PROCEEDINGS OF THE INTERNATIONAL CONFERENCE NANOMATERIALS: APPLICATIONS AND PROPERTIES
Vol. **1** No 2, 02NNBM10(3pp) (2012)

Anionic Micelle-Forming Triblock Copolymers as Nanocontainers for Doxorubicin

L.R. Kunitskaya[1,*], T.B. Zheltonozhskaya[1,†], N.M. Permyakova[1],
R.S. Stoika[2], N.M. Boiko[2], Ye.A. Shlyakhtina[2]

[1] *Kiev National Taras Shevchenko University, 60 Vladimirskaya St, 01033 Kiev, Ukraine*
[2] *Institute of Cell Biology, NAS Ukraine, 14/15 Dragomanova St., 79005 Lvov, Ukraine*

(Received 31 May 2012; revised manuscript received 04 July 2012; published online13 July 2012)

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. Practically the same values of the critical micellization concentration and the Gibbs free micellization energy for both TBCs were found. The anticancer effects of the doxorubicin (DOX)-loaded micelles of the initial and modified triblock copolymers were studied on the tumor cells of human T-leukemia of Jurkat line, transformed leukemia of L929 line and mouse lymphatic leukemia of L1210 line. The fact of high efficacy of DOX/TBC and DOX/TBC-COOH compositions in compare with pure DOX was determined and discussed.

Keywords: Amphiphilic Block Copolymers, Drug, Micellization, Poly(Ethylene Oxide), Polyacrylamide.

PACS number: 62.23.St

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 of toxic and poorly soluble drugs to achieve improved cancer chemotherapy[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 (Figure 1).

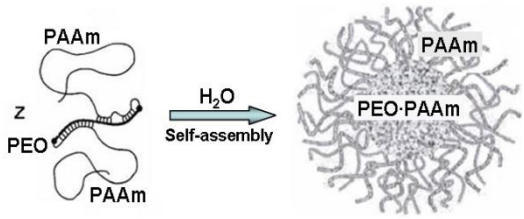


Fig. 1 – Scheme of micelle formation

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

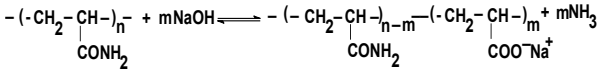
The present study examines the possible use of anionic derivatives of PAAm-*b*-PEO-*b*-PAAm as potential carries for DOX. Major attention is paid to the micelle formation of modified TBC in aqueous solutions and *in vitro* study of antitumor effect of the DOX-loaded modified TBC micelles as compared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

2. EXPERIMENTAL PART

2.1 Materials and Methods

The triblock copolymer was synthesized by the radical block copolymerization of PAAm with PEG initiated by Ce^{IV} ions according to the method described earlier [5]. The sample of PEG with $M_n = 6$ kDa and cerium ammonium nitrate (initiator) from Aldrich (USA) were used for this purpose. The molecular weights of PAAm blocks and TBC macromolecules ($M_{nPAAm} = 117$ kDa, $M_{nTBC} = 240$ kDa) were determined from ^{1}H 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}.

The alkaline hydrolysis reaction was performed in accordance with the following scheme:



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 $P_H \sim 2$. Gel-like TBCs 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 samples were titrated as compared to H_2O using 2N NaOH.

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

* larisa_kunitskaya@ukr.net
† zheltonozhskaya@ukr.net

WINRECODER. In order to define CMCs, the scattering intensities of the vertically polarized light were measured at the $\theta = 90°$ scattering angle and $T = 20°C$.

Biological studies *in vitro* were performed using three cellular models: murine leukemia of L1210 line, transformed leukemia of L929 line and acute T-cell human leukemia of Jukat line. The L1210 and L929 cells were incubated in DMEM medium, Jurkat cells in RPMI medium, in the presence of 10 % bovine serum. Incubation was performed in 96-hole plates ("Falcon", USA) in the CO_2 incubator ("JENCONS NUAIRE", UK).

To compare the action of free DOX, DOX/nanocarrier complex and free nanocarrier, the concentrations of compounds were calculated according to the concentration of DOX. The half-lethal dose (LD_{50}) of DOX for mentioned above cell lines is 1 μg/cm^3. The effectiveness of the investigated substances was determined according to the number of alive cells relative to their number in control (the number of cells in culture medium without addition of the drug). The counting of cell number was carried out in hemocytometer chamber in 24 and 48 hours after incubation with drugs. Trypan blue was used for counting dead cells.

3. RESULTS AND DISCUSSION

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

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.

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

The only modified TBC sample (TBC-COOH) 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 [7 8]. As it follows from Figure 2, 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.

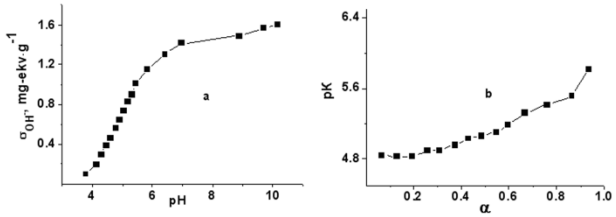


Fig. 2 – Curves of (a) OH^- ion absorbtion and (b) pK dependence from the ionization degree of –COOH groups in TBC-COOH (A = 10,7 %). $C_{TBC-COOH}$ = 2 kg·m^{-3}; T = 25°C

On the basis of Figure 2 a, the hydrolysis degree was calculated according to the (3.1) equation:

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

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 the dissociation degree (α) was calculated from Figure 2 a by an ordinary relation (3.2):

$$pK = pH - \lg\frac{\alpha}{1-\alpha} \qquad (3.2)$$

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

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. Micellization occurs in dilute solutions of block copolymers at a fixed temperature starting from some concentration called the critical micellization concentration (CMC) [5]. The CMCs of TBC and TBC-COOH were determined by SLS. Using CMC and thermodynamic theory of micellization the Gibbs free micellization energy was calculated as:

$$\Delta G^o \approx RT\cdot\ln CMC\,, \qquad (3.3)$$

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.

Table 2 – Micelle formation characteristics

Copolymer	CMC· 108, [1] mol· dm^{-3}	-ΔG°, [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

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 3) has sufficiently developed hydrophobic part and also active hydroxyl-, ether-, carbonyl- and amine groups. It is 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. Moreover, we also relied to improve encapsulation of DOX in result of strong electrostatic interactions between amine and carboxyl groups via introduction of COOH-groups into TBC macromolecule by means of alkaline hydrolysis of PAAm blocks.

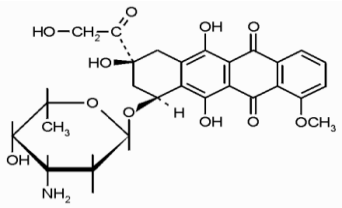


Fig. 3 – Molecular structure of doxorubicin

The anticancer activity of DOX encapsulated by micelles of the initial and modified TBCs was investigated *in vitro* against murine leukemia of L1210 line, transformed leukemia of L929 line and acute T-cell human leukemia of Jukat line (Figure 4-6).

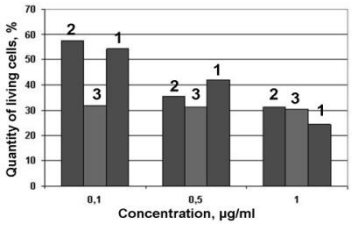


Fig. 4 – Dynamics of living cells of T-cell human leukemia in the presence of DOX-loaded TBC –*2* and TBC-COOH –*3* micelles compared to initial DOX –*1*. The incubation time is 24 h

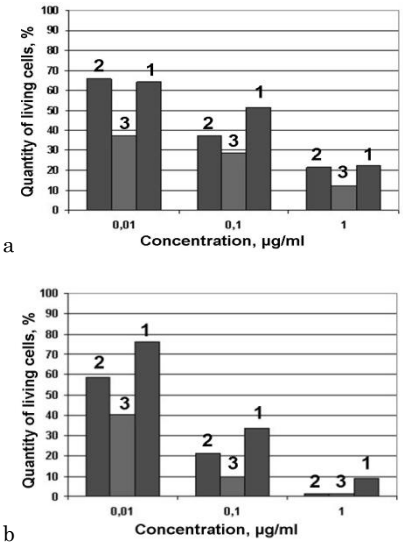


Fig. 5 – Dynamics of living cells of mouse lymphatic leukemia in the presence of DOX-loaded TBC – 2 and TBC-COOH – *3* micelles as compared to pure DOX – *1*. The incubation time is 24 h (a) and 48 h (b)

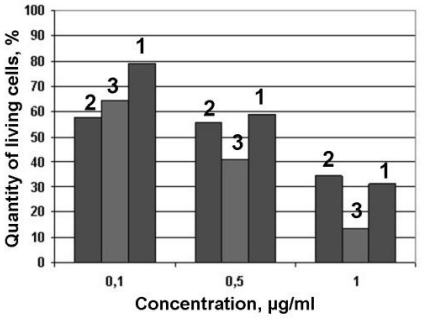


Fig. 6 – Dynamics of living cells of transformed leukemia in the presence of DOX-loaded TBC – *2* and TBC-COOH – *3* micelles unlike to pure DOX – *1*. The incubation time is 24 h

The obtained results suggest that DOX/TBC-COOH composition significantly enhance the antitumor effect of doxorubicin in the range of concentrations 0.10-1.00 μg·ml^{-1} especially through 48 h (the example is shown in Figure 5 b). 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 are characterized practically the same stability in aqueous medium as the micelles of non-modified TBC sample that was confirmed by close CMC and $-\Delta G°$ values.

The composition DOX/TBC-COOH express 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. However, the experimental results can be useful for further development of efficient nanovehicles for delivery of cytotoxic drugs.

REFERENCES

1. Y. Chan, T. Wong, F. Byrne, M. Kavalaris, V. Bulmus, *Biomacromolecules* **9**,1826 (2008)
2. K.T. Oh, E. S. Lee, *Polym Adv. Tech.* **19**, 1907 (2008).
3. Y. Wang, V. Bansal, A. Zelikin, F. Caruso, *Nano Letter* **8**, 1741 (2008)
4. L.R. Kunitskaya, et al. *Mol. Cryst. Liq. Cryst.* **536**, 398 (2011)
5. T.B. Zheltonozhskaya at al. *Mol. Cryst. Liq. Cryst.* **536**, 390 (2011)
6. G. Riess, *Prog. Polym. Sci.* **28**, 1107 (2005).
7. T. Zheltonozhskaya, *Vysokomol. Soed.* **23**, 2425 (1981).