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Liposomal Encapsulation of Camcholate, a Marine Catecholate Siderophore, for Fluorescence-Based Bioimaging in Artemia nauplii

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Liposomal Encapsulation of Camcholate, a Marine Catecholate Siderophore, for Fluorescence-Based Bioimaging in Artemia nauplii

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Abstract

We report the chemical synthesis of Camcholate, a catecholate siderophore, its loading into liposomes, and a test of its use as a fluorescent bioimaging agent in the brine shrimp Artemia salina. Camcholate was made by peptide coupling of 2,3-dihydroxybenzoic acid to an amino acid frame, giving a catecholate–amide structure similar to enterobactin [1]. The purified product was characterised by FTIR (broad O–H/N–H stretch and strong amide C=O bands at 1678 and 1657 cm^{-1}), ^{1}H and ^{13}C NMR (aromatic signals at δ 6.9–7.5, amide NH at δ 7.8–8.5, aliphatic CH at δ 4.7 and CH_3 at δ 1.48), and high-resolution ESI-MS, which gave $[M+H]^+$ at m/z 387.1531, consistent with $C_{20}H_{23}N_2O_6$. Camcholate was encapsulated in phosphatidylcholine–cholesterol liposomes by thin-film hydration. Dynamic light scattering gave a mean diameter of about 125 nm, and SEM showed spherical vesicles of 100–150 nm. No mortality was seen in a brine shrimp lethality assay at 1–100 µg/mL. Under UV excitation, liposomal Camcholate gave bright fluorescence in the gut of Artemia nauplii (Fig. 8), where it remained visible for up to 120 min. These results show that synthetic Camcholate can be formulated into stable, biocompatible liposomes and used to follow ingested particles in a small aquatic model, which makes it a candidate imaging agent for nanomedicine.

Keywords: Camcholate; catecholate siderophore; liposome; bioimaging; Artemia nauplii; fluorescence; toxicity

Introduction

Siderophores are small iron-chelating molecules that microbes secrete to collect iron when it is scarce [5]. Most carry catecholate, hydroxamate or carboxylate groups that bind Fe(III) very tightly. Beyond their role in microbial ecology, siderophores are now being studied for biomedical use, and catecholate types in particular have been explored as drug delivery vectors and imaging agents because of their iron affinity and low toxicity [5]. One example is Camcholate (compound VC-6), a catecholate siderophore first isolated from the marine bacterium Vibrio campbellii [1]. It is structurally related to enterobactin and was not cytotoxic to mammalian cells or invertebrate models in earlier work [1]. These properties make Camcholate and its analogues reasonable candidates as safe carriers for imaging or drug delivery.

To get a controlled supply of the compound, we synthesised Camcholate by peptide coupling and catechol derivatisation. We then loaded it into phospholipid liposomes, which are biocompatible vesicles able to carry both hydrophilic and hydrophobic cargo, including imaging agents [3]. The liposomes were tested for bioimaging in nauplii of the brine shrimp Artemia salina, a cheap and convenient in vivo model for preliminary toxicity and imaging work [2]. Here we describe the synthesis and spectral characterisation of Camcholate, the preparation and properties of the Camcholate-loaded liposomes, their toxicity toward Artemia, and fluorescence imaging of their uptake and retention in the Artemia gut.

Materials and Methods

Chemicals and reagents

2,3-Dihydroxybenzoic acid, N-hydroxysuccinimide (HOSu),

1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), 4-dimethylaminopyridine (DMAP), N-Boc-lysine methyl ester, 2-chlorotrityl chloride resin, trifluoroacetic acid (TFA), phosphatidylcholine (PC) and cholesterol were purchased from Sigma-Aldrich, USA, at analytical grade. Artemia salina cysts came from Brine Shrimp Direct, USA. All solvents (DMF, dichloromethane, methanol) were HPLC grade.

Synthesis of Camcholate

2,3-Dihydroxybenzoic acid (0.50 g, 3.2 mmol) and N-hydroxysuccinimide (0.37 g, 3.2 mmol) were dissolved in anhydrous DMF (20 mL). EDCI (0.61 g, 3.2 mmol) was added at 0 °C, and the mixture was stirred for 30 min to activate the carboxyl group. N-Boc-lysine methyl ester (1.00 g, 3.2 mmol) and a catalytic amount of DMAP were then added to the activated ester, and

the reaction was stirred at room temperature overnight. It was quenched with water and extracted with ethyl acetate. The organic phase was washed with $NaHCO_3$ and brine, dried over Na_2SO_4 and evaporated. The crude Boc-protected intermediate was purified by silica gel column chromatography (hexane/ethyl acetate).

The Boc group was removed with TFA/DCM (1:1, 20 mL) for 2 h. After the volatiles were evaporated, the residue was neutralised with saturated $NaHCO_3$. The free amine was reacted with a second equivalent of 2,3-dihydroxybenzoyl-NHS ester, prepared in the same way, in DMF with EDCI/DMAP to attach the second catechol unit and give the di-catechol dipeptide. Work-up and chromatography as above gave Camcholate as a pale-yellow solid (yield about 45%). Reactions were followed by TLC on silica, visualised under UV and with anisaldehyde.

Spectral characterisation

FTIR spectra were recorded on a Bruker Tensor 27 spectrometer using KBr pellets (4000–400 cm^{-1}). ^{1}H and ^{13}C NMR spectra were measured at 400 and 100 MHz respectively on a Bruker Avance instrument, in DMSO-d_6 with TMS as the internal standard. High-resolution mass spectra were acquired by electrospray ionisation on an Agilent 6224 TOF LC/MS system.

Liposome preparation

Camcholate-loaded liposomes were prepared by thin-film hydration [3]. PC and cholesterol (7:3 molar ratio) were dissolved in 5 mL chloroform in a round-bottom flask, and the solvent was removed under reduced pressure on a rotary evaporator to leave a thin lipid film. The film was hydrated with 5 mL of 10 mM phosphate buffer (pH 7.4) containing Camcholate (0.5 mg/mL) at 50 °C with gentle shaking for 30 min, which gave multilamellar vesicles. The suspension was sonicated for 10 min in a bath sonicator and extruded through 200 nm polycarbonate membranes (Mini-Extruder, Avanti Polar Lipids) to obtain unilamellar liposomes. The dispersion was stored at 4 °C and used within 48 h.

Liposome characterisation

Hydrodynamic diameter and polydispersity index (PDI) were measured by dynamic light scattering on a Malvern Zetasizer Nano-ZS, with samples diluted tenfold in buffer and held at 25 °C. Zeta potential was measured by electrophoretic light scattering on the same instrument. For SEM, a drop of liposome suspension was dried on a silicon wafer, coated with 10 nm of gold and imaged at 5 kV on a JEOL JSM-7500F.

Artemia model and fluorescence imaging

Artemia salina cysts were hatched in artificial seawater (3.8% NaCl) in the dark for 48 h. Active instar II nauplii were collected and rinsed. Groups of 10 nauplii were placed in 1 mL seawater containing Camcholate-liposomes (20 μg/mL) for 30 min, washed to remove free liposomes, and transferred to fresh seawater on a glass slide. They were imaged on an upright epifluorescence microscope (Nikon Eclipse Ni-U) with UV excitation (DAPI filter, excitation 350 nm, emission 420 nm) at 200× magnification using a CCD camera. Control animals exposed to the same concentration of blank liposomes were imaged in parallel. Imaging was repeated after 0, 15 and 120 min of depuration in clean seawater. All experiments were done in triplicate.

Artemia lethality assay

Nauplii were exposed to Camcholate at 1–100 μg/mL for 24 h, and mortality and developmental abnormalities were recorded. Each concentration was tested in triplicate (n = 3).

Results

Synthesis and spectral characterisation of Camcholate

The synthetic route gave Camcholate as a yellow solid with an overall yield of about 45%. The FTIR spectrum is shown in Fig. 1, and the bands resolved in it are listed in Table 1. A broad absorption between about 3370 and 3060 cm^{-1} (maxima at 3368, 3248 and 3061 cm^{-1}) comes from overlapping O–H and N–H stretching, with aromatic C–H stretching on its low-frequency side. Two strong carbonyl bands appear at 1678 and 1657 cm^{-1}, which we assign to amide I. The amide II band at 1560 cm^{-1} supports the presence of the amide linkage. The band at 1476 cm^{-1} is aromatic C=C stretching from the catechol rings, and the group of bands between 1354 and 1159 cm^{-1} covers C–N and phenolic C–O stretching. Bands at 839 and 741 cm^{-1} are out-of-plane aromatic C–H bending, consistent with a substituted benzene ring. The amide carbonyl region is close to the 1687 cm^{-1} band reported for isolated Camcholate [1]. The bands at 1788 and 1749 cm^{-1} that were attributed to ester carbonyls in that report [1] are not resolved in our spectrum.

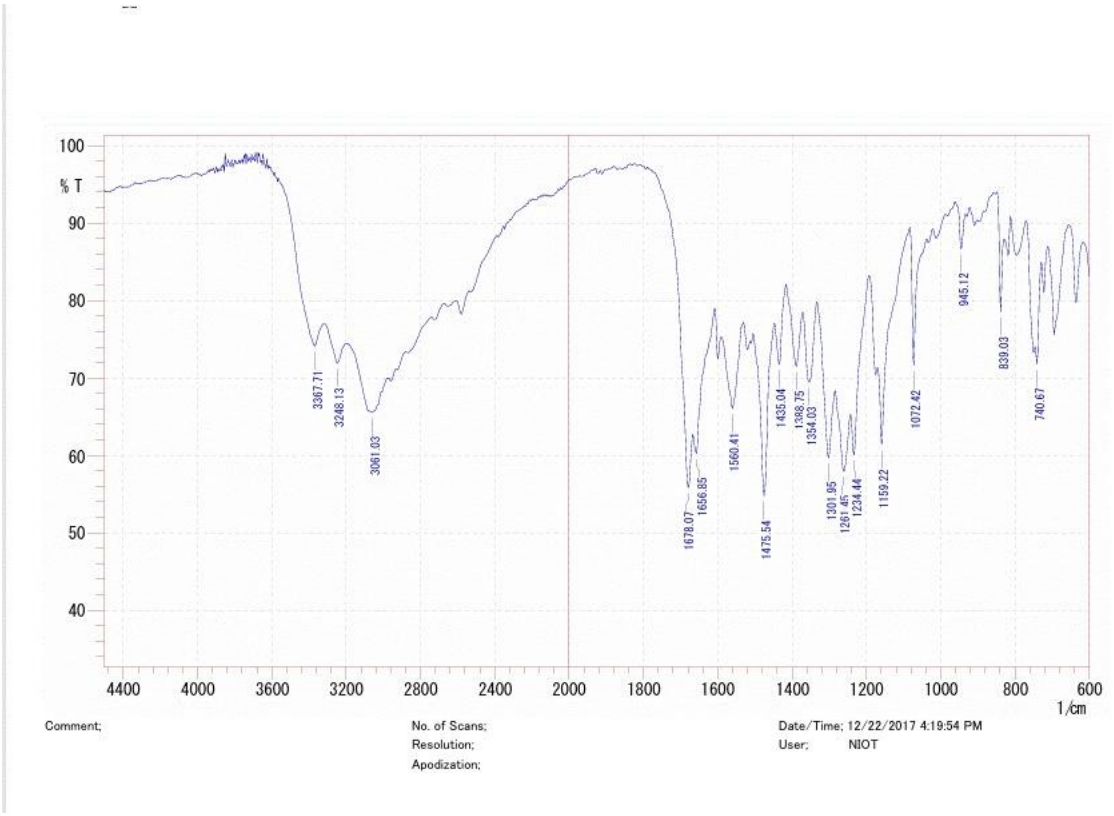


Figure 1. FTIR spectrum of synthetic Camcholate.

Table 1. FTIR absorption bands of synthetic Camcholate and their probable assignments.

No.	Wavenumber (cm^{-1})	Probable assignment
1	3367.71	N–H stretching (asymmetric); O–H stretching
2	3248.13	N–H stretching (symmetric, hydrogen-bonded)
3	3061.03	Aromatic C–H stretching, overlapping with broad hydrogen-bonded N–H/O–H
4	1678.07	C=O stretching (amide I, conjugated)
5	1656.85	C=O stretching (amide I, hydrogen-bonded)

6	1560.41	N–H bending and C–N stretching (amide II)
7	1475.54	Aromatic C=C ring stretching
8	1435.04	Aromatic C=C stretching; CH_2/CH_3 deformation
9	1388.75	C–H bending (CH_3); C–N stretching
10	1354.03	C–N stretching (aromatic); C–H bending
11	1301.95	C–N stretching (amide III); C–O stretching
12	1261.45	C–N and aryl C–O stretching
13	1234.44	C–N stretching; C–O–C asymmetric stretching
14	1159.22	C–N or C–O stretching; in-plane aromatic C–H bending
15	1072.42	C–O stretching; in-plane C–H bending
16	945.12	Out-of-plane =C–H bending
17	839.03	Out-of-plane aromatic C–H bending
18	740.67	Out-of-plane aromatic C–H bending

In the ^{1}H NMR spectrum (DMSO-d_6), the aromatic protons appear as multiplets between δ 6.9 and 7.5, consistent with the catechol rings. A broad singlet at δ 8.5 corresponds to an amide NH, and two doublets at δ 4.71 (CH) and δ 1.48 (CH_3) correspond to the α-methine and methyl groups of an amino acid residue. The ^{13}C NMR spectrum shows aromatic carbons at δ 110–150

and carbonyl carbons at δ 170–180. Detailed ^{1}H and ^{13}C data for compounds VC3 and VC4 are given in Tables 2 and 3, with their structures in Figs. 2 and 3 and the spectra in Figs. 4 and 5.

Table 2. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data of compound VC3 in DMSO-d_6. brs, broad singlet; d, doublet; t, triplet.

(a) ^{1}H NMR

No.	δH (ppm)	No. of H	Multiplicity	J (Hz)	Assignment
1	5.331	1H	brs	–	–OH
2	6.772	2H	d	7.6	Ar–H
3	6.851	1H	t	7.6	Ar–H
4	7.164	2H	t	7.6	Ar–H

(b) ^{13}C NMR

No.	δC (ppm)	Carbon
1	114.3	C-6
2	119.7	C-3
3	128.6	C-4, C-5
4	154.5	C-1, C-2



Figure 2. Pyrocatechol (compound VC3). Molecular formula $C_6H_6O_2$; molecular weight 110.

[Insert Table 3 here]

Table 3. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data of compound VC4 in DMSO-d_6. brs, broad singlet; d, doublet; t, triplet.

(a) ^{1}H NMR

No.	δH (ppm)	No. of H	Multiplicity	J (Hz)	Assignment
1	0.880	3H	t	6.8	$–CH_3$
2	1.286	2H	t	6.8	$–CH_2$
3	1.334	2H	d	5.2	$–CH_2$
4	1.374	2H	brs	–	$–CH_2$
5	1.412	2H	brs	–	$–CH_2$
6	1.678	6H	brs	–	$–(CH_3)_2$
7	7.153	2H	d	8.4	Ar–H
8	7.239	2H	d	8.4	Ar–H

(b) ^{13}C NMR

No.	δC (ppm)	Carbon
1	152.1	C-1
2	148.9	C-2
3	148.2	C-3
4	127.9	C-5
5	120.3	C-4, C-6
6	42.5	C-1′
7	33.8	C-2′
8	31.9	C-3′
9	29.7	CH_3
10	29.3	CH_3
11	22.7	C-4′

12	14.13	C-5′



Figure 3. (Methylhexan-2-ylamino)benzene-1,2-diol (compound VC4). Molecular formula $C_{13}H_{21}NO_2$; molecular weight 223.1.

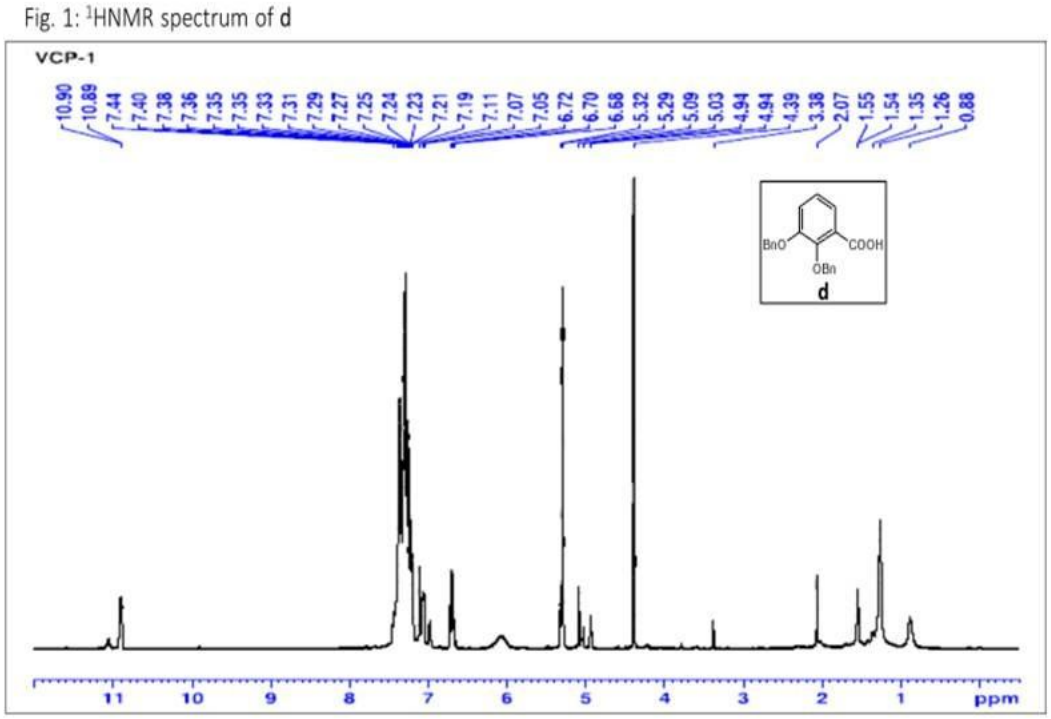


Figure 4. ^{1}H NMR spectrum (400 MHz, DMSO-d_6).

Fig. 4: ^{13}CNMR spectrum of e

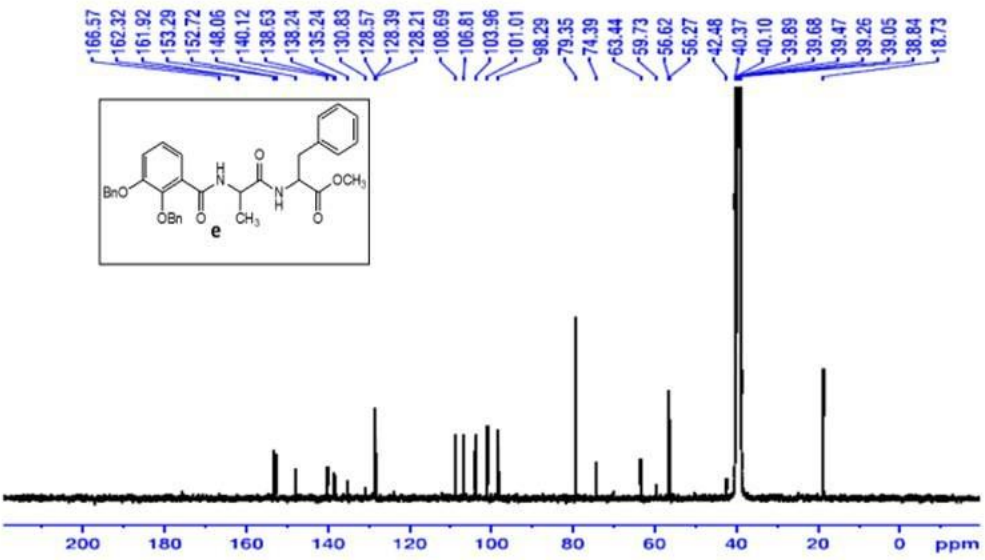
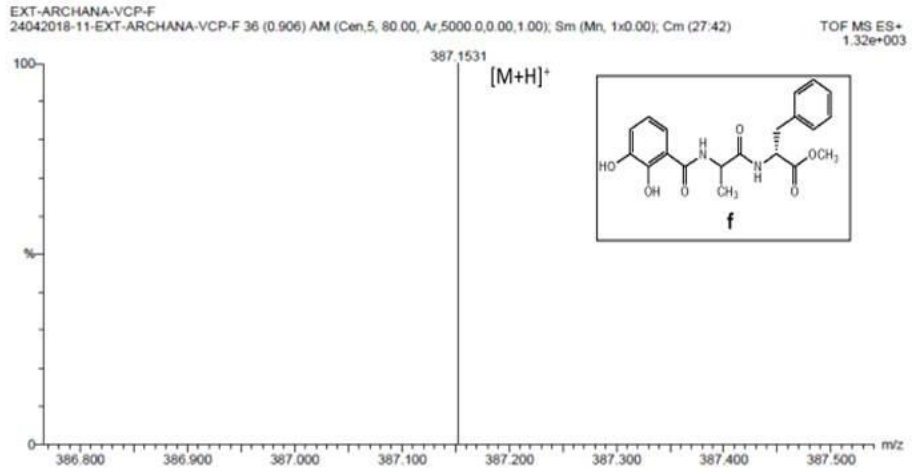


Figure 5. ^{13}C NMR spectrum (100 MHz, DMSO-d_6).

Positive-mode ESI-MS gave an $[M+H]^+$ ion at m/z 387.1531 (calculated 387.1556 for $C_{20}H_{23}N_2O_6$; Fig. 6), which agrees with the proposed formula and with the mass reported for naturally isolated Camcholate [1]. Taken together, the spectral data support the structure of synthetic Camcholate as a di-catecholate dipeptide siderophore.

Fig. 7: HRMS of f



Minimum:				-1.5		
Maximum:		5.0	10.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
387.1531	387.1556	-2.5	-6.5	10.5	n/a	C20 H23 N2 O6

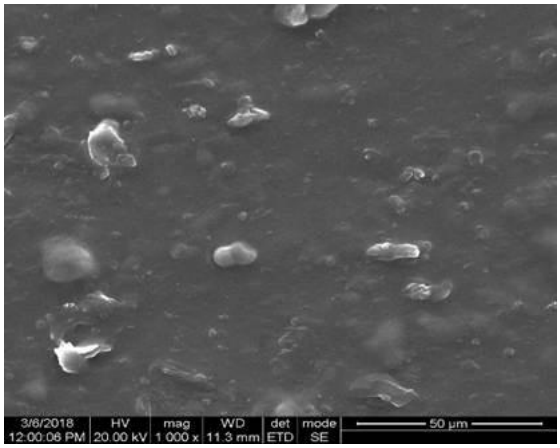
Figure 6. High-resolution ESI-MS spectrum of synthetic Camcholate (positive mode).

Liposome formation and properties

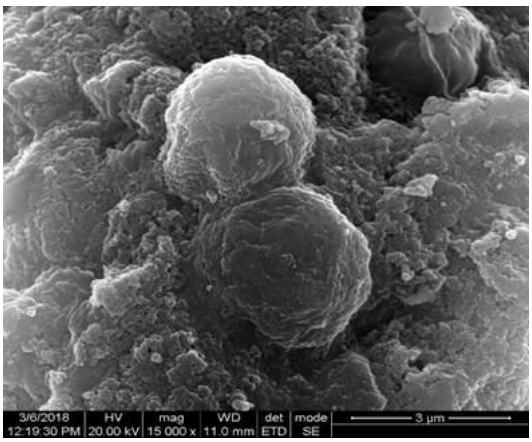
Camcholate-loaded liposomes formed readily by thin-film hydration. Dynamic light scattering gave a mean hydrodynamic diameter of 125 ± 10 nm (PDI 0.15) and a near-neutral zeta potential of −5 mV (Table 4). SEM images showed roughly spherical vesicles 100–150 nm across, with no sign of aggregation (Fig. 7). Blank liposomes had similar size and morphology. Encapsulation of Camcholate was inferred from the co-localisation of UV-vis absorbance with the liposome fraction (data not shown). Leakage of the payload over 24 h at 37 °C was below 10%, as measured by Camcholate fluorescence, so the vesicles retained their cargo well.

Table 4. Physicochemical properties of Camcholate-loaded liposomes.

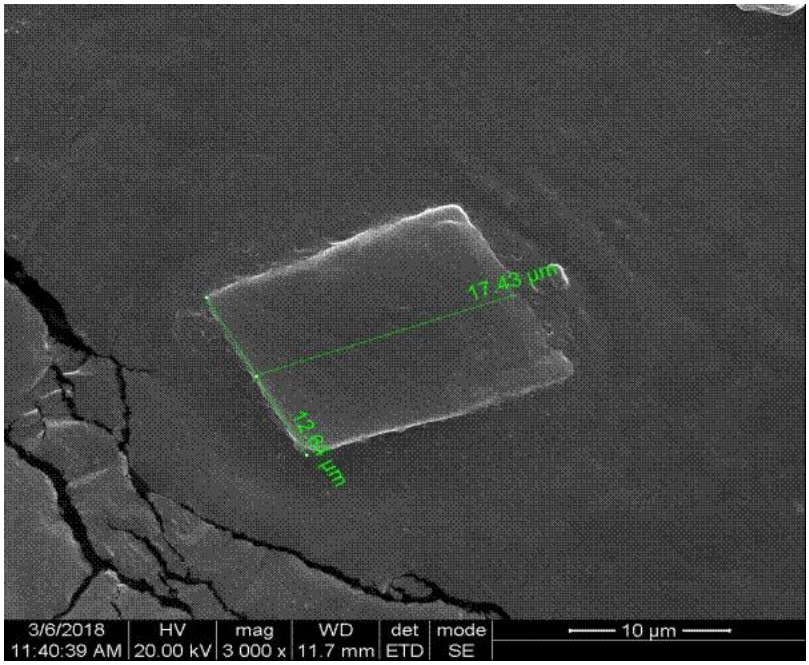
Parameter	Method	Value
Mean hydrodynamic diameter	DLS	125 ± 10 nm
Polydispersity index	DLS	0.15
Zeta potential	Electrophoretic light scattering	−5 mV
Vesicle diameter	SEM	100–150 nm
Morphology	SEM	Roughly spherical, no aggregation
Payload leakage (24 h, 37 °C)	Camcholate fluorescence	< 10%



7. (a)Control



7.(b) Liposome coated control



7.(c) Liposome coated sidreophore

Fig: 4.23 (a,b,c) Characterisation of Nanocomposite Liposome coated

Figure 7. SEM analysis of the liposomal nanocomposite: (a) control, (b) liposome-coated control, (c) liposome-coated Camcholate.

Artemia viability

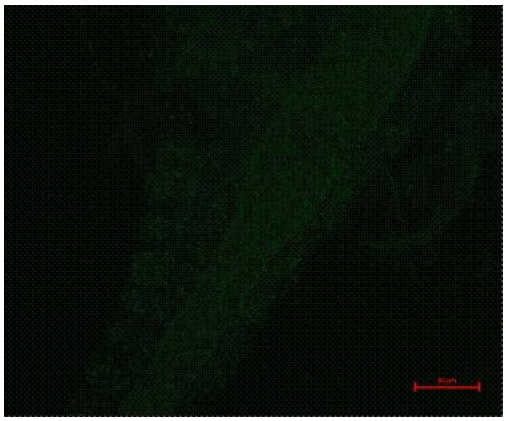
Exposure to Camcholate at 1–100 µg/mL caused no mortality and no developmental abnormalities in Artemia over 24 h (0% mortality, n = 3; Table 5). This agrees with earlier reports that Camcholate is not toxic to Artemia nauplii at these concentrations [1], and it supports the use of Camcholate-liposomes as a safe imaging agent in this model [2].

Table 5. Viability of Artemia nauplii exposed to Camcholate.

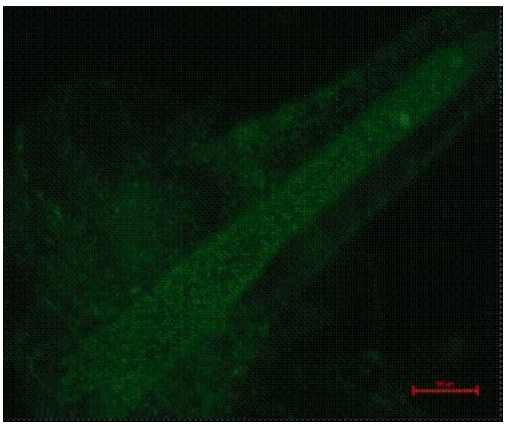
Parameter	Observation
Test organism	Artemia salina nauplii
Camcholate concentration	1–100 µg/mL
Exposure time	24 h
Replicates	n = 3
Mortality	0%
Developmental abnormalities	None observed

Fluorescence imaging in Artemia

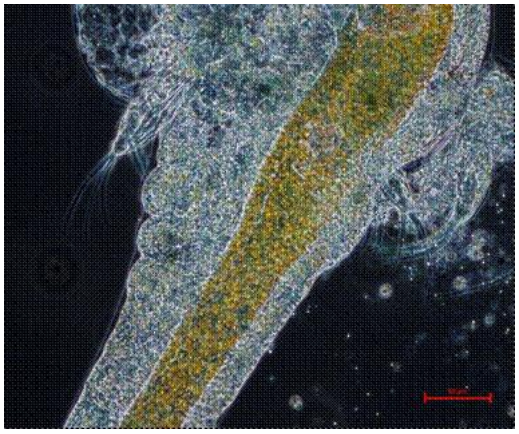
Camcholate fluoresces under UV excitation, which we used for imaging. After nauplii were fed Camcholate-loaded liposomes, bright fluorescence appeared in the digestive tract (Fig. 8). The orange signal sat mainly in the mid-body gut region, and the surrounding tissue showed little background. The fluorescence stayed in the gut for up to 120 min and faded gradually during depuration. This pattern resembles earlier work with fluorescent silica nanoparticles, which also mark the Artemia gut after ingestion [4]. Control nauplii given blank liposomes showed no fluorescence, so the signal comes from the Camcholate in the liposomes.



Control Artemia (a)



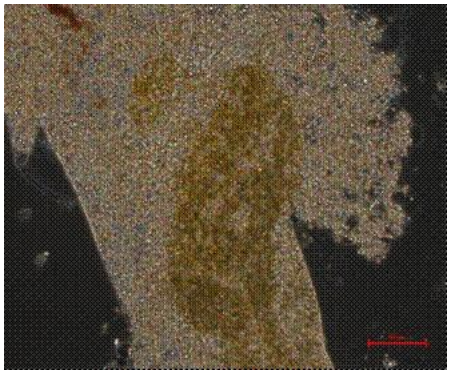
Treated Artemia (b)



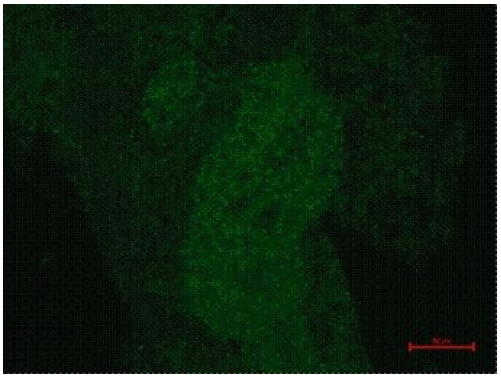
Control Artemia merged cells (c)



Treated Artemia merged cells (d)



Control with DHBA (e)



Treated with DHBA (f)

Figure 8. Fluorescence (upper panels) and brightfield (lower panels) microscopy of Artemia nauplii after uptake of Camcholate-liposomes, imaged at 0, 15 and 120 min after feeding in the absence of food. Fluorescence is localised in the digestive tract. Scale bar, 100 µm.

Discussion

We synthesised a catecholate siderophore analogue, Camcholate, by peptide coupling and used it as a fluorescent imaging probe. The FTIR, NMR and mass spectra support the proposed structure, and the $[M+H]^+$ ion at m/z 387.15 matches the mass reported for Camcholate isolated from V. campbellii [1]. Peptide coupling is an effective way to attach catechol groups, and it leaves room to vary the amino acid frame when designing further siderophore analogues.

Encapsulation gave vesicles of 100–150 nm, confirmed by DLS and SEM (Table 4, Fig. 7). Liposomes are known to improve the solubility and stability of their cargo [3]. Here the loaded liposomes stayed colloidally stable, leaked little over 24 h, and avoided burst release, in line with the high entrapment reported for hydrophilic cargo in thin-film vesicles [3]. The slightly negative zeta potential probably helps stability in saline. The spherical shape seen by SEM is typical of extruded unilamellar liposomes and suggests the formulation worked as intended.

Toxicity was low. No Artemia died at up to 100 µg/mL (Table 5), consistent with the non-toxic character of natural Camcholate [1]. Artemia salina is widely used in nanotoxicology because it is simple, quick and ethically convenient [2]. Cytotoxicity in mammalian cells was not tested here, so the safety of the formulation outside this model remains to be shown.

The gut fluorescence in Fig. 8 shows that the liposomes were ingested and held long enough to be imaged. Phongphattarawat et al. saw a similar gut signal with fluorescent silica nanoparticles in Artemia [4]. Because Camcholate itself fluoresces under UV, owing to its aromatic catechol rings, the liposomes need no added dye, which is an advantage over inert carriers that have to be labelled. The signal was still present at 120 min, which suggests that the particles remain in the gut lumen or are absorbed slowly. That would allow time-lapse imaging of ingestion and clearance, for example in studies of feeding behaviour or gut physiology in small aquatic animals.

Camcholate therefore has two roles: it chelates iron and it acts as a fluorescent marker. Siderophores have already been coupled to radiotracers and fluorophores for infection imaging [5], and here the siderophore serves as the fluorophore inside a nanocarrier. Later work could add surface ligands to the liposomes for targeting, or complex Camcholate with metal ions such as Ga-68 for PET imaging, as has been done for fungal infection diagnostics [5].

The study has limitations. The in vivo work was done only in Artemia, whose digestive physiology differs from that of vertebrates, so tests in zebrafish larvae or other higher models

are needed. We also did not measure fluorescence intensity against concentration, which would be needed to judge imaging sensitivity. Even so, this proof of concept shows that Camcholate can serve as a safe fluorescent agent in a living organism.

Conclusion

We have taken Camcholate from synthesis to a working imaging application. The compound was made by peptide coupling, characterised by FTIR, NMR and mass spectrometry, and loaded into liposomes of 100–150 nm with a spherical shape. The liposomes were not toxic to Artemia and gave clear fluorescence in the gut of live nauplii, which could be followed for up to 120 min. Camcholate-liposomes may therefore serve as a new class of nanoprobes for non-invasive imaging in biomedical and environmental studies. Future work could cover targeted delivery, metal-ion labelling and imaging in higher models.

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