Acta Scientific Pharmaceutical Sciences (ISSN: 2581-5423)

Volume 7 Issue 7 July 2023

Research Article

Doxorubicin Layer-by-layer Nanoparticles for Controlled Delivery and Effective Treatment of Pancreatic Cancer

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Received: May 02, 2023

Published: June 16, 2023

Abstract

Introduction: Layer-by-layer (LbL) nanoparticles consist of a versatile drug delivery technique, for targeted and controlled delivery. The Doxorubicin (DRC) is being widely used for treatment of pancreatic cancer (PCR). The present study was performed to develop and evaluation LbL nanoparticles (NP's) of DRC to provide controlled and targeted delivery for effective treatment of PCR.

Materials and Method: A liposomal based polymeric pH sensitive NP's were prepared by utilizing LbL techniques. The NP's were composed lipids, poly (b-amino ester) (PAE) and hyaluronic acid (HAC). The physiochemical properties of DRC-LbL NPs was evaluated such as particle size, zeta potential, percent entrapment efficiency (%EE), loading of drug and *In-vitro* drug release behavior. The surface morphology of LbL NP's was evaluated by scanning electronic microscopy (SEM). The *in vitro* release of DRC-LbL-NP's was determined by using Dialysis technique at pH 5 and 7.

Results: Results showed that developed DRC-LbL NP's having average particle size of 182.94 ± 4.11 nm, zeta potential (ZP) of -42.78 ± 3.2mV, higher drug entrapment efficiency (EE) of 74.94 ± 3.45. The *in-vitro* drug release from DRC-LbL NP's exhibited sustained release pattern and it was triggered by lower pH. The surface morphology of developed DRC-LbL NP's exhibited smooth and uniform particle size in nano range. The results from *in-vitro* drug release showed that there was significant difference in amount of DRC release in both pH media. In phosphate buffer pH 7.4 the total amount of DRC release was found to be 44.82% after 48 hour of study whereas in pH 5.0 it was recorded 98.24% after 48 hours.

Conclusion: The results from present study conclude that DRC-LbL NPs might be a promising candidate for the effective treatment of pancreatic cancer.

Keywords: Layer-by-layer; Nanoparticles; Doxorubicin; Controlled Delivery; Targeted Delivery

Introduction

Despite the significant advancement in the field of nanotechnology, radiation therapy and surgery chemotherapy is the most widely used method to treat cancer [1]. A number of anticancer drugs like DRC, Paclitaxel and Camptothecin, have been developed and used in clinical practices in these times [2-4]. However, use of these drugs is restricted because of serious side effects, developed drug resistance and low therapeutic efficacy [5,6]. In order to overcome these restrictions nanoparticle drug delivery systems (NDDS) have been extensively studied like NP's, micelles, liposomes and prodrug [7-9]. These NDDS have ability to deliver both hydrophobic and hydrophilic drugs with poor pharmacokinetic and higher cytotoxicity to cancer site [10]. NDDS in the form of LbL NP's are a new breakthrough in the field of nanoparticle dosage forms. Recent research has shown that LbL NPs have the potential to improve *in-vivo* pharmacokinetics, regulate the release of loading agents, and improve molecular-targeting capabilities [11]. The LbL NP's are comprise of a drug containing core, polyelectrolyte multilayer for controlling release of drug and a stealth layer for targeting drug to cancerous site [12].

The cancerous cells observed with lots of specific properties than the normal cells due to disordered metabolic profiles like weakly acidic environment, high specific enzymes and over expressed proteins [13]. For example in most cancerous cells poor oxygen perfusion causes increased level of lactic acid leading to weakly acidic extracellular environment and intracellular microenvironments [14]. The different research studies have reported that weakly acidic microenvironments of cancerous cells could be utilized as signal for anti-cancerous drug delivery and controlled release [15,16]. Further, different cancerous cells such as breast and lunch carcinoma are over-expressed with cell surface marker CD44. As per reported studies CD44 marker in the layer of cancerous cell epidermis could be specifically identified by HAC [17]. These over expressed CD44 markers on the surface of cancerous cells could be utilized as site-specific receptor for targeted drug delivery [18].

In present study liposomal based polymeric NP''s DRC were prepared by LbL techniques for effective treatment of PCR. The developed DRC-LbL NP's utilized specific microenvironment of cancerous cell and over expression of CD44 receptor on cancerous cells for providing controlled and targeted drug delivery.

Materials and Methods

Materials

DRC was received as kind gift sample from Kwality Pharmaceuticals Ltd (Punjab, India). HA, dimethyl sulfoxide (DMSO), dichloromethane (DCM), tetrahydrofuran (THF), poly (β-amino ester) (PAE) and chloroform were procured from Sigma Aldrich India (Banglore, India). L-alpha-phosphatidylcholine (PCH) and 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) and cholesterol were purchased from Lobachemie Pvt. Ltd. (Mumbai, India). All other ingredients and reagents were used as received.

Methods

Deferential scanning calorimeter (DSC) study

To asses any interaction between drug, lipids DSC analyzes (DSC-60, Shimadzu, Kyoto, Japan) of pure FDP, mixture of drug with individual lipid (in 1:1 ratio) was performed. The samples equivalent to 5 mg of drug was packed in aluminum pan and was analyzed over temperature ranging from 25°C to 350°C with 10°C per minute heating rate.

Preparation of DRC-loaded liposomes

For preparation of DRC loaded liposomes all lipids comprising cholesterol, PCH and POPG in ratio of 3:5:2 were dissolved in solvent mixture of chloroform and methanol in ratio of 2:1 v/v in round bottom flask. Now the solvents from the above mixture were removed by help of rotator evaporator at a temperature 50 °C for 60 minutes. After complete evaporation of organic solvents, a lipid film deposited at surface of round bottom flask, which was hydrated with pH 5 citric acid buffer for 80 minutes under sonication at temperature of 60-70°C. The above solution was filtered through 0.2μ membrane filter, and pH was adjusted to pH 6.5 by help of sodium carbonate. DRC was added (1 mg per 20 mg of liposomal suspension) in sodium chloride solution (0.9%w/v) and loaded to liposomes by pH gradient method as reported by Li., *et al*. 1998 [19]. The final DRC loaded Liposomes were achieved after centrifugation filtration (2 times). The DRC loaded liposomes were kept in amber glass vials at -20°C for further experiments.

The DRC loading was also done at 1 mg per 15 mg of liposomes and 1 mg per 20 mg of liposomes.

Formulation of DRC-LbL NP's

To formulate DRC-LbL NP's, 1 ml of DRC loaded liposomes (2 mg/ml) in phosphate buffer solution (pH 7.4) was mixed with PAE (6 mg) and sonicated for 2-3 minutes. The above solution was purified by process of centrifugation at 3000 x g for 10-20 minutes. The HAC layer was deposited in same way briefly HAC (6 mg) was added to above purified NP's in phopshpate buffer pH 7.4, sonicated for 2-3 minutes, and again purified by centrifugation process at 2000 x g for 10- 20 minutes [20].

The Placebo LbL NP's were also prepared by following same method without using the drug.

Characterization of DRC-LbL-NP's

Estimation of %EE and drug loading (%DL)

The percentage EE and DL of developed NP's was estimated by adding 1 ml of DRC-LbL NP's (2mg/ml) to 20 ml of DMSO with slow stirring. The above mixture was then incubated for 1 hour at room temperature. The drug concentration in sample was determined spcectrophotmetrically by using UV-VIS spectrophotometer (Shimadzu 1800, Japan) at 480 nm. The %EE and DL (%) was calculated by using below equations.

DL (%): Weight of loaded DRC / Weight of DRC-LbL NP's

EE (%): Weight of loaded DRC/ DRC initially added

Particle size and Zeta potential (ZP)

The particle size and ZP of DRC-LbL-NP's were determined by using the Zeta sizer (Malvern zetasizer nano ZS, UK). Sample quantity of 1 ml was then diluted to 10 ml with distilled water. The size determination was carried out in samples after 10 minutes of sonication.

Scanning electron microscopy (SEM)

The shape of prepared DRC-LbL-NP's was determined by using SEM (JEOL-JSM7900F, Tokyo, Japan). A small sample quantity was kept on surface of small metal stubs by help of adhesive tape. The sample coating was done with gold by help of sputter coater before testing. The SEM analysis was done by applying fixed voltages.

DRC-LbL NP's pH sensitivity

To determine the pH sensitivity 1ml of DRC-LbL NP's (2 mg/ml) was re-suspended in phosphate buffers of different pH and incubated for 23 hours at 37°C [21]. The particle size, PDI and zeta ZP of the samples were determined as per section 2.2.4.2.

In-vitro drug release

The *in vitro* release of DRC-LbL-NP's was determined by using Dialysis technique [22]. The 2 ml of DRC-LbL NP's (2mg/ml) was mixed with phosphate buffer pH 5 or pH 7 and the mixture was added to dialysis membrane with molecular weight cut of 25,000 to 35,000 Da. The dialysis membrane was then tied by a thread and immersed in a container containing 50 ml of dissolution medium (phosphate buffer of pH 5 or pH 7). The container was previously placed on an electromagnetic stirrer maintained at 37 ± 0.5°C temperature and dissolution medium was stirred at 100 rpm by electromagnetic bead. 1 ml of sample was withdrawn at given time interval for 48 hours and same amount of dissolution medium was used to replenish it at each time intervals. The samples were estimated spectrophotometrically at λ max 480 nm.

Stability studies

The optimized DRC-LbL NP's was evaluated for stability studies packed in amber color glass vials. The NP's were paced at two temperatures i.e. 2-8°C and 25°C/60% RH for a period of 3 months. Initial evaluation parameters like description, particle size, zeta potential, drug content and percent *in-vitro* drug release was again examined after 3 months of stability study for any significant change [23].

Statistical analysis

All the data were statistically analyzed by using Chi-square test with help of Statistical Package for the Social Sciences 18.0 version. For determination of association, $P < 0.05$ was considered statistically significant.

Results and Discussions

DSC study

The DSC graph of DRC (pure drug) exhibited an endothermic peak at 228.6 °C which consistent with DRC melting temperature

suggesting its crystalline nature [24]. The DSC chromatogram of DRC mixture with all individual lipid (Cholesterole, PCH and POPG) also showed endothermic peak close to DRC i.e. 234.8°C for DRC: cholesterol, 232.4 °C for DRC:PCH and 230.9 °C for DRC:POPG mixture (Figure 1). There was non-significant shift of DRC endothermic peak was observed indicating the compatibility of DRC with used lipids.

Figure 1: DSC chromatograph of DRC (a); DRC: Cholesterol (1:1 ratio) (b); DRC: PCH (c); DRC-POPG (d); DRC-LbL-NP's (e).

The DSC chromatograph of DRC LbL NP's showed that it has melting point more than 40°C which confirms DRC LbL NP's solid form at room temperature. The DSC chromatogram of developed DRC-LbL NP's did not showed any endothermic peak at 228.6°C which is characteristics of DRC, thereby confirming the conversion of crystalline to the amorphous form because of loading in to LbL NP's which happened due to complete solubilization or dispersibility of DRC in the lipid matrix.

Preparation of DRC-LbL NP's

In present study after evaluating different methods of preparation and varying type and concentrations of lipids DRC-LbL NP's were successfully obtained. First DRC loaded liposomes were formulated by pH gradient dependent drug loading method. The PAE and HAC layers were coated on liposome by LbL technique via polyelectric interaction.

Characterization of DRC-LbL NP's

Percent entrapment efficiency (%EE) and drug loading (DL)

The %EE of developed DRC-LbL NP's was observed to increase with increase in liposome weight used for DRC loading suggesting DRC loaded in liposomes were increased. However, DL was found to decrease with increase in liposome weight, it might be due to increase in weight of NP's after deposition of PAE and HAC layers. When 20 mg weight of liposome was used the %EE and DL were reported 76.45% and 3.42% respectively. However when liposomes weight reduced to 15 mg the DL was increased to 4.78% and % EE was reduced to 73.46%. The %EE and DL at 10 mg liposome weight were reported as 57.82% and 5.72% respectively (Table 1). Hence, samples prepared at 15 mg of liposome weight with 1 mg of DRC are used in further study.

Formulation variables	%EE	DL (%)	Particle size (nm)	Zeta Potential (mV)	PDI
Liposomes weight with 1 mg of DRC					
10 mg	57.82	5.72	-	-	-
15 mg	73.46	4.78	-	-	-
20 mg	76.45	3.42	-	-	-
DRC-LbL-NP's parameters after each step					
DRC loaded liposomes	-	-	136.67	-58.25	0.215
After PAE layer	-	-	156.78	+12.78	0.243
After HAC layer	-	-	182.94	-42.78	0.264

Table 1: Characteristic of DRC-LbL NP's.

Particle size and zeta potential (ZP)

The average particle size of DRC loaded liposome was found to be 136.67 nm, the particle size was increased to 156.78 nm after deposition of PAE layer. When HAC layer was deposited the particle size of DRC LBL NP's particle size further increased to 182.94 nm (Table 1, Figure 2). The increase in particle size of NP's confirm the successful deposition of functional layers on NP's [21]. The poly dispersibility index (PDI) value after deposition of each functional layer was determined and it was reported to be below 0.3 in all cases. The PDI value below 0.3 indicated uniform particle size distribution of NP's. The ZP of DRC loaded liposomes were recorded -58.25mV that was significantly increased to +12.78mV after deposition of cationic PAE layer. The ZP further decreased to negative – 42.78 mV again after deposition of anionic HAC layer (Table 1). A complete shift in ZP was observed after depiction of each functional layer confirming successful layering of PAE and HAC layer. The overall negative charge on developed DRC-LbL NP's indicated good physical stability. These findings in our study are similar to study reported by Negi., *et al*. 2013 on Lopinavir SLNP's [25].

Figure 2: Average particle size of DRC-LbL NP's.

SEM characterization

The surface morphology of DRC-Lbl NP's revealed that developed NP's were spherical in shape with smooth surface (Figure 3). This confirmed that use method of preparation was able to successfully achieve LbL NP's with uniformly distributed nanoparticles within nano size limit. These finding are in agreement with the study reported by Gondrala., *et al*. 2015 [26].

Figure 3: SEM photograph of DRC LbL NP's.

DRC-LbL NP's pH sensitivity

The weakly acidic environment could ionize tertiary amino group of PAE, leading to pH sensitivity of DRC-LbL NP's. To investigate the pH sensitivity particle size, PDI and ZP of DRC-LbL NP's was determined at different pH range. The average particle size of DRC-LbL NP's was increased significantly with decrease in pH, particularly in weakly acidic conditions. This observation is might be due to protonation of tertiary amino group of PAE layer resulting in shift of solubility characteristic of PAE layer from hydrophobic to hydrophilic leading to swelling of DRC-LbL NP's [27]. The PDI also showed similar pattern with decrease in pH due to loose and swollen morphology of DRC-LbL NP's. The ZP also increased from negative value it positive with decrease in pH due to ionization of tertiary amino group in PAE layer (Table 2). These findings in our study are in agreement to earlier reported study.

pH of buffer solution	Particle size (nm)	Zeta Potential (mV)	PDI
8	210.89	-54.37	0.271
7.4	182.94	-42.78	0.264
6.5	228.67	+5.34	0.291
6.0	247.48	+8.92	0.316
5.5	273.54	+22.56	0.473
5.0	309.69	+31.72	0.487
4.0	356.78	+38.92	0.592

Table 2: pH sensitivity of DRC LbL NP's.

In-vitro drug release

The *in-vitro* drug release of developed DRC-LbL NP's was carried out by dialysis technique in two different pH conditions phosphate buffer pH 7.4 and weakly acidic pH 5.0 (pH of intracellular cancer cell) for period of 48 hours (Figure 4). The results from *in-vitro* drug release showed that there was significant difference in amount of DRC release in both pH media. In phosphate buffer pH 7.4 the total amount of DRC release was found to be 44.82% after 48 hour of study whereas in pH 5.0 it was recorded 98.24% after 48 hours. This observation was might be due to fact that at pH 7.4 PAE layer was not protonated and DRC main part was well protected in liposomal core of DRC-LbL NP's. However at weakly acidic pH 5.0 PAE layer was completely protonated resulting in solubilization of PAE middle layer and which in turn leads to increased release of DRC from unprotected DRC-LbL NP's. These observations in present study confirm the pH triggered release of DRC from DRC-LbL NP's. Further it also suggest that DRC release could be controlled from DRC-LbL pH sensitive NP's based on weakly acidic pH stimulus [28].

Figure 4: *In-vitro* release of DRC from DRC LbL NP's in two different pH.

Stability studies

The developed LbL NP's of DRC were subjected for stabilities studies at 2-8°C (control) and 25°C/60% RH for a period of 3 months. No significant ($p < 0.05$) was observed in physical parameters and drug release studies indicating stability of the formulation (Table 3). Hence, it can be inferred that developed LbL NPs of DRC are stable at the 25°C/60% RH condition and could be stored for their self-life.

Stability condition 2-8°C				
Formulation	DRC LbL NP's			
Time Point	Initial	1 Month	2 Months	3 Months
Appearance	White to off white slight turbid suspension	White to off white slight turbid suspension	White to off white slight turbid suspension	White to off white slight turbid suspension
% EE of DRC LbL NP's	73.46	72.89	71.42	70.13
Particle size (nm)	182.94	180.23	178.24	177.92
Zeta potential (mV)	-42.78	-41.82	-40.56	-39.45
In-vitro drug release (at 48 hours) %	98.24	97.89	96.88	95.89
Stability condition 25C/60%RH				
Time Point	Initial	1 Month	2 Months	3 Months
Appearance	White to off white slight turbid suspension	White to off white slight turbid suspension	White to off white slight turbid suspension	White to off white slight turbid suspension
% EE of DRC LbL NP's	73.46	71.56	70.12	69.39
Particle size (nm)	182.94	179.45	178.09	176.19
Zeta potential (mV)	-42.78	-40.98	-39.89	-37.91
In-vitro drug release (at 48 hours)	98.24	97.89	96.78	95.19

Table 3: Stability observation of developed DRC LbL NP's.

Conclusion

In present study DRC LbL NP's with pH triggered sensitivity were successfully prepared for targeted and controlled drug release. The DRC was effectively loaded on liposomes, which were further layered with PAE and HAC resulting in formulation of DRC-LbL NP's. DRC-LbL NP's exhibited significantly high uptake and effective anti-tumor activity with no variation in the body weight and DRC release was stimulated by weakly acidic environment. The developed DRC-LbL NP's could be expected to be a promising drug delivery system for cancer treatment. In addition, the LbL technique could be an effective tool to formulate functional liposome-polymer hybrid platform for targeted and controlled drug release.

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