media, and it has been developed for the recovery of Aeromonas species from the environment samples such as foods and clinical specimens. EPA method 1605, membrane filtration method, and culture enrichment have also been authenticated for the isolation of AH from drinking water samples, foods, and so forth.

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From the detection point of view, till date, only polymerase chain reaction (PCR) method has been mainly addressed for the recognition of AH. A simple PCR method has been reported for the detection of AH in raw milk within the limit of 2 log_{10} CFU/g, and the detection rate was found to be 23% for this method and 14% for culture method.23 In addition, some microarray-based method is constructed using DNA probes to study the population dynamics of microbial communities, such as marine bacteria in coastal waters in which aeromonads were found to make up a large proportion of the microbial flora.24

Another microarray method has also been reported for the detection of AH cytotoxic enterotoxin-inducing genes in macrophages.25 These methods are only focused on the isolation of *Aeromonas* species from food and water samples.

Among the already-reported PCR techniques, DNA probe microarray-based method is constructed using DNA probes to study the population dynamics of microbial communities, such as marine bacteria in coastal waters in which aeromonads were found to make up a large proportion of the microbial flora.24 In addition, some microarray-based method is constructed using DNA probes to study the population dynamics of microbial communities, such as marine bacteria in coastal waters in which aeromonads were found to make up a large proportion of the microbial flora.24

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containing four $-\text{NH}_2$ groups may be allowed to form amide link with four $-\text{COOH}$ groups of anti-AH, thereby leading to maximum absorbance (Figure 1b). Whereas at higher concentration of AHC (0.01 M), the absorbance intensity was lower which may be because of the poor solubility of AHC. Hence, the precipitate was obtained. Initially, AH has showed a maximum absorbance because of twisted international critical table (ICT) state of the AH structure. After the formation of amide linkage, the peaks at 218 and 277 nm were blue-shifted to 212 and 269 nm, respectively, which may be because of the inhibition of initial twisted intramolecular charge transfer as a result of complex formation between anti-AH (acceptor) and AHC (donor) via amide linkage (Figure 1c), and hence decrement in absorbance was absorbed. We have also cross-checked if any unbound AHC was present in the resulting anti-AH–AHC. Briefly, the settled anti-AH–AHC was washed with phosphate-buffered solution for 5 min and then centrifuged. The resulting supernatant solution was analyzed by UV–vis spectra. No peak corresponding to free AHC appeared for supernatant solution in the UV spectra. This seems to be that all of the 4 equiv of AHC were utilized for the amide linkage. Therefore, the UV–vis spectral shift has clearly evinced and supported the completion of anti-AH tagging with our AHC probe. The corresponding UV data are shown in Figure 1d.

Confirmation of Tagging of AHC with Anti-AH by Emission Analysis. The tagging was further studied with the help of fluorescent emission analysis. Precisely, the initial emission maximum of AHC was analyzed at the absorption maxima of 277 nm in PBS buffer, and the corresponding emission was occurred at 390 and 505 nm. As a result of amide coupling, the emission maxima at 505 nm was blue-shifted to 485 nm along with a drastic decrement in the emission intensity as in the case of UV–vis absorption spectra which may once again confirm the existence of inhibited twisted ICT mechanism during the coupling of AHC with anti-AH. In general, antibody–fluorescent tagging protocol may lead to a blue shift and decrement in fluorescent intensity because of amide coupling. First, the AHC has shown maximum fluorescence because of the twisted ICT state mechanism. After the coupling of anti-AH via amide bond, the initial twisted ICT was inhibited, and the repulsive interaction between the donor–$\text{NH}_2$ and $-\text{COOH}$ of anti-AH may rise the energy level of anti-AH/AHC and hence shift to the lower wavelength was observed (blue shift) The Stoke’s shift of the AHC was found to be 228 nm which shows very excellent luminescent properties of AHC. After the anti-AH was tagged with AHC, the Stoke’s shift was calculated as 216 nm. Generally, proteins, bacterial-based tagging, will give a large Stoke’s shift greater than 200 nm-based. Likewise, our anti-AH tagged with AHC has shown excellent Stoke’s shift, and the respective emission spectra are shown in Figure 2.

Development of Fluorescent Immunoassay for A. hydrophila Using Anti-AH–AHC. Generally, we have to be more aware on Aeromonas colonization as human pathogens because of its association with gastrointestinal diseases. Our developed simple fluorescent immunoassay will be a better analytical tool to quantify the colonies of A. hydrophila. Although varying the concentration of AH from $10^{-1}$ to $10^{-12}$ CFU/g, the emission intensity of peaks at 390 and 485 nm was increased with increasing AH concentrations without any peak shift; however, at higher concentration of AH, the above peaks...
were merged into a single new peak at 411 nm with increment in intensity. The Stoke’s shift of 208 nm from its excitation wavelength was observed during this interaction. The respective emission data along with the linear regression plot are shown in Figure 3. The sensitivity of the developed immunoassay was analyzed using colony counting method based on the dilution of stock culture of A. hydrophila, and the corresponding linear range of detection was found from 4 to 736 CFU/mL with the LOD of 2 CFU/mL.

**Selectivity Study of Anti-AH–AHC Immunoassay toward A. hydrophila.** The important aspect of any immunoassay is its selectivity toward the targeted pathogen among all possible interfering pathogens. The antibody-tagging technology opens a new pathway for the most selectivity toward particular pathogens.40,47 The fluorescent labeling has been keynoted as a very good immunoplatform without using any blocking agents as in the case of electrochemical immunoassay.48 In our present immunoassay, we have tagged the AHC with anti-AH, and hence there is no doubt that the resulting anti-AH–AHC will be attached with its pathogen more specifically. To check the selectivity of the above immunoassay, the pathogens that have an analog behavior to AH, such as Escherichia coli, Bacillus, Pseudomonas aeruginosa, and Staphylococcus aureus were analyzed. There was a slight decrease in the emission intensity of the peaks at 390 and 485 nm and was noted for all other pathogens instead of increase in intensity as in the case of AH. Therefore, the results firmly support the selectivity of anti-AH–AHC toward A. hydrophilaand shown in Figure 4.

**Competitive Performance of the Immunoassay in the Presence of Other Interfering Pathogens.** The selective immunosensing of anti-AH–AHC was proved via a selective recognition study and firmly confirmed the immunocomplex formation only between AH and anti-AH–AHC (Figure 2). The selective nature was again confirmed by performing the competitive analysis using other possible interfering pathogens such as E. coli, Bacillus, P. aeruginosa, and S. aureus (Figure 5). Interaction of all of these pathogens slightly quenches the initial fluorescent intensity of anti-AH–AHC which may be because of nonfluorescent complex formation via FRET quenching.45 The competitive test was performed by incubating 1 equiv of 2 mL of anti-AH–AHC with 1 equiv (10 μL, 10−3 CFU/g) of AH and 1 equiv (10 μL, 10−3 CFU/g) of all interfering pathogens, and the corresponding fluorescent intensity changes were recorded. Remarkably, there was no quenching in the fluorescent intensity for anti-AH–AHC. Hence, the designed immunoassay has proved to be very selective only for the AH in the presence of other interfering pathogens.

**Observation of Fluorescent and Naked Eye Color Changes Using UV–Vis Trans Illuminator.** Generally, fluorescent sensors show excellent color changes toward various analytes such as biomolecules,49 metal ions,50 and pathogens, specifically.51 In our case, we have used AHC as a fluorescent tag because of its excellent quantum yield of 0.90 which is comparable with the standard rhodamine 6G dye. First, the AHC has exhibited orange fluorescent color under UV–vis lamp in MES buffer medium (pH 5.4). After the amide coupling with anti-AH, the initial color was diminished and changed slightly into yellowish green. Finally, after the formation of an immunocomplex between the AH and anti-AH–AHC, a bright-green fluorescent color change was
coupling with anti-AH, it became slightly yellow in color, and then, finally, it turned into deep-bright-green fluorescent after binding with AH (Figure 6b). These results supported the utility of AHC as a fluorescent tag for the detection of AH.

The accumulation of AH in the organs of fish grown in contaminated water (where they were grown for different days) was analyzed through imaging the color change of anti-AH–AHC during binding with AH using a transilluminator. The fluorescent color of anti-AH–AHC increases with the increasing growing time and is shown in Figure 7. All of these observations also supported the accumulation of AH in the fish organs and selectivity of immunoassay toward AH pathogen.

**Sensing Mechanism of the AH/Anti-AH–AHC Fluorescent Immunoassay.** Among all fluorescent-sensing mechanisms, intra/intermolecular charge-transfer-based mechanism has been commonly existing in various fluorescent sensing assays. After tagging, the formation of amide linkage between anti-AH and AHC may be responsible for the spectral shift in both UV–vis as well as fluorescence emission spectra with decrement in absorbance/emission intensity. On the basis of the blue shift, it is confirmed that the synthesized AHC has shown maximum absorbance/emission which may be because of the twisted ICT mechanism among the molecule itself from donor −NH₂ to acceptor anthracene moiety. After tagging with anti-AH, the initial twisted intramolecular charge-transfer mechanism among AH molecule was inhibited because of the amide coupling between donor −NH₂ of AHC and −COOH of anti-AH. Therefore, the donating nature of −NH₂ to anthracene moiety was arrested. As a result, a blue shift was observed which may be because of the electronic charge repulsion between the two strong electron charge entities of the −NH₂ group in AHC and −COOH group in anti-AH via amide bond formation, and this repulsion finally might increase the energy levels of the anti-AH–AHC, which results in the blue shift. Interestingly, when the pathogen AH was incubated with the anti-AH–AHC, the fluorescent intensity was increased enormously. This phenomenon may be because of aggregated induced emission (AIE) of the resulting immuno-complex formation. After the incubation with pathogen, all of the AHC molecules in anti-AH–AHC might come closer to each other, and thereby the anthracene moiety in AHC can undergo dimerization or aggregation. In general, fluorescent intensity of anthracene-containing molecules will be enhanced in solution/solid phase because of the aggregation-induced phenomenon. Therefore, the fluorescent intensity of the immunoassay was increased. Therefore, based on these UV–vis and emission spectral changes, it is confirmed that two interesting mechanisms such as inhibition of twisted ICT and AIE are existing during the formation of anti-AH–AHC and AH/anti-AH–AHC immunocomplexes, respectively, under optimized conditions (Scheme 1).

**Determination of Quantum Yield (ϕ).** Using the emission data, the fluorescence quantum yield (ϕ) at various steps were estimated by integrating the area under fluorescence curves using the following equation. The absorbance value of AHC, anti-AH, and AH/anti-AH–AHC was chosen from Figure 1. The integrated area under the emission spectra of AHC and its complexes were calculated at the excitation wavelengths of 505 nm (AHC), 485 nm (anti-AH–AHC), and 411 nm (AH/anti-AH–AHC), respectively, in a phosphate buffer (pH = 7.4) and compared with quantum yields of rhodamine 6G in ethanol.

\[
\phi = \frac{\int \lambda \text{d} \lambda \text{c} \text{e} \text{s} \nu \text{d} \nu}{\int \lambda \text{d} \lambda \text{e} \text{m} \text{s} \nu \text{d} \nu}
\]

where, ϕ is the quantum yield; I is the integrated area under the corrected emission spectra; A is the absorbance at the

![Figure 6](image_url)

**Figure 6.** Color changes observed during the development of immunoassay: (a) fluorescent color changes (a) and naked eye visualization (b) [(1) AHC, (2) anti-AH–AHC, and (3) AH/anti-AH–AHC].

![Figure 7](image_url)

**Figure 7.** Fluorescent color changes observed in fish organs at different day intervals (a) and at the extract of the corresponding dissected samples in buffer solution (b).
excitation wavelength; $\lambda_{ex}$ is the excitation wavelength; $\eta$ is the refractive index of the solution; and the subscripts $u$ and $s$ refer to the unknown and the standard, respectively. The quantum yield of rhodamine 6G dye was calculated as 0.94 in absolute ethanol medium at an excitation wavelength of 470 nm. The quantum yield of AHC, anti-AH–AHC, and AH/anti-AH–AHC was calculated as 0.80, 0.328, and 0.90, respectively, and calculations were given at the end of the section. The aggregation may lead to an increment in quantum yield after binding with AH.

Analysis of Accumulation of A. hydrophila in Organs of O. mossambicus Fingerlings. The fingerlings of O. mossambicus were grown in A. hydrophila contaminated water for 7 days and the images of growing vessels are shown in the Supporting Information (Figure S1). The detailed protocol was given in the above Materials and Methods section. The accumulation of AH in three different organs such as guts, liver, and tissue of O. mossambicus fingerlings and the remaining portion of AH in growing medium were analyzed. In addition, the photo images of the organs dissected from O. mossambicus fingerlings for 7 days are shown in the Supporting Information (Figure S2).

After dissection, the organs of the fish were collected for each day, and samples were prepared as per the procedure given in Materials and Methods section and analyzed for the AH accumulation using the emission method (Figure 8a–c). Among these organs, higher accumulation of AH was found at guts than at liver and tissue. The results obtained were compared and also confirmed using the colony counting method. The colony formation of AH on a disc was also analyzed at the biologically simulated conditions of fish guts (Figure S3). The calculated CFU values are given in Table 1 and compared with the plate count method.

For cross-checking, the amount of AH in contaminated water was also tested using emission method for each day. The concentration of AH was found higher in the medium on the first day, and it started to decrease as the day goes up to fifth day, whereas in fish organs, a reverse trend was observed. However, after the fifth day, the amount of AH found in fish organs and the medium attained saturation (Figure 8d). Meanwhile, we have also performed the control experiments (Figure 8e). The results obtained from emission and colony-forming techniques have shown very good agreement. No doubt, the real potent application and the other main theme of our work are successfully demonstrated in fish samples.

## CONCLUSIONS

Hence, a very first fluorescent-based immunoassay is developed for the specific and ultrasensitive detection of A. hydrophila. We have designed a natural 4H-chromene core-based, AHC, as a fluorescent tag via the one-pot greener approach using ultrasonication method. Then, it was tagged...
with the anti-AH successfully and applied for the qualitative and quantitative detection of *A. hydrophila* from 4 to 736 CFU/mL with the LOD of 2 CFU/mL. To further enhance the application of our developed immunoassay, it was applied for the real-time quantification of *A. hydrophila* accumulation in organs of *O. mossambicus* fingerlings. Results have shown excellent agreement with the plate-counting method, and we assure that it would be a better immunoanalytical tool for the quantification of *A. hydrophilain* fish and some food samples.

**MATERIALS AND METHODS**

We have used analytical grade solvents and double distilled water throughout the studies. Malononitrile, resorcinol, anthracene-1-carboxaldehyde, HCl, disodium mono hydrogen phosphate, monosodium dihydrogen phosphate, glacial acetic acid, ammonium acetate, sodium acetate, absolute ethanol, high-performance liquid chromatography methanol, ammonium chloride, liquid ammonia, piperidine, NaOH, and KCl were purchased from Sigma-Aldrich Chemical Company. The acetate salts of metal ions analyzed were purchased from Merck and Avra Chemicals. Anti *A. hydrophila* IgG fraction monoclonal antibody [Clone 6B10/AS] was purchased from Santa Cruz Biotechnology Inc, California, via dealers from Synergy Scientific Services Pvt. Ltd., India. Bacterial strains such as *A. hydrophila* (MTCC 735), *Bacillus* (MTCC 430), *S. aureus* (MTCC96), *E. coli* (MTCC 448), and *P. aeruginosa* (MTCC 2534) were originally obtained from Microbial Type Culture Collection Centre, Institute of Microbial Technology, Chandigarh, India. Pure culture of all bacterial strains was procured from MTCC, Pune, and maintained in Luria Bertani medium at 37 °C for 16 h.

NMR studies were carried out using Bruker-400 MHz spectrometer for 1H and 13C NMR analysis using CDCl3 and DMSO-[d6] as solvents containing a trace quantity of tetramethylsilane as the internal standard, and the chemical shifts are reported in parts per million at 25 °C. UV spectra were recorded using Shimadzu single-beam UV–vis spectrophotometer. Finally, fluorescent measurements were done with the help of Cary Eclipse spectrophotometer having a 450 W xenon lamp. Bandwidths of 5 and 2.5 nm were maintained as excitation and emission slit widths, respectively, throughout the experiments.

**Ethical Statement.** Fish were maintained in accordance with the guidelines of the American fisheries society (Guidelines for the use of fish, 2014) and approved by the institutional ethical committee of Madurai Kamaraj University [Internal Research and Review Board (IRB), Ethical Clearance (EC), Biosafety and Animal Welfare Committee].

### Table 1. Comparison of Results Obtained for the Detection *A. hydrophila* in Fingerlings of *O. mossambicus* Fish Organs Current Developed Fluorescence Immunoassay Protocol with the Normal Plate-Count Method

| day order | water sample name of the fish part | control | spiked | name of the fish part | control | spiked | name of the fish part | control | spiked |
|-----------|-----------------------------------|---------|--------|-----------------------|---------|--------|-----------------------|---------|--------|
| 1         | gut                               | control | a        | 25                   | b        | 20                  | control | a        | 8        | b        | 15                   | control | a        | 5         | b        | 4        |
| 2         | gut                               | control | 33               | 42                  | control | 20               | control | 30               | 42                  | control | 5         | control | 5         | 5         |
| 3         | gut                               | control | 50               | 55                  | control | 30               | control | 50               | 65                  | control | 8         | control | 7         | 9         |
| 4         | gut                               | control | 70               | 65                  | control | 50               | control | 80               | 72                  | control | 10        | control | 15        | 15        |
| 5         | gut                               | control | 405              | 385                 | control | 90               | control | 200              | 190                 | control | 12        | control | 20        | 20        |
| 6         | gut                               | control | 726              | 410                 | control | 413              | control | 395              | 395                 | control | 15        | control | 22        | 22        |
| 7         | gut                               | control | 736              | 410                 | control | 413              | control | 395              | 395                 | control | 15        | control | 22        | 22        |

Figure 9. 1H NMR of the synthesized AHC.
The AHC was tagged with anti-AH via standard coupling protocol. The structure of the synthesized 4H-chromene derivative was thoroughly characterized by NMR technique, and the corresponding coupling constant values are given below.

$^{1}$H NMR (400 MHz, DMSO-$d_{6}$): 7.23 (s, 1H), 6.99 (s, 1H, −OH), 6.55 (s, 2H, −NH$_2$), 6.03−5.98 (m, 4H), 5.86−5.77 (m, 4H), 5.25 (s, 2H), 4.77 (s, 1H), 4.52 (d, 1H) and 4.26 (d, 1H) ppm.$^{13}$C NMR (400 MHz, DMSO-$d_{6}$): 157.88 (C1), 156.65 (C4), 156.65 (C5), 152.15 (C2), 151.38 (C3), 102.85 (C4), 156.65 (C5), 93.94 (C6), 30.38 (C7), 57.68 (C8), 159.82 (C9), 150.48 (C12), 132.13 (C13, C17), 137.28 (C14, C16), 127.34 (C15), 120.83 (C27), 129.04 (C20, C21, C22), and 131.04 ppm. C$_{23}$H$_{16}$N$_{2}$O$_{2}$; calcld mass: 364.40. The melting point (uncorrected) of the synthesized AHC was found to be 220 °C (Figures 9 and 10).

Protocol for the Tagging of AHC with the AH Antibody. The AHC was tagged with anti-AH via standard EDC/NHS coupling protocol. In briefly, 1 equiv of anti-AH (1 mL, 100 μg/μL) in MES buffer and 4 equiv (4 mL, 0.001 M) of AHC were mixed, and then 100 μL of each solution of EDC and NHS were added one by one to the above solution. It was gently stirred for 1−2 h and then kept for incubation at 4 °C for 1 day. Then, the resultant tagged anti-AH/AHC (0.002 μg) was allowed to stand at room temperature for 5−10 min, and then it was completely centrifuged at 30 000 rpm for 20 min at 4 °C. The resulting pellets were collected and washed thoroughly with PBS buffer for five times. The unbound AHC was removed by simply washing with PBS buffer and confirmed by analyzing the supernatant solution using UV−vis spectra until the disappearance of standard UV−vis peak of AHC. The final anti-AH/AHC in buffer solution was stored at 4 °C for further studies (Scheme S2).

Bacterial Cultivation. Pure culture of AH bacteria was procured from MTCC1739 strain, Pune, India. It was grown initially in tryptic soya broth and maintained in Luria Bertani medium at 30 °C for 16 h for further studies. The bacterial cells were then separated through centrifugation (6000 rpm, 20 min) and then rinsed thrice with PBS. The culture was serially diluted with physiological saline solution, and the viable cell number was determined via the most probable number method.

Development of Immunosensor (AH/Anti-AH−AHC). Under optimized conditions, 2 mL of AHC/anti-AH (0.002 μg) was mixed thoroughly with 10 μL of AH pathogens diluted in different CFU, and then they were incubated at 4 °C for 25 min. The major absorption maxima at 277 nm were fixed as excitation wavelength, and the corresponding fluorescence spectra were recorded at 411 nm, and all of these experiments were carried out in the phosphate buffer (PBS, pH = 7.4) medium throughout the studies.

Fish Maintenance and Treatment Procedure. Fingerlings of O. mossambicus were grown in artificial condition (plastic turf in laboratory condition) for 1 month, subsequently; they were transferred in to 250 mL conical flask in the presence of sterile water (200 mL) and kept for acclimatization for 15 days inside the sterile laminar hood. After acclimatization, AH was inoculated in seven different conical flasks along with fingerlings of O. mossambicus, and 100 μg/mL concentration of filter-sterilized glucose was added to enhance the growth of AH. From first day to seventh day, fingerlings were sacrificed using sterile dissection apparatus in...
sterile condition, and liver, guts, and tissues samples were collected in sterile PBS (pH = 7.4), and a loop-full suspension was inoculated in the AH growth-specific media to check the increasing number of bacterial colony. O. mosambicus in sterile water without AH was used as a control, and the experiment was performed thrice in a triplicate manner.

**ASSOCIATED CONTENT**

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02467.

Synthetic scheme of AHC, tagging of anti-AH with AHC, fish maintaining, dissection, and plate-count method images for the developed immunoassay (PDF)

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS**

AHC, (S)-2-amino-4-(anthracen-9-yl)-7-hydroxy-4H-chromene-3-carbonitrile

AH, Aeromonas hydrophila

NMR, nuclear magnetic resonance

UV−vis, ultraviolet−visible

CFU, colony forming unit

EPA, environmental protection agency’s

PCR, polymerase chain reaction

DNA, deoxyribonucleic acid

MTCC, Microbial Type Culture Collection and Gene Bank

EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride

NHS, N-hydroxysuccinimide

PBS, phosphate buffer saline

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