Context: Nanocarriers possessing long-circulating abilities could take advantage of the pathophysiology of tumor vasculature to achieve spatial placement. To attain such qualities, the drug carriers should possess suitable physicochemical properties such as size and surface hydrophilicity. Aim: The aim of this study was to prepare poly(ε-caprolactone) nanoparticles (NPs) loaded with vinorelbine bitartrate (VB) and to modify its steric properties using polyethylene glycol and poloxamer. Furthermore, the influence of surface modification of NPs on their physicochemical and cell interactive properties was evaluated. Materials and Methods: NPs were prepared by double emulsion solvent extraction–evaporation technique. The prepared NPs were evaluated for their physicochemical properties, in vitro protein adsorption and cell cytotoxicity. Results and Discussion: The NPs were <250 nm with an entrapment efficiency ranging between 40% and 52%. The zeta potential of the NPs varied from −7.52 mV to −1.27 mV depending on the surface modification. The in vitro release studies exhibited a biphasic pattern with an initial burst release followed by controlled release of the drug over 72 h. The protein adsorption studies revealed that the ability to resist protein adsorption was influenced by the concentration of surface-modifying agents and the amount of proteins available for interaction. The surface-modified NPs produced cell cytotoxicity comparable to free VB at higher concentrations owing to sustained release of the drug into the cellular environment. Conclusion: The results emphasize that surface modification of nanocarriers is an essential and effective tool to dodge opsonization and phagocytosis in the physiological milieu.

Keywords: Cell cytotoxicity, nanoparticles, protein adsorption, surface modification, vinorelbine

INTRODUCTION

The clinical success of nanocarriers based on passive targeting depends on their capability to take advantage of the enhanced permeability and retention (EPR) effect, which is observed to occur unanimously in all types of fast-growing solid tumors with the exception of hypovascular tumors.[1,2] Unlike the normal blood vasculature, which is composed of a nonfenestrated, single layer of endothelial cells with tight junctions, the tumor vasculature shows a defective architecture with large fenestrations about 100–600 nm. The fenestrations are sufficiently large for the extravasation and accumulation of molecules into the vascular bed and the interstitial space.[3,4] Another important feature of solid tumors is the lack of lymphatic drainage that helps in retaining the macromolecules within the tumor tissue.[5]

To exploit the EPR effect, the nanocarriers should remain in the blood circulation long enough to reach the tumor site. However, the prevalence of nanoparticles (NPs) in
blood is greatly impeded by the adsorption of serum proteins, called as opsonization process. The opsonized particles are easily recognized by the receptors on the mononuclear phagocyte system (MPS) and subsequently cleared from the circulation.\[6,7\] These cellular interactions are greatly influenced by their design parameters such as size, surface charge, and surface hydrophobicity.\[8\] The widely used method to avert opsonization is the surface adsorption of hydrophilic shielding groups that overcomes the electrostatic and hydrophobic interactions of the particle with the opsonins.\[9\] Vinorelbine bitartrate (VB) is a semi-synthetic derivative of vinca alkaloids that exhibits effective activity in nonsmall cell lung cancer and advanced breast cancer by disrupting microtubules. The present study was aimed at the formulation of VB loaded poly(ε-caprolactone) (PCL) NPs and to modify the steric properties of the NPs using hydrophilic surface coatings. The prepared NPs were evaluated for physicochemical properties, in vitro protein adsorption and cell cytotoxicity to assess the influence of surface modification on their interactive properties.

**Materials and Methods**

**Materials**

PCL (14 kDa), poloxamer (POL) (Pluronic F68 and F128), and dialysis tubing membrane (MW cutoff: 12,000–14,000) were purchased from Sigma-Aldrich, India. VB was a generous gift from Onco Therapeutics Ltd., Bangalore, India. Polyethylene glycol (PEG) (2 kDa and 4 kDa), polyvinyl alcohol (PVA), and human serum albumin (HSA) were purchased from Merck, Mumbai, India. Eagle’s Minimum Essential Medium (M2279) was purchased from Sigma-Aldrich, India. All other solvents and chemicals used in the study were of analytical grade.

**Methods**

**Preparation of poly(ε-caprolactone) nanoparticles**

VB loaded NPs were prepared by double emulsion (W₁/O/W₂) solvent extraction–evaporation technique.\[10\] Briefly, 20 mg of VB dissolved in one milliliter of ethanol formed the internal aqueous phase (W₁). PCL polymer (200 mg) dissolved in 10 ml of methylene chloride formed the oil phase (O). The internal aqueous phase was then emulsified in the polymeric solution by sonication over an ice bath using an ultrasonic probe (Oscar Ultrasonics, India) at 200 W for 5 min. The pulse was turned off for 2 s with the interval of 2 s to prevent the temperature rise. The resulting primary emulsion was added dropwise into 50 ml of 1% w/v PVA solution in water (W₂) and sonicated 20 times to form a double emulsion (W₁/O/W₂). The emulsion was stirred overnight to evaporate the organic solvent. The NPs were collected by centrifugation (R24-Remi, India) at 12,000 rpm for 30 min followed by washing thrice with distilled water. The produced nanosuspension was freeze-dried for 48 h, at −46°C using a Benchtop lyophilizer (Lyodel, Delvac Pumps Ltd., India) and was stored at 4°C. The preparation of the VB-NP is schematically presented in Figure 1.

![Figure 1: Schematic representation of preparation of poly(ε-caprolactone) nanoparticles of vinorelbine bitartrate](image_url)
Preparation of surface-modified nanoparticles

In this scheme, the steric property of NPs was modified by inclusion of surface-modifying agents (SMA) such as PEG or POL. Briefly, 5% or 10% w/w of SMA (with respect to the weight of the polymer) was co-incorporated along with polymer in methylene chloride to form the oil phase. The internal aqueous phase was then emulsified in the polymeric solution by sonication over an ice bath using an ultrasonic probe at 200 W for 5 min. The rest of the procedure was followed as mentioned above for VB-NP.

Characterization of nanoparticles

Particle size, zeta potential, and surface morphology

The average particle size and zeta potential of VB loaded NPs were determined by using Zetasizer Nano ZS (Malvern Instruments Ltd., UK). The electrophoretic mobility between the electrodes was converted to zeta potential based on Smoluchowski equation. Each sample was determined three times, and the results were expressed as mean value ± standard deviation. The surface morphology of the NP in colloidal suspension was observed by scanning electron microscopy (SEM) (JSM 840A, JOEL, Japan) and transmission electron microscopy (TEM) (H-7500, Hitachi, Japan).

Determination of drug loading parameters

The VB content in the NP formulation was determined by high-performance liquid chromatography (HPLC) assay using HPLC (LC-20AD, Shimadzu, Japan) equipped with a Phenomenex C18 column (250 mm × 4.6 mm, particle size-5 µm) at room temperature. About 10 mg of NPs was solubilized in 1 ml of dichloromethane. The solution was gently stirred to allow the solvent to evaporate and the mobile phase (a mixture of methanol and phosphate buffer pH 5.0 in 60:40% v/v) was added to extract the drug. The solution was then filtered through 0.45 µm filter and subjected to degassing before use. The samples were analyzed at a flow rate of 1.0/min, and the encapsulated drug was detected at 269 nm by a SPD-20A UV detector equipped with Sphinchrom software (Shimadzu, Japan). The drug loading (DL) and encapsulation efficiency (EE) of the VB loaded NPs are calculated according to the following equations (1) and (2), respectively:[11]

\[
DL \% = \frac{\text{Weight of VB in nanoparticles}}{\text{Weight of the nanoparticles}} \times 100 \\
\text{EE} \% = \frac{\text{Weight of drug in nanoparticles}}{\text{Weight of the feeding drug}} \times 100
\]

In vitro release of vinorelbine bitartrate from nanoparticles

The release profile of VB from plain and surface-modified NP was determined by the dialysis method using 0.01 M phosphate buffer saline (PBS) pH 7.4. About 25 mg of NP was accurately weighed and redispersed in 2 ml of PBS by continuous shaking at 37°C. The nanosuspension was then placed in a dialysis tubing membrane bag. The end sealed dialysis bag was then immersed into flasks containing 50 ml of PBS. The flasks were agitated at 100 strokes/min in an incubator shaker (CIS-24, Remi, India) maintained at 37°C for 72 h. At regular time intervals, 0.5 ml aliquots of sample were withdrawn from the flask, and the equivalent volume of fresh media was replaced to maintain pseudo-sink state. The drug quantity in the samples was found by HPLC method as described above. The experiment was conducted thrice, and the results were expressed as cumulative drug release.

In vitro protein adsorption studies

The in vitro protein adsorption study was to quantify the amount of protein adsorbed on the surface of polymeric NPs.[12,13] The VB-NP and surface-modified nanoparticle (SNP) formulations are combined with HSA at different concentration ratios such as 95:5, 90:10, 80:20, and 60:40 v/v. The final volume of the mixture was made up to 1000 µl in microcentrifuge tubes. The prepared samples were incubated for 2 h and centrifuged at 14,000 rpm for 30 min. The supernatant liquid was separated from the NP pellet, and the amount of protein in the supernatant was determined by total protein analysis using Lowry’s assay. The concentration of protein was obtained from the calibration curve made from known concentrations of plasma. The percentage protein bound to the NP was calculated from the equation given below:

\[A_b = 100 - A_u\]

Where \(A_b\) is the percentage protein bound and \(A_u\) is the percentage protein unbound.

Cell cytotoxicity studies

The human breast cancer cell line (MCF-7) was obtained from National Centre for Cell Sciences, India. A cell suspension was formed from the aliquots of stock by detaching the monolayer cells using trypsin-ethylendiaminetetraacetic acid. The viable cells were counted using a hemocytometer and diluted with Eagle’s Minimum Essential Medium containing 5% fetal bovine serum to form a final concentration of 1 × 10^5 cells/ml. The cell suspension (100 µl) was seeded in the 96-well plates and incubated at 37°C for 24 h. Following the incubation period, VB or their NP formulations were added to the culture medium to attain a final volume of 200 µl per well. The medium without drug treatment served as positive control. The cells were exposed for free drug/NP formulations at 37°C for 24 h. After incubation, 15 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
bromide (MTT) (5 mg/ml) in PBS was added to each well and incubated at 37°C for 4 h. The formed formazan crystals were solubilized in 100 μl of dimethyl sulfoxide, and the absorbance was measured at 570 nm using a microplate reader. The absorbance of the test sample was normalized by subtracting with absorbance of respective concentration of blank and the results were expressed as percentage cell inhibition.

RESULTS
Characterization of unmodified and surface-modified poly(ε-caprolactone) nanoparticles
The average particle size of the NPs varied from 160 to 235 nm with a narrow particle size distribution exhibited by the polydispersity index below 0.2. The VB-NPs are slightly larger than the blank NPs (BNP) owing to the inclusion of drug.[12] The representative histograms of BNP, VB-NP, and SNPs are shown in Figure 2. Increasing the concentration of SMA from 5% to 10% decreases the particle size of the NPs as shown in Table 1. The zeta potential of the NPs was negative and varied between −1.27 ± 0.11 mV and −7.52 ± 0.43 mV as shown in Table 1. The small potential difference between BNP and VB-NP is due to the partial adsorption of the drug on the surface of NPs.[14] Inclusion of SMA into the NPs shifted the surface charge toward neutral as shown in Table 1. The representative results of the zeta potential analysis are shown in Figure 3.

The NP formulations on redispersion formed a clear colloidal dispersion as shown in Figure 4a. The morphological studies of the NP using SEM revealed that the particles are uniform, discrete, and spherical with particle size below 200 nm, which is in correlation with the results of particle size analysis. The representative image of VB-NPs is shown in Figure 4b and c. TEM results demonstrated that the NPs were spherical with a smooth surface [Figure 4d]. The SNP covered with PEG/POL chains showed a surface cloud around the core of the NPs. This hydrophilic cloud was prominently displayed on the surface of SNP-4 prepared with

Table 1: Characterization of vinorelbine bitartrate loaded nanoparticles (n=3)

| Code | Nanoparticles formulation | Amount of SMA (%) | Size (nm) | Polydispersity (PDI) | Zeta potential (mV) | Drug loading (%) | Entrapment efficiency (%) |
|------|---------------------------|-------------------|-----------|----------------------|--------------------|------------------|--------------------------|
| BNP  | BNP                       | -                 | 165.8±12.1| 0.067                | −7.52±0.43         | -                | -                        |
| DNP  | VB-NP                     | -                 | 180.5±6.8 | 0.186                | −7.08±0.54         | 4.78±0.23        | 52.63±2.6               |
| SNP1 | PEG<sub>2000</sub>−VB-NP  | 5                 | 224.5±13.4| 0.124                | −4.86±0.22         | 4.48±0.19        | 49.36±2.2               |
| SNP2 | PEG<sub>4000</sub>−VB-NP  | 10                | 206.2±9.6 | 0.110                | −4.72±0.47         | 4.14±0.29        | 45.62±3.2               |
| SNP3 | PEG<sub>8000</sub>−VB-NP  | 5                 | 230.4±7.4 | 0.210                | −2.18±0.18         | 4.25±0.15        | 46.82±1.7               |
| SNP4 | PEG<sub>12000</sub>−VB-NP | 10                | 218.1±10.3| 0.147                | −1.27±0.11         | 3.84±0.24        | 42.34±2.6               |
| SNP5 | POL<sub>16k</sub>−VB-NP   | 5                 | 228.5±8.4 | 0.197                | −4.13±0.56         | 4.26±0.30        | 46.97±3.4               |
| SNP6 | POL<sub>16k</sub>−VB-NP   | 10                | 216.9±9.3 | 0.178                | −3.78±0.22         | 3.84±0.17        | 42.23±1.9               |
| SNP7 | POL<sub>32k</sub>−VB-NP   | 5                 | 236.5±4.3 | 0.166                | −4.02±0.64         | 4.16±0.08        | 45.75±0.9               |
| SNP8 | POL<sub>32k</sub>−VB-NP   | 10                | 229.3±2.7 | 0.172                | −2.38±0.43         | 3.65±0.11        | 40.27±1.2               |

BNP: Blank nanoparticles, VB: Vinorelbine bitartrate, NP: Nanoparticles, PEG: Polyethylene glycol, POL: Poloxamer, SMA: Surface-modifying agents, PDI: Photodynamic inactivation, DNP: Dextran-based nanoparticles, SNP: Surface-modified nanoparticle
PEG (4 kDa; 10%) as shown in Figure 4c. The DL and EE of VB-NP were found to be 4.7% and 52.63%, respectively. VB is an amphoteric molecule. Previous reports have demonstrated that the incorporation of amphoteric drugs into hydrophobic polymer core resulted in low percentage loading since the loading efficiency would depend on the solubility of drug in the polymer matrix.[10,15,16] Increase in the molecular chain length of PEG or POL or increasing their concentration decreased loading and EE of SNPs as shown in Table 1.

**In vitro release of vinorelbine from poly(ε-caprolactone) nanoparticles**

The release of VB from PCL NP in PBS, pH 7.4 was characterized by an initial burst release followed by a steady, controlled release of the drug. The NPs exhibited a burst release up to 34.8% in the first 12 h. The surface-modified NPs presented a faster release as compared to unmodified VB-NP. The NPs released up to 44.5% to 73.3% of their total payload at the end of 72 h based on their composition [Figure 5]. Fitting and analyzing the in vitro release profile using various kinetic models showed the highest correlation coefficient and best fit for the Higuchi model, thus confirming diffusion as the mechanism of drug release from the NPs.

**In vitro protein adsorption studies**

The present study involves the surface engineering of PCL NPs using different hydrophilic polymers. The use of lower concentrations of PEG or POL is not effective in resisting the protein adsorption (data not included). Hence, the results of protein adsorption studies of formulations containing higher concentrations of hydrophilic polymers (SNP-2, SNP-4, SNP-6, and SNP-8) were compared in Figure 6. The most important factor that influences the protein corona-NP composition is the amount of proteins available in the milieu for interaction with the NP surface. Therefore, to study the influence of the amount of protein, different concentration ratios of HSA were incubated with NP formulation. At a low concentration ratio (5:95) of the HSA, the surface adsorption was found to be analogous, but the ability of the NPs to resist or favor the adsorption of proteins

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**Figure 3:** Results of zeta potential analysis of poly(ε-caprolactone) nanoparticles

**Figure 4:** Surface morphology of poly(ε-caprolactone) nanoparticles: Appearance of vinorelbine bitartrate loaded nanosuspension and polyethylene glycol modified nanoparticle formulation (a); scanning electron microscopy images of vinorelbine bitartrate loaded poly(ε-caprolactone) nanoparticles (b and c) transmission electron microscopy image of vinorelbine bitartrate-nanoparticle (d) transmission electron microscopy image of surface modified nanoparticles-4 (e)
was evident as the concentration of HSA available for interaction was increased.

For an equal concentration of HSA (40% v/v), SNP-4 surface modified with PEG (4 kDa) adsorbed 17.78% ± 0.78% and SNP-2 covered with PEG (2 kDa) adsorbed 27.22% ± 0.89%. POL coatings on the NP could equally resist the protein adsorption. SNP-6 coated with Pluronic F-68 showed 26.34% ± 1.3%, whereas SNP-8 made using Pluronic F-127 adsorbed 22.06% ± 1.8% of HSA. The above results show that the ability to resist protein adsorption increases with increasing molecular weight and chain length of the hydrophilic copolymers.[17]

**Cell cytotoxicity studies**

A dose-dependent inhibition of cell viability using MTT assay was observed after 24 h of exposure to free VB and VB-NP formulations. The study exhibited a dose-dependent increase in the growth inhibition. For the same concentration of drug (10 µg/ml), the percentage cell inhibition was 67.54 ± 2.7 for VB-NP, 74.55% ±2.1% for SNP-8, 79.39% ±3.5% for SNP-4, and 83.05% ±2.3% for free VB, as shown in Figure 7. Thus, the order of the antiproliferation activity of the formulations is free VB >SNP-4 >SNP-8 >VB-NP. There was no significant difference in the cell inhibition pattern of the formulation at different concentrations. The representative microscopic fields of MCF-7 cells following drug and NP exposure are shown in Figure 8.

**DISCUSSION**

Multiple emulsion solvent extraction technique is commonly used for the fabrication of NPs from polymers, especially for the loading of hydrophilic drugs.[18] The W₁/O/W₂ emulsion is viewed as a preferable method for the encapsulation of hydrophilic drugs, as employing two immiscible phases restricts the diffusion of drug in the external phase.[19,20]

The size of the NPs (<250 nm) with a hydrophilic surface is more suitable to establish an improved EPR effect due to their ability to remain in blood circulation for a longer duration.[21] Diffusion of the SMA solubilized in the organic phase forms hydrophilic chains on the surface of the NPs, and consequently, the surface-modified NPs (SNPs) are larger than the plain VB-NP. Increasing the concentration of hydrophilic copolymers decreases the particle size of the NPs. This inclination could be attributed to the amphiphilic nature of the SMA, which reduces the interfacial tension between the organic and aqueous phases.[22] All the NP formulations exhibited a negative zeta potential. The negative zeta potential of the BNP is because of the ionized carboxylic groups of PCL in water. The existence of the PEG and POL chains on the surface of the NP core drapes and masks the actual surface charge of the PCL and transforms the potential from a negative value to near zero.[23,24] The DL studies revealed a lower encapsulation of VB into PCL-NPs. VB being an amphoteric molecule has low solubility in the hydrophobic polymer matrix of PCL. Increasing the concentration of SMA may favor the solubility of the drug and its partitioning toward the aqueous phase and therefore resulted in further reduction of drug entrapment inside the
polymeric core. Similar observation was observed in the preparation of PEG-modified solid lipid NPs of VB.

The in vitro release studies showed a biphasic pattern with an early burst release attributed to the diffusion of the drug adhered on the surface of the NPs, which might desorb upon contact with PBS media; the inclusion of SMA leads to the formation of an open hydrophilic framework due to increased hydration. This increases the penetration of the aqueous media facilitates the dissolution of the hydrophilic SMA blocks and the drug from the polymer matrix. For the above-said reason, the increase in the chain density achieved by increasing the concentration of SMA further promotes the release of VB.

Following intravenous administration, the NPs are rapidly cleared as a result of adsorption of plasma proteins which triggers the recognition and uptake of particles by MPS. The complex formed between the NP and the proteins is called as NP-protein corona, and the biological interactions of the NP such as the cellular uptake depend on this protein corona. Precoating or irreversible adsorption of hydrophilic block copolymers can be used as an effective tool against the adsorption of proteins. Hydrophobic NPs possess more protein-binding sites due to the clustering of polymer chains and hence adsorb more albumin than their hydrophilic counterparts. Inclusion of SMA such as PEG and POL in the NP formulations forms a nonionic adsorption layer covers or masks the original charge on the surface of the particles. Thus, the SNP exhibited lower negative charge or near neutral charge [Table 1]. These hydrophilic molecules increase the steric resistance ability by blocking the hydrophobic surface sites from exposure to opsonins. Increase in the molecular chain length and chain density of the hydrophilic polymers on the surface of NPs increase their resistance toward the adsorption of proteins. NPs covered with dense chains of PEG (4 kDa) had the least percentage of protein adsorption but do not completely dodge the adsorption of HSA on its surface. This confirms the presence of other adsorption mechanisms such as hydrophobic interactions, hydrogen bonding, and van der Waals forces at the protein-particle interface.

The in vitro cell cytotoxicity determination on MCF-7 cell assay showed a considerable difference between the free VB and the surface-modified NP formulations. At all concentrations, NPs displayed lower cytotoxicity compared to free VB. The outcome of the present study shows that the cell cytotoxicity of NP formulations increases with higher concentrations and longer time exposure. Previous studies showed resolutely that concentration and incubation time have an influential role in the in vitro cytotoxicity of antineoplastic drugs, i.e., higher concentration and longer incubation results in greater cell inhibition. The results are discernible as free VB is readily available for the cellular uptake by passive diffusion, whereas drug held

| A | B | C | D | E |
|---|---|---|---|---|
| Free VB | VB-NP | SNP-4 | SNP-8 |

*Figure 8: Representative microscopic images of MCF-7 cells after incubation with at concentrations - 0.001 µg/ml (A), 0.01 µg/ml (B), 0.1 µg/ml (C), 1 µg/ml (D), and 10 µg/ml (E). Spherical objects in the image represent dead cells, which float on the surface of the well plate.
up in NP formulations need to be taken up by cells through endocytosis. As the endocytosis progress, VB is released from the NPs, and the concentration of the drug in the cells increases gradually until it reaches a plateau. It could be speculated that if the study point was extended from 24 to 72 h, NP formulations of VB could be more effective than the free drug at similar concentrations, contemplating its sustained release of drug in the cytoplasm rather than extracellular environment.

The results also show that the SNP-4 and SNP-8 produce greater cell inhibition than the uncoated VB-NP. The rationale behind this occurrence may possibly have multiple perspectives. First, the surface-modified NPs exhibit a faster release of VB from its core than unmodified NP, resulting in higher concentrations of the drug within the cells which consequently reduces cell viability. Second, the hydrophilic coating of the NPs associates well with the cell surface, causing greater cellular uptake. Finally, the negative charge of VB-NP might result in electrostatic repulsion with the sialic acid and other negative domains of the cell membrane thwarting them from entering the cell membrane. Thus, higher cytotoxicity comparable to free VB could be achieved by treatment with higher concentrations of surface-modified NPs for a longer duration.

**Conclusion**

The rapid clearance of NPs from the blood circulation due to opsonization and phagocytosis results in low therapeutic concentrations of drug in the tumor tissue. Surface grafting using PEG or suitable polysaccharides provides steric repulsion effects to the nanocarriers to evade protein adsorption and the cells of the MPS. The present study showed that surface modification could have a profound effect on the protein adsorption and cell cytotoxicity. The NPs exhibited less affinity to interact with proteins, even at higher concentrations of protein and yet exhibited superior cell cytotoxicity. Although further studies are required to completely understand its role, the results of this study confirm the importance of steric stabilization in the design of nontargeted and targeted nanomedicines.

**Financial support and sponsorship**

Nil.

**Conflicts of interest**

There are no conflicts of interest.

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