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DESIGN, DEVELOPMENT AND CHARACTERIZATION OF CARFILZOMIB NPDDS

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ABSTRACT

Carfilzomib, a recently FDA-approved proteasome inhibitor, has remarkable anti-myeloma (MM) activity. However, its effectiveness is limited by associated severe side-effects, short circulation half-life, and limited solubility. Here, we report the engineering of liposomal carfilzomib nanoparticles to overcome these problems and enhance the therapeutic efficacy of carfilzomib by increasing tumoral drug accumulation while decreasing systemic toxicity. As an effort to formulate more efficient carfilzomib NPs for systemic administration, this research work is an endeavour to optmize the amount of polymer/ stabilizer, concentration of stabilizer and amount of carfilzomib required to get the ideal NPs using three different biodegradable and biocompatible polymers. The ideal particle size, PCL, PLGA and PLA NPs were prepared and selected for the in-vitro and in-vivo study. PLGA carfilzomib nanoparticles were efficiently taken up by MM cells, demonstrated proteasome inhibition, induced apoptosis, and exhibited enhanced cytotoxicity against MM cells. Based on the results it was observed that quality NPs with 100 % EE, high DC and % recovery were obtained using,20 mg of PCL (PCL/F68/05), 100 mg of PLGA (PLGA/F68/09) and 50 mg of PLA (PLA/F68/07) with 0.5 % PF 68 as stabilizer. The reason for high EE in case of the prepared PNPs may be due to low aqueous solubility of carfilzomib, fast rate of precipitation of polymer during preparation and selection of polymer solvent with high vapour pressure and the low viscosity of the internal phase. The prepared PNPs were characterized for their shape and structure using SEM, TEM and AFM. Taken together, this study establishes the successful synthesis of liposomal carfilzomib nanoparticles that demonstrate improved therapeutic index and the potential to improve patient outcome in MM.

Keywords: Carfilzomib, PLGA, Scanning Electron Microscopy

INTRODUCTION

Design of dosage form is very essential for delivering a drug to achieve effective therapy. Over the years continuous progress is happening in delivery systems. The conventional dosage forms have various shortcomings in therapy. Most important one of these drawbacks is nonselective distribution of drugs, more so serious in cancer chemotheraphy. Carfilzomib is currently marketed as non-aqueous single-dose i.v solution (Taxol®, Bristol-Myers Squibb Co, USA in 5 mL pack) containing 6 mg/mL carfilzomib in 1:1 v/v ratio of Cremophore EL (polyethoxylated castor oil) and dehydrated alcohol. Cremophore EL may produce fatal anaphylactic hypersensitivity reactions, hyperlipidaemia, nephrotoxicity, neuro-toxicity, cardiotoxicity and hypotension at the dosage used in the cancer patients **[1-6]**. In this project attempts have been made to prepare and characterize carfilzomib loaded PLGA, PCL and PLA NPs for better therapy. As an effort to formulate more efficient carfilzomib NPs for systemic administration, this research work is an endeavour to optmize the amount of polymer/ stabilizer, concentration of stabilizer and amount of carfilzomib required to get the ideal NPs using three different biodegradable and biocompatible polymers. The ideal particle size, PCL, PLGA and PLA NPs were prepared and selected for the in-vitro and in-vivo study.

EXPERIMENTAL

Materials and Methods

Carfilzomib was received as gift sample as mentioned below. Polyvinyl alcohol (PVA), (98 % hydrolyzed, molecular weight 13000-23000) were procured from Sigma-Aldrich chemicals, Bangalore, India. Other chemicals are obtained from market and are pharmaceutical and AR grade.

Preparation of PNPs

Carfilzomib loaded PNPs were prepared by, interfacial deposition (nanoprecipitation) and solvent evaporation method (7, 8). These methods were modified according to the present requirement. The PCL and PLA NPs were prepared by nanoprecipitation and PLGA NPs were prepared by solvent evaporation method. In case of nanoprecipitation method, different ratio of carfilzomib and polymer were dissolved in acetone (5 mL) with mild heating and the loss of solvent was adjusted finally. The aqueous phase was prepared by adding different amount (0.25, 0.5, 0.75 and 1 % w/v) of stabilizer (PVA/PF 68) to triple distal water (20 mL).Incase of solvent evaporation method, dichloromethane was used as organic phase to dissolve carfilzomib and polymer. The organic phase was poured into aqueous phase withultra-sonication treatment using a microtip probe sonicator (Microson, Misonix, USA) under controlled temperature (cooling bath maintained at 5°C ± 1.00) with intensity 15 W for 15 min.

Once the organic phase is added to aqueous phase, the milky nanodispersion was formed instantaneously. The formed nanodispersion was continuously stirred for 3 hr using magnetic stirrer (Tarsons, SPINOT digital magnetic stirrer) to evaporate the organic solvent and then the dispersion was subjected to rotavapor (Buchi, Switzerland) at reduced pressure at 40°C for 2 hr to reduce the volume, 10 mL. The nanodispersion was filtered through syringe filter holder (Axiva, India) with 0.44 µm filter membrane (Millipore Co., USA) to remove the free carfilzomib and aggregates. The entire nanodispersion was centrifuged at 14,000 rpm at 15°C for 30 min (Cooling Compufuge, Remi, Mumbai, India). The NPs got settled and supernatant was analyzed for free drug content and the sediment NPs was freeze-dried. Before freeze-drying, pre-freezing of PNPs was done at – 20°C for 18 hr, then the flasks were connected to freeze-drier (Maxi Dry Lyo, Heto, Germany) under vacuum (1 mbar, – 110°C). Freeze-drying was continued until free-flowing PNPs powder was obtained.

Characterization of PNPs

5.4.1 Determination of carfilzomib in PNPs

The EE of the prepared PNPs was determined by direct method. Sediment formed after centrifugation of nanodispersion was digested (30 min) with acetonitrile through sonication (6.5 L Toshibha Laboratory testing instruments, Delhi, India), then suitably diluted with acetonitrile:water (50:50 % v/v) for analysis. The DC in PNPs was determined by taking required amount of freeze-dried PNPs/nanodispersion and digested (30 min) with acetonitrile through sonication. The obtained solution was filtered through 0.44 µm membrane filter and the DC was determined using developed HPLC method. The EE and DC were calculated using standard equation **[1]**.

Surface properties of PNPs

The prepared PNPs were characterized for the size, distribution and PDI by photon correlation spectroscopy using a Zetasizer, Nanoseries (Nano-ZS, Malvern, UK). The surface charge/ZP was measured in water (pH 6.2-7.4) with a Zetasizer, Nanoseries at 25°C. PNPs morphology was examined by scanning electron microscopy (SEM, JSM-7600F), transmission electron microscopy (TEM, Philips, CM200, Netherlands) and atomic force microscopy (AFM, Nanoscope II, USA).

In-vitro dissolution study

To study the release behaviour of PNPs, three millilitre of nanodispersion was spiked into a dialysis bag (Spectrapor, molecular weight cut off: 12,500 Da, USA) which is then sealed and dropped into a beaker containing 200 mL of phosphate buffer (pH 7.4) with 0.5 % (w/v) Tween The whole experiment set-up was placed in a shaker maintained at temperature 37°C±2 with continuous horizontal shaking at 100 rpm. Samples were withdrawn from the beaker at predetermined time points, with

spontaneous replacement of fresh buffer media. Carfilzomib release from the PNPs into the medium was estimated by developed HPLC method. The obtained dissolution data were fitted to various kinetic equations, Zero order, First order, Higuchi, Korsmeyer-Peppas, Hixson-Crowell and Baker-Lonsdate model to find the order and mechanism of carfilzomib release from the PNPs **[9]**.

Stability study

US-FDA has not framed any specific guidelines for NPDDS, production, characterization, handling and use. There is no protocol and limits available from any international bodies to conduct stability studies of these formulations. In the present work, the stability study was performed by keeping the NPs samples in three different conditions, room temperature (15 ± 5°C), refrigerator (5 ± 2°C) and at 37 ± 5 °C over a period of 4 months. The NPs were evaluated at 0, 2 and 4 months for there size, PDI, ZP, DC and in-vitro dissolution. In addition any change in physical appearances was observed and the samples were characterized for their shape and structure using AFM.

RESULT AND DISCUSSION

Preparation of NPs

Based on the initial experiment, organic to aqueous phase ratio, 1:4 was fixed constant throughout the study when other formulation parameters were changed. Carfilzomib loaded PCL and PLA NPs were prepared successfully using simple and fast nanoprecipitation method which is the most reproducible and economical method among all other methods. The nanoprecipitation method produces stable, small and narrow size NPs without using any toxic chlorinated solvents. carfilzomib loaded PLGA NPs were prepared by solvent evaporation method which is an ideal method to incorporate lipophilic drugs and it was the first method developed to prepare NPs. In the nanoprecipitation method when acetone solution of polymers and drug was added to the aqueous phase, there was rapid gradient-driven diffusion of amphiphilic acetone across the interface, which creates a kind of instability at interface and there was marked decrease in the interfacial tension which resulted in the spontaneous emulsification of the acetone solution in the form of nanodroplets.

The formed nanodroplets which contain the dissolved polymer and drug (PCL and PLA) will aggregate as NPs, because of the spontaneous diffusion of acetone and presences of nonsolvent medium (water) to the dissolved polymer. This initial precipitation of polymer forms polymeric nano matrix, further evaporation of the surface solvent by stirring or rotavapor resulted in solidification of the NPs formed **[1]**. In nanoprecipitation method, acetone was selected because it solubilized carfilzomib and PCL and PLA effectively and it took very less time to evaporate acetone during hardening of NPs, which is a very critical step in NPs preparation **[1]**. In case of

emulsion and solvent evaporation method dichloromethane was selected because it has very low water solubility of 2 % w/v when compare to other solvents like ethyl acetate (8.7 % w/v) which is used very often **[10, 11]**.

To get high value of drug encapsulation, the challenging issue in the NPs preparation protocol is to avoid the diffusion of drug along with the solvent to the other phase. The role of surfactant or stabilizer (PVA/PF 68) in nanoprecipitation process is it lowers interfacial energy and helps in the rapid diffusion of acetone to the aqueous phase. The above instantaneous process controls the size of polymer precipitation into NPs. Hence the finest amount of stabilizer in the formula will result in instant and reproducible NPs formation with low PDI with high drug loading capacity **[12]**. Recently in our group, etoposide loaded PLGA NPs wee prepared with very small size, 105.1 ± 2.38 nm with high EE, 78.99 ± 1.04 % by nanoprecipitation technique and prepared PCL NPs were prepared by emulsification and solvent evaporation method using PF 68 as stabilizer with 257 ± 3.96 nm and the EE was 80.15 ± 1.01% respectively **[7]**.

Physiochemical characterization of PNPs

a) Surface properties of PNPs

The detail morphological properties of the prepared PNPs were investigated and presented using the sophisticated microscopic techniques, SEM (Fig. 5.1), TEM **(Fig. 5.2)** and AFM **(Fig. 3)**. The SEM image shows that all the prepared PNPs were spherical in shape with homogeneous solid matrix structure with no evidences of aggregation and crystals of carfilzomib on the surface. From the TEM image, it was evident that the prepared NPs were in fine globular profile and close view of a single NP **(Fig. 5.2)** clearly showed the various degree of smooth structure without any amorphous arrangement. Because of small size of the NPs the close investigation of single particle was done using AFM which gave clear 3D morphological images **(Fig..3)**. The 3D view of multiple particles showed smooth surface, but the single particles showed some roughness on their surface which is physical evidence for diffusion and matrix erosion drug release mechanism **[7, 8]**.

Influences of polymer amount on NPs characterization

a) PCL amount vs. NPs characterization

The effect of polymer, PCL, PLGA and PLA amount on the mean particle size, DC and EE were shown in **Fig. 4**. In PCL NPs, when the polymer amount is increased from 1 (PCL/F68/01) to 3 (PCL/F68/03) mg, there was no significant increase in the size but the PDI was increased (0.04 to 0.12) considerably **(Fig. 5)**. There was no linear (r^2 = 0.7792) increase in particle size when the polymer amount was increased from 1to 200 mg for 1mg of carfilzomib with 0.5 % stabilizer. When the PCL amount was increased from 5 to 40 mg **(Table 1)**, there was significant increase in particle size 135.32 ± 4.99 nm,

157.63 ± 2.34 nm, 174.02 ± 9.34 nm and 187.32 ± 1.35 nm with increase of polymer amount. There was effect on particle size when amount of polymer increased beyond 50 mg. In case of 50 mg of PCL, the particle size observed was 191.04 ± 5.63 nm, and when the polymer amount is doubled to 100 mg (PCL/F68/09), the particle size was only, 203.33 ± 14.01 nm **(Fig.5)**.

However, in present work no such carfilzomib crystals were found on surface of NPs and surface were smooth. Amount of surfactant (PF 68) was also required in much lesser amount (0.5 %) to get NPs. Similarly, in another study by Chakravarthi *et al*. **[15]** produced PLGA NPs by solvent evaporation method using 90 mg of PLGA and obtained larger average particle size (315 nm) where as present method produces NPs of smaller size nanoparticles.

b) PLA amount vs. NPs characterization

In PLA NPs formulations PLA/F68/01 to PLA/F68/13, increase in polymer amount (1 to 200 mg) for 1 mg drug, was found to have linear relationship **(Fig. 5)** with increase in particle size (r^2 = 0.9636) **(Table 3)**. The PDI of the formulations PLA/F68/01 to PLA/F68/13 was less than one, which suggests the homogeneous polydisperse NPs. As like other two polymers, PLA also decreased EE beyond 50 mg of polymer **(Fig.4)**. This confirmed that the polymer amount is the critical parameters in the formulation of polymeric NPs. Using beyond 50 mg of polymer, there were decrease in EE and % recovery **(Fig. 4 and 5)**.

The particle size of PLA/F68/07 formation was 151.23 ± 0.73 nm whose PLA amount was 50 mg, but when the polymer amount was doubled to 100 mg, the obtained particle size was 170.42 ± 6.11 nm. The EE of the formulation PLA/F68/07 was 89.32 ± 1.03 %, but it was decreased to 65.38 ± 1.13 % when the polymer amount was doubled. In case of formulation PLA/F68/13, where the polymer amount was 200mg, there was decrease in EE, 50.05±0.08 % and the particle size, 220.93 ± 11.05 nm. This once again showed that addition of more than 50 mg PLA will be surplus and it will increase the cost of formulation unnecessarily. The DC of prepared formulations was represented in **Table 3**. Already we have showed that, increase in particle size when there was increase in polymer amount in the etoposide loaded polymeric NPs and few other authors were reported this effect in the biodegradable polymers [**7, 10]**. In all the polymers, general observation is that, EE increased with the increase in polymer amount up to a certain limit above which EE decreased **(Fig.4)**. This may be due to insufficient stabilizer with respect increased polymer, which led to agglomeration of NPs and settling at the bottom as agglomerate **(Fig. 5)**.

The influences of polymer amount, nature of stabilizer and its concentration **(Table 1 to 6)** on ZP of the prepared NPs formulations are

give in **Fig 5**. In our study, both the stabilizers PF 68 and PVA imparted negative ZP value; this was due to the presences of terminal carboxylic groups and ionization of groups on the side chain of polymer and surfactant. PF 68 adsorb strongly onto the surface of hydrophobic particles (e.g. PLGA) through their centre block hydrophobic poly (propyl oxide), which leaves the hydrophilic poly (ethyl oxide) side arms in movable state and which extent outwards from the particle surface **[10, 13]**. This hydrophilic tails on each particle surface results in the repulsion effect and provides stability to the system through the steric stabilization with enthalpic and entropic contributions. In case of the PVA, the hydroxyl group in the side chain resulted in the negative ZP value and it was totally a hydrophilic polymer which formed covalent linkage with the polymer through the hydroxyl group and hence it remained attached to the particles surface by forming multipoint linkage **[12]**. In the present NPs preparation method, when the polymer amount was increased, the ZP ranged from, -16.63 to -28.92 for PCL NPs, -19.78 to -25.23 for PLGA NPs and -26.1 to -29.8 for PLA NPs **(Table 5)**.

In case of PCL and PLANPs, stabilizer PF68 produced higher ZP (ranged from 28.24 to-34.62 and-30.24 to -41.53) than PVA which produced ZP ranged for-17.72to-26.03 and 28.11 to -32.72. In case of PLGA NPs, stabilizer PF 68 and PVA produced stable NPs with same ZP value **(Table 4 to 6)**.

5.9 Stability study

The stability study result showed that all the prepared NPs, were stable up to 4 months at 5 ± 2°C and 15 ± 5°C. All the NPs stored at 37 ± 5 °C for 4 months showed some increase in size, due to aggregation of particles **(Table 5.8**). The AFM analysis showed **(Fig. 5.6)** no change of surface character of stored NPs. The in-vitro dissolution study of all stored formulations confirmed no change in release profile. The stability study result confirmed that the prepared PNPs were stable in all the three temperatures tested.

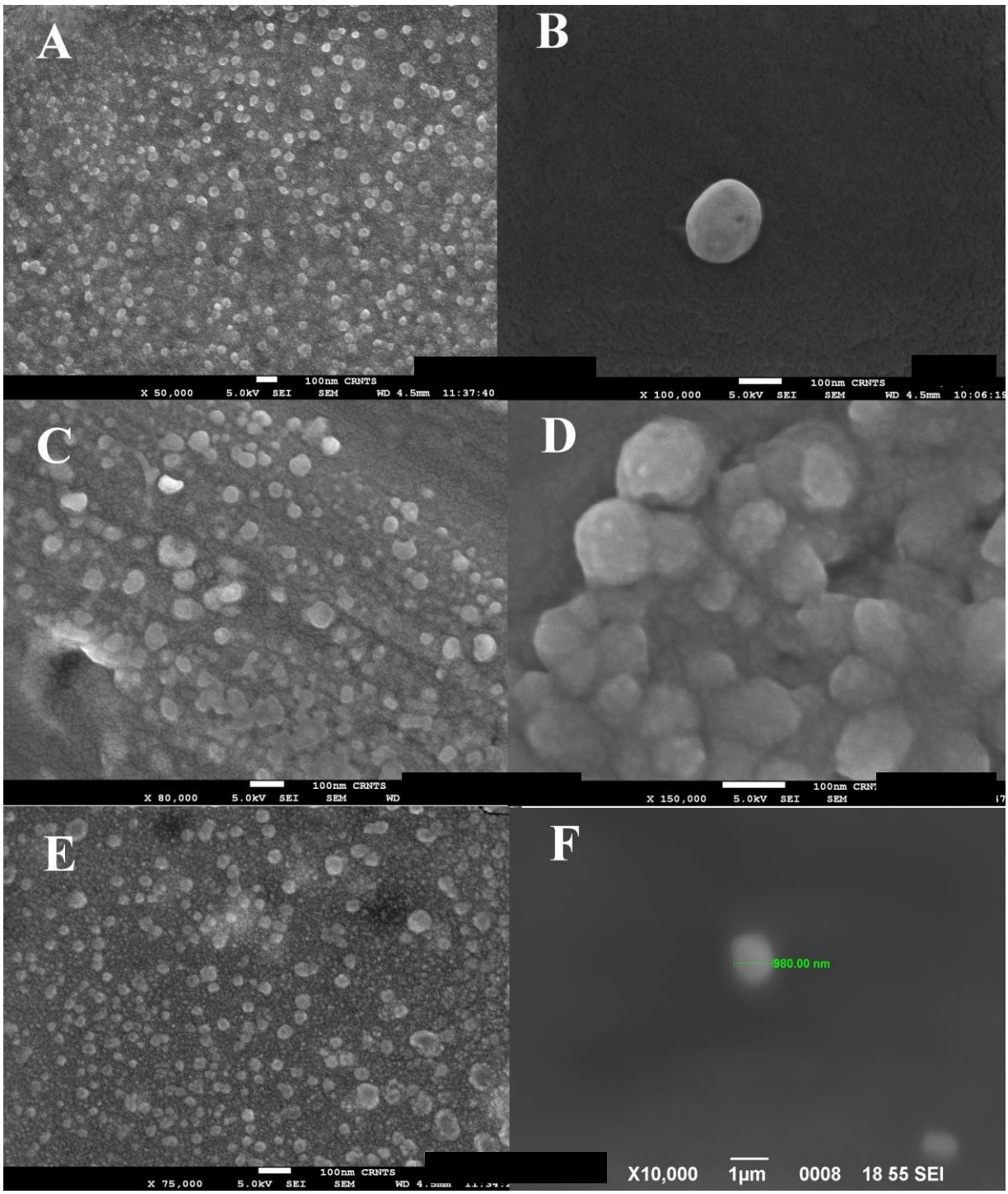

Fig.1: Characterization of carfilzomib loaded PNPs by SEM, cluster and single PCL NPs (A and B), cluster PLGA NPs (C and D) and cluster and single PLA NPs (E and F)

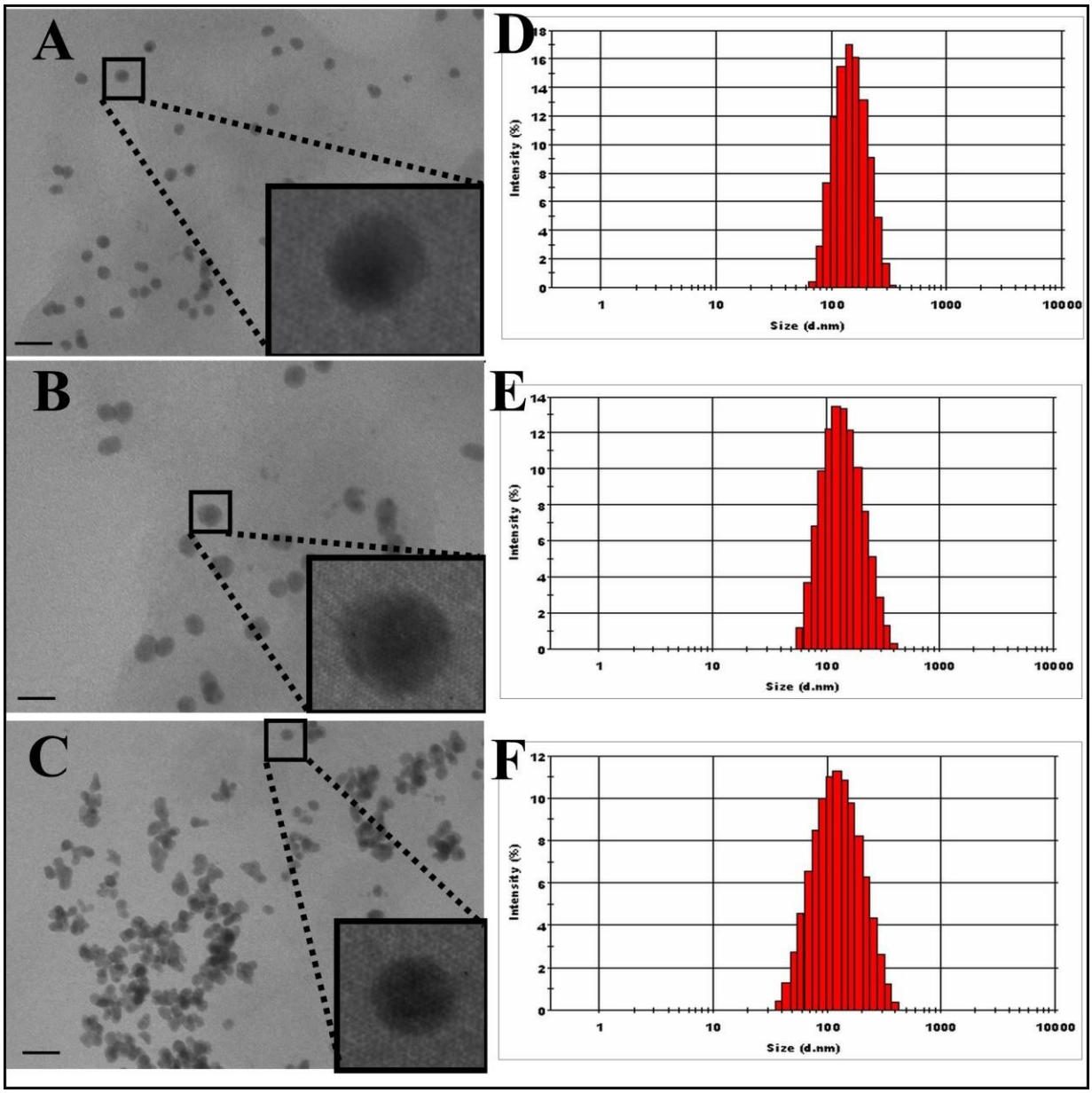

Fig. 2: Characterization of Carfilzomib loaded PNPs by TEM, PCL NPs (A), PLGA NPs (B) PLA NPs (C), particle size distribution of PCL NPs (D), PLGA NPs (E) and PLA NPs (F), bar represents 100 nm

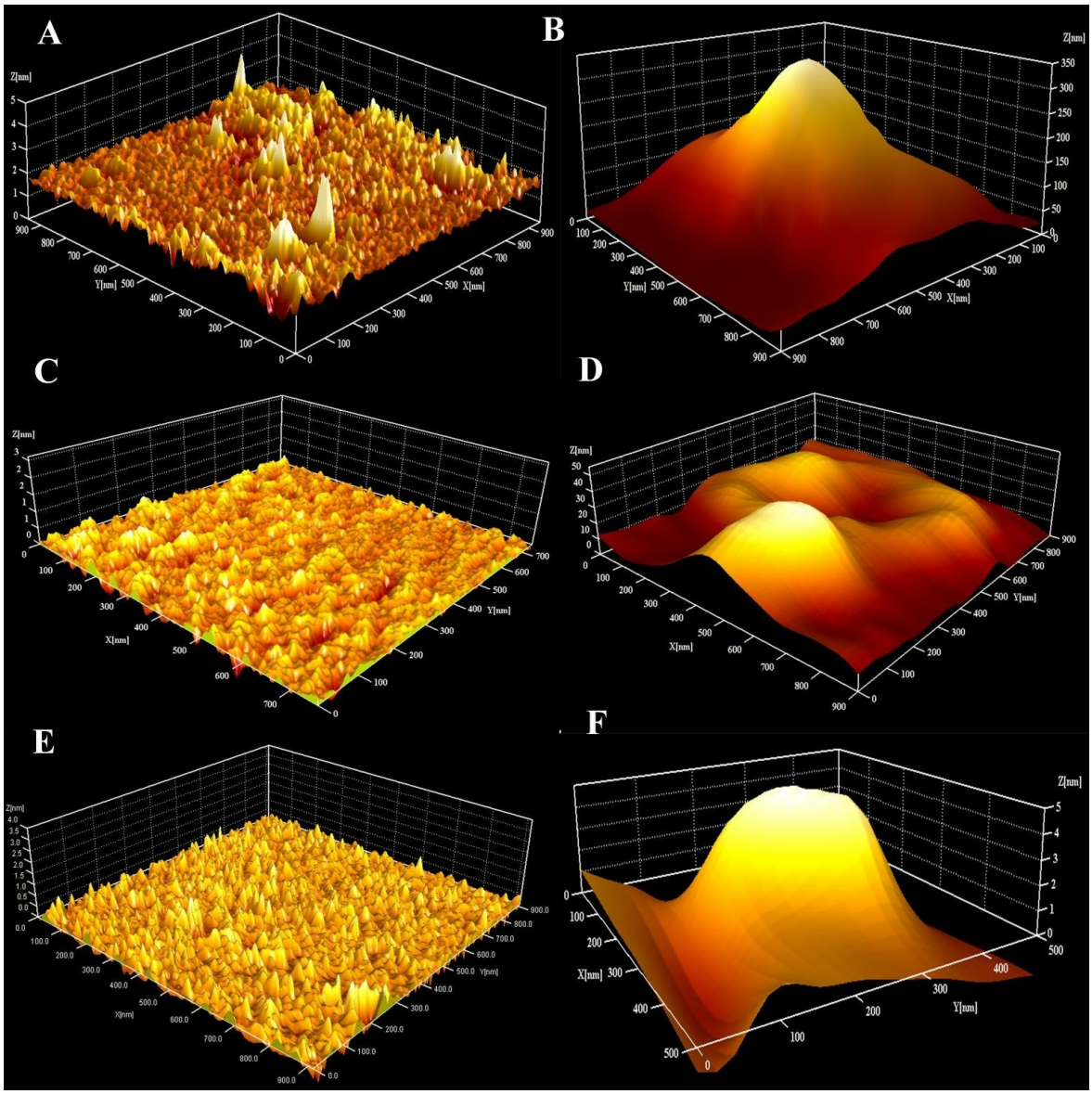

Fig. 3: Characterization of carfilzomib loaded PNPs by AFM, cluster and single PCL NPs (A and B), cluster PLGA NPs (C and D) and cluster and single PLA NPs (E and F)

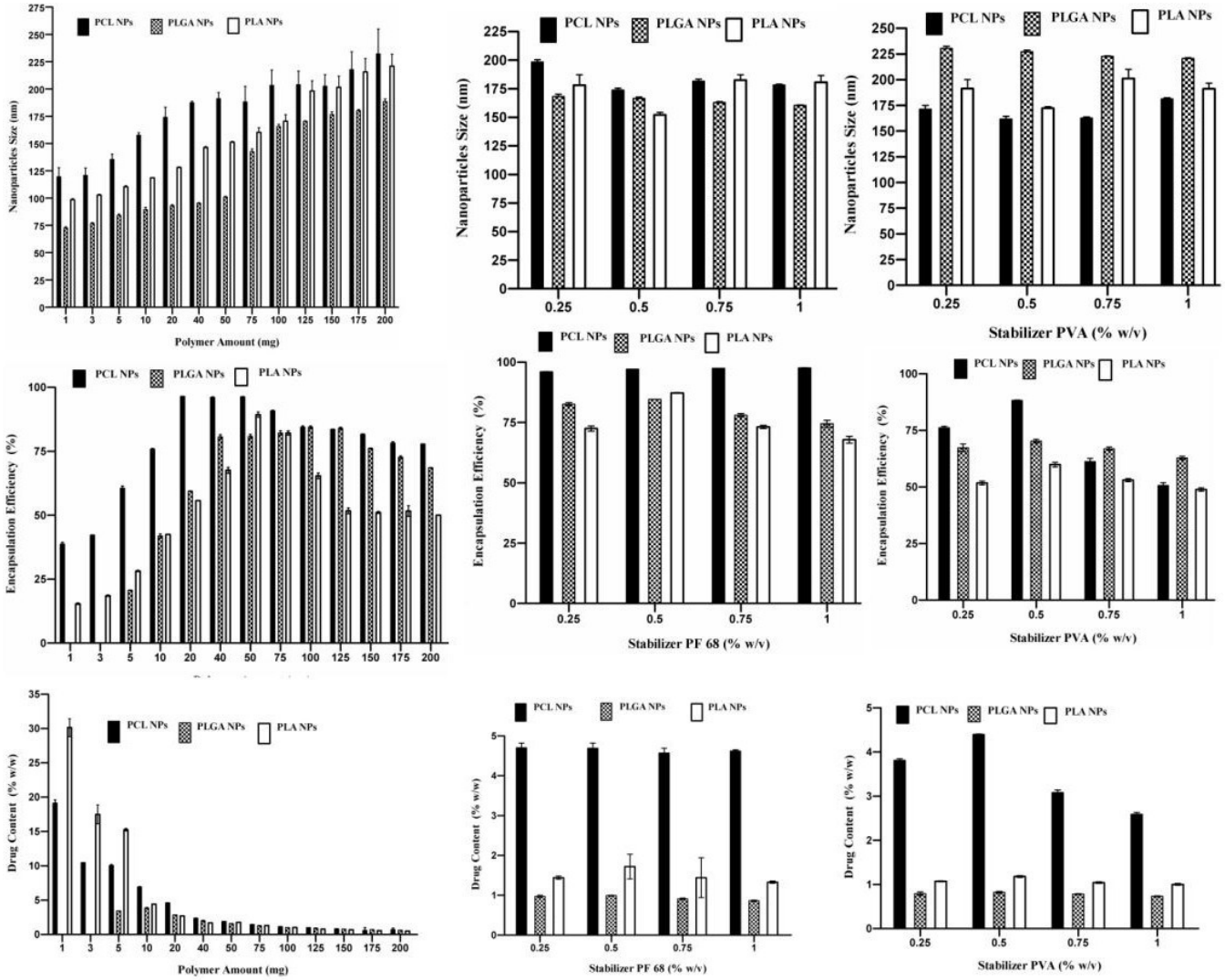

Fig. 4: Influences of polymer amount (A, D and G), PF 68 amount (B, E and H) and PVA amount (C, F and I) on nanoparticle size, EE and DC of carfilzomib loaded PNPs

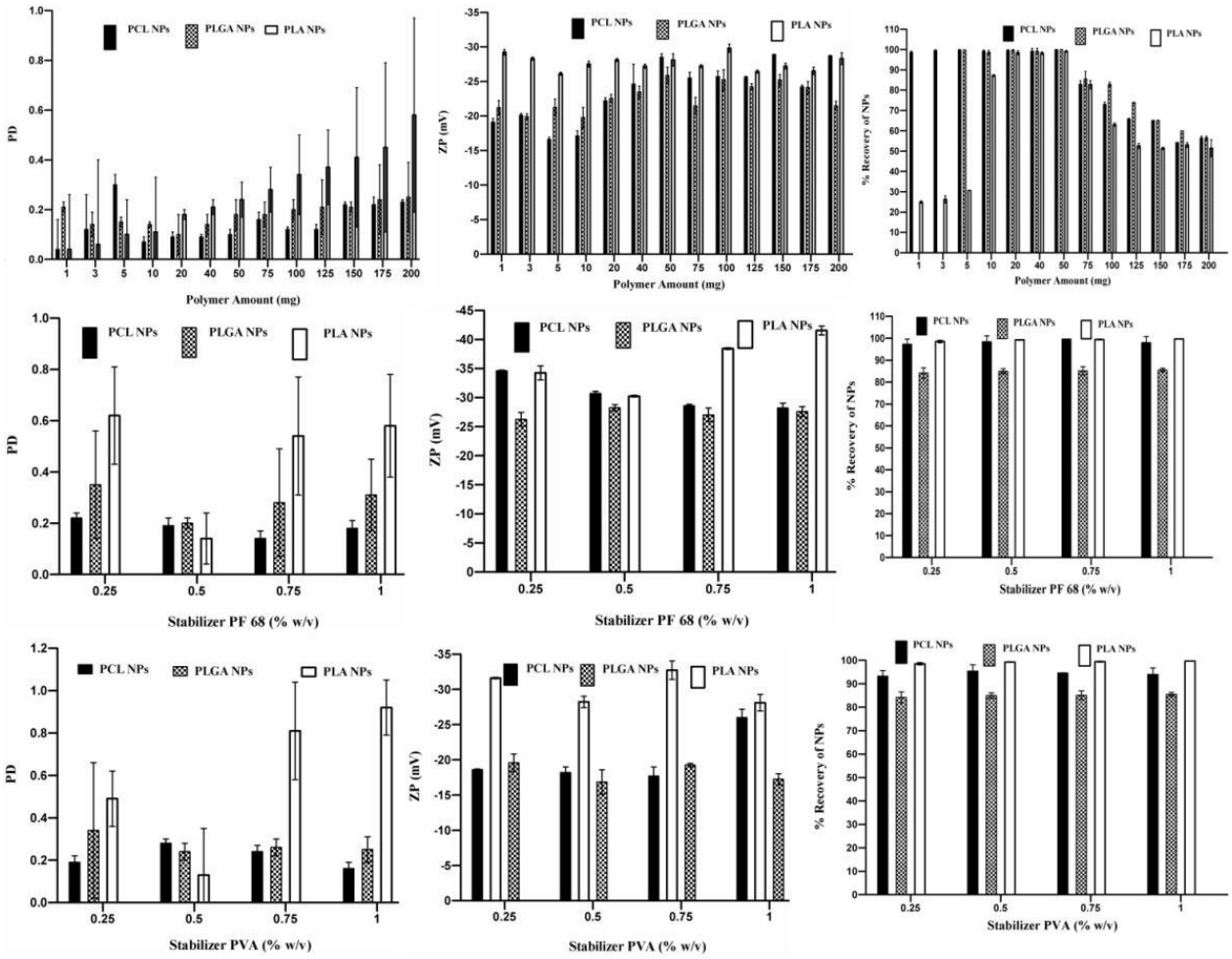

Fig. 5: Influences of polymer (A, B and C), PF 68 (D, E and F) and PVA amount (G, H and I) on PDI, ZP and % recovery of NPs of carfilzomib loaded PNPs

Table 1: Composition and characterization of carfilzomib loaded PCL NPs with variable concentration of polymer PCL*

Batch Code	Drug (mg)	Polymer (mg)	PF 68 (%w/v)	Mean Size (nm ±SD)	Distribution (d. nm)	[a]PDI ±SD	[b]ZP ±SD	[c]EE (%)	[d]DC (% w/w)
PCL/F68/01	1	1	0.5	119.51 ± 8.32	35-250	0.04 ± 0.12	-19.13 ± 0.52	38.68 ± 0.74	19.14 ± 0.47
PCL/F68/02	1	3	0.5	120.83 ± 6.73	35-220	0.12 ± 0.14	-20.14 ± 0.24	42.20 ± 0.08	10.46 ± 0.03
PCL/F68/03	1	5	0.5	135.32 ± 4.99	68–255	0.30 ± 0.04	-16.63 ± 0.31	60.54 ± 0.91	10.03 ± 0.16
PCL/F68/04	1	10	0.5	157.63 ± 2.34	68–342	0.07 ± 0.02	-17.12 ± 0.72	75.78 ± 0.37	6.91 ± 0.06
PCL/F68/05	1	20	0.5	174.02 ± 1.34	79–396	0.09 ± 0.02	-22.22 ± 0.42	96.40 ± 0.13	4.60 ± 0.02
PCL/F68/06	1	40	0.5	187.32 ± 1.35	91-459	0.09 ± 0.01	-24.62 ± 2.89	96.02 ± 0.23	2.36 ± 0.04
PCL/F68/07	1	50	0.5	191.04 ± 5.63	91-459	0.10 ± 0.02	-28.52 ± 0.49	96.25 ± 0.20	1.89 ± 0.02
PCL/F68/08	1	75	0.5	188.13 ± 14.29	68-825	0.16 ± 0.03	-25.54 ± 0.81	90.75 ± 0.30	1.44 ± 0.03
PCL/F68/09	1	100	0.5	203.33 ± 14.01	68-531	0.12 ± 0.01	-25.74 ± 0.81	84.37 ± 0.50	1.14 ± 0.02
PCL/F68/10	1	125	0.5	203.90 ± 12.44	68-615	0.12 ± 0.02	-25.63 ± 0.12	83.55 ± 0.09	1.01 ± 0.01
PCL/F68/11	1	150	0.5	202.52 ± 10.59	68-615	0.22 ± 0.01	-28.92 ± 0.01	81.64 ± 0.18	0.83 ± 0.02
PCL/F68/12	1	175	0.5	217.73 ± 16.43	58-955	0.22 ± 0.03	-24.24 ± 0.25	78.17 ± 0.54	0.57 ± 0.43
PCL/F68/13	1	200	0.5	232.13 ± 22.85	59-825	0.23 ± 0.01	-28.72 ± 0.06	77.82 ± 0.08	0.69 ± 0.25

*Each data represents the average and standard deviation of three independent determinations, [a]Polydispersity index, [b]Zeta potential, [c]Encapsulation Efficiency, [d]Drug Content

Table 2: Composition and characterization of carfilzomib loaded PLGA NPs with variable concentration of polymer PLGA*

Batch Code	Drug (mg)	Polymer (mg)	PF 68 (% w/v)	Mean Size (nm ±SD)	Distribution (d. nm)	[a]PDI ±SD	[b]ZP ±SD	[c]EE (%)	[d]DC (% w/w)
PLGA/F68/01	1	1	0.5	72.56 ± 1.21	40-250	0.21 ± 0.02	-21.21 ± 1.01	-	-
PLGA/F68/02	1	3	0.5	76.63 ± 0.89	50-225	0.14 ± 0.05	-19.89 ± 0.41	-	-
PLGA/F68/03	1	5	0.5	84.12 ± 1.24	45-225	0.15 ± 0.02	-21.24 ±1.20	20.67 ± 0.07	3.43 ± 0.01
PLGA/F68/04	1	10	0.5	89.41 ± 2.26	50-250	0.14 ± 0.01	-19.78 ± 1.45	41.84 ± 0.82	3.84 ± 0.10
PLGA/F68/05	1	20	0.5	92.96 ± 1.01	45-275	0.10 ± 0.08	-22.52 ± 0.58	59.38 ± 0.13	2.83 ± 0.02
PLGA/F68/06	1	40	0.5	95.22 ± 0.56	50-250	0.14 ± 0.04	-23.45 ± 0.85	80.60 ± 0.89	1.98 ± 0.10
PLGA/F68/07	1	50	0.5	100.85 ± 0.85	50-300	0.18 ± 0.06	-25.85 ± 1.20	80.76 ± 0.83	1.58 ± 0.02
PLGA/F68/08	1	75	0.5	142.52 ± 2.56	42-275	0.18 ± 0.05	-21.45 ± 1.23	82.11 ± 0.86	1.26 ± 0.05
PLGA/F68/09	1	100	0.5	165.52 ± 2.10	50-300	0.20 ± 0.04	-25.23 ± 1.45	84.37 ± 0.50	1.01 ± 0.01
PLGA/F68/10	1	125	0.5	170.29 ± 0.45	58-325	0.21 ± 0.11	-24.21 ± 0.45	83.92 ± 0.44	0.90 ± 0.05
PLGA/F68/11	1	150	0.5	176.44 ± 2.56	60-400	0.21± 0.12	-25.21 ± 0.78	76.09 ± 0.18	0.77 ± 0.01
PLGA/F68/12	1	175	0.5	180.11 ± 1.05	42-325	0.24 ± 0.14	-24.12 ± 0.85	72.62 ± 0.54	0.69 ± 0.01
PLGA/F68/13	1	200	0.5	188.45 ± 2.45	40-350	0.25 ± 0.14	-21.48 ± 0.65	68.56 ± 0.08	0.60 ± 0.02

*Each data represents the average and standard deviation of three independent determinations, [a]Polydispersity index, [b]Zeta potential, [c]Encapsulation Efficiency, [d]Drug Content

Table 3: Composition and characterization of carfilzomib loaded PLA NPs with variable concentration of polymer PLA*

Batch Code	Drug (mg)	Polymer (mg)	PF 68 (% w/v)	Mean Size (nm ±SD)	Distribution (d. nm)	[a]PDI ±SD	[b]ZP ±SD	[c]EE (%)	[d]DC (% w/w)
PLA/F68/01	1	1	0.5	98.42 ± 1.02	40-450	0.04 ± 0.22	-29.2 ± 0.42	15.37 ± 0.34	30.13 ± 1.30
PLA/F68/02	1	3	0.5	102.82 ± 0.73	45-390	0.06 ± 0.34	-28.3 ± 0.24	18.52 ± 0.27	17.49 ± 1.37
PLA/F68/03	1	5	0.5	110.31 ± 0.99	55–450	0.10 ± 0.14	-26.1 ± 0.21	28.19 ± 0.29	15.25 ± 0.18
PLA/F68/04	1	10	0.5	118.63 ± 0.24	65–500	0.11 ± 0.22	-27.5 ± 0.42	42.47 ± 0.10	4.41 ± 0.02
PLA/F68/05	1	20	0.5	128.14 ± 0.48	80–450	0.18 ± 0.02	-28.1 ± 0.22	55.70 ± 0.14	2.69 ± 0.02
PLA/F68/06	1	40	0.5	146.33 ± 1.01	90-525	0.21 ± 0.03	-27.2 ± 0.29	67.58 ± 1.18	1.68 ± 0.02
PLA/F68/07	1	50	0.5	151.23 ± 0.73	90-450	0.24 ± 0.07	-28.1 ± 0.89	89.32 ± 1.03	1.77 ± 0.02
PLA/F68/08	1	75	0.5	160.23 ± 4.29	55-500	0.28 ± 0.09	-27.2 ± 0.21	82.11 ± 0.86	1.31 ± 0.04
PLA/F68/09	1	100	0.5	170.42 ± 6.11	60-575	0.34 ± 0.16	-29.8 ± 0.61	65.38 ± 1.13	1.03 ± 0.03
PLA/F68/10	1	125	0.5	198.23 ± 9.14	65-600	0.37 ± 0.15	-26.4 ± 0.22	51.70 ± 1.14	0.78 ± 0.04
PLA/F68/11	1	150	0.5	201.52 ± 10.19	65-650	0.41 ± 0.28	-27.2 ± 0.41	51.05 ±0.42	0.66 ± 0.01
PLA/F68/12	1	175	0.5	215.63 ± 12.13	50-750	0.45 ± 0.34	-26.5 ± 0.55	51.64 ± 2.07	0.55 ± 0.02
PLA/F68/13	1	200	0.5	220.93 ± 11.05	55-700	0.58 ± 0.39	-28.3 ± 0.86	50.05 ± 0.08	0.49 ± 0.04

*Each data represents the average and standard deviation of three independent determinations, [a]Polydispersity index, [b]Zeta potential, [c]Encapsulation Efficiency, [d]Drug Content

Table 4: Composition and characterization of carfilzomib loaded and blank PCL NPs with variable concentration of stabilizers

Batch Code	Drug (mg)	Polymer (mg)	Stabilizer (% w/v)		Mean Size (nm ±SD)	Distribution (d. nm)	[a]PDI ±SD	[b]ZP ±SD	[c]EE (%)	[d]DC (% w/w)
			PF 68	PVA						
PCL/F68/14	1	20	0.25	-	198.32 ± 2.05	91–459	0.22 ± 0.02	-34.62 ± 0.10	96.04 ± 0.02	4.70 ± 0.12
PCL/F68/15	1	20	0.5	-	173.81 ± 1.64	91–342	0.19 ± 0.03	-30.72 ± 0.36	97.09 ± 0.01	4.69 ± 0.13
PCL/F68/16	1	20	0.75	-	181.72 ± 1.82	79–459	0.14 ± 0.03	-28.62 ± 0.25	97.45 ± 0.01	4.57 ± 0.12
PCL/F68/17	1	20	1	-	178.22 ± 0.84	79–397	0.18 ± 0.03	-28.24 ± 0.85	97.64 ± 0.01	4.62 ± 0.03
PCL/F68/18	1	20	0.5	-	184.03 ± 0.34	80–400	0.07 ± 0.02	-28.21 ± 0.42	95.38 ± 0.20	4.84 ± 0.04
PCL/F68/19	2	20	0.5	-	182.24 ± 1.35	90-550	0.28 ± 0.03	-27.31 ± 0.34	96.07 ± 0.18	8.91 ± 0.14
PCL/F68/20	3	20	0.5	-	185.42 ± 0.67	90-500	0.26 ± 0.01	-28.42 ± 0.27	97.05 ± 1.34	12.87 ± 0.06
PCL/F68/21	4	20	0.5	-	184.51 ± 0.32	82-550	0.54 ± 0.41	-30.23 ± 0.87	97.26 ± 1.78	16.61 ± 0.59
PCL/F68/22	5	20	0.5	-	186.42 ± 1.01	90-450	0.45 ± 0.61	-30.83 ± 0.69	98.2 5 ± 0.02	19.99 ± 0.24
PCL/PVA/23	1	20	-	0.25	171.31 ± 3.60	91–342	0.19 ± 0.03	-18.64 ± 0.10	76.17 ± 0.69	3.81 ± 0.04
PCL/PVA/24	1	20	-	0.5	161.71 ± 2.78	79–295	0.28 ± 0.02	-18.21 ± 0.80	88.19 ± 0.24	4.40 ± 0.01
PCL/PVA/25	1	20	-	0.75	162.72 ± 1.02	79–342	0.24 ± 0.03	-17.72 ± 1.30	61.24 ± 1.40	3.08 ± 0.06
PCL/PVA/26	1	20	-	1	181.24 ± 1.30	91–396	0.16 ± 0.03	-26.03 ± 1.17	50.64 ± 1.30	2.59 ± 0.04
PCL/PVA/27	1	20	-	0.5	163.13 ± 1.08	91-350	0.09 ±0.51	-20.12± 1.10	86.70 ± 0.02	4.40 ± 0.03
PCL/PVA/28	2	20	-	0.5	165.62 ± 1.09	91-350	0.13 ±0.25	-19.56± 0.95	86.71 ± 0.02	8.04 ± 0.13
PCL/PVA/29	3	20	-	0.5	161.82 ± 2.05	91-350	0.42 ±0.65	-18.45± 1.32	89.46 ± 0.02	11.86 ± 0.11
PCL/PVA/30	4	20	-	0.5	166.52 ± 1.06	91-350	0.06 ±0.89	-22.52± 0.56	90.06 ± 0.02	15.38 ± 0.28
PCL/PVA/31	5	20	-	0.5	166.12 ± 2.34	80-325	0.08 ±0.54	-20.12± 0.75	96.83 ± 0.01	19.70 ± 0.23
BPCL/F68/01	-	50	0.25	-	181.42 ± 2.21	90-500	0.11 ± 0.03	-41.02 ± 4.11	-	-
BPCL/F68/02	-	50	0.50	-	147.42 ± 3.65	70-350	0.15 ± 0.03	-29.02 ± 1.65	-	-
BPCL/F68/03	-	50	0.75	-	205.61 ± 2.32	50-450	0.21 ± 0.03	-27.13 ± 1.45	-	-
BPCL/F68/04	-	50	1	-	197.61 ± 3.08	50-400	0.14± 0.03	-28.33 ± 1.27	-	-

*Each data represents the average and standard deviation of three independent determinations, [a]Polydispersity index, [b]Zeta potential, [c]Encapsulation Efficiency, [d]Drug Content

Table 5 Composition and characterization of carfilzomib loaded and blank PLGA NPs with variable concentration of stabilizers

Batch Code	Drug (mg)	Polymer (mg)	Stabilizer (% w/v)		Mean Size (nm ±SD)	Distribution (d. nm)	[a]PDI ±SD	[b]ZP ±SD	[c]EE (%)	[d]DC (% w/w)
			PF 68	PVA						
PLGA/F68/14	1	100	0.25	-	168.21 ± 2.10	40-350	0.35 ± 0.21	-16.25 ± 1.21	82.53 ± 0.66	0.97 ± 0.03
PLGA/F68/15	1	100	0.5	-	166.56 ± 1.20	40-365	0.20 ± 0.02	-18.24 ± 0.52	84.60 ± 0.01	0.99 ± 0.01
PLGA/F68/16	1	100	0.75	-	162.89 ± 0.85	50-285	0.28 ± 0.21	-20.01 ± 1.20	78.01 ± 0.70	0.91 ± 0.02
PLGA/F68/17	1	100	1	-	160.52 ± 0.41	40-300	0.31 ± 0.14	-17.58 ± 0.85	74.50 ± 1.45	0.86 ± 0.02
PLGA/F68/18	1	100	0.5	-	169.12 ± 1.01	55-275	0.18 ± 0.08	-20.01 ± 0.56	84.06 ± 1.72	0.99 ± 0.03
PLGA/F68/19	2	100	0.5	-	172.54 ± 0.75	45-350	0.19 ± 0.16	-19.25 ± 1.24	88.15 ± 0.63	2.08 ± 0.02
PLGA/F68/20	3	100	0.5	-	178.52 ± 0.71	40-350	0.21 ± 0.24	-15.24 ±1.56	88.31 ± 1.68	3.11 ± 0.06
PLGA/F68/21	4	100	0.5	-	180.12 ± 1.02	50-325	0.23 ± 0.41	-18.45 ± 0.85	90.89 ± 1.43	4.23 ± 0.09
PLGA/F68/22	5	100	0.5	-	184.41 ± 0.76	30-300	0.23 ± 0.04	-20.14 ± 0.96	90.65 ± 1.62	5.24 ± 0.07
PLGA/PVA/23	1	100	-	0.25	230.41 ± 2.11	55-325	0.34 ± 0.32	-19.58 ± 1.25	67.32 ± 1.66	0.79 ± 0.04
PLGA/PVA/24	1	100	-	0.5	227.23 ± 1.47	48-275	0.24 ± 0.04	-16.85 ±1.74	70.25 ± 0.81	0.82 ± 0.02
PLGA/PVA/25	1	100	-	0.75	222.57 ± 0.54	45-325	0.26 ± 0.04	-19.25 ± 0.25	66.90 ± 0.70	0.78 ± 0.01
PLGA/PVA/26	1	100	-	1	220.54 ± 0.65	40-300	0.25 ± 0.06	-17.25 ± 0.78	62.80 ± 0.76	0.73 ± 0.01
PLGA/PVA/27	1	100	-	0.5	229.32 ± 0.85	45-325	0.14 ±- 0.05	-20.01 ± 1.45	69.19 ± 1.54	0.81 ± 0.04
PLGA/PVA/28	2	100	-	0.5	234.85 ± 0.65	50-300	0.18 ± 0.08	-20.31 ± 1.42	71.69 ± 1.18	1.67 ± 0.01
PLGA/PVA/29	3	100	-	0.5	238.96 ± 0.85	40-300	0.19 ± 0.14	-19.74 ± 0.54	73.91 ± 2.88	2.58 ± 0.16
PLGA/PVA/30	4	100	-	0.5	240.74 ± 0.79	50-325	0.13 ± 0.14	-19.25 ± 0.62	73.40 ± 1.64	3.40 ± 0.05
PLGA/PVA/31	5	100	-	0.5	245.47 ± 1.02	40-325	0.15 ± 0.05	-18.23 ± 0.95	70.24 ± 0.80	4.14 ± 0.03
BPLGA/F68/01	-	50	0.25		109.4 ± 2.85	40-250	0.18 ± 0.23	-34.04 ± 1.21	-	-
BPLGA/F68/02	-	50	0.50		99.1 ± 1.11	70-350	0.11 ± 0.13	-24.51 ± 1.25	-	-
BPLGA/F68/03	-	50	0.75		86.3 ± 2.54	50-350	0.31 ± 0.04	-24.14 ± 1.05	-	-
BPLGA/F68/04	-	50	1		77.6 ± 3.25	50-240	0.18 ± 0.09	-22.32 ± 0.11	-	-

[*]Each data represents the average and standard deviation of three independent determinations, [a]Polydispersity index, [b]Zeta potential, [c]Encapsulation Efficiency, [d]Drug Content

Table 6: Composition and characterization of carfilzomib loaded and blank PLA NPs with variable concentration of stabilizers

Batch Code	Drug (mg)	Polymer (mg)	Stabilizer (% w/v)		Mean Size (nm ±SD)	Distribution (d. nm)	[a]PDI ±SD	[b]ZP ±SD	[c]EE (%)	[d]DC (% w/w)
			PF 68	PVA						
PLA/F68/14	1	50	0.25	-	178.12 ± 9.12	90–760	0.62 ± 0.19	-34.23 ± 1.20	72.50 ± 1.11	1.44 ± 0.04
PLA/F68/15	1	50	0.5	-	152.13± 2.14	95–455	0.14 ± 0.10	-30.24 ± 0.16	87.26 ± 0.13	1.72 ± 0.31
PLA/F68/16	1	50	0.75	-	182.61 ± 4.72	99–559	0.54 ± 0.23	-38.42 ± 0.15	73.15 ± 0.69	1.44 ± 0.50
PLA/F68/17	1	50	1	-	180.72 ± 5.84	80–350	0.58 ± 0.20	-41.53 ± 0.75	67.90 ± 1.42	1.33 ± 0.03
PLA/F68/18	1	50	0.5	-	151.52 ± 1.24	80–400	0.17 ± 0.22	-28.42 ± 0.42	88.34 ± 1.77	1.76 ± 0.03
PLA/F68/19	2	50	0.5	-	152.23 ± 0.35	90-550	0.23 ± 0.05	-29.61 ± 0.54	87.12 ± 2.27	3.40 ± 0.10
PLA/F68/20	3	50	0.5	-	155.41 ± 2.67	90-500	0.36 ± 0.06	-29.61 ± 0.87	65.50 ± 3.39	3.72 ± 0.20
PLA/F68/21	4	50	0.5	-	158.52 ± 1.42	82-550	0.54 ± 0.43	-30.21 ± 0.77	50.70 ± 2.41	3.76 ± 0.20
PLA/F68/22	5	50	0.5	-	160.43 ± 1.45	90-450	0.48 ± 0.62	-30.82 ± 0.49	28.90 ± 0.32	2.63 ± 0.03
PLA/PVA/23	1	50	-	0.25	191.33 ± 8.72	91–840	0.49 ± 0.13	-31.61 ± 0.10	51.80 ± 0.86	1.07 ± 0.01
PLA/PVA/24	1	50	-	0.5	172.32 ± 1.38	80–595	0.13 ± 0.22	-28.22 ± 0.80	59.90 ± 1.00	1.18 ± 0.02
PLA/PVA/25	1	50	-	0.75	201.11 ± 9.02	80–542	0.81 ± 0.23	-32.72 ± 1.30	53.02 ± 0.70	1.04 ± 0.02
PLA/PVA/26	1	50	-	1	191.23 ± 5.30	90–596	0.92 ± 0.13	-28.11 ± 1.17	48.92 ± 0.76	1.00 ± 0.02
PLA/PVA/27	1	50	-	0.5	173.11 ± 0.08	90-550	0.39 ± 0.51	-28.42 ± 1.08	62.50 ± 0.46	1.24 ± 0.02
PLA/PVA/28	2	50	-	0.5	178.44 ± 0.09	90-550	0.41 ± 0.25	-29.22 ± 1.15	62.50 ± 0.58	2.42 ± 0.03
PLA/PVA/29	3	50	-	0.5	182.62 ± 1.05	90-450	0.42 ± 0.65	-27.15 ± 0.31	53.34 ± 1.55	3.03 ± 0.10
PLA/PVA/30	4	50	-	0.5	186.41 ± 2.06	90-450	0.56 ± 0.89	-26.52 ± 0.87	43.20 ± 0.66	3.20 ± 0.05
PLA/PVA/31	5	50	-	0.5	189.22 ± 1.34	80-525	0.58 ± 0.54	-29.26 ± 0.34	27.20 ± 1.05	2.48 ± 0.10
PLA/F68/01	-	50	0.25	-	176.12 ± 2.19	90-600	0.41 ± 0.13	-31.0 ± 1.01	-	-
PLA/F68/02	-	50	0.50	-	149.82 ± 1.25	70-450	0.27 ± 0.23	-29.0 ± 0.21	-	-
PLA/F68/03	-	50	0.75	-	182.62 ± 2.01	50-450	0.29 ± 0.11	-27.1 ± 0.34	-	-
PLA/F68/04	-	50	1	-	179.63 ± 1.11	50-500	0.30 ± 0.21	-28.3 ± 0.23	-	-

*Each data represents the average and standard deviation of three independent determinations, [a]Polydispersity index, [b]Zeta potential, [c]Encapsulation Efficiency, [d]Drug Content

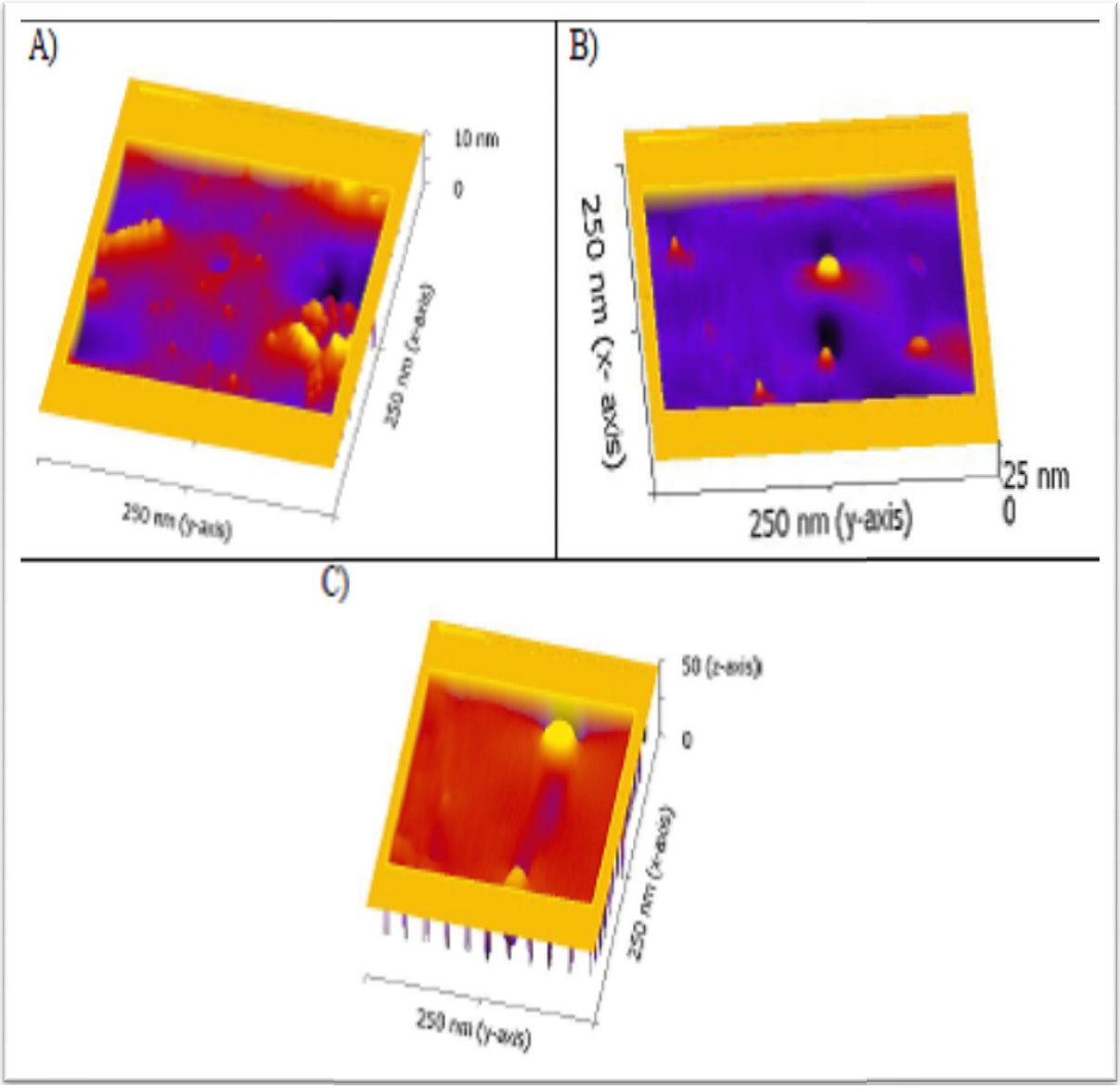

Fig. 6: Stability study results, PCL NPs AFM after 4 month storage (A) PLGA NPs AFM after 4 month storage(B) PLA NPs AFM after 4 month storage (C)

Table 7: Best fitting of in-vitro release data using mathematical modeling

S.NO	Batch	R^2	AIC	MSC	n^z	^{g} T50% (hr)
1	PCL/F68/03	0.9199	66.60	2.32	-	1.80
2	PCL/F68/05	0.9821	101.00	3.92	-	8.11
3	PCL/F68/07	0.9755	126.20	3.92	-	7.45
4	PCL/F68/09	0.9903	101.64	4.54	-	16.02
5	PCL/F68/13	0.9886	95.40	4.40	-	29.70
6	PCL/F68/14	0.9867	50.60	4.13	-	6.94
7	PCL/F68/15	0.9821	66.00	3.90	-	8.40
8	PCL/F68/16	0.9730	71.70	3.50	-	8.72
9	PCL/F68/17	0.9740	70.10	3.50	-	10.40
10	PCL/PVA/23	0.9481	60.03	2.80	-	13.23
11	PCL/PVA/24	0.9740	58.54	3.50	-	19.20
12	PCL/PVA/25	0.9843	45.10	4.00	-	23.70
13	PCL/PVA/26	0.9780	45.02	3.62	-	31.23
14	PLGA/F68/09	0.9886	53.40	4.30	-	6.54
15	PLGA/F68/13	0.9725	69.82	3.43	-	10.00
16	PLGA/F68/14	0.9845	50.80	4.00	-	5.23
17	PLGA/F68/15	0.9886	53.40	4.30	-	6.54
18	PLGA/F68/16	0.9731	62.00	3.50	-	8.13
19	PLGA/F68/17	0.9734	62.52	3.60	-	7.32
20	PLGA/PVA/23	0.9861	53.30	3.90	0.417	7.84
21	PLGA/PVA/24	0.9774	58.00	3.40	0.542	13.22
22	PLGA/PVA/25	0.9847	54.00	3.81	0.611	15.40
23	PLGA/PVA/26	0.9828	62.82	3.72	0.620	15.40
24	PLA/F68/07	0.9820	66.00	4.00	-	7.80
25	PLA/F68/08	0.9816	83.60	3.90	-	8.50
26	PLA/F68/14	0.9789	54.60	3.70	-	5.81
27	PLA/F68/15	0.9820	66.00	4.00	-	7.80
28	PLA/F68/16	0.9758	68.84	3.60	-	8.30
29	PLA/F68/17	0.9781	67.24	3.70	-	9.01
30	PLA/PVA/23	0.9917	39.50	4.70	0.450	5.52
31	PLA/PVA/24	0.9889	50.10	4.14	0.522	8.54
32	PLA/PVA/25	0.9894	49.10	4.30	0.561	9.70
33	PLA/PVA/26	0.9877	58.20	4.10	0.660	12.70

All Polymer and PF 68 variable formulations, follows Baker-Lonsdale model. In PCL polymer NPs, PVA variable formulation follows Higuchi model. In PLGA and PLA polymer NPs, PVA variable formulation follows Korsmeyer-Peppas model. ^{z}Diffusion exponent indicating the drug release mechanism.

Table 8: Stability study results of optimized carfilzomib loaded NPs formulations in three different conditions

S.NO	Stability Conditions	Evaluation Parameters	PCL NPs		PLGA NPs		PLA NPs	
			Observation (months)					
			0	4	0	4	0	4
1	5 ± 2° C	Physical appearances	White	No change	White	No change	White	No change
		Size (nm)	172.03 ± 0.34	175.13 ± 0.81	165.52 ± 2.10	166.23 ± 1.11	152.13 ± 2.10	154.21 ± 0.92
		PDI[a]	0.05 ± 0.53	0.06 ± 0.33	0.20 ± 0.04	0.22 ± 0.05	0.14 ± 0.10	0.24 ± 0.42
		ZP[b]	-25.52 ± 0.44	-26.12 ± 0.14	-25.23 ± 1.45	-24.56 ± 1.23	-30.24 ± 0.16	-30.11 ± 0.89
		DC[c]	4.60 ± 0.12	4.54 ± 0.52	1.65 ± 0.01	1.62 ± 0.22	1.02 ± 0.02	1.01 ± 0.12
2	15 ± 5° C	Physical appearances	White	No change	White	No change	White	No change
		Size (nm)	172.03 ± 0.34	190.13 ± 1.34	165.52 ± 2.10	176.21 ± 1.11	152.13 ± 2.10	165.23 ± 1.11
		PDI	0.05 ± 0.53	0.51 ± 1.42	0.20 ± 0.04	0.52 ± 1.01	0.14 ± 0.10	0.21 ± 0.32
		ZP	-25.52 ± 0.44	-22.52 ± 0.44	-25.23 ± 1.45	-21.89 ± 0.56	-30.24 ± 0.16	-29.11 ± 0.54
		DC	4.60 ± 0.12	4.20 ± 0.42	1.65 ± 0.01	1.59 ± 0.67	1.02 ± 0.02	0.87 ± 0.22
3	37 ± 5 °C	Physical appearances	White	No change	White	No change	White	No change
		Size (nm)	172.03 ± 0.34	193.13 ± 2.34	165.52 ± 2.10	190.23 ± 1.58	152.13 ± 2.10	172.21 ± 1.11
		PDI	0.05 ± 0.53	0.81 ± 0.73	0.20 ± 0.04	0.77 ± 0.24	0.14 ± 0.10	0.67 ± 0.23
		ZP	-25.52 ± 0.44	-21.01 ± 2.44	-25.23 ± 1.45	-20.89 ± 1.01	-30.24 ± 0.16	-23.23 ± 0.32
		DC	4.60 ± 0.12	4.02 ± 0.41	1.65 ± 0.01	1.42 ± 0.40	1.02 ± 0.02	0.67 ± 0.45

[a] Polydispersity index, [b] Zeta potential, [c] Drug loading

CONCLUSION

Successfully small with narrow size distribution PNPs, using PCL, PLGA and PLA polymers were prepared by using nanoprecipitation and solvent evaporation method. Based on the results it was observed that quality NPs with 100 % EE, high DC and % recovery were obtained using,

20 mg of PCL (PCL/F68/05), 100 mg of PLGA (PLGA/F68/09) and 50 mg of PLA (PLA/F68/07) with 0.5 % PF 68 as stabilizer. The reason for high EE in case of the prepared PNPs may be due to low aqueous solubility of carfilzomib, fast rate of precipitation of polymer during preparation and selection of polymer solvent with high vapour pressure and the low viscosity of the internal phase. The prepared PNPs were characterized for their shape and structure using SEM, TEM and AFM. The classy microscopic examination SEM, TEM and AFM analysis revealed the spherical and smooth surface character of the NPs as well as their homogeneous solid matrix without any amorphous arrangements. Though, PVA is the most extensively used stabilizer in the preparation of PNPs, in the present method we studied both PVA and PF 68. Among the two, formulations with PF 68 as stabilizer were selected and used in in-vivo pharmacokinetic and in the in-vivo anti-tumor efficacy study as it provides NPs.

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