Zinc protoporphyrin–trimethylamine-N-oxide complex involves cholesterol oxidation causing Atherosclerosis

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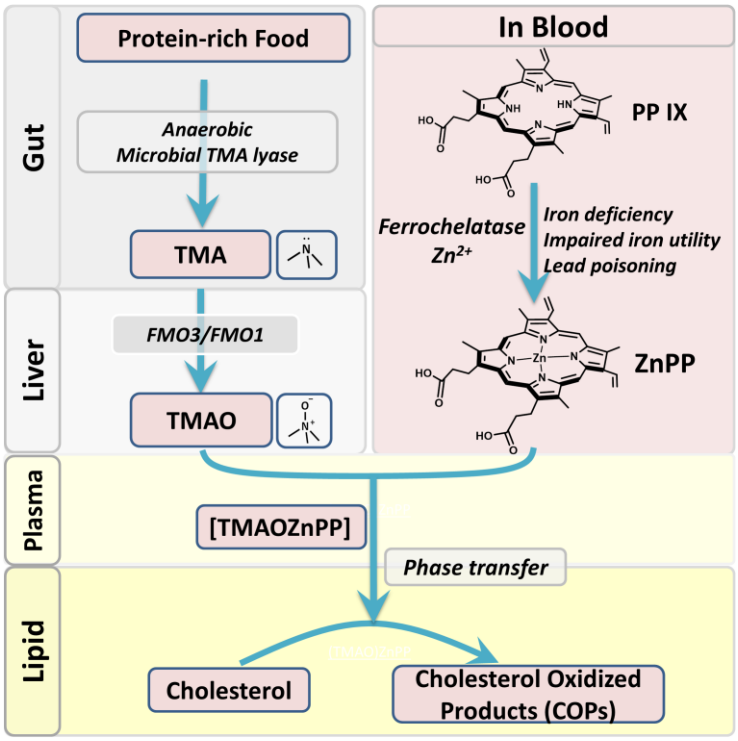
Abstract: Trimethylamine-N-oxide (TMAO) has recently been correlated as biomarker for atherosclerosis. In a model study, we show that the free TMAO when bonded with zinc-protoporphyrin IX, [ZnPP], in blood plasma as [TMAOZnPP] is transported to the lipid site is the reacting species to oxidize cholesterol causing atherosclerosis

Keywords: Atherosclerosis, TMAO, Zinc protoporphyrin, Cholesterol oxidation, Phase transfer.

Trimethylamine-N-oxide (TMAO) present in blood plasma has been implicated in cholesterol oxidation leading plaque formation causing Atherosclerosis[1-3]. The presence of oxidized cholesterol products in plaque are shown in vivo[4-6]. The oxidized LDL in association with macrophages produce foam cell which gradually accumulates to form atherosclerotic plaque[7-11]. This led to heart (coronary heart disease), brain (ischemic stroke), or lower extremities (peripheral vascular disease) diseases[12, 13]. Extensive biomedical research established that choline, L-carnitine, betaine, consumed through dietary sources such as red meat, egg, salt-water fish and dairy products, is metabolized by gut microbes to form trimethylamine(TMA)[14, 15]. TMA catalytically oxidized by hepatic flavin containing monooxygenase (FMO3/FMO1), to trimethylamine-N-oxide (TMAO)[16]. Recently the concentration of TMAO in blood plasma has been correlated as biomarker for atherosclerosis[17, 18]. Cholesterol circulates in plasma as an essential precursor for biosynthesis of steroid hormones, bile acid and vitamin D[19, 20]. But it is not known, how hydrophilic TMAO interacts with lipophilic cholesterol to induce atherosclerosis. Such interaction between TMAO and cholesterol requires a phase transfer mediator. A carrier molecule is essential to transport the hydrophilic TMAO into the lipid site for the oxidation reaction. In this communication we show that the protoporphyrin IX zinc, [ZnPP], complex present in the blood plasma binds TMAO to transport it to the lipid site for the oxidation reaction by a model study. Different stages in the formation of cholesterol oxidized products are briefly shown in scheme-1.



Scheme 1 The pathway of production of free TMAO and [ZnPP] in blood plasma where TMAO bonded [ZnPP] is phase transferred to the lipid phase to oxidize cholesterol to oxysterols.

ZnPP is a metabolite which is produced in trace amount during the last step of heme biosynthesis where insertion of surrogate zinc ion in protoporphyrin IX (PP) occurs as a result of iron inadequacy or impaired iron utility[21]. ZnPP production and build up in human occurs in cases of lead poisoning and anaemia (Scheme-1). Besides, zinc ion can even be inserted non-enzymatically to PP. Interestingly, the blood in healthy individuals contain PP and [ZnPP]; at a ratio of 5 µM : < 50 µM respectively. But [ZnPP] and heme are present in a ratio of 50: 1 x 10^6. Cheng et al. observed that [ZnPP] augments atherosclerosis[22-24]. However, the reason for its involvement in augmenting atherosclerosis has not been clarified. The cholesterol oxidized products (COPs) or oxysterols are regarded to have a potential impact on atherosclerosis. The cholesterol within LDL gets enzymatic oxidation by reactive oxygen species (ROS) or by other chemical processes not clearly understood[25-28]. In enzymatic process its side chain is mainly oxidized forming 22-hydroxycholesterol, 24-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol along with 7-hydroxycholesterol. But in the non-enzymatic process sterol ring is oxidized preferentially at double bond and 7^{th} carbon position resulting products mainly 7-hydroxycholesterol (α & β), 7-ketocholesterol, 5,6-epoxycholesterol (α & β) and cholestan-3,5,6-triol[28]. The involvement of non-enzymatic oxidation of cholesterol may be augmented by TMAO. And this can only be

achieved once the polar TMAO is transported to the non-polar site of cholesterol. Here the role of [ZnPP] as transporter of TMAO to cholesterol site is the possibility.

Therefore, the interaction between [ZnPP] and TMAO has been studied and the results are presented herein. The PP was derivatized to protoporphyrin IX dimethyl ester, [PPDME] just to circumvent the polymerization problem in handling free PP. Then zinc ion was incorporated into [PPDME] to form [ZnPPDME] followed by the formation of its TMAO adduct, [TMAOZnPPDME]. (see ESI) .To understand the details of such interaction, a synthetic analogue, zinc meso-tetraphenylporphyrin – TMAO, [TMAOZnTPP], has also been synthesized. [ZnPPDME] easily transfers TMAO from aqueous phase to non-aqueous phase like dichloromethane (DCM) forming [TMAOZnPPDME]. The phase transfer reaction between TMAO with [ZnPPDME] could be visually recognised (Figure1a). Spectrophotometric study shows that on adduct formation with TMAO the Soret band of [ZnPPDME] is red-shifted by 14 nm; Q-bands are also shifted to higher wavelengths (Figure 1c). Similar changes were observed in the interaction between TMAO and [ZnTPP] (Figure 1d). Due to the non-availability of diffraction quality crystals of [TMAOZnPPDME], we perform the structural study on the synthetic analogue, [TMAOZnTPP], to understand the nature of interaction between TMAO and ZnTPP in the complex, [TMAOZnTPP]. The structure of this complex is shown in Figure 1b.

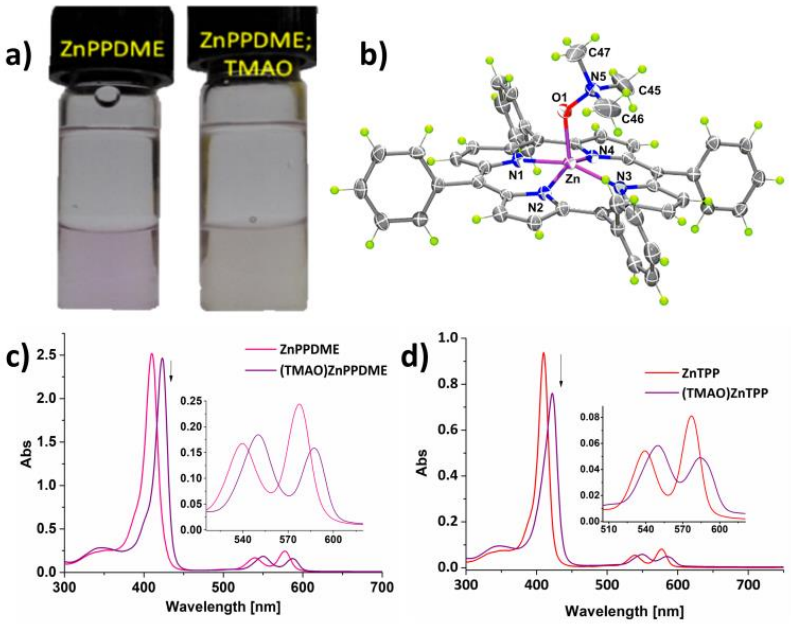


Figure 1 (a) The color change response of [ZnPPDME] (50µM) in DCM upon addition (~150µM) of TMAO in water.(b) ORTEP view of [(TMAO)ZnTPP] ,Zn—O1=2.011(3), solvent molecules have been omitted for clarity; nitrogen (blue); carbon (black); oxygen (red); zinc (pink); hydrogen (green) [CCDC No. 921098].(c) The UV-Vis spectra of a (~10 µM solution); [ZnPPDME] and of [(TMAO)ZnPPDME]) ,inset , Q band profile, (d) The UV-Vis spectra of a (~1.5 µM solution); [ZnTPP] and of [(TMAO)ZnTPP]) ,inset , Q band profile

Infrared spectroscopy of [ZnPPDME] shows a vibration for the ester functional group at 1729 cm^{-1}. The spectrum of [TMAOZnPPDME] shows a signal due to the stretching frequency for N-O at 947 cm^{-1}. This peak indicates the TMAO coordination to [ZnPPDME]. Similarly, the N-O stretching vibration in [TMAOZnTPP] appears at 945 cm^{-1}. X-ray confirms the coordination of oxygen atom of TMAO, ($(CH_3)_3N^+-O^-$) to zinc in [ZnTPP] with the Zn—O distance = 2.011(3) (Figure 1b).

As [ZnPP] and TMAO were separately reported to augment atherosclerosis[17-18,22-24], we design reactions, where cholesterol was allowed to react individually with TMAO and [ZnTPP] respectively and also when mixed together. These reactions were carried out in the dark avoiding any photoreaction of DCM with stirring at room temperature. Respective experiments were also performed when cholesterol, TMAO and [ZnPPDME] were taken together. The progresses of such reactions were monitored with time interval by thin layer chromatography (TLC) from the reaction mixtures and the oxidized products were identified. It is interesting to observe that either TMAO or [ZnTPP] alone does not oxidize cholesterol. The oxidation of cholesterol proceed only when both TMAO and [ZnPPDME] or [ZnTPP] were present. No detectable COPs were found on TLC chromatoplate when cholesterol remained alone. These experiments strongly suggest that the oxidising agent of cholesterol is not free unbound TMAO or [ZnTPP] (proxy of [ZnPP]) but in its bound form with [ZnTPP] or [ZnPPDME]. From the R_f values the main oxidized products were assigned as 7α-hydroxy cholesterol, 7β-hydroxy cholesterol and 7-keto cholesterol along with other less intense products. The dotted circled spot assigned for 7-keto cholesterol can only be found under UV light (365 nm) illumination (Figure 2) .[29]

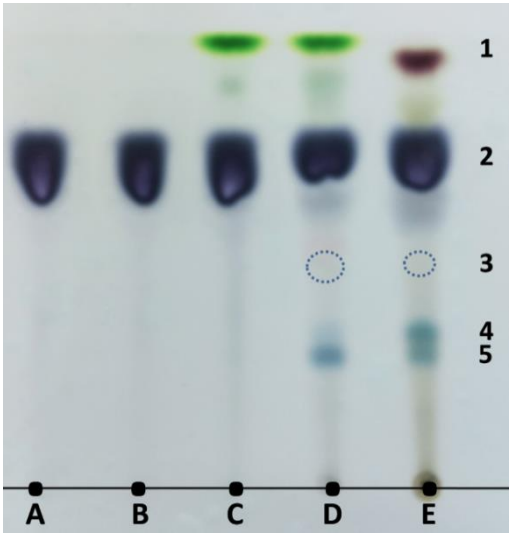


Figure 2 TLC chromatogram of above stated reaction after seven days of A) Cholesterol alone, B) Cholesterol with TMAO, C) Cholesterol with [ZnTPP], D) Cholesterol in presence both [ZnTPP] and TMAO and E) Cholesterol in presence both [ZnPPDME] and TMAO [1—[ZnTPP] (in C and D), [ZnPPDME] (in E), 2—Cholesterol, 3—7-Ketocholesterol only visible under UV light (254nm), 4—7β-hydroxy cholesterol, 5—7α-hydroxy cholesterol]

In atherosclerosis the plaque development requires a long time, therefore, we allowed to retain such reaction mixtures for a month in the dark at room temperature under stirring with frequent taking out aliquot from the respective reaction mixture to monitor the progress of reaction using TLC. We could observe the gradual increment in oxidized product as spot intensity increases with time. The very same results obtained when zinc protoporphyrin dimethyl ester [ZnPPDME] was taken in place of ZnTPP. From TLC spots, we can observe that oxysterols are being produced only when cholesterol is present with both [ZnTPP] and TMAO. Individually neither [ZnTPP] nor TMAO oxidize cholesterol under similar condition. Based on such observation we conclude that it is not free TMAO that can affect the oxidation of cholesterol rather its active state is in the bound form with [ZnTPP] or [ZnPPDME]. The results are shown in Figure 3.

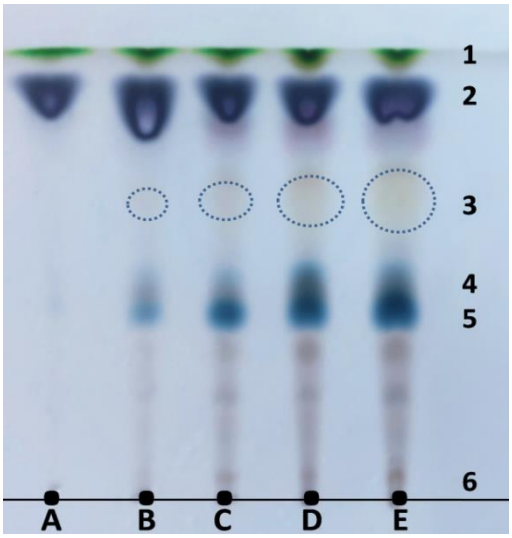


Figure 3. TLC chromatogram; reaction mixture after A) 1, B) 7, C) 14, (D) 21 (E) 28 days. [1—[ZnTPP], 2—Cholesterol, 3—7-Ketocholesterol only visible under UV light (365nm, see ESI), 4—7β-hydroxycholesterol, 5—7α-hydroxycholesterol, 6—Cholestan-3β,5α,6β-triol]

The oxidation of cholesterol under similar conditions has also been examined using circular dichorism (CD) spectroscopy. In this study cholesterol was directly oxidized by di-tert-butyl peroxide (DTBP) to use it as a marker to compare the changes initiated in the reaction mixture of cholesterol with [TMAOZnTPP]. The CD spectra revealed that cholesterol with [TMAOZnTPP] resembles the spectrum of the oxidized product with peroxide showing

significant positive absorption around 228 nm. Rest of the measurement like cholesterol alone, and with TMAO or with [ZnTPP] showed some positive absorption changes in 210-230 nm region but these changes remain inconclusive to draw any significant inference. Here also it is implied that the effecting oxo-transfer agent is the bound [TMAOZnTPP] and not the free TMAO. Such behaviour of free TMAO as not effective oxo-transfer agent has also been noted in our earlier studies on molybdenum enzyme, TMAO reductase.[30] It is suggested that the protonation of TMAO facilitates oxo-transfer reaction.[31-33] So the reduction of electron density on oxygen atom in, TMAO, ($(CH_3)_3N^+–O^-$) either by protonation or by metal-coordination as in the present case facilitates oxygen atom transfer capability of TMAO. Therefore, such possibility does not exclude other biomolecules present in the blood plasma which may activate TMAO similarly.

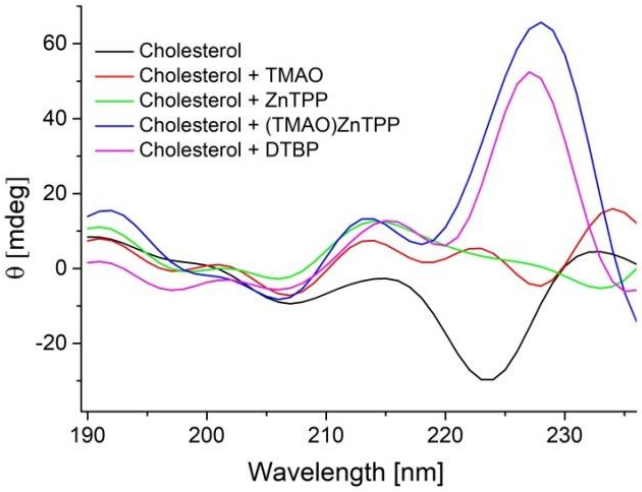


Figure 4. CD spectra of 0.01mmol cholesterol and its reaction mixture with TMAO, [ZnTPP], [TMAOZnTPP] and DTBP respectively recorded in DCM-hexane solvent mixture suggesting solubility of TMAO in presence of cholesterol. Each spectrum was taken after four hr of reaction time.

In the last decade, the atherosclerotic diseases have been correlated extensively with the concentration of free TMAO circulating in blood plasma. This has been used as marker to predict plaque formation as TMAO and linked with cholesterol oxidation. There are reports that the presence of [ZnPP] in blood plasma aggravates atherosclerosis. However, there has not been any report to correlate the functioning of both these jointly to create such problem. We demonstrate here that neither TMAO nor [ZnPP] alone inflicts oxidation of cholesterol. We present here that [ZnPPDME] and its synthetic analogue [ZnTPP] form adduct with TMAO to produce [TMAOZnPPDME] and [TMAOZnTPP] respectively. Such complex compound migrates easily to hydrophobic site suggesting [ZnPP] functions not only as transporter of

TMAO in the cholesterol lipid site but also enhancing the oxo-transfer capacity of ($(CH_3)_3N^+–O^–$) by its coordination to zinc.

Conflicts of interest

There are no conflicts to declare.

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