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A polydiacetylene-based fluorescence assay for the measurement of lipid membrane affinity

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Abbreviations

PDA polydiacetylene

DMPC dimyristoylphosphatidylcholine

FL fluorescence

Chol cholesterol

K_{oct} n-octanol/water partition coefficient

K_m lipid membrane partition coefficient

Keywords

Lipid membrane affinity

Polydiacetylene

Fluorescence

Local anesthetics

Flavonoids

Abstract

The polydiacetylene (PDA) is a promising membrane-screening tool because lipid constituents can be incorporated into the PDA framework to form lipid/PDA vesicles used as lipid bilayers. Previous reports have shown that the colorimetric signals of PDA could be utilized for the measurement of drug-lipid membrane interactions. In this study, the fluorescence signals of PDA vesicles were investigated for the measurement of lipid membrane affinity. Based on the fluorescence response of PDA vesicles (excitation wavelength 485 nm, emission wavelength 560 nm), the half maximal response concentration (EC50) was introduced for the evaluation of drug-membrane interactions. In order to validate this method, local anesthetics and flavonoids were selected as the reference compounds and their log (EC50) values correlated well with other lipid membrane affinity constants. Then the influence of buffer pH conditions and lipid constituents on the membrane affinity were

investigated to show the wide application of this method using tetracaine hydrochloride as the reference compound. The particle size of vesicles before and after addition of tetracaine hydrochloride was determined to observe the extent of vesicle binding of the tested compound. The zeta potential results showed that the electrostatic interaction had less effect on the change of lipid membrane affinity at different pH value. Therefore, the hydrophobic interaction was assumed to play the most important role in the increase of lipid membrane affinity of tetracaine hydrochloride as the increase of buffer pH value. The ratio of Chol in the lipid constituents less affected the affinity of tetracaine hydrochloride, but significantly weakened the sensitivity of PDA-based fluorescence signals. In summary, this work provides a simple, sensitive and reproducible PDA-based fluorescent method for the rapid measurement of lipid membrane affinity.

1. Introduction

The membrane affinity means the binding ability of a drug candidate to cell membranes and the lipid constituents has a major impact on it.[1] Therefore, the measurement of lipid membrane affinity is an important early screening step during drug discovery. The n-octanol/water partition coefficient (K_{oct}) is widely used to represent the lipid membrane affinity, but isotropic phase n-octanol is quite different from the structure of lipid bilayers. Compared with n-octanol, lipid vesicles almost exactly mimic real biological membranes. However, traditional methods for the determination of lipid membrane partition coefficient, equilibrium dialysis and ultracentrifugation,[2-3] are tedious and difficult to be used in the early screening step.

Other existing methods also suffered from different problems, such as poor reproducibility (chromatographic techniques).[4-5] Sensor techniques are widely used in the detection of biological interaction.[6-9] For examining the membrane interactions, polydiacetylene (PDA) vesicles have several advantages over other platforms including molecular structure of the vesicle, ease of synthesis, inexpensive and commercially available monomers, etc.[10-14]

PDA undergoes blue-red color changes and fluorescence transitions due to the distortion of PDA conjugation plane, which may be induced by surface perturbations.[15-19] When using as a membrane-screening tool, lipid constituents are incorporated into the PDA framework to form lipid/PDA vesicles. The interactions of target molecules with lipid/PDA vesicles change the fluidity of the lipid domain and induce distortion of the conjugation plane of PDA.[20] Previous reports have shown that the colorimetric signals of PDA vesicles could be utilized for the measurement of drug-membrane interactions.[10-12] We also have established a quantitative method for the measurement of lipid membrane affinity based on the PDA colorimetric signals in our previous report.[21] The focus in this work was on the fluorescence properties of lipid/PDA vesicles, aiming to take advantage of the significant higher sensitivity of fluorescent signals.

In order to evaluate the applicability of the established PDA-based fluorescence method, local anesthetics and flavonoids were selected as the reference compounds. These drugs were the common-used target drugs for the evaluation of membrane affinity in previous studies.[22-23] Local anesthetics are alkaline and prone to interact with the hydrophobic region of the lipid membrane.[24] Flavonoids are weak acids and

possess the protonophoric properties, which may be related with the ionisable hydroxyl groups.[25] Application of the PDA colorimetric platform on the two types has been hampered by sensitivity problem. To resolve this problem, the PDA-based fluorescent signal was employed for the membrane affinity detection of local anesthetics and flavonoids. Moreover, the influence of pH value and lipid constituents on the membrane affinity was also investigated to show the wide application of this membrane screening method. Based on these studies, this paper presents a reproducible PDA-based fluorescence assay for the measurement of lipid membrane affinity.

2. Material and methods

2.1 Materials

Dimyristoylphosphatidylcholine (DMPC) and 10, 12-tricosadiynoic acid were purchased from NOF (Tokyo, Japan) and Alfa-Aesar (Ward Hill, MA, USA), respectively. Cholesterol (Chol, 5AZZH-OL) was from TCI (Japan). Quercetin, luteolin, apigenin and daidzein were obtained from Yuanye Bio-Technology (Shanghai, China). Tetracaine hydrochloride, mepivacaine hydrochloride, lidocaine hydrochloride, propitocaine hydrochloride and bupivacaine hydrochloride were purchased from Jinan Chenghui–Shuangda Chemical (Jinan, China). All of the above-mentioned materials had purities greater than 98%. All other chemicals were of analytical grade and used as received.

2.2 Vesicles Preparation

The preparation of PDA vesicles has been reported in our previous study.[21] In brief,

DMPC and diacetylene monomer 10,12-tricosadiynoic acid (2:3 molar ratio) were dissolved in a chloroform/ethanol solution (1:1) and dried together in vacuo, followed by addition of deionized water to produce a suspension. The suspension was heated to 70 °C and sonicated in an ultrasonic bath for 5 min. The vesicles solution was cooled to room temperature, kept at 4 °C overnight and then polymerized by irradiation at 254 nm for 5 min with stirring at room temperature to obtain the DMPC/PDA vesicles solution. The vesicles solution was stored at 4 °C before use. When used for the influence of Chol on the membrane affinity, the same procedure was performed as described above except the lipid added in the first step. For example, the molar ratio of Chol, DMPC and PDA was 0.2:0.8:1.5 in the 20 % Chol liposome.

2.3 Preparation of buffer and sample solutions

Tris-HCl buffer solution, with a concentration of 150 mM and pH 6.3 to 9.0, was used for the investigation of pH influence. The stock solutions of local anesthetics and flavonoids were prepared by dissolving the compounds in the deionized water and DMSO, respectively. Different volumes of the stock solution were transferred to make a serious of standard solutions. Samples for the FL studies were prepared by adding 0.2 mL of different standard solutions to 0.2 mL of PDA vesicles solution and 2 mL of Tris-HCl buffer solution. The sample solutions were kept at room temperature for 8-15 min before fluorescence assay. All other solutions were stored at 4 °C.

2.4 Fluorescence measurement and calculation of EC50

Fluorescence measurement of the sample solutions was performed on a 4600 FL spectrometer (Hitachi, Japan). The fluorescence emission intensity at 560 nm with an

excitation wavelength of 485 nm was recorded. Spectral slits of emission and excitation wavelength were both 5 nm and the scan speed was 1200 nm · min^{-1}. FL was defined as $FL = FL_1 - FL_0$, where FL_0 is the initial fluorescence response of vesicles before the addition of test compounds, and FL_1 represents the final fluorescence response of vesicles after the addition of test compounds. EC50 was calculated by processing the FL response versus different concentrations with Graph-prism software (Version 5.01; GraphPad Software Inc., San Diego, CA) basing on the nonlinear regression.

2.5 Determination of zeta potential and size of vesicles

The vesicles size distribution and zeta potential values both before and after addition of tetracaine hydrochloride were measured using dynamic light scattering (Malvern Zetasizer 3000HSA, Malvern Instruments Ltd, United Kingdom). These measurements were performed at 25 °C using a suspension containing 0.1 mM DMPC in 150 mM Tris-HCl buffer solution with different pH value.

3. Results and discussion

3.1 The fluorescence spectra and Calculation of the log (EC50) value

The fluorescence spectra of PDA/DMPC vesicles before and after addition of reference compounds were investigated (Fig. 1). It was found that the maximal fluorescence emission signal at the wavelength of 560 nm exhibited an obvious enhancement with a maximal excitation wavelength of 485 nm after addition of reference compounds. The intrinsic fluorescence of reference compounds had no obvious interference at this wavelength. Therefore, the fluorescence response of

PDA/DMPC vesicles (excitation wavelength 485 nm, emission wavelength 560 nm) was selected as the signal for the further research. Previous reports used to evaluate the membrane affinity by the absolute response of the PDA, such as the first report utilizing PDA/lipid vesicles as membrane-screening tool.[14] Actually, the absolute response of vesicles was also affected by the other properties of the tested compound besides the membrane affinity. It is necessary to use a reliable index to assess the membrane affinity.

We have established a simple quantitative model for the measurement of lipid membrane affinity using the index K_b based on the PDA colorimetric signals in our previous report.[21] However, the K_b values of most tested compounds were negative based on the PDA fluorescence response using double-reciprocal plots. Therefore, the half maximal response concentration (EC50), a more general parameter for evaluating biological interaction,[26,27] was introduced for the evaluation of drug-membrane interactions. As shown in Fig. 2, this index could be calculated according to the compound concentrations and fluorescence responses by the nonlinear regression. It is interesting that the FL response of tetracaine hydrochloride was significantly higher than luteolin, whereas the EC50 value of luteolin was lower than tetracaine hydrochloride at pH 7.6. This is an experimental proof that the absolute response change of PDA/lipid vesicles before and after addition of the tested compound cannot represent its lipid membrane affinity. Therefore, the log (EC50) value was selected for the evaluation of drug-membrane interactions in the next parts.

3.2 Comparison of developed method with classical methods

To validate the performance of PDA-based fluorescence assay for the evaluation of lipid membrane interaction, the log (EC50) values of tested compounds were compared with other lipid membrane affinity constants. Local anesthetics and flavonoids were selected as the reference compounds because these drugs were the common-used target drugs for the evaluation of lipid membrane affinity in previous studies.[22-23] The selected local anesthetics were alkalescent with pKa above 7.6 and the flavonoids were acidic compounds with pKa below 7.6.

For local anesthetics, the log (EC50) values of mepivacaine hydrochloride, lidocaine hydrochloride, propitocaine hydrochloride, bupivacaine hydrochloride and tetracaine hydrochloride were compared with the reported logarithm of the liposome/water partition coefficient (K_m) measured by ultracentrifugation using DMPC liposome.[28] As shown in Fig. 3A, the log (EC50) versus log (K_m) of the five target compounds displayed a favorable linear correlation with R^2=0.9829. When compared with the lipid membrane affinity constants measured by immobilized artificial membrane chromatography (IMAC) and micellar electrokinetic chromatography (MEKC), the favorable linear was good with R^2= 0.9864 and 0.9772, respectively (Fig. 3B & C). Furthermore, the correlation between log (EC50) and the potency of pharmacodynamic effect was also excellent with R^2=0.9749 (Fig. 3D). It has been reported that the potency of local anesthetic drugs partly depend on the membrane affinity.[29] Therefore, the good linear correlation illustrated that the developed PDA-based fluorescence method may be a certain prediction for anesthetic effect. For flavonoids, it has been reported that the lipid membrane partition coefficient (Kd)

improved with the increase of the number of hydroxyl groups.[30] In this study, daidzein (two OH groups), apigenin (three OH groups), luteolin (four OH groups) and quercetin (five OH groups) were selected for examing the influence of OH groups. As shown in Table 1, the affinity of flavonoids increased due to the improvement of the number of free hydroxyl groups. Although a limited number of flavonoids were tested due to the lack of classic data, the results illustrated that the developed method could be used for the research on the lipid membrane affinity of flavonoids.

In conclusion, the log (EC50) value of FL response was demonstrated as an effective, accurate and reproducible index for the assessment of lipid membrane affinity.

3.3 Influence of buffer pH conditions

The buffer pH conditions had an important effect on the lipid membrane affinity of the polar compounds. Tetracaine hydrochloride was a common-used reference for the research on the factors affecting the membrane interaction due to its high affinity and good solubility.[31] Therefore, the membrane affinity of tetracaine hydrochloride at different buffer pH conditions were compared to show the wide application of this method.

As show in Fig. 4, tetracaine hydrochloride was tested from pH 6.3 to pH 9.0 with 6 points to cover the normal physiological pH range. The size distribution of vesicles was determined both before and after addition of tetracaine hydrochloride in order to observe the possible changes of particle size (Fig. 5). It was found the particle size of vesicles became bigger after addition of the reference compound when the pH value was above 7.0. But the size change at pH 6.3 was quite little probably due to the low

lipid membrane affinity of tetracaine hydrochloride at this pH value. Therefore, the changes in the size distribution might reflect the extent of vesicle binding of the tested compounds. To account for the electrostatic effects of the interaction, the zeta potential values of vesicles were detected before and after addition of tetracaine hydrochloride (Fig. 6). The zeta potential decreased with the increase of buffer pH value due to less Tris basic ions binding to the membrane interface. It was observed that the zeta potential increased as the addition of tetracaine hydrochloride at all pH value. Since the addition of the drug does not change the ionic strength and pH value of the solution significantly due to the high salt concentration of buffer, the binding of positively charged tetracaine hydrochloride with the lipid membrane interface should be responsible for the increase of zeta potential. Surprisingly, the change of zeta potential remained almost constant throughout pH range. The most probable explanation for the phenomenon was the electrostatic repulsion between positively charged drug molecules and the Tris basic ions binding to the membrane interface at low pH value, or limited positively charged drug molecules in the buffer at high pH value. The increase of lipid membrane affinity of tetracaine hydrochloride as the increase of buffer pH value might result from more neutral drug molecules formation in the buffer solution and binding to the hydrophobic region of membrane interface. Therefore, hydrophobic interaction between tetracaine hydrochloride and the lipid membrane should play the most important role in the changing of lipid membrane affinity at different pH value, while the electrostatic interaction had less effect on it.

In this part, we illustrated the application of polydiacetylene-based fluorescence

248 method for the study with the buffer pH effect on the lipid membrane affinity of drug
249 molecules. The zeta potential was detected to reflect the intrinsic mechanism of
250 affinity change.

251 ***3.4 Influence of lipid constituents***

252 Besides the phospholipid constituent, Chol was another important lipid constituent in
253 the cell membrane.[32-34] Thus, the effect of different lipid constituents (DMPC/Chol
254 100/0, DMPC/Chol 80/20 and DMPC/Chol 60/40) on the membrane affinity of
255 tetracaine hydrochloride were compared using this PDA-based fluorescent method. As
256 shown in Table 2, the ratio of Chol in the lipid constituents had less effect on the log
257 (EC50) of the reference compound. The structural difference between Chol and
258 phospholipid included the head region: the phospholipid with negatively charged head
259 region and Chol with neutral head region. This result further indicated that
260 hydrophobic interactions between tetracaine hydrochloride and lipid dominate the
261 membrane energy in this case. However, the significant effect of Chol on the FL
262 values could be found in Table 2. The FL response at the same concentration
263 decreased significantly with the increase of the Chol ratio in the lipid constituents.
264 This might result from the fact that Chol has less fluidity than DMPC at room
265 temperature, leading to the less distortion of the conjugation plane of PDA. Therefore,
266 it could be concluded that Chol had less effect on the interaction of target molecules
267 with lipid/PDA vesicles, but weakened the sensitivity of this PDA-based fluorescent
268 platform. This case also indicated that the log (EC50), rather than the FL value,
269 represented the lipid membrane affinity because the PDA sensitivity would be

affected by the lipid constituents.

In summary, the influence of lipid constituents on the membrane affinity can be assessed under the established PDA-based fluorescent platform.

4. Conclusion

This study presents a PDA-based fluorescence assay for the measurement of lipid membrane affinity in drug discovery. As the fluorescence response might be affected by the other properties of test compounds besides membrane affinity or the lipid constituents in the lipid/PDA vesicles, the half maximal fluorescence response concentration (EC50) was introduced for the evaluation of membrane affinity. The log (EC50) values of local anesthetics and flavonoids correlated well with other lipid membrane affinity constants. The lipid membrane affinity of tetracaine hydrochloride increased as the increase of buffer pH value and the electrostatic interaction had less effect on this change. The ratio of Chol in the lipid constituents also less affected the affinity of tetracaine hydrochloride. Therefore, the hydrophobic interaction was assumed to play the most important role in the interaction between tetracaine hydrochloride and the lipid membrane. This work provides a simple, sensitive and reproducible PDA-based fluorescent method for the rapid measurement of lipid membrane affinity and showed its wide application to the study of affinity influencing factors.

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Figure Caption

Fig. 1 Fluorescence emission spectra of PDA with tetracaine hydrochloride (A), PDA with quercetin (B), PDA (C), quercetin (D) and tetracaine hydrochloride (E) at the excitation wavelength of 485 nm. Tetracaine hydrochloride (3 mM) and quercetin (5 mM).

Fig. 2 Concentration-response curve and calculation of EC50 at pH7.6.
(A) Tetracaine hydrochloride; (B) Luteolin.

Fig. 3 Comparison of log (EC50) in the fluorescence DMPC/PDA vesicle system, solute partitioning into DMPC liposome (Km), solute partitioning with IAMC and solute partitioning with MEKC, and potency of anesthetic drugs.
(**A**) Correlation between log (EC50) and solute partitioning into DMPC liposome (Km); (**B**) Correlation between log (EC50) and log (K_{IAMC}); (**C**) Correlation between log (EC50) and log (K_{MEKC}); (**D**) Correlation between log (EC50) and potency of local anesthetic. Values were presented as Mean ± SD.

Fig. 4 The log (EC50) values of tetracaine hydrochloride at different pH value. Values were presented as Mean ± SD.

Fig. 5 Particle size of PDA with or without tetracaine hydrochloride and their differences. Values were presented as Mean ± SD.

Fig. 6 Zeta potential of the test solution with or without tetracaine hydrochloride and their differences. Values were presented as Mean ± SD.

Table 1 The log (EC50) values in the fluorescence vesicle system and comparison with the reported data

Compound	Log(EC50)(mean±SD)	Reference Kd	Free hydroxyl groups
Daidzein	0.2971±0.03	*	2
Apigenin	0.1419±0.02	*	3
Luteolin	-0.1461±0.05	7.1	4
Quercetin	-0.1841±0.06	7.5	5

*, not studied in the previous reference

382 **Table 2 The fluorescence response and log (EC50) values of tetracaine**
383 **hydrochloride for the influence of Chol**

Chol（%）	FL response(mean±SD)	log(EC50) (mean±SD)
0	500.18±1.40	0.2429±0.06
20	433.19±0.76	0.2686±0.03
40	204.17±1.46	0.2275±0.07

384

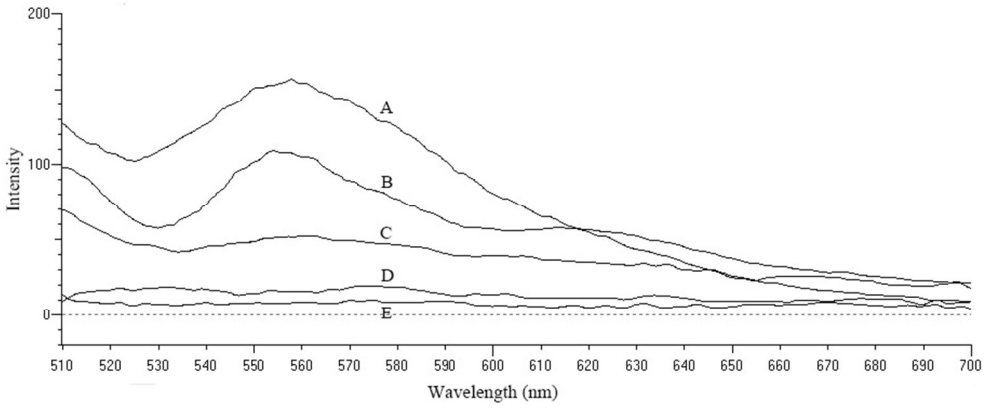

Fig. 1 Fluorescence emission spectra of PDA with tetracaine hydrochloride (A), PDA with quercetin (B), PDA (C), quercetin (D) and tetracaine hydrochloride (E) at the excitation wavelength of 485 nm. Tetracaine hydrochloride (3 mM) and quercetin (5 mM).
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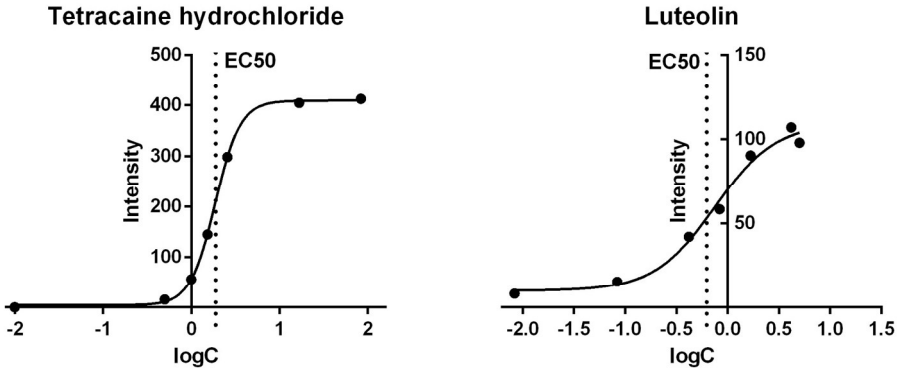

Fig. 2 Concentration-response curve and calculation of EC50 at pH7.6.
(A) Tetracaine hydrochloride; (B) Luteolin.

171x68mm (300 x 300 DPI)

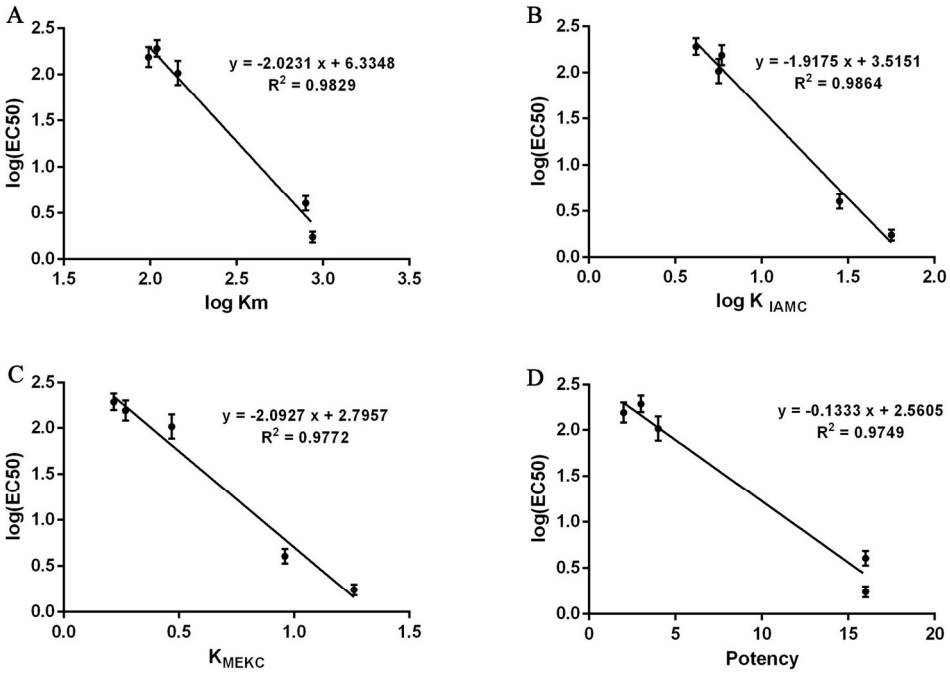

Fig. 3 Comparison of log (EC50) in the fluorescence DMPC/PDA vesicle system, solute partitioning into DMPC liposome (Km), solute partitioning with IAMC and solute partitioning with MEKC, and potency of anesthetic drugs.
(A) Correlation between log (EC50) and solute partitioning into DMPC liposome (Km); (B) Correlation between log (EC50) and log (KIAMC); (C) Correlation between log (EC50) and log (KMEKC); (D) Correlation between log (EC50) and potency of local anesthetic. Values were presented as Mean ± SD.
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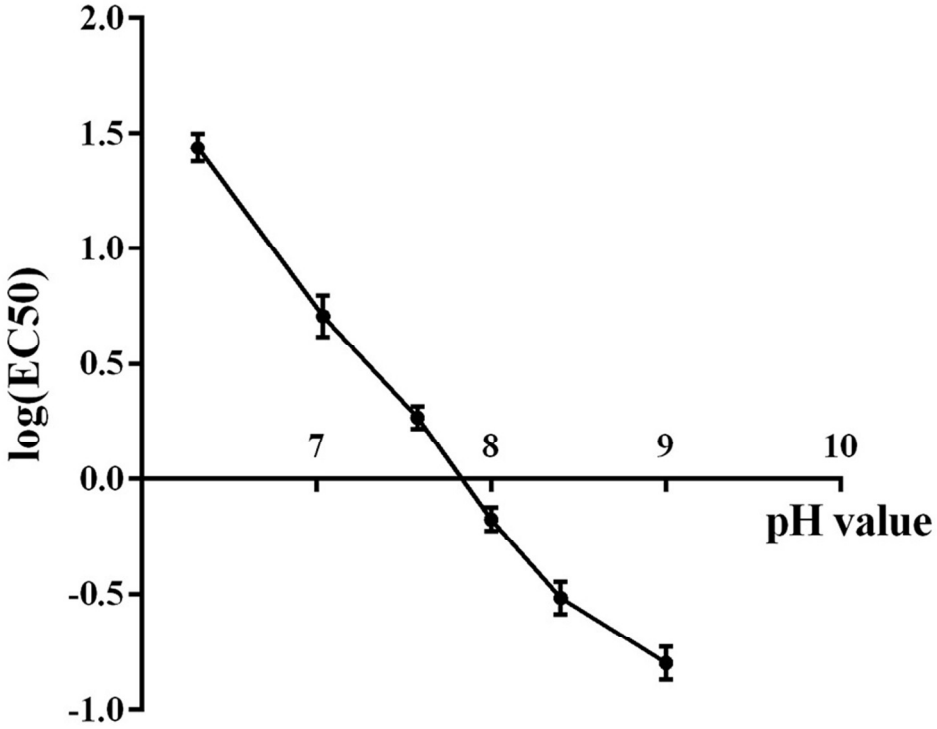

Fig. 4 The log (EC50) values of tetracaine hydrochloride at different pH value. Values were presented as Mean ± SD.
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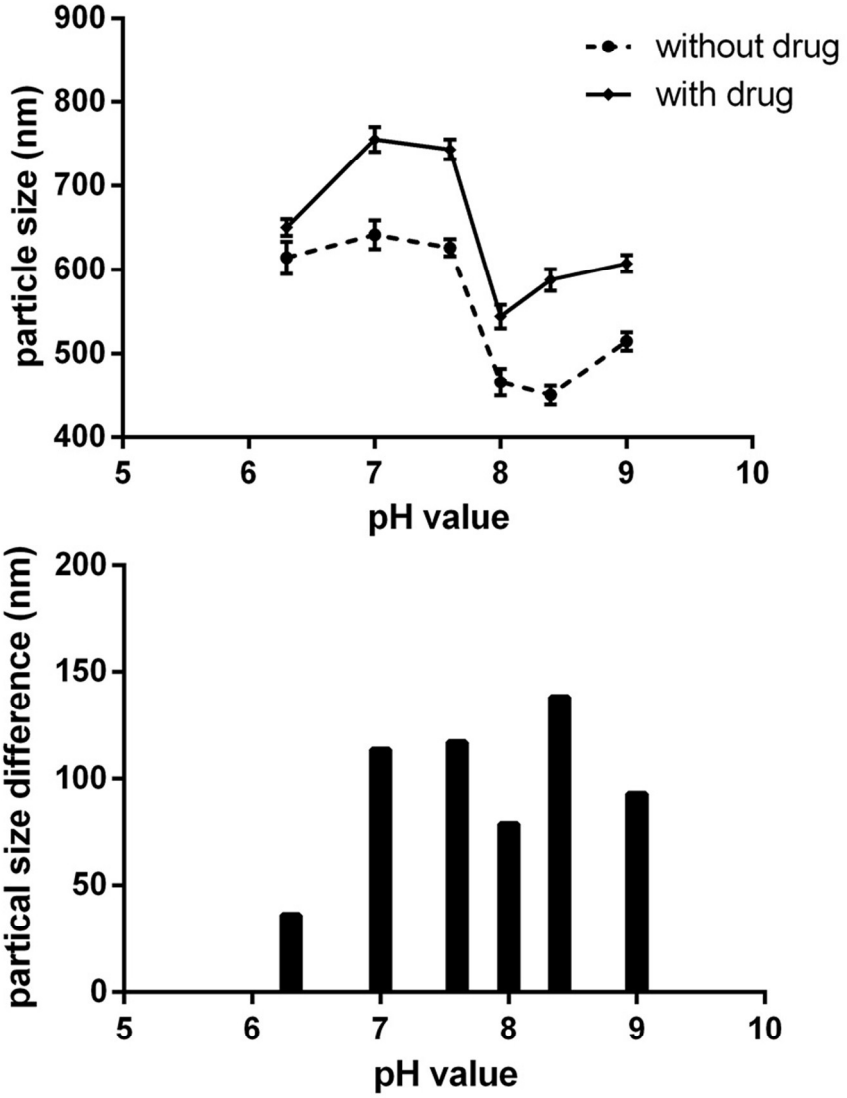

Fig. 5 Particle size of PDA with or without tetracaine hydrochloride and their differences. Values were presented as Mean ± SD.
82x106mm (300 x 300 DPI)

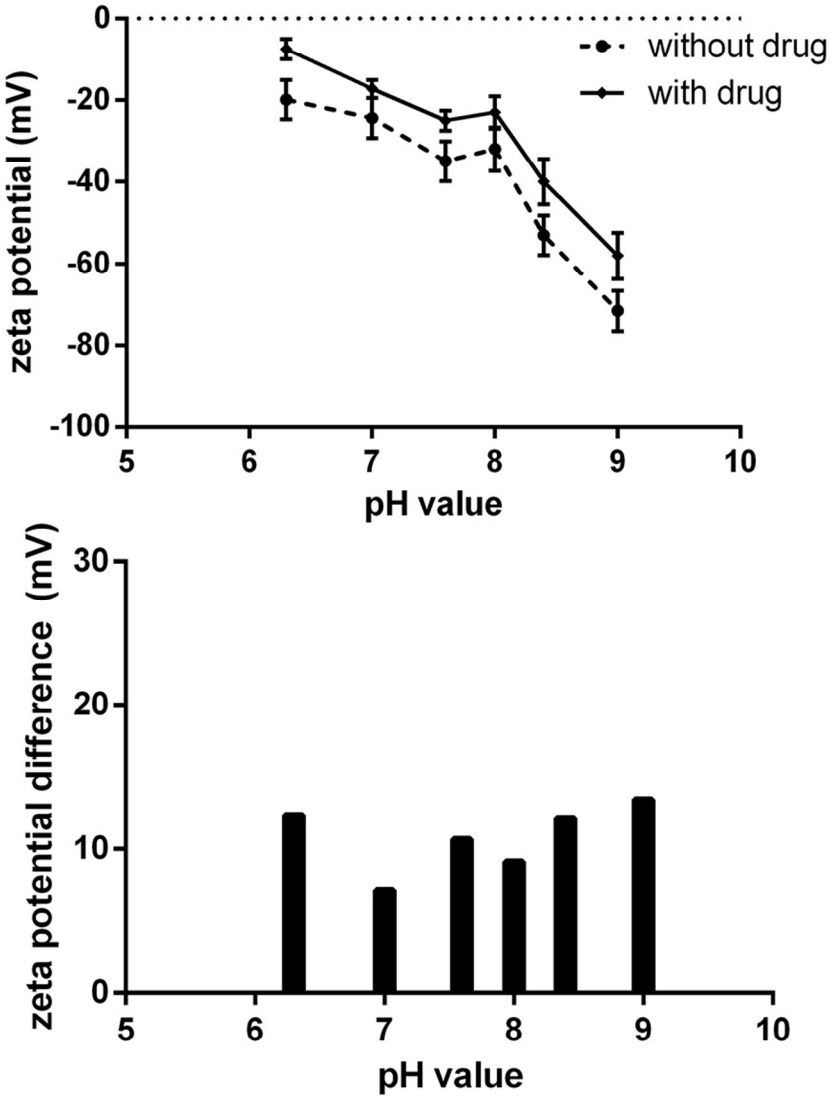

Fig. 6 Zeta potential of the test solution with or without tetracaine hydrochloride and their differences. Values were presented as Mean ± SD.
82x106mm (300 x 300 DPI)