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Development of NIPAAm-PEG acrylate polymeric nanoparticles for co-delivery of paclitaxel with ellagic acid for the treatment of breast cancer

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Abstract: The aim of the current study was to develop a dual-loaded core shell nanoparticles encapsulating paclitaxel (PTX) and ellagic acid (EA) by membrane dialysis method. Based on particle size, polydispersity index (PDI), and entrapment efficiency, the dual drug-loaded nanoparticles (F2) was optimized. The optimized nanoparticles (F2) showed a particle size of 140 ± 2 nm and a PDI of 0.23 ± 3 . The size and the morphology were confirmed by transmission electron microscopy (TEM) and found agreement with the results of dynamic light scattering. The entrapment efficiencies of total drug (PTX and EA), PTX, and EA in the nanoparticles (F2) were measured as 80%, 62.3%, and 37.7%, respectively. The *in vitro* release profile showed a controlled release pattern for 48 h. A higher cytotoxicity was observed with nanoparticles (F2) in comparison to free PTX. The results revealed that co-delivery of PTX and EA could be used for its oral delivery for the effective treatment of breast cancer.

Keywords: core-shell nanoparticle; ellagic acid; MCF-7; MTT assay; paclitaxel.

1 Introduction

We have a carrier system in our hand, which is thermo-sensitive, nanosized, and has the ability to release the drug at a controlled pace. Moreover, its effectiveness on the cell lines has also been confirmed. Although it can

carry a single therapeutic agent, our next challenge is to use it for combination therapy. Moreover, we will use this polymer for making a formulation that will entrap a chemotherapeutic agent with an nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) inhibitor that we have chosen here is also novel, and a volume of research is being carried out for its use in anticancer therapies [1]. The idea behind making such a combination being that, the treatment of malignancies with anticancer drugs and radiation has two main tribulations: time-dependent progress of tumor resistance to therapy and imprecise toxicity toward healthy cells. Paclitaxel (PTX) is a widely used anticancer drug in solid tumors of the ovary, breast, lungs, head, and neck malignancies [2]. It also suffers from the same two shortcomings. Induction of the NF- κ B-dependent pathway plays a major role in its resistance, and therefore, the drug cannot be used alone for treatment of advanced malignancies [3]. Numerous plant-isolated polyphenols (genistein, curcumin, resveratrol, silymarin, caffeic acid phenethyl ester, flavopiridol, emodin, green tea polyphenols, piperine, oleandrin, ursolic acid, punicalagin and with other ellagitannins, ellagic acid (EA) and betulinic acid) were examined for their possible anticancer properties and are observed to be pharmacologically safe. The latest research proved that the plant polyphenols can be used to sensitize cancer cells to chemotherapeutic agent and radiation therapy. For example, ubiquitously expressed transcription factor NFB is engaged with a wide range of cell reactions, including cell cycle control, apoptosis, and stress. Punicalgins and ellagitannins hindered NF- κ B and cell viability of prostate cancer cell lines *in vitro* in a dose-dependent manner [4]. A delayed emergence of LAPC4 androgen-independent xenografts was shown in castrated mice through an induction of apoptosis and inhibition of proliferation. They were also studied in colon cancer where they caused inhibition of the Wnt signaling pathways [4]. Still another action by which these ellagitannins can sharpen the chemotherapy is by COX-2 enzyme prevention. COX-2 is an enzyme expressed in response to inflammation, malignancy, and prostaglandin synthesis and is, at present, under

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investigation for anticancer treatment. Its existence was associated with more aggressive tumor phenotypes and inferior result for breast cancer, colon cancer, head and neck cancers, lung cancer, and pancreatic cancer patients [5]. The drug celecoxib and SC-236 (COX-2 inhibitors) were tested *in vitro* and *in vivo* to sensitize the tumor cells in chemotherapy and radiotherapy, and it was found that SC-236 increased tumor radio response in murine tumor models and in a human glioma xenograft in nude mice, and celecoxib enhanced the response of A431 human tumor xenografts in nude mice to radiation and docetaxel chemotherapy, radiotherapy, or both [6–13]. Keeping such things in mind, we proceeded in making a formulation for the co-administration of PTX and a polyphenolic compound (EA) being taken as a model drug, which can act through any of the above suggested pathways and help in sensitizing the cell toward PTX.

2 Materials and methods

2.1 Materials

N-isopropylacrylamide was procured from Sigma-Aldrich (Saint Louis, MO, USA) and purified by crystallization using *n*-hexane. polyethylene glycol acrylate (PEG acrylate), ferrous ammonium sulfate (FAS), ammonium persulfate (APS), benzene, PTX, 1,4-dioxane, doxorubicin hydrochloride, triethylamine (TEA), pyrene, Dulbecco's modified Eagles' medium (DMEM), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT), N,N-dimethylacetamide (DMAc), dimethylformamide (DMF) and dichloromethane (DCM) were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Fetal serum albumin was provided by Invitrogen ThermoFisher Scientific, NG. MCF-7 human-breast carcinoma cells were bought from National Place for Cell Sciences, Pune, India. All chemicals/substances were of analytical grade and utilized as got.

2.2 Interaction between paclitaxel and ellagic acid

Although a volume of research was already done on the metabolic interaction studies of PTX and EA, we will move in for physical interaction, which is a more important factor for making a formulation. In order to determine any physical/chemical interaction between PTX and EA, the two were triturated in a 1:1 ratio. The fine powder thus obtained was analyzed by differential scanning

calorimeter (Perkin Elmer Pyris 6 DSC, USA) to obtain the thermograms for the individual samples as well as for the blend.

2.3 HPLC analysis

A Shimadzu high-performance liquid chromatography (HPLC) system (Kyoto, Japan), comprising of double plunger reciprocating pump, UV-VIS SPA detector, and 20-ml injection loop, was utilized. The column C₁₈ (Jones Chromatography Ltd. CO, USA) with a 5-mm packing was used for the study. Simultaneous analysis of EA and PTX was performed at a detection wavelength of 230 nm. The samples were prepared in methanol-acetonitrile- phosphoric acid (3:1:3) v/v mixture. A mixture of A (0.05% orthophosphoric acid in water) and B (100% methanol) was used as an elution solvent. The samples were eluted according to the following gradient: 80% A/20% B; 60% A/40% B in 5 min; 0% A/100% B in 10 min, 60% A/40% B in 15 min, and 80% A and finally 20% B in 20 min. Analysis parameters like flow rate and run time were set as 1 ml/min and 30 min, respectively [14]. The generated data was processed using Class VP Chromatography Laboratory Automated software (Shimadzu Corporation).

2.4 Synthesis and characterization of NIPAAm-PEG acrylate

N-iso-propylacrylamide (NIPAAm) was purified by recrystallization using *n*-hexane. The pure NIPAAm was then used for polymerization with PEG-acrylate by a free radical polymerization reaction. APS and ferrous sulfate were used as an initiator and an activator, respectively. Briefly, NIPAAm (8.65 g, 53 mM), PEG acrylate (178.5 µl, 1 mM) was dissolved in deionized water. The mixture was deoxygenated by passing nitrogen gas and stabilizing the same at 35°C for 1 h. FAS and APS were added, and the reaction was initiated and was allowed to undergo for 24 h. The same was terminated by atmospheric oxygen. The reaction mixture was then dialyzed using cellulose membranes 12 kDa (Spectrum Medical Industries, Mumbai, India) to remove all the monomers. The same was then taken for lyophilization, and the powder was then taken for further studies. The proton NMR spectra (¹H-NMR) of the polymer were examined utilizing a Bruker-Avance spectrometer (400 MHz), and deuterated chloroform (Merck, Uvasol) was utilized to dissolve the polymer. FTIR

spectral analysis was carried out utilizing Nicolet, Protege 460 FTIR spectrometer.

2.5 Measurement of lower critical solution temperature (LCST)

The core-shell nanoparticles were developed in phosphate buffer with pH 7.4 and 6.6. The optical transmittance of the solution was estimated by increasing the temperature. The temperature of the solution was varied with the frequency of 0.10°C/min. The transmittance was estimated at 500 nm utilizing a double beam UV/VIS spectrophotometer (Shimadzu, Japan). Another technique was used based upon the variation in size with temperature. Size variation was studied using Malvern Zeta sizer (Malvern Instruments Ltd., Holtville, NY, USA). The LCST values of the polymer were determined at the temperature showing optical transmittance less than 50% [15].

2.6 Preparation of paclitaxel and ellagic acid-loaded core-shell nanoparticles

Core-shell nanoparticles were formed using a dialysis membrane. The amount of NIPAAm-PEG acrylate was specified so, formed (55–70 mg), PTX (3–5 mg), and EA (8–10 mg) were solubilized in 10 ml of DMF, and then, the dialysis of the solution was carried out against deionized water at different temperatures for 24 h utilizing a dialysis membrane (cut off of 12 kDa). The water was replaced every 60 min for the first 12 h. After completion of the dialysis, the solution was collected and filtered, and lyophilized.

2.7 Measurement of size and distribution

The size and size distribution were measured using dynamic light scattering (DLS) through a Malvern Zeta sizer. The DTS software was used for the analysis. Each analysis was measured three times [16].

2.8 Transmission electron microscopy

In order to check any aggregation, transmission electron microscopy (TEM) pictures were taken for that nanoparticulate solution as a drop was fixed on a copper grid and was allowed to dry overnight at room temperature.

The grid was then placed under JEM-2010 microscope (JEOL, Japan) with an electron kinetic energy of 300 keV.

2.9 Encapsulation efficiency of paclitaxel and ellagic acid

Ten grams of powdered nanoformulation was extracted at room temperature with two portions of absolute methanol, 25 ml each [17]. The obtained residue was extracted again under the same conditions with two portions of 25 ml of methanol/acetic acid (80:20, v/v). The obtained extracts were collected and dried in a rotary evaporator at 400°C. The dried residue was solubilized in 5 ml of a methanol/acetic acid/orthophosphoric acid (3:1:2, v/v/v) mixture and analyzed for EA and PTX content by HPLC [14].

2.10 *In vitro* drug release

A known amount of the lyophilized drug-loaded nanoparticles (100 mg) was dispersed in 10 ml of phosphate buffer, pH 7.4, and the solution was divided in 10 microfuge tubes containing 1 ml each [16]. The tubes were kept in a thermostable water bath (Daihan Lab Tech, Korea) set at room temperature. At predetermined time intervals the solution was centrifuged at 3000 rpm to separate the released drug. The released drug was re-dissolved in methanol/Acetic acid/orthophosphoric acid mixture and the concentration was determined with the help of a standard curve being made by HPLC of the standard drug in the same solvent mixture [14]. The percentage of the released drug moieties was determined from the equation:

$$\text{Release (\%)} = \{[\text{Drug}]_{\text{rel}} / [\text{Drug}]_{\text{tot}}\} \times 100$$

where $[\text{Drug}]_{\text{rel}}$ is the concentration of the released PTX and EA collected at time t , and $[\text{Drug}]_{\text{tot}}$ is the total amount of PTX encapsulated in the nanoparticles.

2.11 *In vitro* cytotoxicity studies

In vitro cytotoxicity test of drug alone (free drugs) and drug-loaded nanoparticles were performed against MCF-7 breast cells lines [18, 19]. The cells were seeded onto 96-well plates at a thickness of 10,000 cells for each well and bred for 24 h. Drug alone as well as drug-loaded nanoparticles in DMEM were filtered using a 0.22- μm syringe filter and diluted with growth medium to get

concentrations of 10^{-4} , 10^{-3} , 10^{-2} , 10^{-1} , 1, and 10 mg l⁻¹. The blank nanoparticles were diluted to make the concentrations of 1, 10, 50, 100, and 200 mg l⁻¹. The media in the wells were then replaced by 100 µl of the prepared samples. The plates were again incubated in a CO₂ incubator with CO₂ levels at 5% at 37°C for 48 h. The MTT solution (10 µl) and fresh growth media were further added after 2 days. The plates were again incubated at 37°C for 3 h. The growth media and MTT in each well were then removed. The reaction was then harvested using 100 µl of isopropanol/HCl mixture. The same was then transferred to a fresh 96-well plate. The plates were then assayed at 570/630 nm.

3 Results and discussion

3.1 Interaction between ellagic acid and paclitaxel

For physical/chemical interaction, DSC thermograms of PTX, EA, and the blend (Figure 1) were obtained. The DSC

Figure 1: DSC thermograms of PTX, EA, and its blend.

thermogram of PTX shows a small endothermic peak at 60°C followed by a marked exothermic peak at 350°C. On the other hand, EA shows two marked endothermic peaks (90°C and 260°C). When the blend of the two was taken, two peaks can be clearly differentiated: endothermic at 260°C that could be due to EA and exothermic at 350°C that is probably due to PTX. Further, the literature states that PTX gets metabolized by liver microsomal enzymes to give metabolites like 6- α -hydroxy PTX (6- α -OHP), metabolized by CYP2C8, and C3'-OHP, C2-OHP with another minor metabolites, metabolized by enzyme CYP3A4. EA comes under the category of non-inhibitory CYP3A4 and CYP2C8 phenolic antioxidants [20].

3.2 Synthesis of NIPAAm-PEG acrylate

P(NIPAAm-PEG acrylate) is synthesized by free radical polymerization reaction in the presence of FAS and APS as initiator and activator [21]. The chemical structure and composition of P(NIPAAm-PEG acrylate) were studied by ¹H NMR (Figure 2). The resonance peak obtained at δ 3.652 is attributed to protons of (CH₂CH₂O) from PEG acrylate. The resonance peak at δ 2.538 is from the protons of (CH-C=O). Further, the peak at δ 2.483 is from (HN-CH (CH₃)₂), at δ 1.879 is from (NH), and finally at δ 1.011 is from (HN-CH (CH₃)₂) of NIPAAm. The FTIR spectrum of the polymer is as shown in Figure 3. The peaks obtained are explained:

Figure 2: Chemical shift values from NMR spectra of NIPAAm-PEG acrylate.

Figure 3: FTIR spectra of NIPAAm-PEG acrylate.

3400 cm^{-1} (N-H stretch), 3273 cm^{-1} (N-H stretch) from NIPAAm segment, 3080 cm^{-1} (=C-H stretch of alkene), 2973 cm^{-1} (C-H stretch alkane), 1627 cm^{-1} (C=O stretch of carbonyl) from NIPAAm segment, 1458 cm^{-1} (C-H bend alkane), 1048.81 cm^{-1} (=C-H bend). Disappearance of the peak at 1655 cm^{-1} (C=C-stretch) confirms the breaking of the double bond from NIPAAm.

3.3 LCST of nanoparticles

The copolymer self assembles into core-shell nanoparticles in such a way that the hydrophobic core is well isolated from the outer hydrophilic shell that remains exposed to the aqueous environment, and the structure is deformed releasing the drug above a particular temperature. This particular temperature is referred to as LCST. NIPAAm exhibits the LCST of 32°C. Adding PEG-acrylate to NIPAAm will alter its lower critical solution temperature. The optimization of the parameters was done, keeping in mind that the LCST is so maintained that maximum amount of the drug is released in or near the tumor environment. Both EA solubility and solubility of the proposed polymer are pH dependent. All the LCST studies were, therefore, conducted at a pH value of 7.4. The LCST values depended upon the process parameters like fabrication temperatures, concentration of initiators and activators, pH of the reaction, concentration, and ratio of the monomers being added. By optical transmittance studies of different polymers so formed, the LCST was determined and that polymer was selected that exhibited LCST of 38°C. Figure 4 shows the optical transmittance of the nanoparticulate solutions as a function of temperature.

Figure 4: Lower critical solution temperature of the synthesized polymer at pH 7.4.

3.4 Size, morphology, and stability of the nanoparticles

The size and morphology are the two most important parameters that decide if these particles can be used as a carrier for drugs or not [22]. Blank nanoparticles showed the size of about 123 ± 2 nm. Loaded nanoparticles are slightly bigger in size with relatively narrow size distribution, and the effective size ranges from 140 ± 2 to 230 ± 3 nm. This can be possibly due to nanoparticles with dual drug loading. There is a slight difference in the loaded as well as unloaded nanoparticles. Such small-sized particles makes it ideal for targeting to the tumor as it can undergo passive targeting due to the EPR effect. A TEM picture indicates that particles are spherical in shape, and their size is in good agreement with that measured by DLS (Figure 5). The effect of an initial amount of drug added and polymer concentration can clearly be depicted from Table 1. As the drug:polymer concentration decreases, the size increases. This can be attributed to a decrease in solvent exchange rate due to a higher concentration of the

Figure 5: TEM images of optimized formulae (F2).

polymer. A higher polymer concentration decreases the solvent exchange rate. Another important aspect is stability. Nanoparticles tend to aggregate, and this could lead to occlusion of the blood vessels. These core-shell nanoparticles have PEG-acrylate as the outer part, which is hydrophilic in nature, and therefore, under physiological conditions, they remain in a deaggregated state. This could be seen well from the TEM image, which was taken in phosphate buffer with pH 7.4.

3.5 Encapsulation efficiency

Encapsulation efficiency is affected by the number of fabrication parameters that include initial drug loading, polymer concentration, and micelle fabrication temperature [23]. It was observed that at ambient and higher

temperatures, the nanoparticles have lower encapsulation efficiency when compared to lower temperatures, the reason being that at lower temperatures, the solubility of the polymer is higher, whereas as the temperature increases unlike other solutes, the solubility decreases. Another reason could be attributed to the slow solvent exchange at low temperatures, and therefore, the hydrophobic drug has more time to be incorporated into the core, and loss during the solvent exchange can be minimized. The micelles are loaded by the dialysis process, which involves a solvent exchange mechanism. Both PTX and EA along with the polymer are taken in DMF, and these are then poured in a dialysis bag, which is finally transferred to water. The molecular weight cut of the dialysis membrane was 12 kDa, and the molecular weight of PTX is 853 and of EA is 302. Therefore, certain loss of the drug was inevitable, and maximum encapsulation efficiency achieved was about 74% for PTX and 62.12% EA at 20°C. On the other hand, at 4°C, the encapsulation efficiency was found to be 92.2% and 98.4% for PTX and EA, respectively. Therefore, for further studies, the loading was carried out at lower temperatures.

The effect of initial drug loading and polymer concentration was also studied and it has been found that the lower the drug:polymer concentration ratio is, the higher is the encapsulation efficiency (F1 and F2). If the initial drug loading is higher (F7), the higher concentration gradient leads to a greater loss and, thus, lower encapsulation efficiency. Moreover, as depicted from F5, F6, and F7, on the higher concentrations of the drug, there is hardly any increase in the encapsulation efficiency, even on taking a higher polymer concentration. The reason could be attributed to the fact that when higher concentrations are taken in a dialysis bag, the contents turn turbid, and 10, as well as 20 mg, is much higher than the carrying capacity of the core resulting in a lower encapsulation efficiency. From F3 and F4, it can be predicted that taking a higher concentration of polymer can provide a larger hydrophobic domain for the encapsulation of the hydrophobic drug molecules

Table 1: Size and encapsulation efficiencies of the formulations.

Codes	Mass of the drug (mg) PTX:EA (1:1)	Mass of the polymer (mg)	%EE total, PTX, EA	Size $\pm$ SD (nm)	PDI $\pm$ SD
F1	2	10	67; 58.1; 44.9	150 $\pm$ 1	0.13 $\pm$ 2
F2	2	20	80; 62.3; 37.7	140 $\pm$ 2	0.23 $\pm$ 3
F3	4	16	58; 68.2; 31.8	216 $\pm$ 2	0.38 $\pm$ 1
F4	4	30	74; 58.4; 44.6	142 $\pm$ 1	0.62 $\pm$ 1
F5	10	40	47; 76.4; 23.6	230 $\pm$ 3	0.14 $\pm$ 2
F6	10	50	49; 72.5; 27.5	182 $\pm$ 4	0.13 $\pm$ 1
F7	20	100	47; 78.8; 24.2	155 $\pm$ 3	0.54 $\pm$ 2

(Table 1). Another point to be noted is that PTX being more hydrophobic compared to EA gets incorporated fast compared to EA. The partition coefficient of PTX is 3.5 (log P) and EA is 0.26 (log P); therefore, loss of EA at the time of loading is higher compared to PTX. Based on particle size, polydispersity index, and entrapment efficiency, the formula F2 was optimized and selected for further studies.

3.6 *In vitro* drug release

In vitro release of the PTX and EA from optimized nanoparticles (F2) was studied under physiological conditions, i.e. using PBS at pH 7.4. [24]. As shown in the Figure 6, the release profile is characterized by an initial burst. As can be inferred from the profile, the release was in a very controlled manner. Cumulative percentage release was lesser for PTX compared to EA. In the first 2 h, the release was only $8.982 \pm 0.54\%$ for PTX and $8.23 \pm 0.34\%$ for EA, whereas in 48 h, the release was $61 \pm 2.52\%$ for PTX and 88.64 ± 0.93 for EA.

Figure 6: *In vitro* drug release at different temperature points.

Figure 7: MTT assay to compare the cell viability from free PTX as well as polymeric micelles encapsulated.

3.7 *In vitro* cytotoxicity studies

The cytotoxicity of free drug and drug-loaded nanoparticles (F2) was studied using MTT assay against MCF-7 cell line (Figure 7). PTX and EA-loaded nanoparticles showed a slightly higher cytotoxicity when compared to free drug because of the increased cellular uptake of the nanoparticles. IC_{50} values for free PTX vs. nanoparticles were $80 \mu\text{g/l}$ and $45 \mu\text{g/l}$. It should be noted that blank nanoparticles did not show any cytotoxicity at concentrations up to 200 mg/l .

4 Conclusion

Temperature-sensitive amphiphilic copolymer poly(*N*-isopropylacrylamide-PEG acrylate) was prepared and utilized in the development of core-shell nanoparticles. PTX can be easily loaded into the nanoparticle under the optimized condition that could have potential to increase the bioavailability. The size of the developed nanoparticles can be controlled below 200 nm . The nanoparticles have such an LCST, to the point that in the physiological condition (pH 7.4), the developed nanoparticles were stable. The novel formulation also displayed a better release of the two drugs over a longer period of time at a higher temperature. This one-of-a-kind property can be utilized for intracellular delivery of the anti-cancer drug. The PTX and EA-loaded nanoparticles show prominent cellular uptake and especially higher cytotoxicity against MCF-7 cells in comparison to blank PTX; however, the blank nanoparticles are not cytotoxic at a concentration of up to 200 mg/l . These combinatorial nanoparticles may make an efficient carrier for anticancer drug delivery as they can also assist in overcoming multidrug resistance and making PTX therapy more effective.

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