ARTICLE

Bio-based benzoxazines synthesized in a deep eutectic solvent: A greener approach toward vesicular nanosystems

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Abstract
A green synthesis of benzoxazines, based upon reaction of cardanol with formaldehyde and primary amines, is achieved in high yields using choline chloride-urea mixture as deep eutectic solvent. Then, it is demonstrated how the cardanol-based benxoxazines can be employed as only component for the preparation of a nanovesicular systems.

1 | INTRODUCTION

Recently, deep eutectic solvents (DESs) have attracted considerable attention as biodecstrdable, nontoxic, cheap and recyclable green solvents in separation, organic synthesis and transformations.[1–6] DESs are composed through hydrogen bonding occurring between two main molecular portions, one acting as hydrogen bond donor as, for instance, an acid,[7] alcohol,[8,9] carbohydrate,[10] amide,[11,12] or amine moiety,[4] and the second one, usually a quaternary ammonium salt, operating as hydrogen bond acceptor.[12,13] Abbott et al reported facile synthesis of eutectic liquid from choline chloride [(2-hydroxyethyl) trimethylammonium chloride] and urea as renewable starting materials in a molar ratio of 1:2 showing interesting solvent properties and a melting point lower than those of the individual components.[14] DESs are characterized also by their nonflammability, high thermal

stability, and low volatility features, which promote their use as versatile alternatives to conventional solvents.[15]

On the other hand, 1,3-benzoxazine derivatives (BZs) are one of the most important heterocycles owing to their remarkable antibacterial and antifungal activities,[16–18] anticancer,[19,20] analgesic,[21] antiplatlet,[22] and antimycobacterial activity.[23] In addition, cardanol is a renewable and low-cost organic natural compound that can be easily separated as the main component in 84% via the vacuum distillation of cashew nut shell liquid (CNSL). Cardanol derivatives demonstrated high potential applications like surface coatings,[24–26] surfactants,[27] and pharmaceuticals.[28,29]

Targeted anticancer drug delivery to the infected cells is of fundamental importance in the development of nanomedicine.[30,31] Vesicular systems have shown effectiveness as drug nanocarriers in the treatment of breast cancer,[32] human lung cancer,[33,34] and for pancreatic cancer therapy.[35] Recently, the preparation of multicomponent nanovesicular systems based on renewables like cardanol and cholesterol with embedded minor amounts of various other components has been reported.[36–38]

In this article, for the first time, synthesis of benzoxazines, based on cardanol (both unsaturated and saturated components), formaldehyde, and amines by using a choline chloride-urea mixture as DES is described. The procedure here reported can be considered as a new greener alternative for obtaining mono-and bis-benzoxazines from CNSL components.[39–42] The use of cardanol-based benzoxazines as sole building block for the preparation of mono component nanovesicles is another goal of this work.

2 | RESULTS AND DISCUSSION

Herein, we described a one-pot reaction of naturally occurring cardanol (consisting of unsaturated (**1a** in Scheme 1**)** and saturated (pentadecylphenol) (**1b** in Scheme 1) molecular components), formaldehyde and primary aromatic amines in a suitable molar ratio (1:2:1, respectively) in choline chloride-urea mixture as DES to afford mono-benzoxazine derivative (s **3a-d** in the Scheme 1) through Mannich-like condensation in good to excellent yields (81-88%) (see Scheme 1 and Table 1).

Similarly, saturated phenol **1b** is made to react with ethylene diamine and formaldehyde in choline chloride-urea mixture to give the bis-benzoxazine **4** (Scheme 2).

It is important to remark that all the obtained benzoxazines could be easily separated from the DES medium just by adding water to promote their precipitation. Further purification of the solid products was then accomplished by column chromatography. The DES could be easily recycled for further uses. In particular, after precipitation of products, the aqueous solution of DES was extracted with ethyl acetate (3 × 15 mL), in order to remove all unreacted organic compounds. The aqueous phase was then separated and concentrated under vacuum at 80°C by rotatory evaporation. The wet DES obtained was then ovendried to a constant weight at 80°C and used for further reactions.

The as-obtained benzoxazine (BZs) derivatives resulted as a slightly yellow solid and to confirm their typical structure were characterized by spectroscopic techniques ^{1}H-NMR, ^{13}C-NMR, and IR.[40] ^{1}H NMR displayed two singlet signals centered at 4.65 and 5.39 ppm, characteristic for oxazinic protons, while oxazinic aliphatic carbons showed chemical shifts at 50.24 and 79.3 ppm in ^{13}C NMR spectrum.
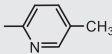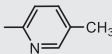
Spectral data of the novel bis-benzoxazine derivative **4** synthetized have been reported in the Supporting Information.

The **3a** and **3c** products were used as main components to prepare nanostructures through a green route. Several formulations were organized starting from lipidic films based on mixing melted BZs (and cholesterol, if present) followed by hydration with a buffer solution, at different temperatures (see Supporting Information). Unlike the previously developed cardanol-based nanoformulations that used a pH 9 borate buffer to ensure the formation of vesicular structures, a pH 7.4 phosphate buffer was chosen for hydration, in order to simulate a physiological environment and to interact
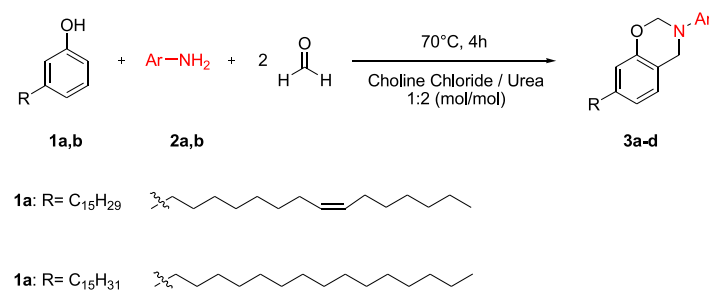
SCHEME 1 Synthesis of benzoxazine derivatives **3a-d** in choline chloride-urea solvent

TABLE 1 Synthesis of benzoxazine derivatives **3a-d** in choline chloride-urea solvent

Entry	1	2	Ar	Product 3	Yield %[a]
1	**1a**	**2a**		3a	85
2	**1b**	**2b**	—〈pyridine〉—CH₃	3b	81
3	**1b**	**2a**		3c	88
4	**1b**	**2b**	—〈pyridine〉—CH₃	3d	85

[a]Determined after isolation by column chromatography on silica gel.

SCHEME 2 Synthesis of bis-benzoxazine derivative **4** in choline chloride-urea solvent
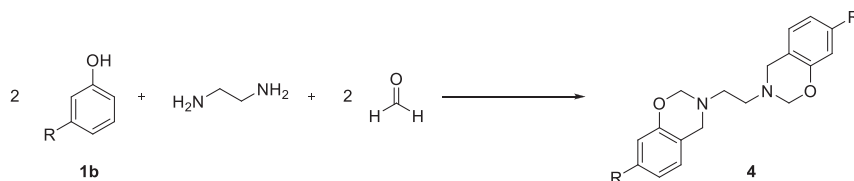
TABLE 2 Formulations and DLS measurements of BZs-based nanovesicles

| Entry	3a (mg)	3c (mg)	Cholesterol (mg)	T (°C)	d (nm)	[BZs] (g/L)	ZP (mV)
A	59	—	33	90	252.7 ± 135.1	1.20	—
B	58	58	—	90	271.2 ± 112.6^{a}	0.84	-83.6 ± 8.1
C	60	60	—	66	275.5 ± 154.5^{a}	0.69	-81.4 ± 7.2
D	61	—	—	50	225.7 ± 135.4	0.39	-69.6 ± 7.7
E	—	61	—	77	207.5 ± 83.9	0.46	-69.3 ± 8.6

[a]Minor population of about 5000 nm.
Abbreviation: DLS, dynamic light scattering.

with heterocyclic oxygen and nitrogen of BZs, thus helping the vesicle arrangements. In Table 2 are summarized the different conditions of BZs-nanovesicle preparation as well as the hydrodynamic diameters (d) and Z-potential (ZP) values obtained by dynamic light scattering (DLS) measurements. Moreover, the BZs concentration ([BZs]) in the nanodispersions was determined by UV-Vis spectrophotometric measurements[42] on lysed vesicle samples (Table 2).

The morphologies of all the nanoformulations were investigated by transmission electron microscopy (TEM), as shown in Figure 1.

The first formulation (entry **A**) was realized according to experimental conditions (molar ratios and temperatures) already used to prepare similar nanosystems,[36–38] by using unsaturated BZ **3a** in place of cardanol oil and mixed with a few amount of cholesterol as cosurfactant. This sample was reasonably comparable in size distribution and stability to cardanol-based nanodispersions, while the morphological analysis displayed nucleated heterostructures around the vesicles. As the unsaturated BZ **3a** is expected to melt at 90°C during the vesicles formation, it can be assumed that it has a lower solvation capacity for cholesterol compared with cardanol oil,
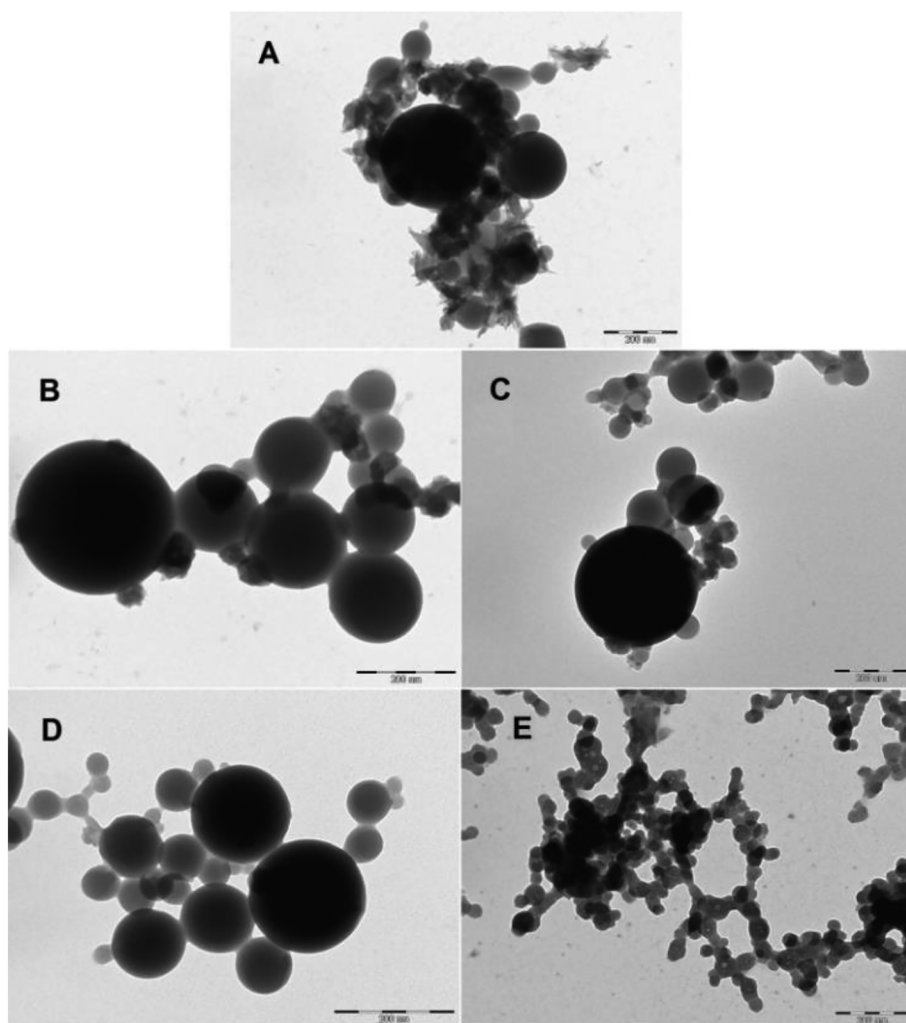
FIGURE 1 Transmission electron microcstrphs of BZs-based nanovesicle formulations A, B, C, D, and E

resulting in a lower retention of cholesterol into vesicular structures when the nanodispersion cools to room temperature, thereby promoting precipitation as heterostructures.

In the following nanodispersions (entries **B** and **C**), the cholesterol was replaced by the saturated-BZ derivative **3c** (in 1:1 molar ratio with unsaturated **3a** derivative) in order to test its efficacy as possible cosurfactant. TEM analysis, as shown in Figure 1 (**B** and **C**) showed nanostructures with regular spherical shape in both cases, with a mean diameter of about 270 nm. In fact, the presence of a larger density of smaller vesicles in the case of entry **C** could be attributed to the larger amount of the cosurfactant **3c** that was not completely embedded into vesicular structures at the operative temperature of 66°C as incompletely melted.

Subsequently, all the other experiments were designed to verify the achievable formation of nanovesicles by using only one BZ(**3a** or **3c**)as vesicular component. In the formulation **D**, for instance, the unsaturated BZ **3a** was the only used and the temperature was lowered to 50°C, anyway ensuring the lipid film formation at milder synthesis conditions. The as-obtained vesicular nanodispersion was not

extremely polydispersed (polydispersity index = 0.17), with a regular spherical shape and a size smaller than the previous samples, however with good stability, as presented in Table 2 and Figure 1.The analogous sample **E** produced by using the saturated BZ **3c** as the single component was prepared at a temperature at which the solid **3c** was completely melted, in order to ensure the lipid film formation. TEM analysis of this sample was thoroughly different from the others, lacking the objective indication of well-defined spherical shapes but rather appearing as loose chains.

3 | CONCLUSIONS

A green synthesis of mono and bisbenzoxazine in choline chloride-urea mixture as DES has been described for the first time. Moreover, the peculiar features of the benzoxazine **3a** and **3c** permitted the preparation of the single-component vesicular nanosystems based on cardanol derivatives. To the best of our knowledge, this last finding can be considered as an alternatively green route to the multicomponent preparation of nanovesicles

concurrently involving cardanol and cholesterol as building blocks. Other innovations related with this new preparative procedure consisted with the use of the mildest process conditions in terms of temperature and use of a pH 7.4 (by phosphate buffer) to mime a physiological environment within producing of the smallest nanovesicles with smallest size known today.

4 | EXPERIMENTAL

4.1 | Reactants

Cardanol oil (distillated, 1a) was kindly offered by Oltremare, while saturated cardanol (1b) was obtained from Sigma-Aldrich office Lecce-Italy (or synthesized as described in the literature (GreenChemDOI: 10.1039/b811504d). Choline chloride (99%), urea (98%), ethyl acetate (99%), formaldehyde (37%), ethylene diamine (99%), aniline (99%), and 2-amino-5-methylpyridine (99%) were obtained also from Sigma-Aldrich.

4.2 | General procedure for the synthesis of monobenzoxazine 3a-d and bis-benzoxazine 4

A mixture of cardanol **1a** or pure hydrogenated cardanol **1b** (0.01 mol), formaldehyde (37% w/w in aqueoussolution, 0.02 mol) and aromatic amine **2a-d** (0.01 mol) in choline chloride-urea mixture as DES (5 mL) was heated under stirring for about 2-4 hours at 60-70°C. For the synthesis of **4**, a mixture of hydrogenated cardanol **1b** (0.02 mol) was reacted with formaldehyde (37% w/w in aqueous solution, 0.04 mol) and ethylene diamine (0.01 mol) in the same reaction conditions. The progress of reactions was checked by TLC. The reaction mixtures were cooled to room temperature and water (10 mL) was added then filtered to remove choline chloride-urea. The formed solids were dissolved in ethyl acetate, dried over sodium sulfate and concentrated in vacuo. The crude products were purified by flash-chromatography (silica gel; petroleum ether/AcOEt 20-80).

4.3 | General procedure for the preparation of benzoxazine-nanovesicles

Lipidic films were formed by heating of BZ (alone or in mixture with cholesterol or other BZs) and by mechanical stirring in the presence of glass beads (10 g) for 1 hour. Hydration of lipidic films was carried out by adding 40 mL of phosphate buffer (pH 7.4, preheated at 50°C) under mechanical stirring (800 rpm) and heating for 1 hour. Then, obtained vesicular nanodispersions were submitted to sonication step for 45 minutes at 40°C and centrifuged (7000 rpm for 15 minutes) to help the separation of smallest vesicles in supernatant collected as sample. Different molar ratios and temperatures are well specified in the Supporting Information.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

Supporting information for

Bio-based benzoxazines synthesized in a deep eutectic solvent: a greener approach toward vesicular nanosystems

Mohamed S. Behalo*, Ermelinda Bloise,Giuseppe Mele, Antonio Salomone, Francesco Messa, Luigi Carbone, Selma Elaine Mazzetto, Diego Lomonaco

Table of Contents

1. General information

All chemicals were purchased from Sigma-Aldrich and used without purification. The deep eutectic solvent choline chloride (ChCl) –urea (1:2) was prepared by gently heating under stirring at 70°C for 10 min until aclear solution was obtained.

Cardanol (CA) was kindly furnished by Amendoas do Brasil (Fortaleza), Cholesterol (CH), $NaH_2PO_4 \cdot H_2O$ and $Na_2HPO_4 \cdot 12H_2O$ were purchased from Sigma-Aldrich (Steinheim, Germany) and used as received. Ultra pure (UP) water delivered by a Zeneer UP 900 Human Corporation system. Phosphate buffer solution pH 7.4 is a UP water solution 5 mM of $NaH_2PO_4 \cdot H_2O$ and $Na_2HPO_4 \cdot 12H_2O$.

FT-IR spectra were recorded on a JASCO FT-IR 660 Plus spectrometer. NMR spectra were recorded on a Bruker Avance 400 (400 MHz) using $CDCl_3$ as solvent. Column chromatography was run using silica gel 60 (70–230 mesh, Vetec), while TLC was conducted on precoated silica gel polyester sheets (Kieselgel 60 F254, 0.20 mm, Merck).

Measurement of dynamic light scattering and electrophoretic light scattering were both carried out on a Malvern Zetasizer Nano ZS90 on diluted samples. The hydrodynamic diameter (d) of nanodispersions have been determined at 25 °C measuring the autocorrelation function at a 90° scattering angle. Cells have been filled with 400 µL of sample solution and diluted to 4 mL with UP water. Each d value is the average of five separate measurements. Values of zeta–potential (ZP) of nanodispersions have been determined at 25 °C filling the Zeta meter cell, equipped with gold-coated electrodes, with 400 µL of sample solutionand diluted to 4 mL with UP water. The voltage ramps were performed according to the indications given by the purveyor.

The morphology of the vesicular nanodispersion were examined using transmission electron microscopy (TEM). Low-magnification TEM analyses were performed on a Jeol JEM-1011 electron microscope operating at 100 kV, equipped with a CCD camera ORIUS 831 from Gatan. TEM samples were prepared by initially mixing dilute vesicles with a few microliters of osmium tetroxide aqueous solution (1% w/v) and then drop-casting them ontocarbon-coated copper grids. Hence, each grid is twice rinsed in UPwater, and afterward, the deposited samples are completely dried at 60°C for one night before examination.

2. Characterization of1,2-Bis(7-pentadecyl-2H-benzo[e][1,3]oxazin-3(4H)-yl)ethane (4)
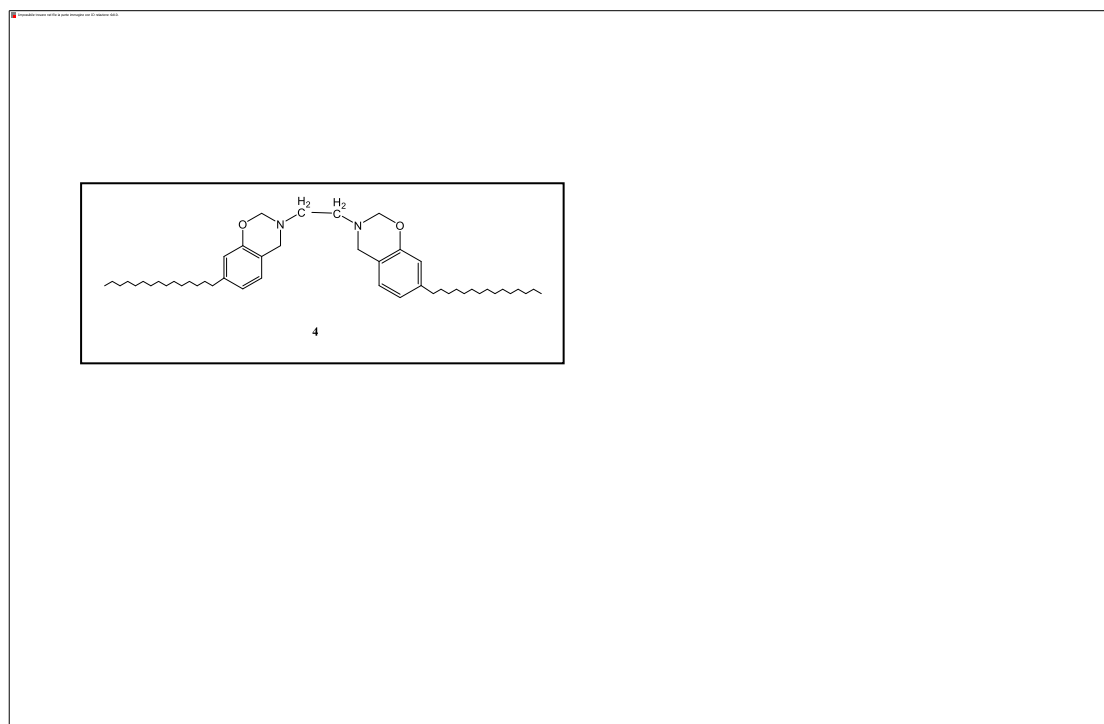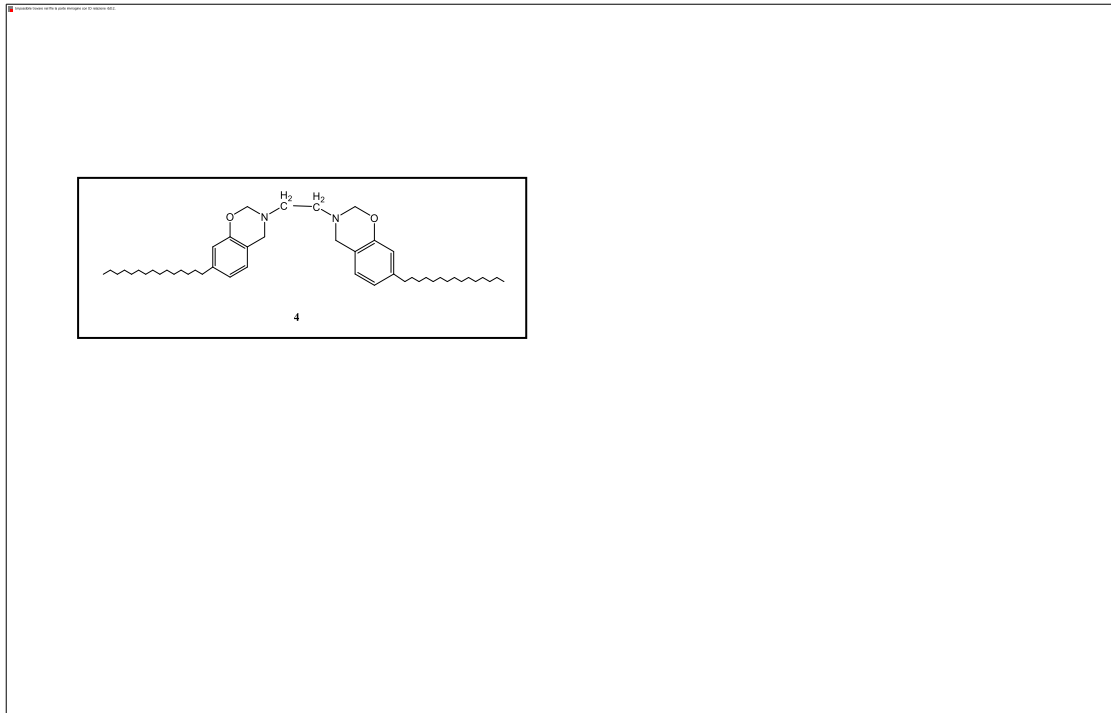
by ^{1}H-NMR and ^{13}C-NMR spectroscopy
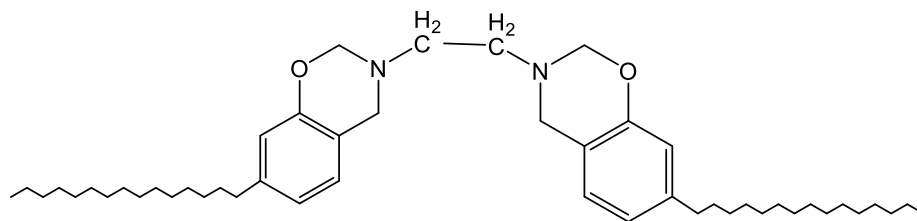
4

^{1}H-NMR (400 MHz, CDCl3): δ = 0.89 (t, 6H, 2CH$_3$), 1.26-1.59 (m,52H, 26 CH$_2$), 2.53 (2 benzylic CH$_2$, *J* = 7.32 Hz, 2H), 3.80 (s, 4H, 2CH$_2$), 4.50 (s, 4H,2 NCH**$_2$**Ar) and 4.85 (s, 4H, 2 OCH**$_2$**N), 6.60 (d, H, 2Ar-H), 6.86 (d, 2H, 2 Ar-H), 7.27 (s, 2H, 2 Ar-H).

^{13}C-NMR (100.64 MHz, CDCl$_3$): δ = 157.19, 144.21, 127.83, 119.19, 118.40, 115.94, 75.80, 57.89, 51.39, 35.51, 31.72, 31.08, 29.49, 29.48, 29.47, 29.45, 29.39, 29.32, 29.18, 29.16, 22.49, 16.50
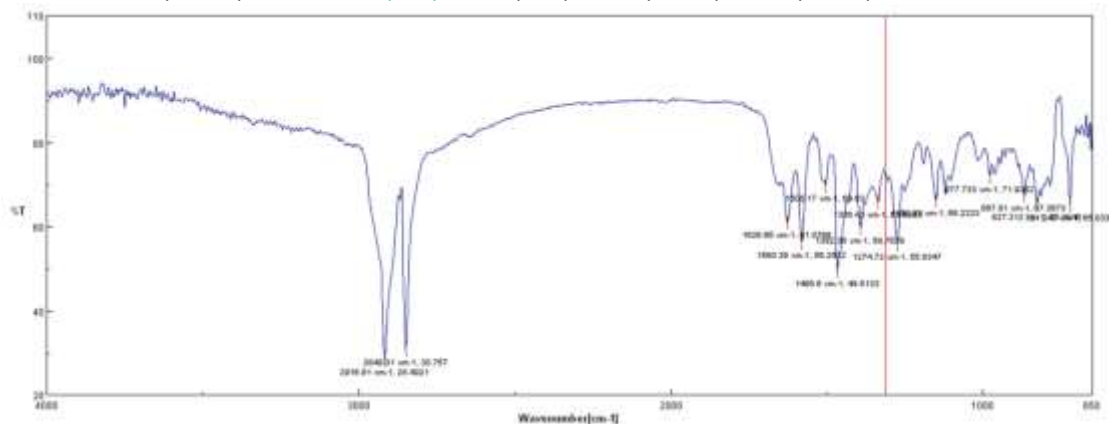

^{1}**H-NMR spectrum of 1,2-Bis(7-pentadecyl-2H-benzo[e][1,3]oxazin-3(4H)-yl)ethane (4)**

13C-NMR NMR spectrum of 1,2-Bis(7-pentadecyl-2H-benzo[e][1,3]oxazin-3(4H)-yl)ethane (4)

3. Characterization of 1,2-Bis(7-pentadecyl-2H-benzo[e][1,3]oxazin-3(4H)-yl)ethane (4) by IR spectroscopy

FT-IR: ν = 3030 (C=C-H), 2920, 2850 (CH$_2$), 1620 (C=C), 1140 (C-N-C), 1255 (C-O-C) cm^{-1}

IR spectrum of 1,2-Bis(7-pentadecyl-2H-benzo[e][1,3]oxazin-3(4H)-yl)ethane (4)

4. Procedure for thepreparation of benzoxazine-nanovesicle formulations

Formulation **A**: A mixture of unsaturated BZ **3a** and CH (molar ratio 1:0.6, respectively) was heated at 90°C for about 1 h, then mixed by mechanical stirring in the presence of glass beads (10 g) for 1 h to form a lipidic film on the flask's wall. Hydration of lipidic film was carried out adding 40 mL of

phosphate buffer (pH 7.4, preheated at 50°C) under mechanical stirring (800 rpm) and heating at 90°C for 1 h. Then, obtained vesicular nanodispersion was submitted to sonication step for 45 min at 40°C and centrifuged (7000 rpm for 15 min) to help the separation of smallest vesicles in supernatant collected as sample.

Formulation **B** and **C**: A mixture of unsaturated BZ **3a** and saturated BZ **3c** derivatives (molar ratio 1:1, respectively) was heated (to 90°C for formulation **B** and to 66°C for formulation **C**) for about 1 h, then mixed by mechanical stirring in the presence of glass beads (10 g) for 1 h to form a lipidic film on the flask's wall. For the formulation **C** a solid residue was noted, probably due to low temperature which clearly does not melt the BZ **3c**. Hydration of lipidic films was carried out adding 40 mL of phosphate buffer (pH 7.4, preheated at 50°C) under mechanical stirring (800 rpm) and heating at 90°C for formulation **B** and to 66°C for formulation **C**, for 1 h. Then, obtained vesicular nanodispersions were submitted to sonication step for 45 min at 40°C and centrifuged (7000 rpm for 15 min) to help the separation of smallest vesicles in supernatant collected as samples.

Formulation **D**: The unsaturated derivative BZ **3a** was heated at 50°C for about 1 h, then mixed by mechanical stirring in the presence of glass beads (10 g) for 1 h to form a lipidic film on the flask's wall. Hydration of lipidic film was carried out adding 40 mL of phosphate buffer (pH 7.4, preheated at 50°C) under mechanical stirring (800 rpm) and heating at 50°C for 1 h. Then, obtained vesicular nanodispersion was submitted to sonication step for 45 min at 40°C and centrifuged (7000 rpm for 15 min) to help the separation of smallest vesicles in supernatant collected as sample.

Formulation **E**: The saturated derivative BZ **3c** was heated at 77°C for about 1 h, until the solid was completely melted, then mixed by mechanical stirring in the presence of glass beads (10 g) for 1 h to form a lipidic film on the flask's wall. Hydration of lipidic film was carried out adding 40 mL of phosphate buffer (pH 7.4, preheated at 50°C) under mechanical stirring (800 rpm) and heating at 77°C for 1 h. Then, obtained vesicular nanodispersion was submitted to sonication step for 45 min at 40°C and centrifuged (7000 rpm for 15 min) to help the separation of smallest vesicles in supernatant collected as sample.

Quantification of BZs concentration in the nanodispersions has been performed by UV-Vis spectrophotometric measurements using a Jasco V-660 spectrophotometer. A calibration curve in the range 0,75−100 mg/L was developed by using the purified unsaturated derivative BZ **3a** as standard. The measurements were carried out after lysis of vesicles obtained by dissolving 1 mL of colloidal solution in a 5 mL of MeOH/CH_2Cl_2 (1:0.25) mixture and then allowing the solvents to evaporate at 70 °C under vacuum and, finally, dissolving the residue in 1 mL of CH_2Cl_2. Hence, 250 μL of concentrated solution was diluted to 4 mL and the absorbance measured at 279 nm, typical of CA-moiety absorption, both for saturated and unsaturated derivative. For formulations **B** and **C**, the calculated BZs concentration is the sum of saturated and unsaturated derivatives.