CHAPTER 5

Synthesis and Study of the Self-assembly of Novel Poly(D,L-Lactide-*co*-Glycolide)-*b*-Poly(*N*-Vinylpyrrolidone) (PLGA-*b*-PNVP) Amphiphilic Diblock Copolymers: Its Antitumor Activity

5.1 Introduction

Polymeric biomaterials are one of the hot and emerging research areas in biomedical science. Among them much progress has been made on polylactic acid (PLA), polyglycolic acid (PGA) and their copolymers poly(D,L-lactide-*co*-glycolide) (PLGA) as both polymers are biocompatible, and biodegradable and moreover they produce non-toxic by-products (i.e. lactic acid and glycolic acid) of metabolic reactions in the body *via* the hydrolysis of ester bonds. [Astente *et al*. (2005), Albertsson *et al*. (2003), Mohamed *et al*. (2008), Middleton *et al*. (2000)] These hydrophobic homo- and copolymers in combination with a hydrophilic polymer are initially used as carriers for the delivery of anticancer and antimalarial drug in controlled manner. [Surolia *et al*. (2012), Dinarvand *et al*. (2011), Kumari *et al*. (2010)] Removal of the coating of hydrophilic polymer from the surface of the drug-loaded hydrophobic polymers during transport leads to the unwanted non-cite-specific drug delivery. [Soppimath *et al*. (2001), Storm *et al*. (1995), Hans *et al*. (2002)] To avoid this problem, hydrophilic groups are covalently bonded with the hydrophobic group. [Ma *et al*. (2008), Prabaharan *et al*. (2009), Tao *et al*. (2012), Kim *et al*. (2005), Simone *et al*. (2007)] Polymeric micelles of amphiphilic block copolymers have gained much interest in biomedical research as drug carriers because of their enhanced solubility and sustained release of drug in to the targeted tissue or, cells. [Du *et al*. (2011), Du *et al*. (2012)] So far to our knowledge, amphiphilic block copolymers with PLGA as a hydrophobic and poly(ethylene oxide) (PEO) and polyethyleneimine (PEI) as hydrophilic polymers have been reported (**Chapter 1**, Literature Review section). [Nam *et.al* (2003), Qian *et al*. (2011), Lin *et al*. (2011), Freichels *et al*. (2012), Kun *et al*. (2014), Zhang *et al*. (2014), Chen *et al*. (2014)]

Among hydrophilic polymers, poly(*N*-vinyl pyrrolidone) (PNVP) can be considered as one of the promising polymers for the biological applications because of its unique physiochemical and biological properties like anti-biofouling, biocompatibility, complexation capability, cryo-protectivity, high water solubility, low toxicity, and lypoprotectivity etc. [Bian *et al*. (2004), Einaga *et al*. (2005), Debuigne *et al*. (2007), Nese *et al*.(2012)] Amphiphilic block copolymers containing a hydrophilic PNVP segment are very important from the biological point of view. [Hira *et al.* (2013), Vishwakarma *et al.* (2015)] Very recently we have reported the synthesis of well-defined amphiphilic poly(ε-caprolactone)-*b*-poly(*N*-vinylpyrrolidone) [Mishra *et al* (2011)] and used the same for controlled delivery of anticancer drug doxorubicin. [Hira *et al*. (2013)]

Since PLGA and PNVP both are biocompatible and the toxicity of PLGA [Astente *et al*. (2005), Albertsson *et al*. (2003), Mohamed *et al*. (2008), Middleton *et al*. (2000), Zhang *et al*. (2014)] and PNVP [Hira *et al.* (2013), Vishwakarma *et al.* (2015)] are low, so the cytotoxicity of PLGA-*b*-PNVP will expectedly be intermediate to its individual polymer blocks. Such PLGA-*b*-PNVP may be useful as nano-carrier for controlled delivery of drug. In such anticipation, here, we have reported the synthesis of PLGA-*b*-PNVP amphiphilic block copolymers *via* the combination of ring opening polymerization, xanthate-mediated reversible addition-fragmentation chain transfer (RAFT) polymerization and alkyne-azide click reaction [**Scheme 5.1**]. First, alkyne-terminated PLGA synthesized by ring opening polymerization with propargyl alcohol. Then, azide-terminated PNVP has been synthesized *via* RAFT polymerization using (*S*)-2-(4-azidobutyl propionate)-(*O*-ethyl xanthate) (**X5**) RAFT agent. Click reaction was then performed between alkyne-terminated PLGA and azide-terminated PNVP for

the synthesis of PLGA-*b*-PNVP diblock copolymer in the presence of CuBr. Further, the self-assembly behaviour of the resultant amphiphilic block copolymers has been studied using fluorescence spectroscopy, transmission electron microscopy (TEM), dynamic light scattering (DLS) and ^{1}H NMR. DOX-loaded polymeric micelles of PLGA amphiphilic block copolymers were prepared and characterized by UV-vis spectroscopy, DLS and TEM. It shows excellent pH-dependent controlled drug delivery properties. DOX-loaded PLGA-*b*-PNVP micelles suppress the lymphoma cells *in vitro* via apoptosis.

5.2 Experimental Section

5.2.1 Materials

Glycolide (Aldrich, St Louis, USA, 99%) was recrystallized from ethyl acetate. Propargyl alcohol (98%, Loba Chemie, India) was distilled over anhydrous magnesium sulphate. CuBr (99%, Aldrich, USA), DBU (1, 8 Diazabicyclo [5.4.0] undec-7-ene, 98%, Aldrich, USA) used as a received. (*S*)-2-(4- azidobutyl propionate)-(*O*-ethyl xanthate (X5) was synthesized as reported earlier by our group. [Patel *et al*. (2013)] Other reagents are used as mentioned in the **2.2.1 section** of **Chapter 2**.

5.2.1 General Methods

All methods are same as **Chapter 2**.

5.2.3 Synthesis of Alkyne-terminated Copolymer of D, L-lactide and Glycolide using Propargyl Alcohol

1.0 g (6.93×10^{-2} mol) of D, L-lactide was placed in a 100 mL Schlenk tube, and dried under vacuum at room temperature (RT) for 3 h. Then, 4.0 mL of dry and degassed CH_2Cl_2 was added to the reaction mixture and stirred until it becomes a

homogeneous solution. To it, 14 μL (2.31×10^{-4} mol) degassed propargyl alcohol followed by 34 μL (2.31×10^{-4} mol) DBU were added under stirring and polymerization was initially continued at RT for 6 min. Then, the degassed solution of 0.27 g glycolide in 2.0 mL CH_2Cl_2 was added to the above-mentioned polymerization mixture and polymerization was continued for another 4 min. Finally, polymerization was quenched by the addition of 39 mg benzoic acid. The polymer was separated from the polymerization mixture via precipitation from cold methanol. The separated polymer was purified by repeated dissolution in CH_2Cl_2 followed precipitation from methanol twice, and finally was dried under vacuum at RT for 24 h. Gravimetric yield = 0.68 g (53.5%).

^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 1.59-1.60(m, $3H_d$), 4.86-4.8 (d, $2H_b$ +$2H_e$), 5.1-5.2 (m, $2H_c$), 4.3-4.4 (d, $2H_f$), 2.53-2.52 (s, $1H_a$)

M_n (NMR) = 6,500 g mol^{-1}, M_n(GPC) = 6,900 g mol^{-1}, M_w/M_n = 1.18

5.2.4 Typical Synthesis of Azide-terminated Poly(NVP) Macro-RAFT agent (run 2, Table 5.1)

A mixture of NVP (2 mL, 2.08 g, 1.87×10^{-2} mol), (*S*)-2-(4- azidobutyl propionate)-(*O*-ethyl xanthate) (36.7 mg, 1.24×10^{-4} mol) and AIBN (4.1 mg, 2.49×10^{-5} mol) were placed in a dry Schlenk tube containing a Teflon-coated magnetic bar and purged with dry nitrogen gas for 30 min. The Schlenk tube was then placed in a thermo-stated bath at 80 ^{o}C for 5 h. The reaction was stopped by dipping the reaction mixture in liquid N_2. The reaction mixture was dissolved in 5 mL THF, and precipitated from 100 mL hexane. The precipitated polymer was collected by centrifugation. The separated polymer was purified by repeated dissolution in THF and precipitation in hexane twice

and finally was dried under vacuum at RT for 24 h. The M_n(GPC) and PDI of the obtained polymer were observed at 9900 g mol^{-1} and 1.30, respectively.

5.2.5 Synthesis of PLGA$_{46}$-*b*-PNVP$_{68}$ Amphiphilic Block Copolymer by Alkyne-Azide Click Reaction (run 1, Table 5.2)

A mixture of alkyne-terminated PLGA [0.1 g, 1. 45 × 10^{-5} mol, calculated with respect to M_n(GPC) = 6900 g mol^{-1}], azide-terminated PNVP [0.167 g, 1.45 × 10^{-5} mol, calculated with respect to M_n(GPC) = 7600 g mol^{-1}] and CuBr (2.0 mg, 1.45 × 10^{-5} mol) in DMF (2.0 mL) was taken in a dry Schlenk tube containing a Teflon-coated magnetic bar, and was purged with dry N_2 gas for 30 min. The polymerization tube was then placed in a thermostated bath preheated at 80 ^oC for 24 h. The reaction was stopped by freezing the polymerization mixture with liquid N_2. Solvent of the reaction mixture was evaporated under vacuum followed by replacement with tetrahydrofuran (THF). CuBr salt was removed from the polymerization mixture by the passage of its THF solution through activated basic alumina. The polymerization mixture was concentrated to 5 mL THF and precipitated from 100 mL hexane. The precipitated polymer was separated by centrifugation and dried under vacuum at RT for 24 h.

M_n(GPC) = 12,300 g mol^{-1}, M_w/M_n = 1.16

5.2.6 Synthesis of PLGA$_{46}$-*b*-PNVP$_{89}$ Amphiphilic Block Copolymer by Alkyne-Azide Click Reaction (run 2, Table 5.2)

This block copolymer was prepared as mentioned in the previous paragraph using a mixture of alkyne-terminated PLGA [0.150 g, 2.174 × 10^{-5} mol, calculated with respect to M_n(GPC) = 6900 g mol^{-1}], azide-terminated PNVP [0.215 g, 2.174 × 10^{-5} mol,

calculated with respect to M_n(GPC) = 9900 g mol^{-1}] and CuBr (3.2 mg, 2.174 × 10^{-5} mol) in DMF (2.0 mL).

M_n(GPC) = 15,300 g mol^{-1}, M_w/M_n= 1.27

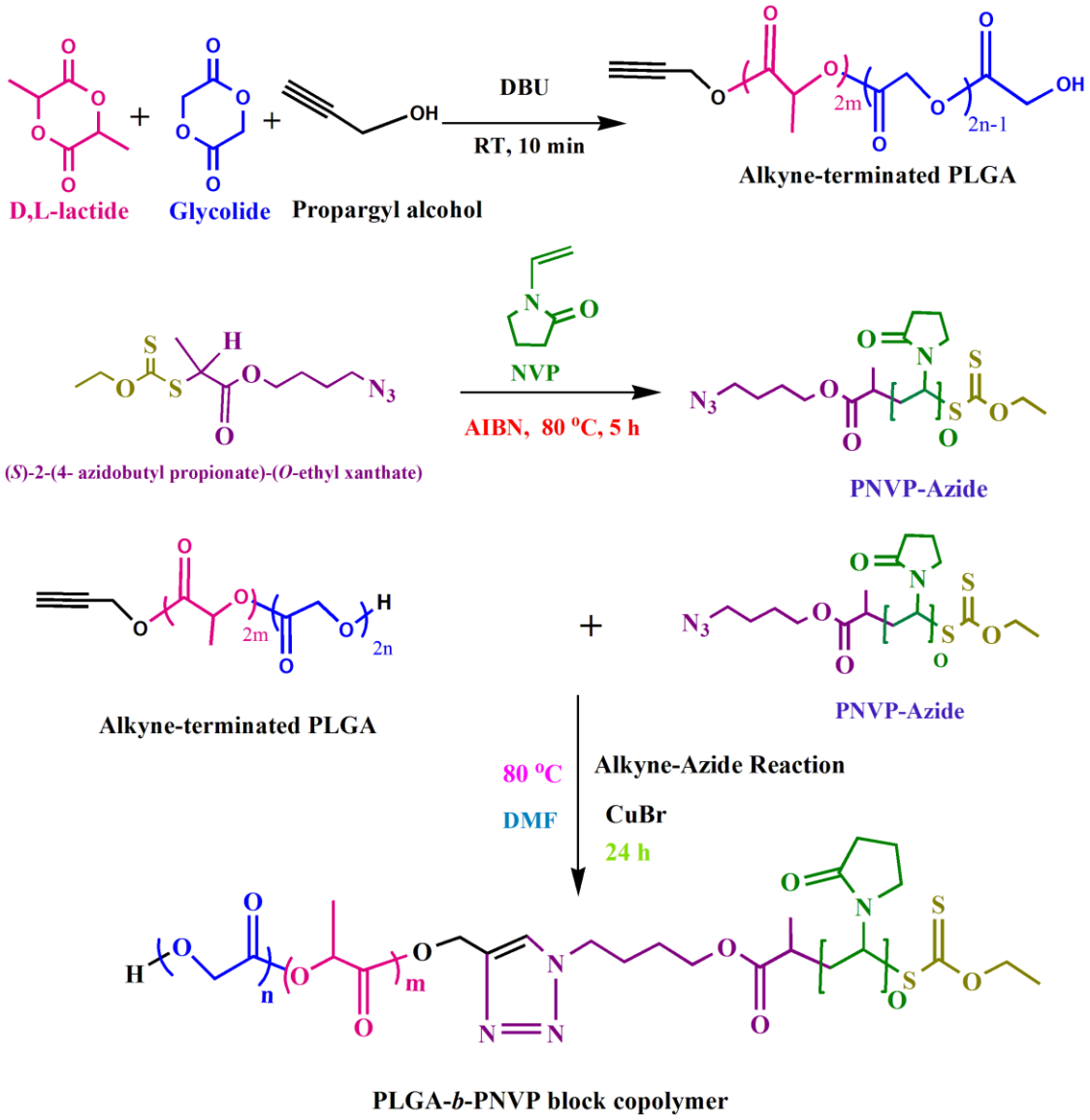
5.2.7 Drug Loading and *In Vitro* Drug Release Study

Procedure is same as described at section 4.2.7 in **Chapter 4**.

5.3 Results and Discussion

5.3.1 Synthesis of Alkyne-terminated PLGA

Alkyne-terminated PLGA was synthesized with 53.5% yield by the ring opening copolymerization of D, L-lactide and glycolide (75/25, w/w) using propargyl alcohol as initiator and DBU as catalyst at RT [**Scheme 5.1**]. Resultant polymer was characterized by ^{1}H NMR, GPC, and FTIR spectroscopy. **Figure 5.1(A)** shows the ^{1}H NMR spectroscopy of the alkyne-terminated PLGA in $CDCl_3$. The peaks at 5.18 and 1.59 ppm correspond to the characteristic methine proton "*c*" and methyl proton "*d*" of the PDLLA backbone chain. The peak at 4.85 ppm corresponds to both methylene proton "*e*" of the PGA backbone chain and methylene proton "*b*" of the alkyne group. The characteristic peak at 2.5 ppm corresponds to the methine proton "*a*" of chain-end alkyne group. So it confirms the formation of PLGA end-functionalized with alkyne group. The M_n(NMR) of this polymer calculated by comparing the average peak areas of the methylene protons ''*c*'' of the PDLLA backbone and "*e*" of the PGA backbone with the peak area of ''*a*'' of the chain-end alkyne group is observed at 6,500 g mol^{-1} . The corresponding GPC chromatogram [M_n(GPC) = 6,900 g mol^{-1} and PDI = 1.18] [**Figure 5.2**] is unimodal with low polydispersity.



Scheme 5.1 Synthesis of PLGA-*b*-PNVP diblock copolymer by click reaction of alkyne-terminated PLGA and azide-terminated PNVP.

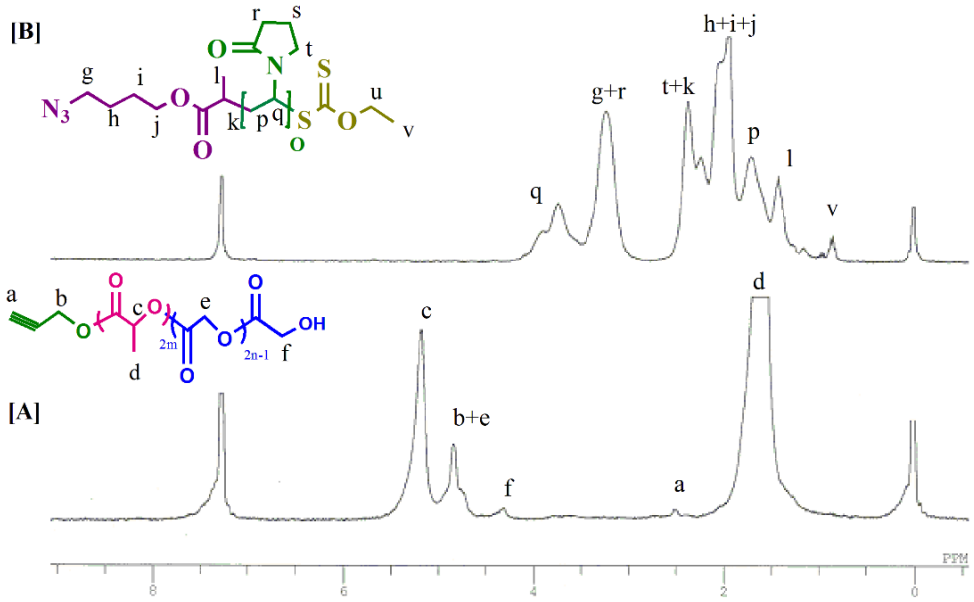


Figure 5.1(A) ^{1}H NMR spectra of alkyne-terminated PLGA, and **(B)** azide-terminated PNVP in $CDCl_3$ at room temperature.

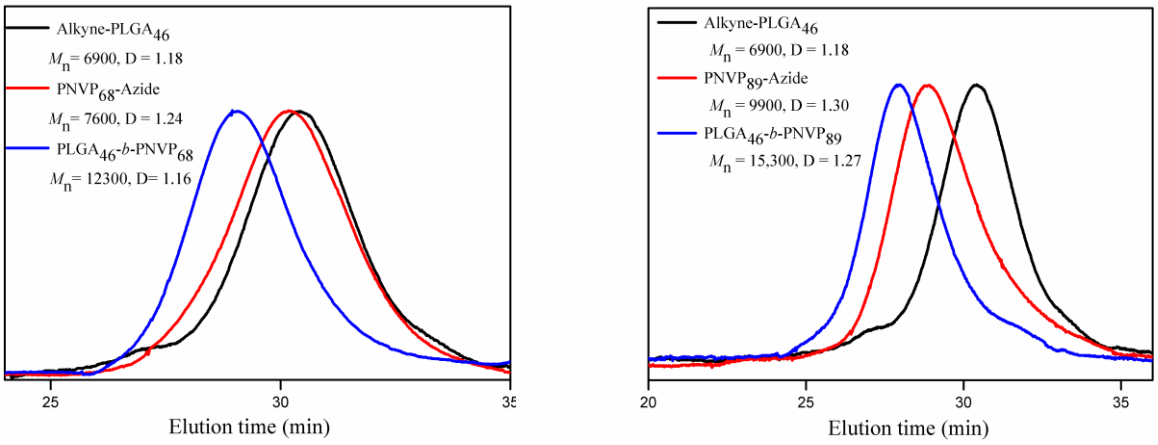


Figure 5.2 Gel permeation chromatograms of alkyne-terminated PLGA, azide terminated PNVP homopolymers and PLGA-*b*-PNVP block copolymers.

FT-IR spectrum of the alkyne-terminated PLGA is shown in **Figure 5.3**. In the spectrum, the characteristic peak at 3514 cm^{-1} is due to –OH end of the polymer chain. The peak at 3300 cm^{-1} is due to the C-H stretching of alkyne. The peaks at 2995 and 2959 cm^{-1} are due to the C-H stretching of CH_3 and CH_2, respectively. The weak peak

at ~ 2100 cm^{-1} is due to the alkyne C-C stretching. The intense peaks at 1755 and 1749 cm^{-1} are due to the C=O stretching of PGA and PDLLA backbone, respectively. The peak at 1419 cm^{-1} is due to the bending of C-O-H. All these results indicate the successful synthesis of alkyne-terminated PLGA.

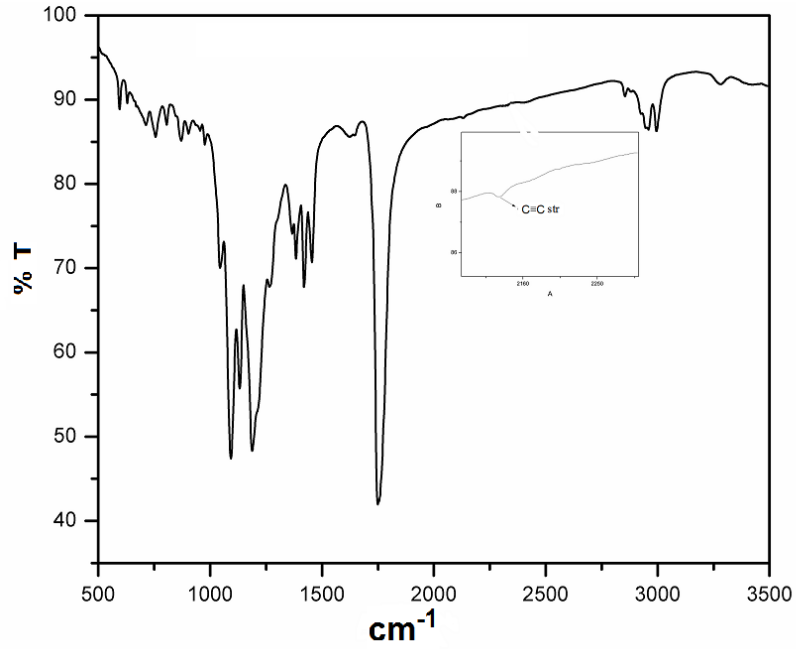


Figure 5.3 FT-IR spectrum of alkyne-terminated PLGA

5.3.2 Synthesis of Azide-terminated Poly (NVP) Macro-RAFT Agent

Two azide-terminated PNVPs [having M_n(GPC) = 7600 g mol^{-1}; PDI = 1.24 and M_n(GPC) = 9900 g mol^{-1}; PDI = 1.30] were prepared by the bulk RAFT polymerization of NVP using AIBN as initiator and (*S*)-2-(4- azidobutyl propionate)-(*O*-ethyl xanthate (**X5**) as RAFT agent at 80°C. The characterization data of these polymers are shown in **runs 1** and **2**, **Table 1**. **Figure 5.1(B)** shows the ^{1}H NMR of azide-terminated PNVP. The peaks "*p*" to "*t*" are the characteristic repeating unit of the NVP as assigned in the figure. Rest peaks are coming from the RAFT agent. All 4-azidobutyl propionate

protons except methyl proton "*l*" are overlapped in the peaks of NVP between 1.42-3.23 ppm. The proton "*l*" is observed at 1.1 ppm. In addition, *O*-ethyl proton "*v*" is observed at 0.9 ppm.

Table 5.1 RAFT Polymerization of NVP mediated with X5[a]

Run	NVP (equiv.)	Time (h)	Conv(%)[b] (NMR.)	$M_{(theory)}$[b]	$M_{n,GPC}$[c]	M_w/M_n[c]
1	60	4	95	6626	7600	1.24
2	150	5	81.2	13794	9900	1.30

[a] Bulk polymerization using X5 : AIBN = 1 : 0.2 with respect to NVP at 80 ^{o}C.
[b] Determined by ^{1}HNMR.
[c] Determined by GPC (DMF, 0.5 mL/min, 40 ^{o}C) calibrated against PMMA standards.

5.3.3 Synthesis of PLGA-*b*-PNVP Block Copolymers by Click Reaction

Amphiphilic $PLGA_{46}$-*b*-PNVP block copolymers were synthesized *via* alkyne-azide click reaction of alkyne-terminated PLGA and azide-terminated PNVP in DMF using CuBr as catalyst with [azide-terminated PNVP]: [alkyne-PLGA]: [CuBr] = 1:1: 1 at 80 ^{o}C for 24 h [**Scheme 5.1, Table 5.2**]. Two different molecular weight azide-terminated PNVPs were used for the synthesis of two block copolymers: $PLGA_{46}$-*b*-$PNVP_{68}$ and $PLGA_{46}$-*b*-$PNVP_{89}$ (**runs 1** and **2**, respectively, **Table 2**). **Figure 5.2** shows clearly the shifting of the GPC chromatogram of block copolymers towards higher molecular weight. The ^{1}H-NMR spectrum of the block copolymer in $CDCl_3$ **Figure 5.4(A)** shows, in addition to the characteristic peaks of the PLGA block, the presence of the characteristic peaks of the PNVP at ~ 2.02, 2.36, 3.20, 3.72 ppm and the peak at around 7.3 ppm corresponding to the triazole ring proton "*e*" also supports the formation of the

block copolymer of PLGA and PNVP through click reaction. The observed mole-fractions of PNVP block in the corresponding block copolymers (**runs 1** and **2**, **Table 5.2**) calculated on the basis M_n(GPC) results are 0.62 and 0.65, respectively. All these results clearly indicate the possible formation of block copolymers of PLGA-*b*-PNVP.

5.3.4 Self-assembly of Amphiphilic PLGA-*b*-PNVP Block Copolymers in Water

It is well known that amphiphilic block copolymers can form nano-structured aggregates *via* self-assembly. The observed suppression of the peaks attributed to the PLGA in the ^{1}H NMR spectrum of $PLGA_{46}$-*b*-$PNVP_{89}$ block copolymer in D_2O [**Figure 5.4(B)**] in comparison to that taken in *d*-chloroform [**Figure 5.4(A)**] indicates the possible formation of micellar aggregates in aqueous solution with PLGA blocks as the core and PNVP blocks as the shell.

Table 5.2 Synthesis of PLGA-*b*-PNVP Block Copolymers[a]

Run	Sample	Alkyne-PLGA (M_n/D[b])	PNVP-Azide (M_n/D[b])	M_n[b](GPC) g mol^{-1}	PDI[b] (GPC)	X_{PNVP}[c] (GPC)	(CMC x10^{-3})[d] (g/L)
1	$PLGA_{46}$-*b*-$PNVP_{68}$	6900/1.18	7600/1.24	12300	1.16	0.62	4.43
2	$PLGA_{46}$-*b*-$PNVP_{89}$	6900/1.18	9900/1.30	15300	1.27	0.65	5.65

[a] Using 1 equivalent CuBr in 2 mL DMF at 80 ^{o}C for 24 h
[b] Determined by GPC(DMF, 0.5 mL/min, 40 ^{o}C) calibrated against PMMA standards
[c] X_{PNVP} = mol-fraction of PNVP
[d] CMC value determined by fluorescence spectrometer

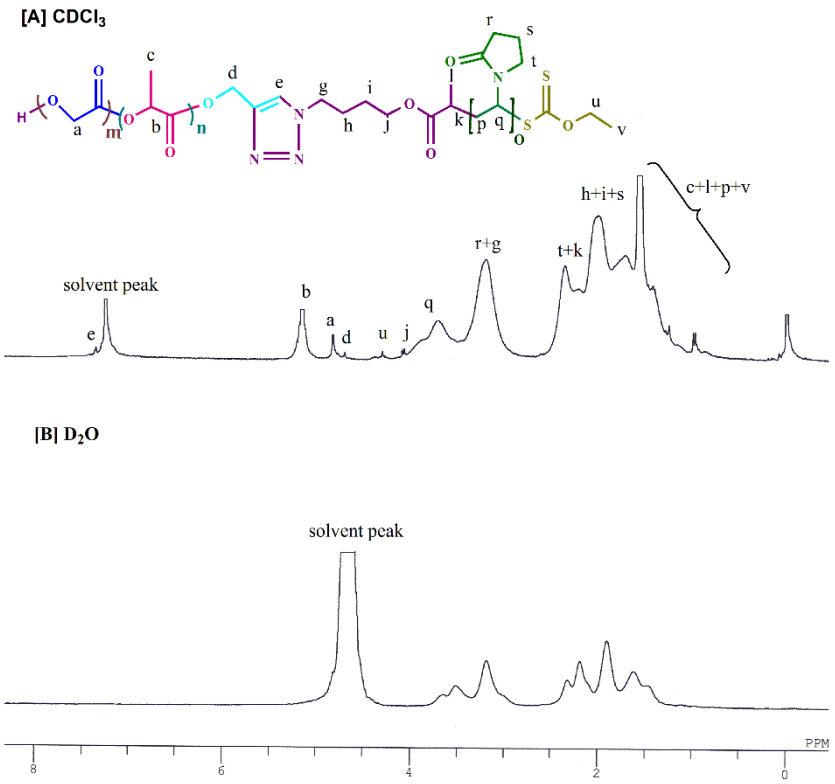
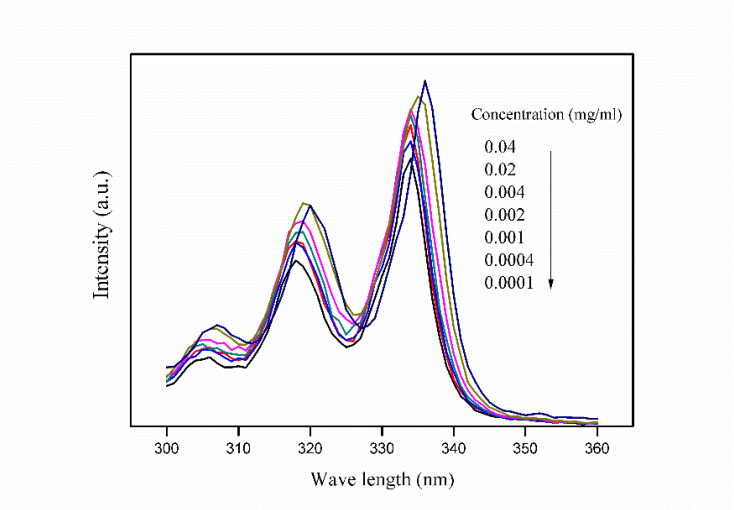
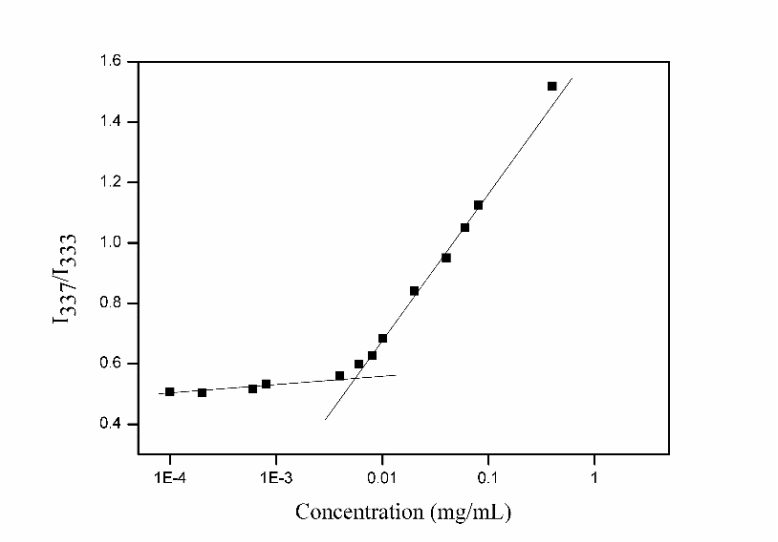


Figure 5.4 ^{1}H NMR spectrum of PLGA$_{46}$-*b*-PNVP$_{89}$ block copolymer in (**A**) *d*-chloroform, and (**B**) D_2O at room temperature.

Fluorescence method is employed to determine the critical micellar concentration (*cmc*) of both block copolymers in water using pyrene as probe. Typical fluorescence excitation spectra (300 - 360 nm) of pyrene (6×10^{-7} M) at different PLGA$_{46}$-*b*-PNVP$_{89}$ [**run 2**, **Table 5.2**] concentrations recorded at an emission wavelength of 394 nm are shown in **Figure 5.5(a)**. **Figure 5.5(b)** shows the corresponding plot of the I_{337}/I_{333} intensity ratio (from fluorescence measurements) *vs.* the log of the polymer concentration (mg/mL) in water. The observed *cmc*s of the block copolymers PLGA$_{46}$-*b*-PNVP$_{68}$ and PLGA$_{46}$-*b*-PNVP$_{89}$ are ~ 4.43×10^{-3} and 5.65×10^{-3} mg/mL, respectively (**runs 1** and **2**, **Table 5.2**).



(a)



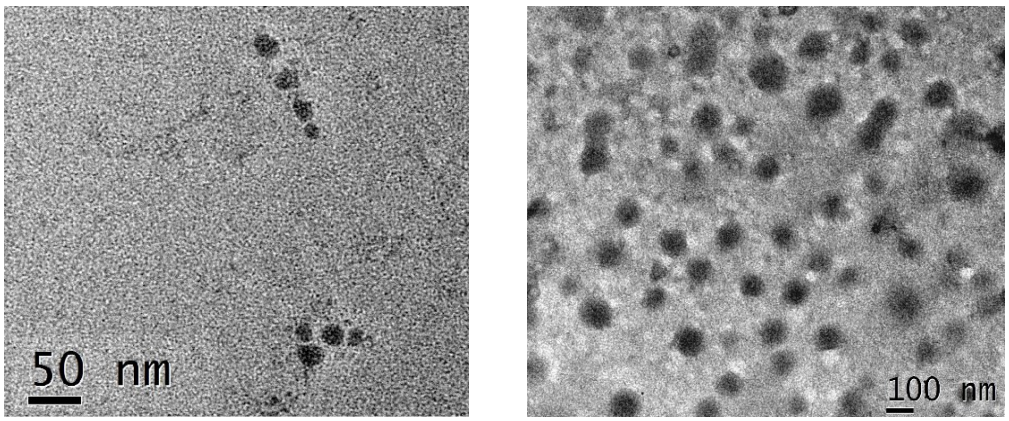
(b)

Figure 5.5 (a) Fluorescence excitation spectra (monitored at λ_{em} = 394 nm) of pyrene (6×10^{-7} M) in the presence of increasing concentration (mg/mL) of $PLGA_{46}$-*b*-$PNVP_{89}$ block copolymer (**run 2**, **Table 5.2**) in water and **(b)** the corresponding semilogarithmic plot of the fluorescence excitation intensity ratio (I_{337}/I_{333}) of pyrene *vs.* the concentration of polymer.

TEM study reveals the formation of spherical micelle of $PLGA_{46}$-*b*-$PNVP_{89}$ from its aqueous solution with 10.6 nm size [**Figure 5.6(a)**]. On the other hand, DLS study reveals the hydrodynamic size of the micelles of the same of 146 nm [**Figure 5.7**].

5.3.5 Drug Loading and Release

In order to explore the possible use of these micelles as nano-carrier for drug delivery, poorly water-soluble anti-cancer model drug doxorubicin (DOX) is encapsulated into the core of the polymeric micelles consisting of hydrophobic block PLGA *via* hydrophobic interaction. The observed drug-loading content (DLC) and drug-loading efficiency (DLE) of DOX in the micelles of $PLGA_{46}$-*b*-$PNVP_{89}$ are 10.8 % and 43.2 %, respectively.



(a) **(b)**

Figure 5.6 The TEM images of **(a)** blank micelles and **(b)** DOX-loaded micelles of $PLGA_{46}$-*b*-$PNVP_{89}$ amphiphilic block copolymer.

TEM study reveals the formation of the spherical DOX-loaded $PLGA_{46}$-*b*-$PNVP_{89}$ micelles with average size of 48.8 nm [**Figure 5.6(b)**]. But DLS study reveals the average hydrodynamic diameter of the same of 189 nm size [**Figure 5.7**]. So, both TEM and DLS studies reveals that DOX-loaded micelles have larger size than the blank micelles. Moreover, the observed larger size in DLS experiment is due to the hydrated state of the micelle. These results suggest that DOX has successfully been encapsulated into the micelles.

The *in vitro* drug release of DOX-loaded $PLGA_{46}$-*b*-$PNVP_{89}$ micelles was carried out at 37 ºC under physiological conditions (PBS, pH = 7.4) and in a slightly acidic environment (PBS, pH = 6.4) and both release profiles are shown in [**Figure 5.8**].

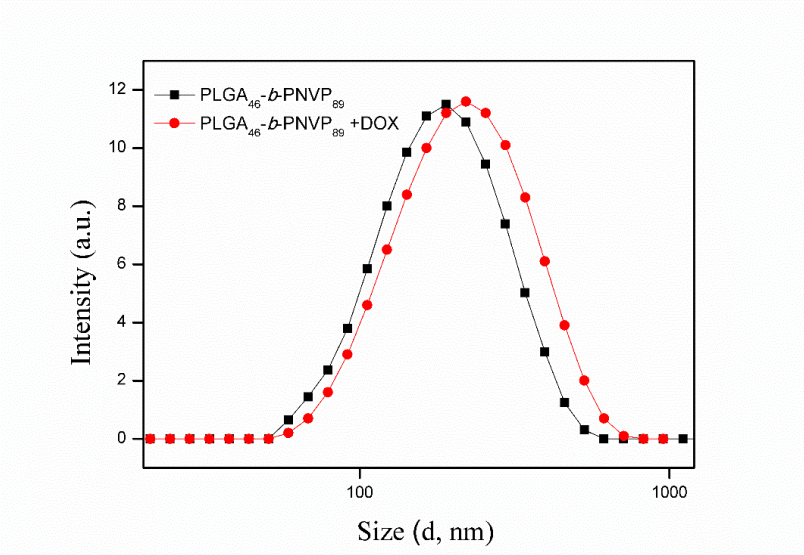


Figure 5.7 Plot of scattering intensity vs. the effective hydrodynamic diameter of without and with DOX loaded micelles of $PLGA_{46}$-*b*-$PNVP_{89}$ block copolymerat 0.1 mg/mL concentration in water at 90° scattering angle.

Interestingly, sustained drug releases are observed at both pHs. The observed DOX release (%) of drug loaded micelles at pH 7.4 and 6.4 are ~4.5 % and ~19.0 %,

respectively. The observed rapid release of DOX-loaded micelles at low pH is due to the presence of NH_2 functional group in DOX as expected. So, the observed accelerated drug release at low pH can be considered as an advantage for the antitumor drug delivery system. Therefore, PLGA-*b*-PNVP amphiphilic block copolymer may find extensive application in sustained drug delivery.

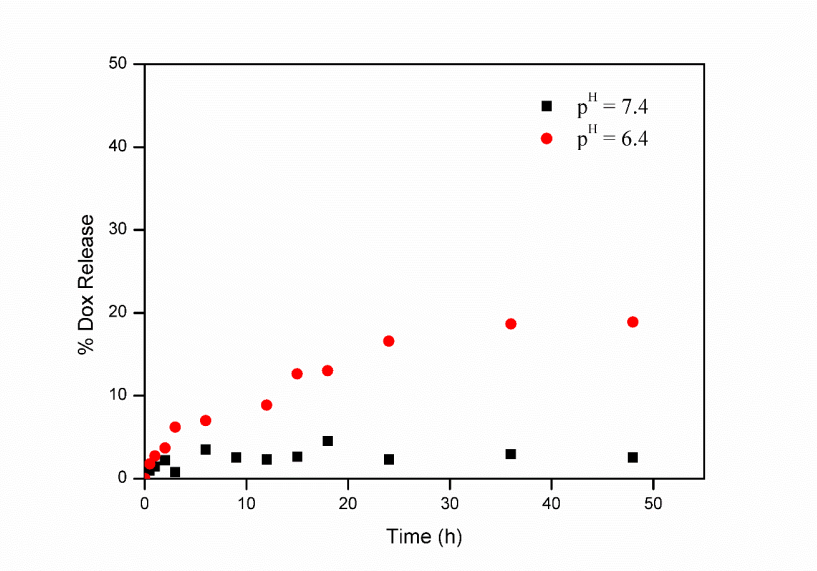


Figure 5.8 *In vitro* DOX release from DOX-loaded $PLGA_{46}$-*b*-$PNVP_{89}$ micelles in PBS (pH = 7.4 and6.4) at 37 ^oC temperature.

5.3.6 Cellular Viability and Toxicity of DOX-loaded $PLGA_{46}$-*b*-$PNVP_{89}$ Micelles

We evaluated the cell viability and toxicity of the free DOX, $PLGA_{46}$-*b*-$PNVP_{89}$ **(**PLGA), blend of PLGA and DOX (blend PLGA-DOX) and DOX-loaded $PLGA_{46}$-*b*-$PNVP_{89}$ micelles (PLGA-DOX) against parental and DOX-resistant human (Raji) and mouse lymphoma cells (DL). We found that increasing DOX concentration gradually decreased the cell viability with all the test samples in all tested cell lines [**Figure 5.9(A, C, E** and **G)**]. Blend of PLGA-DOX behaved almost similarly to that of free DOX in parental DL and Raj cells. [**Figure 5.9(A)** and **(E)**].

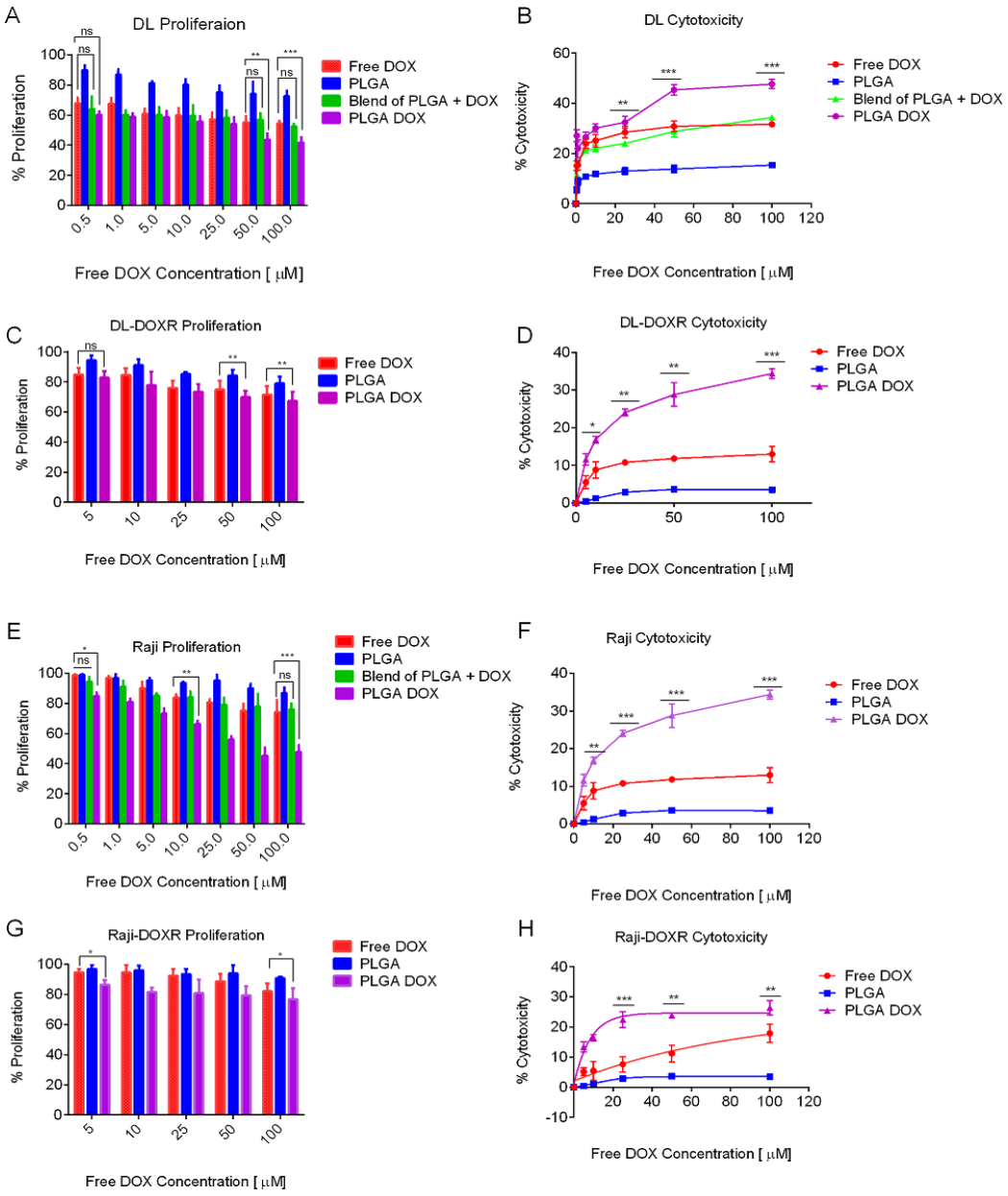


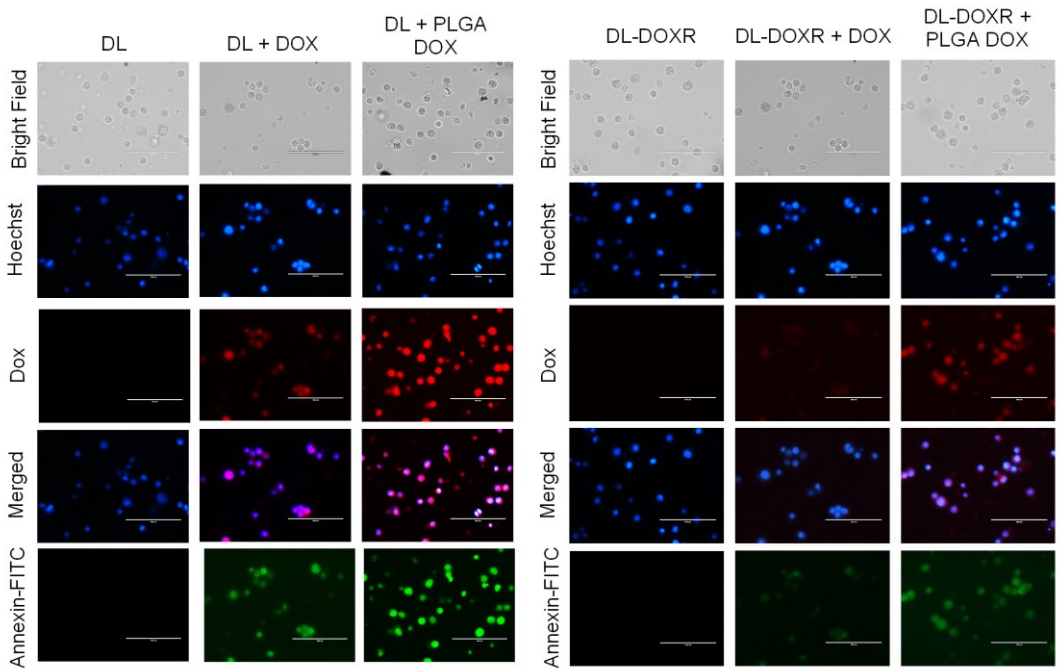
Figure 5.9 Viability and cytotoxicity of tumor cells by free DOX, $PLGA_{46}$-*b*-$PNVP_{89}$ (PLGA), DOX-loaded micelles (PLGA-DOX) and blend PLGA-DOX. In parental DL and Raji (**A**, **E**) and DOX resistant DL and Raji cells (**C**, **G**), viability of tumor cells were studied using XTT assay. An 18 h LDH release assay was used to determine the direct cell lysis (Cytotoxicity) by DOX-loaded PLGA polymeric micelles against parental or, DOX-resistant DL (**B**, **D**) or, Raji cells (**F**, **H**). Data presented as mean ± SD of triplicate determination, n = 3 where n represents the number of times experiment was performed.

Parental DL and Raji cells responded better with significantly higher loss of cell viability in the presence of DOX-loaded micelles compared to free DOX **[Figure 5.9(A)** and **(E)]**. On the other hand, the DOX resistant DL and Raji cells are tolerant to DOX but remain sensitive to DOX-loaded PLGA [**Figure 5.9 (C)** and **(G)**]. Carrier itself has no effect on cell viability in any of the cell line tested.

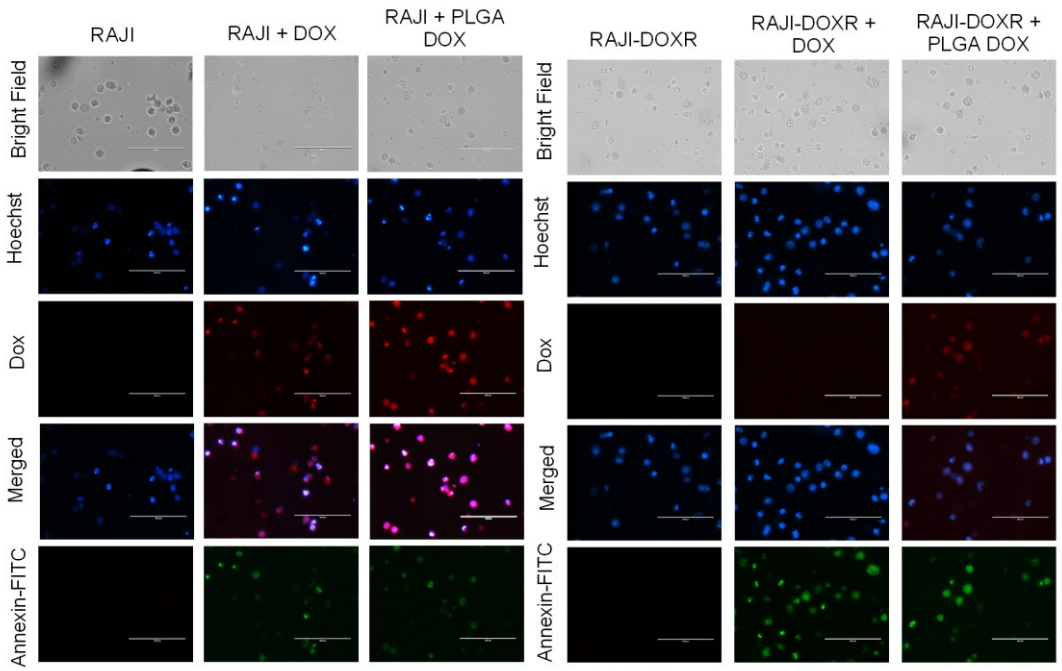
Cell viability could be directly correlated with the cellular toxicity. Increased susceptibility of tumor cells against the drug leads to decreased cytotoxicity or, vice versa. Our results were also supported by the cytotoxicity assay [**Figure 5.9(B)**, **(D)**, **(F)** and **(H)**]. Compared to free DOX and blend PLGA-DOX, DOX-loaded micelles showed higher inhibition of cellular viability in both tested cell lines in concentration dependent manner [**Figure 5.9 (B)** and **(F)**]. Carrier (PLGA) showed almost no toxicity in all cell lines and proves that it's a biocompatible material. Same concentration dependent cell proliferation was found in DOX resistant DL (DL-DOXR) and Raji (Raji-DOXR) cell lines [**Figure 5.9 (D)** and **(H)**]. Our results suggest that DOX-loaded PLGA micelle is highly cytotoxic compared to free DOX and PLGA alone doesn't show any cytotoxicity against the cell lines tested.

5.3.7 Induction of Tumor Cell Apoptosis by DOX-loaded $PLGA_{46}$-*b*-$PNVP_{89}$ Micelles

Our cell viability and cytotoxicity data suggested that DOX-loaded PLGA micelles has significantly higher anti-tumor potential with respect to the reduction in the growth of parental DL and Raji and DOX-resistant DL and Raji cells. This raises the question whether DOX-loaded PLGA micelles also cause apoptosis of tumor cells and if so whether it induces cell death in DOX-resistant tumor cells.



(A)



(B)

Figure 5.10 Microscopic analysis of induction of apoptosis in DL and Raji. Parental DL, DOX-R/DL (**A**) and Parental Raji, DOX-R/Raji (**B**) Cells were treated with free DOX, $PLGA_{46}$-*b*-$PNVP_{89}$ and DOX-loaded $PLGA_{46}$-*b*-$PNVP_{89}$ micelles

Apoptosis was qualitatively measured by monitoring changes in cell size and externalization of phosphatidylserine in DL, Raji, DL-DOXR, and Raji-DOXR cell lines. Interestingly, large numbers of Annexin V positive cells were found in all cell lines upon the treatment of DOX-loaded PLGA micelles. **Figure 5.10 (A)** shows the difference in the tolerance of DL and DL-DOXR against DOX-loaded PLGA micelles. In parental cell line DL, higher Annexin V positive cells were found upon DOX-loaded PLGA treatment over free DOX; whereas, DL-DOXR cell line was tolerable for free DOX, but still susceptible for DOX-loaded PLGA micelles as expected. Same results were found in parental and DOX-resistant Raji cells [**Figure 5.10(B)**]. Our results clearly demonstrate the apoptosis caused by the DOX-loaded PLGA micelles.

5.4 Conclusions

Novel well-defined amphiphilic PLGA-*b*-PNVP block copolymers have successfully been synthesized *via* click reaction of alkyne-terminated PLGA and azide-terminated PNVP. Resultant block copolymers were characterized by ^{1}H NMR and gel permeation chromatography (GPC). The *cmc*s of these block copolymers increases with the increase in PNVP chain length. The hydrophobic anti-cancer drug Doxorubicin has successfully been loaded into the core part of their spherical micelles. *In vitro* drug release study reveals sustained and retarded drug release and also faster release at pH 6.4 than that at pH 7.4. The DOX-loaded micelles show significant cell viability, cytotoxicity, and apoptosis of DL and Raji cells compare to Free DOX. Such PLGA-*b*-PNVP amphiphilic block copolymer may find extensive application in sustained drug delivery, specifically in antitumor drug delivery.