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First Vesicular Self-Assembly of Crotocembraneic Acid, a Nano-Sized Fourteen Membered Macrocyclic Diterpenic Acid

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Study of the spontaneous self-assembly of molecules in aqueous binary solvent mixture yielding vesicular self-assemblies has been an area of intense research in recent years because of its tremendous potential application in controlled-release drug delivery systems, medical implants, tissue engineering, etc. *Crotocembraneic acid* **1**, a nano-sized 14-membered macrocyclic diterpenic acid, is extractable from the leaves of *Croton oblongifolius* Roxb. First self-assembly property of the nano-sized macrocyclic diterpenoid **1** in aqueous binary solvent mixtures is reported here. The diterpenoid spontaneously self-assembled in aqueous binary solvent mixtures yielding vesicles of nano- to micro-meter diameters. The vesicular self-assemblies were capable of entrapping various fluorophores including the anticancer drug doxorubicin.

Spontaneous hierarchical self-assembly of small molecules in liquids yielding self-assemblies of nano- to micro-meter dimensions such as vesicles, fibers, spheres, tubules, etc. has become an area of intense research in recent years for an improved understanding of the structure property relationships and because of their many potential and realized technological applications.[1,2,3,4,5,6] Studies of the vesicular self-assembly of small molecules in aqueous binary solvent mixtures are of special interest because of its tremendous applications in the areas of controlled-release drug delivery systems, medical implants, tissue engineering, etc.[7,8] Literature study reveals that majority of the examples having the aforementioned applications are based upon low molecular weight organic compounds that are often obtained either by multistep chemical synthesis or polymeric systems that are capable of gelling aqueous solvent mixtures.[9,10,11,12] Self-assemblies of molecules from renewable resources are of great significance in recent years because their availability in renewable supply, low toxicity and biodegradability will aid to establish a sustainable society.[13] But, vesicular self-assemblies of molecules from renewable resources in aqueous solvent mixtures are rare.[14,15]

Recently we have reported the spontaneous hierarchical self-assembly of two triterpenic acids and their derivatives that yielded vesicular self-assemblies.[14,16] But, according to our knowledge, there is no report in the literature on the spontaneous self-assembly of naturally occurring diterpenoids. In continuation of our investigations on the utilization of terpenoids as renewables,[17,18,19] herein we report the first self-assembly properties of a nano-sized macrocyclic diterpenoid crotocembraneic acid **1** (Figure 1) in different liquids. The

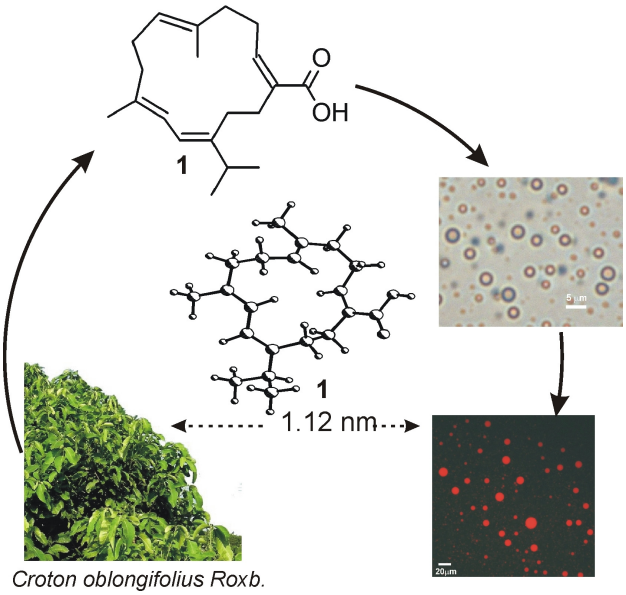

Figure 1. Schematic representation of self-assembly of crotocembraneic acid **1** extractable from *Croton oblongifolius* Roxb yielding vesicular self-assemblies and its use in drug entrapment studies (centre: energy minimized structure of **1**).

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diterpenoid extractable from *Croton oblongifolius* Roxb. spontaneously self-assembled in aqueous binary solvent mixtures yielding vesicular self-assemblies of nano to micrometer diameters. The self-assemblies were capable of entrapping various fluorophores including the anticancer drug doxorubicin making it useful for targeted drug delivery applications.

Crotocembraneic acid **1** was extracted from the leaves of *Croton oblongifolius* Roxb. as a low melting solid following an optimized procedure developed in our laboratory. The molecule has a unique 14-membered macrocyclic structure having four double bonds within the macrocycle with two of them in conjugated and two in the non-conjugated positions. The length of the molecule, obtained by molecular mechanics calculation using Allinger's MMX algorithm, was 1.12 nm (Figure 1 and see supporting information Figure S1).[20] The carboxyl group forming a polar head group and the macrocycle forming a highly hydrophobic tail, crotocembraneic acid **1** turned out to be a unique macrocyclic amphiphile for the study of its self-assembly properties in various liquids. Compound **1** was highly soluble in polar solvents such as ethanol, DMSO, DMF. When a hot and stirred solution of **1** in DMSO (100 μL) contained in a vial was treated with water (50 μL) and the resulting mixture was allowed to cool at room temperature, a colloidal suspension was obtained in 15 min. Such colloidal suspensions were also obtained in DMF-water and ethanol-water mixtures (Table 1). Atomic force microscopy of a dried

Table 1. Self-assembly crotocembraneic acid **1** in aqueous binary solvent mixtures

Entry	Solvent[a]	Conc. (% w/v)	State[b]
1	ethanol-water	2	CS
2	DMSO-water	2	CS
3	DMF-water	2	CS

[a] solvent : water in 2:1 v/v ratio, CS = colloidal suspension

sample prepared from a colloidal suspension of **1** (0.28% w/v) in DMSO-water (2:1 v/v) revealed spherical self-assemblies of 40–50 nm diameters (Figure 2 and see supporting information

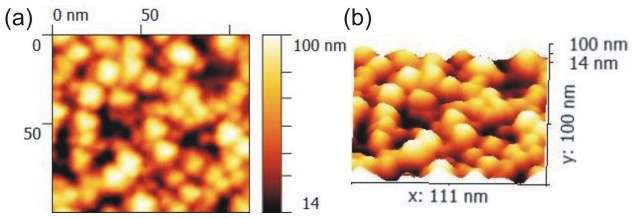

Figure 2. (a,b) AFM images (2D and 3D respectively) of spherical self-assemblies formed from **1** (0.28% w/v) in DMSO-water (2:1 v/v).

Figure S5). The measured heights of the spherical self-assemblies were smaller than the diameters indicating the soft nature of the assemblies.

Dynamic Light Scattering (DLS) studies carried out with a dilute colloidal suspension of **1** (1.18% w/v) in ethanol-water (2:1 v/v) revealed polydisperse spherical self-assemblies with an average size of 200 nm with 15% of the spherical objects having diameter of 42 nm (see supporting information Figure S6). With increasing concentration of **1** to 1.6% w/v and 2.2% w/v, the average size of the self-assemblies increased to 465 nm and 523 nm respectively with a small fraction of the assemblies having micrometer diameters. Optical microscopy (OM) of a colloidal suspension of **1** (2% w/v) in ethanol-water (2:1 v/v), DMSO-water (2:1 v/v) and DMF-water (2:1 v/v) indicated micro-sized spherical self-assemblies of average diameter of 2 μm (Figure 3,a-c and see supporting information

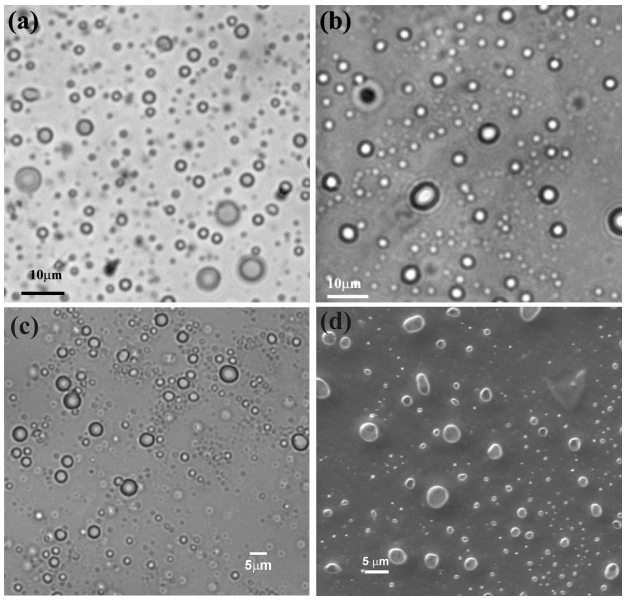

Figure 3. (a-c) Optical micrographs of Crotocembraneic acid **1** in (a) ethanol-water (2% w/v) (b) DMSO-water (2% w/v) (c) DMF-water (2% w/v); (d) SEM of **1** (0.28% w/v) in DMSO-water (2:1 v/v).

Figure S7). The nano-sized self-assemblies could not be observed by OM due to limitation of the technique used. This limitation was overcome by scanning electron microscopy. For example, when the dried self-assemblies of **1** prepared from its colloidal suspension in DMSO-water (0.28% w/v) was examined by SEM (basic), the presence of both nano as well as micrometer sized spherical self-assemblies (Figure 3d and see supporting information Figure S8) were observed. Field emission scanning electron microscopy (FESEM) of the dried self-assemblies of **1** revealed spherical self-assemblies of average size of 30 nm (Figure 4a).

To get further insight into the nature of the spherical self-assemblies, a dried sample prepared from the self-assemblies of **1** (0.28% w/v) in ethanol-water (2:1 v/v) was analyzed by High Resolution Transmission Electron Microscopy (HRTEM).

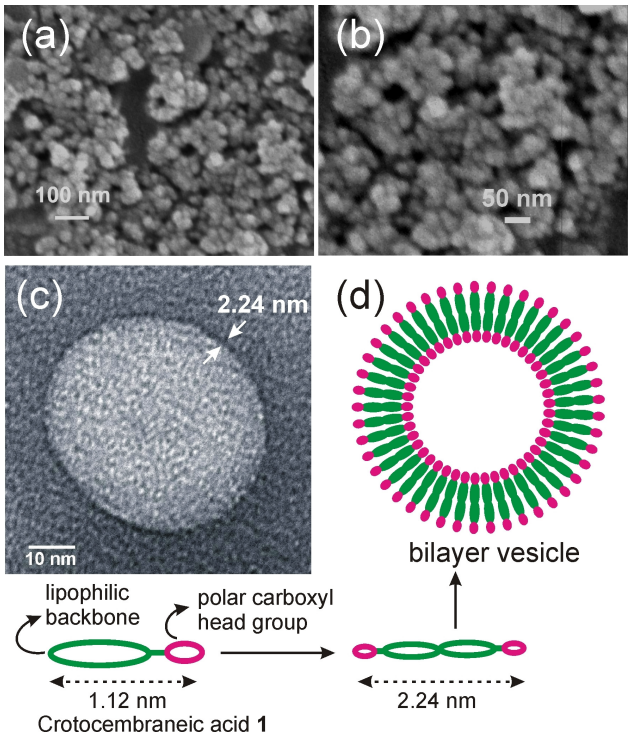

Figure 4. (a,b) FESEM of dried self-assemblies of 1 (0.28% w/v) prepared from its colloidal suspension in DMSO-water (2:1 v/v), (c) HRTEM of dried self-assemblies of **1** prepared from its colloidal suspension in ethanol-water (2:1 v/v), (d) schematic representation of the formation of bilayer membrane yielding vesicular self-assembly of **1.**

Spherical self-assemblies with distinct periphery having a membrane structure indicated the vesicular nature of the spheres. With a membrane thickness of 2.24 nm and the molecular length being 1.12 nm, a bilayer vesicular self-assembly is supported (Figure 4c). Fusion of smaller sized vesicles yielding bigger vesicles has also been observed by HRTEM (see supporting information Figure S9).

The bilayer membrane morphology is supported by X-ray diffraction studies of the self-assemblies of **1** in DMSO-water (1% w/v, 2:1 v/v) where a sharp peak at $2\theta = 4.04^{\circ}$ was observed that corresponds to a d spacing of 2.24 nm (see supporting information Figure S10). Vesicular self-assemblies of amphiphiles in aqueous medium are formed above a critical concentration of the amphiphiles known as critical vesicular concentration (cvc). By using pyrene as a fluorescence probe cvc was determined to be 0.05 mM in DMSO-water (1:9 v/v, supporting information Figure S11).[21]

H-bonding involving the carboxyl groups in addition to the dispersion interactions by the macrocyclic diterpenoid backbones are likely to play a significant role for the self-assembly of the molecules. The stretching frequency of the 'C=O' group in the neat compound appeared at 1683 cm^{-1} whereas the 'C=O' stretching frequencies of the self-assemblies appeared at 1655 and 1658 cm^{-1} at 2% and 0.5% (w/v) in DMSO-water (2:1 v/v) respectively. The stretching frequency of the 'O–H' group in the self-assemblies appeared at 3394, and 3415 cm^{-1} in DMSO-water (2:1 v/v) at 2% and 0.5% (w/v) respectively (see supporting information Figure S12). The lowering of the "-C=O" stretching frequencies in the self-assemblies compared to the neat compound and lowering of the "–O–H" stretching frequency with increasing concentration clearly indicated that the self-assemblies were stabilized by the intermolecular H-bonding among the molecules.

Vesicular self-assemblies of average size smaller than 10 μm are an attractive choice for targeted drug delivery through blood capillaries.[12] Whether the vesicular self-assemblies of **1** are capable of entrapping guest molecules inside, we examined the entrapment of the cationic fluorophores crystal violet (CV) and rhodamine B (Rho-B) and an anionic fluorophore 5,6 carboxy-fluorescein (CF). Interestingly, both the cationic fluorophores as well as the anionic fluorophore were entrapped inside the vesicular self-assemblies of **1** (Figure 5,6). For

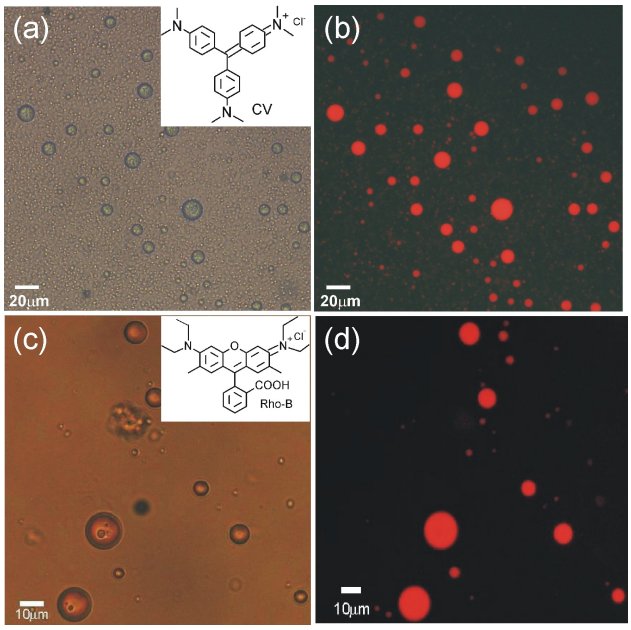

Figure 5. Epifluorescence microscopy images of self- assembled crotocembraneic acid **1** (46.32 mM) in DMSO-water (2:1 v/v) (a,b) containing crystal violet (0.463 mM), (c,d) containing rhodamine B (0.463 mM) (a,c) bright-field images, (b,d) fluorescent images.

example, when a hot solution of crotocembraneic acid **1** (46.32 mM) in DMSO-water (2:1 v/v) containing CV (0.463 mm) was cooled at room temperature and examined by epifluorescence microscopy, bright fluorescence was observed inside the spheres (Figure 5a,b). Similarly, bright fluorescence of Rho-B was also entrapped inside the vesicular self-assemblies of **1** under identical condition (Figure 5c,d). When a hot solution of **1** (26.0 mM) in DMSO-water (2:1 v/v) containing the anionic fluorophore CF (0.26 mM) was cooled at room temperature and examined by epifluorescence microscopy, slow entrapment of fluorophores were observed (Figure 6a,b). The increased intensity of the fluorescence at the periphery indicated slow entrapment of the fluorophores inside the vesicles. All these

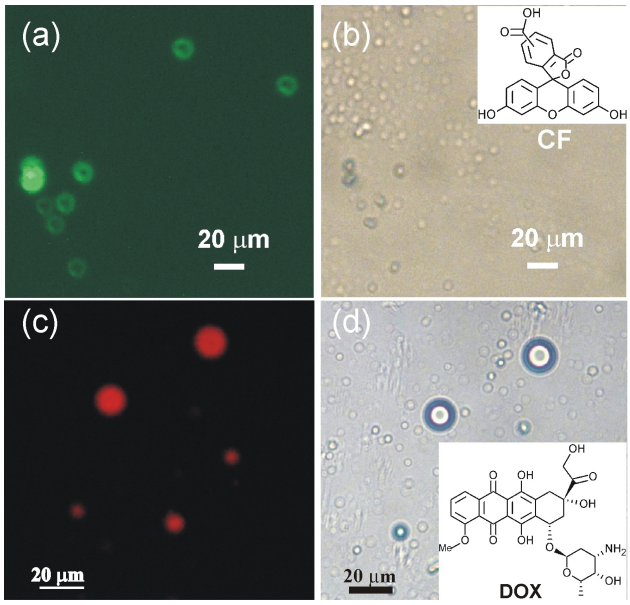

Figure 6. Epifluorescent microscopy images of (a,b) self-assembled **1** (26.0 mM) in DMSO-water (2:1 v/v) containing CF (0.26 mM); (c,d) self-assembled **1** (46.32 mM) in DMSO-water (2:1 v/v) containing doxorubicin (0.46 mM). (a,c) Fluorescent images, (b,d) bright-field image.

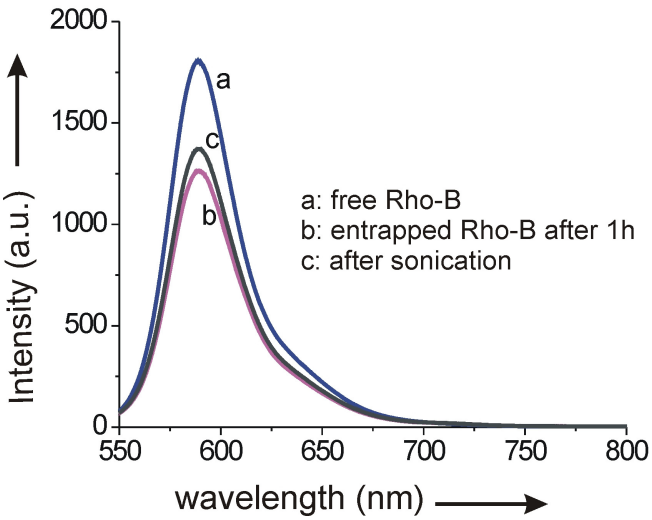

Figure 7. Fluorescence emission spectra ($\lambda_{ex} = 510$ nm) of Rho-B under different experimental conditions showing the effect of entrapment by vesicular self-assemblies and partial release by sonication.

studies also supported the vesicular nature of the spherical self-assemblies. To verify the entrapment of fluorophores inside the vesicular self-assemblies, we treated the Rho-B (0.46 mM) entrapped spherical self-assemblies of **1** (46 mM) with a small amount of triton X-100 (0.46 mM). Lysis of the spherical self-assemblies with concomitant release of the fluorophores confirmed their vesicular nature (see supporting information Figure S13).

Inspired by these observations, we tested the entrapment of the anticancer drug doxorubicin. When a hot solution of **1** (46.32 mM) in DMSO-water (2:1 v/v) containing doxorubicin (0.46 mM) was cooled at room temperature and examined under epifluorescence microscopy after 3 h, bright fluorescence from inside the vesicles confirmed the entrapment of doxorubicin inside the vesicular self-assemblies of **1** (Figure 6c,d). Fluorescence quenching during entrapment of the fluorophores confirmed fluorophore entrapment inside the vesicles (Figure 7). Partial release of the entrapped fluorophores by sonication was also evident from fluorescence emission studies (Figure 7c).

In conclusion, we have reported the formation of vesicular self-assemblies of a naturally occurring macrocyclic diterpenoid crotocembraneic acid in aqueous media yielding vesicular self-assemblies. According to our knowledge, this is the first report of the formation of vesicular self-assemblies of a diterpenoid.

Evidence for the formation of a bilayer vesicular structure has been obtained from HRTEM and X-ray diffraction studies. Thus crotocembraneic acid joins the rare class of macrocyclic natural products yielding vesicular self-assemblies in aqueous binary solvent mixtures. Entrapment of both cationic as well as anionic fluorophores including the anticancer drug doxorubicin inside the vesicles has also been demonstrated. The renewable nature of the diterpenoid and the simplicity of the methods of self-assembly and drug entrapment described here makes our strategy applicable to other naturally occurring diterpenoids some of which are under investigations in our laboratory and will be reported in due course.

Supporting Information Summary

Experimental procedure for the isolation of compound **1**, characterization, energy minimized structure, additional AFM, SEM, TEM, X-ray diffraction, FTIR, optical microscopy images are given in the supporting information.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Crotocembraneic Acid · *Croton oblongifolius* · diterpene · self-assembly · vesicles

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