

**Research Article****Development and characterization of RGD embellished drug-conjugate loaded NPs for dual targeting of tumor cells**

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**Abstract**

**Objective:** The drug targeting strategy is drawn more attention for the effective treatment of various types of lethal diseases such as, cancer. To improve the efficacy of drug the dual targeted delivery system was developed with the help of hyaluronic acid (HA) and RGD peptide. HA targets the overexpressed CD44 receptors while RGD peptide helps to target integrin ( $\alpha_v\beta_3$ ) receptors on the tumor cells. **Material and Methods:** The drug, paclitaxel (Ptx); was used as the model drug and conjugated with HA and then the drug-HA conjugate was entrapped into the RGD decorated nanoparticulate system. The conjugation of the ligand molecules with the drug and carrier system was confirmed by the IR and NMR spectroscopy methods. **Results:** The developed NPs exhibited excellent physical properties like, particle size in nano size range, spherical shape, and high entrapment efficiency which may make drug delivery system more efficient. The cytotoxicity and cell uptake study of the prepared formulations on the cancer cell line (U87MG) (which has overexpressed integrin receptors, showed lowest  $IC_{50}$  value and highest cell uptake) showed better uptake of RGD decorated NPs due to receptor mediated transport across the cells. **Conclusion:** Thus, RGD peptide decorated system developed as the promising candidate for the treatment of cancer cells, effectively.

**Keywords:** RGD peptide, hyaluronic acid, targeted delivery system, integrin receptor, nanoparticles

**Introduction**

Cancer is the major cause for the most of the deaths all around the world. According to the World Health Organization (WHO) cancer is the second most cause of the deaths in 2013 and 2015, which was about 7.6 million and 8.8 million deaths, respectively, and it is estimated that, in 2030 the data will be reached to 11.5 million deaths. In India, the premier research organization, Indian Council of Medical Research, also predicted that more than 17.3 lakh new cases of cancer and 8.8 lakh deaths will be due to the cancer by 2020 (Ali et al., 2011). Chemotherapy is the precious and effective option for the cancer treatment, but, its effectiveness always under the questioning area because of limited half life, poor stability, inferior physicochemical properties, and serious side effects to the normal cells. Various anticancer agents as such or with carrier system which may be either plain or decorated with ligands,

transporters, etc., are used for the tumor treatment (Estanqueiro et al., 2015). In the present research work the drug paclitaxel (Ptx) was used as the model drug which is presently used for the treatment of ovarian, lung, breast cancer and other types of malignant sarcomas. But, it is well known that Ptx possess some serious side effects. To minimize the side effect of the drug, it is conjugated with HA which help to convert hydrophobic drug into hydrophilic moiety. HA have specific affinity to CD44 receptors that are overexpressed on the several types of cancer cells, such as, breast, skin, and lung cancer cells (Bourguignon et al., 2010). HA is a biodegradable and biocompatible glycosaminoglycan polymer composed of repeating disaccharide units of glucuronic acid and N-acetylglucosamine, hence acquired lots of research interest (Robert et al., 2010). In concern of Ptx various carrier systems like, micelles, polymeric nanoparticles (NPs), have already been developed for the delivery of the drug with very less side effects (Soni, 2018). These systems offer several advantages, such as prolonged blood circulation of drug, passive targeting to tumor cells through the enhanced permeation and retention (EPR) effects in the cells and long

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term stability of the drug in the formulation (Elsaesser and Howard, 2012; Jain et al., 2017). Furthermore, these systems can be modified for active targeting by using suitable ligand to target the overexpressed receptors on the tumor cells (Byrne et al., 2008). A number of ligand moieties like lactoferrin, transferrin, RGD peptide, folic acid, etc., have been widely used for the active targeting of overexpressed lactoferrin, transferrin, integrin, folate receptors, respectively. In the current research work, RGD peptide was used as the targeting ligand for transportation of NPs bearing HA-Ptx conjugate. RGD peptide is a tripeptide (arginine-glycine-aspartic acid) sequence, interacted with the integrin receptors, mainly with  $\alpha_v\beta_3$  receptors, which are overexpressed on the angiogenic endothelium of cancer cells (Murphy et al., 2008). The nature of integrin receptors is heterodimeric which composed of glycoprotein and participated in the various cellular processes like, cell-cell interactions, angiogenesis during the cell progression, etc. During the cancer development, the integrins are over-expressed on the stimulated endothelial cells of tumor neovasculature and other tumor cells, including osteosarcomas, neuroblastomas, melanomas, breast cancer, glioblastomas and lung carcinomas (Zitzmann et al., 2002; Asati et al., 2019).

In the current research work, HA-Ptx conjugate loaded RGD decorated NPs were developed for dual targeting to the cancerous cells through the overexpressed CD44 and integrin receptors. Firstly, HA was conjugated with Ptx by using a three steps chemistry approach and the structure of the conjugate were confirmed with the IR and NMR spectroscopic methods. The conjugate loaded nanoparticles (CNPs) were prepared using the modified nanoprecipitation method and RGD peptide was attached to the NPs surface with carbodiimide chemical bonding. The NPs were characterized for the surface morphology, particle size, zeta potential, drug entrapment efficiency while, RGD bonding by IR and NMR spectroscopy and in-vitro release study. The cytotoxicity and cell uptake study of the formulations were performed by using U87MG cancer cell lines for determining the tumor targeting efficiency of the RGD decorated NPs system.

## Material and Methods

### Material

Ptx was obtained as a gift sample from Alchem International Pvt. Ltd., Haryana, India. HA, N-hydroxysuccinimide (NHS), diphenyl phosphoryl succinate (DPP), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), succinic acid and PLGA (Resomer® RG-503H) were procured from Sigma-Aldrich, Germany. Dichloromethane (DCM) and poly vinyl acetate (PVA) were purchased from Thermo Fischer Scientific Pvt. Ltd., India. Adipic dihydrazide (ADH), methanol, acetone and other solvents were purchased from Merck Specialities Pvt.

Ltd., India. All the solvents and chemicals were of analytical grade. Milli-Q water was used during the complete experimental process. Human primary malignant glioblastoma (U87MG) cell line was obtained from National centre for cell science (NCCS) Pune, India.

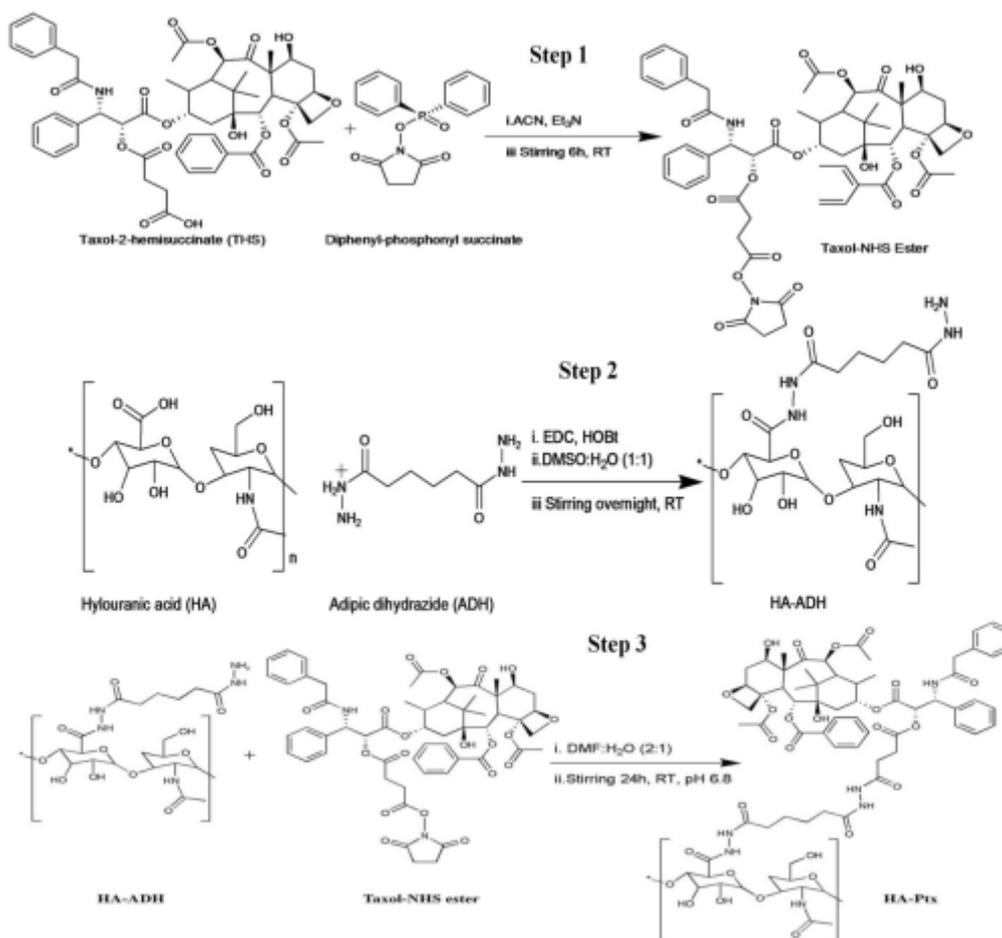
### Synthesis and characterization of HA-Ptx conjugate

The conjugation of HA with Ptx was performed in a three step process. In the first step, the ester form of drug (Taxol-NHS ester) was synthesized by using succinic anhydride and 2-diphenyl phosphonyl succinate (DPP). In the second step, activation of HA was completed with the formation of adipic-dihydrazido functionalized HA (HA-ADH). In the third and final step, both activated moieties were reacted together to produce HA-Ptx conjugate (Xu et al., 2015; Han et al., 2017). The confirmation of conjugation was done by using spectroscopic analysis like, IR and NMR. The summary of the complete reactions is shown in figure 1.

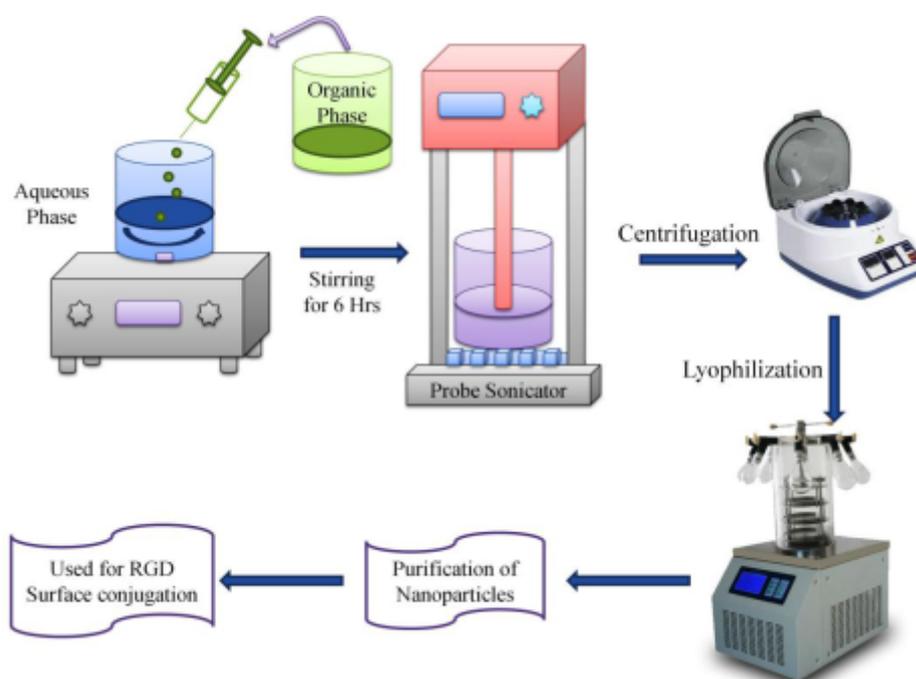
### Preparation and characterization of RGD modified conjugate loaded NPs (RCNPs)

The drug and conjugate loaded NPs (PNPs and CNPs, respectively) were prepared by using modified nanoprecipitation method (Liu et al., 2014). Briefly, the polymer and drug (for PNPs) or conjugate (for CNPs) were dissolved in an organic phase, while surfactant, in different concentrations, was dissolved in an aqueous phase consisting of pH 7.4 phosphate buffer saline. Then, the organic phase was added drop wise to aqueous phase with a syringe under constant stirring (REMI, Mumbai, India) at room temperature (RT;  $25\pm 2^\circ\text{C}$ ). Organic solvent was removed by continuous stirring overnight on magnetic stirrer at RT. The formulations were sonicated under probe sonicator (Soniweld, Imeco Ultrasonics, Mumbai, India) for 5-10 min at the cold temperature. NPs were then recovered from the nanodispersion by centrifugation (Spinwin, India) at 10,000 rpm for 30 min and washed two-three times with distilled water. Finally dispersion was lyophilized (Heto Dry Winner, Denmark) to yield freeze-dried nanoparticles as shown in figure 2.

The prepared formulations with the lower particle size and high entrapment efficiency were used for the surface conjugation with the RGD peptide. The CNPs were diluted in 10 ml phosphate buffer (pH 6.0). EDC and NHS (0.1M and 0.25M) were added (0.155 gm and 0.287 gm) into the buffer solution and incubated for 2 hrs under stirring. 2 mg/ml RGD solution was prepared in DCM and 1 ml quantity of the solution was added into the mixture and kept under mild vortex for overnight (Wang et al., 2011). The conjugation between the PLGA and RGD was taken place with the help of carbodiimide chemistry as shown in figure 3. The solution



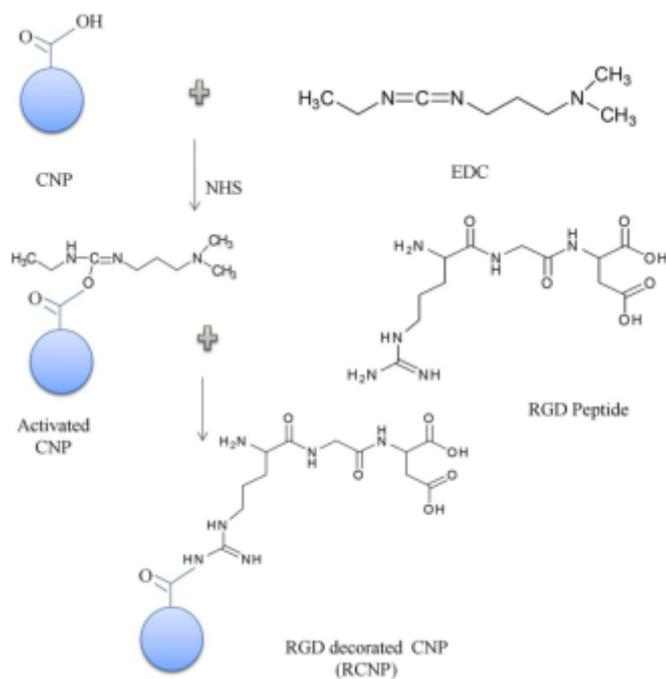
**Figure 1.** Conjugation of HA with Ptx in a three step process



**Figure 2.** Preparation of nanoparticles by modified nanoprecipitation method

mixture was kept overnight under mild vortex. The un-conjugated peptide was removed under centrifugation at 4000 rpm for 30 min

and surface conjugated NPs (RCNPs) were obtained by vacuum drying under rotary evaporator, the product was



**Figure 3.** Chemistry of Surface Conjugation of RGD Peptide to CNPs

purified and the confirmation of the conjugate structure was done by using IR and NMR spectroscopy. Furthermore, all the formulations viz., PNPs, CNPs, and RCNPs, were characterized for particle size, polydispersity index (PDI), zeta potential, entrapment efficiency, surface morphology and *in-vitro* drug release.

### Cytotoxicity study

The various formulations; i.e., PNPs, CNPs, RCNPs and pure conjugate (HA-Ptx) and drug (Ptx) solutions were subjected to cytotoxicity study using SRB assay on the U87MG cell line. Adriamycin (Adr) was used as the positive control sample which inhibits the cell growth completely and high density polyethylene (HDPE) was employed for the negative control. The cell lines were cultured in RPMI-1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For the present screening experiment, cells were inoculated into 96 well microtiter plates in 100  $\mu$ L at plating densities. After cell inoculation, the microtiter plates were incubated at 37°C temperature with 5% CO<sub>2</sub>, 95% air and 100% relative humidity for 24 hrs prior to addition of experimental samples (Soni, 2018).

Samples were dissolved in dimethylsulfoxide (DMSO) to make 100 mg/ml and diluted to 1mg/ml using water and finally added into the microtitre wells within the four concentration ranges, i.e., 10, 20, 40 and 80 $\mu$ g/ml. After 48 hrs of the experiment, the assay was completed with the addition of 30% cold trichloroacetic acid (TCA) and incubated at 4°C for 1 hr. The supernatant was removed and plates were washed with water five

times, and air dried. 0.4% (w/v) SRB solution was prepared with 1% acetic acid and the solution was added into each well plate and incubated at room temperature for about 20 minutes. The plates were washed five times with 1% acetic acid, to recover unbound dye, and air dried. 10 mM trizma base was added to elute out subsequent bound dye and absorbance was determined at 540 nm with 690 nm standard wavelength. Cytotoxicity was estimated with percent (%) growth of cells in the each well plate. The % growth was determined by using the formula given below:

### Cellular uptake study

Coumarin-6 (C-6) loaded formulations were used for the evaluation of cellular uptake of the system in the cancerous cells with the overexpressed integrin receptors on them, i.e., U87MG cells. The qualitative uptake of the systems into the cells or organelles can be determined with confocal microscopy and compared the between the both formulations.

#### (a) Preparation of fluorescence NPs

The Coumarin-6 (C-6) loaded NPs (C6-PNPs) and RGD decorated C-6 loaded PLGANPs (C6-RNPs) were prepared by using the modified nanoprecipitation method as discussed in the previous section. In this research, C-6 was used as the fluorescence probe for the qualitative cellular uptake determination of the NPs by confocal microscopy.

#### (b) Determination of cellular uptake of NPs using confocal microscopy

The cellular uptake study was performed in U87MG cells using a standard procedure. Cells were propagated until density reached 80% confluence. The medium was replaced with samples dispersion and incubated for 2 hrs. After that, dispersion is removed and 1 ml of 70% ethanol solution is added into each well to fix cells. Now the wells with cells in ethanol solution are kept in 37°C for 20 min, then the ethanol solution was removed and PBS was used to wash wells for three times. Subsequently, 10 $\mu$ L of 5 mg/ml propidium iodide (PPI) is added to stain cell nucleolus for 30 min, soon after the stain is washed three times using PBS. The cover slip is carefully removed and inverted on a clean slide and it is observed under fluorescence microscope (BX53, Olympus). The PPI and samples stained cell nucleus will give red color and greenish blue color, respectively (Behzadi et al., 2017).

### Statistical analysis

All data were analyzed by ANOVA, lack of fit tests and multiple correlation coefficients. Student's t test was used to test the statistical significance wherever applicable. Obtained data were expressed as mean $\pm$ SD (n=3).

## Results and discussion

### Characterization of HA-Ptx conjugate

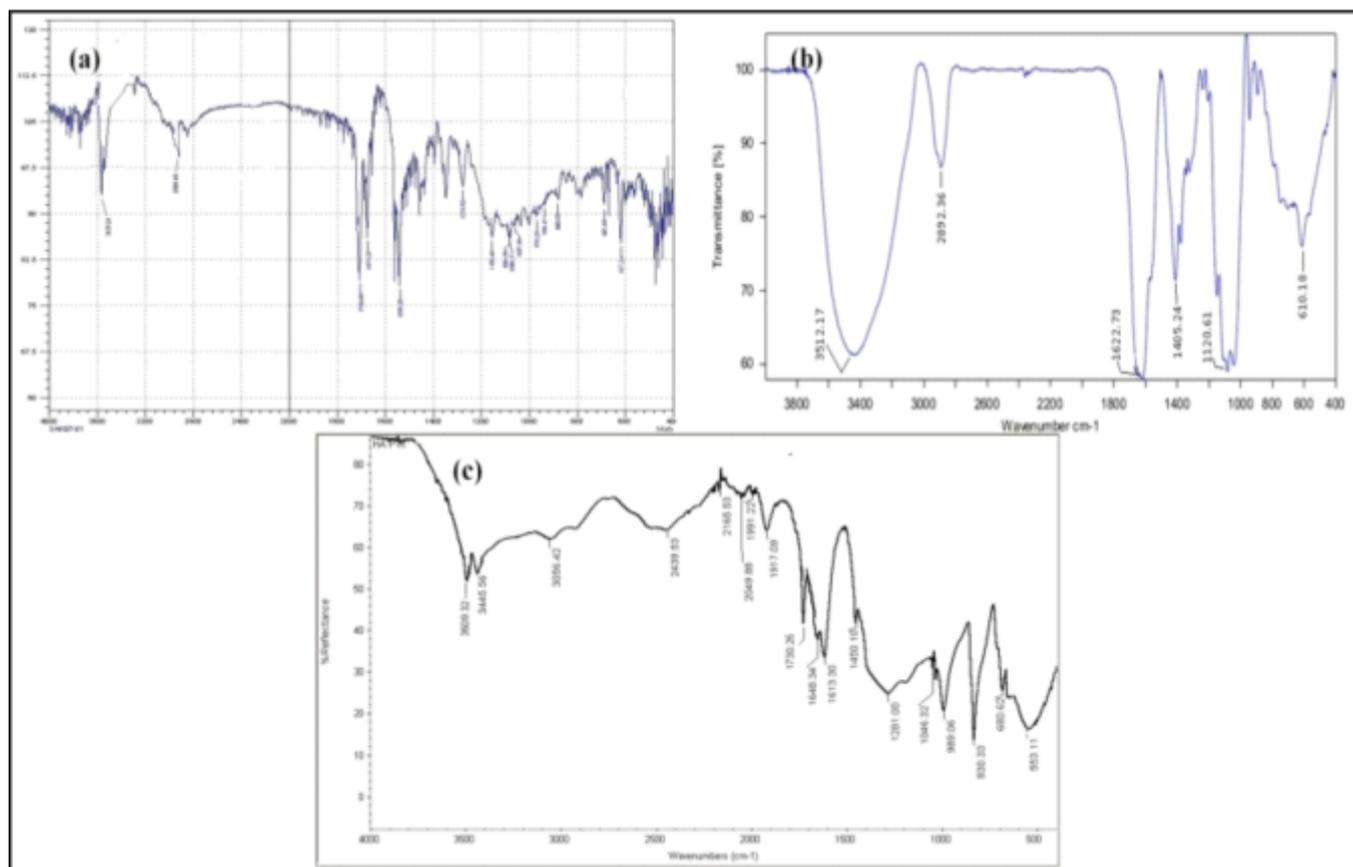
In the first step, ester compound of the drug was formed with the replacement of hydrogen atom of the drug by the succinimide moiety of DPP. In the second step, the HA was activated with the ADH in the presence of EDC and formed the amide bond between the HA and ADH. In the final step, HA molecule was attached to the esterified Ptx by the substitution of succinimide group of the esterified Ptx with the activated HA compound via amide bond formation. The conjugate was characterized by using FTIR spectroscopy. Figure 4(a), 4(b) and 4(c) show the IR spectra of Ptx, HA and HA-Ptx conjugate, respectively. The various peaks are showed in the FTIR spectra of Ptx and HA which demonstrated all the functional groups and bonding present in the structures as described in table 1. Other than these peaks, the characteristic peaks of the conjugate were present in the spectra (Figure 4(c)) confirms the amide bond formation between the HA and Ptx. These peaks are present at 3509, 1730 and 1648  $\text{cm}^{-1}$  which indicate that the N-H, C=O and ester bond stretching. The other peaks in the spectra represent structure of HA and Ptx.

The HA-Ptx conjugation was also confirmed by the  $^1\text{H}$ -NMR spectroscopy as shown in figure 5. The NMR spectra of HA, Ptx and HA-Ptx conjugate were shown in the figure 5 (a), (b) and (c)

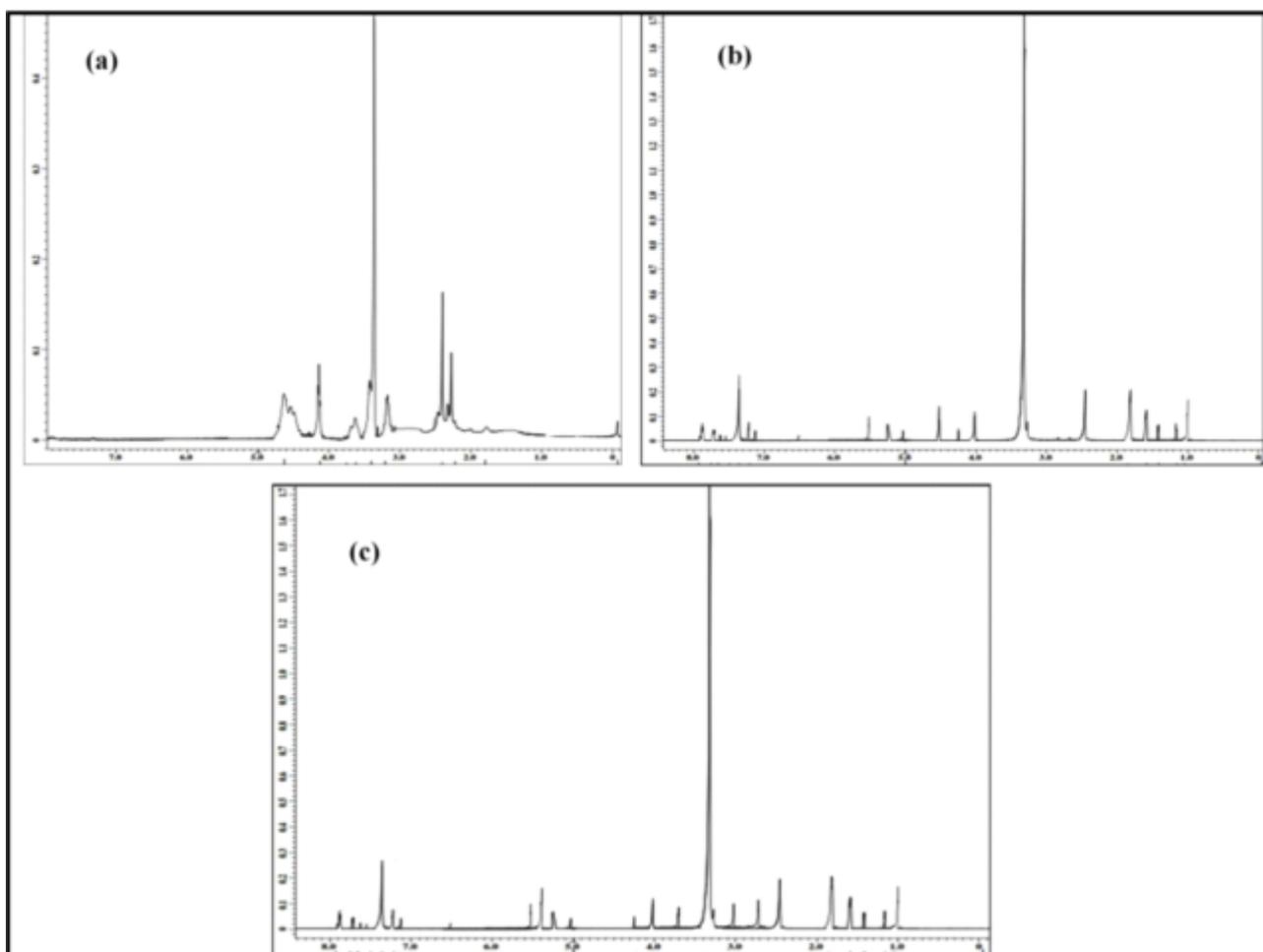
**Table 1.** FTIR Frequency and their respective functional group of Paclitaxel and Hyaluronic acid

Paclitaxel		Hyaluronic acid	
Frequency ( $\text{cm}^{-1}$ )	Functional group	Frequency ( $\text{cm}^{-1}$ )	Functional group
1072.36	C-O stretch	1053.45	C-O stretch (ether)
1108.94	C-O stretch (-OH group)	1406.09	C-O stretch (-OH group)
1276.16	C-O stretch (ester)	1632.56	C=O stretch (ester)
1675.85	C=O stretch (2 <sup>o</sup> amide)	2976.83	C-H stretch
1706.01	C=O stretch (ketone)	3469.68	-OH stretch
1730.08	C=O stretch (ester)		
2920.09	-OH stretch		
3450.07	-NH stretch		

respectively. All the spectra showed the peak at 3.4 ppm which indicated that some of the moisture component was present with the solvent (deuterated DMSO) during the experimental work. The figure 5(a) represents the spectra of HA which showed different peaks at 3.2, 4.1 and 4.6 ppm exhibited the different bonding of ether, C-OH and O=C-NH<sub>2</sub>, respectively. However the figure 5(b) indicated the spectra of Ptx with the presence of different bonds like, -CH<sub>3</sub>-, -CH<sub>2</sub>-, O=C-CH<sub>3</sub>-, -R-OH and O=C-NH<sub>2</sub>, at 1.0, 1.2, 2.5, 4.5 and 7.4



**Figure 4.** FTIR spectra of (a) Pure drug (Ptx), (b) HA and (c) HA-Ptx conjugate



**Figure 5.** NMR spectra of (a) HA, (b) Pure drug (Ptx) and (c) HA-Ptx conjugate

ppm, respectively. The conjugate spectra (Figure 5c) showed that the three or more signals in between 2.5 and 2.9 ppm, confirms the presence of linking chain between the HA and Ptx at the 2'-H of Ptx molecule. The signal at 4.5 ppm of 2'-H in free Ptx spectra (Figure 5b) was shifted to the 5.5 ppm (Figure 5c) of the HA-Ptx spectra also verifies the conjugate bonding between both the moieties.

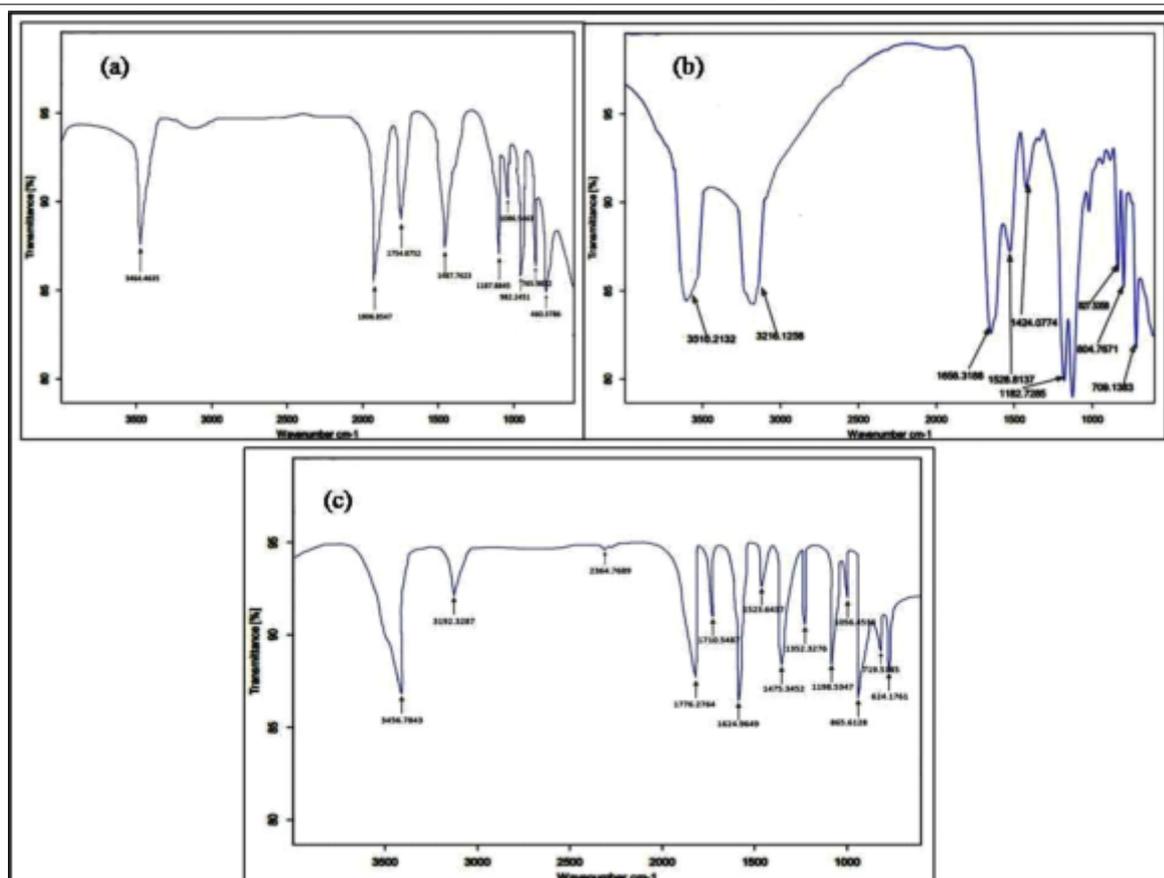
#### Determination of conjugation of RGD peptide to NPs surface

The conjugation of RGD peptide to the PLGA polymer present in the NPs structure was confirmed with the help of FTIR

spectroscopy. Fig. 6(a), 6(b) and 6(c) show the IR spectra of RGD peptide, PLGA and RGD-PLGA conjugate on NPs surface, respectively. The various peaks are showed in the FTIR spectra of RGD peptide and PLGA which demonstrated all the functional groups and bonding present in the structures as described in table 2. The spectra of the conjugate (Fig. 6(c)) shows all the characteristic peaks of the RGD peptide and PLGA polymers were present in the spectra. The amide bond formation between the RGD peptide and PLGA was confirmed by the peaks present at about  $1624\text{ cm}^{-1}$  due to the amide bond stretching.

**Table 2.** IR Frequency and their respective functional groups of RGD peptide and PLGA

RGD Peptide		PLGA	
Frequency ( $\text{cm}^{-1}$ )	Functional group	Frequency ( $\text{cm}^{-1}$ )	Functional group
1182.72	C-N stretch (amide group)	1476.45	C-O stretch (ester)
1424.07	C-H bend	1743.01	C=O stretch
1656.31	C=O stretch	2920.23	C-H stretch
3216.12	-OH stretch (H-bonded)	3510.32	-OH stretch
3510.21	-NH stretch		



**Figure 6.** IR spectra of (a) RGD peptide (b) PLGA and (c) and RGD-PLGA conjugate

The conjugation between the peptide and polymer was also evaluated with the  $^1\text{H}$  - NMR spectroscopy as shown in figure 7. The NMR spectra of PLGA, RGD peptide and RGD-PLGA conjugate were shown in the figure 7 (a), (b) and (c) respectively. The figure 7(a) showed various peaks at 1.5, 4.3 and 7.4 ppm demonstrated the structure of PLGA with the existence of  $-\text{CH}_2-$ ,  $-\text{R-OH}$  and  $\text{O}=\text{C-OH}$  groups in the structure, respectively. While figure 7(b) exhibited the structure of RGD peptide with important NMR peaks present at 1.4, 3.7, 4.8 and 8.0 ppm confirmed the presence of  $-\text{CH}_2-$ ,  $-\text{R-NH}_2$ ,  $\text{O}=\text{C-NH}_2$  and  $\text{O}=\text{C-OH}$  bond in the molecule, respectively. The conjugate spectra (Figure 7c) showed all the signals present as the PLGA and RGD spectra, which confirms the both moieties are available in the final product. The signals at the 4.75 ppm and 8.2 ppm were also present in the figure 7(c) which are characteristic signals of the

conjugate that validate the formation of the conjugate.

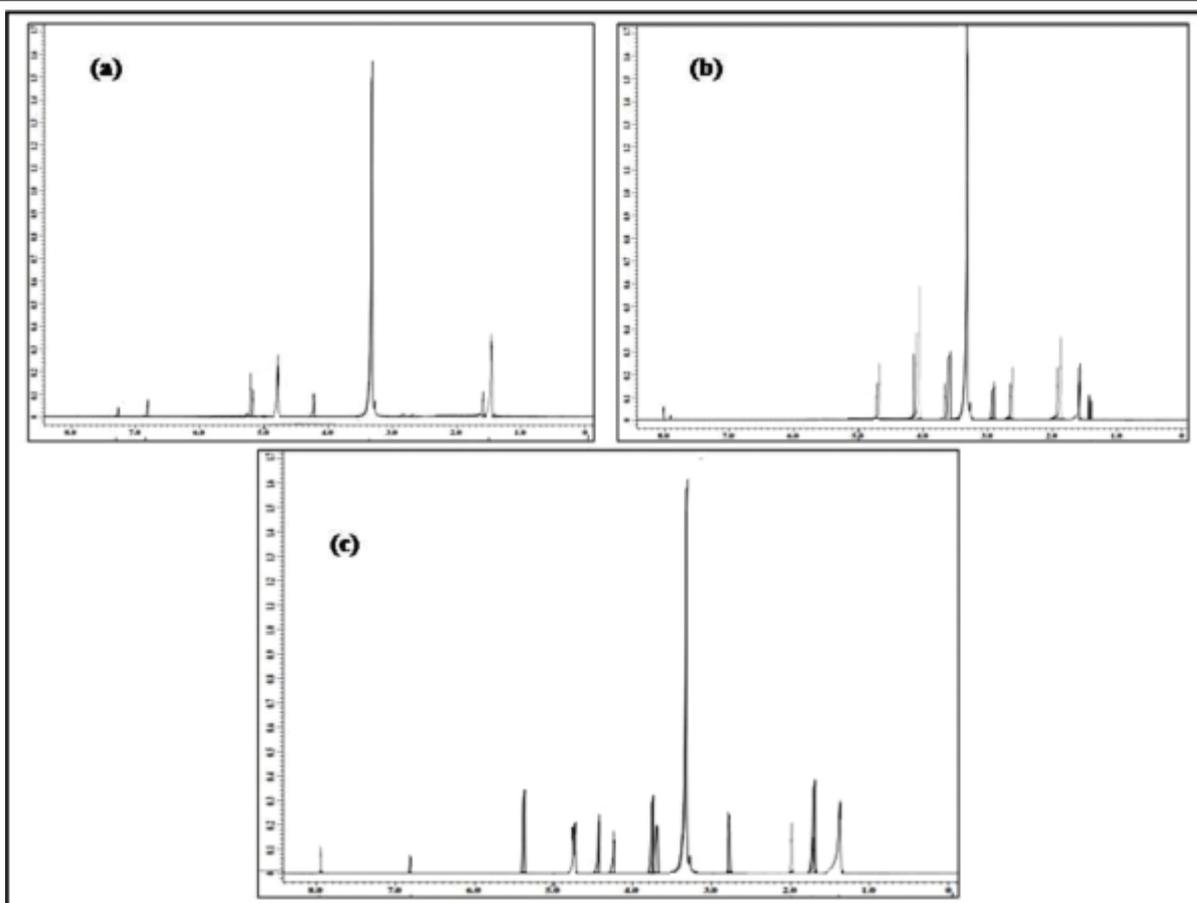
### Characterization of RCNPs

Ptx loaded NPs (PNPs), Conjugate loaded NPs (CNPs) and RGD decorated HA-Ptx conjugate loaded NPs (RCNPs) were prepared using modified nano-precipitation method and characterized for the particle size, PDI, zeta potential, entrapment efficiency and in-vitro release study. The average particle size, PDI, zeta potential and entrapment efficiency were shown in table 3 for the PNPs, CNPs and RCNPs formulations.

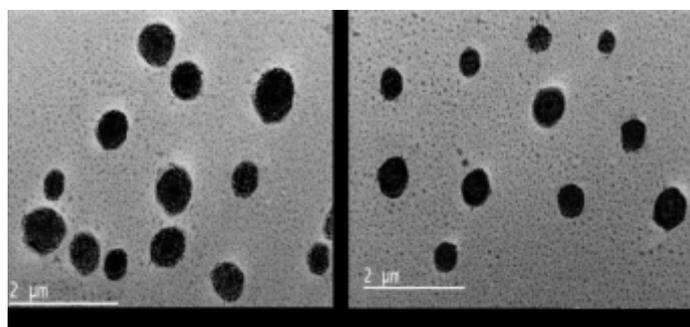
The shape and surface morphology of NPs were determined using TEM and SEM. TEM studies of the CNPs and RCNPs formulation confirmed the particle spherical shape and their discrete and homogeneous dispersion within the size range

**Table 3.** Average Particle size, PDI, Zeta potential and Entrapment efficiency of different formulations

Formulations	Particle size (nm) $\pm$ SD	PDI $\pm$ SD	Zeta potential (mV) $\pm$ SD	Entrapment efficiency (%) $\pm$ SD
PNPs	118.6 $\pm$ 3.1	0.27 $\pm$ 0.03	-37.4 $\pm$ 2.6	71.9 $\pm$ 2.7
CNPs	123.4 $\pm$ 2.4	0.36 $\pm$ 0.06	-34.5 $\pm$ 1.2	70.6 $\pm$ 1.3
RCNPs	127.8 $\pm$ 3.5	0.23 $\pm$ 0.03	-24.6 $\pm$ 2.3	70.3 $\pm$ 1.7



**Figure 7.** NMR spectra of (a) PLGA, (b) RGD peptide and (c) and RGD-PLGA conjugate

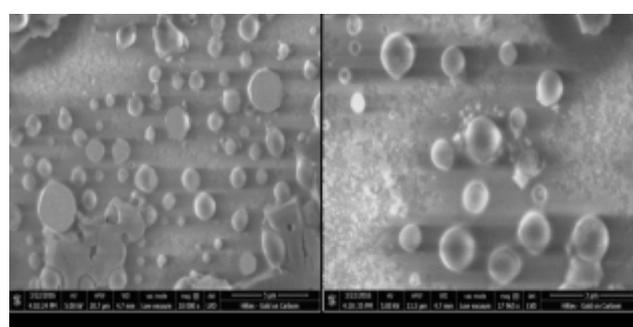


**Figure 8.** TEM photomicrographs of (a) CNPs and (b) RCNPs

of 120–130nm (Figure 8). The surface morphology of the optimized CNPs and RCNPs was also confirmed by SEM and surface of the prepared formulation was found as smooth and spherical in shape (Figure 9).

#### In-vitro release study

In-vitro release study was performed using the dialysis bag method with the help of dialysis membrane (Mw 12000-14000 Da). The release studies were carried out by plotting the graphs with five different kinetic models and equations by using DD solver software. DD Solver software gives various statistical criteria for evaluating the goodness of fit of a model, including the correlation coefficient ( $R_{obs-pre}$ ), the coefficient of



**Figure 9.** SEM photomicrographs of (a) CNPs and (b) RCNPs

determination ( $R_{sq}$ ,  $R^2$ , or COD), the adjusted coefficient of determination ( $R_{sq\_adj}$  or  $R^2_{adjusted}$ ), the mean square error (MSE), the standard deviation of the residuals (MSE\_root or  $Sy.x$ ), SS, WSS, the Akaike Information Criterion (AIC), and the Model Selection Criterion (MSC). Among all these criteria, the most popular ones in the field of dissolution model identification are the  $R^2_{adjusted}$ , the AIC, and the MSC. The best model should be the one with the highest  $R^2_{adjusted}$ , lower AIC value and the largest MSC value. All the resultant graphs were plotted with respect to the time. The important statistical criteria, for all the five models, that were expressed models in table 2 and goodness

**Table 4.** Goodness of Fit table for different dissolution models

Models	R_obs -pre	Rsqr	Rsqr_adj	MSE	MSC	AIC
Zero order	0.8644	0.3262	0.3262	429.4658	-0.0444	132.5240
First order	0.9743	0.9472	0.9472	33.6342	2.5026	94.3190
Higuchi model	<b>0.9936</b>	<b>0.9661</b>	<b>0.9661</b>	<b>21.5925</b>	<b>2.9458</b>	<b>87.6711</b>
Korsmeyer -Peppas model	0.9796	0.9583	0.9551	28.6016	2.6054	92.7762
Hixon Crowell model	0.9896	0.9329	0.9329	42.7654	2.2624	97.9218

of fit of the model was determined. The highest  $R^2_{\text{adjusted}}$  with the minimum AIC and the largest MSC value was found with the Higuchi model in comparison to the other models (Table 4). Therefore, Higuchi model was selected as the best fit model for release study which indicated that the conjugate release from the polymeric matrix dependent on the square root of time, but not dependent on the concentration. The Higuchi model also suggested that the conjugate release from the formulation through the diffusion process for the long period of time.

#### Cytotoxicity study

The cytotoxicity study shows that the RCNPs have better effect on the U87MG cell line when compared with other formulations and drug solutions. The quantitative comparison between the cytotoxicity of the different formulations were performed using the  $IC_{50}$  value which is the minimum concentration of drug required to reduce the cell number upto 50% in a fixed time. Some of the literature suggested that, U87MG cell lines express the integrin receptors in the over expressed manner that is why these cell lines were selected for determining the targeting efficiency of RGD peptide. After 48 hrs of the experiment, the RCNPs and CNPs showed reduction in the cell viability to 50% at very low concentration i.e., less than  $10\mu\text{g/ml}$  and hence exhibited minimum  $IC_{50}$  value among all the other formulations. Although, other formulations also inhibit cell growth, but at lesser extent and thus, they showed higher  $IC_{50}$  value (Figure 10). The  $IC_{50}$  values of different formulations were shown in the table 5 and the lowest  $IC_{50}$  value was found with the RCNPs formulation, may be due to the presence of RGD peptide on the nanoparticle surfaces, which targets the over-expressed integrin receptors on the U87MG tumor cell lines. From the cytotoxicity

**Table 5.**  $IC_{50}$  values of various formulations in the U87MG cell line after 48 hrs (n=3)

Formulation	$IC_{50}$ Value ( $\mu\text{g/ml}$ ) $\pm$ SD
Pure drug (Ptx)	$92.43 \pm 1.96$
Pure conjugate (HA -Ptx)	$67.44 \pm 1.62$
PNPs	$28.61 \pm 2.28$
CNPs	$9.78 \pm 1.07$
RCNPs	$6.57 \pm 1.32$

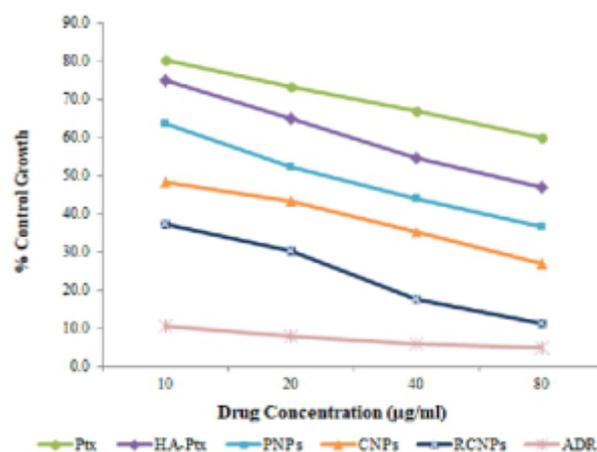
study, we can conclude that the RCNP system is a more effective system for the targeted delivery of drug to the tumor cells. The cytotoxic effect further verified by fluorescence cellular uptake study which will be discussed in the later section.

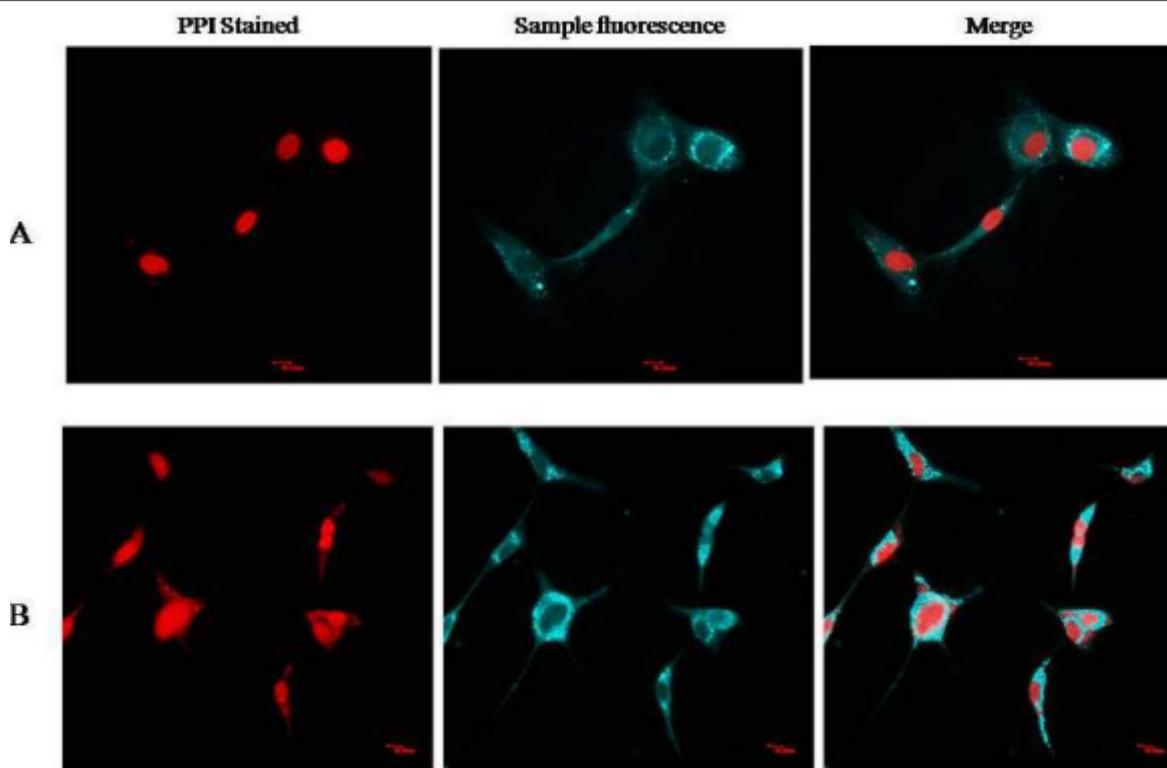
#### Cellular uptake study

The cellular uptake study helps in the determination of therapeutic effects of the formulations. The study suggested that the higher the amount of formulation in the cellular compartment, higher the action of the formulations. Some fluorescent agents like, fluorescein isothiocyanate (FITC), C-6, etc., are used with the formulations for estimation of drug uptake in the cells. In this study, C6 loaded plain NPs and RGD decorated NPs were prepared and their uptake potential was compared in the U87MG cell line. The RGD decorated NPs showed higher fluorescence in the cellular region due to the more uptake of the formulation through the overexpressed integrin receptors on the cells when compared with the C6-PNPs formulation as shown in the figure 11.

#### Conclusion

In the current research work, HA was conjugated with the Ptx and the conjugation was confirmed by IR and NMR spectroscopy. The conjugate loaded polymeric NPs were

**Figure 10.** Cytotoxicity study graph on U87MG cell line after 48 hrs (Mean  $\pm$  SD)



**Figure 11.** Intracellular delivery of two different formulations: C6-PNPs (A) and C6-RNPs (B) in U87MG cells. Cells were treated for 4 hrs with the same concentration and detected by confocal microscopy (Scale = 50 mm).

prepared and RGD peptide was attached to the NP's surface by conjugation chemistry approach. The structure of RGD decorated NPs was also confirmed spectroscopic methods. Finally, the dual targeting delivery system has been developed for the effective treatment of cancer cells with the excellent physicochemical properties like, nano sized particle size, spherical structure and high entrapment efficiency. The *in-vitro* release pattern showed that the formulations follow the Higuchi model which indicated that the conjugate release from the polymeric matrix dependent on the square root of time, but not dependent on the concentration. The *in-vitro* cytotoxicity and cellular uptake study suggested that the prepared formulation may be suitable carrier for targeted drug delivery to tumor cells by using the overexpressed integrin receptors. The RCNPs showed the highest cytotoxicity and cell uptake because of the RGD peptides which are present on the surface of NP formulation. The receptor mediated transport will be acquired via integrin receptors which help in the targeted delivery for the anticancer drug to reach the appropriate site of action hence, potentiate the drug efficacy and reduce the systemic side effects. Therefore, the RGD decorated NPs developed as most promising delivery system for the delivery of chemotherapeutic agent to the cancer cells effectively.

#### Conflicts of interest

The authors disclose no conflicts of interest.

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