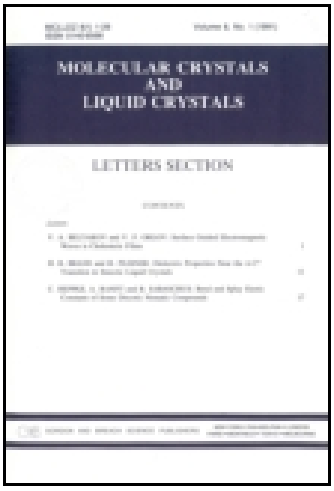


Molecular Crystals and Liquid Crystals
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gmcl20

Double Hydrophilic Block Copolymers for Doxorubicin Delivery

Larisa Kunitskaya [a] , Tatyana Zheltonozhskaya [a] , Natalia Permyakova [a] & Natalia Kobylinska [b]

[a] Taras Shevchenko National University of Kiev, Faculty of Chemistry, Macromolecular Chemistry Department , 60 Vladimirskaya Str, 01033 , Kiev , Ukraine

[b] Taras Shevchenko National University of Kiev, Faculty of Chemistry, Analytical Chemistry Department , 60 Vladimirskaya Str, 01033 , Kiev , Ukraine

Published online: 28 Mar 2014.

To cite this article: Larisa Kunitskaya , Tatyana Zheltonozhskaya , Natalia Permyakova & Natalia Kobylinska (2014) Double Hydrophilic Block Copolymers for Doxorubicin Delivery, Molecular Crystals and Liquid Crystals, 590:1, 164-171, DOI: 10.1080/15421406.2013.874179

To link to this article: http://dx.doi.org/10.1080/15421406.2013.874179

Mol. Cryst. Liq. Cryst., Vol. 590: pp. 164–171, 2014

ISSN: 1542-1406 print/1563-5287 online
DOI: 10.1080/15421406.2013.874179

Double Hydrophilic Block Copolymers for Doxorubicin Delivery

LARISA KUNITSKAYA,[1,*] TATYANA ZHELTONOZHSKAYA,[1] NATALIA PERMYAKOVA,[1] AND NATALIA KOBYLINSKA[2]

[1]Taras Shevchenko National University of Kiev, Faculty of Chemistry, Macromolecular Chemistry Department, 60 Vladimirskaya Str, 01033, Kiev, Ukraine
[2]Taras Shevchenko National University of Kiev, Faculty of Chemistry, Analytical Chemistry Department, 60 Vladimirskaya Str, 01033, Kiev, Ukraine

The anionic derivative of asymmetric triblock copolymer containing biocompatible chemically complementary polyacrylamide and poly(ethylene oxide) (PAAm-b-PEO-b-PAAm) was obtained. The micellization of the initial (TBC) and modified (TBC-COOH) copolymer samples in aqueous solution were investigated. The similar values of the critical micellization concentration and the Gibbs free micellization energy for initial and modified TBCs were found. The anticancer effects of the doxorubicin (DOX)-loaded micelles of the initial and modified triblock copolymers were studied. The fact of higher efficacy of DOX/TBC and DOX/TBC-COOH compositions in compare with pure DOX was determined and discussed.

Keywords Amphiphilic block copolymers; doxorubicin; micellization; poly (ethylene oxide); polyacrylamide

1. Introduction

Amphiphilic block copolymers are widely studied for pharmaceutical application. Polymeric micelles self-assembled from amphiphilic block copolymers have been intensively investigated as nano-carrier systems for tumor-targeted drug delivery. Numerous attempts have been made to improve the therapeutic index of toxic and poorly soluble drugs by modifying its mode of delivery [1–3]. The continuous development of new drug carriers is driven by the need to maximize therapeutic activity while minimizing negative side effects. It was shown earlier [4, 5] that asymmetric diblock (DBC) and triblock (TBC) copolymers contained biocompatible chemically complementary polyacrylamide and poly(ethylene oxide) (PAAm-*b*-PEO-*b*-PAAm) or its monomethyl ether (MOPEO-*b*-PAAm) formed special micellar structures in aqueous solutions

Such structures contain hydrophobic "core" formed by hydrogen-bonded PEO and PAAm chains and hydrophilic "corona" of the surplus segments of PAAm. Essential influence of the anticancer agent doxorubicin (DOX) on the micellization process due to the interaction between DOX and copolymer micelles was established. This opened

*Address correspondence to Larisa Kunitskaya. Tel.: +380442393390; fax: +380442393100. E-mail: larisa_kunitskaya@ukr.net

new prospects for using such copolymers as nanocontainers for DOX and other toxic drugs.

The present study examines the possible use of anionic derivatives of PAAm-*b*-PEO-*b*-PAAm as potential carries for DOX. Major attention is paid to the micelle formation of modified TBC in aqueous solutions and *in vitro* study of antitumor effect of the DOX-loaded modified TBC micelles as compared to the initial one and free DOX.

2. Experimental

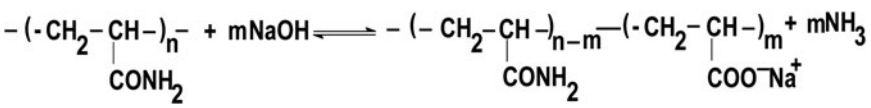
*2.1. Synthesis and Characterization of Triblock Copolymer PAAm-*b*-PEO-*b*-PAAm*

The triblock copolymer was synthesized by the radical block copolymerization of acrylamide (AAm) from "Merck" (Germany) and PEG (Mn = 6 kDa) from Aldrich (USA) initiated by Ce^{IV} ions according to the method described earlier [4, 5]. The initial AAm had been twice re-crystallized from chlorophorm before a synthesis. The reaction blend was mixed in the deionized water and an inert atmosphere at 20°C during 24 h. Homopolymerization of AAm was carried out in the same conditions at the presence of ethanol as a source of the terminal hydroxyl groups. The synthesized samples of TBC and PAAm were re-precipitated by acetone, then dissolved in deionized water and freeze dried.

The molecular weights of PAAm blocks and TBC macromolecules (M_{nPAAm} = 117 kDa, M_{nTBC} = 240 kDa) were determined from ^1H NMR spectra recorded in D_2O at C = 1 kg·m^{-3} and a room temperature using a Varian Mercury-400 spectrometer operating at 400 MHz. The structure of TBC was conformed by FTIR spectrum measured by a Nexus-470 Nicolet (USA) spectrometer with a resolution 4 cm^{-1}.

*2.2. PAAm-*b*-PEO-*b*-PAAm Alkaline Hydrolysis*

The TBC and PAAm alkaline hydrolysis reaction was performed in accordance with the Scheme 1:



Scheme 1. The hydrolysis reaction for studied materials.

Aqueous solutions of TBC and individual PAAm (M_v = 3500 kDa) with C = 10 kg·m^{-3} were stirred at the presence of NaOH (C_{NaOH} = 5 mol·l^{-1}) at T = 50°C. Sample splitting was realized via 10, 60, 75, 240 and 480 minutes. The samples of anionic derivatives were modified into H-form by the acidification up to pH ~ 2. Gel-like TBCs and PAAm were re-precipitated by acetone, dissolved in water and freeze-dried. Kinetic investigations of the alkaline hydrolysis process were performed by potentiometric titration. The initial and modified TBC and PAAm samples were titrated as compared to H_2O using 2N NaOH.

2.3. Static Light Scattering Measurements

The critical micellization concentration (CMC) of TBCs was determined by the static light scattering (SLS) using a modernized light scattering instrument FRS-3 (Russia) contained WP7113VGC/A light-emitting diode (λ = 520 nm) from "Kingbright," ADC-CPUTM

Table 1. – Parameters of TBC and PAAm alkaline hydrolysis

Copolymer	t, [1] min	$\sigma_{lim}\cdot10^3$, [2] g-ekv·g-1	A, [3] %	$V_h\cdot10^4$, [4] s-1
TBC-COOH	0	0.18	1.4	1.6
	10	1.51	10.7	
	60	2.85	20.3	
	75	3.15	22.4	
	240	4.82	34.4	
	480	5.39	38.5	
PAAm	10	0.29	10.2	0.4
	240	0.66	23.4	

[1] The hydrolysis time. [2] The limit value of hydroxyl ion absorption. [3] The hydrolysis degree.
[4] The initial rate of hydrolysis.

controller from "Insoftus" (Ukraine) and the computer program WINRECODER. In order to define CMCs, the scattering intensities of the vertically polarized light were measured at the $\theta = 90^\circ$ scattering angle and T = 20°C.

2.4. Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectra of TBC and PAAm were recorded using a Nexus-470 Nicolet (USA) spectrometer with a resolution 4 cm^{-1}. All the spectra were recalculated in the dependences of the optical density (D) versus the wavenumber (ν) using the relation: $D = \log T_0/T$, where T_0 and T are the maximum and current values of the transmittance in a certain spectrum.

3. Results and Discussion

3.1. Alkaline Hydrolysis Reaction Peculiarities

The results of TBC hydrolysis as compare to PAAm is shown in Table 1.

It is seen that the alkaline hydrolysis process developed with higher rate in TBC than in pure PAAm.

Although several modified TBC samples were obtained just one TBC-COOH sample belonging to 10 min of hydrolysis time was chosen for our further experiments and tested by potentiometric titration. The titration curve for the modified TBC was used to calculate the hydroxyl ion absorption [6]. As it follows from Figure 1, the TBC-COOH absorption curve has S-shaped character. The limit value of the hydroxyl ion absorption (σ_{lim}) corresponded to the quantity of carboxyl groups in the polymer sample, which was achieved at pH = 8.5–9.0.

On the basis of Figure 2a, the hydrolysis degree was calculated according to the following equation [6]:

$$A = \frac{\sigma_{lim}\cdot 10^{-3}}{\frac{w'_{PAAm}}{71} + \sigma_{lim}\cdot 10^{-3}}\cdot 100\% \tag{3.1}$$

where w'_{PAAm} is the weight fraction of PAAm chains in the modified TBC-COOH sample. The dependence of negative logarithm of the apparent dissociation constant (pK) versus

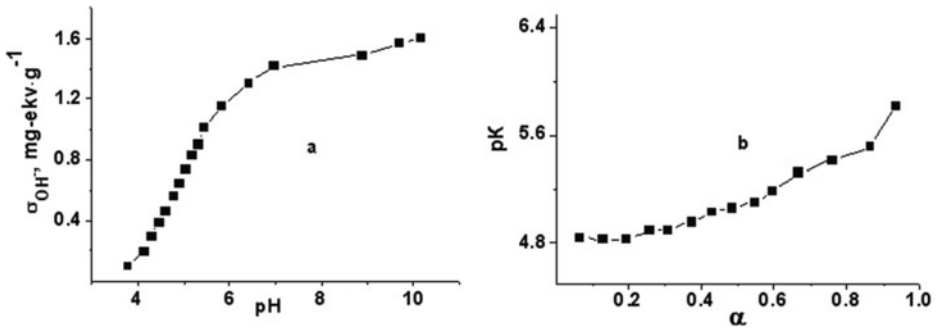


Figure 1. Curves of (a) -OH ion absorbtion and (b) pK dependence from the ionization degree of −COOH groups in TBC-COOH (A = 10,7%). $C_{TBC-COOH} = 2\ kg{\cdot}m^{-3}$; T = 25°C.

the dissociation degree (α) was calculated from Figure 2 by an ordinary relation [6]:

$$pK = pH - \lg \frac{\alpha}{1-\alpha} \tag{3.2}$$

Here $\alpha = \sigma_c/\sigma_{lim}$, where σc is the current hydroxyl ion absorption at a certain pH. As we can see from Figure 2b, TBC-COOH demonstrates the properties of a weak polyelectrolyte without any conformation transition during its ionization.

3.2. Behavior of TBC-COOH in Aqueous Solution

In the movement of using the initial and modified TBCs as nanocarriers for target delivery of toxic and poorly soluble drugs, it was important to examine the micelle formation of mentioned block copolymers in water solution. As it known, micellization occurs in dilute solutions of block copolymers at a fixed temperature starting from some concentration called the critical micellization concentration (CMC) [7]. The CMCs of TBC and TBC-COOH were determined by SLS. Using CMC and thermodynamic theory of micellization

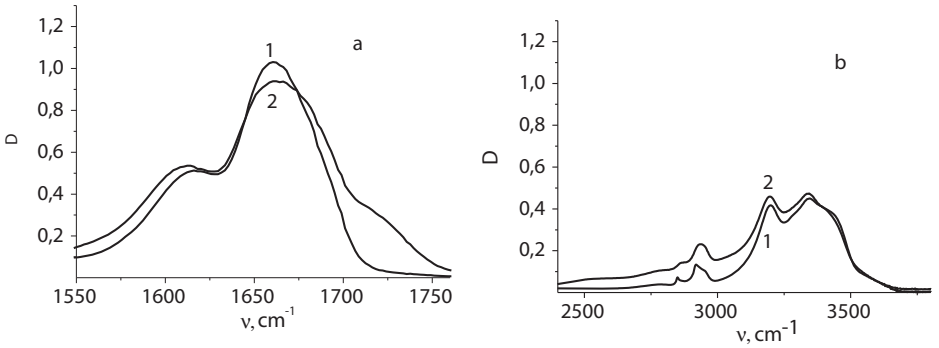


Figure 2. Reduced IR spectra of PAA (*1*) and TBC-COOH (*2*) films in the Amide I and Amide II regions (*a*) and in the region of C-H, N-H, O-H vibration bands (*b*).

Table 2. – Micelle formation characteristics

Copolymer	CMC· 10^8, [1] mol· dm^{-3}	$-\Delta G^\circ$,[2] kJ·mol^{-1}
TBC	37.8	36.15
TBC-COOH	39.8	36.14

[1] The critical micellization concentration. [2] The Gibbs free micellization energy.

the Gibbs free micellization energy was calculated as [7]:

$$\Delta G^o \approx RT \cdot \ln CMC \tag{3.3}$$

Both the parameters are submitted in Table 2 and show practically the same CMC and -ΔG° values for initial and modified TBC samples. Thereby, such insignificant substitution of amide groups with carboxyl ones in PAAm blocks of TBC does not lead to additional micelle stabilization in aqueous medium.

3.3. The Hydrogen Bond System in Hydrolyzed Triblock Copolymer

Detailed studies of the H-bonds structure in the PAAm-*b*-PEO-*b*-PAAm triblock copolymers films have been described earlier [8]. The changes in the hydrogen bond system in TBC-COOH sample, which are conditioned by the presence in the copolymer molecular structure of some –COOH groups, were established by FTIR spectroscopy. Two of the most important regions of FTIR spectra of PAA and TBC-COOH are shown in Figure 2.

The region of $\nu_{C=O}$ vibrations and Amide II (Fig. 2**a**) contained many overlapped vibration bands, corresponded to free and hydrogen-bonded amide and carboxyl groups. Thereby, the computer processing of this region (1570–1800 cm^{-1}) was carried out taking into consideration the established earlier hydrogen bond structures with participation of amide groups [9] and also the appearance in TBC-COOH spectrum (Fig. 2**a**, spectrum 1) a new $\nu_{C=O}$ vibration band of –COOH groups (∼1720 cm^{-1}). The results of computer processing carried out with WINSPECTRUM program are presented in Figure 3 and Table 3.

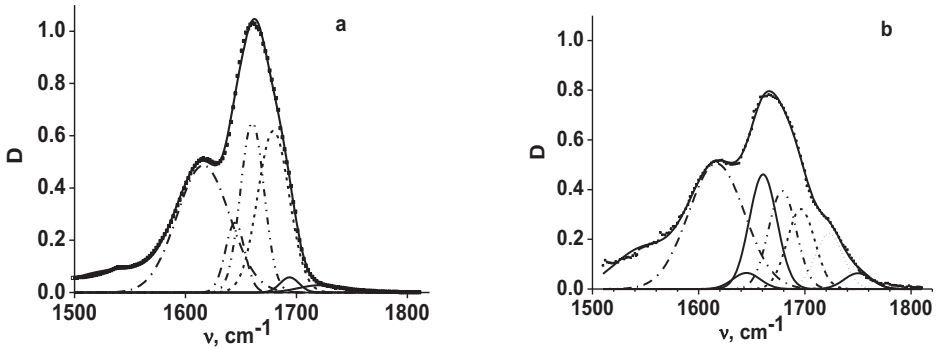


Figure 3. Result of computer processing of the IR spectra of PAA (*a*) and TBC-COOH (*b*) in the Amide I and Amide II regions.

Table 3. – Parameters of the Amide I and Amide II absorption bands for PAA and TBC-COOH

	ν, [1] cm^{-1}		$\Delta\nu_{1/2}$, cm^{-1}		A_i, [2] cm^{-2}		δ_A, [3] cm^{-2}	
Vibration mode	PAA	TBC-COOH	PAA	TBC-COOH	PAA	TBC-COOH	PAA	TBC-COOH
$\nu_{C=O}$	—	1750	—	30,61	—	2,07	—	0,12
(−COOH)	1721	1723	45,35	30,61	1,35	7,29	0,15	0,14
$\nu_{C=O}$	1704	—	0	—	0	—	0	—
(Amide I)	1694	1696	20,02	30,61	1,23	10,48	0,15	0,18
	1679	1679	33,24	30,61	21,9	12,86	0,16	0,24
	1660	1661	24,85	30,61	17,2	15,03	0,12	0,26
δ_{O-H} (H_2O)	1645	1645	20,46	30,61	5,82	2,11	0,09	0,22
$\delta_{N-H}+\nu_{C-N}$ (Amide II)	1616	1617	55,88	62,09	28,9	33,36	0,23	0,20

[1] The band location; [2] The integral intensities; [3] The standard deviation

Using integral intensities (A_i) of $\nu_{C=O}$ vibration bands, we calculated the contributions (α_i) of these bands in the total intensity of $\nu_{C=O}$ vibrations and the effective length (β) of trans-multimers of amide groups according to the equations [8]:

$$\alpha_i = A_i / \sum_i A_i \tag{3.4}$$

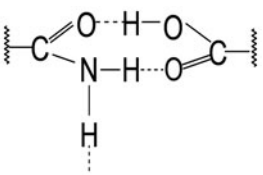
$$\beta = A_{1679}/A_{1694} \tag{3.5}$$

These results are presented in Table 4. It is seen that the appearance in TBC macromolecules of some −COOH groups resulted to decrease in the quantity of *cis-trans*-multimers of amide groups (the band at 1660 cm^{-1}), increase in the quantity of *trans*-multimers of amide groups (the band ∼1694 cm^{-1}) but with essential reduction in their effective length (the value β). Moreover, significant growth of the 1722 cm^{-1} band intensity for the hydrogen-bonded

Table 4. – Reduced apparent integral vibration coefficients of the $\nu_{C=O}$ absorption band and the effective length of *trans*-multimers of amide groups for PAA and TBC-COOH

	α_i [1]						
Polymer	∼1660 cm^{-1}	∼1679 cm^{-1}	∼1694 cm^{-1}	∼1704 cm^{-1}	∼1722 cm^{-1}	∼1747 cm^{-1}	β [2]
PAAm	0.41	0.53	0.03	0	0.03	0	17.8
TBC-COOH	0.31	0.27	0.22	0	0.15	0.04	1.23

[1] The contribution of C=O oscillation intensities of the carbonyl groups. [2] Effective length of the amide trans multimers



Scheme 2. Mixed cyclic dimers of carboxyl and amide groups.

−COOH groups and the displaying small quantity of free carboxylic groups (the band ∼1747 cm^{-1}) was observed.

The appearance of intense $\nu_{C=O}$ vibration band at 1722 cm^{-1} together with characteristic wide $\nu_{O\text{-}H}$ vibration band in the region of 2200–2700 cm^{-1} pointed out the participation of –COOH groups in the following hydrogen bond structure named as the mixed cyclic dimers of carboxyl and amide groups (Scheme 2).

Such conclusion confirms also well-known literature data about complex formation between low-molecular-weight primary amides and carboxyl acids [10]. Thus, the substitution of amide groups by some carboxyl ones intensifies the complex formation between modified and non-modified units and facilitates to form more compact macromolecular structure.

3.4. Anticancer Activity of DOX Encapsulated by TBC and TBC-COOH

In order to estimate the binding ability of the initial and modified TBCs in respect of toxic hydrophobic drugs, the anticancer activity one of the most effective antitumor agent DOX was studied in the presence of the mentioned block copolymers. DOX molecule (Figure 4) has sufficiently developed hydrophobic part and also active groups, which can interact with TBC macromolecules. It was reasonable to suppose that –OH groups of DOX are able of hydrogen-bonding interaction with ether groups of PEO block and amide groups of PAAm blocks of TBC. We also tended to improve encapsulation of DOX in result of strong electrostatic interactions between amine and carboxyl groups via introduction of COOH-groups into TBC macromolecule through the alkaline hydrolysis of PAAm blocks.

The anticancer activity of DOX encapsulated by micelles of the initial and modified TBCs was investigated *in vitro* against three types of leukemia cells. The obtained results suggest that DOX/TBC-COOH composition significantly enhance the antitumor effect of

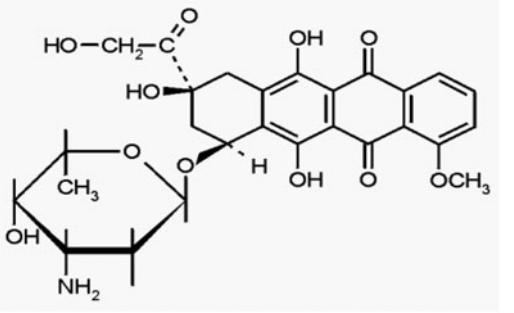


Figure 4. Molecular structure of doxorubicin.

doxorubicin in the range of concentrations 0.10–1.00 $\mu g \cdot ml^{-1}$ especially through 48 h. The anticancer activity of this composition is higher than that of DOX/TBC composition and pure DOX practically in all cultural mediums under study. It is connected with increase in the binding capability of the modified P(AAm-*co*-AAc) blocks of TBC with respect to DOX molecules due to strong electrostatic interactions of amine and carboxylic groups.

4. Conclusion

The anionic derivative of PAAm-*b*-PEO-*b*-PAAm triblock copolymer with relatively small hydrolysis degree forms micellar structures because of intramolecular complex formation of P(AAm-*co*-AAc) and PEO blocks. They demonstrate the similar stability in aqueous medium as the micelles of non-modified TBC that confirmed by close CMC and $-\Delta G^{\circ}$ values.

The partial TBC hydrolysis decreases the quantity of cis-trans-multimers of amide groups, increases the quantity of trans-multimers of amide groups with essential reduction in their effective length and intensifies the complex formation between modified and non-modified TBC units.

The composition DOX/TBC-COOH expresses essentially stronger anticancer activity than DOX/TBC composition and pure DOX against all the tumor lines tested. At the same time, the DOX/TBC composition turns out to be more effective than pure DOX at the long-term incubation (48 h). Optimization of doses and study of the mechanism of DOX/TBC-COOH action is under way.

References

[1] Chan, Y., Wong, T., Byrne, F., Kavalaris, V., & Bulmus, V. (2008). *Biomacromolecules*, *9*, 1826.
[2] Oh, K. T., & Lee, E. S. (2008). *Polymers for Advanced Technologies*, *19*, 1907.
[3] Wang, Y., Bansal, V., Zelikin, A., & Caruso, F. (2008). *Nano Letter*, *8*, 1741.
[4] Kunitskaya, L. R., *et al*. (2011). *Mol. Cryst. Liq. Cryst*., *536*, 398.
[5] Zheltonozhskaya, T. B., *et al*. (2011). *Mol. Cryst. Liq. Cryst*., *536*, 390.
[6] Zheltonozhskaya T. (1981). *Vysokomolekuliarnyje Soedineniya*, *23*, 2425.
[7] Riess, G. (2005). *Prog. Polym. Sci*., *28*, 1107.
[8] Ahlberg, G., *et al*. (1969). *The Theory of Splines and Their Application*, Academic Press: USA.
[9] Permyakova, N. M., Zheltonozhskaya, T. B., Fedorchuk, S. V., Zagdanskaya, N. E., & Syromyatnikov, V. G. (2007). *Mol. Cryst. Liq. Cryst*., *468*, 53.
[10] Bellamy, L. J. (1975). *The Infra-red Spectra of Complex Molecules*, Chapman and Hall: London.