Clinics in Oncology

Review Article
Published: 08 Aug, 2017

Encapsulation of Doxorubicin in PLGA Nanoparticles Enhances Cancer Therapy

*Lakshmi Priya Krishnamoorthy, Rajesh Kannan Moorthy, Devan Umapathy, Mahesh Kumar Kannan, Nithya Ganesan and Antony Joseph Velanganni Arockiam**

Department of Biochemistry, School of Life Sciences, Bharathidasan University, India

Abstract

The anticancer effect of doxorubicin loaded PLGA nanoparticles on p-Dimethylaminoazobenzene (p-DAB) induced liver cancer in male albino rats has been studied. Nanoparticles of poly (D, L-Lactic-co-glycolic acid) (PLGA) were developed by nanoprecipitation method as delivery system for doxorubicin. Doxorubicin was chemically conjugated to a terminal end group of PLGA. Group I animals were fed with feed and water ad libitum and served as control. Group II-IV animals were received p-DAB intraperitoneal injection at 20 mg/kg body weight once in a week for two months. Then group III animals received intravenous injection of doxorubicin (240 µg/kg body weight) in a tail vein daily. Group IV animals were received intravenous injection of PLGA nanoencapsulated doxorubicin (420 µg of equivalent doxorubicin/kg body weight) in tail vein daily and stopped to the conclusion of the experiment. Doxorubicin loaded PLGA nanoparticles were characterized by X-Ray Diffractometer (XRD), Transmission Electron Microscopy (TEM) and Fourier Transmission Infra-Red microscopy (FTIR). The elevated levels of protein, SGOT, SGPT in the p-DAB administered group were significantly reduced by doxorubicin loaded PLGA nanoparticles than free doxorubicin. In vivo anticancer activity showed that administration of doxorubicin loaded PLGA nanoparticles had comparable activity to that of free doxorubicin.

Keywords: P-dimethylaminoazobenzene; Doxorubicin; Nanoparticles; liver cancer; Poly (D); L-lactic-co-glycolic acid

Abbreviations

PLGA: Poly (D, L-Lactic-co-Glycolic Acid); P-DAB: P-Dimethylaminoazobenzene; XRD: X-ray Diffractometer; TEM: Transmission Electron Microscopy; FTIR: Fourier Transmission Infra-Red microscopy; PVA: Poly Vinyl Alcohol; SOD: Super Oxide Dismutase; SGPT: Serum Glutamate Pyruvate Transaminase; SGOT: Serum Glutamate Oxaloacetate Transaminase; FDA: Food and Drug Administration

Introduction

Hepatocellular Carcinoma (HCC) is the major health problem of the world. Its incidence is increasing in both developing and developed countries. HCC represents the third cause of cancer-related deaths [1]. P-Dimethylaminoazobenzene (p-DAB) is reported to induce damage in liver and caused tumors in several species of experimental animals by different route of administration [2]. Current cancer treatments can only prolong the life of the patients, but do not cure, because of the high toxicity and poor specificity of currently used drugs. Chemotherapeutic drugs are systemically active and cannot target cancer cells. Increasing the chemotherapeutic dosages is not possible. High dosage of chemotherapeutic drugs cause severe side effects like destruction of bone marrow cells which impairs the erythrocytes production, cardiotoxicity, nephrotoxicity, hepatotoxicity and hematotoxicity. To overcome these problems nanporticles are used in the cancer treatment. A nanoparticle-mediated drug delivery system can eliminate drug or drug carrier side effects significantly [3,4]. The major advantage of nanotechnology is targeted drug delivery to the site of the disease. This can be achieved either by passive targeting of drugs to the site of action or by active targeting of the drug [5]. Passive targeting exploits the anatomical differences between normal and diseased tissues to deliver the drugs to the required site, because the physiology of diseased tissues may be altered in a variety of physiological conditions through the enhanced permeability and retention (EPR) effect. The thermoplastic aliphatic polyesters like poly (lactide-co-glycolide) (PLGA), the copolymer of lactide and glycolide have generated tremendous interest due to their favorable properties such as good biocompatibility, biodegradability and mechanical strength [6].

OPEN ACCESS

***Correspondence:**
Antony Joseph Velanganni Arockiam, Department of Biochemistry, Bharathidasan University, India, Tel: 0431-2407071; Fax: 0431-2407045; E-mail: ajvelanganni@gmail.com

Received Date: *19 May 2017*
Accepted Date*: 16 Jun 2017*
Published Date*: 08 Aug 2017*

Citation:
Krishnamoorthy LP, Moorthy RK, Umapathy D, Kannan MK, Ganesan N, Arockiam AJV. Encapsulation of Doxorubicin in PLGA Nanoparticles Enhances Cancer Therapy. Clin Oncol. 2017; 2: 1325.

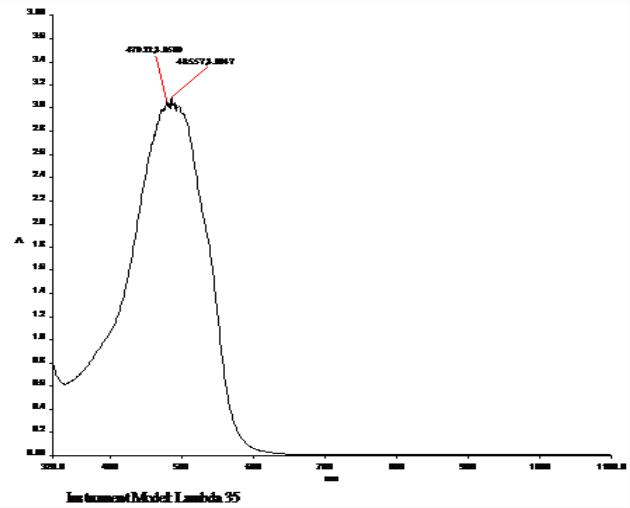

Figure 1: UV-Visible spectral peak of total drug.

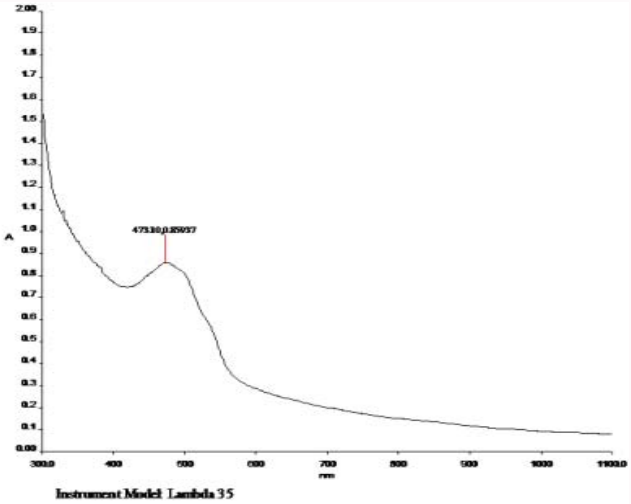

Figure 2: UV-Vis spectral peak of Free Drug.

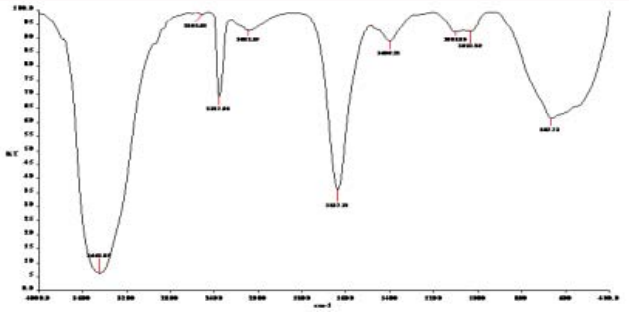

Figure 3: FTIR analysis of Free Drug.

They are easy to formulate into different devices for carrying a variety drug classes such as vaccines, peptides, proteins and micro molecules. Also, they have been approved by the Food and Drug Administration (FDA) for drug delivery [7]. Doxorubicin (Adriamycin) is a potent anti-neoplastic agent isolated from Streptomyces peuceticus. Doxorubicin was chemically conjugated to a terminal end group of PLGA by an ester linkage and the doxorubicin–PLGA conjugate was formulated into nanoparticles. A carboxylic acid end group of

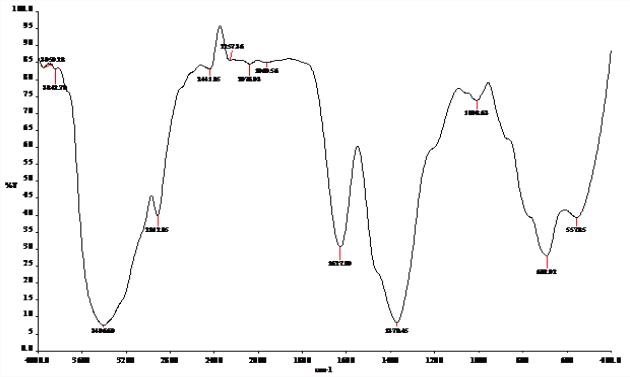

Figure 4: FTIR analysis of PLGA Polymer.

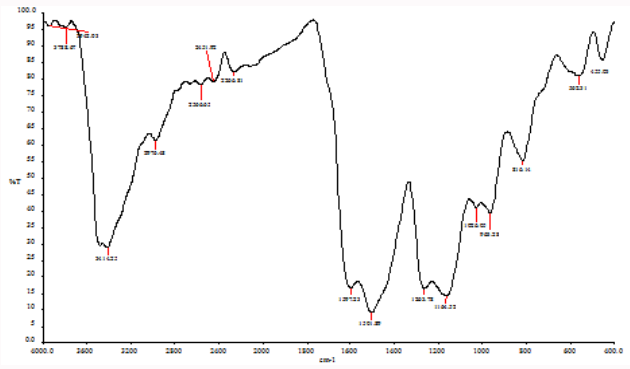

Figure 5: FTIR analysis of Doxorubicin loaded PLGA.

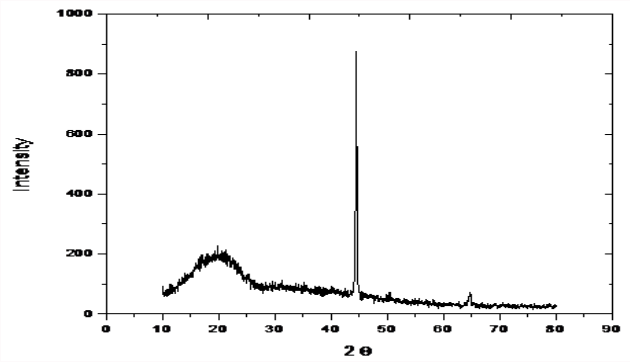

Figure 6: XRD analysis of Doxorubicin loaded PLGA.

PLGA was conjugated to a primary hydroxyl group of doxorubicin. The primary amine group of doxorubicin was protected during the conjugation process and then deprotected. The nanoparticles containing the conjugate exhibited sustained doxorubicin release profiles over a month period [8]. Hence, the present work has been carried out to study the effect of doxorubicin-loaded PLGA on p-DAB induced liver cancer in rats.

Materials and Methods

Materials

Wistar male albino rats weighing 150 gm - 200 gm, reared and maintained under the supervision of IAEC in the animal house of the Bharathidasan University, were used for the study. P-Dimethylaminoazobenzene (DAB) was purchased from LOBA Chemie Pvt. Ltd, Mumbai-400 005. PLGA polymer was purchased

Table 1: FTIR analysis of Functional Groups of Doxorubicin.

Wave number (cm^{-1})	Chemical Bonding
667.73	Alkyne C-H
1031.2	Aromatic C-H
1098.86	Aromatic C-H
1400.12	Carboxylic acids
1637.12	Alkenyl C=C stretch
2085.67	NH_3 structure
2357.89	Charged amines $C=NH^+$
3445.65	H-bonded OH stretch

Table 2: FTIR analysis of Functional Groups of PLGA Polymer.

Wave number (cm^{-1})	Chemical Bonding
692.02	Thioether CH_2-S
1008.63	Cyclohexane ring
1370.45	Methyl C-H bend
1627.9	Alkenyl C=C stretch
1960.56	Aromatic combination bands
2257.36	Saturated nitriles C≡N
2441.85	Charged amines $C=NH^+$
3406.6	OH stretch

Table 3: FTIR analysis of Functional Groups of Doxorubicin loaded PLGA.

Wave number (cm^{-1})	Chemical Bonding
963.28	Trans C-H stretch
1026.05	Cyclohexane ring vibrations
1164.52	Aromatic C-H
1263.78	Esters of aromatic acid
1501.89	Aromatic ring stretch
1597.23	Aromatic ring stretch
2266.65	Diazonium salts $(R-C=N=N)^+$
2451.92	Charged amines $C=NH^+$
2566.65	Thiols S-H stretch
2976.48	Alkanes $-CH_3$
3414.25	H bonded OH stretch

from Sigma Aldrich Co., 3050 Spruce Street, St.Louis, MO 63103 USA 314-771-5765. Polyvinyl alcohol (PVA) was purchased from Hi Media Laboratories Pvt. Ltd, Mumbai-400 086. Doxorubicin hydrochloride was purchased from Fresenius Kabi Oncology Ltd, HP-173 205, India.

Experimental Design

All animals were acclimatized for 15 days and fed *ad libitum* with rat feed and water. After acclimatization, animals were divided into four groups consisting of 5 rats each. Group I animals were served as normal control and received food and water for 90 days. Group II animals were served as disease control and received DAB (20 mg/Kg body weight) once in a week, intraperitoneally for 2 months [9,10]. Group III animals received DAB (20 mg/kg body weight) intraperitoneally, once in a week for 2 months and after received intravenous injection of doxorubicin hydrochloride (240 µg/kg body weight) in a tail vein daily and stopped to the conclusion of the experiment [11]. Group IV animals were received DAB (20 mg/kg body weight) intraperitoneally, once in a week for 2 months and after received intravenous injection of PLGA nanoencapsulated doxorubicin hydrochloride (420 µg of equivalent doxorubicin/kg body weight) in tail vein daily and stopped to the conclusion of the experiment.

Preparation of nanoparticles and encapsulation

The modified method of was used for nanoencapsulation [12]. The nanoprecipitation method was used for the formation of drug-encapsulated PLGA nanoparticles. PLGA polymer (40 mg) was dissolved in acetone. Then the polymer solution containing same amount of drug was added drop-wise into PVA aqueous solution and stirred magnetically at room temperature until complete evaporation of the organic solvent. Then, the nanoparticle suspension was centrifuged by cooling centrifuge at 12,000 rpm for 1 hour. The separated nanoparticles were redispersed and centrifuged three times in distilled water to remove free drug completely. Finally, nanoparticles were dried via vaccum drier at room temperature for 5 minutes, and then were characterized.

Results and Discussion

Evaluation of Doxorubicin Encapsulation

Nanoparticles were centrifuged at 12,000 g at 30 min in cooling centrifuge, and then supernatants of Doxorubicin solutions were measured by UV-spectrophotometer at 473 nm. Figure 1 and 2 showed the OD values of total drug (485.57) and free drug (473.10). Calculations were performed by using the calibration curve, and encapsulation efficiency was calculated as described [13].

Doxorubicin encapsulation efficiency (%) = Total Doxorubucin – Free Doxorubicin X 100/Total Doxorubucin

= 20 mg – 5.0 mg X 100/20 mg

= 75

In the present study, Doxorubicin encapsulation efficiency was as 75 for 20 mg of PLGA polymer. However, that the encapsulation efficiency of chitosan nanoparticles (5.0 mg/mL 5-FU) was 29.98 ± 0.8 [14].

FTIR Analysis

Infrared spectroscopy was used to study the interactions between the drug and the polymers. In order to investigate possible molecular interaction between drug and the polymer, FTIR spectroscopy (Perkin-Elmer, Spectrum RXI) of the Doxorubicin, PLGA polymer and Doxorubicin-loaded PLGA nanoparticles (NPs) was carried out. The FTIR spectra were collected in the range of 4000 cm^{-1} – 400 cm^{-1} at room temperature. FTIR analysis of free drug (Figure 3) showed distinct peaks at wave numbers 667.73 cm^{-1}, 1031.20 cm^{-1}, 1098.86 cm^{-1}, 1400.12 cm^{-1}, 1637.12 cm^{-1}, 2085.67 cm^{-1}, 2357.89 cm^{-1}, 3445.65 cm^{-1}. The peaks corresponding to Alkyne C-H, Aromatic C-H, Aromatic C-H, Carboxylic acids, Alkenyl C=C stretch, NH_3 structure, Charged amines $C=NH^+$, H-bonded OH stretch (Table 1).

FTIR analysis of PLGA polymer (Figure 4) showed distinct peaks at wave numbers 692.02 cm^{-1}, 1008.63 cm^{-1}, 1370.45 cm^{-1}, 1627.90 cm^{-1}, 1960.56 cm^{-1}, 2257.36 cm^{-1}, 2441.85 cm^{-1}, 3406.60 cm^{-1}. The peaks corresponding to thioether CH_2-S, cyclohexane ring, methyl C-H bend, alkenyl C=C stretch, aromatic combination bands, saturated nitriles C≡N, charged amines $C=NH^+$, OH stretch (Table 2).

FTIR analysis of Doxorubicin loaded PLGA (Figure 5) showed distinct peaks 963.28 cm^{-1}, 1026.05 cm^{-1}, 1164.52 cm^{-1}, 1263.78 cm^{-1},

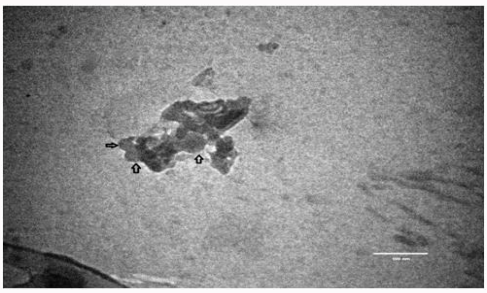

Figure 7: Transmission Electron Microscopy of Doxorubicin loaded PLGA.

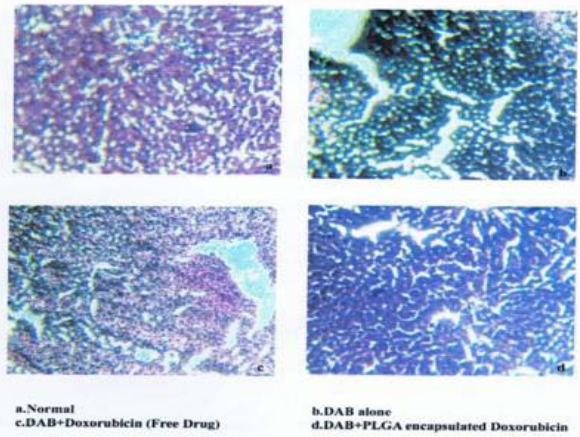

Figure 8: Histopathological Appearance of Rat Liver.

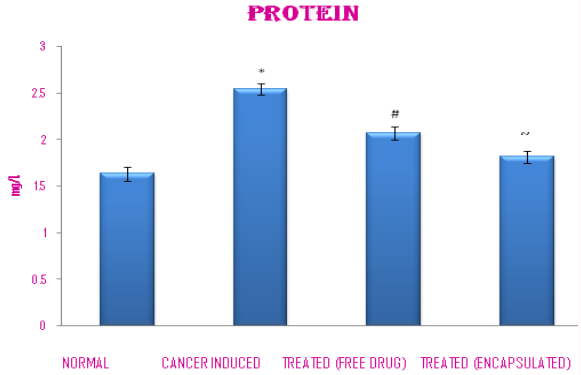

Figure 9: Effect of Doxorubicin on Protein Levels of Rats Administered With p-DAB.

1501.89 cm^{-1}, 1597.23 cm^{-1}, 2266.65 cm^{-1}, 2451.92 cm^{-1}, 2566.65 cm^{-1}, 2976.48 cm^{-1}, 3414.25 cm^{-1}. The peaks corresponding to trans C-H stretch, cyclohexane ring vibrations, aromatic C-H, esters of aromatic acid, aromatic ring stretch, aromatic ring stretch, diazonium salts $(R\text{-}C{=}N{=}N)^+$, charged amines $C{=}NH^+$, thiols S-H stretch, alkanes $-CH_3$, H bonded OH stretch. Doxorubicin was chemically conjugated to a terminal end group of poly (D, L-lactic-co-glycolic acid) [PLGA] by an ester linkage. 1263.78 cm^{-1} showed that ester linkage (Table 3 and 4) [15]. Reported that the spectra of the PLGA NPs and Alpha 1-antitrypsin-loaded PLGA NPs showed two distinct peaks at wave numbers 1300 cm^{-1} - 1450 cm^{-1} and 1720 cm^{-1} - 1800 cm^{-1}, which were assigned to δCH and V(C=O).

XRD Analysis

Showed the diffraction lines Figure 6 and 7, which were observed

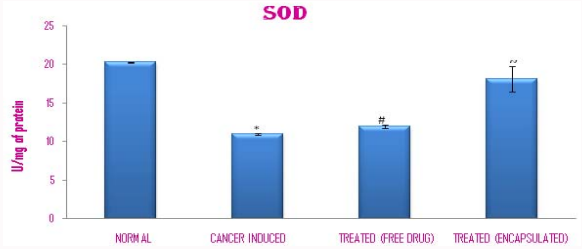

Figure 10: Effect of Doxorubicin on Super Oxide Dismutase Levels of Rats Administered with p-DAB.

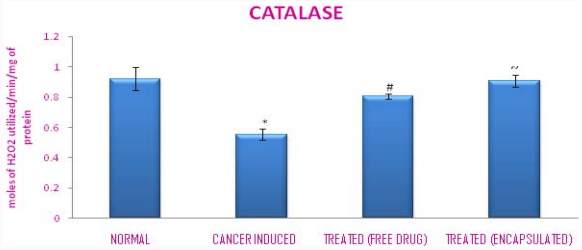

Figure 11: Effect of Doxorubicin on Catalase Levels of Rats Administered With p-DAB.

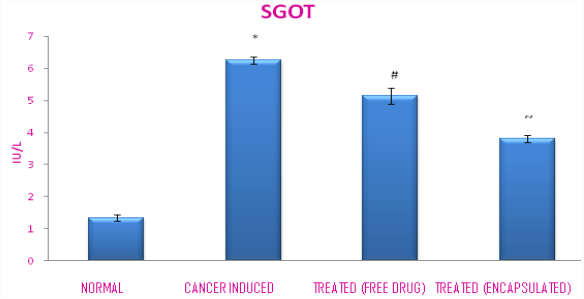

Figure 12: Effect of Doxorubicin on SGOT Levels of Rats Administered With p-DAB.

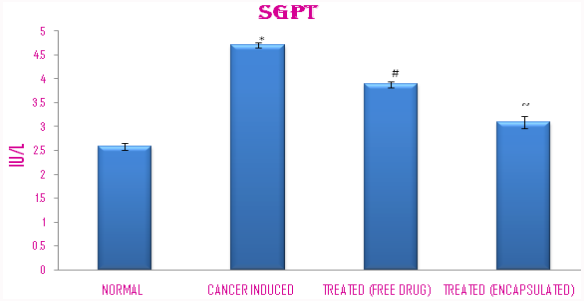

Figure 13: Effect of Doxorubicin on SGPT Levels of Rats Administered Wit p-DAB.

at 2θ angle 16.780°, 20.440° and 21.100° and 23.060°. By using 2θ angle, the size of the nanoparticle was calculated. Debye-Scherrer's formula was used to calculate the size of the nanoparticle [16]. Reported that the XRD pattern of pure silver ions was known to display peaks at 2θ= 7.9°, 11.4°, 17.8°, 30°, 38°, and 44°.

TEM Analysis

Microphotographs of the Doxorubicin loaded PLGA were obtained by transmission electron microscopy (Zeiss). Figure 7 showed the Transmission Electron Microscope (TEM) image of the

Table 4: Measurement of the Doxorubicin loaded PLGA nanoparticles by using Debye-Scherrer's equation.

S.NO	2θ	FWHM	β=π/180*FWHM[a]	Cos θ	D=Kλ/β*Cos θ
1	16.36	0.118	$2.0532X10^{-3}$	0.9898	68.22 nm
2	16.62	0.118	$2.0532X10^{-3}$	0.9895	68.25 nm
3	16.78	0.118	$2.0532X10^{-3}$	0.9893	68. 26 nm
4	17.04	0.118	$2.0532X10^{-3}$	0.9889	68.29 nm
5	17.26	0.141	$2.4534X10^{-3}$	0.9887	68.29 nm
6	18.06	0.118	$2.0532X10^{-3}$	0.9876	68.38 nm
7	18.8	0.118	$2.0532X10^{-3}$	0.9866	68.45 nm
8	18.98	0.118	$2.0532X10^{-3}$	0.9863	68.47 nm
9	19.82	0.118	$2.0532X10^{-3}$	0.9851	68.55 nm
10	20.44	0.165	$2.8710X10^{-3}$	0.9841	49.07 nm
11	20.76	0.165	$2.8710X10^{-3}$	0.9836	49.10 nm
12	21.1	0.141	$2.4534X10^{-3}$	0.9833	57.48 nm
13	21.36	0.118	$2.0532X10^{-3}$	0.9827	68.72 nm
14	23.06	0.118	$2.0532X10^{-3}$	0.9798	68.92 nm
AVERAGE SIZE					64.82 nm

Table 5: Effect of Doxorubicin on Protein Levels of Rats Administered with p-DAB.

GROUP	STATUS	PROTEIN LEVEL
I	Normal Rats	1.632 ± 0.073
II	DAB alone	2.540 ± 0.060
III	DAB+Doxorubicin (Free Drug)	2.072 ± 0.068*
IV	DAB+ PLGA Encapsulated Doxorubicin	1.816 ± 0.0650*

Table 6: Effect of Doxorubicin on Super Oxide Dismutase levels of rats administered with p-DAB.

GROUPS	STATUS	SOD LEVEL
I	Normal Rats	20.230 ± 0.080
II	DAB alone	10.966 ± 0.099
III	DAB+Doxorubicin (Free Drug)	11.964 ± 0.211*
IV	DAB+PLGA Encapsulated Doxorubicin	18.058 ± 1.636*

Table 7: Effect of Doxorubicin on Catalase Levels of Rats Administered With p-DAB.

GROUPS	STATUS	CATALASE LEVELS
I	Normal Rats	0.92 ± 0.076
II	DAB alone	0.55 ± 0.035
III	DAB+Doxorubicin (Free Drug)	0.80 ± 0.018*
IV	DAB+PLGA Encapsulated Doxorubicin	0.90 ± 0.038*

Table 8: Effect of Doxorubicin on SGOT Levels of Rats Administered with p-DAB.

GROUPS	STATUS	SGOT LEVELS
I	Normal Rats	1.334 ± 0.096
II	DAB alone	6.266 ± 0.110
III	DAB+Doxorubicin (Free Drug)	5.144 ± 0.262*
IV	DAB+PLGA Encapsulated Doxorubicin	3.804 ± 0.105*

Doxorubicin loaded PLGA nanoparticles. It revealed that the NPs have spherical shape. The resulting NPs were predominantly spherical and of uniform size and shape [17]. Reported that the imaging of the GC–BTNPs by TEM revealed well-dispersed structures [18]. Reported that the TEM of freeze- fractured samples showed that the particles had a central cavity surrounded by a polymer wall.

Table 9: Effect of Doxorubicin on SGPT Levels of Rats Administered with p-DAB.

GROUPS	STATUS	SGPT LEVELS
I	Normal Rats	2.578 ± 0.072
II	DAB alone	4.706 ± 0.055
III	DAB+Doxorubicin (Free Drug)	3.888 ± 0.064*
IV	DAB+PLGA Encapsulated Doxorubicin	3.088 ± 0.123*

Histopathological Appearance of Rat Liver

Normal rats show more compact and well-distributed junctional complexes (Figure 8a). In DAB-administered rats, hepatic cells exhibit damaged cell structure (Figure 8b). However, animals treated with doxorubicin (Figure 8c) show high cell density with compact junctional complexes than DAB-administered animals. The animals treated with doxorubicin loaded PLGA show higher cell density with compact junctional complexes (Figure 8d) than doxorubicin treated animals. Velanganni and Balasundaram reported that hepatic cells exhibit loss of contact inhibition (polarity) and damaged central vein of liver lobules in DAB-administered rats (Figure 9).

Biochemical Analysis

The Figure 9 showed the effect of doxorubicin on protein levels of rats administered with p-DAB. Protein level was estimated by Lowry's method [19]. Protein level, which increased after DAB administration, was found to be lowered significantly (P <0.001) by Doxorubucin loaded PLGA when compared to free Doxorubicin (Table 5). Figure 10 and 11 showed the effect of doxorubicin on SOD and catalase levels of rats administered with p-DAB respectively. SOD was estimated [20,21]. Catalase level was estimated by Sinha's method [22]. Antioxidants SOD and catalase, which were decreased after DAB administration and found to be increased after the administration of Doxorubicin and Doxorubicin loaded PLGA. Doxorubicin loaded PLGA increased the antioxidants levels significantly (P <0.001; P <0.005) when compared to free doxorubicin (Table 6 and 7).

Figure 12 and 13 showed the effect of doxorubicin on SGOT and SGPT levels of rats administered with p-DAB respectively. SGOT and SGPT levels were estimated by Reitman's method. SGOT and SGPT levels, which increased after DAB administration, were found to be lowered significantly (P <0.005) by Doxorubicin loaded PLGA when compared to free Doxorubicin. Reported that the levels of GSH, ALP, GST and bilirubin were increased after DAB administration and these were found to be lowered by vitamins A, C and E (Table 8 and 9).

Conclusion

The *in vivo* anti-tumor activity of the PLGA loaded doxorubicin nanoparticle was higher than the free doxorubicin. Hence, Doxorubicin loaded PLGA nanoparticle can be used to improve the therapeutic efficacy of Doxorubicin in the treatment of cancer.

Acknowledgement

Ms. K. Lakshmi Priya is thankful to DST for the award of INSPIRE Fellowship and DST-FIST Sponsored Dept. of Biochemistry, Bharathidasan University, Tiruchirappalli for providing the platform to do the research work.

References

1. Arjmand Sareh, Elham Bidram, Abbas Sahebghadam Lotfi, Hamid Mahdavi. Evaluation of Poly (D, L-lactide-co-glycolide) for Nanoencapsulation of Alpha 1-antitrypsin and *In Vitro* Release Study, International Journal of Bioscience, Biochemistry and Bioinformatics. 2011.

2. Aydin Seda Tigl R, Mehlika Pulat. 5-Fluorouracil Encapsulated Chitosan Nanoparticles for pH-Stimulated Drug Delivery: Evaluation of Controlled Release Kinetics, J Nanomaterials. 2012.
3. Cheung TK, Lai CL, Wong B CY, Fung J, Yuen MF. Clinical features, biochemical parameters, and virological profiles of patients with hepatocellular carcinoma in Hong Kong. Aliment Pharmacol Ther. 2006; 24(4): 573-583.
4. Ciofani Gianni, Serena Danti, Delfo D'Alessandro, Stefania Moscato, Mario Petrini, Arianna Menciassi. Barium Titanate Nanoparticles: Highly Cytocompatible Dispersions in Glycol-chitosan and Doxorubicin Complexes for Cancer Therapy, Nanoscale Research Letter. 2010; 5(1): 1093-1101.
5. Derakhshandeh K, Erfan M, Dadashzadeh S. Encapsulation of 9-nitrocamptothecin, a novel anticancer drug, in biodegradable nanoparticles: factorial design, characterization and release kinetics. Eur J Pharm Biopharm. 2007; 66(1): 34-41.
6. Derakhshandeh Katayoun, Marzieh Soheili, Simin Dadashzadeh, Reza Saghiri. Preparation and *in vitro* characterization of 9-nitrocamptothecin-loaded long circulating nanoparticles for delivery in cancer patients. Int J Nanomedicine. 2010; 5: 463-471.
7. Dubey Manish, Seema Bhadauriaa, Kushwahb BS. Green synthesis of nano silver particles from extract of Eucalyptus hybrida (Safeda) leaf, Digest Journal of Nanomaterials and Biostructures. 2009; 4(2): 537-543.
8. Yoo HS, Lee KH, Oh JE, Park TG. *In vitro* and *in vivo* anti-tumor activities of nanoparticles based on doxorubicin-PLGA conjugates. J Control Release. 2000; 68(3): 419-431.
9. Hyuk Sang Yoo, Keun Hyeung Lee, Jong Eun Oh, Tae Gwan Park. Biodegradable Nanoparticles Doxorubicin-PLGA conjugate for Sustained Release. Pharm Res. 1999; 16(7): 1114-1118.
10. IARC. Para-Dimethylaminoazobenzene. In Some Aromatic Azo Compounds. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, vol. 8. Lyon, France: International Agency for Research on Cancer. 1975; 125-146.
11. Jin S, Ye K. Nanoparticle-mediated drug delivery and gene therapy. Biotechnol Prog. 2007; 23(1): 32-41.
12. Kumar CSSR, Hormes J, Leuschner (Eds). Nanofabrication Towards Biomedical Applications, Techniques, Tools, Applications, and Impact, Weinheim, WILEY-VCH Verlag GmbH&Co.KGaA. 2005.
13. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement woth the Folin-Phenol reagents, J Biol Chem. 1951; 193(1): 265-275.
14. Markland S, Markland G. Involvement of the superoxide anion radical in the auto oxidation of pyrogallol and a convenient assay for super oxide dismutase, Eur J Biochem. 1974; 47(3): 469-474.
15. Jain RA. The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. Biomaterials. 2000; 21(23): 2475-2490.
16. Reitman S, Frankel S. *In vitro* determination of transaminases in serum, Am J Clin Pathol. 1957; 28(1): 56-63.
17. Sinha AK. Colorimetric assay of catalase. Anal Biochem. 1972; 47(2): 389-394.
18. Suphiya Parveen, MS, Ranjita Misra, MS, Sanjeeb K. Sahoo Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging, Nanomedicine. Nanomedicine. 2012; 8(2): 147-166.
19. Velanganni AAJ, Balasundaram C. Protective effect of vitamin A, ascorbic acid and Æ-tocopherol on 2, 4-dimethylaminoazobenzene-induced hepatoma in rats. Current Science. 2013; 85(2).
20. Watnasirichaikul Suchat, Nigel M, Davies, Thomas Rades, Ian G. Tucker Preparation of Biodegradable Insulin Nanocapsules from Biocompatible Microemulsions. Pharma Res. 2000; 17(6).
21. WHO, Monographs on the evolution of carcinogenic risk of chemicals to man. 1975; 8.