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In-Vitro Cytotoxicity and Cellular Uptake Studies of carfilzomib nanoparticles

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ABSTRACT
Drug-loaded nanoparticles have established their benefits in the fight against multiple myeloma; however, ligand-targeted nanomedicine has yet to successfully translate to the clinic due to insufficient efficacies reported in preclinical studies.In this study,carfilzomib nanoparticles targeting multiple myeloma are prepared from PLGA,PLA AND PCL purified constituents to ensure increased consistency over standard synthetic methods. These nanoparticles are then tested both in vitro for uptake to cancer cells and in vivo for accumulation at the tumor site and uptake to tumor cells. Finally, drug-loaded nanoparticles are tested for long-term efficacy in a month-long in vivo study by tracking tumor size and mouse health.In overall the cell uptake study established the efficient uptake/target ability of PCL, PLGA and PLA NPs in breast cancer cells, MCF-7.However, biodistribution and tumor suppression studies established tumor-targeted nanoparticles to have significantly increased in vivo tumor accumulation, tumor cell uptake, and tumor suppression. These results both highlight a promising cancer treatment option in Carfilzomib PLGA nanoparticles and emphasize that targeting success in vitro does not necessarily translate to success in vivo.
Keywords*: carfilzomib, Cytotoxicity,PLGA,PLA*

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INTRODUCTION

The therapeutic efficacy of several types of drugs, particularly anti-cancer drugs, depends on the cellular uptake efficiency. It is expected that in nanoparticulate systems the cellular uptake is enhanced and thus can provide enhanced efficacy. The cellular uptake study in suitable cancer cell is essential to study enhanced uptake and efficacy. Staining the cells/organelles with suitable dye and incubating with the particles labeled with appropriate probe molecule, which can be studied using confocal microscope is the traditional technique for qualitative study of cellular or organ uptake of particles. In case of quantification for the amount of particles taken up by the cancer cells, microplate reader is the universal basic techniques available. Once the NPs reach the cancer cells, it is expected to inhibit the cell proliferation which results in cell death. This cytotoxicity of the drug loaded NPs is studied extensively by in-vitro 3-(4, 5- Dimethylthiazol-2yl)-2, 5, diphenyltetrazolium bromide (MTT) assay [1-5].
In this work, the cytotoxicity of the prepared carfilzomib loaded NPs were studied and the Coumarin 6 (C6) loaded PCL, PLGA and PLA NPs were also subjected for particle uptake study.

MATERIAL AND METHODS

Pure drug carfilzomib and selected nanoparticulate systems, prepared and mentioned in Chapter 5 were used for cytotoxicity study. Breast cancer cell line MCF-7 was obtained from National Centre for Cell Sciences, Pune, India. Chemicals, MTT, fetal bovine serum (FBS), minimal essential medium (MEM), Coumarin 6 (C6), Triton X-100 and propidium iodide (PPI) were obtained from Sigma–Aldrich, Bangalore, India.
Cytotoxicity study
The cytotoxicity of the prepared carfilzomib loaded PNPs were measured in MCF-7 cancer cell line using MTT assay at SCTIMST, Thiruvananthapuram, India with ethical approval. The MCF-7 cells were maintained in MEM, supplemented with 10% FBS and antibiotics, 1% penicillin/streptomycin under the conditions of 5 % CO2 and > 90% humidity at 37°± 1 C. Antibiotics was added to maintain sterility.

Samples of cytotoxicity study

Negative control (NC)sample

NC samples are one where theoretically no cytotoxicity effect should be observed. NC sample was included in the study with the intension to prove that the MTT procedure is selective. High density polyethylene is used as negative control. It is prepared by incubating 0.2 gm of high density polyethylene with culture medium containing serum at 37° ± 1 C for 24 ±2hr.

Positive control (PC) sample

PC samples are one which should completely inhibit the cell viability. PC sample was included in the study expecting that it will inhibit the cell growth, to confirm that the MTT procedure is sensitive and selective to cytotoxic material. The positive control used in this study is dilute phenol which is prepared by diluting phenol stock solution (13 mg/ml) to 1.3 mg/ml with culture medium containing serum.

Carfilzomib loaded PNPs-Testsamples

The cytotoxicity of the prepared carfilzomib loaded PCL, PLGA and PLA NPs and commercial formulation (CRE-MR) were measured at three different concentrations, 10, 20 and 40 µg/mL. ***MTT assay procedure***

To determine the cell viability, MCF-7 cells were sub cultured and transferred to 96-well plate to ensure 1 X 10^4 cells per well in 100 µL MEM containing 10 % FBS. Medium was changed every day until 80% confluence was reached. Then the medium was replaced with 100 µL of test samples of 10 µg/mL concentration. The plate was incubated for 24, 48, and 72 hr, then the well content was removed and the wells were washed three times using PBS. MTT reagent, (50 µL from 1mg/mL) was added to the well and incubated for 2 hr.

Now, MTT was removed and 100 µL of isopropanol was added to the wells and swayed before the plate was observed for optical density (OD) using spectrophotometer reader (Bio-Tek Instruments, Vermont, USA) at 570 nm. In addition to this, the cells were observed under microscope for there morphological changes. MCF-7 cells without test samples treatment were used as control cells. The cell viability was determined by using the formula

Cell viability (%) = (OD of test cells/OD negative control cells) X 100

Cellular uptake studies of NPs

Preparation of fluorescence PNPs

The Coumarin 6 (C6) loaded PCL and PLA NPs were prepared by nanoprecipitation and C6 loaded PLGA NPs were prepared by solvent evaporation method. The fluorescence PNPs were prepared as per the carfilzomib loaded NPs procedure. The fluorescence PNPs prepared was characterized for their size, polydispersity index, zeta potential and drug content to study the cellular uptake efficiency of the prepared PNPs in MCF-7 cells. The shape and size of the C6 loaded NPs were further confirmed through AFM analysis. The cellular uptake efficiency of these prepared C6 loaded PCL, PLGA and PLA NPs were determined by confocal microscopy and micro plate reader technique.

Determination of cellular uptake of NPs by confocal microscopy

The particle cellular uptake study was performed in MCF-7 cells using standard procedure (5, 8). MCF-7 cells were sub cultured and seeded at a density of $1x10^4$ cells/cm^2 in well plate. After 80% confluence, the medium was replaced with C6 loaded PCL, PLGA and PCL NPs dispersion and incubated for 2 hr. After that, dispersion was removed and 1 ml of 70% ethanol solution was added into each well to fix cells. Now the wells with cells in ethanol solution were kept in 37ºC for 20 min, then ethanol solution was removed and PBS was used to wash wells for three times. Subsequently, 10 µL of 5 mg/ml PPI was added to stain MCF-7 cell nucleolus for 30 min, soon after the stain was washed three times using PBS and it is observed under confocal laser scanning microscop (LSM 510 Meta). The C6 loaded particles and PPI staining cell nucleus showed green color and red color, respectively.

Determination of cellular uptake of NPs by micro plate reader

The cells were seeded in the 96-well plate at a density of 1 X 104 cells per well. Medium was changed until 80% confluence was reached. The medium was then replaced with 100 µL medium with C6 loaded PCL, PLGA and PCL NPs of three different concentrations 50, 75 and 100 µg/ml. The plate was incubated for 2 hr and the cellular uptake efficiency of C6 loaded NPs was determined by micro plate reader.

For each PNPs sample, one control sample without cells was added in the well. After predetermined time interval, NPs suspension was removed and the wells were washed three times using PBS. Now, 100 µl 0.5% triton X-100 in NaOH was added to break the cells and then the plate was measured using a micro plate reader. The excitation wavelength and emission wavelength was 430 and 485 nm, respectively, for C6. The cellular uptake efficiency of PNPs was given by the ratio between the amount of NPs taken up in cells and the amount of NPs in control well.

RESULT AND DISCUSSION

Cytotoxicity study

As it is important to use sterilized material in cell cytotoxicity study, the prepared PNPs were sterilized by filtering through membrane filtration (6). The cytotoxicity effect of prepared three PNPs was compared to that of commercial formulation, CRE-MR. The concentrations of carfilzomib 10, 20 and 40 µg/mL was preferred for cytotoxicity study since the plasma level of carfilzomib is likely to be in this range (7, 8). The MTT test is an assay to identify the proliferation and cell viability by measuring the mitochondrial activity of cells. Metabolically active cells are able to convert the yellow water-soluble tetrazolium salt MTT to water insoluble dark blue formazan by reductive cleavage of the tetrazolium ring (9).

The % cell viability vs. incubation time graph (Fig. 6.1, 6.2 and 6.3) clearly illustrated carfilzomib loaded PNPs showed strong inhibitory effects on the proliferation of MCF-7 cells when compared to commercial formulation. The negative sample analysis showed almost 80-90 % cell viability in all the three time points studied.

The positive control sample analysis evidently showed that the procedure adopted allows the cytotoxic compound phenol to inhibit MCF-7 cells proliferation completely. The increased cytotoxicity of carfilzomib loaded PNPs than the CRE-MR is attributed to higher intracellular uptake and drug concentration. The reason for increased cytotoxicity of PNPs could be due to enhanced cell permeation of NPs. The mechanism of enhanced cell permeation is probably due to hydrophilic surface nature of NPs which reduces MDR. Further, the stabilizer used in NPs preparation, PF 68 is also a potent inhibitor of P-gp and CYP3A4 (1), which might have helped in enhanced permeation. The profiles suggest cytotoxic effect is dependent on both, carfilzomib concentration as well as time of incubation.

Table .1 :IC50 (µg/mL) of MCF-7 cells after 24, 48 and 72 hr of incubation with 10 µg/mL concentration of carfilzomib loaded PNPs and commercial carfilzomib formulation

S.NO	NPs Formulation	IC50 (µg/mL) at different incubation time (hr)		
		24	48	72
1	CRE-MR	19.51	12.27	8.74
2	carfilzomib PCL NPs	14.30	10.28	7.63
3	carfilzomib PLGA NPs	13.32	9.88	7.21
4	carfilzomib PLA NPs	9.79	8.18	6.80

Among the PNPs, carfilzomib loaded PLA NPs exhibited maximum inhibitory effect. This could be due to increase in cellular uptake of carfilzomib loaded PLA NPs observed in the particle cellular uptake study. The % cell viability profile illustrated that when the concentration of carfilzomib loaded PNPs was increased from 10 to 40µg/mL, the cytotoxicity action increases. When the contact time of NPs and MCF-7 cells were increased the proliferation of cells decreased as a result of sustained drug release from PNPs, which is not observed in CRE MR. The cytotoxicity of carfilzomib, depends on sustain therapeutic concentration rather than maximal plasma concentration, since carfilzomib need cells to enter M phase [10, 11].

The IC50 of the evaluated formulations in MCF-7 cells after incubation for 24, 48 and 72 hr is represented in Table .1. IC50 is defined as the drug concentration at which 50 % of the cells in culture have been killed in a particular time period. IC50 values for commercial formulation (CRE-MR) were found to be 19.51 µg/mL, 12.27 µg/mL and 8.74 µg/mL at 24, 48 and 72 hr incubation respectively. However these IC50 values were found to be lower for NPs formulation with lowest value for PLA NPs with 9.79, 8.18 and 6.80 µg/mL at 3 different incubation.

Periods (Table.1). PCL NPs showed maximum IC50 value among 3 NPs with 14.50 µg/mL,

10.28 µg/mL and 7.63 µg/mL respectively. There was not much difference in IC50 value at 72 hr incubation as in 72 hr all the carfilzomib are released by 72 hr. During MTT assay, the morphological examination of the cells was observed at 24, 48 and 72 hr through phase- contrast photomicrographs. The morphological examination of cells incubated withPNPs and CRE-MR showed alterations in cell shape compared to negative control and controlcells.

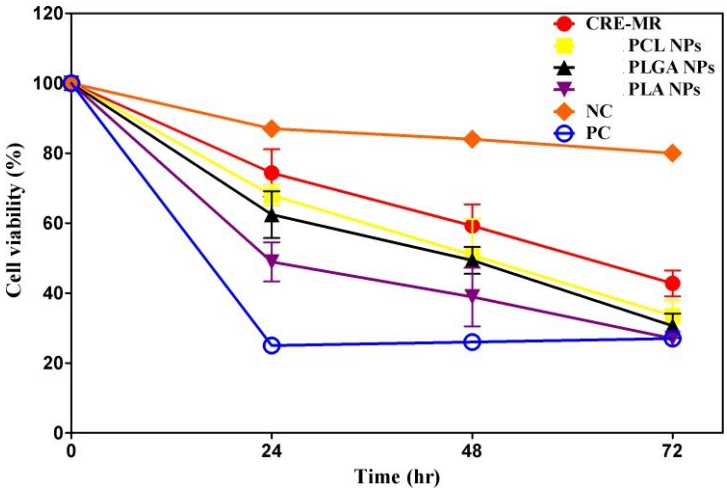


Fig. .1 Cell viability of MCF-7 cells treated with commercial product, carfilzomib(CZB) loaded PNPs at concentration 10 µg/ml. Each data point showed average of five samples

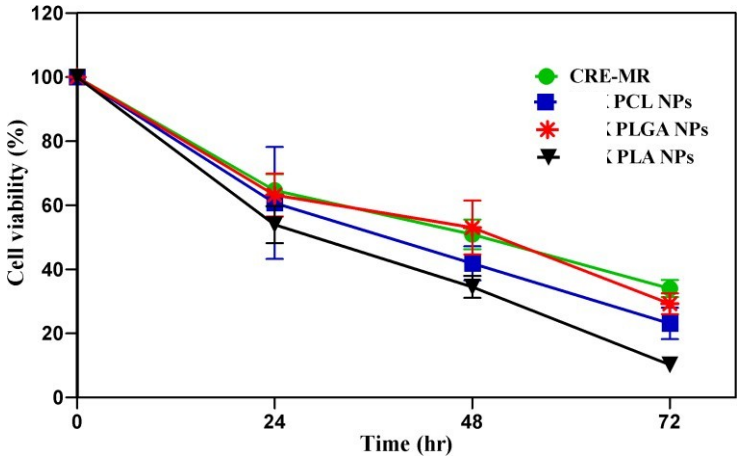


Fig. .2 Cell viability of MCF-7 cells treated with commercial product, carfilzomib (CZB) Loaded PNPs at concentration 20 µg/ml. Each data point showed average of five samples

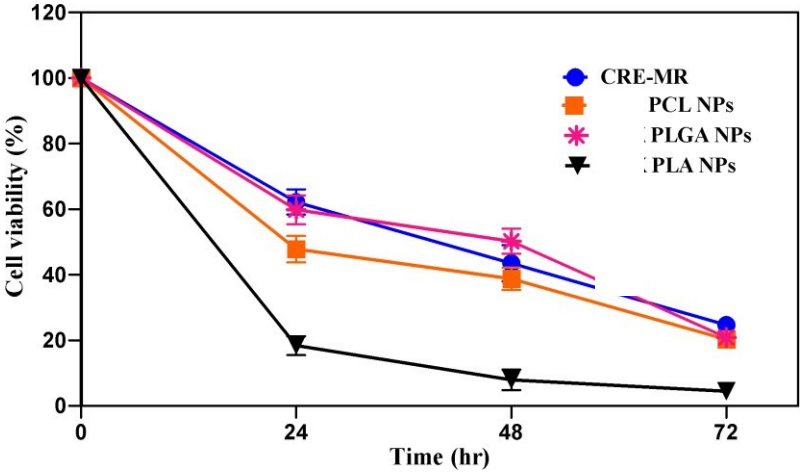


Fig..3 Cell viability of MCF-7 cells treated with commercial product, carfilzomib (CZB) Loaded PNPs at concentration 40 µg/ml. Each data point showed average of five samples

The negative control cells did not show any change in shape when compared to control cells, this showed that the MTT assay is specific to proliferation inhibiting samples. In

positive control group, the shape of the cells was altered drastically or in other hand, phenol inhibited the proliferation of MCF-7 cells.

Cellular uptake studies of NPs

a) Preparation of Fluorescence PNPs

Successfully, C6 loaded PCL, PLGA and PLA NPs were prepared using the optimized procedure of carfilzomib loaded PNPs as mentioned in Chapter 5. The size and PDI of the prepared C6 loaded PCL, PLGA and PLA NPs were, 171.8 ± 0.80 nm, 0.20 ± 0.03, 165.8 ± 0.14 nm, 0.24 ± 0.12, 154.5 ± 2.20 nm and 0.11 ± 0.01 respectively. The ZP of the prepared C6 loaded PNPs were -25.0 ± 0.9, -21.2 ± 1.20 and -26.0 ± 0.83 respectively. The shape and surface morphology of the prepared C6 loaded PNPs were observed using AFM and they are represented in Fig. 6. The C6 loaded PNPs characterization resembles the carfilzomib loaded PNPs and it is used in the particle uptake study.

b) Confocal microscopic observation of cellular uptake of NPs

The improved drug targeting ability of NPs towards cancer cells depends on internalization and continued retention of particles in target cells. The particle uptake study in cancer cells will be a proof of concept for the better therapeutic effect of drug loaded NPs over the conventional chemotheraphy.

The fluorescein isothiocyanate channel was used to observe the NPs and neutral red channel was used to examine the MCF-7 cells. The C6 loaded NPs were represented in green color and the PPI stained MCF-7 cell nucleus was shown in red color. The confocal fluorescence 2D images of MCF-7 cells with C6 labelled NPs after 2hrincubation is represented in Fig. 7. The 3D view of confocal image of particle uptake evidently confirmed that the C6 loaded PNPs (green color) is located closely around the nucleus (red color), indicating the internalization of NPs.

In addition the cellular uptake is visualized by overlaying images obtained by fluorescein isothiocyanate channel (green), propidium iodide channel (red) and background (black) in Fig 8. The particle uptake image was displayed in three orthogonal projections xy, xz and yz respectively to verify whether the C6 NPs are located outside the top surface of the cells or entrapped intracellularly Fig 8 [12]. The orthogonal images (Fig.9) illustrates that the NPs were indeed entrapped within the intracellularspaces.

c) Determination of cellular uptake of NPs by micro plate reader

The quantitative analysis of cellular uptake of C6 loaded PNPs after 2 hr of incubation with MCF-7 cells were represented in Fig.9. It can be observed that when the concentration was increased from 50 to 100 µg/mL the carfilzomib loaded PCL, PLGA and PLA NPs uptake increased respectively. The increasing order of carfilzomib loaded NPs uptake observed after 2 hr incubation with MCF-7 cells at 100 µg/mL were, PCL NPs<PLGA NPs<PLA NPs respectively. Among the PNPs studied for cellular uptake, PLA NPs were efficiently taken up by the MCF-7 cells at all the concentrations studied. Researchers demonstrated that the degree of internalization of NPDDS into cancer cells is inversely proportional to the particle size (13, 14). In the present work, PLA NPs cellular uptake is highest, due to small size, 151.13 ± 2.44 nm particles. The particle size of PLGA and PCL NPs was 166.56 ± 1.20 nm and 174.0 ± 9.34 nm respectively. Hence cellular uptake of PCL and PLGA NPs are lower compared to PLA NPs in MCF-7cells.

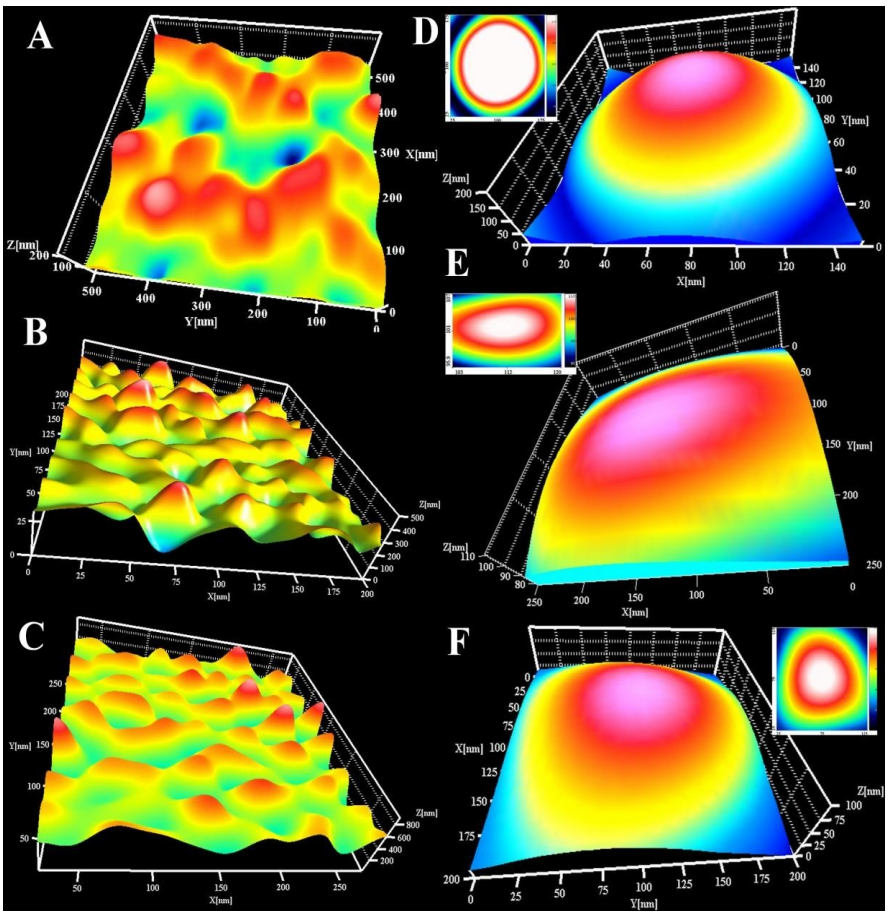


Fig. 4 Characterization of Coumarin 6 loaded PNPs by AFM, cluster, single 3D and 2D PCL NPs (A and D) cluster, single 3D and 2D PLGA NPs (B and E) and cluster, single 3D and 2D PLA NPs (C and F)

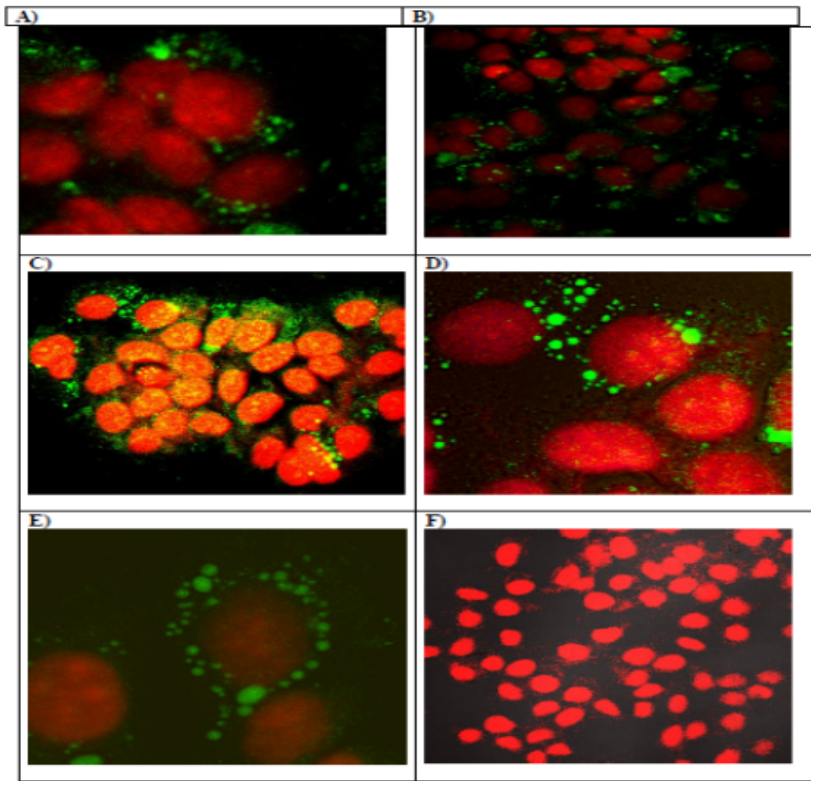


Fig 5 Confocal fluorescence 2D images of MCF-7 cells with Coumarin 6 labeled NPs after 2 hr exposure, PCL NPs (A) PLGA NPs (B) PLA NPs (C-E) blank cells (F)

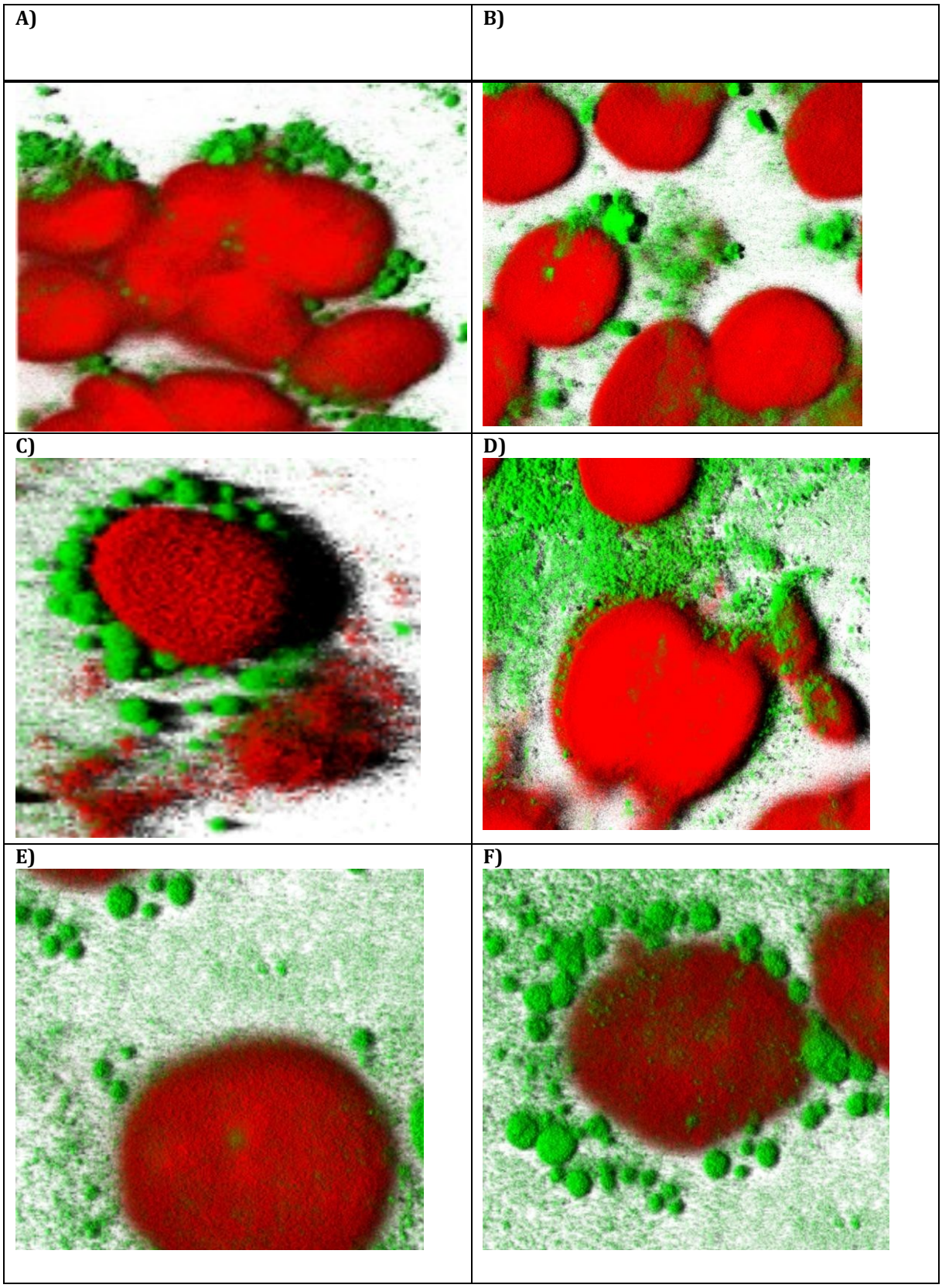
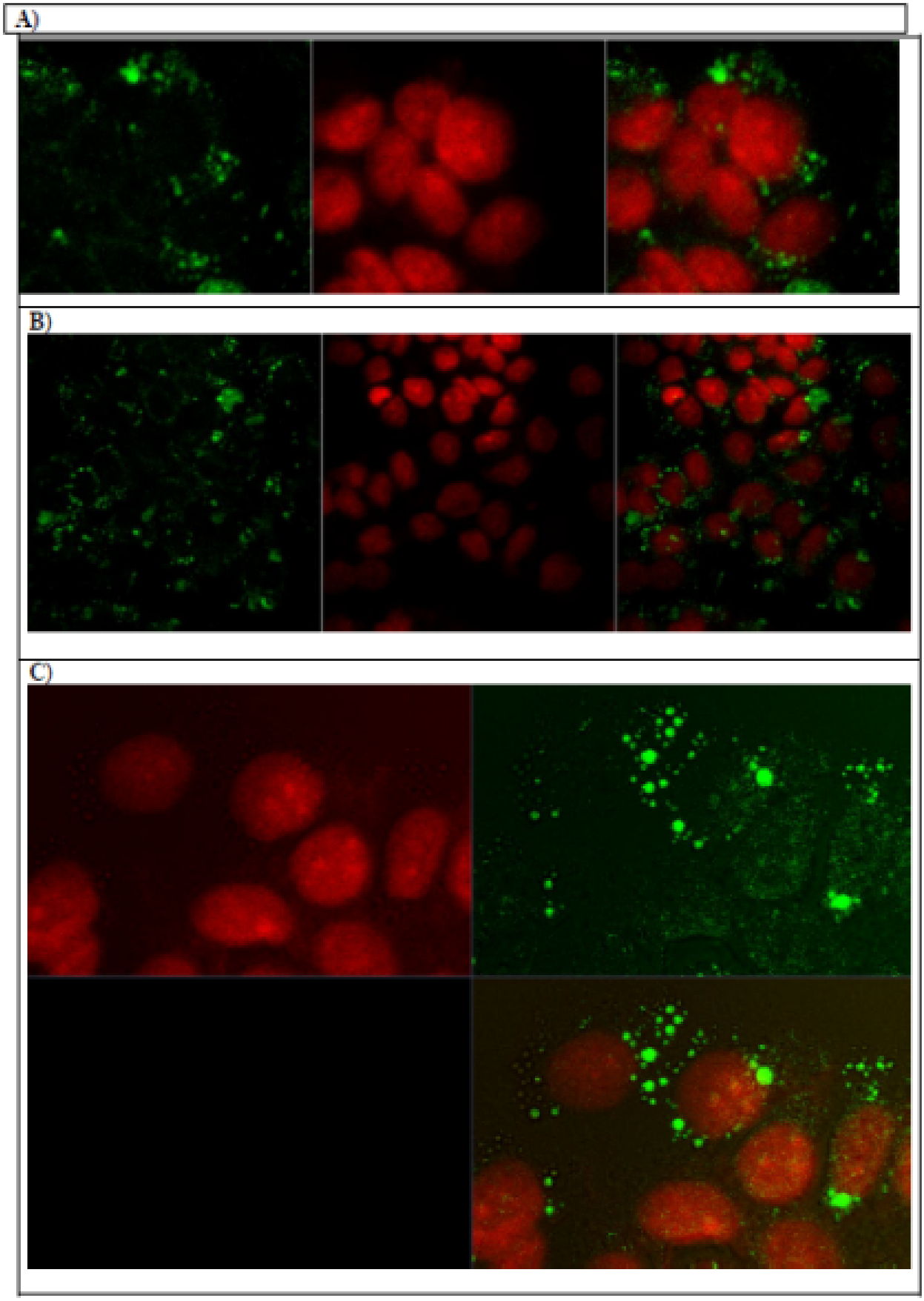


Fig .6 Confocal fluorescence 3D images of MCF-7 cells with Coumarin 6 labeled NPs after 2 hr exposure, PCL NPs (A and B) PLGA NPs (C and D) PLA NPs (E and F)


A)
B)
C)

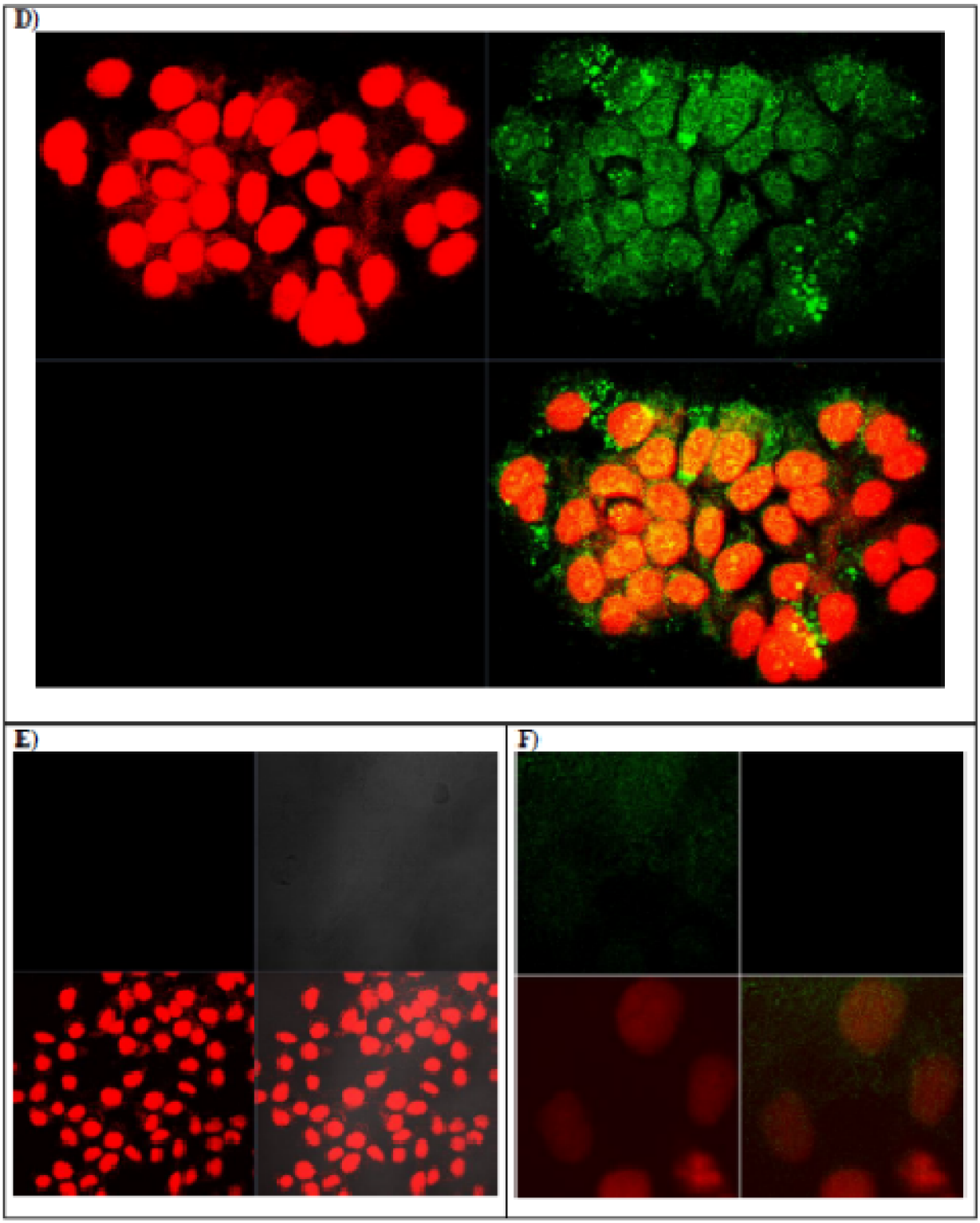
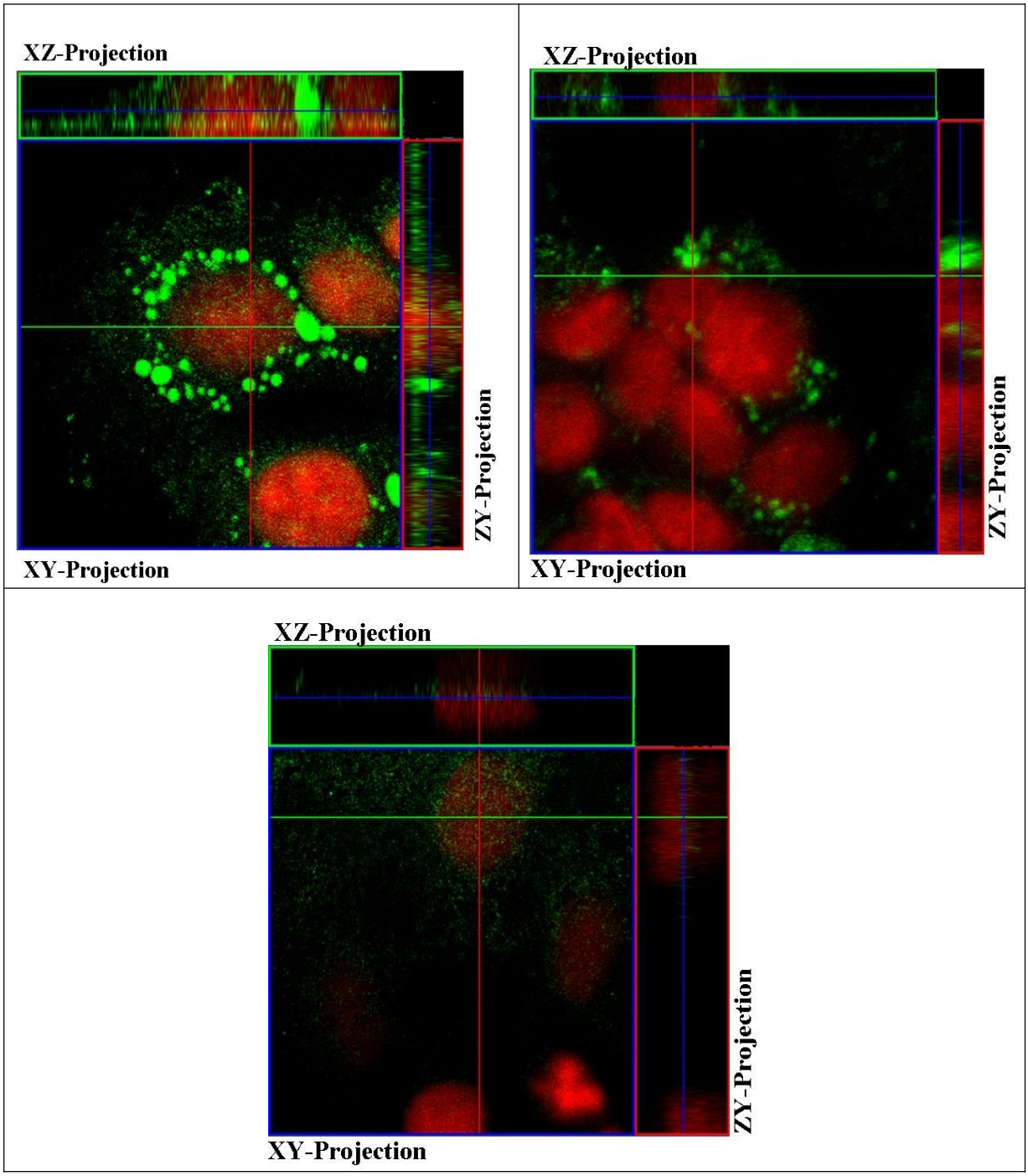


Fig .7 Confocal fluorescence microscopy 2D image of MCF-7 cellular uptakes of, PCL NPs (A) PLGA NPs (B) PLA NPs (C and D) blank MCF-7 Cells (E) free Coumarin 6 incubatedfor 2 hr (F). The cellular uptake is visualized by overlaying images obtained by fluorescein isothiocyanate channel (green), propidium iodide channel (red), background (black) and combination of all three images



A) **C)** **B)**

Fig .8 Confocal microscopy confirms internalization of NPs by MCF-7 cells. The cell uptake image is displayed in three orthogonal projections XY, XZ and YZ respectively. PLA NPs (A) PCL NPs (B) can be seen within the cytoplasm on the Z-sections (XZ & ZY projection), free Coumarin 6 incubated with MCF-7 cells illustrates less uptake of Coumarin 6 (C)

The orthogonal image of MCF-7 cells incubated with free C6 (Fig.8 C) demonstrated that only C6 loaded PNPs can reach cytoplasm of cancer cells more than the free C6. In general, endocytosis is considered to be the main mechanism for the cells to take up NPs. Particles less than 150 nm reaches cells through pinocytosis and particles larger than 200 nm were taken up by cells through phagocytosis (5, 11). In this study, the nonspecific phagocytosis played major role in the engulfing of C6 PNPs by MCF-7cells.

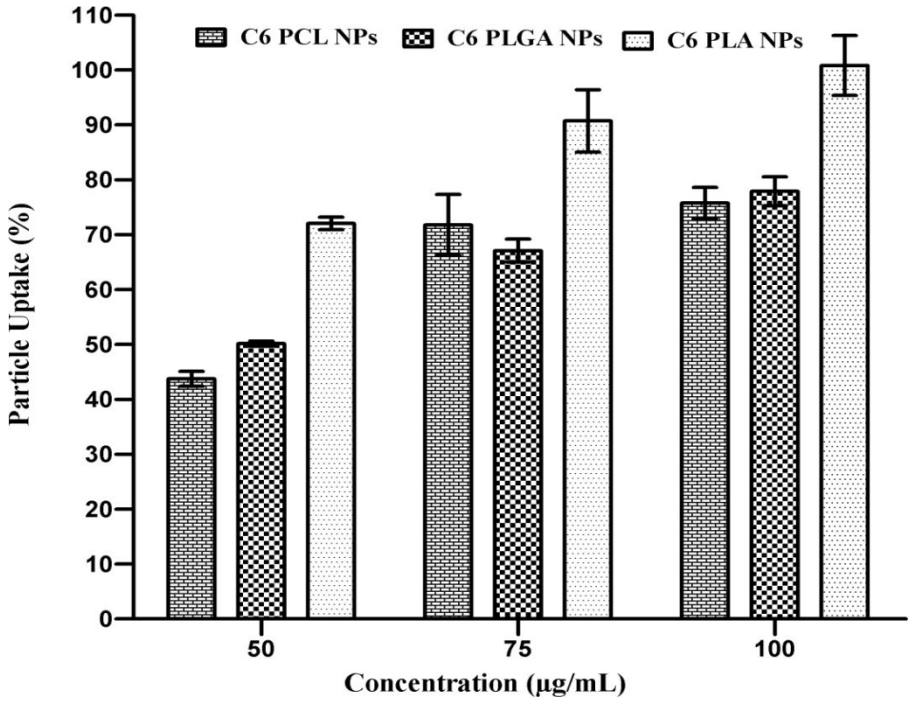


Fig .9 Particle uptake of NPs using MCF-7 cells

CONCLUSION

The cytotoxicity study result demonstrated that the prepared carfilzomib loaded NPs inhibited proliferation of MCF-7 cells more than the commercial formulation, CRE-MR. In addition, the IC50 values of PNPs were low for NPs when compared to CRE-MR. This demonstrated the efficiency of carfilzomib loaded NPs in uptake and cytotoxicity. In overall the cell uptake study established the efficient uptake/target ability of PCL, PLGA and PLA NPs in breast cancer cells, MCF-7. The orthogonal projection of MCF-7 cellular uptake confocal fluorescence image, illustrated that maximum NPs reached the cytoplasm of the cells. The 2D and 3D view confocal fluorescence microscopic image showed that the PNPs were positioned closely around the nucleus. As in this study drug carfilzomib was not used during identification problem, study is to be done with drug.

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