Facile Fluorescence Monitoring of Gut Microbial Metabolite Trimethylamine N-oxide via Molecular Recognition of Guanidinium-Modified Calixarene

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Abstract

Detection and quantification of trimethylamine N-oxide (TMAO), a metabolite from gut microbial, is important for the disease diagnosis such as atherosclerosis, thrombosis and colorectal cancer. In this study, a novel method was established for the sensing and quantitative detection of TMAO via molecular recognition of guanidinium-modified calixarene from complex matrix.

Methods: Various macrocycles were tested for their abilities to serve as an artificial TMAO receptor. Using the optimized receptor, we developed an indicator displacement assay (IDA) for the facile fluorescence detection of TMAO. The quantification of TMAO was accomplished by the established calibration line after excluding the interference from the various interfering substances in artificial urine.

Results: Among various macrocycles, water-soluble guanidinium-modified calix[5]arene (GC5A), which binds TMAO in submicromolar-level, was identified as the optimal artificial receptor for TMAO. With the aid of the GC5A•Fl (fluorescein) reporter pair, TMAO fluorescence "switch-on" sensing was achieved by IDA. The fluorescence intensity increased linearly with the elevated TMAO concentration. The detection was not significantly interfered by the various interfering substances. TMAO concentration in artificial urine was quantified using a calibration line with a detection limit of 28.88 ± 1.59 µM, within the biologically relevant low µM range. Furthermore, the GC5A•Fl reporter pair was successfully applied in analyzing human urine samples, by which a significant difference in fluorescence response was observed between the [normal + TMAO] and normal group.

Conclusion: The proposed supramolecular approach provides a facile, low-cost and sensitive method for TMAO detection, which shows promise for tracking TMAO excretion in urine and studying chronic disease progression in humans.

Key words: calixarene, trimethylamine N-oxide, gut microbiota, fluorescence sensing, indicator displacement assay

Introduction

Trimethylamine N-oxide (TMAO) is the most focused metabolite originated from intestinal microorganisms, and its precursor, trimethylamine (TMA), is transformed from dietary carnitine, choline or choline-containing compounds by host intestinal bacteria [1, 2]. TMA enters the liver via the portal circulation, where it is rapidly converted into a water-soluble compound, TMAO, by host hepatic
flavin monooxygenases [1]. In recent years, the mysterious veil of TMAO in the development of numerous diseases has been gradually uncovered [3-7]. In vivo animal studies have shown that TMAO directly increased the reactivity and thrombotic potential of platelets, which are important risk factors for complications of cardiovascular metabolic diseases (e.g., heart attack and stroke) [3]. In people, TMAO is mostly eliminated from body though urine, and is also excreted though breathe and sweat [8]. As a noninvasive test method, urine testing is of great significance for the diagnosis and risk prediction of clinical diseases. Consequently, quantifying TMAO in urine becomes an indispensable and urgent clinical task that facilitates the early diagnosis of disease.

Some sophisticated methods have been reported for quantifying TMAO in biological matrices. With the high selectivity, sensitivity and throughput, mass spectrometry has been extensively applied in clinical detection. Undertaken to determine TMA reduced from TMAO by the guidance of complex protocols, gas chromatography-mass spectrometry (GC-MS) method suffered from the complicated and laborious procedures, time consumption, and incompleteness of TMAO transformation, limiting its clinical use [9]. To our knowledge, many researchers preferred utilization of liquid chromatography tandem mass spectrometry (LC-MS/MS) for determining TMAO, employing a stable isotopically labelled standard [10-16], which required synthetic stable isotope markers, specially trained personnel, and specialized and costly analytical instruments that were not widely available in clinical diagnostic laboratories. Other studies have employed proton nuclear magnetic resonance spectroscopy (1H NMR) to determine TMAO, which underwent poor sensitivity, instability of determining results subjected to pH variation [17-19]. To address these issues, the development of a novel, simple, inexpensive and rapid method for TMAO detection is urgently needed.

The well-developed artificial receptors with discrete cavities, macrocyclic hosts of cyclodextrin, calixarene and cucurbituril types, selectively bind certain guests. With the help of the intriguing host-guest properties between macrocycles and biological substrates, the molecular recognition by macrocycles in aqueous media has attracted a great deal of attention. It has been used in disease diagnosis and therapy [20-30], enhancement of drugs’ solubility and stability [31-34], and regulation of protein-protein interactions [35, 36], among others.

Recently, researchers have increasingly paid attention to calixarene macrocycles in the biomedical field on account of their recognition and assembly properties [24-26, 37-41]. To the best of our knowledge, despite the significant achievements in the molecular recognition of macrocycles, the binding and detection of TMAO by macrocycle have not been reported yet. In this study, we explored the facile fluorescence monitoring for TMAO on the basis of indicator displacement assay (IDA). Water-soluble guanidinium-modified calix[5]arene (GC5A) was screened as an artificial receptor from various water-soluble macrocycles, including cyclodextrins, p-sulfonatocalixarenes and cucurbiturils. Benefiting from submicromolar binding of TMAO by GC5A, the fluorescence “switch-on” sensing of TMAO was realized with high sensitivity. It was impressed us that the quantitative detection of TMAO was achieved in artificial urine within the biologically relevant low µM range without tedious sample pretreatment, conducing to point-of-care testing. Also, the fluorescence response of human urine samples was proved to be significantly different between the [normal + TMAO] and normal group.

Materials and Methods

Materials

Unless otherwise specified, all chemicals used in this study were commercially available. TMAO 2H2O and p-dimethylaminobenzonitrile (DMABN) were purchased from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Sodium chloride, a-cyclodextrins (a-CD), β-cyclodextrins (β-CD), γ-cyclodextrins (γ-CD), cucurbit[6]uril hydrate (CB6), cucurbit[7]uril hydrate (CB7), cucurbit[8]uril hydrate (CB8), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) and deuterium oxide (D2O) were obtained from Sigma-Aldrich Co., Ltd. (St. Louis, Missouri, USA). Fluorescein (Fl), 8-hydroxyxyprene-1,3,6-trisulfonate (HPTS), acridine orange (AO), 2-(p-tolidiyl)naphthalene-6-sulfonic acid (2,6-TNS), glutamic acid (Glu), aspartic acid (Asp) and fumaric acid were purchased from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA) and creatinine were purchased from J&K Chemical Co., Ltd (Beijing, China). N,N’-dimethyl-2,7-diazapyrenium (Me2DAP) was purchased from Tianjin Heowns Biochemical Technology Co., Ltd. (Tianjin, China). Bovine serum albumin (BSA) and creatinine were purchased from J&K Chemical Co., Ltd (Beijing, China). 

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Preparation of HEPES buffer solution

HEPES buffer solution (10 mM, pH 7.4) was obtained by dissolving 2.38 g HEPES in approximately 900 mL ultra-pure water. The solution was titrated with sodium hydroxide to pH 7.4 at 25 °C, which was then diluted to 1 L with ultra-pure water to make a 10 mM HEPES buffer solution. The pH value of the buffer solution was checked with a Sartorius PB-20 pH-meter adjusted by three standard buffer solutions.

Preparation of artificial urine solution

Artificial urine solution was obtained as previously reported [45], which was briefly prepared by dissolving urea (36.40 g), sodium chloride (15.00 g), sodium phosphate (monobasic, 9.60 g), potassium chloride (9.00 g), creatinine (4.00 g) and BSA (100 mg) in 2 L ultra-pure water. The artificial urine solution was verified to pH 6.0 with hydrochloric acid or sodium hydroxide and stored at 4 °C.

Preparation of urine samples

Written informed consent was signed by five healthy volunteers (22-33 years old), who provided urine samples maintained at −20 °C until analysis. The supernatant of urine samples was collected after 10 min centrifugation (14000 rpm) and diluted twofold with 10 mM HEPES buffer solution (pH 7.4) before analysis.

Fluorescence spectroscopy

The steady-state fluorescence spectra were recorded in a conventional 10 × 10 × 45 mm³ quartz cell (light path = 10 mm) by Varian Cary Eclipse spectrometer (Agilent Technologies Inc., USA). In order to eliminate the affect on the measured result caused by temperature variation, a Varian Cary single-cell Peltier accessory was equipped.

The excitation wavelengths for Fl, DMABN, 2,6-TNS, HPTS, LCG, DSMI, AO, and Me2DAP were 500, 300, 350, 405, 368, 450, 450 and 335 nm, respectively [38]. For the direct host-guest titrations at 25 °C in 10 mM HEPES buffer solution (pH 7.4), the dye solutions (0.50 – 10.00 µM) were excited at their respect excitation wavelengths to afford a emission, and the sequential changes of fluorescence intensity at various concentrations of hosts were used to determine the host-guest association constant (K_a). The data were fitted according to a 1:1 host-guest (with the exception of 1:2 for SC6A•LCG) binding stoichiometry [39, 46, 47].

The competitive fluorescence titrations in 10 mM HEPES buffer solution (pH 7.4) at 25 °C were carried out by gradual addition of competitor (TMAO) with known concentration to solutions containing hosts and dyes with appropriate concentrations, which gave rise to variation of the fluorescence intensities of dyes. The association constants of TMAO with hosts were obtained by fitting fluorescence intensities at their emission wavelengths (Fl: 513 nm, LCG: 505 nm) and TMAO concentrations according to the 1:1 competitive binding model [48]. In the course of the titrations, the concentration of hosts and dyes were kept constant.

The calibration line was established at 25 °C according to the fluorescence intensities of GC5A•Fl reporter pair by gradual addition of TMAO with known concentrations in HEPES buffer (10 mM, pH 7.4) and artificial urine solution, respectively.

NMR spectroscopy

1H NMR spectra were recorded on a Bruker AV400 spectrometer (Bruker Group, Switzerland) using fumarpic acid (δ = 6.70 ppm) as an external reference. 2D ROESY spectrum was recorded on a Zhongke-Niujin BIXI-I 400 spectrometer (Zhongke-Niujin, Wuhan, China). Samples of GC5A•TMAO complex, GC5A and TMAO for NMR measurement were prepared in D2O. All NMR spectra were acquired at 298 K in the solution state.

Theoretical Calculations

A Gaussian 09 program density was employed to perform functional theory calculations [49]. Based on density, the solvation model was undertaken in all calculations [50]. At the B3LYP/6-31G(d) level, geometry optimization was carried out by using Grimme’s D3 dispersion correction [51, 52]. Default convergence criteria were used for the optimization in Gaussian 09.

Data analysis

Mean values of fluorescence titrations, calibration lines and limit of detection were determined from at least three experiments, and errors were given as standard deviations (± SD).

The fitting of data from direct host-guest titrations and competitive titrations were performed in a nonlinear manner [39], and the fitting modules were downloaded from the website of Prof. Nau’s group (http://www.jacobs-university.de/ses/wnau) under the column of “Fitting Functions”. It was introduced in detail as following.

For analyzing the host-guest fluorescence titrations as described by equation 1, we considered that a guest (G) formed a 1:1 host•guest complex with a host (H) at an association constant (K_a), which satisfied the respective law of mass action relating to the equilibrium concentrations of free host, [H], free
guest, [G], and host•guest complex [HG]. Also, the relationship between the total concentrations of host, [H]₀ and guest, [G]₀ and their equilibrium concentrations were introduced by the law of mass conservation (equation 2). Here, [G]₀ was the initial concentration of guest as a known experimental parameter, which was kept constant in the titration process. Furthermore, equation 1 and 2-1 were employed to deduce equation 3.

When the fluorescence titration was performed, the intensity of fluorescence (F) corresponded to the combined intensity of the guest and the host•guest complex, which were described by their molar fractions (equation 4). Both F_{HG} and F_{G} were the known experimental parameters, in which F_{HG} was the fluorescence intensity when all guests were complexed and F_{G} when they were uncomplexed. The equation 5 deduced by equation 2-2, 3 and 4, explained the relationship between K_a and variables ([H]₀) in fluorescence titration [47]. In the light of equation 5, K_a was obtained by fitting the data of fluorescence intensity and total host concentration.

\[
[H]+[G] \xrightarrow{K_a} [HG]
\]
\[
K_a = \frac{[HG]}{[H][G]}
\]  
(1)
\[
[G] = [G]_o - [HG]
\]  
(2-1)
\[
[H] = [H]_o - [HG]
\]  
(2-2)
\[
[HG] = \frac{K_a[H][G]_o}{1 + K_a[H]}
\]
(3)
\[
F = \frac{[HG]}{[G]_o} F_{HG} + \frac{[G]}{[G]_o} F_{G}
\]
(4)
\[
F = F_{HG} + (F_{HG} - F_{G}) \frac{K_a[H]}{1 + K_a[H]}
\]
(5)

For the analysis of the competitive titrations (equation 6), we also considered a competitor (TMAO) that could bind to a host’s cavity in a 1:1 stoichiometry at an association constant (K_T). Free host, [H], free competitor, [T], and host•TMAO complex [HT] obeyed the respective law of mass action referring to the equilibrium concentrations. Also, [H]₀ and the total concentrations of TMAO, [T]₀, and their equilibrium concentrations satisfied the law of mass conservation (equation 7).

\[
[H] + [T] \xrightarrow{K_T} [HT]
\]
\[
K_T = \frac{[HT]}{[H][T]}
\]
(6)
\[
[H] = [H]_o - [HG] - [HT]
\]  
(7-1)
\[
[T] = [T]_o - [HT]
\]  
(7-2)
\[
F_C = \frac{[HG]}{[G]_o} F_{HG} + \frac{[G]}{[G]_o} F_{G}
\]
(8)
\[
F_C = F_{G} + (F_{HG} - F_{G}) \frac{K_a[H]}{1 + K_a[H]}
\]
(9)

\[
0 = A[H]^2 + B[H] + C[H] + D, \quad \text{where}
\]
\[
A = K_a K_T
\]
\[
B = K_a + K_T + K_a [G]_o + [T]_o - [H]_o
\]
\[
C = K_T [T]_o - [H]_o
\]
\[
D = -[H]_o
\]
(10)

Results and Discussion

Screening of TMAO artificial receptors from classical water-soluble macrocycles

IDA (Scheme 1) was popularized by Anslyn and co-workers, the synthetic receptors with different styles were employed for molecular sensing via competitive binding assays [20]. In this classical model, introduction of a competitive analyte causes the shift of fluorescence intensity due to extrusion of fluorescence indicator from the host, which feasibly paved the way for determining analytes without chromophores or chemical labeling. Importantly, the sifting of host and indicator with the appropriate binding affinity must be accomplished, allowing for the linear fluorescence response of indicator along with increase of analyte concentration. Therefore, the key step to develop a successful IDA for TMAO is to screen artificial receptors with strong and selective binding towards TMAO.

The most popular water-soluble macrocyclic receptors commercially available [46], including cyclodextrins (α-, β-, and γ-CD), p-sulfonatocalix[n]-arenes (SCnAs, n = 4, 5, 6) and cucurbit[n]urils (CBn, n = 6, 7, 8), were selected for sensing and quantification of TMAO. As for the CD family, the fluorescence response was not influenced by the gradual addition of TMAO after screening of three reporter pairs, α-CD•DMABN [55], β-CD•2,6-TNS [56] and γ-CD•
HPTS [57] (Figures S1 – S3, Table 1). Unfortunately, similar result happened to the SCnA family except for SC4A (Figures S4 – S6, Table 1). LCG showed slight response by gradual addition of TMAO to the SC4A•LCG reporter pair [38, 39, 46], which arose from fluorescence enhancement in view of LCG extrusion from SC4A. The binding affinity of SC4A towards TMAO was 65.51 ± 3.61 M$^{-1}$, which cannot satisfy the implementation of IDA. Benefiting from extremely strong binding capability, the CB family came into our sight, with the highest binding affinity of 7.2 × 10$^{17}$ M$^{-1}$ between CB7 and diamantane quaternary diammonium ion [58]. Therefore, the feasibility of TMAO quantification was demonstrated by employing CB6•DSMI [59], CB7•AO [60] and CB8•Me$_2$DAP [61]. However, the binding towards TMAO was not obtained by the CB family using the competitive fluorescence titrations (Figures S7 – S9, Table 1). In general, failure of TMAO quantification was due to the unsatisfactory non-covalent interactions, such as hydrogen bonding, hydrophobic interaction and van der Waals forces, between host and guest. In our opinion, the hydrophobic cavities of CDs and CBs families cannot afford the ideal binding affinity with water-soluble TMAO [32]. SCnAs with negative charge cannot successfully encapsulate TMAO, a neutral polar compound [62]. This study revealed that the molecular recognition of TMAO by artificial receptors posed a challenge to us. It drove us to search for the ideal reporter pair for TMAO quantification.

Table 1. The reporter pairs of fluorescent dyes and macrocycles utilized for detecting TMAO by IDA.

| Reporter pair               | $K_a$ (dye) / M$^{-1}$ | $\lambda_{ex}$ (nm) | $K_a$ (TMAO) / M$^{-1}$ b, c |
|-----------------------------|------------------------|---------------------|-----------------------------|
| α-CD DMABN                  | (2.58 ± 0.03) × 10$^2$ | 350 (525)           | –                          |
| β-CD 2,6-TNS                 | (1.88 ± 0.36) × 10$^3$ | 350 (485)           | –                          |
| γ-CD HPTS                    | (5.61 ± 0.26) × 10$^3$ | 405 (435)           | –                          |
| SC4A LCG                    | (1.26 ± 0.13) × 10$^7$ | 368 (505)           | (6.55 ± 0.36) × 10$^1$     |
| SC5A LCG                    | (1.48 ± 0.21) × 10$^6$ | 368 (505)           | –                          |
| SC6A$^a$ LCG                | (4.96 ± 0.62) × 10$^7$ | 368 (505)           | –                          |
| CB6 DSMI                    | (1.68 ± 0.26) × 10$^6$ | 450 (582)           | –                          |
| CB7 AO                      | (9.04 ± 0.54) × 10$^4$ | 450 (510)           | –                          |
| CB8 Me$_2$DAP               | (1.27 ± 0.42) × 10$^5$ | 335 (449)           | –                          |

*a, $\lambda_{ex}$ represents the fluorescence excitation wavelength. b, $\lambda_{em}$ represents the maximum fluorescence emission wavelength. c, $K_a$ represented the binding affinity of macrocycle towards TMAO. d, “−” represented no binding detected. e, data fit with the host:guest 1:2 binding model.

Scheme 1. Schematic illustration for IDA operating principle of TMAO fluorescence “switch-on” sensing by the GC5A•Fl reporter pair and chemical structures of the tested reporter pairs.
The study on formation of the GC5A•TMAO complex

GC5A, a new water-soluble calixarene, was synthesized and first applied in detecting lysophosphatidic acid in blood as a biomarker of cancer via fluorescence IDA by our group [26]. Also, GC5A was successfully employed in identifying six glycosaminoglycans [25]. Furthermore, the $K_a ((5.0 \pm 1.0) \times 10^6 \text{M}^{-1})$ of GC5A•Fl was validated by the fluorescence titration and UV-Vis titration [26]. Reasonably, GC5A-based molecular recognition and sensing were performed to determine TMAO with the aid of Fl as the indicator for IDA [26]. Importantly, the fluorescence intensity of Fl was not affected by gradual addition of TMAO (0 – 9.48 mM) (Figure S10). As shown in Figure 1, the displacement of Fl with TMAO from the GC5A•Fl reporter pair was monitored by the increase in fluorescence signal. Fortunately, GC5A afforded a relatively strong binding affinity $(1.61 \pm 0.04) \times 10^4 \text{M}^{-1}$ towards TMAO, which shed light on the puzzling question for TMAO quantification.

The encapsulation of TMAO into GC5A resulted in forming the GC5A•TMAO complex, which was validated by $^1$H NMR spectroscopy (Figure 2). As soon as TMAO has been titrated into GC5A, the signal assigned to TMAO methyl protons shifted downfield ($\Delta \delta = 0.23 \text{ppm}$). The shift of TMAO protons were possibly caused by the intermolecular hydrogen bonding and ring currents effect, which resulted in shift to downfield and upfield, respectively [63-65]. Compared to the negatively charged or uncharged calixarenes [39, 63, 66], the GC5A with positively charged substituents had the lower electrostatic potential, leading to a relatively weak ring currents effect [26]. Therefore, the shift was dominated by the intermolecular hydrogen bonding, which was lessened under the influence of the aromatic ring currents of GC5A. The signals of the protons from GC5A did not shift appreciably, because TMAO lacked groups (such as aromatic rings) that could result in significant shielding or deshielding. The assumed binding geometry was further demonstrated by the 2D ROESY spectrum (Figure S11). The intermolecular correlation was observed between the methyl protons of TMAO and aromatic protons of GC5A. Geometry optimization of the GC5A•TMAO complex revealed the formation of hydrogen bonding interaction and the size/shape matching between the GC5A cavity and TMAO (Figure 2B inset), which was consistent with the NMR information [51, 67-69].

Quantification of TMAO by the GC5A•Fl reporter pair

As shown in Figure S12, the elevated TMAO concentration was accompanied by an increased fluorescence signal ($R^2 = 0.980$), which supported fluorescence “switch-on” sensing of TMAO in the GC5A•Fl (0.80/1.00 µM) reporter pair. The limit of detection (LOD) of TMAO was achieved at 8.98 ± 0.06 µM in the HEPES buffer solution using the $3\sigma/\text{slope}$ method [70, 71]. Subsequently, the feasibility of TMAO quantification was tested in artificial urine. It was very important to verify whether the matrix substances posed interference or not, including creatinine, urea, chlorine, bovine serum albumin (BSA), glutamic acid (Glu) and aspartic acid (Asp) (Figure 3A), conducing to evaluating the sensing selectivity of GC5A•Fl reporter pair to TMAO. No

Figure 1. Fluorescence competitive titration in the GC5A•Fl (0.80/1.00 µM) reporter pair with TMAO (up to 2.52 mM) at $\lambda_{ex} = 500 \text{nm}$ (A) and competitive titration curve ($\lambda_{em} = 513 \text{nm}$) and fitting data according to a 1:1 competitive binding model (B) in HEPES buffer solution (10 mM, pH 7.4) at 25 °C.

Figure 2. $^1$H NMR spectra (400 MHz, 298 K) of TMAO (2.00 mM) (A), TMAO (0.40 mM) with addition of GC5A (2.00 mM) (B) and GC5A (2.00 mM) (C) using fumaric acid (peak E in NMR spectra) as an external reference in D$_2$O. The optimized structure of the GC5A•TMAO complex at the B3LYP-D3(BJ)/6-31G(d)/SMD (water) level of theory shown in the inset of Figure 2B. Some hydrogen atoms were omitted for clarity.
obvious interference was detected upon addition of matrix substances into the GC5A•Fl reporter pair.

The artificial urine was employed to verify application of GC5A•Fl in TMAO quantification. Accompanied by the increased TMAO concentration, the elevated fluorescence signal was linearly monitored in artificial urine in the range of 0 to 1.22 mM ($R^2 = 0.999$, Figure 3B), which also demonstrated that the interference from the matrix of artificial urine could be excluded in the GC5A•Fl (10.00/5.00 µM) reporter pair. The TMAO quantification was proved to be sensitive by obtaining LOD low to 28.88 ± 1.59 µM in artificial urine. Furthermore, the GC5A•Fl reporter pair was successfully applied in analyzing TMAO in authentic human urine samples from the healthy volunteers. According to the reported TMAO concentration (approximately 0.20 mM) in urine from infarcted patients [72], the corresponding TMAO was added to the samples of normal group. As shown in Figure 4, the significant difference in fluorescence response was identified between the [normal + TMAO] and normal group.

In general, we herein achieved sensitive detection of TMAO, arising from the binding capability of GC5A towards TMAO as well as the remarkable fluorescence regeneration of Fl from the GC5A•Fl reporter pair. A calibration line was set up for accurately determining TMAO concentration down to the low µM range, based on the linear relationship between the fluorescence intensity and TMAO concentration, principally conducing to track TMAO excreted in urine. Also, a successful application was performed in human urine samples to distinctly identify the difference of fluorescence response between the [normal + TMAO] and normal group.

**Conclusions**

In conclusion, we have established an IDA method for the fluorescence “switch-on” sensing and quantitative assay of TMAO in artificial urine via the GC5A•Fl reporter pair. To accurately determine concentrations of TMAO in the low µM range for practical diagnostic purpose, a calibration line of TMAO in artificial urine was successfully established. The feasibility was approved by application in detecting TMAO in authentic human urine samples from the healthy volunteers. In comparison to the previous methods, this study provides a low-cost, easy-to-operate, label-free and sensitive method for detecting TMAO, which may offer an alternative for TMAO detection in the clinical study. This method shows promise for application in tracking TMAO excretion and studying chronic disease progression in humans.

**Abbreviations**

AO: acridine orange; Asp: aspartic acid; BSA: bovine serum albumin; CB: cucurbituril; CD: cyclo-dextrin; DMABN: p-dimethylaminobenzonitrile; D$_2$O: deuterium oxide; DSMI: trans-4-[4-(dimethylamino)-styril]-1-methylpyridinium iodide; 2D ROESY: two dimensional rotating-frame nuclear Overhauser effect spectroscopy; Fl: fluorescein; GC5A: guanidinium-modified calix[5]arene; GC-MS: gas chromatography-mass spectrometry; Glu: glutamic acid; $^1$H NMR:
proton nuclear magnetic resonance; HEPS: 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; HPTS: 8-hydroxy-1,3,6-trisulfonate; IDA: indicator displacement assay; LC-MS/MS: liquid chromatography tandem mass spectrometry; LCG: lucigenin; LOD: limit of detection; Me2DAP: N,N’-dimethyl-2,7-diazapyrenium; SCnα: p-sulfonatocalix[n]arene; SD: standard deviations; TMA: trimethylamine; TAOM: trimethylamine N-oxide; 2,6-TNS: 2-(p-toluidinyl)-naphthalene-6-sulfonic acid.

Supplementary Material

Supplementary figures and tables. http://www.thno.org/v09p4624s1.pdf

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Competing Interests

The authors have declared that no competing interest exists.

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