Vesicle formation by amphiphilic 10-alkyl-3-methyl isoalloxazine in aqueous medium

Abha Awasthi, Shveta Chaudhary and S M S Chauhan

Department of Chemistry, University of Delhi, Delhi 110 007

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The synthetic 10-alkyl isoalloxazines have been found to form vesicles in aqueous and binary solvent systems and confirmed by UV-visible, fluorescence, transmission electron microscopy and quasi elastic light scattering experiments. The mean external diameters of vesicles have been calculated for isoalloxazine with different carbon atom chain at position 10 by transmission electron microscopy and quasi elastic laser light scattering. The gel to liquid phase transition of liposomes measured by differential scanning calorimetry shows reproducible endothermic peak which lies well in the range of typical aqueous vesicles.

The 10-substituted isoalloxazines, FMN and FAD are present as prosthetic groups in various flavoenzymes and flavoproteins. The substitution of different alkyl group at 10 position of isoalloxazines increases the amphiphilicity of flavin nucleus. Bioorganic approaches to design and construction of synthetic cells have been focus of much current interest. Lipids being predominant constituents of biological membrane, their important functions are derived from their propensity to self organize. Such self assembly is due to amphiphilic nature of lipids. Most of the synthetic amphiphilic molecules organize in aqueous as well as organic solvents depending on their structure and solvent polarity. The unusual behaviour of 10-dodecyl-3-methyl isoalloxazine has been reported in the binary solvent systems by UV-visible spectral analysis. But no study has been done so far regarding the amphiphilic nature of flavin leading to the supramolecular structures such as micelles, monolayer, bilayer, multilayer, rods and vesicles. This paper deals with the formation of vesicles by synthetic amphiphilic isoalloxazine in aqueous medium and binary solvent systems. The vesicle formation by aggregation of flavins has been supported using Transmission electron microscopy and Quasi elastic laser light scattering. The gel to liquid thermotropic transition by isoalloxazine vesicles is monitored by differential scanning calorimetry.

All chemicals are of reagent grade and solvents were distilled before use. The 10-alkyl isoalloxazines used in this work were synthesized according to the protocol as given by Chauhan and co-worker and characterized by IR, UV-visible, H NMR and mass spectral techniques.

10-Hexyl-3-methyl isoalloxazine (la)
Yield: 1.25 g (80%); M.P : 180°C (dec.); IR (Nujol) : 760, 960, 1040, 1100, 1190, 1270, 1500, 1540, 1580, 1610, 1650, 1710 cm⁻¹; UV (CHCl₃) λ_max (E_max mM): 228.6 (31.20), 268.0 (70.20), 330.1 (18.72), 406.0 (17.19), 444.2 (21.84) and 466.8 (15.21) nm; UV (MeOH) λ_max (E_max mM): 228.0 (12.36), 265.8 (20.85), 333.6 (4.48) and 435.2 (5.56) nm; H NMR (CDCl₃) : 0.90 (3H, t, -CH₃), 1.35-1.70 (8H, m, -(CH₂)₄), 3.45 (3H, m, N-CH₃), 4.70 (2H, t, N-CH₂), 7.4-8.2 (4H, m, 6, 7, 8 and 9 aromatic protons); Analysis : Found C, 65.40; H, 6.4 and N, 17.95; Calculated C, 65.38; H, 6.4 and N, 17.90 (for C₁₇H₂₀N₂O₂).

10-Decyl-3-methyl isoalloxazine (lb)
Yield: 1.40 g (80%); M.P : 156°C (lit. mp 155°C); IR (Nujol) : 940, 1040, 1170, 1180, 1280, 1460, 1510, 1540, 1580, 1610, 1650 and 1710 cm⁻¹; UV(CHCl₃) λ_max (E_max mM): 226.5 (16.28), 263.0 (33.97), 328.0 (9.90), 417.1 (9.30), 444.2 (11.89) and 462.7 (8.39) nm; UV (MeOH) λ_max (E_max mM): 239.0 (15.64), 270.0 (26.48), 331.0 (45.55) 430.0 (6.24) nm; H NMR (CDCl₃) : 0.90 (3H, t, -CH₃), 1.3 (16H, m, -(CH₂)₄), 3.50 (3H, s, N-CH₃) 4.70 (2H, t, -CH₂), 7.4-8.2 (4H, m, 6, 7, 8 and 9 aromatic protons); Analysis : Found C, 65.38; H, 6.4 and N, 17.90 (for C₁₉H₂₂N₂O₂).
N-CH$_2$) and 7.5-8.2 (4H, m, 6, 7, 8 and 9 aromatic protons); Analysis: Found C, 68.40; H, 7.61 and N, 15.3; Calculated C, 68.40; H, 7.60 and N, 15.2 (for C$_2$H$_{32}$N$_4$O$_2$).

10-Dodecyl-3-methyl isoalloxazine (1c)

Yield: 1.57 g (80%); M.P: 177-178°C (lit. mp 177-179°C); IR (Nujol): 940, 1040, 1180, 1280, 1390, 1460, 1510, 1540, 1580, 1610, 1650 and 1710 cm$^{-1}$; UV(CHCl$_3$) $\lambda_{max}$ (E$_{max}$ mM): 226.5 (7.61), 270.0 (18.27), 330.1 (4.265), 419.2 (4.520), 446.14 (5.178) and 446.7 (3.890) nm; UV(MeOH) $\lambda_{max}$ (E$_{max}$ mM): 221.0 (13.94), 266.8 (27.01), 332.2 (5.64), 434.0 (7.34) nm; $^1$HNMR (CDCl$_3$): 0.88 (3H, t, -CH$_3$), 1.18-1.60 (20H, m, -(CH$_2$)$_{10}$), 3.49 (3H, s, N-CH$_3$), 4.65 (2H, t, N-CH$_2$) and 7.45-8.15 (4H, m, 6, 7, 8 and 9 aromatic protons); Analysis: Found C, 69.6; H, 8.01 and N, 14.15; Calculated C, 69.6; H, 7.60 and N, 14.14 (for C$_{29}$H$_{44}$N$_4$O$_2$).

10-Hexadecyl-3-methyl isoalloxazine (1d)

Yield: 1.8g (80%); M.P: 140°C; IR (Nujol): 960, 1040, 1100, 1180, 1280, 1380, 1460, 1520, 1560, 1590, 1610, 1650 and 1710 cm$^{-1}$; UV(CHCl$_3$) $\lambda_{max}$ (E$_{max}$ mM): 228.6 (3.466), 268.0 (8.138), 330.1 (2.034), 419.2 (2.034), 437.8 (2.637) and 466.85 (1.917) nm; UV(MeOH) $\lambda_{max}$ (E$_{max}$ mM): 227.2 (10.59), 266.8 (16.55), 332.2 (3.66) and 435.4 (4.35) nm; $^1$HNMR (CDCl$_3$): 0.88 (3H, t, -CH$_3$), 1.20-1.62 (28H, m, -(CH$_2$)$_{16}$), 3.42 (3H, s, N-CH$_3$), 4.68 (2H, t, N-CH$_2$) and 7.87-8.27 (4H, m, 6, 7, 8 and 9 aromatic protons); Analysis: Found C, 71.69; H, 8.85 and N, 12.35; Calculated C, 71.68; H, 8.80 and N, 12.31 (for C$_{34}$H$_{56}$N$_4$O$_2$).

10-Octadecyl-3-methyl isoalloxazine (1e)

Yield: 1.8 g (75%); M.P: 156-157°C; IR (Nujol): 940, 1040, 1180, 1280, 1390, 1460, 1510, 1540, 1580, 1610, 1650 and 1710 cm$^{-1}$; UV (CHCl$_3$) $\lambda_{max}$ (E$_{max}$ mM): 245.2 (100.30), 328.0 (55.28), 417.1 (50.75), 446.1 (59.39), and 466.8 (44.96) nm; UV (MeOH) $\lambda_{max}$ (E$_{max}$ mM): 238.0 (14.679), 338.0 (1.402) and 432.0 (1.358) nm; $^1$HNMR (CDCl$_3$): 0.95 (3H, t, -CH$_3$), 1.25-1.70 (32H, m, -(CH$_2$)$_{16}$), 3.45 (3H, s, N-CH$_3$), 4.65 (2H, t, N-CH$_2$) and 7.45-8.20 (4H, m, 6, 7, 8 and 9 aromatic protons); Analysis: Found C, 72.51; H, 9.2 and N, 11.6; Calculated C, 72.50; H, 9.1 and N, 11.5 (for C$_{35}$H$_{58}$N$_4$O$_2$).

The UV-visible and fluorescence spectra of isoalloxazine (5 $\times$ 10$^{-5}$ M) in binary solvent systems were carried out on Shimadzu UV 260 spectrophotometer and Jobin Yvon-Jy-3CS spectrofluorimeter respectively.

Preparation of isoalloxazine vesicles

The 10-alkyl isoalloxazine were used to prepare vesicles by the standard sonication method$^9$. The thin film of isoalloxazine (0.5 mM) was dispersed in deionised water (5% methanol) by sonication (190 W, 30±5°C, 4 h) on a bath type sonicator. Sonic dispersal was centrifuged to obtain clear solution at 2300 rpm at 5°C. The supernatant was subjected to gel permeation chromatography (g.p.c) on Sepharose 4B as outlined by Razin$^7$. The sample for the electron microscopy was prepared with the droplet techniques using aqueous 1% unbuffered uranyl acetate as a negative stain and formver carbon as supporting film. The electron microscopy was carried out on JEOL JEM 2000 EX transmission electron microscope (TEM) at accelerating voltage of 80 KV with different optical magnification depending upon the clarity of picture. Images were recorded on Kodak EM film no. 4489.

The mean external diameter of the isoalloxazine vesicles was measured on Brookhaven 9000, Quasi elastic light scattering instrument. Gel to liquid phase transition of flavins were measured on Mettler TA 3000 differential scanning calorimeter.

Incorporation of fluorescein in isoalloxazine vesicles

The fluorescein functionalized vesicles were prepared by the above method. The thin film of 1c (0.5 mM) was hydrated by 2 ml deionized water (5% MeOH) containing fluorescein (0.005 M). This suspension was sonicated, centrifuged and gel permeated on Sepharose 4B column (1 cm x 5 cm). The column was eluted with deionized water, different fractions of 1 ml each were collected. The formation of vesicles was monitored at 300 nm in UV-visible spectrophotometer.

Hydrophilic lipophilic balance (HLB) and critical aggregational concentration (cac)

The hydrophilic lipophilic balance (HLB) of the amphiphiles is an important factor which affects their solubility, emulsion capability and critical aggregational concentration (cac)$^{10,11}$. The cac of amphiphiles is affected by the chain length of
The cac value of amphiphile is lowered by lengthening of the hydrocarbon tail\textsuperscript{12}. The HLB and cac of amphiphilic isoalloxazines are calculated by following equations\textsuperscript{13,14} and presented in Table 1.

\[
\text{HLB} = \frac{\text{Mol. wt. of hydrophilic group}}{\text{mol. wt. of amphiphile}} \times 100/5 \quad \ldots (1)
\]

\[
1/\text{HLB} = 0.01076 / (\log_{10}(\text{cac} + 1.671)) + 0.05 \quad \ldots (2)
\]

Polar to apolar surface ratio (a/p) of amphiphilic molecules also decides their aggregation, like if this ratio is 0.7, this can lead to the formation of micelles and if it is nearly equal to 1, this can form vesicles as in phospholipids. The lowering in cac values of amphiphilic isoalloxazine are due to lengthening in hydrocarbon tail.

Thus, HLB, a/p and cac values of isoalloxazines suggest that these can form supramolecular structure like vesicles.

The UV-visible spectra of 10-alkyl isoalloxazines are very sensitive to the solvent polarity and show three absorption maxima \( S_1, S_2 \) and \( S_3 \) respectively\textsuperscript{5,6}. Depending on the microenvironment and solvent polarity, three types of \( S_3 \) peak in UV-visible spectra are observed i.e type A (guassian type) as in polar solvents like ethanol, type B (three band vibration structure) as in hydrophobic environment or apolar solvents and type C (a peak with two shoulders) as in solvent of intermediate polarity like DMF. The natural isoalloxazine FMN and FAD show guassian type \( S_3 \) in aqueous medium\textsuperscript{5,6} but the UV-visible studies of 10-alkyl isoalloxazine \( 1c \) and \( 1d \) in 40:60 ethanol:water binary solvent system show spectra of type B which on increasing water ratio above 60% becomes more clear. Similar type of results have been observed in different ratios of pyridine:water (Fig. 1). This change in UV-Visible spectra may be due to self organization in aqueous media\textsuperscript{15,16}.

Fluorescence spectra of \( 1c \) and \( 1e \) show peak at 509 nm in 100% ethanol while in 40:60 of ethanol:water and 100 % water this peak splits into two 513 nm and 533 nm with decreased r.i (Fig. 2). Micellar media show increased relative intensity of same isoalloxazine.

The UV-visible and fluorescence spectra indicate intramolecular interaction and aggregational behaviour of synthetic isoalloxazine. However the isoalloxazines with chain length \(<10\) at N-10 position does not show unusual behavior in UV-visible and fluorescence spectra. Shinkai and coworkers\textsuperscript{5} have

<table>
<thead>
<tr>
<th>10-alkyl isoalloxazine</th>
<th>HLB</th>
<th>a/p</th>
<th>cac (10(^{-4}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 1a )</td>
<td>13.6</td>
<td>0.38</td>
<td>1.95</td>
</tr>
<tr>
<td>( 1b )</td>
<td>12.6</td>
<td>0.66</td>
<td>1.35</td>
</tr>
<tr>
<td>( 1c )</td>
<td>11.1</td>
<td>0.79</td>
<td>1.01</td>
</tr>
<tr>
<td>( 1d )</td>
<td>9.70</td>
<td>1.05</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Fig. 1 — UV-visible spectra of 3-methyl-10-dodecyl isoalloxazine \( (1c) \) in pyridine : water \( (\ldots , 100\% \text{H}_2\text{O}, (\ldots), 90\% \text{H}_2\text{O}, (\ldots), 80\% \text{H}_2\text{O}, (\ldots), 50\% \text{H}_2\text{O}, (\ldots), 0 \text{H}_2\text{O}) \)

Fig. 2 — Fluorescence spectra of \( 1c \) in 100 % EtOH, \( (a) \); \( 1e \) in 100 % EtOH, \( (b) \); \( 1c \) in 40 % EtOH, \( (c) \) and \( 1e \) in 40 % EtOH, \( (d) \)
suggested the stacking mode of aggregation of 10-dodecyl-3-methyl isoalloxazine but there is no suggestion for the formation of organized structure out of it. Wieczorek and Drabent proposed the micelle formation by 10-dodecyl-3-methyl isoalloxazine but evidence was not furnished. The theoretical calculations as well as unusual UV-visible and fluorescence results indicate that these amphiphilic isoalloxazine form vesicles in the aqueous medium. So the vesicle solutions of 10-alkyl isoalloxazines were subjected to TEM and QELS.

Transmission electron microscopy (TEM)

The unilamellar liposomes can be obtained by sonication of suspended amphiphiles or by injection of ethanolic or etheral solutions into aqueous media. The liposomes of 10-alkyl isoalloxazines were prepared by sonication method. The negatively stained electron micrographs of 1c and 1e show circular unilamellar vesicles of diameter $150\pm5$ and $100\pm5$ nm respectively (Fig. 3). The vesicle size of other isoalloxazines are presented in Table 2. The aqueous dispersion of vesicles were stable upto 4 weeks.

Quasi elastic light scattering (QELS)

The dynamic quasi elastic light scattering method is a widely used technique to determine the mean external hydrodynamic diameters in organized media including micelles and vesicles. The mean external diameter of homogeneously dispersed

*Fig. 3 — Electron micrograph of (a): 1c (0.5 mM) vesicles in aqueous solution stained by uranyl acetate. (b): 1e (0.5 mM) vesicles in aqueous solution stained by uranyl acetate*

*Fig. 4 — Mean external hydrodynamic diameter of 1c and 1e calculated by quasi elastic light scattering*
particles of isoalloxazine measured by QELS are found to be in agreement with the diameter of vesicles obtained from TEM (Fig. 4, Table 2).

**Differential scanning calorimetry (DSC)**

The vesicles generally display gel to liquid thermotropic phase transition as a result of which vesicles provide the effective barrier for the transport of water soluble solutes. Thus gel to liquid phase transition of isoalloxazine vesicles were measured by differential scanning calorimetry. Differential scanning heating curve of le and ld displayed reproducible endothermic peak with $\Delta H = 24 \text{kJ/mole}$ and $\Delta H = 20 \text{kJ/mol}$ at 169°C and 172°C respectively (Fig. 5) which corresponds to vesicle formation. The $\Delta H$ value lies well in the range of typical aqueous vesicles.

**Incorporation of fluorescein dye in isoalloxazine vesicles**

Fluorescein incorporated isoalloxazine vesicles were prepared by sonication method and subjected to gel permeation chromatography on Sepharose 4B.

<table>
<thead>
<tr>
<th>10-alkyl isoalloxazine</th>
<th>Diameter* (nm)</th>
<th>Diameter* (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>la</td>
<td>865</td>
<td>850-900</td>
</tr>
<tr>
<td>lb</td>
<td>990</td>
<td>950-1000</td>
</tr>
<tr>
<td>lc</td>
<td>144</td>
<td>150-160</td>
</tr>
<tr>
<td>ld</td>
<td>432</td>
<td>450-500</td>
</tr>
<tr>
<td>le</td>
<td>600</td>
<td>550-600</td>
</tr>
</tbody>
</table>

*Quasi elastic light scattering; # Transmission electron microscopy

The coelution curve was plotted by monitoring the UV-visible spectra of different fractions eluted at 300 nm for vesicles and at 488 nm for fluorescein. The total bed volume of column was 4.32 ml and void volume was 1.73 ml. The apparent internal volume

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**Fig. 5** — Differential scanning heating curve of le

**Fig. 6** — Gel permeation chromatography curve for 3-methyl-10-dodecyl isoalloxazine vesicles (le) [(a), without fluorescein; (b), with fluorescein]

**Fig. 7** — Time dependent UV-visible spectra of fluorescein incorporated into le vesicles on addition of 5 μl Triton X-100 (0.1 mM) [Curves 1, 0 hr; 2, 1 hr; 3, 3 hr; 4, 6 hr; 5, 10 hr; 6, 20 hr; 7, 24 hr]
was found to be 2.59 ml. The coelution curve plotted between absorbance versus fraction number or total volume eluted from the column indicates that the blank vesicles and fluorescein functionalized vesicles have the same retention time (Fig. 6). Thus, the coelution of fluorescein with isoalloxazine vesicles clearly indicate that the dye is incorporated within the vesicle. The fraction with a maximum concentration of fluorescein functionalized vesicles were treated with 5 μl Triton X-100 (0.01 M) and subjected to time dependent UV-visible spectroscopy (Fig. 7). The decrease of O.D of 488 nm peak with time was observed (Fig. 8). The change in the UV-visible spectra of fluorescein incorporated in the above case suggests the formation of vesicles.

Thus, in the present study synthetic isoalloxazines are behaving as an ‘ideal amphiphile’ or ‘new lipid’. They are forming vesicles in aqueous medium due to the hydrophobic forces between the alkyl chains. Scheme 1 shows the schematic presentation of vesicle formation by synthetic amphiphilic isoalloxazine.

**Acknowledgement**

This work was supported by Council of Scientific and Industrial Research (CSIR) and Department of Science and Technology (DST), New Delhi, India. We thank Prof A N Maitra for quasi elastic light scattering and Dr P Dureja for differential scanning calorimetry.

![Graph](image1)

Fig. 8 — Plot of optical density of λ<sub>max</sub> vs time of fluorescein incorporated in vesicles on addition of 5 μl Triton X-100 (0.1 mM).

![Scheme](image2)

Scheme 1 — Schematic presentation of vesicle formation by synthetic amphiphilic isoalloxazine
References